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Symbiosis

Multidisciplinary approach to describe Trebouxia diversity within lichenized fungi Buellia zoharyi from the Canary Islands --Manuscript Draft--

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Full Title:	Multidisciplinary approach to describe Trebouxia diversity within lichenized fungi Buellia zoharyi from the Canary Islands	
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	Generalitat Valenciana (PROMETEO/2017/039)	Dr. Patricia Moya
Response to Reviewers:	<p>Ref: Ms. No. SYMB-D-20-00159</p> <p>Title: Temperature as a bioclimatic factor affecting association patterns in Buellia zoharyi from the Canary Islands</p> <p>Note for the Editor: Dear David there was a mistake related to the author's order in the submission menu, the correct order is the one that appear in the doc. Sorry for this inconvenience</p> <p>Authors: Arantzazu Molins¹, Salvador Chiva¹, Ángeles Calatayud², Francisco Marco³, Francisco García-Breijo^{4,5}, José Reig-Armiñana^{1,4}, Pedro Carrasco³, Patricia Moya¹</p> <p>Editor comments</p> <p>Please consider the comments and recommendations for revision by the Reviewers. The recommendations for changes, though in some cases substantial, are well worth making as I am sure the paper will be much improved as a consequence. If there are any recommendations you do not wish to comply with, please identify these and state</p>	

your reasons in an E-mail with the attached electronic version of your revised manuscript.

Thank you very much for your recommendations. Substantial changes have been made in the manuscript. See def version and with CC attached

Reviewer Comments:

Reviewer 1

Line 93: check ever – present

Done. See line 92

Line 120: occur instead of occurs

Done. See line 116

Line 253 algal strain instead of algae strain

Done. See line 277

Reviewer 2

I would suggest that this be published without the additional physiological measurements. These need to be much more intensively and carefully studied before we can know if the observed patterns have anything to do with the ability of the strains to display differences in acclimation and adaptation to temperature.

Thank you very much for your recommendations. We have reorganized, reduced and re-written the complete manuscript. We decided not to exclude the physiological measurements but we have changed the approach of this part of the study, because as the referee mentioned additional measurement should be included to confirm the influence of the temperature in the photobiont adaptation. However, we consider that each *Buellia zoharyi* photobionts showed different Chlorophyll a fluorescence responses to different temperatures (20° C and 17° C) as according to the Fv/Fm measurements (Fig. 5a). As shown in this Fig. *Trebouxia* sp. 'arnoldoi' and *T. cretacea* were significantly affected by temperature, with the lowest Fv/Fm values at 17° C. However, *Trebouxia asymmetrica* showed similar values at 20° C and 17° C (0.66 ± 0.02 and 0.62 ± 0.05), respectively, without significant differences between them. Therefore, those differences are described in the manuscript.

I certainly don't believe that the data presented justify the title of the m/s: "Temperature as a bioclimatic factor affecting association patterns in *Buellia zoharyi*". I personally would doubt if temperature has much influence, but in any event, much more work is needed.

As the referee recommended we have changed the title: Multidisciplinary approach to describe *Trebouxia* diversity within lichenized fungi *Buellia zoharyi* from the Canary Islands

Briefly, my feeling is that the differences in temperature between the sites (17 and 20°C) are too small to have selected different photobionts. Studies that have focused on thermal adaptation have typically used populations of lichens growing in areas with much greater differences in temperature. For example, Sahu et al. (2019) measured NPQ etc., in *Phaeophyscia hispidula* and *Flavoparmelia caperata* growing along a large altitudinal gradient, with average temperatures between sites varying by more than 20°C. In the present study with *Buellia*, differences in photobiont composition would seem to be unlikely to be related to such small differences in temperature when other factors e.g. N supply may vary between sites.

As mentioned before, we agree with the referee that additional measurement should be included to confirm the influence of the temperature or other bioclimatic factors in the photobiont adaptation hypothesis.

We know, Sahu et al (2019) studied variations in microclimatic attributes and their effects on photosynthetic efficiency and analyzed four elevational ranging from 1950 to 3508 m. They detected that extreme variations in air temperature (5.75–31. 65° C) and other factors were imperative in controlling species richness, distribution and photosynthetic quenching of lichen flora in the region. However, in this work, *Buellia zoharyi* populations included in this study grown in areas with similar ecological conditions except for the temperature although other factors could be influence on their performance

Moreover Casano et al (2011), as mentioned in the manuscript (see lines 527-539). To search for physiological differences between the two *R. farinacea* phycobionts, studied the effects of temperature and light on the growth and photosynthetic traits of isolated and cultured *T. jamesii* and *Trebouxia* sp. TR9 algae. They showed the fresh weight reached by both microalgae after 30 days at 17°C or 20°C and 15–100 $\mu\text{mol m}^{-2}\text{s}^{-1}$ of PAR. Both species grew better at the lower temperature; however, at 20°C the negative impact of the high temperature was less on TR9 than on TR1, whose growth was almost inhibited. In fact, since the low algal biomass at 20°C significantly increased the experimental error of fluorescence measurements, rendering non-reliable data, so the experiments in Casano et al (2011) were carried out only in cultures growing at 17°C. Therefore, the influence of only 3-4 °C of temperature in the *Trebouxia* spp. was previously reported by the mentioned authors.

On the contrary, we did not find such differences in the growth temperature, and thus, we performed the experiments at both 17° C (mean annual temperature in Fuerteventura and Lanzarote) and 20° C (mean annual temperature in Tenerife) to study the photosynthesis response to different temperatures (20° C and 17° C). In the same way, I cannot accept that experiments that quantified the responses of the photobionts to the two temperatures can explain the observed differences in photobiont composition. Thus, while it is interesting that strains cultured at 17oC compared with 20oC display slightly lower Fv/Fm values, it is hard to really interpret this observation without more data. It certainly does not correlate simply with NPQ values.

See previous answer

From the data in Figure 5, it appears that algae cultured at 17oc have slightly higher ETR rates, which is not consistent with the Fv/Fm data. In general, I found the logic in the paragraphs discussing the fluorescence data muddled and difficult to follow, and furthermore, does not refer back to the figures. But ultimately, based on the data presented, photobiont composition cannot be related to the response of the photobionts to temperature.

We have rewritten this part of the document to clarify it

I am rather skeptical that individual spot measurements of phytohormone levels will tell us much about the stress tolerance / differences in the different photobionts. The work of Pichler et al. (2020) cited by the authors illustrates one possible approach. Here, first secretion of hormones was studied, and second changes in the levels of the hormones induced by light and desiccation. In theory, a study comparable to this with the different strains / geographical isolates could be valuable, but the data presented are not helpful. In conclusion therefore, my recommendation therefore is that the physiology sections are deleted and the m/s be re-submitted without them.

Thank you very much for your recommendations. Due to the scarce information related to the phytohormone content in microalgae available in the literature, we agree with the referee that a study comparable to Pichler et al. (2020) with different strains / geographical isolates exposed to several growth conditions (control and stress), would be valuable to understand photobiont adaptation, given the well know relationship between phytohormones and adaptation to environmental conditions. However, in the present study this analysis was included as a preliminary technique in order to complement the photobiont characterization. In this sense, we have been able to detect different phytohormone profiles for the three isolated photobionts, an observation that could suggest different signaling needs for each of them. We consider this data an interesting start point for further studies that could help to determine how phytohormone signaling could contribute to explain the local adapted photobiont hypothesis. In order to clarify our point of view to a future reader, we have reordered the information regarding phytohormone data in material and methods, results and discussion in the manuscript.

Minor Comments

In general replace phycobiont with photobiont

Done. See new manuscript

Line 99 Meaning of "outcompete" not clear

We have changed adapt and outcompete for thrive. See line 98

Line 123 Meaning of "communities" not clear - "populations"?

Done. See line 119

Line 126 something is missing before "population"

Done. See line 122

Line 127"have" rather than "showed"
Rephrased. See lines 123-125

Line 128a "biocrust" is not an ecological condition
Rephrased. See line 125

Line 139"document" rather than "depict"
We have modified depict for describe. See line 136

Line 144"They were characterized by means of molecular, microscopy and spectrometry." Please turn into a grammatical sentence.
Done. See line 141

Line 291"grau"?
Grade. See line 247

Line 306replace "with" with "with the"
Done. See line 262

Line 402First paragraph of the Discussion should summarize the findings of the study, not the aims!
Thank you for the suggestion. We have rewritten these paragraph. See lines 406-409

Line 420better give area in square km
Sentence rewritten. See lines 418-419

Line 486"resolutive", odd word, please re-phrase
Sentence deleted.

Line 538"heterogeneities of Chl fluorescence" - is this data really used? If not, maybe delete reference to it.
We agree with the referee. Thank you for the recommendation. We have deleted this paragraph and reference

Line 554 "which could indicate sensitivity to photoinhibition" - rather an odd statement, given that the photobionts were cultured at 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$?
Sentence deleted

Line 937Caption to Figure 5. "Two areas of interest (AOI) were selected for Chl fluorescence measurements, one in the central part and one in the outer zones in order to evaluate spatial heterogeneity. The values of chlorophyll fluorescence parameters were means of two AOI (internal and external); no significant differences were obtained between internal and external areas." This text seems to be in the wrong place?
Paragraph included in material and methods. See lines 299-303

[Click here to view linked References](#)

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4 1 Journal: Symbiosis
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6 2 Article type: Special Issue Festschrift Barreno
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8 3 **Title:** Multidisciplinary approach to describe *Trebouxia* diversity within lichenized fungi *Buellia*
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10 4 *zoharyi* from the Canary Islands
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51 27 **Running title:** *Trebouxia* species within *Buellia zoharyi* from the Canary Islands
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4 **29 Abstract**

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6 30 The Canary Islands are famous for their extraordinary biodiversity; however, lichenized algae have
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8 31 only been studied partially. *Buellia zoharyi* is a circum-Mediterranean/Macaronesian species that
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10 32 usually occurs in semi-arid areas of the Mediterranean, but occasionally some interesting
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12 33 communities of this species grow on basaltic lava flows in Lanzarote, Fuerteventura and Tenerife.
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14 34 Those three locations showed similar ecological conditions, but different mean annual temperatures.
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16 35 Here we applied a multidisciplinary approach to describe microalgae diversity from *B. zoharyi*
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18 36 covering the entire described range of distribution in the Canary Islands. Photobionts were
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20 37 characterized in symbiosis using molecular and microscopic techniques. Different *Trebouxia* spp.
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22 38 were detected as primary photobiont in each island (*Trebouxia cretacea*-Fuerteventura, *T.*
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24 39 *asymmetrica*-Lanzarote and *Trebouxia* sp. `arnoldoi`-Tenerife). Coexistence of various *Trebouxia*
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26 40 spp. within a thallus were detected by using specific primers-PCR. Those three photobionts were
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28 41 isolated and cultured under laboratory conditions. Different phytohormone profiles were obtained in
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30 42 the isolated strains which suggest different internal signalling needs. In addition, we characterized
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32 43 the response of the isolated strains to different temperatures using chlorophyll fluorescence. *T.*
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34 44 *asymmetrica* did not modify their F_v/F_m values with respect to temperature acclimation. In contrast,
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36 45 *Trebouxia* sp. `arnoldoi` and *T. cretacea* were more sensitive to changes in growing temperature
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38 46 decreasing F_v/F_m at 17° C. Our results indicate that *B. zoharyi* is flexible regarding the photobiont
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40 47 choice depending on the region, and suggest that bioclimatic factors could influence the
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42 48 myco/photobiont association patterns.

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50 **Keywords:** coexistence, isolation, microalgae, photosynthesis, symbiosis, ultrastructure

51
52 **Declarations**

53 **Funding** Funding for field and laboratory work for this study was provided by the Ministerio de
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53 **Competing interests** The authors declare no competing interests

54 **Availability of data and material** GenBank

55 **Code availability** LSU rDNA: MT458607-MT458609; nrITS MT458610-MT458618

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4 60 **Authors' contributions** PM, AM and SC conceived the study and designed the laboratory part of
5
6 61 the study. SC, PM and AM carried out laboratory work. FG-B and JR-A analyzed microscopic
7
8 62 images. AC and PM performed photosynthesis measurements. FM and PC performed
9
10 63 phytohormone quantifications. AM and PM analyzed all data and wrote the manuscript. All authors
11
12 64 edited and approved the final version of the manuscript.

12 65 **Acknowledgments**

14 66 Dr. Arnaldo Santos was involved in the design of the surveys and the sample collection, also in the
15
16 67 ecology and bioclimatic information of the locations. Daniel Sheerin revised the English
17
18 68 manuscript. We dedicate this article to Eva Barreno in honour of her retirement.
19
20 69

21 70 **Introduction**

23 71 The Canarian Archipelago contains eight volcanically active islands proximal to the African
24
25 72 continent and the High Atlas Mountains. The origin and magmatic evolution of these islands have
26
27 73 been contentious issues for several decades and make this particular archipelago unique for
28
29 74 biodiversity studies. The Canary Islands are famous for their extraordinary diversity in lichenized
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31 75 and lichenicolous fungi (Van den Boom and Etayo 2006; Hernández Padrón and Pérez Vargas
32
33 76 2010). The diversity of lichenized algae has only been studied partially for *Tephromela atra*
34
35 77 (Muggia et al. 2010), *Ramalina farinacea* (Casano et al. 2011; del Campo et al. 2013; Moya et al.
36
37 78 2017), *Lecanora rupicola* (Blaha et al. 2006), *Parmotrema pseudotinctorum* (Molins et al. 2013;
38
39 79 Škaloud et al. 2018), *Stereocaulon vesuvianum* (Vancurová et al. 2015), *Psora decipiens* (Moya et
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41 80 al. 2018) and *Cladonia* spp. (Moya et al. 2015).

41 81 Lichen symbioses are currently considered as microecosystems in which multispecies assemblages
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43 82 are hosted in the lichen thallus (holobiont), formed by the two major lichen symbionts, e.g. the
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45 83 mycobiont (fungal partner) and the photobiont (a population of photosynthetic green or blue-green
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47 84 algae). According to the most recent investigations, many other fungi (Muggia and Grube 2018;
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49 85 Smith et al. 2020), non-photosynthetic bacteria (e.g. Aschenbrenner et al. 2014; Grube et al. 2015;
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51 86 Cernava et al. 2017; Sierra et al. 2020) and photosynthetic green microalgae co-inhabit within the
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53 87 lichen thalli, giving rise to the peculiar phenotypes, and which may contribute to the diverse
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55 88 patterns of secondary metabolites (Spribille 2018) and environmental adaptation. In terms of lichen
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57 89 photobionts, intrathalline microalgal diversity, e.g. multiple photobiont species within a single
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59 90 lichen thallus, has previously been observed in a number of lichen symbioses (Dal Grande et al.
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61 91 2014; Muggia et al. 2013; Moya et al. 2017; Škaloud et al. 2018). In some cases, algae with
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63 92 different physiological performances are present in lichen thalli, potentially facilitating the success

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93 of these lichens in a wide range of habitats and geographic areas and/or in changing environmental
94 conditions (Casano et al. 2011; del Hoyo et al. 2011).

95 Muggia et al. (2020) highlight the need for an integrative taxonomic approach, incorporating
96 morphological and physiological data from axenic cultures with genetic data, to establish a robust,
97 comprehensive taxonomy for *Trebouxia*. Complementation with ecophysiological studies needs to
98 be employed to explain how lichens thrive in extreme environments (Sadowsky and Ott 2012).
99 Lichen photobionts show a particular photosynthesis performance and have evolved alternative
100 photosynthetic-machinery protective mechanisms in response to their special ecophysiology
101 (Gasulla et al. 2019). The photosynthesis responses to abiotic factors, like temperature and light
102 conditions in axenic cultures of photobionts, reflects the ecophysiological plasticity of this
103 symbiosis as a mechanism, allowing the lichen to adapt to changing and often stressful
104 environments (Casano et al. 2011).

105
106 Phytohormones are chemical messengers involved in several physiological, stress response and
107 biochemical processes of higher plants at very low concentrations. Phytohormone composition has
108 been characterized in only a few algae (Yokoya et al. 2010; Gupta et al. 2011; Wang et al. 2014), but
109 recently, Pichler et al. (2020) studied phytohormone composition in aeroterrestrial
110 *Trebouxiophyceae*, including three lichen- forming microalgae. The results obtained in this study
111 contribute to valuable baseline information for further studies into the roles of phytohormones in
112 microalgae. Including this technique to characterize isolated symbiotic microalgae could help to
113 understand the ecologically adapted photobionts hypothesis.

114
115 *Buellia zoharyi* is a circum-Mediterranean/Macaronesian species forming crustose placodioid
116 lichens that usually occur in biocrusts in semi-arid areas of the Mediterranean region (Gutiérrez-
117 Carretero and Casares-Porcel 2011). Specifically, this species predominantly grows on gypsum soils
118 (Crespo and Barreno 1975; Barreno 1994; Trinkaus and Mayrhofer 2000), but occasionally some
119 interesting populations of this species grow on basaltic lava flows in the Canary Islands (Etayo
120 2011; Giralt and Van den Boom 2011; Roux and Poumarat 2015). The presence of this lichen
121 species in the Canary Islands was reported in Lanzarote (Trinkaus and Mayrhofer 2000),
122 Fuerteventura (Van den Boom and Etayo 2006) and Tenerife (Chiva et al. 2019), and populations in
123 the other four Islands must be scarce or even non-existent. These three locations have similar
124 ecological conditions e. g. the same high irradiance (5.5 – 5.7 KWh/m²) moreover, in the three
125 locations *B. zorayi* grown forming biocrusts in areas with sparse vascular vegetation. However, the
126 WordClim database reported different mean annual temperatures (MAT): 17° C in Fuerteventura

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127 and Lanzarote, and 20° C in Tenerife (<https://www.worldclim.org>; Hijmans et al. 2005). Chiva et al.
128 (2019) analyzed mycobiont diversity in *B. zoharyi* in the Mediterranean region including those three
129 Canary Island locations. They found low genetic diversity and two geographically differentiated
130 haplogroups: one including populations from the Iberian Peninsula to Azerbaijan, and the other
131 from the southern Iberian Peninsula, North Africa and the Canary Islands. Complementary analyses
132 of *B. zoharyi* concerning the range of associated photobionts and their ecophysiological traits will
133 be necessary to identifying historical and ecological factors at the basis of the evolutionary history
134 of this group of soil- dwelling taxa.

135
136 Here we applied a multidisciplinary approach to describe microalgae diversity from *B. zoharyi*
137 settled in the Canary Islands. Populations included in this study cover the entire described range of
138 distribution in the archipelago. Photobionts were characterized in symbiosis using molecular and
139 microscopic techniques. Coexistence of various *Trebouxia* spp. were detected by using specific
140 primers-PCR. Representative photobionts were isolated and cultured under laboratory conditions.
141 We characterized the isolated strains using molecular, microscopy and spectrometry techniques.
142 Their responses to different temperatures were monitored using chlorophyll fluorescence to answer
143 if this bioclimatic factor could influence the myco/photobiont association patterns.

144
145 **Material and methods**

146 **Sampling and DNA extraction**

147 In this study, 46 thalli of *Buellia zoharyi* collected from Lanzarote, Tenerife and Fuerteventura were
148 analyzed (Table 1S). We included fresh (N=43) and herbarium samples (N=3). Fresh specimens
149 were air-dried for one day after sampling and then stored at -20°C. Lichen thalli were examined
150 under a stereomicroscope to remove soil particles and were immersed sequentially in ethanol and
151 NaOCl (Arnold et al. 2009) to remove surface contaminants and to ensure the intrathalline origin of
152 the sequenced microalgae. Fragments from different parts of each thallus were randomly excised
153 and pooled together. Total genomic DNA was isolated and purified using the DNeasy Plant Mini kit
154 (Qiagen, Hilden, 121 Germany) following the manufacturer’s instructions.

155
156 **Primary photobiont PCR amplification and Sanger sequencing**

157 Two algal loci were amplified; a region of the chloroplast LSU rDNA gene using the algal specific
158 primer pair 23SU1 and 23SU2 (del Campo et al. 2010a) and the nrITS (internal transcribed spacer)
159 using the primer pair nr-SSU-1780 (Piercey-Normore and DePriest 2001) and ITS4 (White et al.
160 1990). PCR reactions were performed following Moya et al. (2018). The PCR products were

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161 visualized on 2% agarose gels and purified using the Gel Band Purification Kit (GE Healthcare Life
162 Science, Buckinghamshire, England). The amplified PCR products were sequenced with ABI
163 3730XL using the BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City,
164 California). Sanger sequences were visualized and manually evaluated with Chromas 2.6.6.0
165 (<http://technelysium.com.au/wp/chromas/>).

166
167 ***Trebouxia* photobiont phylogenetic analysis**

168 The nrITS and LSU rDNA datasets were collapsed using TCS 1.21 (Clement et al. 2002). The
169 dataset included the newly determined nrITS and LSU rDNA sequences from thalli and isolated
170 photobiont (accession numbers LSU rDNA: MT458607-MT458609; nrITS: MT458610-
171 MT458618), and a selection of 30 *Trebouxia* species sequences available from the Culture
172 Collection of Algae at Goettingen University (SAG), from the Culture Collection of Algae at the
173 University of Texas (UTEX) and from the Symbiotic Microalgal Collection of the University of
174 Valencia (ASUV, *Trebouxia* sp. TR9 and *Trebouxia crespoana*). We included *Asterochloris*
175 *mediterranea* as an outgroup.

176 A multiple alignment was built using MAFFT 7.0 (Kato and Standley 2013) using default
177 parameters. Aligned sequences were improved by eliminating the ambiguous regions using Gblocks
178 0.91b (Castresana 2000) with the least stringent parameters. This software allows conflicting
179 regions in the alignment to be automatically removed. Both loci were concatenated, yielding an
180 alignment of 1329 characters. The final matrix contained 42 ITS rDNA and 33 LSU rDNA
181 sequences.

182 For each locus, the most appropriate substitution model was estimated using the Akaike information
183 criterion (AIC) using JModelTest 2.1.4 (Darriba et al. 2012). The most appropriate nucleotide
184 substitution models for nrITS and LSU rDNA were GTR+I+G and GTR+G, respectively. The
185 phylogenetic trees of both loci were inferred by Bayesian inference (BI) and Maximum Likelihood
186 (ML) approaches carried out on partitioned datasets using the different substitution models selected
187 by JModelTest. ML analysis was implemented in RAxML 8 (Stamatakis 2014) using the
188 GTRGAMMA substitution model. Bootstrap support (BS) was calculated based on 1,000
189 pseudoreplicates (Stamatakis et al. 2008). BI was carried out in MrBAYES 3.2 (Ronquist et al.
190 2012). Settings included two parallel runs with six chains over 20 million generations, starting with
191 a random tree and sampling after every 200th step. We discarded the first 25% of the data as burn-in,
192 and the corresponding posterior probabilities (PPs) were calculated from the remaining trees.
193 Estimated sampled sized (EES) values above 200, and potential scale reduction factor (PSRF)

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194 values approaching 1,000, were considered indicators of chain convergence. The phylogenetic tree
195 was visualized in FIGTREE 1.4.2 (Rambaut 2014; <http://tree.bio.ed.ac.uk/software/figtree/>). All
196 analyses were run at the CIPRES Science Gateway 3.3 webportal (Miller et al. 2010).

197

198 **Microscopic examinations “in thallus”.**

199 The ultrastructure of the photobionts was characterized by transmission electron microscopy (TEM)
200 from selected thalli (LA7, LA22, TE1, TE10 and FU1). For TEM, the cells were fixed and
201 dehydrated as described in Molins et al. (2018a). Samples were embedded in Spurr’s resin
202 according to the manufacturer’s instructions. Sections (90 nm) were cut and mounted as described
203 in Moya et al. (2018). The sections were observed with a JEOL JEM-1010 (80 kV) electron
204 microscope, equipped with a MegaView III digital camera and ‘AnalySIS’ image acquisition
205 software. TEM examinations were carried out at the SCSIE Service of the University of Valencia.

206

207 **Secondary *Trebouxia* strains detected by specific PCR primers**

208 To detect the presence of secondary *Trebouxia* species in each thallus, PCR were performed using
209 as templates each PCR from the primary photobiont (obtained with the primer pair nr-SSU-1780 /
210 ITS4) and re-amplifying with specific primer pairs (Table 2S). These specific forward and reverse
211 primers were designed in this study based on the nrITS sequences obtained with the primer pair nr-
212 SSU-1780 / ITS4 in *B. zoharyi* from the Canary Islands, which included *Trebouxia asymmetrica*,
213 *Trebouxia cretacea* and *Trebouxia* sp. ‘*arnoldoi*’.

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215 **Isolation, PCR identification and propagation of microalgae strains.**

216 Photobionts were isolated from selected thalli (LA7, TE1 and FU1) using two protocols: a) the
217 micromethod described by Gasulla et al. (2010), where the resulting algal suspension was diluted
218 with sterile water and spread using the streak method on sterile 1.5% agar Bold's Basal Media Petri
219 dishes (BBM) (Bold 1949; Bischoff and Bold 1963); and b), the method described in Muggia et al.
220 (2014) where tiny clumps of the algal layer were inoculated directly into BBM. The isolated algae
221 were maintained under a $50 \mu\text{mol} / \text{m}^{-2}\text{s}^{-1}$ photosynthetic photon flux density (PPFD) with a 12 h
222 photoperiod at 20°C. Subsequent subcultures were performed until we obtained a unialgal culture
223 and fast PCR microalgae identification was performed directly from the colonies as described in
224 Molins et al. (2018b).

225

226 **Microscopic investigations of photobionts “in culture”.**

227 Light microscopy (LM) and Epifluorescent Microscopy (EFM) was performed on selected unialgal
228 cultures on the 21st day of cultivation at 20° C. All LM and EFM observations were carried out with
229 an Olympus Provis AX 70 fluorescence microscope equipped with an Infinity 2–3 C Lumenera®
230 digital camera and analyzed with “Infinity Analyze” Software. For EFM, an Olympus U-ULS 100
231 HG epifluorescence system with U-MWBV (excitation filter 400–440 nm, dichroic mirror 455 nm,
232 barrier filter 475 nm) cubes was used.

233

234 **Phytohormone content determination**

235 Endogenous phytohormone levels of 21-day-old microalgae cultures grown at 20° C were
236 determined according to the protocol of Durgbanshi et al. (2005). Lyophilized microalgae samples
237 were processed by extraction in 5 ml of distilled water after fortifying them with internal standards:
238 [²H₆] - abscisic acid (ABA) (100 ng, prepared as described in Gómez-Cadenas et al. (2002)),
239 dihydrojasmonic acid (JA) (100 ng, synthesized in the laboratory by catalytic hydrogenation
240 according to Kristl et al. (2005)), [²H₂]-indole-3-acetic acid (IAA) (10 ng, Sigma-Aldrich) and [²H₄]
241 -salicylic acid (SA) (100 ng, Sigma-Aldrich). The extracts were centrifuged at 4000 x g, at 4° C for
242 45 min. Subsequently, the supernatant was collected in clean tubes and the pH adjusted to 3.0 using
243 a 30% (v / v) acetic acid solution. The acidified extracts were partitioned twice with 3 ml of ethyl
244 ether (ACS grade, Scharlau, Barcelona, Spain). The upper organic phase was recovered in a clean
245 vial, combining both partitions and drying them under vacuum using an evaporation centrifuge
246 coupled to a cold trap (RC 10.22 and RT 10.90, Jouan, Saint-Herblain Cedex, France). The dried
247 residue was resuspended by adding 100 μL of methanol (HPLC grade, Scharlau) in the test tube by
248 ultrasound for 10 min. Subsequently, the final volume of 1 mL was completed with pure water

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249 (MiliQ). The resulting solution was filtered using regenerated cellulose filters with a pore diameter
250 of 0.2 μm before analysis. Phytohormone analysis was performed using an HPLC device (Alliance
251 2860, Waters Corp., Milford, USA) coupled to a tandem mass spectrometer with an electrospray
252 interface (Quattro LC, Micromass, Manchester, UK). The samples were injected and separated by a
253 reverse phase column (Kromasil 100, C18, 5 μm , 100 \times 2.0 mm, Scharlau) using a linear gradient of
254 methanol and ultrapure water, supplemented with acetic acid to a final concentration of 0.01% (v/v)
255 and a flow of 0.3 mL / min. Discrimination and detection of each analyte was carried out following
256 the fragmentation pattern and the characteristic retention time. The ionization and collision
257 conditions for each compound were optimized by direct infusion of pure standards (approximately 5
258 mg/L). The quantification of the analytes of interest was performed using the response factor
259 (analytical area/area) by interpolation in a calibration curve injected alternatively in the samples.
260 Chromatogram processing, integration and quantification were performed using MassLynx 4.0
261 software. The profiles of the relative content of phytohormones between strains were determined
262 with the Heatmapper web tool (Babicki et al. 2016).

263

264 **Measurement of chlorophyll fluorescence imaging from isolated algae**

265 Fast PCR was performed directly from the unialgal cultures as described in Molins et al. (2018b) to
266 ensure the purity and identity of the selected aliquot used for chlorophyll (Chl) fluorescence images
267 (CFI) measurements.

268 A 50 ml aliquot of these actively growing algae, resuspended in liquid BBM medium, was
269 inoculated on cellulose–acetate disks placed on agarized BBM medium and then cultured for 21
270 days under two conditions, 17°C and 20°C with a 50 $\mu\text{mol m}^{-2}\text{s}^{-1}$ PPFD under a 12 h/12 h light/dark
271 cycle.

272 CFI was performed using an imaging-PAM fluorometer (Walz, Effeltrich, Germany), in order to
273 investigate the behavior of Chl fluorescence parameters in the three algae at different light
274 intensities and grown under different temperature conditions (17°C and 20°C). Algae samples were
275 layered on filter paper that was kept moist with distilled water in order to maintain the cells in a
276 fully hydrated state. The algae membranes were darkened for 30 min prior to measurement. Chl *a*
277 fluorescence determinations were obtained from $n=4$ samples for each algal strain and temperature.
278 The minimum (dark) fluorescence (F_o) was obtained by applying measuring light pulses at a low
279 frequency (1 Hz). The maximum fluorescence (F_m) was determined by applying a saturating blue
280 pulse (10 Hz). The maximum quantum yield of PSII photochemistry (Roháček 2002), often called
281 the F_v/F_m ratio, was determined as $F_m - F_o/F_m$. Then, a light curve with actinic illumination from 0

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282 to 964 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ was switched on, and saturating pulses were applied for 20 s in order to
283 determine the maximum fluorescence yield (F'_m) intensity, and the steady-state fluorescence value
284 that is immediately prior to the saturating pulse is F_s . Calculation of quenching due to non-
285 photochemical dissipation of absorbed light energy (NPQ) was determined at each saturating pulse,
286 according to the equation $\text{NPQ} = (F_m - F'_m)/F_m$ (Bilger and Björkman 1991). The actual quantum
287 efficiency of PSII photochemistry, Φ_{PSII} , was calculated according to Genty et al. (1989) by the
288 formula: $(F'_m - F_s)/F'_m$, and the coefficient of photochemical quenching (Van Kooten and Snel
289 1990) was determined as $qP = (F'_m - F_s)/(F'_m - F'_o)$. Excitation pressure on PSII (QA), which reflects
290 the proportion of the primary quinone electron acceptor of PSII that is in the reduced state, was
291 calculated as $1 - qP$ (Demmig-Adams et al. 1996). The relative electron transport rate (ETR) was
292 calculated as $\Phi_{\text{PSII}} \times \text{PAR} \times 0.84 \times 0.5$ (Schreiber et al. 1986). To determine F'_o correctly, it would
293 be necessary to switch off the actinic light and quickly reoxidise the PSII acceptor side with the help
294 of far-red light, but this is not feasible with imaging-PAM as far-red light would penetrate the CCD-
295 detector and cause serious disturbances to fluorescence images (see <http://www.walz.com>). The
296 value of F'_o was estimated using the approximation of Oxborough and Baker (1997), $F'_o = F_o/(F_v/F_m$
297 $+ F_o/F'_m)$. For each interval, saturation pulse images and values of various Chl fluorescence
298 parameters were captured (Calatayud et al. 2006).
299 Two AOI were selected one in the central part and other in the outer algae zones in order to evaluate
300 spatial heterogeneity CFI parameters between both AOI. After comparing both AOI for each algae
301 strain and temperature, not differences were observed for any Chl fluorescence parameters (data not
302 shown). Then, the values of Chl fluorescence parameters displayed in the figures (4a and 5) are
303 means of both AOI.

304 305 **Statistical analyses**

306 Data was analysed by one-way Analysis of Variance (ANOVA) followed by post-hoc comparisons
307 by Tukey's HSD test. A probability value < 0.05 was considered statistically significant.
308 Calculations were performed using R Software.

309 310 **Results**

311 **Primary *Trebouxia* diversity detected by Sanger sequencing (nrITS)**

312 The concatenated aligned algal nrITS + LSU rDNA fragment was 1329 bp in length. BI and ML
313 phylogenetic hypotheses were topologically congruent. In the 46 samples, we detected three

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4 314 *Trebouxia* species (Fig. 1). According to the clade code introduced for *Trebouxia* by Leavitt et al.
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6 315 (2015), and subsequently applied by Moya et al. (2017) and Muggia et al. (2020), these *Trebouxia*
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8 316 species belong to clade ‘A’ *arboricola/gigantea* type, precisely: *Trebouxia asymmetrica* in thalli
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10 317 from Lanzarote, *Trebouxia cretacea* in Fuerteventura, and *Trebouxia* sp. ‘*arnoldoi*’ in Tenerife.
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13 319 **Ultrastructural characterization of *Trebouxia***

15 320 Transmission electron microscopy (TEM) analyses of photobionts based on the ultrastructure of
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17 321 pyrenoids (Py) and plastids (Chl) distinguished at least three different *Trebouxia* morphotypes.
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19 322 Morphological characteristics of each morphotype, in detail, can be seen in Fig. 2 and Fig. 1S.
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21 323 One morphotype was found in *Buellia zoharyi* cells from Lanzarote (Fig. 2a; b). They showed a
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23 324 single central Py related to the *gigantea* type described by Friedl (1989) with pyrenoglobuli (Pg)
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25 325 uniformly distributed within the Py matrix. Lobulated-stellate chloroplast with abundant lax
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27 326 thylakoid membranes forming stacks of 4-5 membranes (grana).

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29 327 The second morphotype was found in *B. zoharyi* cells from Fuerteventura (Fig. 2c, d). These cells
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31 328 presented a single central Py related to the *impressa/gigantea* type described by Friedl (1989), with
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33 329 a highly lobulated chloroplast with abundant dense thylakoid membranes forming stacks of 2-3
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35 330 membranes.

36 331 The third morphotype was detected in *B. zoharyi* cells from Tenerife (Fig. 2e, f). They presented a
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38 332 single central Py related to the *impressa/gigantea* type described by Friedl (1989). The chloroplast
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40 333 morphology was similar to the second morphotype (lobulated with dense abundant thylakoid
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42 334 membranes forming stacks of 2-3 membranes). Large peripheral vesicles surrounding the
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44 335 chloroplast were present.

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47 337 ***Trebouxia* multiplicity revealed by specific PCR primers**

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49 338 Herbarium samples were excluded from this analysis. Specific forward and reverse primers were
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51 339 designed in this study based on the nrITS sequences obtained with the primer pair nr-SSU-1780 /
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53 340 ITS4 in *B. zoharyi* from the Canary Islands, which included *Trebouxia asymmetrica*, *Trebouxia*
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55 341 *cretacea* and *Trebouxia* sp. ‘*arnoldoi*’

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57 342 All the samples from Lanzarote (n=23) showed *T. asymmetrica* as the primary photobiont, and
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59 343 specific PCR detected *T. cretacea* in 22 thalli. *Trebouxia* sp. ‘*arnoldoi*’ was not detected (Fig. 3;
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61 344 Table 3S). Samples from Fuerteventura (n=7) showed *T. cretacea* as the primary photobiont. The
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4 345 presence of secondary algae was detected only in three thalli: two of them revealed *T. asymmetrica*
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6 346 as the secondary photobiont and one *Trebouxia* sp. `arnoldoi´ (Fig. 3; Table 3S). Samples from
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8 347 Tenerife (n=13) had *Trebouxia* sp. `arnoldoi´ as the primary photobiont. All the samples only
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10 348 showed *T. asymmetrica* as the secondary photobiont. *T. cretacea* was not detected (Fig. 3; Table 3S).

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13 350 **Identification and morphological characterization of isolated *Trebouxia* spp. from *Buellia***
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15 351 ***zoharyi***

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17 352 To corroborate the purity and identity of the unialgal selected aliquot, fast PCR was performed
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19 353 directly from the cultures as described in Molins et al. (2018b). Sequences obtained were included
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21 354 in the phylogeny indicated as isolated photobiont (Fig. 1). We detected three *Trebouxia* spp.: *T.*
22
23 355 *asymmetrica*, *T. cretacea* and *Trebouxia* sp. `arnoldoi´.

24
25 356 All three *Trebouxia* spp. presented mature vegetative cells, mostly unicellular and spherical as seen
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27 357 using LM and EFM (Fig. 2S). Tetrads and octads were only observed in *T. cretacea*. The spherical
28
29 358 vegetative cells were 14–16 (19) µm in diameter. All cells showed the characteristic *Trebouxia*
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31 359 central chloroplast dissected into lobes (Fig. 2S).

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34 361 **Phytohormone profiles of *Buellia zoharyi* photobionts**

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36 362 Phytohormone endogenous content was also determined to characterize *Trebouxia* strains cultured
37
38 363 in BMM at 20° C for 21 days. Mass spectrometry analysis showed detectable levels of IAA, ABA,
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40 364 JA conjugated to the amino acid Isoleucine (JA-Ile) and methyl jasmonate (Me-JA) (Fig. 4). The
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42 365 *Trebouxia* strains isolated from *B. zoharyi* showed differences in their phytohormone profiles:

- 43 366 1) *T. asymmetrica* and *T. cretacea* presented similar levels of IAA and ABA (Fig. 4a, b),
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45 367 *Trebouxia* sp. `arnoldoi´ showed a different profile, with about double the ABA endogenous
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47 368 content compared to the other strains (Fig. 4b), and higher levels of IAA (about five times)
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49 369 than *T. asymmetrica* and *T. cretacea* (Fig. 4a).
- 50
51 370 2) SA and JA-Ile levels also showed a different profile between microalgae strains, with
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53 371 similar levels for *T. cretacea* and *Trebouxia* sp. `arnoldoi´, while *T. asymmetrica* showed
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55 372 the highest level of SA (6 times greater; Fig. 4c) and higher levels of JA-Ile than *T. cretacea*
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57 373 (about 400% more; Fig.4d).

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4 374 3) Me-JA content of the *Trebouxia* strains showed another different profile, with similar levels
5 375 for *T. asymmetrica* and *Trebouxia* sp. `arnoldoi`, which were about 2.6 times higher than
6 376 the levels observed for *T. cretacea* (Fig. 4d).
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11 378 **Chlorophyll *a* fluorescence response to different temperatures (20° C and 17° C) of *Buellia***
12 ***zoharyi* photobionts**
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15 380 According to the F_v/F_m measurements (Fig. 5a), *Trebouxia* sp. `arnoldoi` was significantly affected
16 381 by temperature, with the lowest F_v/F_m values at 17° C. Similarly, in *T. cretacea*, F_v/F_m showed a
17 382 significant decrease from 0.65 ± 0.02 to 0.55 ± 0.03 with a lower value at 17° C However,
18 383 *Trebouxia asymmetrica* showed similar values at 20° C and 17° C (0.66 ± 0.02 and 0.62 ± 0.05),
19 384 respectively, without significant differences between them. The highest F_v/F_m values were obtained
20 385 for *T. asymmetrica* and *T. cretacea* at 20° C.
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26 386 The observation of color changes, ranging from black (0.000) to pink (1.000), revealed that the
27 387 images for F_v/F_m showed a uniform color for algae discs, and that different intensities of blue colors
28 388 associated with higher values of F_v/F_m (Fig. 4b) for *T. asymmetrica* and *T. cretacea* at 20° C, and
29 389 with the lowest F_v/F_m values (green) for *Trebouxia* sp. `arnoldoi` at 17° C (Fig. 5b).
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33 390 All NPQ values increased with respect to light intensity, but with a different shape and magnitude
34 391 depending on the *Trebouxia* species and growth temperature (Fig. 6a). In the case of *Trebouxia* sp.
35 392 `arnoldoi` and *T. asymmetrica* NPQ exhibited similar values for both temperature growth regimes
36 393 showing the same kinetic shape, but *Trebouxia* sp. `arnoldoi` showed the highest NPQ values. *T.*
37 394 *cretacea* at 20° C and *T. asymmetrica* displayed similar NPQ values and at the end of light-curve
38 395 kinetics *T. cretacea* showed the highest NPQ values.
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44 396 Regarding ETR (Fig. 6b), their values were slightly higher for *Trebouxia* strains growing at 20 ° C
45 397 compared with 17° C. Among species, *T. cretacea* displayed the highest ETR values, mainly at the
46 398 end of light curve kinetic at 20° C and the minimum values were observed in *Trebouxia* sp.
47 399 `arnoldoi` at 17° C.
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51 400 Photosystem II excitation pressure, expressed as 1-qP (photochemical quenching; Fig. 6c),
52 401 increased gradually with light intensity in the three algal strains. In general, *T. asymmetrica* and *T.*
53 402 *cretacea* showed similar values, however *Trebouxia* sp. `arnoldoi` showed slightly values except at
54 403 high irradiance where *Trebouxia* sp. `arnoldoi` at 17° C showed the highest values.
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4 **405 Discussion**

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6 406 This study applied a multidisciplinary approach to describe microalgae diversity from *B. zoharyi*
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8 407 growing in the Canary Islands. Our results indicate that *B. zoharyi* is flexible regarding the
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10 408 photobiont choice depending on the region, and suggest that bioclimatic factors could influence the
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12 409 myco/photobiont association patterns.

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14 411 According to Beck et al. (2002), ‘selectivity’ in lichens refers to the taxonomic range of partners
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16 412 that are selected by one of the bionts, while ‘specificity’ should be used for the symbiotic
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18 413 association, and depends on the range and taxonomic relatedness of acceptable partners. Lichens
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20 414 with high selectivity may associate with a limited number of photobionts. Numerous lichen-forming
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22 415 fungi have been shown to associate with identical species of *Trebouxia*, while others exhibited
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24 416 higher photobiont flexibility (Kroken and Taylor 2000; Ohmura et al. 2006, 2018; Doering and
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26 417 Piercey-Normore 2009; Leavitt et al. 2013, 2015; Lindgren et al. 2014). Our results indicate that *B.*
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28 418 *zoharyi* are flexible in their photobiont choice, as they associate with three *Trebouxia* species in 300
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30 419 km (distance from Iguete de San Andrés; Tenerife to Los Valles; Lanzarote).

31 420 Photobiont diversity can be shaped by the reproductive and dispersal strategies of the mycobiont
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33 421 (Cao et al. 2015, Steinová et al. 2019), geography (Muggia et al. 2014, Werth and Sork 2014,
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35 422 Leavitt et al. 2015), growth substrate (Bačkor et al. 2010, Leavitt et al. 2013, Muggia et al. 2014)
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37 423 and macroclimate (Lu et al. 2018, Singh et al. 2018). Lichens that reproduce sexually via
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39 424 independent dispersal of fungal spores, undergo a process of re-lichenisation. This means that the
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41 425 germinating spore of the mycobiont can easily exchange its autotrophic partner, in contrast to
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43 426 asexually reproducing lichens distributing both partners together, which allows the continuation of
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45 427 the symbiosis without the need to re-associate with another biont (Beck et al. 1998, 2002; Romeike
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47 428 et al. 2002; Sanders and Lücking 2002). Lichens that depend on the cyclical establishment of
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49 429 fungal-photobiont associations to colonize varied wide-ranging habitats might require a relatively
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51 430 higher flexibility in the specificity and ecological selection of their photobionts. This flexibility
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53 431 would facilitate successful re-lichenizations by allowing for alternative partnerships in each habitat
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55 432 (Romeike et al. 2002

56 433 The dispersal of *B. zoharyi* over medium to long distances can be accomplished by either meiotic
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58 434 ascospores produced in fertile thalli, or thallus fragments detached from fertile and sterile ones
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60 435 (Barreno 1994; Casares and Llimona 1983). Both strategies are suitable for long- distance
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62 436 dispersal, even across the Mediterranean Sea, based on evidence of other lichens showing widely
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64 437 disjunct populations (Alors et al. 2017; Fernández- Mendoza and Printzen 2013; Garrido-

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438 Benavent et al. 2018). Moreover, increased connectivity among the Canary Islands, Africa and the
439 Iberian Peninsula possibly occurred during Pleistocene glaciations, when the distance between the
440 Eastern most island (Fuerteventura) and the Moroccan coast was reduced to 60 km (Fernández-
441 Palacios and Whittaker 2008). Chiva et al. (2016) showed *T. cretacea* as the predominant
442 photobiont in 117 thalli of *B. zoharyi* from the Iberian Peninsula and Morocco. The colonization of
443 the Canary Islands by *B. zoharyi* must have originated from the Moroccan coast to Fuerteventura
444 (20.7 Ma), currently the eastern island, only 100 km from the African coast, evidenced as the
445 maintenance of the symbiont pattern (*T. cretacea*). Carracedo (1994), dated the origin of Lanzarote
446 in 15.5 Ma and Tenerife in 11.6 Ma. *B. zoharyi* must have colonized the Canary Islands
447 subsequently and adopted ecologically adapted photobionts (*T. asymmetrica* and *Trebouxia* sp.
448 `arnoldoi`) in those Islands.

449 The diversity of photobionts has only recently been explored by environmental DNA metabarcoding
450 approaches, and has focused on species within the Mediterranean basin to date (Moya et al. 2017;
451 Dal Grande et al. 2018; Smith et al. 2019). In contrast to high-throughput sequencing approaches,
452 traditional and largely applied DNA barcoding using Sanger sequencing was able to detect only the
453 principal photobiont in the thalli (Paul et al. 2018; Moya et al. 2020). In this study, the authors
454 determined if the second most abundant microalga exceeded 30% of the total HTS reads in a
455 sample, Sanger sequencing generally failed and generated ambiguous Sanger sequences showing
456 double peaks. Moreover, in the present study no samples with double peaks were found in the
457 electropherogram, the co-occurrence of multiple *Trebouxia* inside a thallus was performed by using
458 specific primers. This approach may limit the detection of further associated algae due to specificity
459 and thus, it should be used as a complement to traditional Sanger sequencing when it is not possible
460 to perform HTS approaches. However, all analyses performed using the multi-copy nrITS showed
461 methodological limitations that potentially bias the results presented, due to the variation in the
462 copy numbers across microalgae species. Therefore, the relative abundance of algal groups inferred
463 in this study with specific primers does not accurately describe the true relative abundance of
464 lichen-associated algae, given the potential for a very wide range of nrDNA copy numbers of these
465 algal groups. Even with these limitations, specific PCR primers revealed the presence of other
466 secondary photobionts which would be available in the substrate. The presence of multiple algal
467 species, and the different dominance of one of them in each species, implies the selection for a
468 particular algal species by the mycobiont (Peksa and Škaloud 2011; Dupont et al. 2016). To
469 corroborate this hypothesis, a complementary HTS approach, both from the lichen and from the
470 substrate, should be included.

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4 471 A reliable definition of photobiont species requires the description of morpho-anatomical traits both
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6 472 axenically cultured and in symbiosis. However, such traits are absent for the majority of lineages
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8 473 described in molecular phylogenetic analyses (Muggia et al. 2020). In this study, photobionts from
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10 474 Lanzarote, Fuerteventura and Tenerife were characterized in *Buellia zoharyi* thalli using
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12 475 transmission electron microscopy (TEM). The three morphotypes structurally characterized
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14 476 corresponded to each *Trebouxia* species molecularly described for each Island. These three
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16 477 *Trebouxia* species belong to clade 'A' *arboricola/gigantea* (Fig. 1) and showed, by TEM, a similar
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18 478 pyrenoid type (Fig. 2 and Fig. 1S), corresponding to the *impressa/gigantea* type described by Friedl
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20 479 (1989). Although Lanzarote's morphotype showed a high similarity to the *gigantea* type, this trait
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22 480 does not allow us to differentiate each morphotype. In contrast to the pyrenoid, the chloroplast
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24 481 thylakoid arrangements clearly differentiated the three morphotypes. This coincides with further
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26 482 studies (Molins et al. 2018a) and evidences that the thylakoid arrangement is a key complement to
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28 483 the pyrenoid structure to characterize *Trebouxia* species. Current knowledge concerning *Trebouxia*
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30 484 phylogenetic relationships highlights the pressing needs to revise the original classification of the
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32 485 group proposed by Friedl (1989) as that classification does not match with Muggia et al.'s (2020)
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34 486 clade delimitation. The combination of molecular analyses together with ultrastructural techniques
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36 487 should be initiated to clarify taxonomic concepts to delimit new taxa of microalgae, and particularly
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38 488 in the case of *Trebouxia* diversity (Muggia et al. 2016, 2020; Moya et al. 2017; Molins et al. 2018b).
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40 489 Here we detected the new *Trebouxia* sp. '*arnoldoi*' which did not match any previously described
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42 490 *Trebouxia* spp. This provisional name has been proposed until it can be formally described:
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44 491 '*arnoldoi*' refers to the Canarian botanist Arnaldo Santos.

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46 492 Isolation procedures should be included in photobiont diversity studies to perform morphological
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48 493 and physiological analyses. In this study, the *Trebouxia* strains isolated from *B. zoharyi* were
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50 494 visualized from unialgal cultures using light microscopy (LM) and epifluorescent microscopy
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52 495 (EFM) and its phytohormone composition has been included to characterize them. This technique
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54 496 has been recently performed by Pichler et al. (2020) with the lichen forming algae *Trebouxia* sp.,
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56 497 *Trebouxia decolorans* and *Asterochloris glomerata*. Under controlled experimental conditions, *T.*
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58 498 *cretacea*, *T. asymmetrica* and *Trebouxia* sp. '*arnoldoi*' showed characteristic profiles of endogenous
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60 499 content in phytohormones (Fig. 4). IAA levels found in *Trebouxia* sp. '*arnoldoi*' (Fig. 4a) are similar
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62 500 to those observed in *Trebouxia* sp., *T. decolorans* and *A. glomerata* grown in solid media (Pichler et
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64 501 al. 2020). ABA production has been also detected in in these three microalgae (Pichler et al. 2020)
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66 502 as well as in *Trebouxia* sp. TR9 (Hinojosa-Vidal et al. 2018) isolated from *Ramalina farinacea*
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68 503 (Pichler et al. 2020). The ABA levels observed in the *Trebouxia* strains tested in this study (Fig. 4b)
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70 504 are in the same range of magnitude as the levels in *Trebouxia* sp. TR9, *Trebouxia* sp. and *T.*

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505 *decolorans* (Hinojosa-Vidal et al. 2018; Pichler et al. 2020). In contrast, the endogenous bioactive
506 form of JA, JA-Ile (Fonseca et al. 2009), was found in all three *Trebouxia* strains from *B. zoharyi*
507 (Fig. 4d), while no detectable endogenous levels of JA have been found by Pichler et al. 2020.)In
508 addition, SA and Me-JA were also detected in the *Trebouxia* strains isolated in this study but they
509 were not previously described in lichen-forming algae, although Me-JA has been detected in
510 *Chorella* (Ueda et al. 1991).

511 In conclusion, the different phytohormone profiles obtained in the three *Trebouxia* strains isolated in
512 this study (Fig. 4f), and in the other lichen forming algae mentioned above, suggest that each
513 microalgae strain could present different internal signalling needs. On the other hand, it cannot be
514 ruled out that a part of the production of these phytohormones could also be secreted to the external
515 environment and play a role in external signalling mechanisms with the other members of the
516 thallus symbiotic system. Related to this, the extracellular release of IAA, ABA and JA in the
517 lichen-forming algae *Trebouxia* sp., *T. decolorans* and *Asterochloris glomerata*, has recently been
518 described (Pichler et al. 2020). The determination of the role of these phytohormones in the internal
519 signalling mechanisms of the photobionts, as well as in the coordination mechanisms with the rest
520 of the elements of the lichen symbiosis, is a question that needs future studies.

521 The worldwide distribution of lichen species was also hypothesized to be strongly correlated with
522 the ecological specialization and the physiological performances of the photobionts (Casano et al.
523 2011; Peksa and Škaloud 2011). In lichens, different degrees of mycobiont-photobiont specificity is
524 known (Leavitt et al. 2015; Beck et al. 2019; Steinová et al. 2019) and in some cases these
525 relationships have been correlated to the suitability and the physiological performance of the
526 photobionts in diverse, or even changeable, environmental settings (Vančurová et al. 2015; Steinová
527 et al. 2019).

528 The lichen *Ramalina farinacea* (L.) Ach. has proved to be a suitable reference and model species
529 for studying microalgal diversity, as it recurrently shows the co-occurrence of at least two
530 photobionts (*Trebouxia* sp. TR9 and *T. jamesii*) inside the thalli using microscopy techniques,
531 culture isolations and molecular characterization with different genetic markers (del Campo et al.
532 2010b, 2013). Moreover, the predominant photobiont differs between different populations; *T.*
533 *jamesii* being the most abundant in the Iberian Peninsula and *Trebouxia*. sp. TR9 in the Canary
534 Islands. Several studies have further demonstrated that these two photobionts respond differently to
535 abiotic stresses (Casano et al. 2011). Casano et al. (2011) also analysed the effects of temperature
536 and light on the growth and the photosynthetic traits of isolated photobionts from *R. farinacea*
537 (*Trebouxia* sp. TR9 and *T. jamesii*). They found that both species grew better at the lowest
538 temperature and performed the experiments only in cultures grown at 17° C. On the contrary, we did

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4 539 not find such differences in the growth temperature, and thus, we performed the experiments at both
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6 540 17° C (mean annual temperature in Fuerteventura and Lanzarote) and 20° C (mean annual
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8 541 temperature in Tenerife) to study the photosynthesis response to different temperatures (20° C and
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10 542 17° C).

11 543 The maximum quantum yield of primary photochemistry in dark-adapted leaves (F_v/F_m) in healthy
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13 544 plants reach F_v/F_m values around 0.840, but for lichens this parameter ranges between 0.550 and
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15 545 0.700 (Casano et al. 2011; Gasulla et al. 2019). In this study F_v/F_m values ranged between 0.51-
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17 546 0.66. The decline in F_v/F_m might be a result of an increase in non-photochemical processes in the
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19 547 light harvesting antennae of PSII associated with a photochemical quenching down-regulation,
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21 548 photodamage of PSII reaction centres, or both (Osmond et al. 1993). In this study, we observed that
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23 549 *T. asymmetrica* did not modify their F_v/F_m values with respect to temperature acclimation. In
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25 550 contrast, *Trebouxia* sp. *arnoldoi* and *T. cretacea* were more sensitive to changes in growing
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27 551 temperature, showing a significant decline in F_v/F_m when grown at 17° C with respect to 20° C. In
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29 552 addition, these species showed the lowest ETR, mainly from 400 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ to the end of
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31 553 light curve. The electron transport rate was saturated at approximately the same irradiance (700
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33 554 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) in all *Trebouxia* species (except *T. cretacea*) under both temperatures; similar
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35 555 results were obtained in *Trebouxia* sp. TR9 and *T. jamesii* (Casano et al. 2011). A decrease in ETR
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37 556 and an increase in 1-qP indicate a reduction in the quantum yield of PSII and a reduction state of the
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39 557 first electron acceptor of PSII, QA, respectively. In this circumstance, non-photochemical processes
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41 558 (NPQ) must be increased to guarantee excitation energy dissipation (Havaux et al. 1991) such as
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43 559 occurred in *Trebouxia* sp. *arnoldoi* where higher NPQ and 1-qP, and the lowest ETR, was
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45 560 observed. NPQ plays an important role in plants against excess radiation. In these algae strains from
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47 561 *Buellia zoharyi*, NPQ increases in all of them reducing the excitation pressure on reaction centres,
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49 562 thereby decreasing the possibility of photodamage (Papageorgiou and Govindjee 2014). These
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51 563 different behaviours between algae strains grown under two temperatures have been observed by
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53 564 Casano et al. (2011) in *Trebouxia* sp. TR9 and *T. jamesii* where *Trebouxia* sp. TR9 performed better
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55 565 under high temperatures, indicating a different capacity to adaptation.

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57 566 Our results indicate that *B. zoharyi* is flexible regarding the photobiont choice depending on the
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59 567 region, and suggest that bioclimatic factors could influence the myco/photobiont association
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61 568 patterns due to the different photosynthesis response to different temperatures (20° C and 17° C).

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860 **Fig. 1** *Trebouxia* phylogenetic analysis. Rooted ITS1-5.8S-ITS2 + LSU rDNA gene tree
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862 UTEX, *Trebouxia* sp. TR9 and *Trebouxia crespoana* from ASUV retrieved from the GenBank.
863 Newly generated sequences are marked as ex. *Buellia zoharyi*_locality_haplotype_code_frequency.
864 Three *Trebouxia* species detected were indicated. Values at nodes indicate statistical support
865 estimated by two methods: bootstrap support (BS, RAxML analysis) and posterior probabilities (PP,
866 MrBayes analysis). Scale bar shows the estimated number of substitutions per site.

867 **Fig. 2 a, b** Cross-section of *B. zoharyi* thalli from Lanzarote by TEM. **a** Photobionts of *B. zoharyi*
868 inside thallus, **b** Detail of pyrenoid. **c, d** Cross-section of *B. zoharyi* thallus from Fuerteventura by
869 TEM. **c** Photobionts of *B. zoharyi* inside thallus, **d** Detail of pyrenoid. **e, f** Cross-section of *B.*
870 *zoharyi* thalli from Tenerife by TEM. **e** Photobionts of *B. zoharyi* inside thallus, **f** Detail of
871 pyrenoid. Abbreviations: Py, Pyrenoid; Pg, Pyrenoglobuli; Chl, Chloroplast; PV, Peripheral vesicles.
872 Bars 500 nm, 800 nm, 1 μ m and 2 μ m.

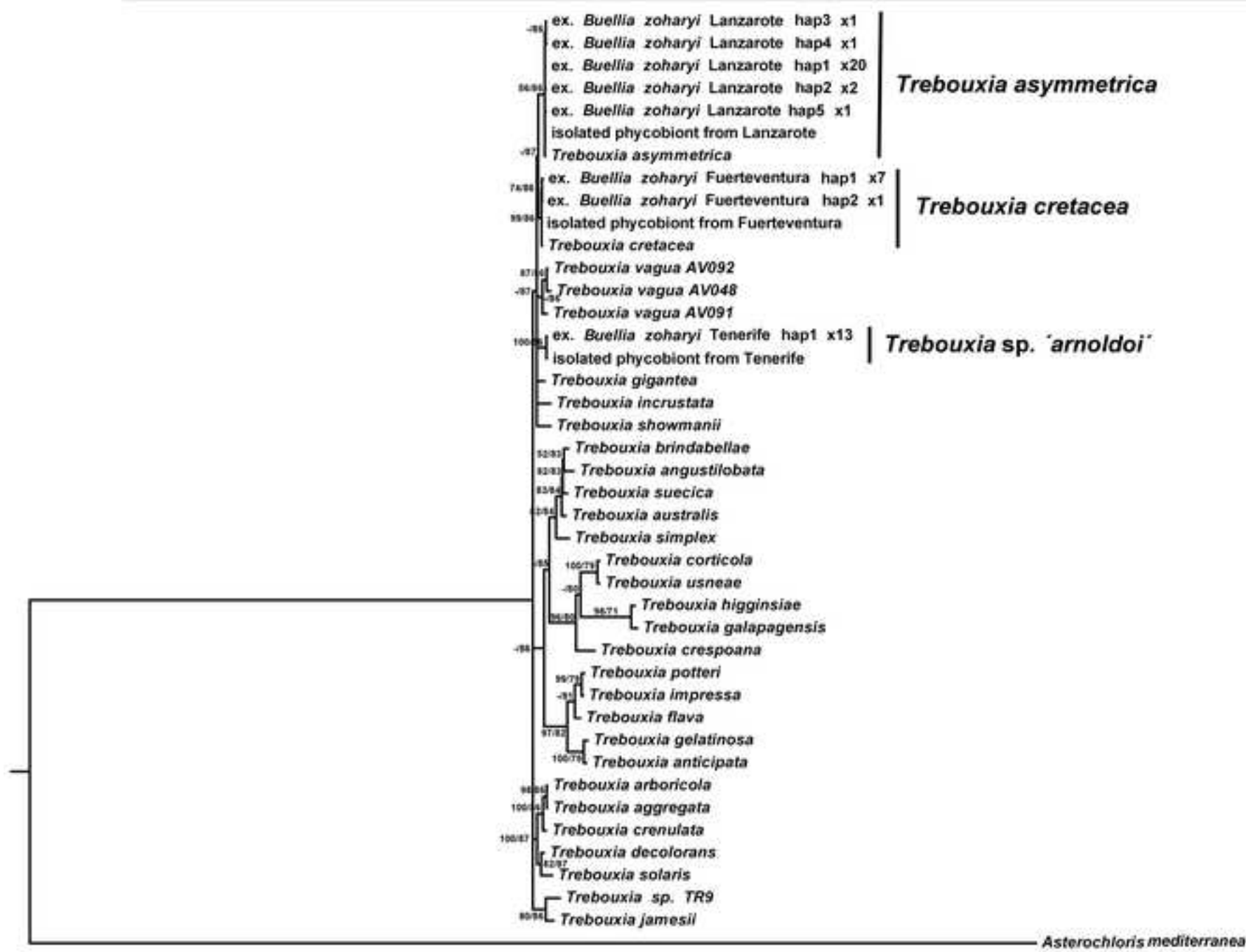
873 **Fig. 3** Algal multiplicity detected by PCR depicted in the localities included in this study: Tenerife,
874 Lanzarote and Fuerteventura. Inner, outer circle and numbers represent the primary and secondary

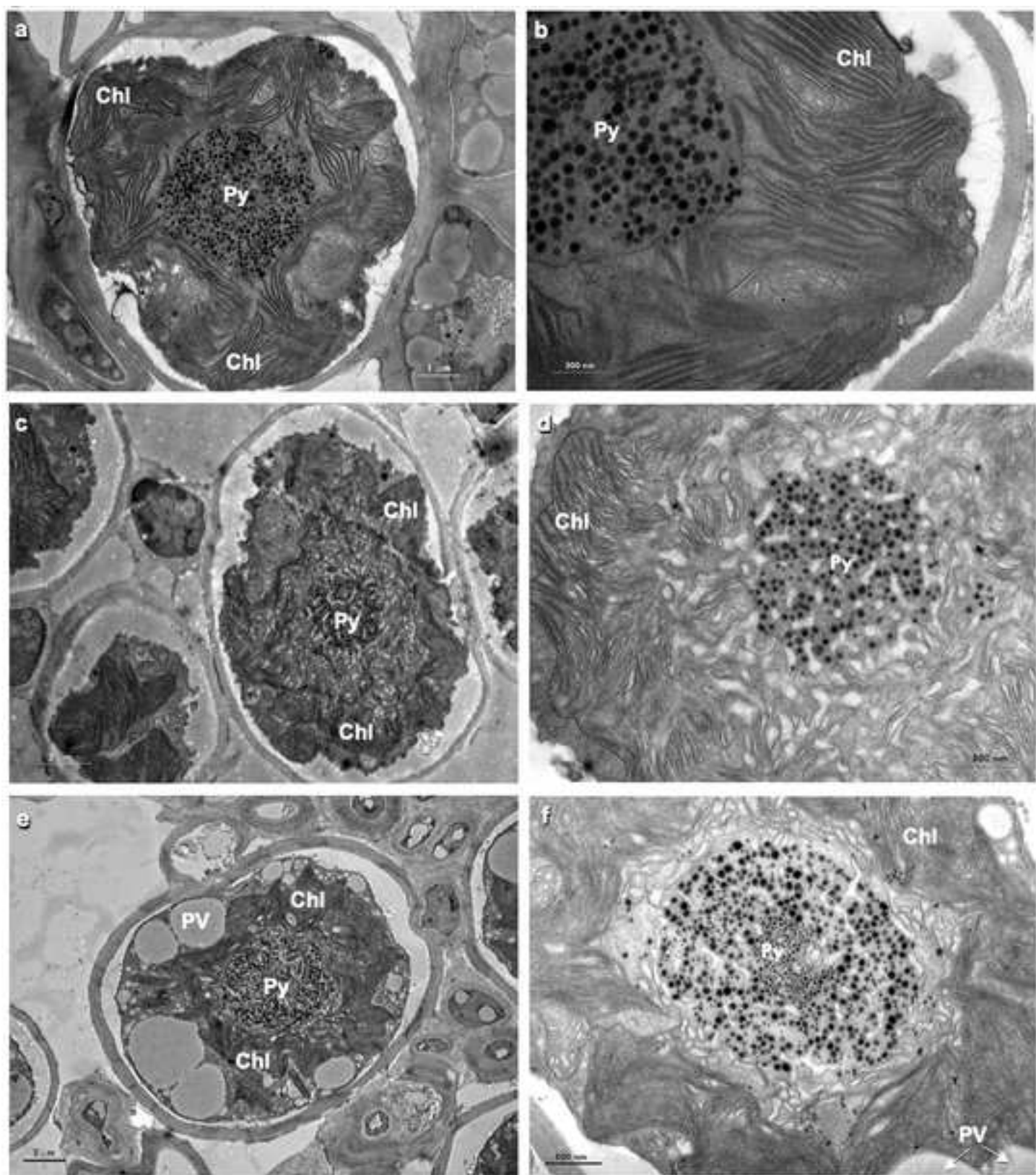
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4 875 photobiont detected, respectively. The colour coding for each *Trebouxia* is shown on the bottom
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6 876 left-hand side of the figure.

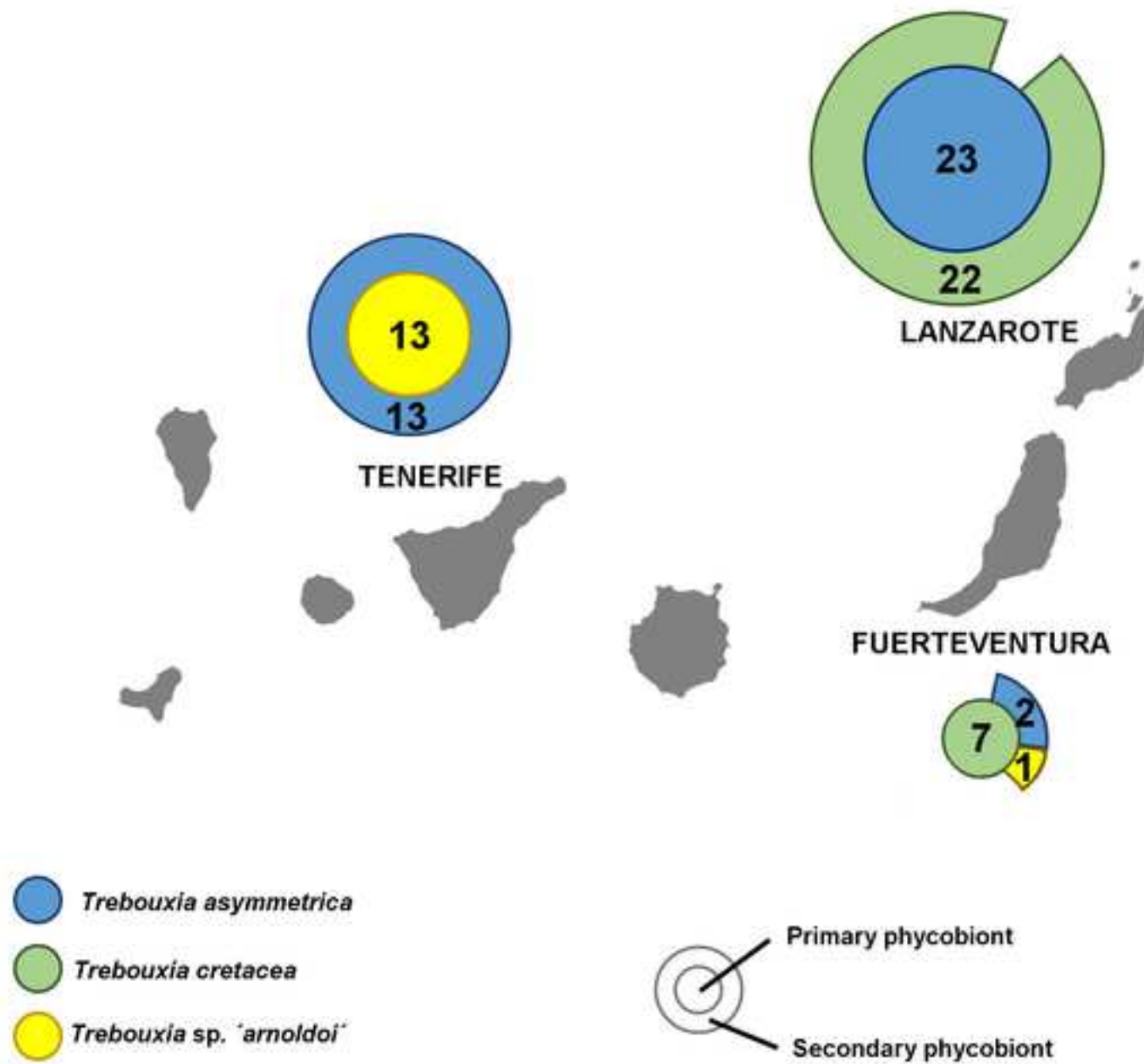
7
8 877 **Fig. 4** Phytohormone profiles of axenic cultures of the different *Trebouxia* strains isolated from
9
10 878 *Buellia zoharyi*. Microalgae were grown in BBM medium for 21 days at 20° C. Three cultures were
11 879 sampled for each condition and phytohormone levels were quantified: **a** indole-3-acetic acid (IAA);
12
13 880 **b** abscisic acid (ABA); **c** salicylic acid (SA); **d** jasmonic acid conjugated to isoleucine (JA-Ile) and
14
15 881 **e** methyl jasmonate (Me-JA). Graphs show the mean ± standard deviation. Significant differences
16 882 between strains are indicated with letters (ANOVA, Tukey HSD test, $p < 0.05$). **f** Heatmap analysis
17
18 883 of the phytohormone content of each *Trebouxia* strain. Scale bar (Z-score) indicates the relative
19
20 884 abundance of a particular phytohormone in each strain.

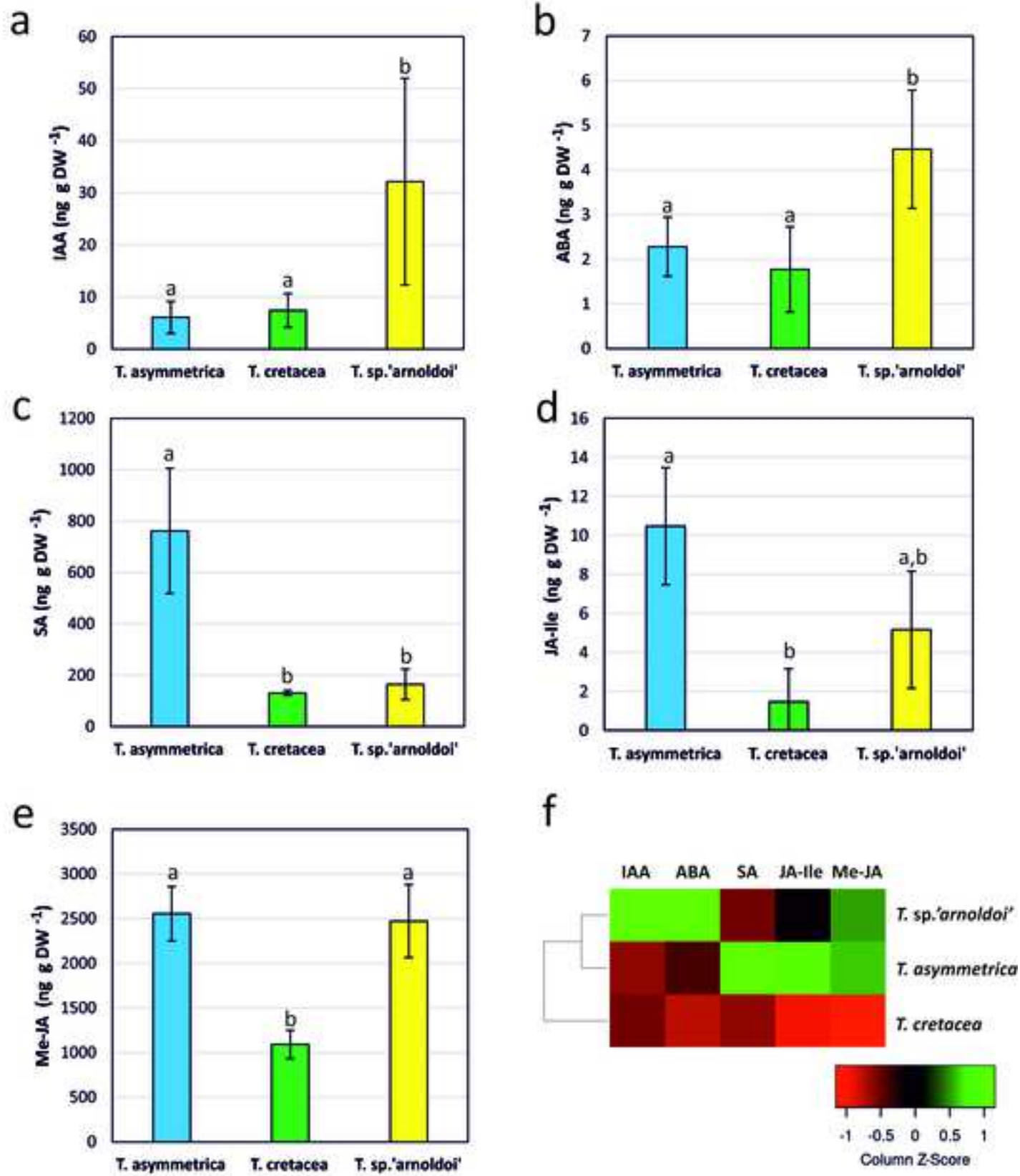
21
22 885 **Fig. 5 a** Maximum fluorescence yield (F_v/F_m) of the different *Trebouxia* strains isolated from
23
24 886 *Buellia zoharyi* growing in BBM medium at 17° C and 20° C. Bars represent means ± SE, $n = 4$.
25 887 Significant differences between treatments and species are indicated with letters (ANOVA, Tukey
26
27 888 HSD test, $p < 0.05$). **b** Chlorophyll fluorescence images for F_v/F_m of the different *Trebouxia* strains
28
29 889 isolated from *Buellia zoharyi* growing in BBM medium at 17° C and 20° C. The color scale bar
30 890 shown at the bottom of the figures stands for values from 0 (black) to 1 (pink).

31
32 891 **Fig. 6 a** PPF response curves of the non-photochemical quenching (NPQ), **b** relative electron
33
34 892 transport rate (ETR), and **c** PSII photooxidative pressure (1-qP) in the different *Trebouxia* strains
35
36 893 isolated from *Buellia zoharyi* growing in BBM medium at 17° C and 20° C. Bars represent means ±
37
38 894 SE, $n = 4$.

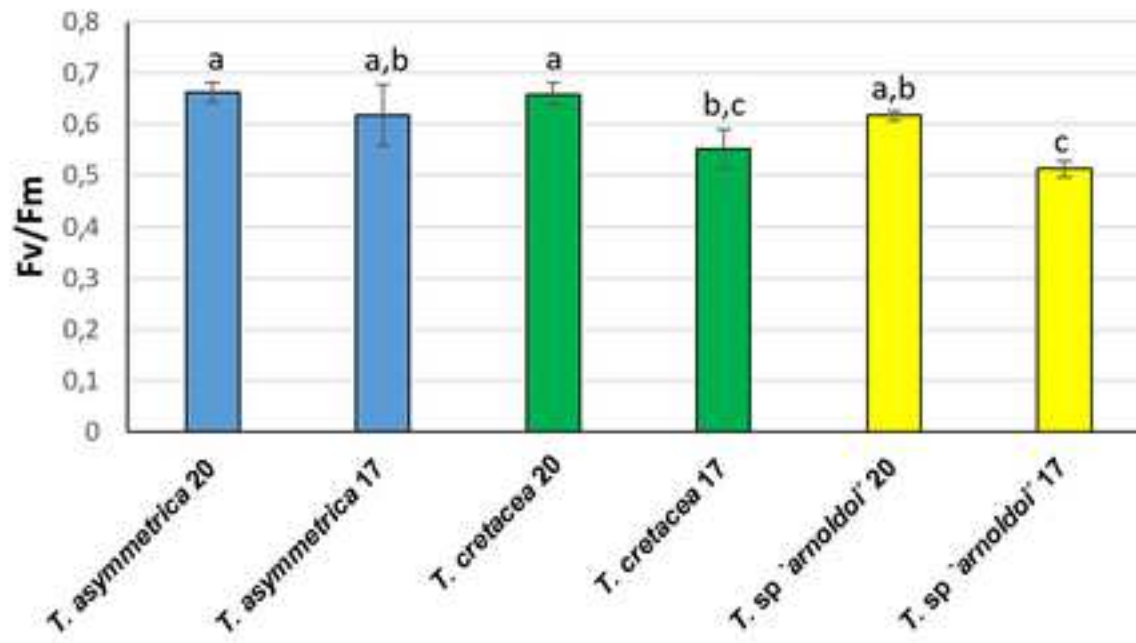




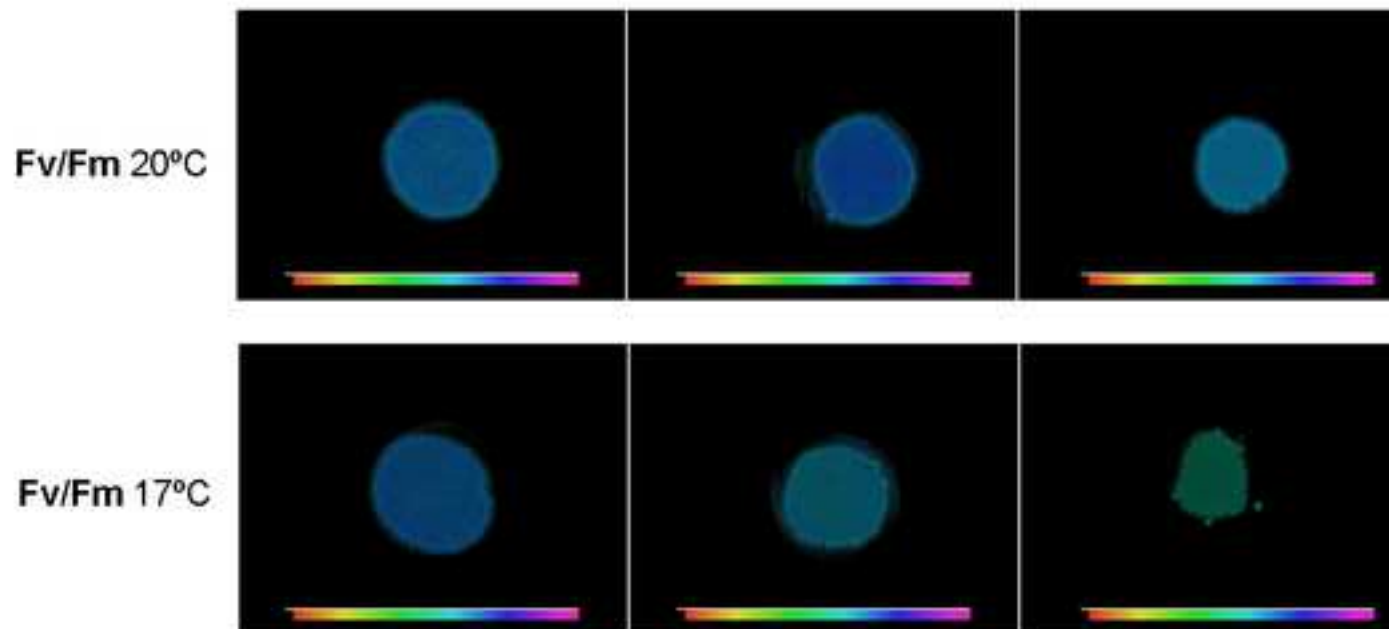


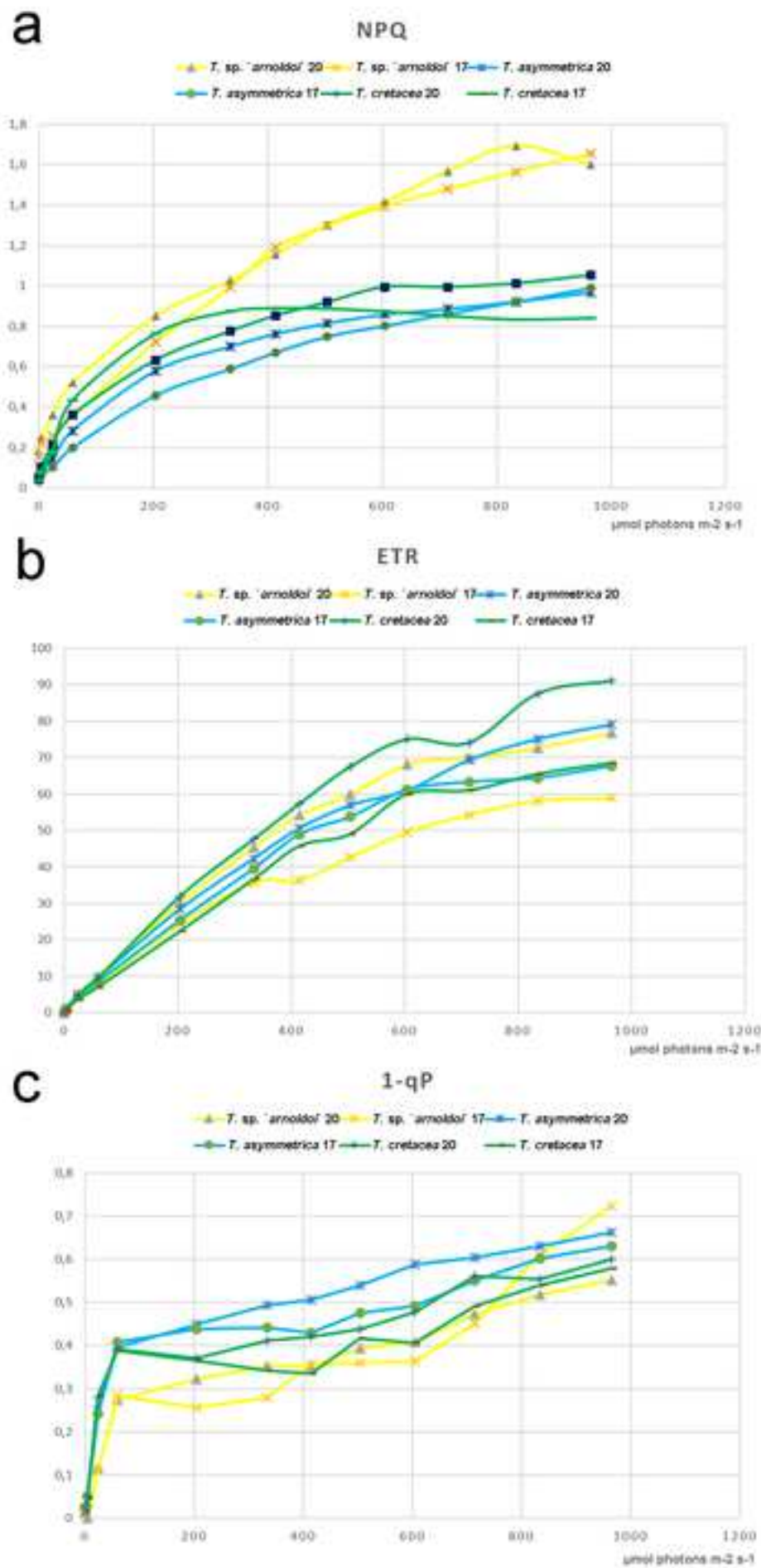


a



b








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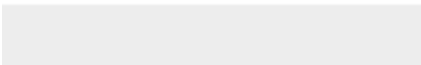

		Primary phycobiont
Locality	Sample code	LSU rDNA
Lanzarote, Los Valles	LA 1	T. asymmetrica
Lanzarote, Los Valles	LA 2	T. asymmetrica
Lanzarote, Los Valles	LA 3	T. asymmetrica
Lanzarote, Los Valles	LA 4	T. asymmetrica
Lanzarote, Los Valles	LA 5	T. asymmetrica
Lanzarote, Los Valles	LA 6	T. asymmetrica
Lanzarote, Los Valles	LA 7	T. asymmetrica
Lanzarote, Los Valles	LA 8	T. asymmetrica
Lanzarote, Los Valles	LA 9	T. asymmetrica
Lanzarote, Los Valles	LA 10	T. asymmetrica
Lanzarote, Los Valles	LA 11	T. asymmetrica
Lanzarote, Los Valles	LA 12	T. asymmetrica
Lanzarote, Los Valles	LA 13	T. asymmetrica
Lanzarote, Los Valles	LA 14	T. asymmetrica
Lanzarote, Los Valles	LA 15	T. asymmetrica
Lanzarote, Los Valles	LA 16	T. asymmetrica
Lanzarote, Los Valles	LA 17	T. asymmetrica
Lanzarote, Los Valles	LA 18	T. asymmetrica
Lanzarote, Los Valles	LA 19	T. asymmetrica
Lanzarote, Los Valles	LA 20	T. asymmetrica
Lanzarote, Haria, Peñas del Chache	LA 21	T. asymmetrica
Lanzarote, Haria, Peñas del Chache	LA 22	T. asymmetrica
Lanzarote, Haria, Peñas del Chache	LA 23	T. asymmetrica
Lanzarote, Haria, Peñas del Chache	LA 24	T. asymmetrica
Lanzarote, Haria, Peñas del Chache	LA 25	T. asymmetrica
Tenerife, Iguete de San Andrés	TE 1	T. sp. `arnoldoi´
Tenerife, Iguete de San Andrés	TE 2	T. sp. `arnoldoi´
Tenerife, Iguete de San Andrés	TE 3	T. sp. `arnoldoi´
Tenerife, Iguete de San Andrés	TE 4	T. sp. `arnoldoi´
Tenerife, Iguete de San Andrés	TE 5	T. sp. `arnoldoi´
Tenerife, Iguete de San Andrés	TE 6	T. sp. `arnoldoi´
Tenerife, Iguete de San Andrés	TE 7	T. sp. `arnoldoi´
Tenerife, Iguete de San Andrés	TE 8	T. sp. `arnoldoi´
Tenerife, Iguete de San Andrés	TE 9	T. sp. `arnoldoi´
Tenerife, Iguete de San Andrés	TE 10	T. sp. `arnoldoi´
Tenerife, Iguete de San Andrés	TE 11	T. sp. `arnoldoi´
Tenerife, Iguete de San Andrés	TE 12	T. sp. `arnoldoi´
Tenerife, Iguete de San Andrés	TE 13	T. sp. `arnoldoi´
Fuerteventura, Betancuria	FU 1	T. cretacea
Fuerteventura, Betancuria	FU 2	T. cretacea
Fuerteventura, Betancuria	FU 3	T. cretacea
Fuerteventura, Betancuria	FU 4	T. cretacea
Fuerteventura, Betancuria	FU 5	T. cretacea
Fuerteventura, Betancuria	FU 6	T. cretacea
Fuerteventura, Betancuria	FU 7	T. cretacea

Fuerteventura, Betancuria	FU 8	T. cretacea
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T. cretacea	NOT ANALYZED (HERBARIUM)			
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Ref: Ms. No. SYMB-D-20-00159

Title: Temperature as a bioclimatic factor affecting association patterns in *Buellia zoharyi* from the Canary Islands

Note for the Editor: Dear David there was a mistake related to the author's order in the submission menu, the correct order is the one that appear in the doc. Sorry for this inconvenience

Authors: Arantzazu Molins¹, Salvador Chiva¹, Ángeles Calatayud², Francisco Marco³, Francisco García-Breijo^{4,5}, José Reig-Armiñana^{1,4}, Pedro Carrasco³, Patricia Moya¹

Editor comments

Please consider the comments and recommendations for revision by the Reviewers. The recommendations for changes, though in some cases substantial, are well worth making as I am sure the paper will be much improved as a consequence. If there are any recommendations you do not wish to comply with, please identify these and state your reasons in an E-mail with the attached electronic version of your revised manuscript.

Thank you very much for your recommendations. Substantial changes have been made in the manuscript. See def version and with CC attached

Reviewer Comments:

Reviewer 1

Line 93: check ever – present

Done. See line 92

Line 120: occur instead of occurs

Done. See line 116

Line 253 algal strain instead of algae strain

Done. See line 277

Reviewer 2

I would suggest that this be published without the additional physiological measurements. These need to be much more intensively and carefully studied before we can know if the observed patterns have anything to do with the ability of the strains to display differences in acclimation and adaptation to temperature.

Thank you very much for your recommendations. We have reorganized, reduced and re-written the complete manuscript. We decided not to exclude the physiological measurements but we have changed the approach of this part of the study, because as the referee mentioned additional measurement should be included to confirm the influence of the temperature in the photobiont adaptation. However, we consider that each *Buellia zoharyi* photobionts showed different Chlorophyll *a* fluorescence responses to different temperatures (20° C and 17° C) as according to the F_v/F_m measurements (Fig. 5a). As shown in this Fig. *Trebouxia* sp. 'arnoldoi' and *T. cretacea* were significantly affected by temperature, with the lowest F_v/F_m values at 17° C. However, *Trebouxia asymmetrica* showed similar values at 20° C and 17° C (0.66 ± 0.02 and 0.62 ± 0.05), respectively, without significant differences between them. Therefore, those differences are described in the manuscript.

I certainly don't believe that the data presented justify the title of the m/s: "Temperature as a bioclimatic factor affecting association patterns in Buellia zoharyi". I personally would doubt if temperature has much influence, but in any event, much more work is needed.

As the referee recommended we have changed the title: Multidisciplinary approach to describe *Trebouxia* diversity within lichenized fungi *Buellia zoharyi* from the Canary Islands

Briefly, my feeling is that the differences in temperature between the sites (17 and 20oC) are too small to have selected different photobionts. Studies that have focused on thermal adaptation have typically used populations of lichens growing in areas with much greater differences in temperature. For example, Sahu et al. (2019) measured NPQ etc., in Phaeophyscia hispidula and Flavoparmelia caperata growing along a large altitudinal gradient, with average temperatures between sites varying by more than 20oC. In the present study with Buellia, differences in photobiont composition would seem to be unlikely to be related to such small differences in temperature when other factors e.g. N supply may vary between sites.

As mentioned before, we agree with the referee that additional measurement should be included to confirm the influence of the temperature or other bioclimatic factors in the photobiont adaptation hypothesis.

We know, Sahu et al (2019) studied variations in microclimatic attributes and their effects on photosynthetic efficiency and analyzed four elevational ranging from 1950 to 3508 m. They detected that extreme variations in air temperature (5.75–31.65 °C) and other factors were imperative in controlling species richness, distribution and photosynthetic quenching of lichen flora in the region. However, in this work, *Buellia zoharyi* populations included in this study grown in areas with similar ecological conditions except for the temperature although other factors could be influence on their performance

Moreover Casano et al (2011), as mentioned in the manuscript (see lines 527-539). To search for physiological differences between the two *R. farinacea* phycobionts, studied the effects of temperature and light on the growth and photosynthetic traits of isolated and cultured *T. jamesii* and *Trebouxia* sp. TR9 algae. They showed the fresh weight reached by both microalgae after 30 days at 17°C or 20°C and 15–100 $\mu\text{molm}^{-2}\text{s}^{-1}$ of PAR. Both species grew better at the lower temperature; however, at 20°C the negative impact of the high temperature was less on TR9 than on TR1, whose growth was almost inhibited. In fact, since the low algal biomass at 20°C significantly increased the experimental error of fluorescence measurements, rendering non- reliable data, so the experiments in Casano et al (2011) were carried out only in cultures growing at 17°C. Therefore, the influence of only 3-4 °C of temperature in the *Trebouxia* spp. was previously reported by the mentioned authors.

On the contrary, we did not find such differences in the growth temperature, and thus, we performed the experiments at both 17° C (mean annual temperature in Fuerteventura and Lanzarote) and 20° C (mean annual temperature in Tenerife) to study the photosynthesis response to different temperatures (20° C and 17° C).

In the same way, I cannot accept that experiments that quantified the responses of the photobionts to the two temperatures can explain the observed differences in photobiont composition. Thus, while it is interesting that strains cultured at 17oC compared with 20oC display slightly lower Fv/Fm values, it is hard to really interpret this observation without more data. It certainly does not correlate simply with NPQ values.

See previous answer

From the data in Figure 5, it appears that algae cultured at 17°C have slightly higher ETR rates, which is not consistent with the Fv/Fm data. In general, I found the logic in the paragraphs discussing the fluorescence data muddled and difficult to follow, and furthermore, does not refer back to the figures. But ultimately, based on the data presented, photobiont composition cannot be related to the response of the photobionts to temperature.

We have rewritten this part of the document to clarify it

I am rather skeptical that individual spot measurements of phytohormone levels will tell us much about the stress tolerance / differences in the different photobionts. The work of Pichler et al. (2020) cited by the authors illustrates one possible approach. Here, first secretion of hormones was studied, and second changes in the levels of the hormones induced by light and desiccation. In theory, a study comparable to this with the different strains / geographical isolates could be valuable, but the data presented are not helpful. In conclusion therefore, my recommendation therefore is that the physiology sections are deleted and the m/s be re-submitted without them.

Thank you very much for your recommendations. Due to the scarce information related to the phytohormone content in microalgae available in the literature, we agree with the referee that a study comparable to Pichler et al. (2020) with different strains / geographical isolates exposed to several growth conditions (control and stress), would be valuable to understand photobiont adaptation, given the well known relationship between phytohormones and adaptation to environmental conditions. However, in the present study this analysis was included as a preliminary technique in order to complement the photobiont characterization. In this sense, we have been able to detect different phytohormone profiles for the three isolated photobionts, an observation that could suggest different signaling needs for each of them. We consider this data an interesting start point for further studies that could help to determine how phytohormone signaling could contribute to explain the local adapted photobiont hypothesis. In order to clarify our point of view to a future reader, we have reordered the information regarding phytohormone data in material and methods, results and discussion in the manuscript.

Minor Comments

In general replace phycobiont with photobiont

Done. See new manuscript

Line 99 Meaning of "outcompete" not clear

We have changed adapt and outcompete for thrive. See line 98

Line 123 Meaning of "communities" not clear - "populations"?

Done. See line 119

Line 126 something is missing before "population"

Done. See line 122

Line 127 "have" rather than "showed"

Rephrased. See lines 123-125

Line 128 a "biocrust" is not an ecological condition

Rephrased. See line 125

Line 139 "document" rather than "depict"

We have modified depict for describe. See line 136

Line 144 "They were characterized by means of molecular, microscopy and spectrometry." Please turn into a grammatical sentence.

Done. See line 141

Line 291 "grau"?

Grade. See line 247

Line 306 replace "with" with "with the"

Done. See line 262

Line 402 First paragraph of the Discussion should summarize the findings of the study, not the aims!!

Thank you for the suggestion. We have rewritten these paragraph. See lines 406-409

Line 420 better give area in square km

Sentence rewritten. See lines 418-419

Line 486 "resolutive", odd word, please re-phrase

Sentence deleted.

Line 538 "heterogeneities of Chl fluorescence" - is this data really used? If not, maybe delete reference to it.

We agree with the referee. Thank you for the recommendation. We have deleted this paragraph and reference

Line 554 "which could indicate sensitivity to photoinhibition" - rather an odd statement, given that the photobionts were cultured at 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$?

Sentence deleted

Line 937 Caption to Figure 5. "Two areas of interest (AOI) were selected for Chl fluorescence measurements, one in the central part and one in the outer zones in order to evaluate spatial heterogeneity. The values of chlorophyll fluorescence parameters were means of two AOI (internal and external); no significant differences were obtained between internal and external areas." This text seems to be in the wrong place?

Paragraph included in material and methods. See lines 299-303

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1 Journal: Symbiosis

2 Article type: Special Issue Festschrift Barreno

3 **Title:**

4 Multidisciplinary approach to describe *Trebouxia* diversity within lichenized fungi *Buellia*

5 *zoharyi* from the Canary Islands

6 ~~Temperature as a bioclimatic factor affecting association patterns in *Buellia zoharyi* from the~~

7 ~~Canary Islands~~

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31 Running title: *Trebouxia* species within *Buellia zoharyi* from the Canary Islands

32 **Abstract**

33 The Canary Islands are famous for their extraordinary biodiversity; however, lichenized algae have
34 only been studied partially. *Buellia zoharyi* is a circum-Mediterranean/Macaronesian species that
35 usually occurs in semi-arid areas of the Mediterranean, but occasionally some interesting
36 communities of this species grow on basaltic lava flows in Lanzarote, Fuerteventura and Tenerife.
37 Those three locations showed similar ecological conditions, but different mean annual temperatures.
38 Here we applied a multidisciplinary approach to describe microalgae diversity from *B. zoharyi*
39 covering the entire described range of distribution in the Canary Islands. ~~Phycobiont~~Photobionts
40 were characterized in symbiosis ~~by means of using~~ molecular and microscopic techniques. Different
41 *Trebouxia* spp. were detected as primary ~~phycobiont~~photobiont in each island (*Trebouxia cretacea*-
42 Fuerteventura, *T. asymmetrica*-Lanzarote and *Trebouxia* sp. `arnoldoi`-Tenerife). Coexistence of
43 various *Trebouxia* spp. within a thallus were detected by using specific primers-PCR. Those three
44 ~~phycobiont~~photobionts were isolated and cultured under laboratory conditions. ~~To relate the~~
45 ~~temperature with the presence of a specific *Trebouxia* species, Different phytohormone profiles~~
46 ~~were obtained in the isolated strains which suggest different internal signalling needs. In addition,~~
47 ~~we characterized their~~ response of the isolated strains to different temperatures ~~were monitored~~
48 using chlorophyll fluorescence. ~~*T. asymmetrica* did not modify their Fv/Fm values with respect to~~
49 ~~temperature acclimation. In contrast, *Trebouxia* sp. `arnoldoi` and *T. cretacea* were more sensitive to~~
50 ~~changes in growing temperature decreasing Fv/Fm at 17° C. Phytohormone profiles of isolated~~
51 ~~phycobionts were performed to delve deeper into the ecologically adapted phycobiont hypothesis.~~
52 Our results indicate that *B. zoharyi* is flexible regarding the ~~phycobiont~~photobiont choice;
53 depending on the region ~~or the environmental conditions~~, and ~~suggest clearly influenced by that~~
54 ~~bioclimatic factors could influence the myco/photobiont association patterns the temperature. The~~
55 ~~results point to habitat specific adaptations which lead to similar behaviour in phycobionts which~~
56 ~~are genetically different. The physiological adaptations of lichen phycobionts may substantially~~
57 ~~contribute to the stress resistance strategy and the colonization capacity.~~

58

59 Keywords: coexistence, isolation, microalgae, photosynthesis, symbiosis, ultrastructure

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61 **Declarations**

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Funding Funding for field and laboratory work for this study was provided by the Ministerio de Economía y Competitividad (MINECO and FEDER, Spain) (CGL2016-79158-P) and Prometeo Excellence in Research Program (Generalitat Valenciana, Spain) (PROMETEOII/2013/021; PROMETEO/2017/039). Daniel Sheerin revised the English manuscript.

Competing interests The authors declare no competing interests

Availability of data and material GenBank

Code availability LSU rDNA: MT458607-MT458609; nrITS MT458610-MT458618

Authors' contributions PM, AM and SC conceived the study and designed the laboratory part of the study. SC, PM and AM carried out laboratory work. FG-B and JR-A analyzed microscopic images. AC and PM performed photosynthesis measurements. FM and PC performed phytohormone quantifications. AM and PM analyzed all data and wrote the manuscript. All authors edited and approved the final version of the manuscript.

ACKNOWLEDGMENTS

Dr. Arnoldo Santos (~~Tenerife~~) was involved in the design of the surveys and the sample collection, also in the ecology and bioclimatic information of the locations. Daniel Sheerin revised the English manuscript. We dedicate this article to Eva Barreno in honour of her retirement.

Introduction

The Canarian Archipelago contains eight volcanically active islands proximal to the African continent and the High Atlas Mountains. The origin and magmatic evolution of these islands have been contentious issues for several decades and make this particular archipelago unique for biodiversity studies. The Canary Islands are famous for their extraordinary diversity in lichenized and lichenicolous fungi (Van den Boom and Etayo 2006; Hernández Padrón and Pérez Vargas 2010). The diversity of lichenized algae has only been studied partially for *Tephromela atra* (Muggia et al. 2010), *Ramalina farinacea* (Casano et al. 2011; del Campo et al. 2013; Moya et al. 2017), *Lecanora rupicola* (Blaha et al. 2006), *Parmotrema pseudotinctorum* (Molins et al. 2013; Škaloud et al. 2018), *Stereocaulon vesuvianum* (Vancurová et al. 2015), *Psora decipiens* (Moya et al. 2018) and *Cladonia* spp. (Moya et al. 2015).

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93 Lichen symbioses are currently considered as microecosystems in which multispecies assemblages
94 are hosted in the lichen thallus (holobiont), formed by the two major lichen symbionts, e.g. the
95 mycobiont (fungal partner) and the photobiont (a population of photosynthetic green or blue-green
96 algae). According to the most recent investigations, many other fungi (Muggia and Grube 2018;
97 Smith et al. 2020), non-photosynthetic bacteria (e.g. Aschenbrenner et al. 2014; Grube et al. 2015;
98 Cernava et al. 2017; Sierra et al. 2020) and photosynthetic green microalgae co-inhabit within the
99 lichen thalli, giving rise to the peculiar phenotypes, and which may contribute to the diverse
100 patterns of secondary metabolites (Spribille 2018) and environmental adaptation. In terms of lichen
101 ~~phycobiontphotobionts~~, intrathalline microalgal diversity, e.g. multiple ~~phycobiontphotobiont~~
102 species within a single lichen thallus, has previously been observed in a number of lichen symbioses
103 (Dal Grande et al. 2014; Muggia et al. 2013; Moya et al. 2017; Škaloud et al. 2018). In some cases,
104 algae with different physiological performances are ~~ever-~~ present in lichen thalli, potentially
105 facilitating the success of these lichens in a wide range of habitats and geographic areas and/or in
106 changing environmental conditions (Casano et al. 2011; del Hoyo et al. 2011).

107 Muggia et al. (2020) highlight the need for an integrative taxonomic approach, incorporating
108 morphological and physiological data from axenic cultures with genetic data, to establish a robust,
109 comprehensive taxonomy for *Trebouxia*. Complementation with ecophysiological studies needs to
110 be employed to explain how lichens ~~thrive adapt and outcompete~~ in extreme environments
111 (Sadowsky and Ott 2012). Lichen ~~phycobiontphotobionts~~ show a particular photosynthesis
112 performance and have evolved alternative photosynthetic-machinery protective mechanisms in
113 response to their special ecophysiology (Gasulla et al. 2019). The photosynthesis responses to
114 abiotic factors, like temperature and light conditions in axenic cultures of ~~phycobiontphotobionts~~,
115 reflects the ecophysiological plasticity of this symbiosis as a mechanism, allowing the lichen to
116 adapt to changing and often stressful environments (Casano et al. 2011). ~~The study of biomass-~~
117 ~~accumulation and photosynthetic traits, including the photochemical efficiency of photosystem II-~~
118 ~~(PSII), the reduction oxidative state of QA, and non-photochemical quenching (NPQ) denote which-~~
119 ~~holobiont has improved its ecological fitness (Casano et al. 2011).~~

121 Phytohormones are chemical messengers involved in several physiological, stress response and
122 biochemical processes of higher plants at very low concentrations. Phytohormone composition has
123 been characterized in only a few algae (Yokoya et al. 2010; Gupta et al. 2011; Wang et al. 2014), but
124 recently, Pichler et al. (2020) studied phytohormone composition in aeroterrestrial
125 *Trebouxiophyceae*, including three lichen-forming microalgae. The results obtained in this study
126 contribute to valuable baseline information for further studies into the roles of phytohormones in

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127 microalgae. Including this technique to characterize isolated symbiotic microalgae could help to
128 understand the ecologically adapted ~~phyeobiont~~photobionts hypothesis.

130 *Buellia zoharyi* is a circum-Mediterranean/Macaronesian species forming crustose placodioid
131 lichens that usually occurs in biocrusts in semi-arid areas of the Mediterranean region (Gutiérrez-
132 Carretero and Casares-Porcel 2011). Specifically, this species predominantly grows on gypsum soils
133 (Crespo and Barreno 1975; Barreno 1994; Trinkaus and Mayrhofer 2000), but occasionally some
134 interesting ~~population~~communities of this species grow on basaltic lava flows in the Canary
135 Islands (Etayo 2011; Giralt and Van den Boom 2011; Roux and Poumarat 2015). The presence of
136 this lichen species in the Canary Islands was reported in Lanzarote (Trinkaus and Mayrhofer 2000),
137 Fuerteventura (Van den Boom and Etayo 2006) and Tenerife (Chiva et al. 2019), and populations in
138 the other four Islands must be scarce or even non-existent. ~~¶~~These three locations ~~have~~showed
139 similar ecological conditions e. g. the same high irradiance (5.5 – 5.7 KWh/m²) moreover, in the
140 three locations *B. zorayi* grown forming similar ecological conditions, such as biocrusts in areas
141 with sparse vascular vegetation and high irradiance (5.5 – 5.7 KWh/m²). However, the WorldClim
142 database reported different mean annual temperatures (MAT) ~~in the three locations~~: 17° C in
143 Fuerteventura and Lanzarote, and 20° C in Tenerife (<https://www.worldclim.org>; Hijmans et al.
144 2005). Chiva et al. (2019) analyzed mycobiont diversity in *B. zoharyi* in the Mediterranean region
145 including those three Canary Island locations. They found low genetic diversity and two
146 geographically differentiated haplogroups: one including populations from the Iberian Peninsula to
147 Azerbaijan, and the other from the southern Iberian Peninsula, North Africa and the Canary Islands.
148 Complementary analyses of *B. zoharyi* concerning the range of associated ~~phyeobiont~~photobionts
149 and their ecophysiological traits will be necessary to identifying historical and ecological factors at
150 the basis of the evolutionary history of this group of soil- dwelling taxa.

152 Here we applied a multidisciplinary approach to ~~describe~~microalgae diversity from *B. zoharyi*
153 ~~growing settled~~ in the Canary Islands. Populations included in this study cover the entire described
154 range of distribution in the ~~Canary Islands archipelago~~. ~~Phyeobiont~~Photobionts were characterized
155 in symbiosis ~~by means of using~~ molecular and microscopic techniques. Coexistence of various
156 *Trebouxia* spp. were detected by using specific primers-PCR. Representative ~~phyeobiont~~photobionts
157 were isolated and cultured under laboratory conditions. ~~We -They were~~ characterized the isolated
158 strains by means of using -molecular, microscopy and spectrometry techniques. Their responses to
159 different temperatures were monitored using chlorophyll fluorescence to answer if this bioclimatic
160 factor could influence the myco/photobiont association patterns. answer if temperature drives the

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~~presence of a specific *Trebouxia* species in each location, and whether or not the complementary physiological behaviour of each algal species, globally improves the ecological fitness of the holobiont under global warming temperature conditions.~~

Material and methods

Sampling and DNA extraction

In this study, 46 thalli of *Buellia zoharyi* collected from Lanzarote, Tenerife and Fuerteventura were analyzed (Table 1S). We included fresh (N=43) and herbarium samples (N=3). Fresh specimens were air-dried for one day after sampling and then stored at -20°C. Lichen thalli were examined under a stereomicroscope to remove soil particles and were immersed sequentially in ethanol and NaOCl (Arnold et al. 2009) to remove surface contaminants and to ensure the intrathalline origin of the sequenced microalgae. Fragments from different parts of each thallus were randomly excised and pooled together. Total genomic DNA was isolated and purified using the DNeasy Plant Mini kit (Qiagen, Hilden, 121 Germany) following the manufacturer's instructions.

Primary ~~phyeobiont~~photobiont PCR amplification and Sanger sequencing

Two algal loci were amplified: a region of the chloroplast LSU rDNA gene using the algal specific primer pair 23SU1 and 23SU2 (del Campo et al. 2010a) and the nrITS (internal transcribed spacer) using the primer pair nr-SSU-1780 (Piercey-Normore and DePriest 2001) and ITS4 (White et al. 1990). PCR reactions were performed following Moya et al. (2018). The PCR products were visualized on 2% agarose gels and purified using the Gel Band Purification Kit (GE Healthcare Life Science, Buckinghamshire, England). The amplified PCR products were sequenced with ABI 3730XL using the BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California). Sanger sequences were visualized and manually evaluated with Chromas 2.6.6.0 (<http://technelysium.com.au/wp/chromas/>).

***Trebouxia* ~~phyeobiont~~photobiont phylogenetic analysis**

The nrITS and LSU rDNA datasets were collapsed using TCS 1.21 (Clement et al. 2002). The dataset included the newly determined nrITS and LSU rDNA sequences from thalli and isolated ~~phyeobiont~~photobiont (accession numbers LSU rDNA: MT458607-MT458609; nrITS: MT458610-MT458618), and a selection of 30 *Trebouxia* species sequences available from the Culture Collection of Algae at Goettingen University (SAG), from the Culture Collection of Algae at the University of Texas (UTEX) and from the Symbiotic Microalgal Collection of the University of

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194 Valencia (ASUV, *Trebouxia* sp. TR9 and *Trebouxia crespoana*). We included *Asterochloris*
195 *mediterranea* as an outgroup.
196 A multiple alignment was built using MAFFT 7.0 (Katoh and Standley 2013) using default
197 parameters. Aligned sequences were improved by eliminating the ambiguous regions using Gblocks
198 0.91b (Castresana 2000) with the least stringent parameters. This software allows conflicting
199 regions in the alignment to be automatically removed. Both loci were concatenated, yielding an
200 alignment of 1329 characters. The final matrix contained 42 ITS rDNA and 33 LSU rDNA
201 sequences.
202 For each locus, the most appropriate substitution model was estimated using the Akaike information
203 criterion (AIC) using JModelTest 2.1.4 (Darriba et al. 2012). The most appropriate nucleotide
204 substitution models for nrITS and LSU rDNA were GTR+I+G and GTR+G, respectively. The
205 phylogenetic trees of both loci were inferred by Bayesian inference (BI) and Maximum Likelihood
206 (ML) approaches carried out on partitioned datasets using the different substitution models selected
207 by JModelTest. ML analysis was implemented in RAxML 8 (Stamatakis 2014) using the
208 GTRGAMMA substitution model. Bootstrap support (BS) was calculated based on 1,000
209 pseudoreplicates (Stamatakis et al. 2008). BI was carried out in MrBAYES 3.2 (Ronquist et al.
210 2012). Settings included two parallel runs with six chains over 20 million generations, starting with
211 a random tree and sampling after every 200th step. We discarded the first 25% of the data as burn-in,
212 and the corresponding posterior probabilities (PPs) were calculated from the remaining trees.
213 Estimated sampled sized (EES) values above 200, and potential scale reduction factor (PSRF)
214 values approaching 1,000, were considered indicators of chain convergence. The phylogenetic tree
215 was visualized in FIGTREE 1.4.2 (Rambaut 2014; <http://tree.bio.ed.ac.uk/software/figtree/>). All
216 analyses were run at the CIPRES Science Gateway 3.3 webportal (Miller et al. 2010).

Microscopic examinations “in thallus”.

19 The ultrastructure of the ~~phyceobiont~~photobionts was characterized by transmission electron
20 microscopy (TEM) from selected thalli (LA7, LA22, TE1, TE10 and FU1). For TEM, the cells were
21 fixed and dehydrated as described in Molins et al. (2018a). Samples were embedded in Spurr’s resin
22 according to the manufacturer’s instructions. Sections (90 nm) were cut and mounted as described
23 in Moya et al. (2018). The sections were observed with a JEOL JEM-1010 (80 kV) electron
24 microscope, equipped with a MegaView III digital camera and ‘AnalySIS’ image acquisition
25 software. TEM examinations were carried out at the SCSIE Service of the University of Valencia.

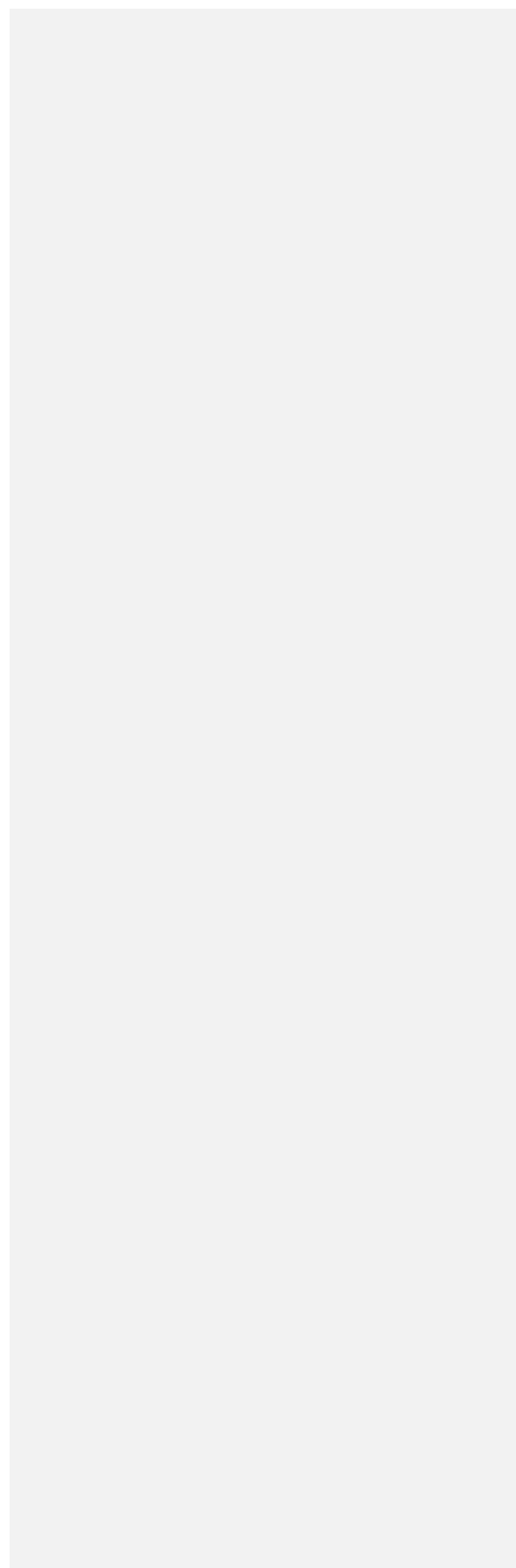
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227 **Secondary *Trebouxia* strains detected by specific PCR primers**

228 To detect the presence of secondary *Trebouxia* species in each thallus, PCR were performed using
229 as templates each PCR from the primary ~~phycobiont~~ photobiont (obtained with the primer pair nr-
230 SSU-1780 / ITS4) and re-amplifying with specific primer pairs (Table 2S). These specific forward
231 and reverse primers were designed in this study based on the nrITS sequences obtained with the
232 primer pair nr-SSU-1780 / ITS4 in *B. zoharyi* from the Canary Islands, which included *Trebouxia*
233 *asymmetrica*, *Trebouxia cretacea* and *Trebouxia* sp. '*arnoldoi*'.

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235 **Isolation, PCR identification and propagation of microalgae strains.**

236 **Phyco**~~biont~~**Photobiont**s were isolated from selected thalli (LA7, TE1 and FU1) using two protocols:
237 a) the micromethod described by Gasulla et al. (2010), where the resulting algal suspension was
238 diluted with sterile water and spread using the streak method on sterile 1.5% agar Bold's Basal
239 Media Petri dishes (BBM) (Bold 1949; Bischoff and Bold 1963); and b), the method described in
240 Muggia et al. (2014) where tiny clumps of the algal layer were inoculated directly into BBM. The
241 isolated algae were maintained under a 50 $\mu\text{mol}/\text{m}^2\text{s}^{-1}$ photosynthetic photon flux density (PPFD)
242 with a 12 h photoperiod at 20°C. Subsequent subcultures were performed until we obtained a
243 unialgal culture and fast PCR microalgae identification was performed directly from the colonies as
244 described in Molins et al. (2018b).

245
246 **Microscopic investigations of phyco**~~biont~~**photobiont**s “in culture”.

247 Light microscopy (LM) and Epifluorescent Microscopy (EFM) was performed on selected unialgal
248 cultures on the 21st day of cultivation at 20° C. All LM and EFM observations were carried out with
249 an Olympus Provis AX 70 fluorescence microscope equipped with an Infinity 2–3 C Lumenera®
250 digital camera and analyzed with “Infinity Analyze” Software. For EFM, an Olympus U-ULS 100
251 HG epifluorescence system with U-MWBV (excitation filter 400–440 nm, dichroic mirror 455 nm,
252 barrier filter 475 nm) cubes was used.

253
254 **Phytohormone content determination**

255 Endogenous phytohormone levels of 21-day-old microalgae cultures grown at 20° C were
256 determined according to the protocol of Durgbanshi et al. (2005). Lyophilized microalgae samples
257 were processed by extraction in 5 ml of distilled water after fortifying them with internal standards:
258 [²H₆] - abscisic acid (ABA) (100 ng, prepared as described in Gómez-Cadenas et al. (2002)),
259 dihydrojasmonic acid (JA) (100 ng, synthesized in the laboratory by catalytic hydrogenation
260 according to Kristl et al. (2005)), [²H₂]-indole-3-acetic acid (IAA) (10 ng, Sigma-Aldrich) and [²H₄]
261 -salicylic acid (SA) (100 ng, Sigma-Aldrich). The extracts were centrifuged at 4000 x g, at 4° C for
262 45 min. Subsequently, the supernatant was collected in clean tubes and the pH adjusted to 3.0 using
263 a 30% (v / v) acetic acid solution. The acidified extracts were partitioned twice with 3 ml of ethyl
264 ether (ACS grade, Scharlau, Barcelona, Spain). The upper organic phase was recovered in a clean
265 vial, combining both partitions and drying them under vacuum using an evaporation centrifuge
266 coupled to a cold trap (RC 10.22 and RT 10.90, Jouan, Saint-Herblain Cedex, France). The dried
267 residue was resuspended by adding 100 μL of methanol (HPLC grade, Scharlau) in the test tube by
268 ultrasound for 10 min. Subsequently, the final volume of 1 mL was completed with pure water

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269 (MiliQ). The resulting solution was filtered using regenerated cellulose filters with a pore diameter
270 of 0.2 µm before analysis. Phytohormone analysis was performed using an HPLC device (Alliance
271 2860, Waters Corp., Milford, USA) coupled to a tandem mass spectrometer with an electrospray
272 interface (Quattro LC, Micromass, Manchester, UK). The samples were injected and separated by a
273 reverse phase column (Kromasil 100, C18, 5 µm, 100 × 2.0 mm, Scharlau) using a linear gradient of
274 methanol and ultrapure water, supplemented with acetic acid to a final concentration of 0.01% (v/v)
275 and a flow of 0.3 mL/min. Discrimination and detection of each analyte was carried out following
276 the fragmentation pattern and the characteristic retention time. The ionization and collision
277 conditions for each compound were optimized by direct infusion of pure standards (approximately 5
278 mg/L). The quantification of the analytes of interest was performed using the response factor
279 (analytical area/area) by interpolation in a calibration curve injected alternatively in the samples.
280 Chromatogram processing, integration and quantification were performed using MassLynx 4.0
281 software. The profiles of the relative content of phytohormones between strains were determined
282 with the Heatmapper web tool (Babicki et al. 2016).

284 **Measurement of chlorophyll fluorescence imaging from isolated algae**

285 Fast PCR was performed directly from the unialgal cultures as described in Molins et al. (2018b) to
286 ensure the purity and identity of the selected aliquot used for chlorophyll (Chl) fluorescence images
287 (CFI) measurements.

288 A 50 ml aliquot of these actively growing algae, resuspended in liquid BBM medium, was
289 inoculated on cellulose–acetate disks placed on agarized BBM medium and then cultured for 21
290 days under two conditions, 17°C and 20°C with a 50 µmol m⁻²s⁻¹ PPFD under a 12 h/12 h light/dark
291 cycle.

292 CFI was performed using an imaging-PAM fluorometer (Walz, Effeltrich, Germany), in order to
293 investigate the behavior of Chl fluorescence parameters in the three algae at different light
294 intensities and grown under different temperature conditions (17°C and 20°C). Algae samples were
295 layered on filter paper that was kept moist with distilled water in order to maintain the cells in a
296 fully hydrated state. The algae membranes were darkened for 30 min prior to measurement. Chl *a*
297 fluorescence determinations were obtained from n= 4 samples for each algae strain and
298 temperature. ~~Two areas of interest (AOI) were selected for Chl fluorescence measurements, one in~~
299 ~~the central part and the other in the outer zones in order to evaluate spatial heterogeneity.~~ The
300 minimum (dark) fluorescence (F_o) was obtained by applying measuring light pulses at a low
301 frequency (1 Hz). The maximum fluorescence (F_m) was determined by applying a saturating blue

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302 pulse (10 Hz). The maximum quantum yield of PSII photochemistry (Roháček 2002), often called
303 the F_v/F_m ratio, was determined as $F_m - F_o/F_m$. Then, a light curve with actinic illumination from 0
304 to $964 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ was switched on, and saturating pulses were applied for 20 s in order to
305 determine the maximum fluorescence yield (F'_m) intensity, and the steady-state fluorescence value
306 that is immediately prior to the saturating pulse is F_s . Calculation of quenching due to non-
307 photochemical dissipation of absorbed light energy (NPQ) was determined at each saturating pulse,
308 according to the equation $\text{NPQ} = (F'_m - F'_s)/F'_m$ (Bilger and Björkman 1991). The actual quantum
309 efficiency of PSII photochemistry, Φ_{PSII} , was calculated according to Genty et al. (1989) by the
310 formula: $(F'_m - F_s)/F'_m$, and the coefficient of photochemical quenching (Van Kooten and Snel
311 1990) was determined as $qP = (F'_m - F_s)/(F'_m - F'_o)$. Excitation pressure on PSII (QA), which reflects
312 the proportion of the primary quinone electron acceptor of PSII that is in the reduced state, was
313 calculated as $1 - qP$ (Demmig-Adams et al. 1996). The relative electron transport rate (ETR) was
314 calculated as $\Phi_{\text{PSII}} \times \text{PAR} \times 0.84 \times 0.5$ (Schreiber et al. 1986). To determine F'_o correctly, it would
315 be necessary to switch off the actinic light and quickly reoxidise the PSII acceptor side with the help
316 of far-red light, but this is not feasible with imaging-PAM as far-red light would penetrate the CCD-
317 detector and cause serious disturbances to fluorescence images (see <http://www.walz.com>). The
318 value of F'_o was estimated using the approximation of Oxborough and Baker (1997), $F'_o = F_o/(F_v/F_m$
319 $+ F_o/F'_m)$. For each interval, saturation pulse images and values of various Chl fluorescence
320 parameters were captured (Calatayud et al. 2006).

Two AOI were selected one in the central part and other in the outer algae zones in order to evaluate spatial heterogeneity CFI parameters between both AOI. After comparing both AOI for each algae strain and temperature, not differences were observed for any Chl fluorescence parameters (data not shown). Then, the values of Chl fluorescence parameters displayed in the figures (4a and 5) are means of both AOI.

Phytohormone content determination

~~Endogenous phytohormone levels of 21 day old microalgae cultures grown at 20° C were determined according to the protocol of Durgbanshi et al. (2005). Lyophilized microalgae samples were processed by extraction in 5 ml of distilled water after fortifying them with internal standards: [2H6] abscisic acid (ABA) (100 ng, prepared as described in Gómez-Cadenas et al. (2002)), dihydrojasmonic acid (JA) (100 ng, synthesized in the laboratory by catalytic hydrogenation according to Kristl et al. (2005)), [2H2] indole 3 acetic acid (IAA) (10 ng, Sigma Aldrich) and [2H4] salicylic acid (SA) (100 ng, Sigma Aldrich). The extracts were centrifuged at 4000 x g, at 4° C for 45 min. Subsequently, the supernatant was collected in clean tubes and the pH adjusted to 3.0-~~

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336 using a 30% (v/v) acetic acid solution. The acidified extracts were partitioned twice with 3 ml of
337 ethyl ether (ACS grade, Scharlau, Barcelona, Spain). The upper organic phase was recovered in a
338 clean vial, combining both partitions and drying them under vacuum using an evaporation-
339 centrifuge coupled to a cold trap (RC 10.22 and RT 10.90, Jouan, Saint Herblain Cedex, France).
340 The dried residue was resuspended by adding 100 µL of methanol (grau HPLC, Scharlau) in the test
341 tube by ultrasound for 10 min. Subsequently, the final volume of 1 mL was completed with pure
342 water (MiliQ). The resulting solution was filtered using regenerated cellulose filters with a pore
343 diameter of 0.2 µm before analysis. Phytohormone analysis was performed using an HPLC device
344 (Alliance 2860, Waters Corp., Milford, USA) coupled to a tandem mass spectrometer with an
345 electrospray interface (Quattro LC, Micromass, Manchester, UK). The samples were injected and
346 separated by a reverse phase column (Kromasil 100, C18, 5 µm, 100 × 2.0 mm, Scharlau) using a
347 linear gradient of methanol and ultrapure water, supplemented with acetic acid to a final
348 concentration of 0.01% (v/v) and a flow of 0.3 mL/min. Discrimination and detection of each
349 analyte was carried out following the fragmentation pattern and the characteristic retention time.
350 The ionization and collision conditions for each compound were optimized by direct infusion of
351 pure standards (approximately 5 mg/L). The quantification of the analytes of interest was performed
352 using the response factor (analytical area/area) by interpolation in a calibration curve injected
353 alternatively in the samples. Chromatogram processing, integration and quantification were
354 performed using MassLynx 4.0 software. The profiles of the relative content of phytohormones
355 between strains were determined with Heatmapper web tool (Babicki et al. 2016).

357 **Statistical analyses**

358 Data was analysed by one-way Analysis of Variance (ANOVA) followed by post hoc comparisons
359 by Tukey's HSD test. A probability value < 0.05 was considered statistically significant.
360 Calculations were performed using R Software.

361 **Statistical analyses**

362 Data was analysed by one-way Analysis of Variance (ANOVA) followed by post-hoc comparisons
363 by Tukey's HSD test. A probability value < 0.05 was considered statistically significant.
364 Calculations were performed using R Software.

366 **Results**

367 **Primary *Trebouxia* diversity detected by Sanger sequencing (nrITS)**

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368 The concatenated aligned algal nrITS + LSU rDNA fragment was 1329 bp in length. BI and ML
369 phylogenetic hypotheses were topologically congruent. In the 46 samples, we detected three
370 *Trebouxia* species (Fig. 1). According to the clade code introduced for *Trebouxia* by Leavitt et al.
371 (2015), and subsequently applied by Moya et al. (2017) and Muggia et al. (2020), these *Trebouxia*
372 species belong to clade ‘A’ *arboricola/gigantea* type, precisely: *Trebouxia asymmetrica* in thalli
373 from Lanzarote, *Trebouxia cretacea* in Fuerteventura, and *Trebouxia* sp. ‘*arnoldoi*’ in Tenerife.

374 **Ultrastructural characterization of *Trebouxia***

375 Transmission electron microscopy (TEM) analyses of ~~phyceobiont~~photobionts based on the
376 ultrastructure of pyrenoids (Py) and plastids (Chl) distinguished at least three different *Trebouxia*
377 morphotypes. Morphological characteristics of each morphotype, in detail, can be seen in Fig. 2 and
378 Fig. 1S.

379 One morphotype was found in *Buellia zoharyi* cells from Lanzarote (Fig. 2a; b). They showed a
380 single central Py related to the *gigantea* type described by Friedl (1989) with pyrenoglobuli (Pg)
381 uniformly distributed within the Py matrix. Lobulated-stellate chloroplast with abundant lax
382 thylakoid membranes forming stacks of 4-5 membranes (grana).

383 The second morphotype was found in *B. zoharyi* cells from Fuerteventura (Fig. 2c, d). These cells
384 presented a single central Py related to the *impressa/gigantea* type described by Friedl (1989), with
385 a highly lobulated chloroplast with abundant dense thylakoid membranes forming stacks of 2-3
386 membranes.

387 The third morphotype was detected in *B. zoharyi* cells from Tenerife (Fig. 2e, f). They presented a
388 single central Py related to the *impressa/gigantea* type described by Friedl (1989). The chloroplast
389 morphology was similar to the second morphotype (lobulated with dense abundant thylakoid
390 membranes forming stacks of 2-3 membranes). Large peripheral vesicles surrounding the
391 chloroplast were present.

392
393 ***Trebouxia* multiplicity revealed by specific PCR primers**

394 Herbarium samples were excluded from this analysis. Specific forward and reverse primers were
395 designed in this study based on the nrITS sequences obtained with the primer pair nr-SSU-1780 /
396 ITS4 in *B. zoharyi* from the Canary Islands, which included *Trebouxia asymmetrica*, *Trebouxia*
397 *cretacea* and *Trebouxia* sp. ‘*arnoldoi*’

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398 All the samples from Lanzarote (n=23) showed *T. asymmetrica* as the primary
399 ~~phyeobiontphotobiont~~, and specific PCR detected *T. cretacea* in 22 thalli. *Trebouxia* sp. `arnoldoi`
400 was not detected (Fig. 3; Table 3S). Samples from Fuerteventura (n=7) showed *T. cretacea* as the
401 primary ~~phyeobiontphotobiont~~. The presence of secondary algae was detected only in three thalli:
402 two of them revealed *T. asymmetrica* as the secondary ~~phyeobiontphotobiont~~ and one *Trebouxia* sp.
403 `arnoldoi` (Fig. 3; Table 3S). Samples from Tenerife (n=13) had *Trebouxia* sp. `arnoldoi` as the
404 primary ~~phyeobiontphotobiont~~. All the samples only showed *T. asymmetrica* as the secondary
405 ~~phyeobiontphotobiont~~. *T. cretacea* was not detected (Fig. 3; Table 3S).

407 **Identification and morphological characterization of isolated *Trebouxia* spp. from *Buellia*** 408 ***zoharyi***

409 To corroborate the purity and identity of the unialgal selected aliquot, fast PCR was performed
410 directly from the cultures as described in Molins et al. (2018b). Sequences obtained were included
411 in the phylogeny indicated as isolated ~~phyeobiontphotobiont~~ (Fig. 1). We detected three *Trebouxia*
412 spp.: *T. asymmetrica*, *T. cretacea* and *Trebouxia* sp. `arnoldoi`.

413 All three *Trebouxia* spp. presented mature vegetative cells, mostly unicellular and spherical as seen
414 using LM and EFM (Fig. 2S). Tetrads and octads were only observed in *T. cretacea*. The spherical
415 vegetative cells were 14–16 (19) µm in diameter. All cells showed the characteristic *Trebouxia*
416 central chloroplast dissected into lobes (Fig. 2S).

418 **Phytohormone profiles of *Buellia zoharyi* photobionts**

419 Phytohormone endogenous content was also determined to characterize *Trebouxia* strains cultured
420 in BMM at 20° C for 21 days. Mass spectrometry analysis showed detectable levels of IAA, ABA,
421 JA conjugated to the amino acid Isoleucine (JA-Ile) and methyl jasmonate (Me-JA) (Fig. 4). The
422 *Trebouxia* strains isolated from *B. zoharyi* showed differences in their phytohormone profiles:

- 423 1) *T. asymmetrica* and *T. cretacea* presented similar levels of IAA and ABA (Fig. 4a, b),
424 *Trebouxia* sp. `arnoldoi` showed a different profile, with about double the ABA endogenous
425 content compared to the other strains (Fig. 4b), and higher levels of IAA (about five times)
426 than *T. asymmetrica* and *T. cretacea* (Fig. 4a).
- 427 2) SA and JA-Ile levels also showed a different profile between microalgae strains, with
428 similar levels for *T. cretacea* and *Trebouxia* sp. `arnoldoi`, while *T. asymmetrica* showed

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the highest level of SA (6 times greater; Fig. 4c) and higher levels of JA-Ile than *T. cretacea* (about 400% more; Fig. 4d).

3) Me-JA content of the *Trebouxia* strains showed another different profile, with similar levels for *T. asymmetrica* and *Trebouxia* sp. '*arnoldoi*', which were about 2.6 times higher than the levels observed for *T. cretacea* (Fig. 4d).

Chlorophyll *a* fluorescence response to different temperatures (20° C and 17° C) and phytohormone profiles of *Buellia zoharyi* photobionts.

According to the F_v/F_m measurements (Fig. 54a), *Trebouxia* sp. '*arnoldoi*' was significantly affected by temperature, with the lowest F_v/F_m values at 17° C. Similarly, in *T. cretacea*, F_v/F_m showed a significant decrease from 0.65 ± 0.02 to 0.55 ± 0.03 with a lower value at 17° C. However, *Trebouxia asymmetrica* showed similar values at 20° C and 17° C (0.66 ± 0.02 and 0.62 ± 0.05), respectively, without significant differences between them). In *T. cretacea*, F_v/F_m showed a significant decrease from 0.65 ± 0.02 to 0.55 ± 0.03 with a lower value at 17° C. The highest F_v/F_m values were obtained for *T. asymmetrica* and *T. cretacea* at 20° C.

The mean of images pixels from CFI for F_v/F_m inside the two areas did not display significant differences between internal and external areas (data not show). The observation of color changes, ranging from black (0.000) to pink (1.000), revealed that the images for F_v/F_m showed a uniform color for algae discs, and that different intensities of blue colors associated with higher values of F_v/F_m (Fig. 4b) for *T. asymmetrica* and *T. cretacea* at 20° C, and with the lowest F_v/F_m values (green) for *Trebouxia* sp. '*arnoldoi*' at 17° C (Fig. 54b).

All NPQ values increased with respect to light intensity, but with a different shape and magnitude were observed depending on the *Trebouxia* species and growth temperature (Fig. 65a); however, in the case of *Trebouxia* sp. '*arnoldoi*' and *T. asymmetrica* NPQ exhibited similar values for both temperature growth regimes showing the same kinetic shape, but *Trebouxia* sp. '*arnoldoi*' showed the highest NPQ values the NPQ value increased steeply at higher light intensities at both temperatures showing the same kinetic shape. *T. asymmetrica* NPQ exhibited similar values for both temperature growth regimes. For *T. cretacea* at 20° C and *T. asymmetrica* displayed similar NPQ values and at the end of light-curve kinetics *T. cretacea* showed the highest NPQ values.

T. cretacea, the highest NPQ values at the end of light curve kinetic were for the algae grown at 20° C. Nevertheless, *T. asymmetrica* NPQ exhibited similar values for both temperature growth regimes.

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461 Regarding ETR (Fig. 65b), their values were slightly higher for *Trebouxia* strains growing at 20 °C
462 compared with 17° C. Among species, *T. cretacea* displayed the highest ETR values, mainly at the
463 end of light curve kinetic at 20° C and the minimum values were observed in *Trebouxia* sp.
464 'arnoldoi' at 17° C.

465
466 Photosystem II excitation pressure, expressed as 1-qP (photochemical quenching; Fig. 65c),
467 increased gradually with light intensity in the three algal strains, with In general, *T. asymmetrica* at
468 20° C and *T. cretacea* showed slightly higher similar values, however *Trebouxia* sp.
469 'arnoldoi' showed slightly values except at high irradiance where compared to *Trebouxia* sp.
470 'arnoldoi' at 20° C. *Trebouxia* sp. 'arnoldoi' at 17° C showed the highest values at high irradiance.

471 Phytohormone endogenous content was also determined for *Trebouxia* strains cultured in BMM at
472 20° C for 21 days. Mass spectrometry analysis showed detectable levels of IAA, ABA, JA
473 conjugated to the amino acid Isoleucine (JA-Ile) and methyl jasmonate (Me JA) (Fig. 6). The
474 *Trebouxia* strains isolated from *B. zoharyi* showed some differences in their phytohormone profiles.
475 While *T. asymmetrica* and *T. cretacea* presented similar levels of IAA and ABA (Fig. 6a, b),
476 *Trebouxia* sp. 'arnoldoi' showed a different profile, with about double the ABA endogenous content
477 compared to the other strains (Fig. 6b), and higher levels of IAA (about five times) than *T.*
478 *asymmetrica* and *T. cretacea* (Fig. 6a). SA and JA-Ile levels showed a different profile between
479 microalgae strains, with similar levels for *T. cretacea* and *Trebouxia* sp. 'arnoldoi', while *T.*
480 *asymmetrica* showed the highest level of SA (6 times greater; Fig. 3c) and higher levels of JA-Ile
481 than *T. cretacea* (about 400% more; Fig. 6d). Finally, Me JA content of the *Trebouxia* strains showed
482 another different profile, with similar levels for *T. asymmetrica* and *Trebouxia* sp. 'arnoldoi', which
483 were about 2.6 times higher than the levels observed for *T. cretacea* (Fig. 6d).

484
485 **Discussion**

486
487 This study applied a multidisciplinary approach to describe microalgae diversity from *B.*
488 *zoharyi* growing in the Canary Islands. Our results indicate that *B. zoharyi* is flexible regarding the
489 photobiont choice depending on the region, and suggest that bioclimatic factors could influence the
490 myco/photobiont association patterns. Phycobionts were characterized in symbiosis by means of
491 molecular and microscopic techniques. Representative phycobionts were isolated and cultured, and
492 were characterized by different procedures. Their response to different temperatures were monitored

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493 ~~using chlorophyll fluorescence to discover if temperature drives the presence of a specific~~
494 ~~Trebouxia species in each location, and if the complementary physiological behaviour of each algal~~
495 ~~species explains how lichens adapt and outcompete in different environments.~~
496 ~~▲~~
497 According to Beck et al. (2002), ‘selectivity’ in lichens refers to the taxonomic range of partners
498 that are selected by one of the bionts, while ‘specificity’ should be used for the symbiotic
499 association, and depends on the range and taxonomic relatedness of acceptable partners. Lichens
500 with high selectivity may associate with a limited number of ~~phycobiontphotobionts~~. Numerous
501 ~~lichen-forming fungi~~mycobionts have been shown to associate with identical species of *Trebouxia*,
502 while others exhibited higher ~~phycobiontphotobiont~~ flexibility (Kroken and Taylor 2000; Ohmura et
503 al. 2006, 2018; Doering and Piercey-Normore 2009; Leavitt et al. 2013, 2015; Lindgren et al.
504 2014). Our results indicate that *B. zoharyi* are flexible in their ~~phycobiontphotobiont~~ choice, as they
505 associate with three *Trebouxia* species in 300 km (distance from Igueste de San Andrés; Tenerife to
506 Los Valles; Lanzarote) in a delimited geographic area (approx. 1000 km).
507 ~~PhycobiontPhotobiont~~ diversity can be shaped by the reproductive and dispersal strategies of the
508 mycobiont (Cao et al. 2015, Steinová et al. 2019), geography (Muggia et al. 2014, Werth and Sork
509 2014, Leavitt et al. 2015), growth substrate (Bačkor et al. 2010, Leavitt et al. 2013, Muggia et al.
510 2014) and macroclimate (Lu et al. 2018, Singh et al. 2018). ~~An important mechanism controlling~~
511 ~~symbiotic associations may be the symbiont's reproductive mode.~~ Lichens that reproduce sexually
512 via independent dispersal of fungal spores, undergo a process of re-lichenisation. This means that
513 the germinating spore of the mycobiont can easily exchange its autotrophic partner, in contrast to
514 asexually reproducing lichens distributing both partners together, which allows the continuation of
515 the symbiosis without the need to re-associate with another biont (Beck et al. 1998, 2002; Romeike
516 et al. 2002; Sanders and Lücking 2002). Lichens that depend on the cyclical establishment of
517 fungal ~~phycobiontphotobiont~~ associations to colonize varied wide-ranging habitats might require a
518 relatively higher flexibility in the specificity and ecological selection of their
519 ~~phycobiontphotobionts~~. This flexibility would facilitate successful re-lichenizations by allowing for
520 alternative partnerships in each habitat (Romeike et al. 2002). ~~However, some exceptions to this~~
521 ~~rule have been described; even asexually reproducing lichens, such as the *Lepraria* species, have~~
522 ~~been shown to switch their algal partners (Nelsen and Gargas 2008). In populations of *Physconia*~~
523 ~~*grisea* with a vegetative propagation strategy, mycobionts associate with more than one phycobiont~~
524 ~~genotype (Wornik and Grube 2010). It was also reported that both sexual and vegetative~~
525 ~~reproduction allows lichens to generate almost the same amount of diversity to adapt to their~~
526 ~~environments (Cao et al. 2015). Moreover, *Protoparmeliopsis muralis*, which does not produce~~

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vegetative propagules, exhibited a low selectivity level (Guzow-Krzemińska 2006; Muggia et al. 2013).

The dispersal of *B. zoharyi* over medium to long distances can be accomplished by either meiotic ascospores produced in fertile thalli, or thallus fragments detached from fertile and sterile ones (Barreno 1994; Casares and Llimona 1983). Both types of reproductive strategies are suitable for long- distance dispersal, even across the Mediterranean Sea, based on evidence of other lichens showing widely disjunct populations (Alors et al. 2017; Fernández- Mendoza and Printzen 2013; Garrido- Benavent et al. 2018). Moreover, increased connectivity among the Canary Islands, Africa and the Iberian Peninsula possibly occurred during Pleistocene glaciations, when the distance between the Eastern most island (Fuerteventura) and the Moroccan coast was reduced to 60 km (Fernández- Palacios and Whittaker 2008). Chiva et al. (2016) showed *T. cretacea* as the predominant phycobiont/photobiont in 117 thalli of *B. zoharyi* from the Iberian Peninsula and Morocco. The colonization of the Canary Islands by *B. zoharyi* must have originated from the Moroccan coast to Fuerteventura (20.7 Ma), currently the eastern island, only 100 km from the African coast, evidenced as the maintenance of the symbiont pattern (*T. cretacea*). Carracedo (1994), dated the origin of Lanzarote in 15.5 Ma and Tenerife in 11.6 Ma. *B. zoharyi* must have colonized the Canary Islands subsequently and adopted ecologically adapted phycobiont/photobionts (*T. asymmetrica* and *Trebouxia* sp. `arnoldoi`) in those Islands.

The diversity of phycobiont/photobionts has only recently been explored by environmental DNA metabarcoding approaches, and has focused on species within the Mediterranean basin to date (Moya et al. 2017; Dal Grande et al. 2018; Smith et al. 2019). In contrast to high-throughput sequencing approaches, traditional and largely applied DNA barcoding using Sanger sequencing was able to detect only the principal phycobiont/photobiont in the thalli (Paul et al. 2018; Moya et al. 2020). In this study, the authors determined if the second most abundant microalga exceeded 30% of the total HTS reads in a sample, Sanger sequencing generally failed and generated ambiguous Sanger sequences showing double peaks. Moreover, in the present study no samples with double peaks were found in the electropherogram, the co-occurrence of multiple *Trebouxia* inside a thallus was performed by using specific primers. This approach may limit the detection of further associated algae due to specificity and thus, it should be used as a complement to traditional Sanger sequencing when it is not possible to perform HTS approaches. However, all analyses performed using the multi-copy nrITS showed methodological limitations that potentially bias the results presented, due to the variation in the copy numbers across microalgae species. Therefore, the relative abundance of algal groups inferred in this study with specific primers does not accurately describe the true relative abundance of lichen-associated algae, given the potential for a very

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561 wide range of nrDNA copy numbers of these algal groups. Even with these limitations, specific
562 PCR primers revealed the presence of other secondary ~~phyceobiont~~photobionts which would be
563 available in the substrate. The presence of multiple algal species, and the different dominance of
564 one of them in each species, implies the selection for a particular algal species by the mycobiont
565 (Peksa and Škaloud 2011; Dupont et al. 2016). To corroborate this hypothesis, a complementary
566 HTS approach, both from the lichen and from the substrate, should be included.

567 A reliable definition of ~~phyceobiont~~photobiont species requires the description of morpho-anatomical
568 traits both axenically cultured and in symbiosis. However, such traits are absent for the majority of
569 lineages described in molecular phylogenetic analyses (Muggia et al. 2020). In this study,
570 ~~phyceobiont~~photobionts from Lanzarote, Fuerteventura and Tenerife were characterized in *Buellia*
571 *zoharyi* thalli using transmission electron microscopy (TEM). ~~Phyceobionts were also visualized~~
572 ~~from unialgal cultures using light microscopy (LM) and epifluorescent microscopy (EFM). To~~
573 ~~delimit algal species, LM and EFM techniques were less resolutive than TEM. In this study, The~~
574 ~~three morphotypes were characterized structurally by means of transmission electron microscopy,~~
575 ~~corresponding~~ to each *Trebouxia* species molecularly described for each Island. These three
576 *Trebouxia* species belong to clade 'A' *arboricola/gigantea* (Fig. 1) and showed, by TEM, a similar
577 pyrenoid type (Fig. 2 and Fig. 1S), corresponding to the *impressa/gigantea* type described by Friedl
578 (1989). Although Lanzarote's morphotype showed a high similarity to the *gigantea* type, this trait
579 does not allow us to differentiate each morphotype. In contrast to the pyrenoid, the chloroplast
580 thylakoid arrangements clearly differentiated the three morphotypes. This coincides with further
581 studies (Molins et al. 2018a) and evidences that the thylakoid arrangement is a key complement to
582 the pyrenoid structure to characterize *Trebouxia* species. Current knowledge concerning *Trebouxia*
583 phylogenetic relationships highlights the pressing needs to revise the original classification of the
584 group proposed by Friedl (1989) as that classification does not match with Muggia et al.'s (2020)
585 clade delimitation. The combination of molecular analyses together with ultrastructural techniques
586 should be initiated to clarify taxonomic concepts to delimit new taxa of microalgae, and particularly
587 in the case of *Trebouxia* diversity (Muggia et al. 2016, 2020; Moya et al. 2017; Molins et al. 2018b).
588 Here we detected the new *Trebouxia* sp. '*arnoldoi*' which did not match any previously described
589 *Trebouxia* spp. This provisional name has been proposed until it can be formally described:

590 ~~"arnoldoi"~~ *arnoldoi* refers to the Canarian botanist Arnoldo Santos.

591 ~~Incorporating morphological and ultrastructural traits from axenic cultures of candidate species~~
592 ~~circumscribed using molecular sequence data will be fundamental to facilitate a robust taxonomy~~
593 ~~within an integrative framework (Škaloud et al. 2018). Thus, isolation procedures should be~~

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594 included in ~~phycobiont~~ photobiont diversity studies, ~~to properly concatenate molecular markers and~~
595 to perform ~~morphological and physiological studies~~ analyses. In this study, the *Trebouxia* strains
596 isolated from *B. zoharyi* were visualized from unialgal cultures using light microscopy (LM) and
597 epifluorescent microscopy (EFM) and its phytohormone composition has been included to
598 characterize them. ~~This technique has been recently performed by Pichler et al. (2020) with the~~
599 ~~lichen forming algae~~ *Trebouxia* sp., *Trebouxia decolorans* and *Asterochloris glomerata*. Under
600 controlled experimental conditions, *T. cretacea*, *T. asymmetrica* and *Trebouxia* sp. '*arnoldoi*'
601 showed characteristic profiles of endogenous content in phytohormones (Fig. 4). IAA levels found
602 in *Trebouxia* sp. '*arnoldoi*' (Fig. 4a) are similar to those observed in *Trebouxia* sp., *T. decolorans* and
603 *A. glomerata* grown in solid media (Pichler et al. 2020). ABA production has been also detected in
604 in these three microalgae (Pichler et al. 2020) as well as in *Trebouxia* sp. TR9 (Hinojosa-Vidal et al.
605 2018) isolated from *Ramalina farinacea* (Pichler et al. 2020). The ABA levels observed in the
606 *Trebouxia* strains tested in this study (Fig. 4b) are in the same range of magnitude as the levels in
607 *Trebouxia* sp. TR9, *Trebouxia* sp. and *T. decolorans* (Hinojosa-Vidal et al. 2018; Pichler et al.
608 2020). In contrast, the endogenous bioactive form of JA, JA-IIe (Fonseca et al. 2009), was found in
609 all three *Trebouxia* strains from *B. zoharyi* (Fig. 4d), while no detectable endogenous levels of JA
610 have been found by Pichler et al. 2020. In addition, SA and Me-JA were also detected in the
611 *Trebouxia* strains isolated in this study but they were not previously described in lichen-forming
612 algae, although Me-JA has been detected in *Chorella* (Ueda et al. 1991).
613 In conclusion, the different phytohormone profiles obtained in the three *Trebouxia* strains isolated in
614 this study (Fig. 4f), and in the other lichen forming algae mentioned above, suggest that each
615 microalgae strain could present different internal signalling needs. On the other hand, it cannot be
616 ruled out that a part of the production of these phytohormones could also be secreted to the external
617 environment and play a role in external signalling mechanisms with the other members of the
618 thallus symbiotic system. Related to this, the extracellular release of IAA, ABA and JA in the
619 lichen-forming algae *Trebouxia* sp., *T. decolorans* and *Asterochloris glomerata*, has recently been
620 described (Pichler et al. 2020). The determination of the role of these phytohormones in the internal
621 signalling mechanisms of the photobionts, as well as in the coordination mechanisms with the rest
622 of the elements of the lichen symbiosis, is a question that needs future studies.

623
624 In this study, further isolation, cultivation and physiological studies of three different *Trebouxia*
625 species, should shed light on the ecological plasticity of the entire holobiont. The worldwide
626 distribution of lichen species was also hypothesized to be strongly correlated with the ecological
627 specialization and the physiological performances of the ~~phycobiont~~ photobionts (Casano et al.

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628 2011; Peksa and Škaloud 2011). In lichens, different degrees of mycobiont-~~phyceobiont~~photobiont
629 specificity is known (Leavitt et al. 2015; Beck et al. 2019; Steinová et al. 2019) and in some cases
630 these relationships have been correlated to the suitability and the physiological performance of the
631 ~~phyceobiont~~photobionts in diverse, or even changeable, environmental settings (Vančurová et al.
632 2015; Steinová et al. 2019).

633
634 The lichen *Ramalina farinacea* (L.) Ach. has proved to be a suitable reference and model species
635 for studying microalgal diversity, as it recurrently shows the co-occurrence of at least two
636 ~~phyceobiont~~photobionts (*Trebouxia* sp. TR9 and *T. jamesii*) inside the thalli using microscopy
637 techniques, culture isolations and molecular characterization with different genetic markers (del
638 Campo et al. 2010b, 2013). Moreover, the predominant ~~phyceobiont~~photobiont differs between
639 different populations; *T. jamesii* being the most abundant in the Iberian Peninsula and *Trebouxia*.
640 sp. TR9 in the Canary Islands. Several studies have further demonstrated that these two
641 ~~phyceobiont~~photobionts respond differently to abiotic stresses (Casano et al. 2011). ~~The variety of~~
642 ~~ecological contexts in which *R. farinacea* proliferates, reflects the ecophysiological plasticity of this~~
643 ~~symbiosis as a mechanism allowing the lichen to cope and thus to adapt to changing and often~~
644 ~~stressful environments. (Casano et al. 2011; del Hoyo et al. 2011). Specifically, Casano et al. (2011)~~
645 ~~also analysed the effects of temperature and light on the growth and the photosynthetic traits of~~
646 isolated ~~phyceobiont~~photobionts from *R. farinacea* (*Trebouxia* sp. TR9 and *T. jamesii*). They found
647 that both species grew better at the lowest temperature and performed the experiments only in
648 cultures grown at 17° C. On the contrary, we did not find such differences in the growth
649 temperature, and thus, we performed the experiments at both 17° C (mean annual temperature in
650 Fuerteventura and Lanzarote) and 20° C (mean annual temperature in Tenerife) to study the
651 photosynthesis response to different temperatures (20° C and 17° C) ~~if temperature drives the~~
652 ~~presence of a specific *Trebouxia* species in each location.~~

653
654 ~~Chlorophyll fluorescence images in algae strains had allowed the study of the spatial~~
655 ~~heterogeneities of Chl fluorescence signatures over the whole area which cannot be detected~~
656 ~~through the conventional point measurements by non-imaging Chl fluorescence (Gorbe and~~
657 ~~Calatayud 2012). One of the most common parameter used to evaluate the physiological fitness of~~
658 ~~vegetable samples by fluorescence is the maximum quantum yield of PSII photochemistry,~~
659 ~~measured in the dark-adapted state, F_v/F_m . F_v/F_m images from lichen algae displayed homogeneity~~
660 ~~values; however, heterogeneity was shown in e.g. *Usnea antarctica* thallus associated with anatomy~~
661 ~~and its growth (Barták et al. 2004).~~

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662 The maximum quantum yield of primary photochemistry in dark-adapted leaves (F_v/F_m) in healthy
663 plants reach F_v/F_m values around 0.840, but for lichens this parameter ranges between 0.550 and
664 0.700 (Casano et al. 2011; Gasulla et al. 2019). In this study F_v/F_m values ranged between 0.51-
665 0.66. The decline in F_v/F_m might be a result of an increase in non-photochemical processes in the
666 light harvesting antennae of PSII associated with a photochemical quenching down-regulation,
667 photodamage of PSII reaction centres, or both (Osmond et al. 1993). In this study, we observed that
668 *T. asymmetrica* did not modify their F_v/F_m values with respect to temperature acclimation. In
669 contrast, *Trebouxia* sp. "~~arnoldoi~~" *arnoldoi* and *T. cretacea* were more sensitive to changes in
670 growing temperature, showing a significant decline in F_v/F_m when grown at 17° C with respect to
671 20° C, ~~which could indicate sensitivity to photoinhibition~~. In addition, these species showed the
672 lowest ETR, mainly from 400µmol photons m⁻² s⁻¹ to the end of light curve. The electron transport
673 rate was saturated at approximately the same irradiance (700 µmol photons m⁻² s⁻¹) in all *Trebouxia*
674 species (except *T. cretacea*) under both temperatures; similar results were obtained in *Trebouxia* sp.
675 TR9 and *T. jamesii* (Casano et al. 2011). A decrease in ETR and an increase in 1-qP indicate a
676 reduction in the quantum yield of PSII and a reduction state of the first electron acceptor of PSII,
677 QA, respectively. In this circumstance, non-photochemical processes (NPQ) must be increased to
678 guarantee excitation energy dissipation (Havaux et al. 1991) such as occurred in *Trebouxia* sp.
679 *arnoldoi* where higher NPQ and 1-qP, and the lowest ETR, was observed. NPQ plays an important
680 role in plants against excess radiation. In these algae strains from *Buellia zoharyi*, NPQ increases in
681 all of them reducing the excitation pressure on reaction centres, thereby decreasing the possibility of
682 photodamage (Papageorgiou and Govindjee 2014). These different behaviours between algae strains
683 grown under two temperatures have been observed by Casano et al. (2011) in *Trebouxia* sp. TR9
684 and *T. jamesii* where *Trebouxia* sp. TR9 performed better under high temperatures, indicating a
685 different capacity to adaptation.

~~Under controlled experimental conditions, *Trebouxia* strains tested in this study showed
characteristic profiles of endogenous content in phytohormones (Fig. 6). IAA levels found in
Trebouxia sp. '*arnoldoi*' (Fig. 6a) are similar to those observed in the lichen forming algae *Trebouxia*
sp., *Trebouxia decolorans* and *Asterochloris glomerata* grown in solid media (Pichler et al. 2020).
ABA production has been also detected in other isolated lichen forming algae, such as *Trebouxia* sp.
TR9 (Hinojosa Vidal et al. 2018) as well in the previously mentioned *Trebouxia* sp., *T. decolorans*
and *A. glomerata* (Pichler et al. 2020). The ABA levels observed in the *Trebouxia* strains tested in
this study (Fig. 6b) are in the same range of magnitude as the levels in *Trebouxia* sp. TR9,
Trebouxia sp. and *T. decolorans* (Hinojosa Vidal et al. 2018; Pichler et al. 2020). In contrast, the
endogenous bioactive form of JA, JA-IIc (Fonseca et al. 2009), is also found in all three *Trebouxia*~~

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696 strains from *B. zoharyi* (Fig. 6d), while no detectable endogenous levels of JA have been found in
697 *Trebouxia* sp., *T. decolorans* and *A. glomerata* (Pichler et al. 2020). In addition, SA and Me JA were
698 also detected in the *Trebouxia* strains isolated in this study. Regarding these phytohormones, there is
699 an absence of previous studies that describe their presence in lichen-forming algae, although Me JA
700 has been detected in *Chorella* (Ueda et al. 1991).

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701 The different phytohormone profiles obtained in the three *Trebouxia* strains isolated in this study
702 (Fig. 6f), and in the other lichen-forming algae mentioned above, suggest that each microalgae
703 strain could present different internal signalling needs, which could be caused by the adaptation to a
704 different symbiotic environment in each lichen thalli. On the other hand, it cannot be ruled out that a
705 part of the production of these phytohormones could also be secreted to the external environment
706 and play a role in external signalling mechanisms with the other members of the thallus symbiotic
707 system. Related to this, the extracellular release of IAA, ABA and JA in the lichen-forming algae
708 *Trebouxia* sp., *T. decolorans* and *Asterochloris glomerata*, has recently been described (Pichler et al.
709 2020). The determination of the role of these phytohormones in the internal signalling mechanisms
710 of the phycobionts, as well as in the coordination mechanisms with the rest of the elements of the
711 lichen symbiosis, is a question that needs future studies.

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712 Our results indicate that *B. zoharyi* is flexible regarding the photobiont choice depending on the
713 region, and suggest that bioclimatic factors could influence the myco/photobiont association
714 patterns due to the different photosynthesis response to different temperatures (20° C and 17° C).
715 Our results clearly indicate that association patterns in each Island could be driven by phycobiont-
716 physiological features and/or mycobiont specialization (Ortiz Álvarez et al. 2015). The mycobiont
717 provides a “growth chamber” (Honegger 2009) with suitable conditions for physiological activity of
718 the photosynthetic partner. However, special adaptations in the physiology of the phycobiont are
719 postulated to explain the ability of the phycobiont to cope with severe environmental conditions.
720 The results point to habitat-specific adaptations which lead to similar behaviour in phycobionts
721 which are genetically different. The physiological adaptations of lichen phycobionts may
722 substantially contribute to the stress-resistance strategy and the colonization capacity. This may be
723 particularly relevant in changing conditions predicted by the climate change scenario.

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50 1031 **Fig. 1** *Trebouxia* phylogenetic analysis. Rooted ITS1-5.8S-ITS2 + LSU rDNA gene tree
51 1032 representing 41 *Trebouxia* sequences, including 28 well- accepted *Trebouxia* species from SAG and
52 1033 UTEX, *Trebouxia* sp. TR9 and *Trebouxia crespoana* from ASUV retrieved from the GenBank.
53 1034 Newly generated sequences are marked as ex. *Buellia zoharyi*_locality_haplotype_code_frequency.
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1035 Three *Trebouxia* species detected were indicated. Values at nodes indicate statistical support
1036 estimated by two methods: bootstrap support (BS, RAxML analysis) and posterior probabilities (PP,
1037 MrBayes analysis). Scale bar shows the estimated number of substitutions per site.

1038 **Fig. 2 a, b** Cross-section of *B. zoharyi* thalli from Lanzarote by TEM. **a** ~~PhyeobiontPhotobionts~~ of
1039 *B. zoharyi* inside thallus, **b** Detail of pyrenoid. **c, d** Cross-section of *B. zoharyi* thallus from
1040 Fuerteventura by TEM. **c** ~~PhyeobiontPhotobionts~~ of *B. zoharyi* inside thallus, **d** Detail of pyrenoid.
1041 **e, f** Cross-section of *B. zoharyi* thalli from Tenerife by TEM. **e** ~~PhyeobiontPhotobionts~~ of *B. zoharyi*
1042 inside thallus, **f** Detail of pyrenoid. Abbreviations: Py, Pyrenoid; Pg, Pyrenoglobuli; Chl,
1043 Chloroplast; PV, Peripheral vesicles. Bars 500 nm, 800 nm, 1 μ m and 2 μ m.

1044 **Fig. 3** Algal multiplicity detected by PCR depicted in the localities included in this study: Tenerife,
1045 Lanzarote and Fuerteventura. Inner, outer circle and numbers represent the primary and secondary
1046 ~~phyeobiontphotobiont~~ detected, respectively. The colour coding for each *Trebouxia* is shown on the
1047 bottom left-hand side of the figure.

1048 **Fig. 4** Phytohormone profiles of axenic cultures of the different *Trebouxia* strains isolated from
1049 *Buellia zoharyi*. Microalgae were grown in BBM medium for 21 days at 20° C. Three cultures were
1050 sampled for each condition and phytohormone levels were quantified: **a** indole-3-acetic acid (IAA);
1051 **b** abscisic acid (ABA); **c** salicylic acid (SA); **d** jasmonic acid conjugated to isoleucine (JA-Ile) and
1052 **e** methyl jasmonate (Me-JA). Graphs show the mean \pm standard deviation. Significant differences
1053 between strains are indicated with letters (ANOVA, Tukey HSD test, $p < 0.05$). **f** Heatmap analysis
1054 of the phytohormone content of each *Trebouxia* strain. Scale bar (Z-score) indicates the relative
1055 abundance of a particular phytohormone in each strain.

1056 **Fig. 5** **a** Maximum fluorescence yield (F_v/F_m) of the different *Trebouxia* strains isolated from
1057 *Buellia zoharyi* growing in BBM medium at 17° C and 20° C. Bars represent means \pm SE, n = 4.
1058 Significant differences between treatments and species are indicated with letters (ANOVA, Tukey
1059 HSD test, $p < 0.05$). **b** Chlorophyll fluorescence images for F_v/F_m of the different *Trebouxia* strains
1060 isolated from *Buellia zoharyi* growing in BBM medium at 17° C and 20° C. The color scale bar
1061 shown at the bottom of the figures stands for values from 0 (black) to 1 (pink).

1062 **Fig. 6** **a** PPF response curves of the non-photochemical quenching (NPQ), **b** relative electron
1063 transport rate (ETR), and **c** PSII photooxidative pressure (1-qP) in the different *Trebouxia* strains
1064 isolated from *Buellia zoharyi* growing in BBM medium at 17° C and 20° C. Bars represent means \pm
1065 SE, n = 4. ~~Two areas of interest (AOI) were selected for Chl fluorescence measurements, one in the~~
1066 ~~central part and one in the outer zones in order to evaluate spatial heterogeneity. The values of~~

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chlorophyll fluorescence parameters were means of two AOI (internal and external); no significant differences were obtained between internal and external areas.

Fig. 6 Phytohormone profiles of axenic cultures of the different *Trebouxia* strains isolated from *Buellia zoharyi*. Microalgae were grown in BBM medium for 21 days at 20° C. Three cultures were sampled for each condition and phytohormone levels were quantified: **a** indole 3-acetic acid (IAA); **b** abscisic acid (ABA); **c** salicylic acid (SA); **d** jasmonic acid conjugated to isoleucine (JA-Ile) and **e** methyl jasmonate (Me JA). Graphs show the mean ± standard deviation. Significant differences between strains are indicated with letters (ANOVA, Tukey HSD test, $p < 0.05$). **f** Heatmap analysis of the phytohormone content of each *Trebouxia* strain. Scale bar (Z-score) indicates the relative abundance of a particular phytohormone in each strain.

