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RESEARCH

# Physiological responses and gene expression of *Gracilaria chilensis* under infection of the green epiphyte *Ulva* sp.

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## Abstract

*Ulva* sp. infection in seaweed is considered one of the most harmful infections, since it penetrates deep into the host cell wall and disorganizes the cortical tissue. In *Gracilaria chilensis* farms, epiphytism is one of the major biological problems and strong fluctuations in landings of this rhodophyte have been documented in the past decades. The present study evaluated the damage of *Ulva* sp. infection through histological assessment, germination of the epiphytic algal spores on *G. chilensis*, the photosynthetic response, and gene expression associated with stress after exposure to epiphyte infection. The histological results showed significant and a characteristic damage according to the type of infection described by this species. In addition, a higher infection in farmed than in natural individuals was observed, along with increased photoinhibition of the maximum fluorescence quantum yield ($F_v/F_m$). Two genes, one related to photosynthesis (*PSBO*) and the other responsible for neutralizing free superoxide radicals (*SOD-C*), followed the same pattern, although due to the high variance no significant differences were detected. *VBPO*, a gene related to the halo-organic compounds synthesis, showed a higher expression in infected thalli, independent of the population type, suggesting a higher protection of the alga from oxidative damage. This study confirms the sensibility of farmed populations of *G. chilensis* to infection by *Ulva* sp. and the existence of an active response to early infection.

**Keywords** Epiphytes · Gene expression · Gracilariales · Rhodophyta · Photosynthetic activity

## Introduction

The damage caused by an epiphyte to its basiphyte can be highly variable and is mainly influenced by the type of anatomical association and the incidence of the epiphyte (Leonardi et al. 2006). Characterization of the epiphyte-host interaction allowed the classification of five different types of anatomical relationships. The higher the type of infection, the higher the damage to the host cell. In infection types I and II, epiphytes are restricted to the host surface without causing any damage to the tissue. Infection types III and IV include epiphytes that alter the outer layers of the host (i.e., cuticula and cortical tissue). In contrast, type V infection corresponds to epiphytes that penetrate deeply into host tissues, seriously damaging the host's cells in the area of infection (Leonardi et al. 2006). Depending on the type of infection, the noxious effects of epiphytes include interference with the growth rate and photosynthetic performance of the basiphyte, loss of its biomass or even whole or partial mortality of host seaweed (Davis et al. 1989; González et al. 1993; Pang et al. 2011; Borlogan et al. 2016; Usandizaga et al. 2023). For example, in the red alga *Gracilaria chilensis, Ectocarpus* sp., a brown algal epiphyte classified as causing type I infection, did not generate any significant negative effect on its physiology, while infection with *Acrochaetium* sp. (infection type II) generated a release of antioxidant and chemopreventive compounds in this basiphyte (Usandizaga et al. 2023).

*Gracilaria chilensis* farming is an important economic activity in Chile, mainly for agar production (Santelices

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and Doty 1989; Buschmann et al. 2008). Over the years, these crops have suffered from the influence of different types of epiphytes, which have led to negative effects on algal production. The most common epiphytes found on farmed *G. chilensis* are red algae of the order Ceramiales (such as *Polysiphonia*, *Ceramium*, *Antithamnion*. and *Callithamnion*; Westermeier et al. 1991; Buschmann et al. 1994, 1997). However, in these populations, other epiphytes have been observed, such as the brown algae *Giffordia* and *Ectocarpus* (Küschel and Buschmann 1991; Usandizaga et al. 2018), diatoms such as *Melosira* (Matamala and Sanhueza 1988) and green algae such as *Ulva* (Matamala and Sanhueza 1988; Buschmann et al. 1992; Buschmann and Gómez 1993) and *Rhizoclonium* (Aroca et al. 2020; Leal et al. 2020). Some of these genera, such as *Ectocarpus* or *Ulva*, have also been reported in farms located in northern Chile, outside the natural distribution range (Leonardi et al. 2006). Other pathogens are included in the phycopathological atlas published by Murúa et al. (2024). The nutrients and the physicochemical characteristics of the overlying water, varying throughout the year, play a fundamental role in shaping the composition and diversity of these epiphyte communities. Indeed, environmental conditions characteristic of austral spring to mid-autumn seem to promote the proliferation of epiphytic algae in *G. chilensis* stands (González et al. 1993).

It has been suggested that populations of *G. chilensis* are sensitive to epiphytic invasion (Aroca et al. 2020; Leal et al. 2020) and infection (Usandizaga et al. 2023). However, defense mechanisms, through the release of antioxidant and chemopreventive compounds (e.g., phenolic compounds; Usandizaga et al. 2023) or oxylipins (Lion et al. 2006), have been shown in *G. chilensis*. The study of gene expressions involved in this response to infection could be of help to understand better the signalling pathways that are activated, and that could be key defense of macroalgae against epiphytes. Studies focusing on the effect of metal pollution on *Gracilaria tenuistipitata* have listed genes involved in chronic stress in these red algae (Tonon et al. 2018). These authors evaluated genes involved in cellular defense mechanisms and photosynthesis in *G. tenuistipitata* exposed to high concentrations of Cd and Cu. Four genes implicated in broad cellular stress responses (*GLC*, Glutamate-cysteine ligase; *TRX*, Thioredoxin; *SOD-C*, Superoxide dismutase; *VBPO*, Vanadium-dependent bromoperoxidase) and one that influences the performance of the photosynthetic apparatus (*PSBO*, Oxygen-evolving photosystem II complex) seem of particular importance in general stress responses. The GLC enzyme is involved in the glutathione (GDH) synthesis, which prevents cellular damage (Tonon et al. 2018). TRX proteins act as a redox regulator, especially in stressful situations (Arnér and Holmgren 2000). SOD catalyzes the dismutation (decomposition) of superoxide radicals into oxygen and hydrogen peroxide. This last reaction is considered an important antioxidant defense in cells exposed to oxygen (McCord and Fridovich 2014). It was also suggested that VBPO could be involved in general response to stress, since this enzyme consumes $H_2O_2$ and is involved in cell wall biosynthesis (Roeder et al. 2005). PSBO protein protects the manganese cluster, which catalyzes water-splitting in photosystem II (Najafpour et al. 2015). Altogether, these genes could be useful indicators of *G. chilensis* sensitivity to early epiphyte infections and help screen the variability of more or less resistant strains by identifying early signs of cellular stress and more or less active antioxidant response.

*Ulva* has been identified as a genus of highly harmful epiphytes, not only because of its type of infection (type IV, disruption of host's cell wall, disorganizing the cortical tissue; Leonardi et al. 2006) but also because the growth of *Ulva* epiphytes can reduce photosynthesis and hinder the normal growth of cultivated seaweed basiphytes (e.g., *Sargassum fusiforme*, Xu et al. 2022). Other detrimental effects on commercial seaweeds have also been reported related to biochemical composition and nutrient competition with the basiphyte (Chen et al. 2015; Sun et al. 2022). These negative effects have been associated with *Ulva* sheet-like morphological structure, associated with high carbon fixation rates and nutrient uptake capacities (Cao et al. 2022). Detrimental effects have been reported in *G. chilensis* populations infected by *Ulva*, as growth inhibition (Friedlander et al. 1996, 2001) and losses of biomass production (Buschmann and Gómez 1993).

We assessed the susceptibility of different populations of *G. chilensis* to *Ulva* epiphyte infection and determined the physiological responses and gene expression after the stress. We addressed the hypothesis that farmed strains are generally more infected by epiphytes than natural populations, either because of a loss of sensitivity (i.e. no antioxidant response is activated following early infection stages, but evidence of damage is detected) or, on the contrary, an increased activation of antioxidant response, compared to wild strains, that allow them to tolerate the presence of epiphyte germlings (i.e. no damage perceived). A third possibility would be that *G. chilensis* is stressed by the settlement of *Ulva* epiphytes but is unable to activate efficiently antioxidant responses. In this case, we expect both antioxidant gene activation and damage detection.

## Materials and methods

### Samples collection

*Gracilaria chilensis* samples were collected from one natural population, located in Lenga (36° 76'S, 73° 17'W, Region of BioBio) and one farm, located in Achao (42° 46'S, 73°

48'W, Region of Los Lagos). Thalli in the natural population presented a holdfast attached to rocks and shells, while in the farm they were buried in the muddy substrate. In the farm, the samples (N = 100) were collected more than 10 m apart to avoid sampling the same clone (Guillemin et al. 2008). In the natural population, individuals correspond to distinct holdfasts.

*Ulva* sp. was collected in Algarrobo (33° 36'S, 71° 68'W, Region of Valparaiso), a locality in the central Chilean coast, in November 2022. All samples were taken to the laboratory in hermetic bags and kept at low temperatures using icepacks during transport.

## Obtention of *Gracilaria chilensis* unialgal cultures and phase determination

*Gracilaria chilensis* unialgal cultures were obtained as described previously by Usandizaga et al. (2023). Briefly, all fragments were cut into smaller pieces (approx. 10 cm) and sonicated twice for 20 s with distilled and filtered seawater. To ensure the absence of epiphytes, the thalli were observed under a microscope and brushed. Then, 1 cm apical fragments were cut and placed in Petri dishes with sterile Provasoli's enriched seawater medium (Provasoli 1968). The cultures were maintained at irradiance 20 µmol photons $m^{-2}$ $s^{-1}$; photoperiod 12:12 (light/dark); temperature 11 °C and weekly change of the medium. After three months, only cultures free of epiphytes were retained.

Since haploid and diploid phases have been shown to present significant physiological differences in *G. chilensis* (Guillemin et al. 2012a; Gallegos Sánchez et al. 2018), which could confound the effects of the experimental manipulations, only tetrasporophytic individuals were selected for the infection experiment. Mature tetrasporophytic individuals were identified under a Stemi DV4 stereoscopic microscope (Zeiss) by direct observation of the reproductive structures. To identify the phase of vegetative thalli, molecular markers were used (Guillemin et al. 2012b).

## Inoculation of *Ulva* sp. and quantification of settlement and germination

Mature *Ulva* sp. thalli were cut into smaller pieces, placed in Petri dishes and then washed and brushed, first with fresh water and then with filtered seawater. The fragments were slightly dehydrated at room temperature and then placed in 250-mL flasks containing 100 mL of cold filtered seawater (4 °C). A minimum of 1 h was allowed for sporulation. The zoospores were counted on an aliquot fixed in Lugol's solution using a Neubauer chamber. The spore concentration was adjusted to 80,000 spores per inoculum.

Thirty tetrasporophytes from each population were infected with *Ulva* sp. For each individual, three pseudoreplicate apical thalli were used for epiphyte inoculation and three were kept as epiphyte-free controls. All *G. chilensis* thalli started at an initial length of 1 cm. Five days after inoculation, the germination rate was estimated by counting all germinated spores of *Ulva* sp. along two transects on each *G. chilensis* thallus. The length (cm) of the basiphyte was evaluated at the end of the experiment. Epiphyte development and cortical damage were observed under an Axiocam 208 mounted on a microscope (Zeiss), on manually generated cross-sections of one of the 3 pseudoreplicates. The experimental procedure lasted 12 days starting with the selection of apical thallus fragments of *G. chilensis*.

To estimate *Ulva* sp. spore germination rate independent of the basiphyte (i.e., on the inert substrate) and determine if the presence of *G. chilensis* increase spore germination, an additional inoculum of 80,000 *Ulva* sp. spores was seeded in 30-mL Petri dishes without any *G. chilensis* thalli (N = 3).

## Detection of damage to the photosystem: Fluorescence measurements

Five algal thalli were selected randomly per population at the end of the experiment (i.e. 12 days) and were incubated for 20 min in the dark before measurement of the maximal quantum yield of fluorescence ($F_v/F_m$, an indicator of the maximal quantum efficiency and photoinhibition, Schreiber et al. 1995) with a Junior PAM (Walz GmbH, Germany). The electron transport rate (ETR, µmol electrons $m^{-2}$ $s^{-1}$) was determined after exposure to 12 increasing irradiances of blue light, every 20 s. ETR was calculated according to Schreiber et al (1995) as follows:

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

where $\Delta F/F_{\mathrm{m}}$ is the effective quantum yield, $E$ is the incident PAR irradiance, $A$ is the absorptance of the thallus and $F_{\mathrm{II}}$ (chlorophyll fraction related to PSII 400–700 m) was 0.15 according to Grzymski et al. (1997) and Figueroa et al. (2003) for red macroalgae. The initial slope of the ETR ($\alpha_{\mathrm{ETR}}$) and the maximum ETR ($\mathrm{ETR}_{\mathrm{max}}$) were calculated by the tangential function reported by Eilers and Peeters (1988). Maximum non-photochemical quenching ($\mathrm{NPQ}_{\mathrm{max}}$) was used as an estimator of photoprotective response and was estimated from the tangential function of NPQ versus irradiance function (αNPQ) according to Eilers and Peeters (1988). These parameters are indicative of the performance

of the photosystem, which is highly sensitive to oxidative stress, and therefore indirectly related to cellular damage.

## Detection of the activation of antioxidant genes' expression: RT-qPCR

Five genes mentioned above were selected from the list of genes potentially involved in *G. tenuistipitata* response to stress published by Tonon et al. (2018). We also selected two housekeepers as reference genes (elongation factor I alpha and tubulin; Tonon et al. (2018)). The sequences of the superoxide dismutase (*SOD-C*), vanadium-dependent bromoperoxidase (*VBPO*), thioredoxin (*TRX*), glutamate-cysteine ligase (*GCL*), oxygen-evolving photosystem II complex (*PSBO*), elongation factor I alpha (*EF1*) and tubulin (*TUB*) were retrieved from the recently sequenced genome of *G. chilensis* (Lipinska et al. 2023; https://rhodoexplorer.sb-roscoff.fr/home/). The primers were designed using Primer3 software (https://bioinfo.ut.ee/primer3/), with melting temperatures between 60 °C and 61 °C, and amplicon length ranging from 100 to 190 bp selected as restrictive parameters for primer selection. Other parameters were kept at the default setting. Primer sequences and amplicon length are listed in Table 1.

## RNA and DNA sample preparation

RNA extraction was performed using the RNAeasy Plant Mini Kit (Qiagen) and the kit RNase free DNase (Qiagen) was used to stabilize the RNA. RNA quantity and quality was evaluated using a Qubit RNA HS assay kit (Invitrogen, ThermoFisher). Twelve biological samples of good quality were used for qRT-PCR per population (i.e., 6 control and 6 infection samples per population). Selected samples were the ones presenting the highest RNA quantity and quality. cDNA was obtained using the Revert aid Rt Kit (ThermoFisher).

Gene level expression was analyzed using quantitative polymerase chain reaction (qPCR) performed using the Maxima SYBR Green kit (K0222, ThermoFisher), according to the manufacturer's instructions. The reactions were carried out in a StepOnePlus thermocycler (Applied Biosystems) with the following steps: a holding stage of 50 °C for 2 min and 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The melt curve used 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. Starting concentration per sample was measured to calculate the relative gene expression. For all samples, 3 technical replicates were used for qRT-PCR.

## Statistical analysis

The differences in germination rate among substrates (i.e., inert, natural and farmed *G. chilensis*) were tested using a one-way ANOVA. The rest of the physiological and molecular parameters of interest (i.e., growth, photosynthetic response and gene expression) were analyzed using a two-way ANOVA (Treatment x Population, Treatment: epiphyte-free control or epiphyte infected; Population: natural or farm) followed by Tukey's HSD tests (R 3.2.4 version) (Cayuela 2011). Levene and Shapiro-Wik tests were used to test the assumptions of homogeneity and normal distribution, respectively. For variables for which non-normal

**Table 1** Primer sequences used for real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) in *Gracilaria chilensis*. Gene Name, code of predicted transcript (see https://rhodoexplorer.sb-roscoff.fr/home/), Primer Sequences (5′ to 3′), and Amplicon Size (bp) are given. F: forward, R: reverse

| Gene_Name | Predicted transcripts | Oligonucleotide_primer_sequence | Amplicon_size |
|---|---|---|---|
| Elongation factor I alpha (EF1) | GChil3786 | F: GTGTCAAGCAGCTTATTGTTGG | 100 pb |
| | | R: ATGGTGGTGACTTCAGCGCG | |
| Tubulin (TUB) | Gchil9565 | F: ACAGTGCGGAAACCAGGT | 156 pb |
| | | R:. GGGACATAGCGACCGTTG | |
| Superoxide dismutase (SOD-C) | Gchil8143 | F:. TACCTATGGCGATCTCTCT | 153 pb |
| | | R: ATACCTGTGTCCTCGTCCAC | |
| Oxygen-evolving photosystem II complex (PSBO) | Gchil6734 | F: CCGCCACCACAGTCCAATTA | 122 pb |
| | | R: AAAGTCGCCTCCAAATTCGG | |
| Vanadium-dependent bromoperoxidase (VBPO) | Gchil985 | F: TCTATTTGACGAACTTGACAAGG | 103 pb |
| | | R: CTCGCTTCTAGCCTGTCCGTTG | |
| Thioredoxin (TRX) | Gchil7505 | F: ACAGCATCAGACTCCACCAA | 150 pb |
| | | R: GCATTGCAACGACGGAGATA | |
| Glutamate-cysteine ligase (GCL) | Gchil7135 | F: TGGGTCTTCTTACTCTTGGCA | 150 pb |
| | | R: CCTCATCGCCCCATTTCAAA | |

Elongation factor I alpha and tubulin were used as endogenous controls

residuals and/or heterocedasticity were detected, the data were transformed. A Yeo-Johnson transformation was used for infection analysis, a logarithmic transformation was used for $ETR_{max}$ while Box-Cox transformations were used for growth, number of germinated spores, $F_v/F_m$ and gene expression before analyses.

## Results

### Histological observation

Histological observations confirmed the physical connections between the basiphyte, *G. chilensis,* and its epiphyte, *Ulva* sp. (Fig. 1). Significant damage could be observed in *G. chilensis*, where *Ulva* rhizoids disorganized the cortical tissue (Fig. 1A). The rhizomatous holdfast of *Ulva* sp. disrupted the outer layer of the host cell wall (Fig. 1B); penetration was intercellular.

### Differences in epiphyte infection between farmed and natural populations of *G. chilensis*

Germination of *Ulva* sp. spores on *G. chilensis* thalli from the farmed population ($18.12 \pm 7.15$) was significantly higher than the one obtained on thalli from the natural population ($6.69 \pm 2.52$) (Fig. 2; $F_{2,178} = 9.73$; $p < 0.001$) and in the Petri plate ($6.11 \pm 0.84$) ($F_{2,178} = 9.73$; $p = 0.033$). No significant differences were found between germination on thalli from the natural population and on the inert substrate (Fig. 2; $F_{2,178} = 9.73$; $p = 0.944$).

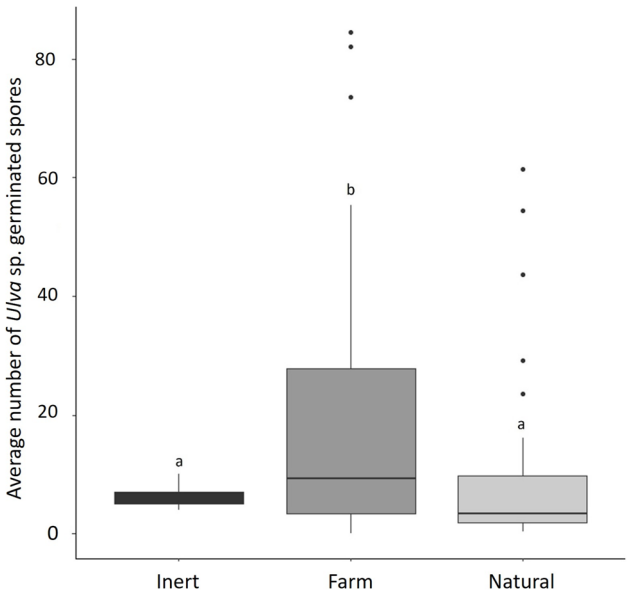


**Fig. 2** Average number of spores of *Ulva* sp. germinated on inert substrate and on thalli from cultivated and natural populations of *Gracilaria chilensis*. Lowercase letters indicate differences at $p < 0.05$. Box plot whiskers show the 1%–99% range values; the horizontal line in each box plot is the median, and the colored segment shows the quartile range (25%–75%). Values outside of the whisker range are plotted as dots

### Effect of epiphyte infection on *G. chilensis* growth and photosynthesis

Whatever the experimental condition, a lower growth was observed for the thalli sampled from the natural population of Lenga than those from the farm of Achao (natural infected: $1.52 \pm 0.07$ cm; natural non-infected: $1.50 \pm 0.14$ cm; farmed infected: $2.04 \pm 0.20$ cm; farmed non infected: $1.72 \pm 0.19$ cm; Fig. 3). Thalli sampled on the farm presented a slightly but significantly higher growth

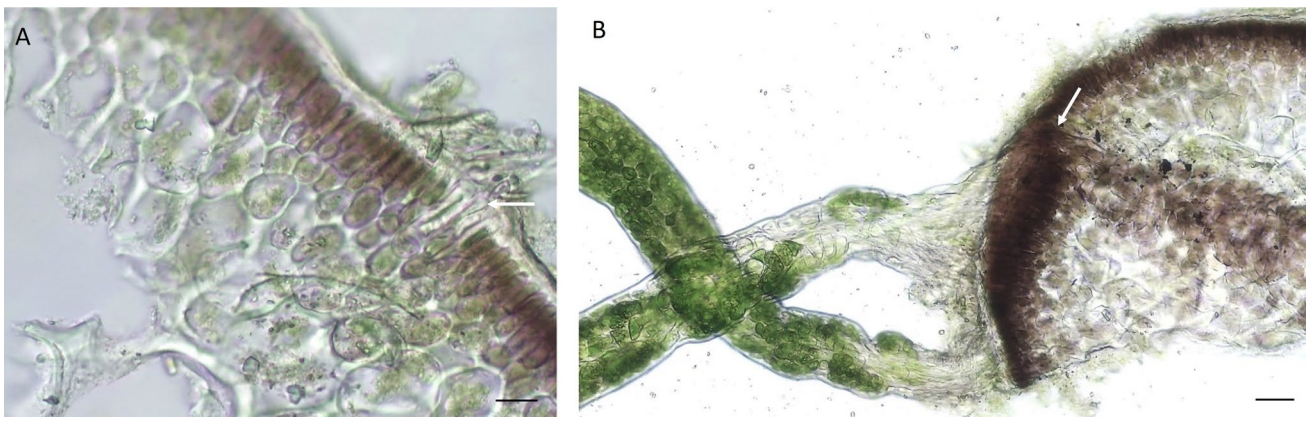


**Fig. 1** Cross-sections of thalli of *Gracilaria chilensis* infected by *Ulva* sp. after three weeks; (**A**) a close-up of the rhizoid cells penetrating the *G. chilensis* cell wall (**B**). The white arrow indicates the entry of the *Ulva* sp. rhizoids into *G. chilensis* tissue. Scale bars: 20 µm

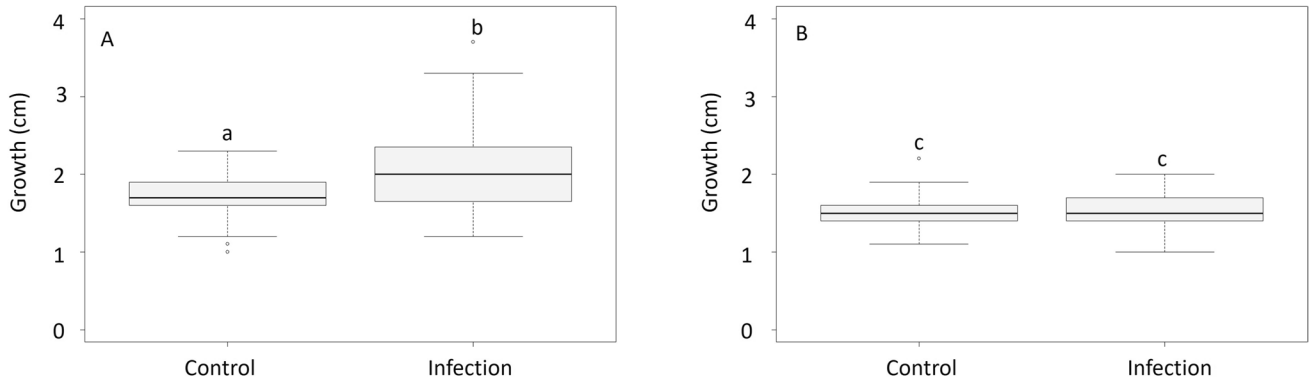


**Fig. 3** Growth (cm) of farmed (**A**) and natural (**B**) populations of *Gracilaria chilensis* of the infected and epiphyte-free control thalli (i.e. *Gracilaria* thalli without *Ulva* sp.). Box plot whiskers show the 1%–99% range values; the horizontal line in each box plot is the median, and the colored segment shows the quartile range (25%–75%). Lowercase letters indicate differences at $p < 0.05$

**Table 2** Photosynthetic parameters measured in *Gracilaria chilensis* natural and farmed population without epiphyte infection (A) and with *Ulva* spp. infection (B)

| A | Natural population | Farmed population |
|---|---|---|
| $ETR_{max}$ (µmol electrons $m^{-2}$ $s^{-1}$) | $77.41 \pm 27.95^{a}$ | $71.47 \pm 15.57^{a}$ |
| $F_v/F_m$ | $0.53 \pm 0.03^{a}$ | $0.49 \pm 0.03^{bc}$ |
| αETR (µmol photons $m^{-2}$ $s^{-1}$) | $0.26 \pm 0.08^{ab}$ | $0.23 \pm 0.05^{a}$ |
| $NPQ_{max}$ | $0.78 \pm 0.12^{a}$ | $0.65 \pm 0.12^{b}$ |
| B | | |
| $ETR_{max}$ (µmol electrons $m^{-2}$ $s^{-1}$) | $88.21 \pm 24.01^{ab}$ | $115.36 \pm 34.41^{b}$ |
| $F_v/F_m$ | $0.51 \pm 0.03^{ab}$ | $0.45 \pm 0.06^{c}$ |
| αETR (µmol photons $m^{-2}$ $s^{-1}$) | $0.26 \pm 0.06^{ab}$ | $0.29 \pm 0.05^{b}$ |
| $NPQ_{max}$ | $0.76 \pm 0.14^{ab}$ | $0.68 \pm 0.13^{b}$ |

when infected than non-infected ($F_{1,173} = 11.27$; $p = 0.001$; Fig. 3). No significant difference in terms of growth rate was detected between infected and non-infected thalli from the natural population ($F_{1,121} = 0.466$; $p = 0.496$).

Both the maximum electron transport rate ($ETR_{max}$) and maximum fluorescence quantum yield ($F_v/F_m$) were affected by the experimental treatment (Table 2 and 3; $F_{1,50} = 11.913$; $p = 0.001$; $F_{1,54} = 23.917$; $p < 0.0001$). $ETR_{max}$ values were higher for infected than non-infected thalli and $F_v/F_m$ values were lower for infected than non-infected thalli (Table 2). Whatever the experimental treatment, the farmed population of Achao presented lower values of $F_v/F_m$ than the natural population, suggesting higher photoinhibition in farmed thalli (Table 2 and 3; $F_{1,54} = 23.917$; $p < 0.0001$). The interaction between experimental treatment and the population type significantly affected the photosynthetic efficiency ($_{\alpha}$ETR), with significantly higher values in infected thalli than in non-infected thalli from the farmed population (Table 2 and 3; $F_{1,56} = 4.771$; $p = 0.033$). Significantly higher values of $NPQ_{max}$ were observed in the natural population than in the farm (Table 2), however post-hoc analysis revealed that this difference was reduced when infected by *Ulva* sp. (Table 3).

## Gene expression analysis

qRT-PCR results showed that *VBPO* gene expression increases with infection, and consistently across populations, unlike the *PBSO* gene, which decreases with infection, regardless of the population studied. The other 3 genes have a different behavior between populations: *TRX* and *SOD* decrease with infection only in farm, while *GLC* has

**Table 3** Results of ANOVA analysis of the photosynthetic parameters for *Gracilaria chilensis* thalli submitted to *Ulva* spp. epiphyte infection. Treatment (T; Control, Epiphyte infection treatment), population type (P.T; natural or farmed) were considered as fixed factors

| | Treatment (T) | | | Population type (P.T.) | | | (T x P.T) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Df | F | p | Df | F | p | Df | F | p |
| $ETR_{max}$ | 1 | 11.913 | **0.001**** | 1 | 2.228 | 0.142 | 1 | 3.099 | 0.084 |
| $F_v/F_m$ | 1 | 8.102 | **0.006**** | 1 | 23.917 | **< 0.001***** | 1 | 0.389 | 0.536 |
| αETR | 1 | 3.778 | 0.057 | 1 | 0.022 | 0.882 | 1 | 4.771 | **0.033*** |
| $NPQ_{max}$ | 1 | 0.267 | 0.607 | 1 | 13.813 | **< 0.001***** | 1 | 1.759 | 0.190 |

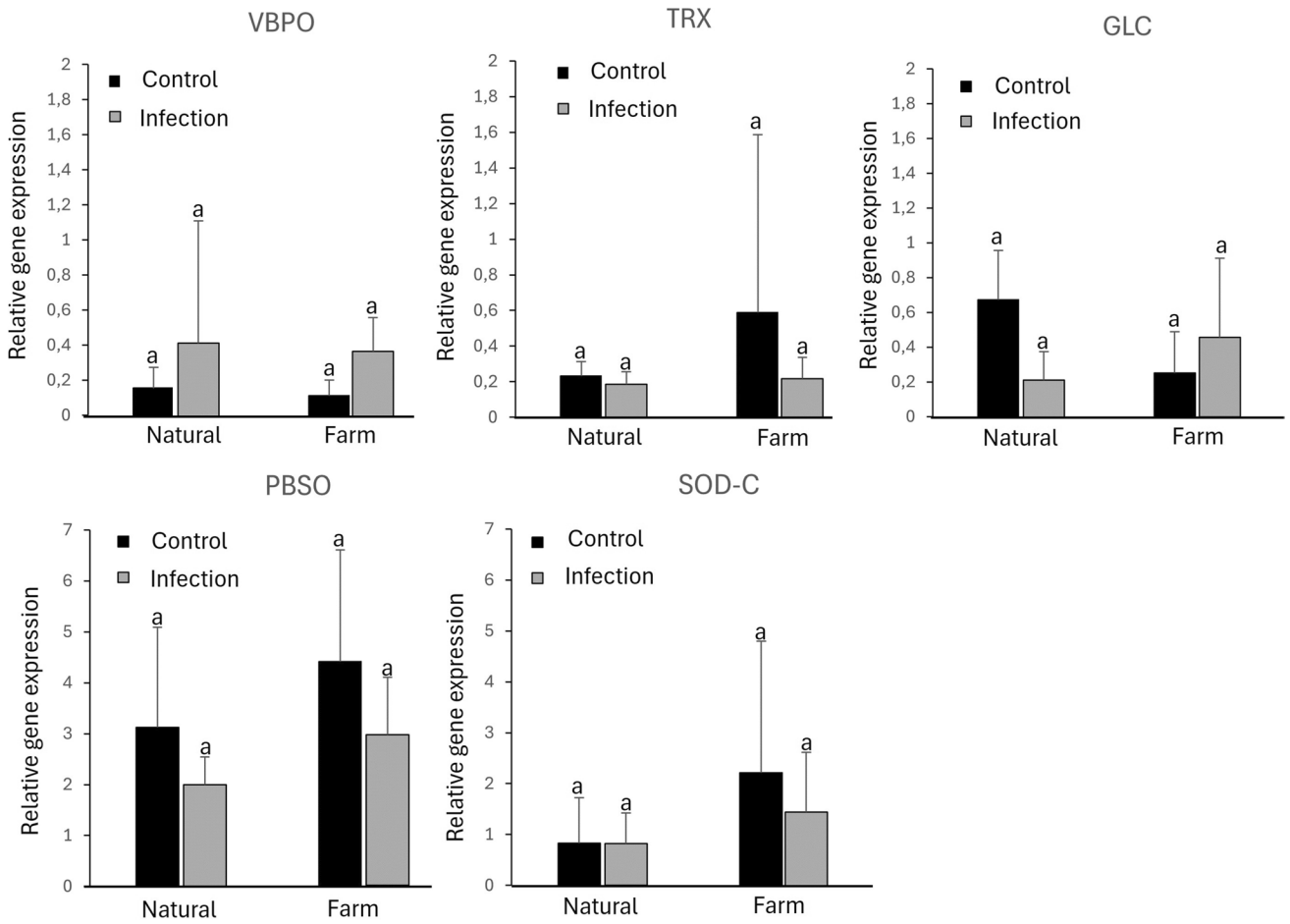


**Fig. 4** Gene expression ratios from natural and farmed populations in control an infection condition. The lowercase refers to the values between populations and treatment, these *p* values were calculated for each gene independently. Results shown are average ± standard deviation

an inverted pattern with under expression in infected natural populations, but overexpression in infected farm (Fig. 4). The variance between strains was particularly high in some cases, such as *VBPO* infected natural population, *TRX* control farm, or *SOD* farm (both control and infected), which explains the lack of statistical power to discriminate these trends.

## Discussion

The higher susceptibility of farmed individuals of *G. chilensis* to epiphyte infection was confirmed in these trials, in agreement with other field observations and laboratory experiments, showing a general pattern in which farmed populations are affected by different epiphyte species (Buschmann and Gómez 1993; Buschmann et al. 1997; Usandizaga et al. 2018, 2023). This is the first study to explore the effects of a type V epiphyte as per the Leonardi et al. (2006) classification, that is, with effects of tissue disorganization and mechanical damage evident from the very early stages of infection. In this context, we expected *Ulva* sp. could be a good model to test the response of *G. chilensis* to epiphyte infections experimentally and decipher the possible mechanisms explaining the enhanced susceptibility of farmed individuals.

*Physiological responses differ among populations*. The initial stage of *Ulva* sp. (spore germination) did affect the physiology of *G. chilensis*, specifically in farmed population. This result is consistent with another study, where the presence of epiphytes (e.g. *Acrochaetium* sp.) in their initial stage (settled or germinated spores) had a negative effect on *G. chilensis* photosynthetic performance (Usandizaga et al. 2023). In that study, other mechanisms were activated due to the stress of the algae, such as the increased production of phenolic compounds, which generally showed that infected *G. chilensis* individuals had a higher defense capacity. Therefore, these results, together with those obtained in this study, confirm that *G. chilensis*

responds rapidly to an early infection regardless of the type of epiphyte.

In our study, the photosynthetic capacity was improved (higher values of electron transport, $ETR_{max}$ and energy utilization efficiency, αETR) in infected farmed populations of *Gracilaria*, which could be related to the higher growth observed. The increase of $ETR_{max}$ can be explained by a better redox condition, due to the activation of antioxidant mechanisms. Considering that the infection increases the antioxidant capacity, it is possible that once epiphyte spores settle and germinate, and before causing severe damage, an active response to the infection is generated in *G. chilensis* that perhaps anticipates oxidative stress. Studies in *G. chilensis* have shown that agar oligosaccharides are released when an epiphyte or bacteria from the surface of the algae attacks the cell wall, inducing a fast oxidative burst in which $H_2O_2$ (i.e., oxidative stress response) is released into the medium as a defense mechanism. This mechanism involves an agar oligosaccharide oxidase located in the cell wall (Weinberger et al. 2005). A comparative study of agar oligosaccharide responses within the Gracilariaceae revealed that two mechanisms of $H_2O_2$ release exist in the *Gracilaria* "sensu lato" major clade: i) direct oxidation of agar oligosaccharides; ii) oxidative burst triggered by oligoagar (Weinberger et al. 2010). In the case of *G. chilensis* the first mechanism would occur and that's how oxidative stress would begin.

In both red and brown algae, endogenous elicitors act as signals that stimulate defense responses. These elicitors are often cell wall degradation products due to the activity of lytic enzymes secreted by harmful organisms (Weinberger 2007). Weinberger et al. (2011) demonstrated that an extract from infected alga could trigger a defense response in a non-infected one. The metabolic response consisted of the activation of metabolic pathways (a change in the metabolic profile was detected in those elicited by the extract), which led to an increase of the lipoxygenase and phospholipase activity (lipid oxidizing enzymes), which also produces $H_2O_2$, as part of the active response against epiphytes. Our results demonstrated that there is an active response in other genes that are part of the antioxidant machinery. This could eventually explain the improvement in the redox condition that favours $ETR_{max}$.

On the contrary, the natural population showed very similar $F_v/F_m$ values with or without epiphyte infections. Comparable non-photochemical quenching values (i.e., $NPQ_{max}$) were also observed, meaning that photoprotection mechanisms were not activated.

*Gene expression differ among populations*. It has been hypothesized that ROS produced by oxidative burst might be an internal signal to induce the expression of genes with defense-related function (Cosse et al. 2009). Our study showed that *VBPO* gene expression tended to increase in those *G. chilensis* individuals subjected to *Ulva* sp. infection in both natural and farmed populations. Specifically, *VBPO* is related to the synthesis of halo-organic compounds that may have an active role in pathogen and herbivore deterrence (Al-Adilah et al. 2022) and contribute to the consumption of peroxide (Yotsukura et al. 2010). Studies in *Saccharina japonica* have already shown that the increase of *VBPO* expression has two main roles, protecting the alga from oxidative damage and producing antimicrobial products that prevent growth inhibition due to the adherence of microalgae and/or bacteria on the surface of the lamina (Yotsukura et al. 2010). In our study, the generally higher expression level of *VBPO* in the infected thalli agrees with an increased protection against epiphyte spore settlement and germination, and against the early triggering of oxidative stress. *PSBO* gene had a slightly lower expression when the thalli were infected with *Ulva* sp., regardless of population type. PSBO is an extrinsic subunit of the membrane-associated photosynthetic redox enzyme photosystem II that plays a central role in the stabilization of the catalytic manganese cluster, which is the primary site of water splitting during the photosynthetic process (Popelkova and Yocum 2011), promoting a stable oxygen release (Enami et al. 2008). When photosynthetic rates are high, more electrons are required from water splitting. In this case, a positive correlation between *PSBO* expression and $ETR_{max}$ would have been expected. The opposite trend suggests that downregulation of PSBO gene expression may contribute to lower production of $O_2$, therefore reducing the production of damaging intracellular superoxide radicals during the oxidative burst. This hypothesis should be further tested to better understand the complex nature of antioxidant mechanisms during early epiphyte infections.

*SOD* and *TRX* expression, in turn, seem to decrease with infection only in farms. *TRX* gene is related to antioxidant functions (Fierro et al. 2017) and it has been reported as necessary to maintain cellular functions during stress conditions (e.g. desiccation) in the red alga *Pyropia orbicularis* and the brown alga *Scytosiphon lomentaria* (Pearson et al. 2010; Contreras-Porcia et al. 2011). SOD-C is a periplasmic antioxidant protein containing Cu, $Zn^{2+}$ cofactors. In bacteria, it has been shown to protect from external damage factors (exogenously generated $O_2$) and contributes to bacteria resistance to oxidative damage (Keith and Valvano 2007). In seaweed, Aguilera et al. (2002) showed that those seaweeds more exposed to highlight presented higher SOD activities. Interestingly, in that study, the authors suggest that permanent exposure to some external factors, such as low water temperature, causes a higher background level of ROS (reactive oxygen species), requiring a more efficient detoxification system, which is reflected by increased SOD activity. In Chile, farms are subject to significant variations in epiphyte loads, which could have generate a higher ROS background level in these populations compared to the natural ones. Finally, GLC encodes an enzyme critical for

synthesizing glutathione (GSH). GSH is potentially essential during increased oxidative stress, providing the glutathione used in redox control to prevent oxidative stress (Collén et al. 2007). Therefore, it appears intuitive that in our study, the infected farm showed a slight overexpression of GLC.

## Conclusion

This study confirms the increased sensibility of farmed populations of *G. chilensis* to infection by *Ulva* sp. and the species' ability to respond to early infections. Unfortunately, due to the high variances observed in gene expression, no conclusive impact of infection on the expression of photosynthetic-related genes and genes involved in cellular defense mechanisms was observed. Even considering these limitations, the expression patterns observed for the *VBPO* and *PSBO* genes are very encouraging, and further enquiries are now needed to test their potential as biomarkers to monitor the physiological responses during epiphytic infection. Nevertheless, more specific molecular biomarkers of epiphytic infection should be explored to better understand the reduced defense reactions of cultivated populations and elucidate the possible mechanisms or pathways that are activated to cope with damage of their cell wall structure.

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**Data Availability** Data are available from the corresponding author upon reasonable request.

## Declarations

**Competing Interests** The authors declare no competing interests.

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