Microalgae cross-fertilization: short-term effects of *Galdieria phlegrea* extract on growth, photosynthesis and enzyme activity of *Chlorella sorokiniana* cells

Giovanna Salbitani¹ · Petronia Carillo² · Catello Di Martino³ · Francesco Bolinesi¹ · Olga Mangoni¹ · Francesco Loreto¹ · Simona Carfagna¹

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**Abstract**

*Galdieria* spp. (Rhodophyta) are polyextremophile microalgae known for their important antioxidant properties in different biological systems. Nowadays, the beneficial and bio-stimulant effect of microalgal extracts is widely tested on crops. Here, for the first time, potential positive effects of aqueous extracts from *Galdieria* were tested on a second microalgal culture systems. *Chlorella sorokiniana* cultures were supplemented with *Galdieria phlegrea* extracts (EC) and the short-term (48 h) effects of extract addition on growth and biochemical and physiological parameters were monitored and compared to those of non-supplemented *Chlorella* (CC). Growth of *Chlorella* was improved in EC as shown by higher optical density and cells number in the enriched cultures. In addition, EC appreciably increased the pigments (chlorophyll (*a* and *b*) and carotenoids) contents of *Chlorella* cells. Increase of photosynthetic pigments was associated with higher photosynthesis and lower non-radiative dissipation of light in EC as indicated by chlorophyll fluorescence parameters. Reduced activities of antioxidant enzymes (SOD, CAT and APX), but increased total antioxidant capacity (ABTS) were observed in EC, suggesting that this culture was under a low oxidative status, but can activate antioxidant defences if exposed to oxidative stress. In conclusion, a short-term positive effect of the addition of *G. phlegrea* extracts on growth and physiology of *C. sorokiniana* was demonstrated.

**Keywords** Antioxidant enzymes · Chlorophyll fluorescence · *Chlorella sorokiniana* · Fertilization · *Galdieria phlegrea* · Photosynthesis

**Introduction**

Microalgae are photosynthetic organisms able to colonize a wide range of habitats, from aquatic environments comprising lakes, ponds, rivers and oceans, to soils. Thus microalgae represent a rich biodiversity (~800,000 estimated and ~ 40,000 described species) (Hu et al. 2008; Ronga et al. 2019). Microalgae utilization by humans dates back 2000 years. However, microalgal biotechnology only really started developing in the middle of the last century (Spolaore et al. 2006). The microalgae-based industry for biomass and bio-products has considerably gained value over the last years. Nowadays, there are numerous commercial applications of these microorganisms including human and animal food supplementation, cosmetics, biofuels, pharmaceuticals and cosmeceuticals (Khan et al. 2018; Vona et al. 2018; Colla and Rouphael 2020; Levasseur et al. 2020; Napolitano et al. 2020). In addition, microalgal factories do not compete with terrestrial crops for land use. Rather, they represent an excellent alternative for cultivation and exploitation of marginal areas with environmental and economic benefits. Among the most noteworthy and exploited microalgae, *Chlorella* spp. ( Chlorophyceae) are widely used as food and as a recognized source of interesting molecules such as vitamins, proteins, lipids, pigments and minerals (Miyazawa et al. 2013; Safi
et al. 2014; Saini et al. 2020). As food or food ingredient *Chlorella* spp. have been “Generally Recognized As Safe” (GRAS) by the US Food and Drug Administration (FDA) and by the European Food Safety Authority (EFSA) (García et al. 2017; Wells et al. 2017; Caporgno et al. 2019). In particular, *Chlorella* represents an interesting source of chlorophylls and carotenoids. These pigments are well-known antioxidant molecules whose production can be enhanced by cultivation conditions (Salbitani et al. 2020a, b).

Interest on *Chlorella* spp. also arose due to their resistance to high light conditions typical of photobioreactors and due to their high productivity with a world annual dried biomass production of about 5000 t (Cazzaniga et al. 2014; Li et al. 2018; Levasseur et al. 2020).

Recently algae are also gaining interest as plant biostimulants, with the main goal of helping sustainable agricultural intensification by improving nutrients use efficiency and abiotic stress tolerance (Carillo et al. 2020 and references therein). This possibility captured the attention of farmers and agrochemical industries aiming to sustainably secure future crop yield stability (Ronga et al. 2019). According to Renuka et al. (2018) microalga-derived products can indeed facilitate nutrient uptake, improving the physiological status of plants and their tolerance to abiotic stress. Nowadays, aqueous extracts from microalga have been effectively employed as biostimulants to several crops such as tomatoes, sugar beets and peppers (Chanda et al. 2019; Puglisi et al. 2020). Microagal extracts can stimulate germination, seedling, lateral root growth, aboveground growth and biomass in several crops (Garcia-Gonzalez and Sommerfeld 2016; Barone et al. 2018; Chiaiese et al. 2018; El Arroussi et al. 2018). Among the compounds with biostimulant action in algal extracts there are phytohormones or hormone-like substances (Stirk et al. 2013), mineral nutrients, amino acids, betaines, peptides or proteins, vitamins (Khan et al. 2009), polysaccharides and polyamines (Mògor et al. 2018; Chanda et al. 2019).

To date, however, the beneficial effect of microalgal extracts has only been tested on plant crops and never on other microalga cultures. In this study we fill the gap, testing for the first time the possible use of a microalgal extract from *Galdieria phlegrea* to improve growth and metabolism of cultures of *Chlorella sorokiniana*, one of the most commercially exploited microalga.

We used *G. phlegrea* because extracts of this extremophilic microalga have shown important antioxidant properties in different biological systems (Carfagna et al. 2015; Bottone et al. 2019). The extremophilic red microalgae of the genus *Galdieria* are also attracting the attention of the scientific community for phycocyanin production (Carfagna et al. 2018) or, when grown mixo- or hetero-trophically, for purifying urban or food waste (Selvaratnam et al. 2014; Sloth et al. 2017; Salbitani and Carfagna 2020, 2021). *Galdieria phlegrea* (Cyanidiophyceae) is a species isolated in cryptoendolithic environments at Phlegrean Fields (Naples, Italy), adapted to relatively dry conditions and to reduced light intensities (Pinto et al. 2007; Carfagna et al. 2018). Extremophilic organisms, such as *Galdieria* spp., have developed special mechanisms that allow cells to grow and thrive in harsh environment. Although the molecular strategies for survival in hostile environments are not fully explained yet, it is known that these organisms produce biomolecules and peculiar biochemical pathways of great biotechnological interest (Rampelotto 2013).

In the present research we preliminary assessed the effects of aqueous *G. phlegrea* extracts to improve cultivation of another microalga (*C. sorokiniana*). In particular, we evaluated *G. phlegrea* short-term beneficial effects on growth, photosynthetic efficiency, and antioxidant response of *C. sorokiniana* cells. The applied aim of this research was to demonstrate that microalgae can be exploited as bioactive molecule sources not only for plant crops but also for improving microalgae cultivation systems.

### Materials and methods

#### Algal strains and cultivation

Experiments were performed with pure cultures of *Galdieria phlegrea* (strain 009) from the ACUF (Algal Collection University of Federico II, http://www.acuf.net/index.php?lang=en). *Galdieria* was grown in Allen’s medium (Allen 1959). The pH was set at 1.5 with sulfuric acid and controlled daily. The cultures were kept at 34.0 ± 1.5 °C in a thermostatic chamber (Angelantoni CH 770), under continuous light (light intensity 100 μmol photons m⁻² s⁻¹) and flushed with filtered (sterile filter CA 0.22 μm) natural air.

*Chlorella sorokiniana* strain 211/8K (CCAP of Cambridge University), was grown in the basal medium at pH 6.5 as previously described by Salbitani et al. (2014). The cultures were kept at 30.0 ± 1.5 °C, under continuous light (LED panels, light intensity 100 μmol photons m⁻² s⁻¹) and flushed with filtered (sterile filter CA 0.22 μm) air.

When the *Chlorella* culture was in the exponential growth phase it was divided into two sub-cultures: 1) Control Culture (CC), maintained in basal medium; 2) Enriched Culture (EC), maintained in basal medium enriched with *Galdieria* extract. The aqueous extract was added to EC as 12 μg of *Galdieria* proteins per mL of *Chlorella* culture. This was T0, when the number of cells in the culture was around 1.0-2.0 × 10⁶ cells mL⁻¹. Further information about extract preparation is in the next section.

The growth of *C. sorokiniana* was monitored for 48 h as changes in optical density (OD₅₆₀), cells number, cellular size, growth rate. The cells number and cells size of
Chlorella were determined by Countess II FL automated cell counter (Thermo Fisher Scientific) equipped with a fluorescence filter (Ex 628/40, Em 692/40; EVOS Light Cube for Cy5; Thermo Fisher Scientific Inc.).

The growth rate of Chlorella was calculated using the following formula: \( \mu = \ln(N_2/N_1)/t \), where \( t \) is the time (days) of observation, \( N_1 \) is the cell concentration (cell mL\(^{-1} \)) at the beginning of the experiment period, and \( N_2 \) is the cell concentration at the end of the experimental period.

Preparation of Galdieria crude extract and protein determination

Galdieria cells (from 300 mL of culture) were harvested by low-speed centrifugation (4000 x\( g \) for 8 min) and washed twice in distilled water to remove culture medium components. The packed cells were re-suspended in 5 mL of cold extraction buffer (50 mM phosphate pH 7.5) and broken by passing them twice through a French cell press (1000 psi). The homogenate was centrifuged at 12,000 x\( g \) and 4 °C for 30 min, and the clear supernatant was used as crude extract. In the extracts the concentration of proteins was determined using the Bio-Rad protein assay based on the Bradford method (1976), using bovine serum albumin (BSA) as the standard.

Soluble carbohydrates and polysaccharides analysis in Galdieria

Soluble sugars were determined according to Carillo et al. (2019). The method was modified as it follows. Aliquots of 50 μL of crude extract were suspended in 250 μL of ethanol (98%, v/v), incubated for 20 min at 80 °C in a water bath and centrifuged at 14,000 x\( g \) for 10 min at 4 °C. The clear supernatants were separated from the pellets and stored in 1 mL tubes at 4 °C. The pellets were then treated with two subsequent extractions with 150 μL of 80% ethanol (v/v) and 250 μL of 50% ethanol (v/v). Each extraction was followed by an incubation for 20 min in a water bath at 80 °C, and a centrifugation at 14,000 x\( g \), for 10 min at 4 °C. At this stage, the supernatants of the first and the two subsequent extractions were pooled and stored at -20 °C until analysis. The pellets of the ethanolic extraction were heated at 90 °C for 2 h in 500 μL of 0.1 M KOH. After cooling the samples were put in ice and acidified to pH 4.5 with 80 μL of 1 M acetic acid. An aliquot of 400 μL of acidified samples was added to 400 μL of 50 mM sodium acetate pH 4.8 containing 0.2 U α-amylase and 2 U amyloglucosidase and incubated at 37 °C for 18 h. The samples were vortexed and then centrifuged at 13,000 x\( g \) for 10 min at 4 °C and the supernatant containing the glucose derived from hydrolysed polysaccharides was used for measurement. Soluble glucose as well as glucose originating from saccharides hydrolysis were analysed enzymatically according to Carillo et al. (2019).

Determination of photosynthetic pigments in Chlorella

Cells from 10 mL of culture were collected by a low-speed centrifugation (4000 x\( g \) for 8 min). Chlorophylls (Chl \( a \) and Chl \( b \)) and total carotenoids (Car) were extracted with N,N-dimethylformamide and assayed according to Inskipp and Bloom (1985) and Wellburn (1994), respectively.

Fluorescence parameters determination in Chlorella

In order to define adaptation and photosynthetic capacity in control and enriched cells, samples were analyzed with an IMAGING-PAM M-Series Chlorophyll Fluorometer (Walz). An aliquot of 15 mL of Chlorella cultures was separated from the medium by filtration on 0.2 μm pore size Sartorius polyamide membrane. Filtered microalgae were acclimated in the dark for 30 min before analysis. After dark adaptation, the maximal quantum efficiency of PSII in the dark (\( F_v/F_{m} \), where \( F_v \) is the variable and \( F_{m} \) is the maximal fluorescence in dark-adapted organisms) was measured and filtered Chlorella was then exposed to growing actinic light (AL). AL was increased between 1-700 μmol photons m\(^{-2} \) s\(^{-1} \) of photosynthetically active radiation (PAR) at 20 s-long steps, to measure light responses. The parameters measured at each step of the light response were: 1. the effective quantum efficiency of PSII (\( \Phi_{PSII} \), \( \Phi_{PSII} = (F_{m}′ - F_v)/F_{m}′ \), where: \( F_{m}′ \) is light-adapted maximum fluorescence and \( F_v \) is the light-adapted steady-state fluorescence; 2. the electron transport rate of PSII (ETR). ETR = \( \Phi_{PSII} \times \) PAR × 0.84 × 0.5 where the two numeric coefficients correct for light absorbance and the partitioning of light between the two photosystems, respectively; 3. The non-photochemical energy loss (quenching) in PSII or light-dependent heat dissipation quantum efficiency of PSII (NPQ). NPQ = \( F_v/F_{m}′ - F_v/F_{m} \).

Antioxidant activity determination in Chlorella

Algal cells (250 mL) were harvested by low-speed centrifugation (4500 x\( g \) for 8 min), re-suspended in 3 mL of cold extraction buffer (50 mM phosphate pH 7.5) and broken by passing twice through a French Press cell (1000 psi). The homogenate was centrifuged at 12,000 x\( g \) for 30 min at 4°C and the clear supernatant was used as crude extract. According to the method of Re et al. (1999), antioxidant activities were determined by decolourisation of the ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] determined at 734 nm. For this study, ascorbic acid (Asa) was used as antioxidant standard (0-20 μM). For determining antioxidant enzymes activities, algal cells (300 mL) were
harvested by low-speed centrifugation (4500 x g for 8 min), re-suspended in 3 mL of cold extraction buffer (according to enzyme assay) and broken by passing twice through a French Press cell (1000 psi). The catalase (CAT, EC 1.11.1.6) activity was measured by a Catalase Assay kit (MyBioSource, MBS841637) as reaction of H2O2 with OxiRed probe. The ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined by detecting the decrease of ascorbate at 290 nm by APX assay Kit (MyBioSource, MBS2548460). The superoxide dismutase (SOD, EC 1.15.1.1) activity was measured as blue formazan production by SOD Assay Kit (MyBioSource, MBS2548473).

Statistical analyses

Data analyses were carried out using Sigmaplot 14 software. Data of the mean ± standard deviation of three independent experiments were presented. Significant differences (*p < 0.05, **p < 0.01, ***p < 0.001) were determined by Student’s t test or one-way analysis of variance (ANOVA) was performed with a Tukey post-hoc test.

Results

Effect of Galdieria extract supplementation on Chlorella cell growth

After 24 h from the addition of Galdieria extract significant increases of cells number (p<0.01) and optical density (p<0.001) were observed in the Enriched Culture (EC) with respect to the Control Culture (CC). After 48 h, the optical density of the cultures and the number of the cells of the EC was 1.4 and 1.8 times higher than in CC, respectively (Fig. 1A-B). An important enlargement in the average cell diameter (from 3.45 ± 0.22 μm at T0 to 4.98 ± 0.02 μm (p<0.001)) was observed 2 h the extract addition in EC. EC cell size was significantly (p<0.01) higher than in CC also after 24 h but dropped to values comparable to those measured in CC after 48 h (Fig. 1C).

The specific growth rate was measured over the entire experimental time (24 and 48 h) and resulted significantly higher (p<0.001) in EC compared to CC at the end of the experiment (Table 1).

Growth of Chlorella supplemented with glucose

In the crude extracts of Galdieria fructose was 1.22 ± 0.16 mg mL⁻¹, glucose 0.49 ± 0.02 mg mL⁻¹ and polysaccharides 0.36 ± 0.03 mg mL⁻¹. In general, the total saccharides amount (glucose, fructose and polysaccharides), added to EC through crude extracts of Galdieria, ranged from 2 to 6 mg mL⁻¹.

To verify the possible mixotrophic effect induced by saccharides present in the Galdieria extract, we grew Chlorella

Table 1 Means of specific growth rates for Chlorella sorokiniana grown in control and supplemented medium. The values reported are the average of three separate experiments ± Standard Deviation (SD). Superscript letters indicate statistical significance. Significant differences between EC and CC at the same time-point were determined by Two-way ANOVA with post-hoc Tukey HSD Test. Different letters identify statistically different means (p < 0.05). Upper case letters indicate the comparison between the different growth conditions (CC and EC). Lower case letters indicate the comparison among values of the same growth conditions but at different periods (0-24 and 0-48 h).

| Specific Growth Rate (day⁻¹) | 0-24 h | 0-48 h |
|-----------------------------|--------|--------|
| CC                          | 0.72 ± 0.09ab | 0.66 ± 0.045ab |
| EC                          | 1.35 ± 0.125aAB | 0.96 ± 0.045aBA |

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cells under supplementation of 6 mg mL\(^{-1}\) glucose (a total saccharides concentration corresponding to that supplied by *Galdieria* extract). CC and the glucose cultures (GC) showed a very similar trend (data not shown) and at the end of the experimental period similar growth rate was observed (CC 0.60 ± 0.09; GC 0.61 ± 0.03).

**Effect of *Galdieria* extract supplementation on *Chlorella* pigment content**

The amount of chlorophyll *a* (Chl *a*) of EC was significantly higher than in CC (*p*<0.001) already 24 h after extract addition (Fig. 2A). After 48 h, the content of Chl *a* was about 2.5 times higher in EC (10.4 ± 0.06 μg mL\(^{-1}\)) than in CC (4.1 ± 0.19 μg mL\(^{-1}\)). The chlorophyll *b* (Chl *b*) content remained low in both EC and CC in the first 24 h, but sharply increased in the subsequent 24 h in EC, to a concentration 5.8-fold higher in EC (11.3 ± 0.02 μg mL\(^{-1}\)) than in CC (1.94 ± 0.05 μg mL\(^{-1}\)) after 48 h (Fig. 2B).

The levels of carotenoids significantly increased in EC with respect to CC. Carotenoids were 1.6-fold higher in EC than in CC (*p*<0.001) already after 24 h and continued to increase along the experimental period in EC, reaching a maximum concentration of 2.28 ± 0.01 μg mL\(^{-1}\) after 48 h (Fig. 2C).

In addition, Chl *a*/Chl *b* and total Chl/Car ratios were estimated at 0, 24 and 48 h both in CC and EC (Table 2). The ratio Chl *a*/Chl *b* was higher in EC at 24 h, while the tot Chl/Car was greater at 24 and 48 h respect to CC.

**Effect of *Galdieria* extract supplementation on fluorescence parameters in *Chlorella***

Fluorescence parameters were measured to assess possible differences in photosynthetic efficiency between CC and EC of *Chlorella sorokiniana* 48 h after beginning the experiment. All parameters showed a noteworthy improved photochemical efficiency in EC. These include the maximal quantum yield of PSII in the dark (*F*\(_{v}/F_\text{m}^\prime\), Fig. 3A-B) and the parameters measured during the light responses: the photochemical yield (*Φ*\(_{\text{PSII}}\), Fig. 3C), and the electron transport rate (ETR, Fig. 3E). Improved photochemical efficiency also resulted in a reduced non-photochemical quenching (NPQ) in EC compared to CC (Fig. 3D).

**Antioxidant properties and antioxidant enzymes activities in *Chlorella* extracts**

The antioxidant capacity of *Chlorella* cellular extracts was measured as ABTS radical scavenging activity at the end of the experiment (48 h) and is shown in Table 3. The antioxidant capacity of EC cellular extracts was 32 ± 2.67 μmol Eq. Asa mg\(^{-1}\)prot, a significantly (*p*<0.05) higher value (18%) than in CC extracts.

The antioxidant enzymatic activities of superoxide dismutase (SOD), catalase (CAT) and ascorbic peroxidase (APX) were also evaluated at 48 h (Table 3). All antioxidant enzymes showed reduced activities in EC than in CC cells (-26%, -28%, -45%, respectively).

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**Table 2** Chla/Chlb and tot Chl/Car ratio in *Chlorella sorokiniana* (CC and EC) at 0, 24 and 48 h from *Galdieria* extract supplementation

| Time (h) | Chla/Chlb | tot Chl/Car |
|----------|-----------|-------------|
|          | CC        | EC          | CC       | EC       |
| 0        | 1.3       | 1.3         | 6        | 6        |
| 24       | 1.35      | 2.55        | 4.2      | 7.9      |
| 48       | 2         | 1.04        | 7        | 13.5     |

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**Fig. 2** Pigment contents in *Chlorella sorokiniana* cells. Chlorophyll-\(a, b\) and total carotenoids were measured in CC (dark green) and EC (light green) cells. Error bars represent SD (n=3). Significant differences between EC and CC at the same time-point were determined by Student’s *t* test and indicated by asterisks (*p*<0.05, **p*<0.01, ***p*<0.001).
Discussion

Just like plant crops, today microalgae play an important role in the global economy and industrial microalgal cultivation for biomass production has spread noticeably over the last years. Large scale cultivation of microalgae in bioreactors produces biomass for a wide range of applications such as biofuel, animal and human food, health, cosmetics and pharmaceutics (Khan et al. 2018).

Strategies to enhance biomass production are fundamental for improving the economic system revolving on microalgae cultivation. Conventional methods to increase algal growth or bioproducts accumulation principally are based on nutrients (e.g., nitrogen and phosphorus) or environmental factors (e.g., temperature, light, salinity, inorganic carbon source) manipulation (Salbitani et al. 2015, 2019, 2020a, b; Chu 2017; Ramanna et al. 2017). Our study demonstrates that production of economically important microalgae can also be enhanced using other microalgae as positive effectors. In particular, addition of *G. phlegrea* extracts rapidly and noteworthy improved many growth parameters of *C. sorokiniana* cells, such as optical density, cells number, growth rate. We discuss below the possible mechanisms behind the observed effects. We also caution that the recorded beneficial effect could depend on the quality of the *Galdieria* extract, containing carbon, nitrogen and

\[ \text{Fv/Fm value of } Chlorella \text{ sorokiniana control culture (CC, dark green) and enriched cultures (EC, light green) at 48 h (A). Image representing } F_{\text{v}}/F_{\text{m}} (B) \text{ of CC and EC was obtained by Imaging-PAM;} \]

\[ \text{The false-colour scale ranging from black (0) to purple (1) is indicated under the image. Light response curves of } \Phi_{\text{PSII}} (C), \text{ NPQ (D) and ETR (E) in CC and EC } Chlorella \text{ sorokiniana cells at 48 h. Error bars represent SD (n=3). Significant differences between EC and CC at the same light intensities were determined by One-way ANOVA with post-hoc Tukey HSD Test (*p<0.05, **p<0.01)} \]

|            | CC       | EC       |
|------------|----------|----------|
| ABTS (μmol Eq. Asa mg⁻¹ prot) | 27.01 ± 1.10<sup>a</sup> | 32.4 ± 2.67<sup>b</sup> |
| SOD (U mg⁻¹ prot)       | 10.27 ± 0.11<sup>a</sup> | 7.64 ± 0.31<sup>b</sup> |
| CAT (mU mg⁻¹ prot)       | 77 ± 7.27<sup>a</sup> | 56 ± 5.06<sup>b</sup> |
| APX (U mg⁻¹ prot)       | 0.96 ± 0.04<sup>a</sup> | 0.52 ± 0.04<sup>b</sup> |
antioxidant molecules, as reported elsewhere (Carfagna et al. 2016; Salbitani et al. 2022).

Interestingly, the fastest effect (observed only at 2 h after extract addition) was a temporary increase in diameters of EC cells, which swelled to a diameter 45% higher than in CC. This sudden and temporary swelling could be related to lipid or carbohydrate (mainly starch) accumulation in Chlorella (Takeshita et al. 2014), as cellular carbon storage. However, accumulation of carbohydrate or lipid restrains cell growth rate due to a temporary cellular replication break (Pancha et al. 2014; Salbitani et al. 2020b). In our experiment, such a growth slowdown was not observed. Rather, cells numbers and all growth parameters of EC were always higher than in CC at all checked times. The increase in cells size could also be due to an improved availability of phytohormones or hormone-like molecules added into culture by Galdieria extract. There are no data about phytohormone accumulation in Galdieria spp. However, the synthesis of auxin n-indole-3-acetic acid, cytokinins and gibberellins has been reported in Cyanidioschyzon merolae, a rhodophyte like Galdieria spp. (Lu and Xu 2015). A possible mixotrophic effect could be also considered. The concentration of saccharides (glucose, fructose, polysaccharides), added with Galdieria extract to EC, corresponded to 2-6 mg mL-1. According to Zhang et al. (2014), in mixotrophic Chlorella pyrenoidosa cultures, glucose is the best growth-promoting carbon source compared with different saccharides at similar concentrations. It is now well established that glucose can stimulate the mixotrophic growth of Chlorella spp. (Cheirsilp and Torpee 2012; Yeh and Chang 2012; Zhang et al. 2014) and some Chlorella strains even grow heterotrophically on glucose (Dani et al. 2020). However, our results indicate that growth with glucose supplementation (6 mg mL-1) was not sufficient to trigger mixotrophy in C. sorokiniana.

In C. sorokiniana cells, as well as in green algae in general, Chl a and Chl b make up the bulk of pigments. In microalgae several environmental factors are known to affect chlorophyll biosynthesis and accumulation: light, temperature and nutrient availability among the others (Da Silva Ferreira and Sant Anna 2017). Physiological response of microalgae to external stimuli can influence the pigment constituents and their ability to perform photosynthesis. The extract addition to culture medium led to important increase of Chl a and Chl b levels in EC cells. The chlorophyll contents represent a valid indicator of the physiological status in microalgae (Jayasankar and Valsala 2008; Srinivasan et al. 2018; Salbitani et al. 2021) and the high chlorophyll content is interpreted as indicating improved health of EC cells. As for the reason of chlorophylls rise, this could be due to i) ex novo biosynthesis powered by the nitrogen supply present in the added extract; ii) reduced chlorophyll degradation, and thus, delayed senescence. Indeed, in higher plants the application of microalgae extracts and the consequently higher chlorophyll concentration is both associated with delayed senescence (Mutale-joan et al. 2020; Colla and Rouphael 2020; Lee et al. 2020) and improved nitrogen uptake and use (Di Mola et al. 2019; Mutale-joan et al. 2020). While we cannot dissect among these two possible causes of chlorophyll increase, this is to our knowledge the first report indicating such an effect in a microalgal culture enriched with a second microalga. In addition, differences in Chl a/Chl b ratio emerged between CC and EC. The increase at 24 h in EC could indicate (i) a decrease in the unit size of photosystems, or (ii) a decrease in the number of PS II units (Smith et al. 1990; Jeong et al. 2018). The boost of Chl a/Chl b ratio denotes a good status of the cells and directly influence the photosynthetic capacity of the alga. At 48 h the ratio returns to a value comparable to CC, this probably due to the exhaustion of the beneficial effect of the added extract.

The Galdieria extract addition also considerably increased the levels of total carotenoids of C. sorokiniana cultures already after 24 h. In the plant cell carotenoids perform important roles such as membrane stabilization, light harvesting, energy dissipation, antioxidant activity and scavengers of reactive oxygen species (ROS). Increasing carotenoid content was indeed associated with a decrease of enzymatic antioxidants. In general, the ratio of tot Chl/Car is commonly used as an indicator of cellular responses to environmental stress such as excessive temperature, pH or light intensity (Booth et al. 2022). The highest value of the tot Chl/Car ratio (24-48 h) found in EC could indicate a healthy state of well-being of the cells and a reduced presence of ROS at cellular level, this confirmed by antioxidant measurements (see below).

In plant cells, high pigment content correlates with improved light absorption rate, in turn promoting better conversion of light to biochemical energy by photosynthesis (de Mooij 2016). Photosystems I and II are the photosynthetic functional units, inserted in the photosynthetic membranes, and directly responsible of such conversion. Consistently, in our study all chlorophyll fluorescence parameters indicating efficiency of PSII, the effective quantum yield ΦPSII, and the linear electron transport rate ETR, were higher in EC than in CC cultures. Interestingly, the superior performances of EC cultures were observed already at low light intensity. This indicates that EC cultures can make better use of light at growing conditions, which likely is a consequence of higher chlorophyll level and associated photosystems. Higher ETR, in particular, is a direct indicator of photosynthesis as it represents the number of electrons feeding photosynthesis and photospiration (Genty et al. 1989). High ETR matches the boost of growth observed in EC Chlorella. Even the maximal quantum yield (Fv/Fm) was slightly but significantly improved in EC Chlorella. Fv/Fm is a very conserved
parameter and the higher value suggests a more stable assembly of PSII and lower photoinhibition in EC cultures (Guidi et al. 2019). Better linear electron transport was also reflected in a reduction of non-photochemical quenching (NPQ) in EC. This was expected as NPQ reveals the amount of photochemical energy that is dissipated non-radiatively (mainly as heat) when linear electron transport cannot be further driven (e.g., under high light) or is limited by stress conditions. Lower NPQ therefore mirrored higher ΦPSII in EC than in CC cultures of Chlorella.

Finally, the effect of Galdieria extract supplementation on antioxidant enzymes was also evaluated. In plant cells such efficient antioxidant defence system is often able to remove ROS and avoid oxidative damage (Tattini et al. 2015). Higher antioxidant activities and antioxidant enzyme activities are indeed associated with higher stress tolerance also in unicellular algae (Vega et al. 2005; Salbitani et al. 2015). The first-line scavengers in the detoxification of ROS in plant cells are SODs, metalloenzymes that produce \( \text{H}_2 \text{O}_2 \) by the dismutation reaction of superoxide anion (\( \text{O}_2^- \)), which is formed from aerobic metabolism (Chatzikonstantinou et al. 2017). Cellular accumulation of \( \text{H}_2 \text{O}_2 \) raises the possibility of hydroxyl radical production via the Fenton reaction, in turn eliciting cellular oxidative damage. CAT and APX enzymes are important antioxidant components responsible for cellular \( \text{H}_2 \text{O}_2 \) removal.

After Galdieria extract addition, a decrease of all enzymatic activities (SOD -26%, CAT - 28%, and APX -45%) was observed in EC compared to CC of Chlorella. However, the total antioxidant capacity (ABTS) was higher in EC than in CC cells. Such a consistent and strong reduction of the activity of enzymatic antioxidants may therefore indicate a lower oxidative status in the EC cells, forming less intracellular \( \text{H}_2 \text{O}_2 \), rather than a dangerous reduction of the antioxidant machinery. The higher ABTS indeed suggests that pigments may complement enzymatic antioxidants for photosynthesis protection, if/when needed. The low oxidative status of EC cultures is interpreted as another consequence of improved pigment content and more efficient light conversion by the photosynthetic machinery in EC cells.

**Conclusions**

In conclusion, the addition of Galdieria phlegrea extracts to Chlorella sorokiniana cultures causes a series of positive physiological changes into the cells, such as improved pigment content, antioxidant capacity, photosynthesis, and growth. These beneficial effects do not seem attributable to the onset of mixotrophic conditions, but to the bio-stimulant properties of Galdieria extracts.

**Author contribution** Conception and design: GS, SC; Conducting experiments: GS, PC, FB; Analysis and interpretation of the data: GS, FL, SC; Drafting of the article: GS, FL, SC; Critical revision of the article: GS, CDM, FL, OM, SC. All authors have read and approved the final document.

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**Data availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Declarations**

**Conflict of interest** The authors declare no competing interests.

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