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RESEARCH

Contrasting response of *Gracilaria chilensis* (Gracilariales, Rhodophyta) life cycle stages to epiphyte infection

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Abstract

The red seaweed *Gracilaria chilensis*, a species extensively cultivated in Chile for agar extraction, was subjected to a bioassay to determine the susceptibility of tetrasporophytes, female and male gametophytes collected from natural and cultivated populations, to the red epiphyte *Acrochaetium* sp. and the brown epiphyte *Ectocarpus* sp. The settlement, attachment and germination of epiphytic algal spores on *G. chilensis* thalli were evaluated, and the photosynthetic responses and the concentration of total phenolic compounds were determined as a possible response of *G. chilensis* to biotic stress. The results showed that when the thalli were exposed to *Acrochaetium* infection, female individuals had a significantly lower percentage of germinated spores than other phases of the life cycle. After infection with *Ectocarpus* spores, males showed the highest % germination of the epiphyte. For both epiphytes, the response of tetrasporophytes from natural and cultivated populations shows a similar trend. The total content of phenolic compounds showed that, in general, the individuals infected with *Acrochaetium* had a higher defense capacity, whereas the infection with the brown alga did not induce a significant release of phenolic compounds. Despite the heterogeneous results observed for photosynthetic activity, a higher photoinhibition of the maximum fluorescence quantum yield (F_v/F_m) was observed in thalli with the *Acrochaetium* epiphyte, confirming that *G. chilensis* was subjected to stress after infection. Taken together, these observations may suggest that the cultivation of females could be of long-term benefit to farms by reducing biomass losses under stressful conditions and epiphyte invasions on farms.

Keywords Epiphytes · Gracilariales · Rhodophyta · Life cycle · Photosynthetic activity · Phenolic compounds

Introduction

Epiphyte infestations in commercial seaweed farming may have a major impact on crop productivity, resulting in reduced yield (Lüning and Pang 2003; Critchley et al. 2004; Hurtado et al. 2006; Vairappan 2006; Mulyaningrum et al. 2019), reduced growth rate due to competition for light and nutrients with the host (Buschmann and Gómez 1993), altered reproductive effort of the basiphyte (e.g. *Ascophyllum nodosum* affected by *Vertebrata lanosa*; Kraberg and Norton 2007), degraded agar quality and skewed economics (Behera et al. 2022). Epiphytic infestations attracted the attention of various researchers after outbreaks occurred in commercial farms of *Kappaphycus* in the 1970s (Doty and Alvarez 1975), causing retarded growth and significant loss of biomass (Behera et al. 2022). Subsequently, outbreaks were also reported in other red seaweeds namely *Gelidium* (Melo et al. 1991) and *Gracilaria chilensis* in Chile (Leonardi et al. 2006; Leal et al. 2020). These reports address the question of the causes of epiphytism in seaweed farms.

Gracilaria chilensis is the most important seaweed cultivated for agar extraction in Chile (Buschmann et al. 2017; Camus et al. 2018). Total landings varied between 35 and 137 t year^{-1} (2000–2021, Servicio nacional de pesca y acuicultura). Strong fluctuations during the last two decades (www.sernapesca.cl) were associated with variations in external market demand, but also with the presence of epiphytic proliferation in the farms. The most common epiphytic pests

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found in *Gracilaria* cultures include diatoms (Buschmann et al. 1995), *Ectocarpus* spp. (Kuschel and Buschman 1991), encrusting *Erythrocladia* sp., filamentous *Ceramium rubrum*, *Polysiphonia* spp. and *Acrochaetium* sp. (Leonardi et al. 2006), green algae and cyanobacteria (Buschmann and Gómez 1993; Buschmann et al. 1995; Leonardi et al. 2006) as well as invertebrates (e.g., polychaeta, gastropods) and pathogenic amoebae (Correa and Flores 1995). In addition, since 2013 the green alga *Rhizoclonium* sp. has been detected in a large area of *G. chilensis* farms in the Los Lagos region (one of the main areas of *Gracilaria* production, Ávila et al. 2019), causing severe production losses (i.e., > 90%; Aroca et al. 2020; Leal et al. 2020). These different epiphyte species differ not only in the level of damage they can cause to the basiphyte, from weak attachment to the host surface and no associated damage to host tissues (i.e. infection level I, Leonardi et al. 2006), to deep penetration into the host cortex, reaching the medullary tissue and causing destruction of host cells in the area of infection (i.e. infection level V, Leonardi et al. 2006), but also in their relative abundance and seasonal dynamics (Leonardi et al. 2006).

The widespread occurrence of infections by algal epiphytes in *Gracilaria* farms negatively affects the growth rate of the crop, leading to biomass losses due to increased resistance and consequently to products of lower economic value (Buschmann and Gómez 1993; Buschmann et al. 1993, 1997). Epiphytes may also disrupt physiological processes such as photosynthetic capacity, leading to photoinhibition in the seaweed (Dawes et al. 2000; Friedlander et al. 2001; Leal et al. 2020; Behera et al. 2022). At the same time, the host can alter and/or induce the production of harmful allelochemicals (i.e. chemical compounds that inhibit the growth of other species) as a defense mechanism (Svirski et al. 1993; Friedlander et al. 1996). In species of the genus *Gracilaria*, this mechanism is activated in response to the presence of epiphytes through the activation of an oxidative burst and the release of phenolic compounds, which are considered to have high antioxidant and chemopreventive activities in seaweeds (Rönnberg and Ruokolahti 1986; Bravo 1998). The oxidative burst activates metabolic pathways involving oxidized lipids, which trigger a defense response capable of reducing spore settlement and/or germination of *Acrochaetium* sp. and *C. rubrum* (Lion et al. 2006; Weinberger et al. 2011).

Gracilaria chilensis has a complex life cycle with two free-living isomorphic macroscopic generations: female and male gametophytes (haploid phase) and tetrasporophytes (diploid phase). Tetrasporophytes are the dominant phase found in cultivated farms, which are maintained by vegetative propagation through manual fragmentation of thalli and partial burial in the substrate. Despite reaching their reproductive state, tetraspores are unable to settle to form gametophytes due to lack of suitable substrate. This raises the question of whether the gametophytic phase might have a different susceptibility to epiphytes. On the other hand, natural populations are maintained by sexual reproduction and spore recruitment (Guillemin et al. 2008), and fertile male and female gametophytes are often found (Meneses 1996). Changes in phenotypic traits have been reported as a consequence of an unconscious domestication process carried out by artisanal farmers over 40 years (Valero et al. 2017): faster growth (Alveal et al. 1997), lack of reproductive structures, high prevalence of epiphytes (Westermeier et al. 1991, 1993; Candia et al. 2006; Leonardi et al. 2006; Aroca et al. 2020; Leal et al. 2020) and low genotypic diversity (Guillemin et al. 2008) in farms compared to natural populations. It has been proposed that greatly reduced genetic and/or genotypic diversity in farms could lead to increased susceptibility to epiphytes (Hurtado et al. 2015). Alternatively, some trade-offs between fast growth and other traits may have evolved in the context of incipient domestication. This seems to explain the selection of asexual diploid individuals in farms (Guillemin et al. 2014). Trade-offs could also explain the occurrence of epiphytes if resistance and/or defense traits have been altered by misdirected selection (i.e. only for growth). Alternatively, the presence of epiphytes could result from environmental and demographic changes associated with management conditions (e.g. high density of individuals in a non-native environment). In order to distinguish between genetic and environmental causes of epiphytism on farms, it is necessary to characterize the sources of variability in the response of basiphytes to epiphytes.

In this study we analyze epiphyte-basiphyte interactions in *G. chilensis* from natural and cultivated populations. We address both the effect of different epiphytes (i.e. the red alga *Acrochaetium* sp. and the brown alga *Ectocarpus* sp.) on physiological traits (i.e. photosynthetic efficiency and total phenolic concentration) of *G. chilensis*, and the effect of the population source of the basiphytes on the colonization and germination rates of the epiphytes. We also compare haploid and diploid individuals of the life cycle of *G. chilensis* to test the hypothesis that selection for diploids in farms could be explained by a higher susceptibility of haploids to epiphytes. The choice of *Acrochaetium* sp. was based on previous knowledge that it triggers a molecular response in *G. chilensis* (Lion et al. 2006; Weinberger et al. 2011), in addition to having a low impact (i.e. infection type II, indicating strong attachment but no visible damage to host tissues; Leonardi et al. 2006). *Ectocarpus* sp. was chosen because it produces infection type I, i.e. loose attachment and no damage to host tissues (Leonardi et al. 2006; Behera et al. 2022), and is therefore expected to induce neither defense responses nor physiological changes in the host.

Materials and methods

Algal material sampling

Gracilaria chilensis thalli were collected from natural and farmed populations, located along the Chilean coast during the winter-spring season. Three natural (Putemun, Maullín and Lenca) and three cultivated (Quetelmahue, Achao and Lenga) populations were sampled, all separated by more than 10 km (Fig. 1). The distinction between the two types of populations was based on whether *G. chilensis* thalli were actively planted by farmers or not. In addition, thalli in natural populations are attached to rocks and pebbles by a holdfast, whereas in farmed populations they are unattached but embedded in sandy or muddy bottom in farms. The collected thalli were separated by at least 10 m to avoid sampling fragments of the same clone in each population (following the criteria of Guillemin et al. 2008).

Two epiphyte species were used in the infection experiments: *Acrochaetium* sp. and *Ectocarpus* sp. The strain of *Acrochaetium* sp. was obtained from *Gracilaria chilensis*-infected thalli collected in Lenca in January 2020. *Ectocarpus* sp. was collected in a natural area, in Chañaral, a locality in northern Chile, in 2006.

Acquisition of *Gracilaria* unialgal cultures

Once in the laboratory, all *Gracilaria* individuals were first separated and cut into 10 cm fragments. All fragments were then sonicated twice for 20 s, first in distilled water and then in filtered seawater. After this step, the thalli were examined under a Stemi DV4 stereoscopic microscope (Zeiss) to find epiphytes on the surface of the fragments and brushed. Finally, 1 cm apical fragments were excised and placed in small Petri dishes with sterile culture medium (Provasoli's enriched seawater medium (PES; Provasoli 1968)). Once a week for 3 months, the fragments were sonicated, observed under the microscope, brushed and the apical part was again excised until it regenerated completely free of epiphytic algae. Unialgal cultures were kept in active growth using standard culture conditions in PES medium. The culture conditions in the laboratory were as follows: irradiance 20 µmol photons m^{-2} s^{-1}; photoperiod 12:12 (light/dark); temperature 11 °C and weekly change of PES culture medium.

Determination of phase and sex

For all vegetative *Gracilaria* thalli, phase and sex were determined using the molecular markers developed by Guillemin et al. (2012). Briefly, males and females show the

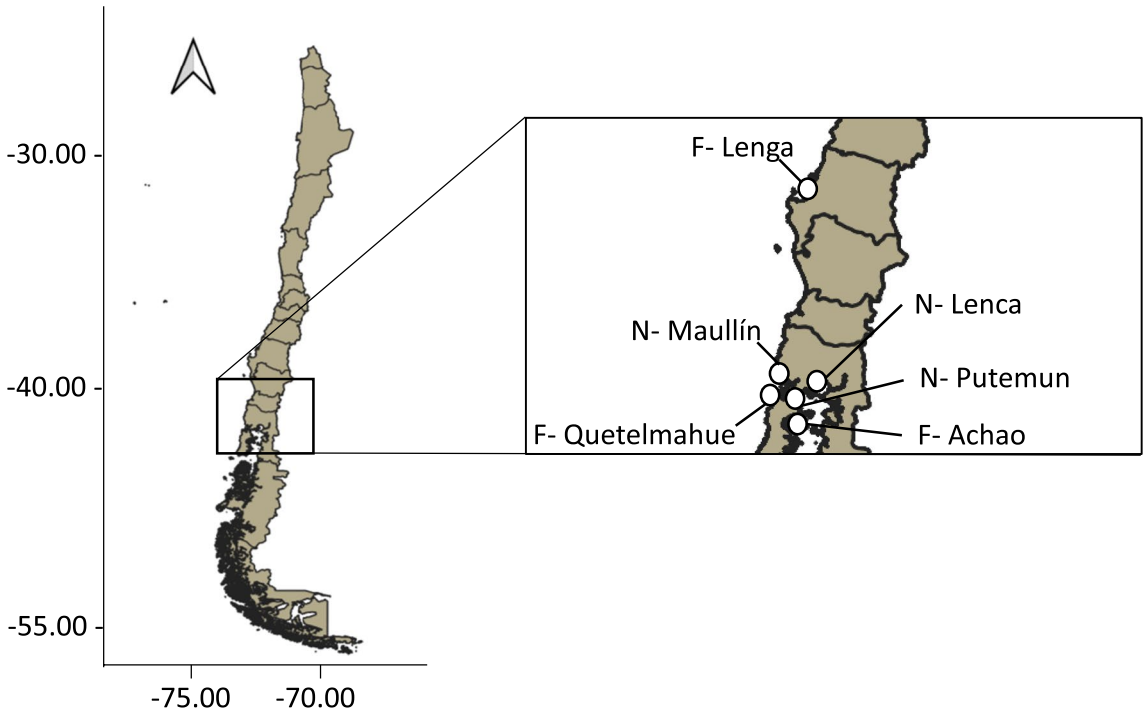

Fig. 1 Map of Chile showing sampling location of the 3 farmed populations (F-), and the 3 natural populations (N-) sampled in two regions along the Pacific coast

amplification of sex-specific PCR fragments, which are distinguishable by simple electrophoresis due to their different molecular size. The diploid tetrasporophyte amplifies both. The amplification of the SCAR-G16-486 marker occurs in males, whereas the amplification of the SCAR-D12-386 marker occurs only in females (Guillemin et al. 2012). For the farmed populations, the few identified haploid males and females were discarded. For the natural populations, female and male gametophytes and tetrasporophytes were included in the bioassays. Mature individuals were observed under a Stemi DV4 stereoscopic microscope (Zeiss) to determine phase and sex by direct observation of reproductive structures.

Inoculation of epiphytes

Acrochaetium sp.

Vegetative thalli of *Acrochaetium* sp. kept in small Petri dishes with PES culture medium were scraped and transferred to new Petri dishes and after 6–7 days the *Acrochaetium* thalli were reproductively mature. Both vegetative and reproductive thalli are tufts with numerous erect filaments, but the reproductive thalli have lateral ramifications. Once the reproductive structures were formed, *Acrochaetium* thalli were transferred to new dishes with fresh medium to stimulate sporulation. Before inoculation of *G. chilensis* with *Acrochaetium* sp., the spore concentration was determined by counting the number of spores in 3 aliquots of 10 µL of concentrated medium. The inoculation of spores was adjusted to achieve 20,000 spores per inoculum.

Ectocarpus sp.

In *Ectocarpus* sp. the biomass was increased by fragmentation of the thalli of about 0.5 mm. Reproductive structures appeared after 7–10 days. Spore release was stimulated by dehydration for 30 min followed by rehydration with culture medium at 10 °C. As the zoospores of *Ectocarpus* sp. are mobile, they were counted on an aliquot fixed in Lugol's solution using a hemocytometer (Neubauer chamber). The inoculation of spores was adjusted to obtain 80 000 spores per inoculum.

Inoculation and early development of epiphytes

Thalli of *G. chilensis* from both natural and cultivated populations were exposed independently to each epiphyte species. For each epiphyte-host combination, 10 individuals per population, ploidy and sex of *G. chilensis* were used (i.e. for each farmed population: nt = 10 tetrasporophytes, and for each natural population: nt = 10 tetrasporophytes, nf = 10 females and nm = 10 males). Each individual was fragmented into clonal replicates used either for inoculation of epiphytes or as epiphyte-free controls. Spores of *Acrochaetium* sp. were added to each set of clonal fragments (n = 3) from each *Gracilaria* individual and kept in individual Erlenmeyer flasks filled with 100 mL sterile seawater. In the case of *Ectocarpus* sp., due to the mobile spores, we used Petri dishes for both the control and thalli. All *G. chilensis* thalli started at an initial length of 1 cm, randomly selected from each individual. Each inoculated (i.e. treatment) and un-inoculated (i.e. control) flask was left on a shaker for 24 h. Settlement rate was then quantified by counting all spores settled along two transects on the 1 cm surface of each *G. chilensis* thallus. Germination rate was estimated by counting all germinated spores on the two transects two days later. The experiment lasted 12 days starting with the fragmentation of *Gracilaria* thalli.

Photosynthetic parameters determination of the basiphyte

For physiological analysis, 9 individuals from each natural population (3 individuals from each life cycle phase: males, females and tetrasporophytes) and 3 individuals from each cultured population (tetrasporophytes) were analyzed for each epiphyte treatment at the end of the experiment. Algal thallus fragments were incubated for 20 min in the dark before measuring the maximum fluorescence quantum yield (F_v/F_m, an indicator of maximum quantum efficiency, Schreiber et al. 1995) using a Junior PAM (Walz GmbH, Germany). The electron transport rate (ETR, µmol electrons m^{-2} s^{-1}) was determined from rapid light curves (RLC) after 20 s exposure to 12 increasing irradiances of blue light provided by the Junior PAM. The ETR was calculated according to Schreiber et al. (1995) as follows:

$$\mathrm{ETR} = \Delta F/F_{\mathrm{m}} \cdot E \cdot A \cdot F_{\mathrm{II}} \left(\mu\mathrm{mol\ electrons\ m^{-2}\ s^{-1}}\right) \quad (1)$$

where $\triangle F/F'_{\mathrm{m}}$ is the effective quantum yield, E is the incident PAR irradiance expressed in µmol photons m^{-2} s^{-1}, A is the thallus absorbance and F_{II} is the fraction of chlorophyll related to PSII (400-700 m), using 0.15 for red algae according to Grzymski et al. (1997) and Figueroa et al. (2003).

As an estimator of photosynthetic efficiency, the initial slope of the ETR (α_{ETR}) and the maximum ETR ($\mathrm{ETR}_{\mathrm{max}}$) were obtained from the tangent function reported by Eilers and Peeters (1988), and the saturated irradiance (Ek_{ETR}) was calculated from the intercept between these two parameters. α increases as antenna size decreases due to disorganization of accessory pigments, indicating faster light saturation, while $\mathrm{ETR}_{\mathrm{max}}$ is an indicator of electron transport chain saturation, which may be due to the inefficiency of PSI in recycling electrons generated by PSII under oxidative stress (Hurd et al. 2014). Maximum non-photochemical quenching ($\mathrm{NPQ}_{\mathrm{max}}$), used here as an estimate of the photoprotective

response to high light stress) was obtained from the tangent function of NPQ versus irradiance (αNPQ) according to Eilers and Peeters (1988).

Determination of phenolic compounds of the basiphyte

For the determination of phenolic concentration, 9 individuals from natural populations (3 individuals from each life cycle phase: males, females and tetrasporophytes) and 3 individuals from farms (tetrasporophytes only) were analyzed for each epiphyte treatment at the end of the experiment. For each thallus fragment, 0.1—0.2 g fresh weight was ground in a mortar with sand using 2.5 mL of 80% methanol. This mixture was then centrifuged at 3000 rpm for 5 min and the supernatant was collected to measure the total phenolic content using Folin-Ciocalteu reagent. Gallic acid (G-7384) was used as a standard. Finally, the absorbance was measured at 700 nm. The phenolic concentration was expressed as mg gallic acid eauivalents (GAE) g^{-1} fresh weight.

Statistical analysis

All analyses were performed using the statistical program R 3.6.3 (R Core Team 2016). The assumptions of homogeneity of variances and normal distribution were tested using the Levene test and the Shapiro–Wilk test, respectively. Where data did not follow a normal distribution or were not homoscedastic, they were transformed using logarithm or using the transformation of the Box-cox family. The effect of the epiphyte infection on the measured variables (spore settlement, germination, photosynthetic responses and phenolic compounds content) was evaluated for each phase (i.e., females, males and tetrasporophytes) of the natural populations and for tetrasporophytes only when comparing natural and farmed populations. These were tested with two-way ANOVA followed by the Tukey multiple comparison tests.

Results

Sex determination of thalli sampled on farmed and natural populations

From 300 *Gracilaria chilensis* thalli collected in cultivated populations, we obtained 82.9%, 94.3% and 40.0% of tetrasporophytes in Achao, Quetelmahue and Lenga, respectively (Supplementary Table 1). On the other hand, among the 400 *G. chilensis* individuals collected in natural populations, the distribution of tetrasporophytes and gametophytes was highly variable, with male gametophytes being the least represented (Supplementary Table 1).

Gracilaria chilensis response against epiphytes

The average number of settled and germinated spores of *Acrochaetium* sp. epiphyte on *G. chilensis* was 1.3–2.8 and 0.6–6.0 times higher compared to the glass control (i.e. *Acrochaetium* sp. spores settled in Petri dishes without *G. chilensis* thalli), respectively (Figs. 2A and 3A). On the contrary, the colonization and germination of *Ectocarpus* spores on *G. chilensis* were 0.4–0.6 and 0.2–0.5 times lower, respectively, compared to the control (i.e. *Ectocarpus* spores colonized on Petri dishes without *G. chilensis* thalli) (Figs. 4A and 5A), even though a higher concentration of *Ectocarpus* spores was inoculated on the petri dishes.

In the *Acrochaetium* sp. bioassays, the mean number of settled spores was significantly different (i.e. nearly twofold) between diploid individuals from natural (76.63 ± 36.18) and cultivated populations (42.53 ± 17.88) ($F_{1,76} = 11.695$; $p < 0.001$). Furthermore, significant differences were observed between the different phases of the natural populations ($F_{2,86} = 11.174$; $p < 0.001$). Interestingly, females had a lower number of settled spores than males (37.12 ± 29.45 and 68.56 ± 36.52, respectively) ($F_{2,86} = 11.174$; $p = 0.002$) and tetrasporophytes ($F_{2,86} = 11.174$; $p < 0.001$). However, there was no significant difference between males and tetrasporophytes ($F_{2,86} = 11.174$; $p = 0.641$) (Fig. 2B). On the contrary, no significant differences were observed between individuals from natural populations (females: 13.72 ± 10.38; males: 12.27 ± 7.64, tetrasporophytes: 13.66 ± 11.63) ($F_{2,87} = 0.201$; $p = 0.818$) and tetrasporophytes from natural and farmed populations (13.66 ± 11.63 and 15.73 ± 7.80, respectively) ($F_{1,58} = 3.822$; $p = 0.055$) in the *Ectocarpus* sp. bioassays (Fig. 4B).

The germination rate of *Acrochaetium* sp. was significantly lower for female gametophytes (8.69 ± 6.24) than for the other life cycle stages (males: 59.70 ± 60.18; tetrasporophytes: 34.36 ± 22.99) ($F_{2,87} = 7.247$; $p = 0.001$), while it did not differ between farmed and natural diploids (20.23 ± 15.96 and 34.36 ± 22.99, respectively) ($F_{1,58} = 0.018$; $p = 0.893$) (Fig. 3B). In the *Ectocarpus* sp. bioassay, males (12.24 ± 13.39) from natural populations had a statistically higher germination rate than females (5.58 ± 4.96) and tetrasporophytes (5.64 ± 7.87) ($F_{2,87} = 6.238$; $p = 0.003$). There were no significant differences between the diploid individuals of the natural and cultivated populations (3.78 ± 3.01) ($F_{1,58} = 2.77$; $p = 0.101$) (Fig. 5B).

Photosynthetic response of *Gracilaria chilensis* to epiphytes

After inoculation of *G. chilensis* with *Acrochaetium* sp., photosynthetic parameters showed that there was a significant effect of group (with higher values in the control group compared to the treatment) on maximum electron transport rate (ETR_{max}) (up to 1.54 times higher), maximum fluorescence

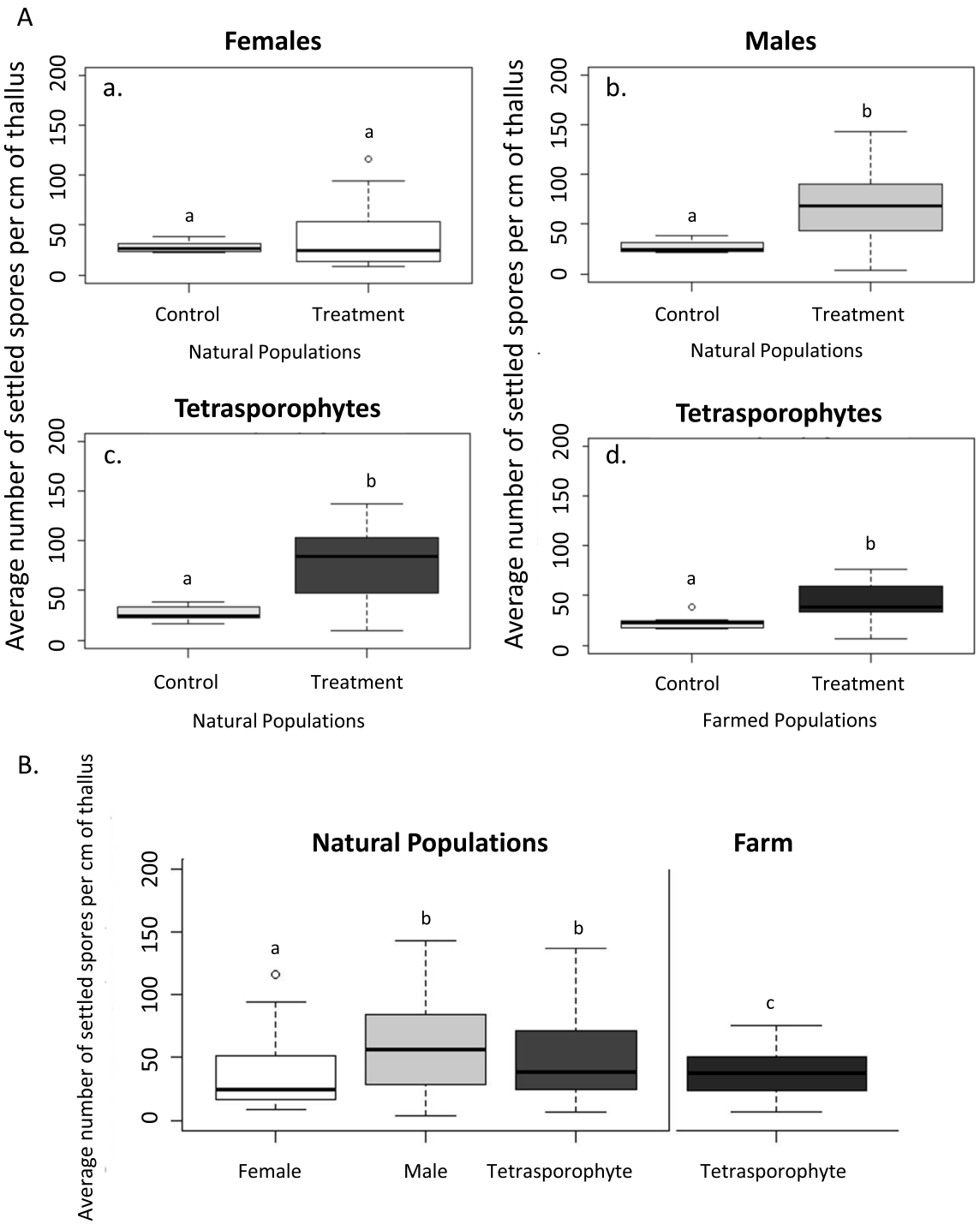

Fig. 2 **A**: Average number of spores of *Acrochaetium* sp. settled per cm of thallus of female (a) and male (b) gametophytes, and tetrasporophytes of *Gracilaria chilensis* from natural (c) and farmed (d) populations in the treatment and control (i.e. *Acrochaetium* spores on glass substrate of a Petri plate). **B**: Average number of settled spores of *Acrochaetium* sp. per cm of thallus of *G. chilensis* on female and male gametophytes, and tetrasporophytes from natural and farmed populations. Lowercase letters indicate differences at $p < 0.05$. Box plot whiskers show the 1%–99% range values; the horizontal line in each box plot shows the median, and the colored segment shows the quartile range (25%–75%). Values outside of the whisker range are plotted as dots

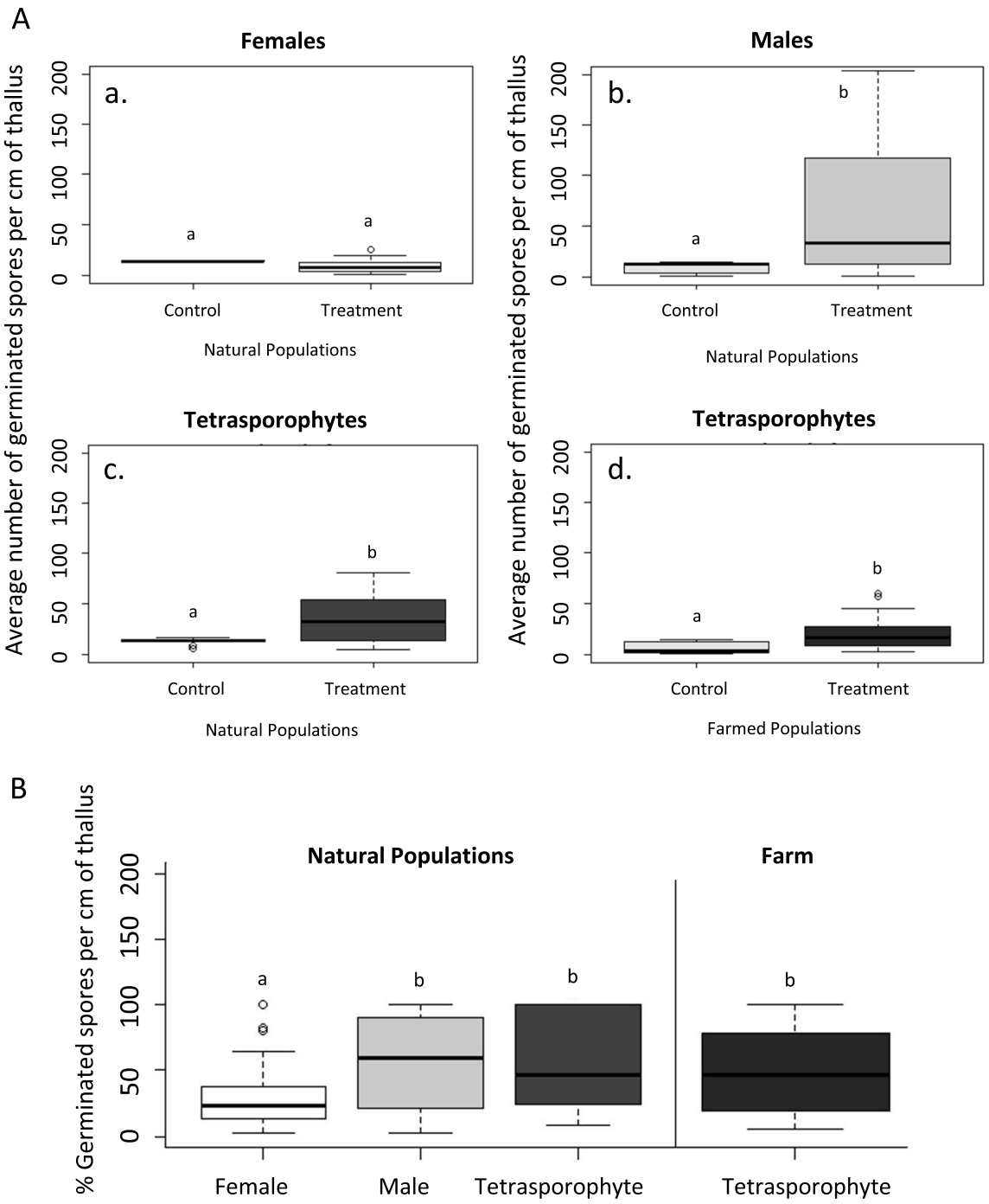

Fig. 3 **A**: Average number of spores of *Acrochaetium* sp. germinated per cm of thallus of female (a) and male (b) gametophytes, and tetrasporophytes of *Gracilaria chilensis* from natural (c) and farmed (d) populations in the treatment and control (i.e. *Acrochaetium* spores on glass substrate of a Petri plate). **B**: Average number of settled spores of *Acrochaetium* sp. per cm of thallus of *G. chilensis* on female and male gametophytes, and tetrasporophytes from natural and farmed populations. Box plots descriptors as in Fig. 2

quantum yield (F_v/F_m) (up to 1.20 times higher) and non-photochemical quenching (NPQ_{max}) (up to 1.37 times higher) (see Table 1A). There was also a significant effect of population type only on F_v/F_m (up to 1.17 times higher in natural populations than in farms) and a significant interaction of group and population type on ETR_{max} ($F_{1,104} = 23.\ 901$; $p < 0.0001$), F_v/F_m ($F_{1,104} = 5.268$; $p = 0.023$) and photosynthetic efficiency (α_{ETR}) ($F_{1,104} = 11.823$; $p = 0.009$) measured in tetrasporophyte individuals of *G. chilensis* (Tables 1A and 2A). F_v/F_m was significantly lower, up to 1.2 times lower in

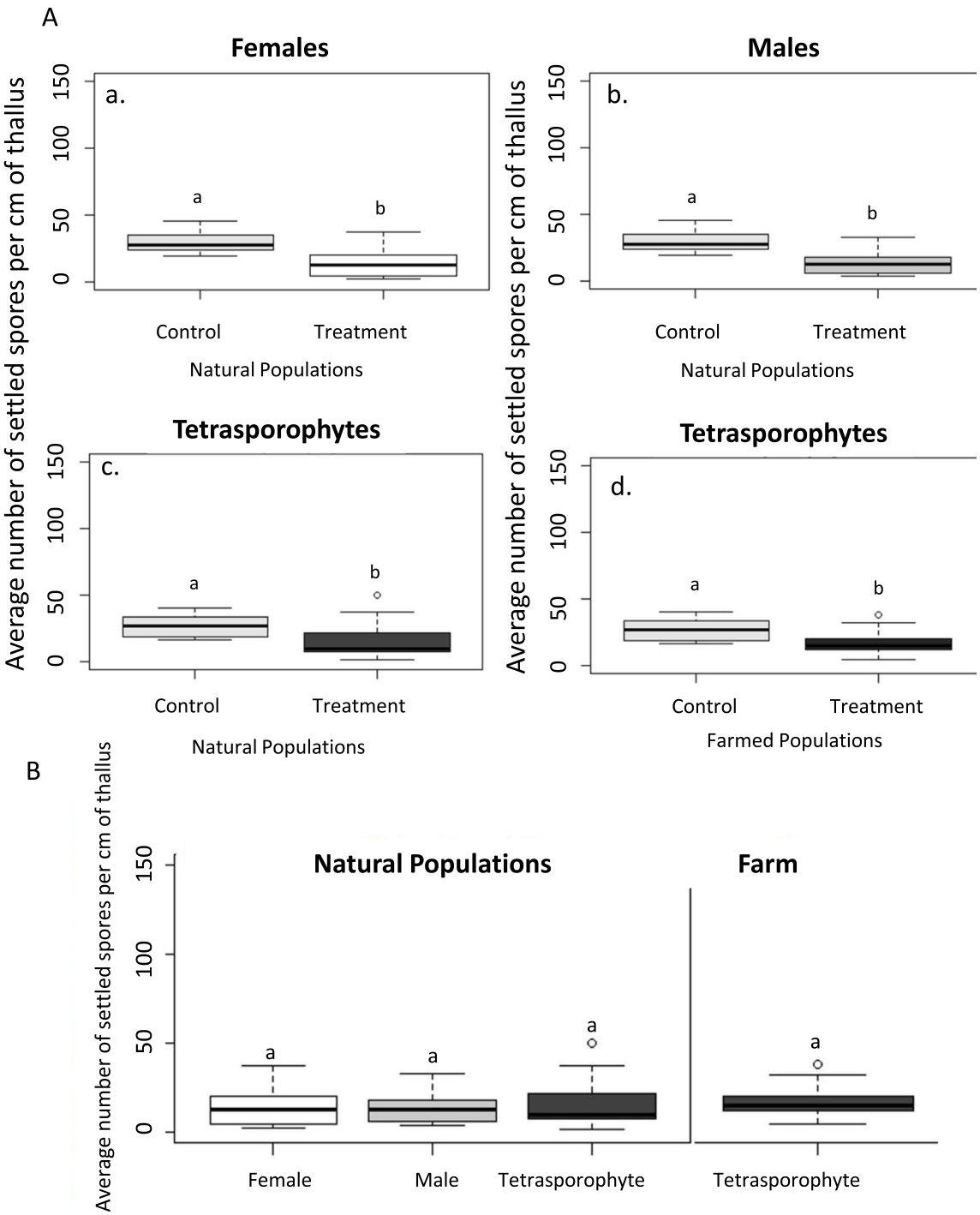

Fig. 4 **A**: Average number of spores of *Ectocarpus* sp. settled per cm of thallus of female (a) and male (b) gametophytes, and tetrasporophytes of *Gracilaria chilensis* from natural (c) and farmed (d) populations in the treatment and control (i.e. *Ectocarpus* spores on glass substrate of a Petri plate). **B**: Average number of settled spores of *Ectocarpus* sp. per cm of thallus of *G. chilensis* on female and male gametophytes, and tetrasporophytes from natural and farmed populations. Box plots descriptors as in Fig. 2

thalli with epiphytes than in the control group, while cultured populations showed higher photoinhibition (1.2 times lower F_v/F_m value) than natural populations (Table 1A). Non-photochemical quenching (NPQ_{max}) was significantly higher in the control (up to 1.37 times higher), while no significant differences between populations ($F_{1,104} = 0.386$; $p = 0.535$) or interaction between group and population type ($F_{1,104} = 3.142$; $p = 0.079$) were found for tetrasporophyte individuals (Table 2A). The comparison between the different phases of the life cycle of *G. chilensis* of the

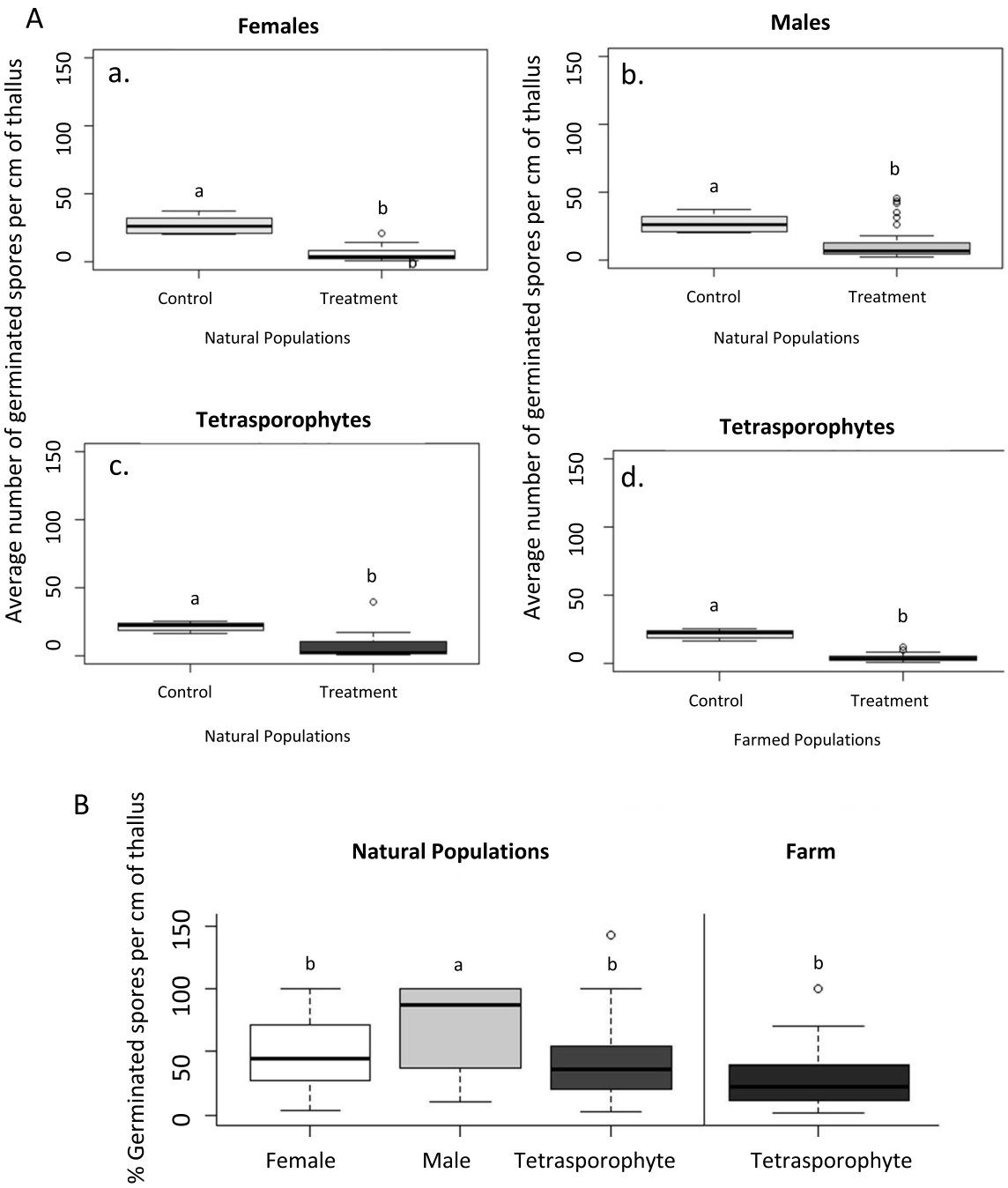

Fig. 5 **A**: Average number of spores of *Ectocarpus* sp. germinated per cm of thallus of female (a) and male (b) gametophytes, and tetrasporophytes of *Gracilaria chilensis* from natural (c) and farmed (d) populations in the treatment and control (i.e. *Ectocarpus* spores on glass substrate of a Petri plate). **B**: Average number of settled spores of *Ectocarpus* sp. per cm of thallus of *G. chilensis* on female and male gametophytes, and tetrasporophytes from natural and farmed populations. Box plots descriptors as in Fig. 2

natural populations showed that for all the photosynthetic parameters evaluated there was a significant main effect of epiphyte infection (see Table 2B), with higher values (up to 1.54 times) in the control group for almost all the photosynthetic parameters, except for α_{ETR} (see Table 1A). On the other hand, phase had a significant effect only on F_v/F_m ($F_{2,156} = 5.163$; $p = 0.007$) and NPQ_{max} ($F_{2,156} = 11.772$; $p < 0.0001$) (Table 2B).

Concerning the infection with *Ectocarpus* sp., the maximum electron transport rate (ETR_{max}) was the only parameter that showed up to 1.6 higher values in the control than in the treatment (Table 1B) in all phases of the cultivated and natural populations. The maximum quantum yield

Table 1 Photosynthetic parameters measured in *Gracilaria chilensis* natural and farmed thalli infected with *Acrochaetium* sp. (A) and *Ectocarpus* sp. (B). Data are given as mean ± S.E. (n = 3 for each phase and population). Distinct lowercase letters denote significant differences after Tukey test

A		Natural populations			Farmed populations
		Females	Males	Tetrasporophytes	Tetrasporophytes
ETR_{max}	C	83.37 ± 14.07^{a}	69.94 ± 21.68^{ab}	86.46 ± 23.49^{a}	74.29 ± 19.79^{a}
	T	57.73 ± 19.98^{b}	74.02 ± 50.37^{ab}	55.93 ± 17.72^{b}	86.44 ± 32.14^{a}
F_v/F_m	C	0.56 ± 0.02^{a}	0.57 ± 0.03^{a}	0.55 ± 0.03^{ac}	0.55 ± 0.02^{c}
	T	0.47 ± 0.09^{b}	0.54 ± 0.04^{ac}	0.52 ± 0.05^{bc}	0.46 ± 0.09^{d}
α	C	0.21 ± 0.05^{a}	0.22 ± 0.08^{a}	0.21 ± 0.04^{a}	0.23 ± 0.06^{a}
	T	0.25 ± 0.10^{ab}	0.28 ± 0.07^{b}	0.28 ± 0.06^{b}	0.22 ± 0.10^{a}
NPQ_{max}	C	0.78 ± 0.07^{a}	0.87 ± 0.26^{a}	0.77 ± 0.10^{a}	0.78 ± 0.11^{a}
	T	0.59 ± 0.17^{b}	0.78 ± 0.14^{a}	0.65 ± 0.16^{b}	0.57 ± 0.18^{b}
B		Natural populations			Farmed populations
		Females	Males	Tetrasporophytes	Tetrasporophytes
ETR_{max}	C	83.37 ± 14.07^{a}	69.94 ± 21.68^{c}	86.46 ± 23.49^{a}	74.29 ± 19.79^{a}
	T	56.71 ± 12.68^{bc}	57.93 ± 15.79^{bc}	54.20 ± 15.66^{b}	49.13 ± 13.14^{b}
F_v/F_m	C	0.56 ± 0.02^{a}	0.57 ± 0.03^{ab}	0.55 ± 0.03^{a}	0.55 ± 0.02^{a}
	T	0.59 ± 0.01^{b}	0.58 ± 0.02^{b}	0.56 ± 0.06^{a}	0.55 ± 0.04^{a}
α	C	0.21 ± 0.05^{a}	0.22 ± 0.08^{a}	0.21 ± 0.04^{a}	0.23 ± 0.06^{c}
	T	0.31 ± 0.05^{b}	0.29 ± 0.05^{b}	0.24 ± 0.09^{ac}	0.25 ± 0.07^{c}
NPQ_{max}	C	0.78 ± 0.07^{a}	0.87 ± 0.26^{a}	0.77 ± 0.10^{a}	0.78 ± 0.11^{a}
	T	0.80 ± 0.09^{ab}	0.82 ± 0.09^{a}	0.76 ± 0.18^{b}	0.78 ± 0.11^{ab}

C Control = *G. chilensis* thalli without *Acrochaetium* sp. epiphyte; *T* Treatment = *G. chilensis* thalli with *Acrochaetium* sp. epiphyte infection

Table 2 Results of ANOVA analyses of the photosynthetic parameters for *Gracilaria chilensis* thalli submitted to *Acrochaetium* sp. epiphyte infection. Group (G; Control, Epiphyte infection treatment), population type (P.T; natural or farmed), and phase (P; male and female gametophytes, and tetrasporophytes) were considered as fixed factors. (**A**) Results for diploids individuals of natural and farmed populations. (**B**) Results for females and male gametophytes, and tetrasporophytes from natural populations

A	Group (G)				Population type (P.T)				(G x P.T)		
	Df	F	p	Result	Df	F	p	Result	Df	F	p
ETR_{max}	1	4.90	**0.029***	C > T	1	2.46	0.1192	F = N	1	23.90	**< 0.0001*****
F_v/F_m	1	35.84	**< 0.0001*****	C > T	1	7.07	**0.009****	F < N	1	5.27	**0.023***
α	1	3.87	0.051	C ≤ T	1	2.39	0.125	F = N	1	11.82	**0.009*****
NPQ_{max}	1	48.70	**< 0.0001*****	C > T	1	0.39	0.535	F = N	1	3.14	0.079
B	Group (G)				Phase (P)				(G x P)		
	Df	F	p	Result	Df	F	p	Result	Df	F	p
ETR_{max}	1	35.50	**< 0.0001*****	C > T	2	0.17	0.847	T = H = M	2	6.52	**0.002****
F_v/F_m	1	37.98	**< 0.0001*****	C > T	2	5.16	**0.007****	M > H; T = H and M	2	3.49	**0.033***
α	1	24.72	**< 0.0001*****	C < T	2	2.06	0.131	T = H = M	2	1.24	0.291
NP_{max}	1	34.57	**< 0.0001*****	C > T	2	11.77	**< 0.0001*****	T = H; M > H and T	2	2.72	0.069

Bold significant at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. *F* Farms, *N* Natural populations, *C* Control, *T* Treatment.

of fluorescence (F_v/F_m) showed very similar values in all phases from both populations (Table 1B), with significant differences between control and treatment only for females ($F_{2,156} = 5.737$; $p = 0.004$) (Table 3). Similarly, the initial slope of the ETR (α_{ETR}) was statistically up to 1.5 lower in the control only (Table 1B). Finally, the results of maximal non-photochemical quenching (NPQ_{max}) showed no significant differences between tetrasporophytes of both populations ($F_{1,104} = 0.284$; $p = 0.595$) (Table 3A).

Bold significant at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. *F* Farms, *N* Natural populations, *C* Control, *T* Treatment.

Total phenolic content

The total phenolic content (TPC) in response to epiphytic infection is shown in Tables 4 and 5. In the *Gracilaria* control (i.e. *G. chilensis* cultivated without epiphytes), significant differences in TPC were observed, with tetrasporophytes from

Table 3 Results of ANOVA analyses of the photosynthetic parameters for *Gracilaria chilensis* thalli submitted to *Ectocarpus* sp. epiphyte infection. Group (G: Control or Epiphyte infection treatment), population type (P.T: natural or farmed), and phase (P: males, females or tetrasporophytes) were considered as fixed factors. (A) Results for diploids individuals of natural and farmed populations. (B) Results for females, males and tetrasporophytes from natural populations

A	Group (G)				Population type (P.T)				(G x P.T)		
	Df	F	p	Result	Df	F	p	Result	Df	F	p
ETR_{max}	1	68.71	**< 0.0001*****	C > T	1	5.84	**0.017***	F < N	1	0.54	0.462
F_v/F_m	1	10.45	**0.002****	C ≤ T	1	5.20	**0.024***	F < N	1	2.52	0.115
α	1	4.96	**0.027***	C < T	1	6.10	**0.014***	F < N	1	0.01	0.925
NPQ_{max}	1	0.11	0.744	C = T	1	0.28	0.595	F = N	1	0.00	0.969
B	Group (G)				Phase (P)				(G x P)		
	Df	F	p	Result	Df	F	p	Result	Df	F	p
ETR_{max}	1	72.86	**< 0.0001*****	C > T	2	2.89	0.058	T = H = M	2	4.62	**0.011***
F_v/F_m	1	26.43	**< 0.0001*****	C ≤ T	2	5.74	**0.004****	M = H; T < H and M	2	0.48	0.617
α	1	43.94	**< 0.0001*****	C < T	2	6.88	**0.001****	M = H; M > T and H	2	4.91	**0.008****
NPQ_{max}	1	0.39	0.531	C = T	2	4.06	**0.019***	M = H; T = H; T < M	2	0.37	0.692

Table 4 The total phenolic content (mg (GAE) g^{-1} f.w.) in *Gracilaria chilensis* natural and farmed thalli infected with *Acrochaetium* sp. (A. I.) and *Ectocarpus* sp. (E. I.). Data are given as mean ± S.E. (n = 3 for each phase and population). Distinct lowercase letters denote significant differences after Tukey test

A	Population	Females	Males	Tetrasporophytes
Control	Natural	1.04 ± 0.09^a	0.62 ± 0.04^a	0.23 ± 0.14^b
	Farm			0.31 ± 0.26^b
A. I	Natural	1.65 ± 0.12^a	0.85 ± 0.06^a	1.92 ± 0.22^a
	Farm			1.92 ± 0.17^a
E. I	Natural	0.57 ± 0.05^a	0.43 ± 0.05^a	0.56 ± 0.05^a
	Farm			0.63 ± 0.06^a

natural populations reaching the lowest values, followed by tetrasporophytes from cultivated populations, females and males. For thalli of *Gracilaria chilensis* exposed to *Acrochaetium* sp. or *Ectocarpus* sp. no statistical differences between phases or populations were observed (Tables 4 and 5). However, higher values were found in tetrasporophytes from natural and farmed populations in the case of the red epiphyte and in farmed tetrasporophytes in the case of the brown epiphyte (Table 4).

Discussion

Epiphyte germinating spores trigger a response in Gracilaria chilensis The results presented here confirm that the presence of epiphytes in their initial stage, i.e. settled and recently germinated spores, not only has an effect on the physiology of their basiphyte, but ultimately triggers a response that can be interpreted as a defense mechanism. The significant change in all photosynthetic parameters of *G. chilensis* (except NPQ_{max} after infection by *Ectocarpus* sp.) compared to epiphyte-free controls was associated with highly significant changes in antioxidant capacity, measured here as total phenolic compounds. Photosynthetic parameters were estimated from the fluorescence of photosystem II, which is highly sensitive to redox balance. Negative effects of epiphytes on F_v/F_m have been previously reported in some

Table 5 Results of ANOVA analyses on the total phenolic content of *Gracilaria chilensis* thalli submitted to epiphyte infection. Group (G: Control or Epiphyte infection treatment), population type (P.T: natural or farmed), and phase (P: males, females or tetrasporophytes) were considered as fixed factors. (A) Results for diploids individuals of natural and farmed populations. (B) Results for females, males and tetrasporophytes from natural populations

A	Group (G)				Population type (P.T)				Group x Population type (G x P.T)			
	SumSq	Df	F	p	SumSq	Df	F	p	SumSq	Df	F	p
A. I	38.93	1	66.03	**< 0.0001*****	1.15	1	1.95	0.172	0.17	1	0.28	0.597
E. I	1.32	1	36.31	**< 0.0001*****	0.06	1	1.76	0.194	0.00	1	0.03	0.872
B	Group (G)				Phase (P)				Group x Phase (G x P)			
	SumSq	Df	F	p	SumSq	Df	F	p	SumSq	Df	F	p
A. I	11.85	1	17.92	**0.0001*****	8.48	2	6.41	**0.004****	11.55	2	8.73	**0.0008*****
E. I	0.65	1	5.41	**0.0257***	3.44	2	14.24	**< 0.0001*****	5.66	2	23.41	**< 0.0001*****

Bold significant at $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

seaweeds (e.g. *Kappaphycus alvarezii*, Pang et al. 2011; Borlongan et al. 2016) and aquatic plants (e.g. *Potamogeton crispus*, Chen et al. 2007), showing that the higher the epiphytic load, the greater the reduction in photosynthetic performance of the basiphytes. In *G. chilensis*, it has been shown that mechanical damage associated with type IV or V epiphytes (following Leonardi et al. 2006), such as *Ceramium rubrum* and *Polysiphonia* sp., causes oxidative stress that triggers an oxylipin-mediated defense response (Lion et al. 2006). A defense response was also observed when infected by less aggressive epiphytes such as *Acrochaetium* sp. (epiphyte type II in Leonardi et al. 2006), induced by the presence of oligoagar, which triggered the production of reactive oxygen species (ROS) in the cell wall (Weinberger et al. 2005). This mechanism is similar to the defense of *G. conferta* against agar-degrading bacteria, which was associated with an oxidative burst following the release of oligoagar (Weinberger and Friedlander 2000) and suggests that these epiphytes somehow degrade components of the cell wall, although no effect was observed at the histological level (Leonardi et al. 2006). Taken together, these results suggest that *G. chilensis* is sensitive to the presence of epiphytes, even if they are colonizing the basipetal surface with little or no visible effect, and even at the very early stage of development of these epiphytes (i.e. spore germination).

The response depends upon the epiphyte species However, the two epiphyte species induced a different magnitude of effect on both photosynthetic parameters and the total phenolic content. While *Acrochaetium* sp. was associated with an increase in total phenolic compounds, suggesting a rapid activation of synthesis pathways, infections by *Ectocarpus* sp. were associated with a decrease, suggesting a consumption of part of these compounds. Changes in photosynthetic parameters could be explained by these changes in the redox balance: the decrease in F_v/F_m when infected with *Acrochaetium* sp. but the slight increase when infected with *Ectocarpus* sp. could suggest that the former could have induced the release of ROS inside the cell, whereas the latter would have generated ROS only in the cell wall. ROS in the cell wall is expected to oxidize phenolic compounds present there, which may have induced de novo synthesis within the cell, thereby locally increasing its antioxidant capacity to the benefit of PSII quantum efficiency. However, this hypothesis remains to be tested as only the total phenolic content of the organism was measured in this study. While both ETR_{max} and α changed according to the presence of photosystem stress in the presence of both epiphytes, NPQ_{max} was only reduced when infected by *Acrochaetium* sp., again suggesting that the damage to PSII efficiency may have been so mild that it did not trigger the activation of photoprotective mechanisms when infected by *Ectocarpus* sp., a type I epiphyte.

The settlement fate of *G. chilensis* spores differed unexpectedly between the two epiphyte species. Settlement and germination rates of *Ectocarpus* sp. were significantly reduced compared to an inert substrate, strongly suggesting that *G. chilensis* releases compounds that somehow repel this epiphyte. The identification of these compounds and their biosynthetic pathways may provide some useful opportunities for epiphyte control in agricultural settings. On the contrary, *Acrochaetium* sp. was clearly favored by the biotic surface of the basiphyte. However, this difference between the two species is difficult to reconcile with the physiological response of the basiphytes, which seem to react to their presence regardless of their developmental behavior.

Gracilaria chilensis from natural populations and farms had minor differences in physiological and defense responses There were only minor reductions in photosynthetic parameters when comparing the effect of epiphyte infection between tetrasporophytes from farms and natural populations. This effect was similar for both epiphyte species. Although some of these differences were statistically significant, the magnitude of the effect appeared to be marginal. The differences in colonization and germination rates of epiphytes on farmed and natural individuals were also marginal. This means that the effects of incipient domestication (i.e. selection for fast growers at the expense of reproductive investment) and massive clonal propagation (Guillemin et al. 2008) were not accompanied by relevant changes in traits associated with defense against epiphytes. What was observed was a reduction in the variance of the responses across farms, which could be explained by the presence of reduced genetic diversity in farms due to clonal propagation. However, this variance could also be due to genetic variance underlying the defense-related traits, which should be estimated in future studies with a specific experimental design to explore the possibilities of selective breeding for improved resistance in farms.

The response to epiphytes differs among life cycle stages of G. chilensis The present study confirmed that different life cycle stages of *G. chilensis* respond differently to epiphyte infection. The effect was significant for the total phenolic content and all photosynthetic parameters except ETR_{max} under infection with either *Acrochaetium* sp. or *Ectocarpus* sp. Infected males had the lowest total phenolic content, but this was not associated with significant differences in photosynthetic parameters compared to infected females and tetrasporophytes. However, they had the highest levels of spore colonization and germination, together with tetrasporophytes from natural populations, suggesting that the antioxidant response could be explained by a correlation between epiphyte load and physiological stress. This poor performance of males was also observed in terms of growth

rate and survival in natural populations (Guillemin et al. 2014; Vieira et al. 2018a; b; 2020), and probably explains their absence or strong underrepresentation in farms (Guillemin et al. 2008). Females had the greatest capacity to repel spore colonization and inhibit spore germination, a result that was not correlated with physiological and antioxidant parameters. This capacity was statistically higher than in any other life cycle stage, although the differences were marginal in magnitude compared to cultured tetrasporophytes. These differences may explain, at least in part, the greater variance in all parameters, and particularly in spore settling and germination, discussed above. Therefore, the genetic basis of the response to epiphyte infection may be sex-linked.

Concluding remarks

A high prevalence of epiphytes is often found in *G. chilensis* farms where farmers' cultivation practices favor the presence of tetrasporophytes. This study found that females, which are usually absent from farms, are the most resistant to epiphyte infections. Other studies have also shown that females are more resistant to stressful environments (Vieira et al. 2018a). Taken together, these observations may suggest that the cultivation of females could have a long-term advantage for farms: even though tetrasporophytes grow faster than other life cycle stages, females could reduce biomass losses under stressful conditions and epiphyte invasions in farms. This hypothesis needs to be tested under field conditions.

The ability of *G. chilensis* to detect the presence of early stages of epiphyte infection and to activate some physiological responses points to the role of agar degradation (Weinberger and Friedlander 2000). It has been possible to induce an effective defense against epiphytes in the laboratory by treating *G. chilensis* with oligoagar (Weinberger et al. 2005), suggesting that a defense mechanism exists. In addition to studying its genetic background (as discussed above), triggering such a defense capacity under field conditions is another perspective that should be explored. Since there are currently no chemical pesticides that could act against epiphytes (with reference to their industrial use in land plant agriculture), and their use in the natural environment surrounding marine farms would likely have strong negative effects, stimulating the defense of cultivated algae could be a promising strategy.

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Declarations

Competing interests The authors declare no competing interests.

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