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Article

Plant Biostimulant Based on Biomass of *Chlorella sorokiniana* Treated with SL-6 Strigolactone Mimic

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Abstract: This study aimed to evaluate the impact on *Chlorella sorokiniana* NIVA-CHL 176 growth and the plant biostimulant functions of the microalgal extract of the strigolactone mimic SL-6, in comparison with the strigolactone analog GR24. Three molar SL-6 concentrations were tested: 10^{-7} M, 10^{-8} M, and 10^{-9} M, respectively. Five parameters of the microalgal growth were assessed: optical density, turbidity, biomass production, chlorophyll fluorescence, and pigment concentration. Results after 15 days of culturing revealed that the SL-6 treatments significantly enhanced biomass production, chlorophyll synthesis, and photosystem II activity. Treatment with SL-6 c3 (10^{-9} M) exhibited significant increases in chlorophyll *a* (27.55%), chlorophyll *b* (24.81%), carotenoids (12.31%), and total pigments (24.00%) compared to the solvent control (DMSO). Experimental treatment SL-6 c2 (10^{-8} M) followed next and recorded notable enhancements in chlorophyll *a* (25.80%), carotenoids (9.77%), and total pigments (20.08%). Conversely, higher doses of GR24 and SL-6 (10^{-7} M) inhibited the microalgal growth, reducing cell density, biomass production, and pigment synthesis. The microalgal extracts acted as a plant biostimulant stimulating root and shoot elongation and proton pump functioning in the presence and absence of salt stress. The extracts from SL-6 bio-stimulated *C. sorokiniana* NIVA-CHL 176 are more active as plant biostimulants.

Keywords: microalgal biostimulant, chlorophyll fluorescence; light stress; mung seedling; proton pump

1. Introduction

Microalgae cultivation is more efficient in harvesting light and using mineral nutrients than terrestrial plants [1,2]. Their metabolic flexibility, i.e., ability to use organic nutrients in mixotrophic and heterotrophic modes [3,4] is exploited in bioremediation and wastewater treatment [5-7], and valorization of various agro-industrial by-products, such as whey [8,9], sugar cane vinasse [10,11], wine lees [12-14], ethanol thin-stillage [15], hydrolyzed lignocellulosic biomass [16] or food waste [17]. Microalgal biomass is a feedstock for energy- and material-driven biorefinery systems [18,19]. Due to its high content of bioactive ingredients, the microalgal biomass is a renewable resource for high-value-adding bioproducts, e.g., dietary supplements [20,21] and plant biostimulants [22-24].

Plant biostimulants represent a category of agricultural inputs, defined by their functions related to the improvement of “one or more of the following characteristics of the plant and/or the plant rhizosphere: (1) nutrient use efficiency, (2) tolerance resistance to (a)biotic stress, (3) quality characteristics, or (4) availability of confined nutrients in the soil or rhizosphere”[25]. Various types

of extracts from microalgae demonstrated the agricultural functions specific to plant biostimulants, i.e., increased nutrient uptake and nutrient use efficiency [26], enhanced tolerance to different abiotic stress, e.g., water stress [27] or drought [28,29], improved yield quality [30], and enhanced growth and photosynthetic performance [31]. Microalgae were used as terrestrial plant models to screen the bioactive components influencing photosynthesis performance, e.g., whey hydrolysate peptides [32] or hydrophobic contaminants [33].

Strigolactones (SLs) are exo- and endo-signals produced by terrestrial plants, initially identified in root exudates as inducers of seed germination in the *Striga* parasitic plant [34]. The initially discovered SLs have a complex structure, with a complex ABC-ring system linked through an enol-ether bridge to the bioactiphore, a methyl-butenolide D-ring [35]. Studies from the last decade have demonstrated a complex landscape of strigolactone signal structures. Besides the strigolactone with the complex ABC-ring (called "canonical strigolactones"), more simple molecules that maintain the bioactive D-ring were discovered [36]. As endo-signals, SLs coordinate plant response to (a)biotic stress [37,38] and integration of metabolic and nutrition signals [39]. SLs functions as exo-signals (ecomones) are both for target organisms, i.e., as pheromones (for detection of neighboring plants) and synomones (exo-signals for arbuscular mycorrhizae fungi, nitrogen-fixing bacteria, and other plant-beneficial microbes) and for eavesdropping organisms, as kairomones (exo-signals for parasitic plants, nematodes and fungal plant pathogens) [40,41].

Due to the difficulty of preparing natural SLs, which are complex and easily degradable structures, synthetic strigolactones were proposed [42]. The synthetic analog GR24 [43] is among the most used synthetic strigolactone for various studies demonstrating agricultural functions similar to plant biostimulants. The exogenous application of the strigolactone analogs GR24 enhanced plant tolerance to drought [44], salt stress [45], heat [46], low temperature [47], and toxic elements such as cadmium (Cd) [48]. Exogenous strigolactone application increased mineral nutrient uptake, including due to the stimulation of mycorrhizal symbiosis [49]. Exogenous application of GR-24 improves artemisinin production in *Artemisia annua* [50]. The mechanisms involved in the plant biostimulant-like effects of exogenous strigolactone are related to enhanced photosynthetic efficiency and decreased oxidative stress due to the activation of the antioxidant systems in plants [51,52].

Although strigolactones are specific endo- and exo-signals in multicellular photosynthetic organisms, with the first appearance in the green lineage on stoneworts, liverworts and mosses [53], the strigolactones were proved to be active on microalgae as well. 5-Deoxystrigol (5-DS), a canonical strigolactone, applied at 10^{-11} M concentration, improved the efficiency of a co-culture *Scenedesmus obliquus* – *Ganoderma lucidum* in biogas upgrading and wastewater purification [54]. The application of the strigolactone analog GR24 was demonstrated to improve the efficiency of microalgal-based technologies for biogas upgrading and removing nutrients/pollutants from digestate. GR24, applied at a concentration of 10^{-7} M, increased the growth rate and daily productivity of *C. vulgaris* FACHB-8 cultivated in a medium that simulates biogas digestate and in the presence of biogas. The increase was due to an enhanced photosynthetic performance [55].

In microalgae, SLs have been reported to have functions similar to those exerted on terrestrial plants, enhanced response to abiotic stress (as endo-signals), and induction of symbiotic interactions (i.e., synomones function). SLs could be considered as "microalgal biostimulants", a mirror term for products that have microalgal biotechnological functions analog to agricultural function of plant biostimulants. Some of these functions are increased nutrient use efficiency (including due to the enhanced photosynthesis performance), enhanced tolerance to abiotic stress, improved yield quality (bioactive compound accumulation in biomass), enhanced bioavailability of confined nutrients (due to promotion of the symbioses with others organisms) [56]. Other reported "microalgal biostimulant" products are humic substances [57,58] and protein hydrolysates prepared from waste chicken feathers [59]. Microalgal biostimulants are one of the solutions to the challenges that limit microalgal-based biotechnologies development.

The cost of synthesis of strigolactone analogs is still very high. For the scale-up applications, compounds that are easily accessible in significant amounts and safe are needed. Our group

demonstrated that strigolactone mimics, synthetic molecules easier to synthesize, which keep only the butenolide active D-ring (more similar to the non-canonical natural strigolactone structure) are active on microalgae. The SL-F3 mimic, 3-(4-methyl-5-oxo-2,5-dihydrofuran-2-yl)-3H-benzothiazol-2-one (7), at a concentration of 10^{-7} M, increased the biomass accumulation due to an improved photosynthetic efficiency. The SL mimics supported microalga adaptation to the light stress determined by the continuous illumination [60].

We have previously reported the synthesis of another SL mimic, SL-6, active in inducing parasitic seed germination and modification of phytopathogenic fungi branching [61]. We improved the process for multi-gram synthesis, and we demonstrated that SL-6 has a synomone function, increasing the biofilm formation by nodulation-enhancing bacteria [62]. We recently assessed SL-6 for ecotoxicological impact on marine and freshwater organisms, including microalgae, *Raphidocelis subcapitata* and *Skeletonema pseudocostatum*. The estimated EC50 was 1.21 mg/L after 48 h exposure, corresponding to 0.4×10^{-5} M [63].

This study aimed to investigate the effect of SL-6, at concentrations significantly lower than EC50, on the growth of *C. sorokiniana* NIVA-CHL 17 and the plant biostimulant effect of the extract prepared from SL-6 treated microalgae in comparison to non-treated microalgae. The *C. sorokiniana* NIVA-CHL 17 extract was reported to stimulate the root elongation of *Arabidopsis thaliana* (ecotype Columbia) and the yield and photosynthetic performance of lettuce (*Lactuca sativa* L. cv. Finstar) [31]. We used GR24 as a positive control due to its demonstrated effect on microalgae from the *Chlorella* genus [64]. The microalgal extract was tested on mung seedlings in the presence and absence of salt stress to check the plant biostimulant effects. To the best of our knowledge, this is the first study to investigate (SLs) biostimulated microalgal biomass as a source of extract with (enhanced) plant biostimulant functions.

2. Results

2.1. Microalgal Growth Parameters

2.1.1. Optical Density

The optical density showed statistically significant effects of both the GR24 analogue and SL-6 mimic on *C. sorokiniana* compared to controls (Figure 1).

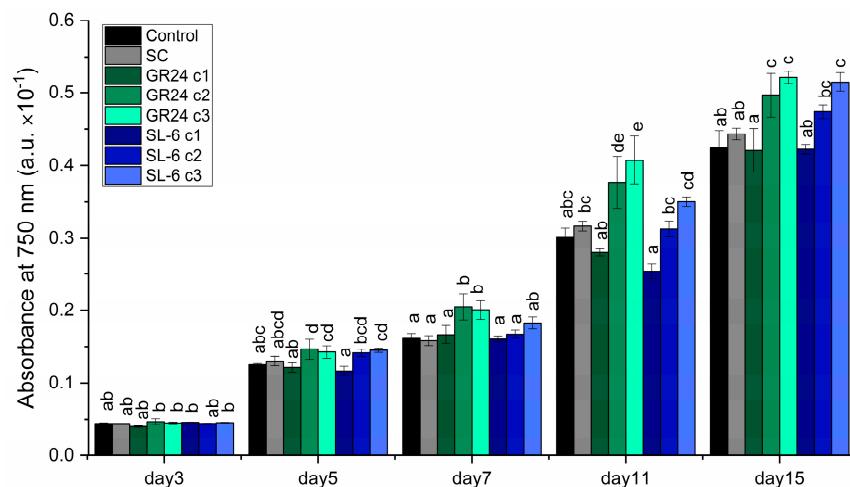


Figure 1. Optical density of *C. sorokiniana* cultures during 15 days of culturing. Control - growth media without treatment; SC-DMSO 0.1%, c1: 1×10^{-7} M, c2: 1×10^{-8} M, c3: 1×10^{-9} . Values represent means \pm standard errors (n = 3); different letters show statistically significant differences at $p < 0.05$.

From the fifth day of culturing marginal significant differences emerged between the control group and SC group and the experimental treatments with synthetic SLs. After a week of culturing, the growth rate was significantly stimulated by c2 and c3 of GR24 and marginally significant stimulated by the lowest concentration of mimic SL-6, c3. At the end of the culturing time, at day 15, the growth rates of the variants with GR24 c2 and GR24 c3 were $12.11 \pm 8.25\%$ and $17.68 \pm 2.54\%$, respectively, higher compared to SC. The growth rates of the variants with SL-6 c2 and SL-6 c3 were $7.00 \pm 0.80\%$ and $16.26 \pm 5.00\%$, respectively, higher than SC. The stimulatory effect of SL-6 on *C. sorokiniana* was slightly lower, but similar to the stimulatory effect of GR24.

The highest concentration tested, c1, (1×10^{-7} M) of both GR24 and SL-6 induced similar or lower optical densities compared to SC, which indicates a tendency towards inhibition of microalgal growth of applied synthetic strigolactones at higher concentrations.

2.1.2. Microalgal Culture Turbidity

The turbidity measurements recorded values that followed the same trend as optical density (Figure 2).

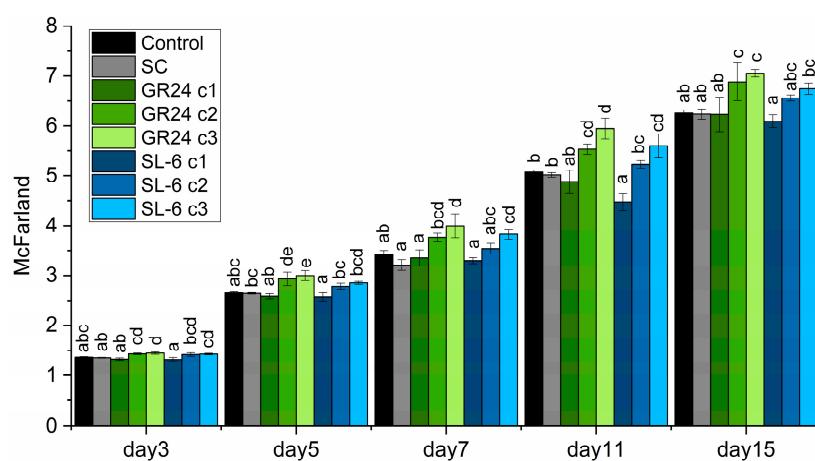


Figure 2. Turbidity of *C. sorokiniana* cultures during 15 days of culturing. Control - growth media without treatment; SC-DMSO 0.1%, c1: 1×10^{-7} M, c2: 1×10^{-8} M, c3: 1×10^{-9} . Values represent means \pm standard errors (n = 3); different letters show statistically significant differences at $p < 0.05$.

The lower molar concentrations of 10^{-8} and 10^{-9} M stimulated the microalgal cell density with statistical significance from day 5 to day 15, compared to SC. The treatments with SL-6 c2 and c3 recorded slightly lower turbidity levels than the same concentrations of GR24, but higher than controls, starting from day 7 to the end of the culturing period. On day 15 the treatment with SL-6, 1×10^{-9} M exhibited McFarland values increased with $8.17 \pm 1.3\%$ compared to SC.

The treatments with the highest dose of both strigolactones consistently exhibited the lowest turbidity values, lower than controls, a trend observed from day 5 to the end of the experiment, especially in the case of SL-6.

2.1.3. Biomass Quantification

The microalgal biomass resulted after two weeks of culturing was increased compared to control when the lower concentrations of SLs were applied (Figure 3). Similar to previous parameters, SL-6 induced slightly lower biomass than GR24, but higher than the controls. The lowest production of microalgal biomass was recorded in the variant with 10^{-7} M for both GR24 and SL-6, a little lower than the controls.

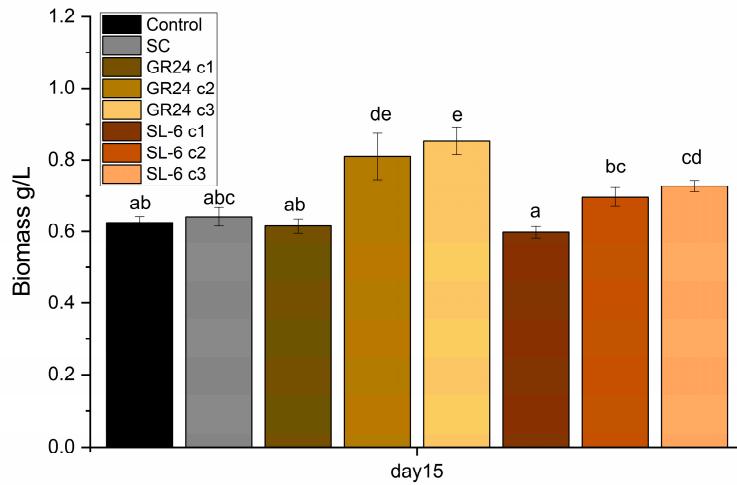


Figure 3. Biomass quantity of *C. sorokiniana* cultures. Control - growth media without treatment; SC-DMSO 0.1%, c1: 1×10^{-7} M, c2: 1×10^{-8} M, c3: 1×10^{-9} M. Values represent means \pm standard errors (n = 3); different letters show statistically significant differences at $p < 0.05$.

Comparing each treatment with SC, we observed distinct responses: GR24 c1 and SL-6 c1 decreased slightly the biomass by $3.96 \pm 4.85\%$, and $6.74 \pm 4.1\%$, respectively; GR24 c2 and c3 increased the biomass by $26.35 \pm 12.47\%$ and $33.17 \pm 10.1\%$, respectively; SL-6 c2 and c3 increased the biomass by $8.47 \pm 2.55\%$ and $13.53 \pm 5.53\%$, respectively.

2.1.4. Extracted Pigments Concentration

Microalgal biomass is a source of valuable compounds such as pigments, therefore we analyzed the chlorophyll and carotenoids concentrations within the cells.

Results followed the same trend as biomass development, where higher dose treatments slightly inhibited pigment formation whereas lower concentrations stimulated chlorophyll synthesis (Figure 4).

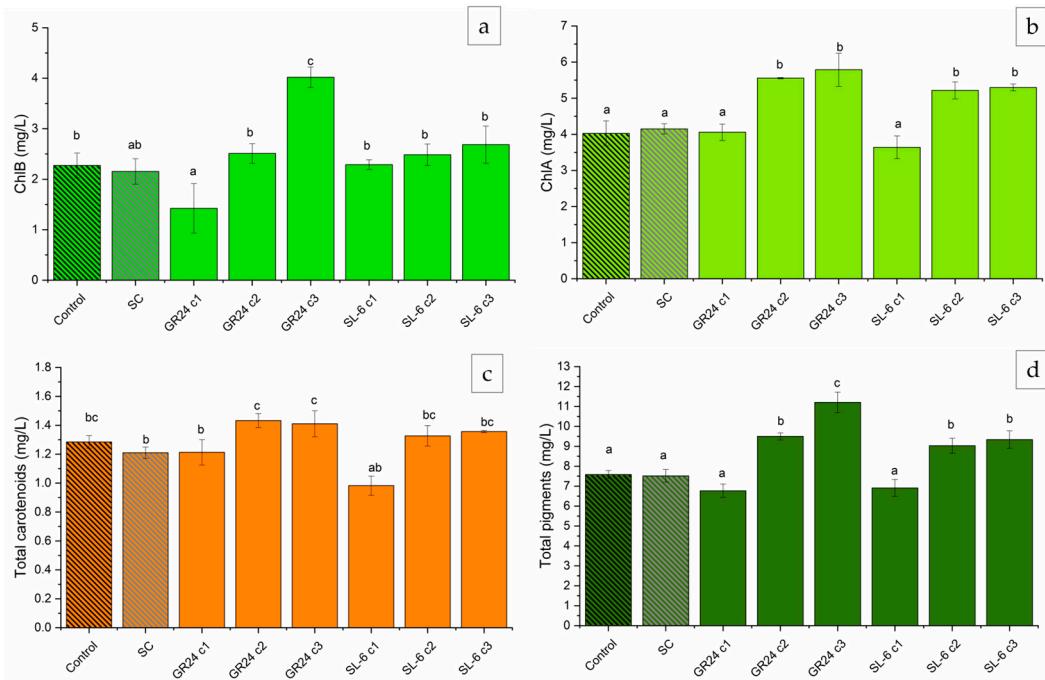


Figure 4. Pigment concentrations. (a) Chlorophyll *a*, (b) Chlorophyll *b*, (c) Total carotenoids, (d) Total piments. Control - growth media without treatment; SC-DMSO 0.1%, c1: 1×10^{-7} M, c2: 1×10^{-8} M, c3: 1×10^{-9} . Values represent means \pm standard errors (n = 3); different letters show statistically significant differences at p < 0.05.

The strigolactone analog GR24 c2 (1×10^{-8} M) and c3 (1×10^{-9} M) induced substantial increases in ChlA compared to SC, of 33.88 ± 4.18 % and 39.49 ± 11.4 %, respectively (Figure 4a). SL-6 c2 and c3 showed increases in ChlA content by 25.80 ± 8.12 % and 27.55 ± 8.24 %, respectively, relative to SC. The ChlB production was stimulated the most by the treatment with GR24 c3, with a difference of approximately 86.14 ± 15.17 % compared to SC (Figure 4b). SL-6 c2 and c3 exhibited marginally significant increases in ChlB by 6.38 ± 2.4 % and 24.81 ± 4.45 %, respectively, relative to SC.

Chlorophyll *a* and chlorophyll *b* are pigments crucial for photosynthesis in microalgae, contributing to adaptability to various environmental conditions [65]. The treatments with SL-6 c2 and SL-6 c3 increased the total carotenoids by 9.77 ± 5.2 % and 12.31 ± 5.6 %, respectively, compared to the solvent control (Figure 4c). The same concentrations of the analogue strigolactone GR24 recorded even more notable increases in total carotenoids compared to SC, with approximately 18.57 ± 6.71 % and 16.64 ± 4.11 %, respectively. SL-6 c1 (10^{-7} M) exhibited a decrease in total carotenoids by about 19.00 ± 6.23 % compared to SC, whereas the same concentration of GR24 did not have an effect on the total carotenoids. The total pigments followed the same trend as its components. The lower doses, c2 and c3, of GR24 used in *C. sorokiniana* cultures recorded significant increases in total pigments compared to SC, with differences of 26.52 ± 6.82 % and 48.89 ± 14.75 %, respectively. The mimic SL-6 showed increases in the total extracted pigment quantities, by 20.08 ± 9.60 % and 24.00 ± 9.23 %, when c2 and c3 were applied, respectively, compared to SC. The highest concentration of both the analogue GR24 and mimic SL-6 tested decreased the total pigments extracted by 8.15 ± 7.89 %, but not statistically significant (Figure 4d).

After normalization to the microalgal biomass within each treatment, the pigment content showed slightly different results compared with the non-normalized concentrations (Figure 5).

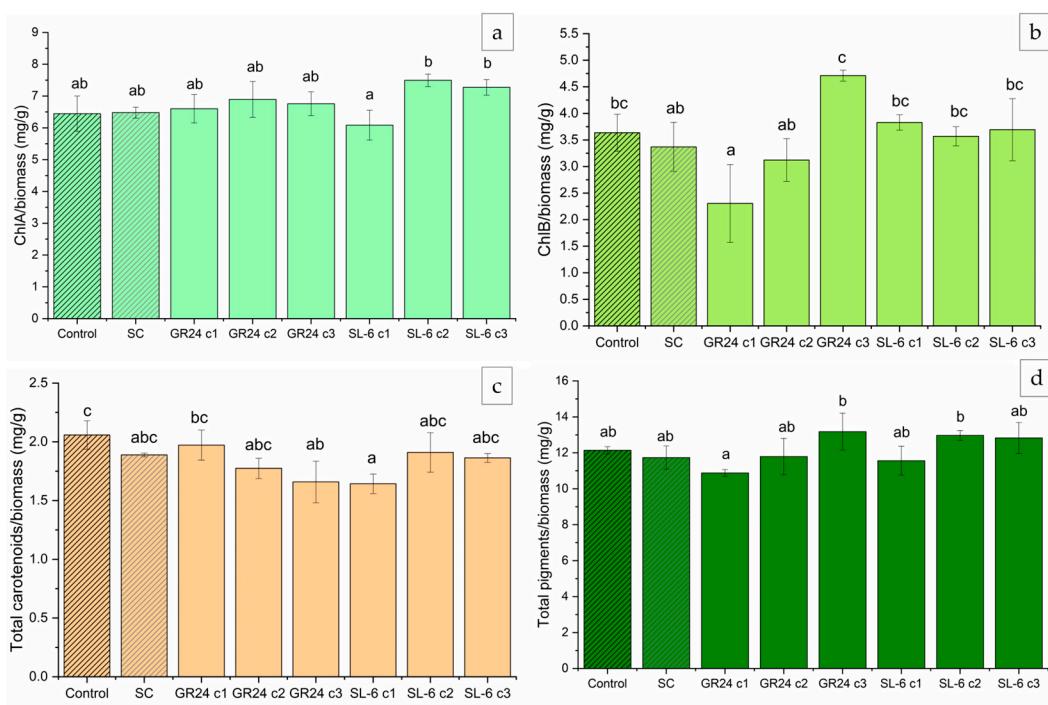


Figure 5. Pigment concentrations in dependence of microalgal biomass. (a) Chlorophyll *a*, (b) Chlorophyll *b*, (c) Total carotenoids, (d) Total piments. Control - growth media without treatment; SC-DMSO 0.1%, c1: 1×10^{-7} M, c2: 1×10^{-8} M, c3: 1×10^{-9} M. Values represent means \pm standard errors (n = 3); different letters show statistically significant differences at p < 0.05.

Both lowest concentrations of GR24 c2 (1×10^{-8} M) and c3 (1×10^{-9} M) induced slight increases in ChlA compared to SC, of 6.60 ± 3.00 % and 4.42 ± 1.40 %, respectively, but not statistically significant (Figure 5a). SL-6 c2 and c3 showed higher increases in the ChlA content of 15.70 ± 4.10 % and 12.30 ± 5.24 % from CS, respectively, compared to GR24 (Figure 5a).

The ChlB content was the highest upon the treatment with GR24 c3, with a 41.72 ± 18.10 % increase compared to SC (Figure 5b). Higher doses of GR24 recorded decreases in ChlB content per gram of microalgal biomass. SL-6 c1 and c3 showed marginally significant increases in ChlB by 7.85 ± 5.4 % and 12.87 ± 8.45 %, respectively, relative to SC (Figure 5b).

The total carotenoid content was slightly lower than control in the case of GR24 c3 and SL6 c1, which showed a rather opposite behaviour between the two compounds with concentration. The highest concentration of GR24 c1 (1×10^{-7} M) induced a small increase with 4.4 ± 3.4 % compared to SC. SL-6 c2 and c3 did not show a significant increase in carotenoid production (2.3 ± 1.5 % of SC for SL-6 c2).

The total pigments biosynthesized by the microalgal cultures per gram of biomass were the highest for the treatment with GR24 c3 (1×10^{-9} M) with 12.85 ± 5.46 % more than SC, followed by the treatment with SL-6 c2 and c3 with 10.78 ± 3.45 % and 9.81 ± 3.79 % more than SC, respectively. These results were marginally significant.

Considering the significance of photosystem II (PSII) and the quantum yield in microalgae photosynthesis, these results provide valuable insights into the efficiency of light energy conversion in *C. sorokiniana* under different treatments (Figure 6).

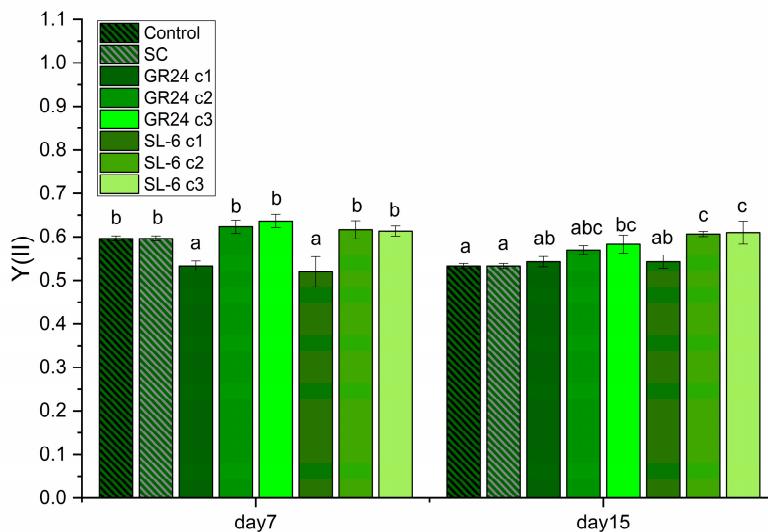


Figure 6. Chlorophyll fluorescence of microalgal cultures. Control - growth media without treatment; SC-DMSO 0.1%, c1: 1×10^{-7} M, c2: 1×10^{-8} M, c3: 1×10^{-9} M. Values represent means \pm standard errors ($n = 3$); different letters show statistically significant differences at $p < 0.05$.

Treatments with lower SL doses resulted in higher chlorophyll fluorescence values compared to the solvent control at both time points, which suggests potentially enhanced PSII activity and improved quantum yield. This could indicate increased photosynthetic efficiency and confirms the stimulated biomass production in these treatments. Conversely, treatments that induce lower fluorescence values may indicate reduced PSII activity and lower quantum yield, possibly leading to decreased photosynthetic efficiency and growth. SL-6 increased significantly the *C. sorokiniana* quantum yield when the lower doses (c2 and c3) were applied, more than GR24, after 15 days of culturing.

We performed Pearson correlation between the parameters determined above to understand how these parameters relate to each other (Figure 7).

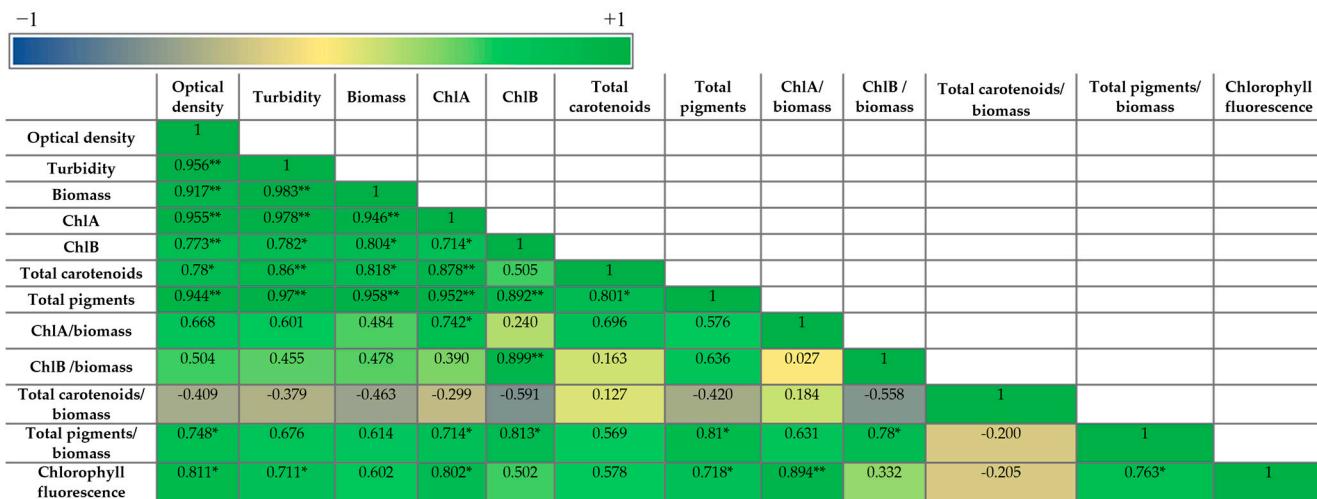


Figure 7. Pearson correlation between the growth parameters and pigment production of *C. sorokiniana* grown in the absence and presence of GR24 and SL-6; ** Correlation is significant at the 0.01 level; * Correlation is significant at the 0.05 level.

As expected, all the parameters of the microalgal growth correlated with each other and with the pigment concentration (per liter), as the pigment concentration is a function of the total biomass. The highest growth – pigment correlation was with ChlA and total Chl, and the lowest correlation was with ChlB. This indicates that the effect of SL on the production of pigments depends on the pigment type.

A positive correlation was observed between these parameters and the chlorophyll fluorescence as well, especially for optical density, turbidity, and ChlA that showed a statistically significant correlation. The correlation with the normalized pigments (level of expression) presented some differences compared with the pigment concentrations. The growth parameters showed statistically significant positive correlation only of the optical density with the total pigments/biomass. The total carotenoids/biomass had a negative correlation with almost all the other parameters, but not statistically significant. Other statistically significant correlations were between total pigments/biomass and ChlA, ChlB, ChlB/biomass and between the chlorophyll fluorescence and ChlA/biomass, total pigments/biomass.

In our experimental findings, treatments with SL-6, particularly at concentrations c2 (1×10^{-8} M) and c3 (1×10^{-9} M) demonstrated significant increases in *C. sorokiniana*'s growth, biomass production, pigment synthesis, and *Chl a* photosynthetic yield (i.e., Chlorophyll fluorescence)

The results showed that lower concentrations of both tested synthetic strigolactones, GR24 and SL-6, stimulated microalgal growth, while higher concentrations inhibited it. Specifically, GR24 c2 (1×10^{-8} M) increased growth by $12.11 \pm 8.25\%$, and GR24 c3 (1×10^{-9} M) by $17.68 \pm 2.54\%$ compared to the solvent control (SC). Similarly, SL-6 c2 increased growth by $7.00 \pm 0.8\%$, and SL-6 c3 by $16.26 \pm 5\%$. This suggests that at optimal concentrations, strigolactones enhance microalgal cell division and proliferation, which is crucial for biomass accumulation. These findings are aligning with similar growth-promoting effects of synthetic strigolactone on microalgae, reported in the literature [55,60,66]

2.2. Mung Seedlings Bioteats

2.2.1. Effects on Seedlings Growth

The extract of the non-treated microalgal culture (CSk) slightly increased the root (radicle) and hypocotyl length compared to control C, without any treatment, both in the absence and presence of salt (Figure 8).

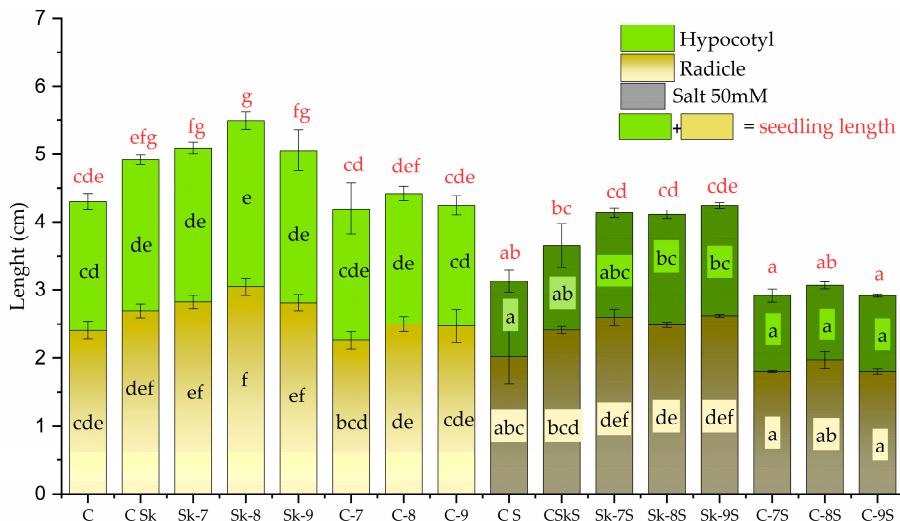


Figure 8. Mung seedlings measurements. C, control with water; CSk, control with untreated *C. sorokiniana* biomass extract; Sk-7, extract from *C. sorokiniana* biomass treated with SL-6 c1 (1×10^{-7} M); Sk-8, extract from *C. sorokiniana* biomass treated with SL-6 c2 (1×10^{-8} M); Sk-9, extract from *C. sorokiniana* biomass treated with SL-6 c3 (1×10^{-9} M); C-7, control for SL-6 c1 solution; C-8, control for SL-6 c2 solution; C-9, control for SL-6 c3 solution; CS, control with water and salt stress; CSkS, control for untreated *C. sorokiniana* biomass extract with salt stress; Sk-7S, extract from *C. sorokiniana* biomass treated with SL-6 c1 (1×10^{-7} M) with salt stress; Sk-8S, extract from *C. sorokiniana* biomass treated with SL-6 c2 (1×10^{-8} M) with salt stress; Sk-9S, extract from *C. sorokiniana* biomass treated with SL-6 c3 (1×10^{-9} M) with salt stress; C-7S, control for SL-6 c1 solution with salt stress; C-8S, control for SL-6 c2 solution with salt stress; C-9S, control for SL-6 c3 solution with salt stress; Values represent means \pm standard deviations. Different letters indicate statistically significant differences between samples at $p < 0.05$.

The root length of mung seedlings was further increased significantly by treatments with extracts of microalgae stimulated by 1×10^{-7} , 1×10^{-8} , 1×10^{-9} M SL-6, i.e., Sk-7, Sk-8, and Sk-9, respectively, compared to CSk. Sk-8 showed the highest increase, 26.46 ± 6.25 % of C whereas Sk-9 and Sk-9 induced similar values. Under stress conditions (50 mM salt), the radicle length decreased overall compared to unstressed seedlings. Treatments with Sk-7 S, Sk-8 S, and Sk-9 S determined significant increases, slightly higher than the non-treated microalgae (CSkS), reaching the control without salt and higher, indicating resilience under stress (Figure 8). The differences between the three concentrations of SL-6 were not significant.

In the case of hypocotyl, CSk, Sk-7, and Sk-9 variants had marginally significant higher length values (with approx. 5%) compared to control C and there was no statistical difference between the three treatments. Sk-8 induced a significant increase of 13.62 ± 3.54 % compared to C.

When salt stress was applied, treatments Sk-7 S, Sk-8 S, and Sk-9 S showed significant increases in hypocotyl length compared to CS, and marginally significant compared to CSkS. The differences between the three SL-6 concentrations were not statistically significant. The seedling length without salt stress varied between 4.19 ± 0.24 and 5.49 ± 0.12 cm among controls and treatments. When salt stress was applied, the seedlings length fell in between 2.91 ± 0.03 and 4.25 ± 0.06 cm. Without salt stress, the longest seedlings were recorded for variant Sk-8 and with salt stress for variant Sk-9, with 27.40 ± 5.47 % higher than C and 35.80 ± 7.60 % higher values than CS.

The SL-6 controls did not have a significant effect, neither in the absence nor in the presence of salt stress.

2.2.2. Acidification of the Growth Medium

Proton pumps are integral to plant physiology, affecting nutrient uptake, growth, pH regulation, and stress responses. Acid pH induces the activation of expansins, which induces root growth [67].

Proton pumps extrude H^+ ions into the soil, lowering the pH around the roots. This acidification can help in nutrient solubilization and uptake [68].

The CSk treatment resulted in a $13.6 \pm 7.5\%$ increase in the total extracellular H^+ levels compared to C, and the treatments with Sk-7 and Sk-8 exhibited a $19.7 \pm 4.3\%$ and $28.0 \pm 6.7\%$ respectively, higher extracellular H^+ levels, in the absence of salt stress. Sk-9 showed a similar behavior to CSk (Figure 9a). The controls with SL-6 decreased slightly the total H^+ levels compared to control C.

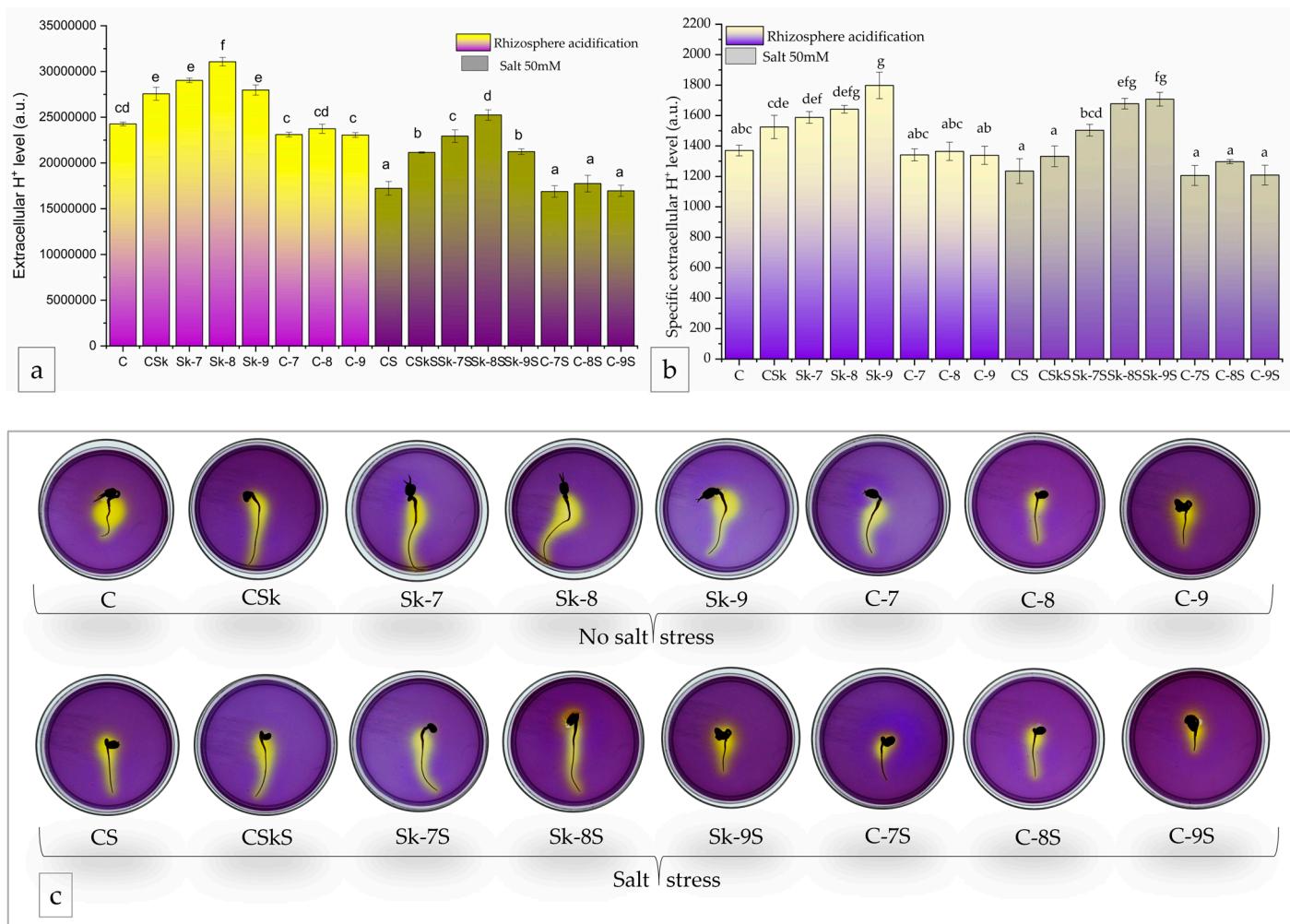


Figure 9. Proton pump activity; (a) Total extracellular H^+ level; (b) Specific extracellular H^+ level; (c) petri dishes after medium acidification test; C, control with water; CSk, control for untreated *C. sorokiniana* biomass extract; Sk-7, extract from *C. sorokiniana* biomass treated with SL-6 c1 (1×10^{-7} M); Sk-8, extract from *C. sorokiniana* biomass treated with SL-6 c2 (1×10^{-8} M); Sk-9, extract from *C. sorokiniana* biomass treated with SL-6 c3 (1×10^{-9} M); C-7, control for SL-6 c1 solution; C-8, control for SL-6 c2 solution; C-9, control for SL-6 c3 solution; CS, control with water and salt stress; CSkS, control for untreated *C. sorokiniana* biomass extract with salt stress; Sk-7S, extract from *C. sorokiniana* biomass treated with SL-6 c1 (1×10^{-7} M) with salt stress; Sk-8S, extract from *C. sorokiniana* biomass treated with SL-6 c2 (1×10^{-8} M) with salt stress; Sk-9S, extract from *C. sorokiniana* biomass treated with SL-6 c3 (1×10^{-9} M) with salt stress; C-7S, control for SL-6 c1 solution with salt stress; C-8S, control for SL-6 c2 solution with salt stress; C-9S, control for SL-6 c3 solution with salt stress; values represent means \pm standard deviations. Different letters indicate statistically significant differences between samples at $p < 0.05$.

When salt stress was applied, there was a similar behavior, i.e., all four microalgal extract treatments (CSkS, Sk-7S, Sk-8S, Sk-9S) enhanced medium acidification in a similar trend. In particular, treatments with Sk-8S and Sk-7S had the highest effect and enhanced the H^+ values with $46 \pm 8.5\%$ and $33 \pm 6.4\%$, respectively, compared to CS. The Sk-8S treatment brought the H^+ values slightly higher than the control without salt.

The specific extracellular H⁺ level resulted after normalizing the total H⁺ level to the root area (Figure 9b).

The highest specific H⁺ was recorded for the treatment with SK-9, both with and without salt stress, and the values were significantly higher than C(S) and CSk(S).

In order to understand better the relation between the SL effects on microalgae and the microalgal effects on the mung seedlings, we performed Pearson correlation, separately for the seedlings without stress (Figure 10a) and the salt-stressed seedlings (Figure 10b).

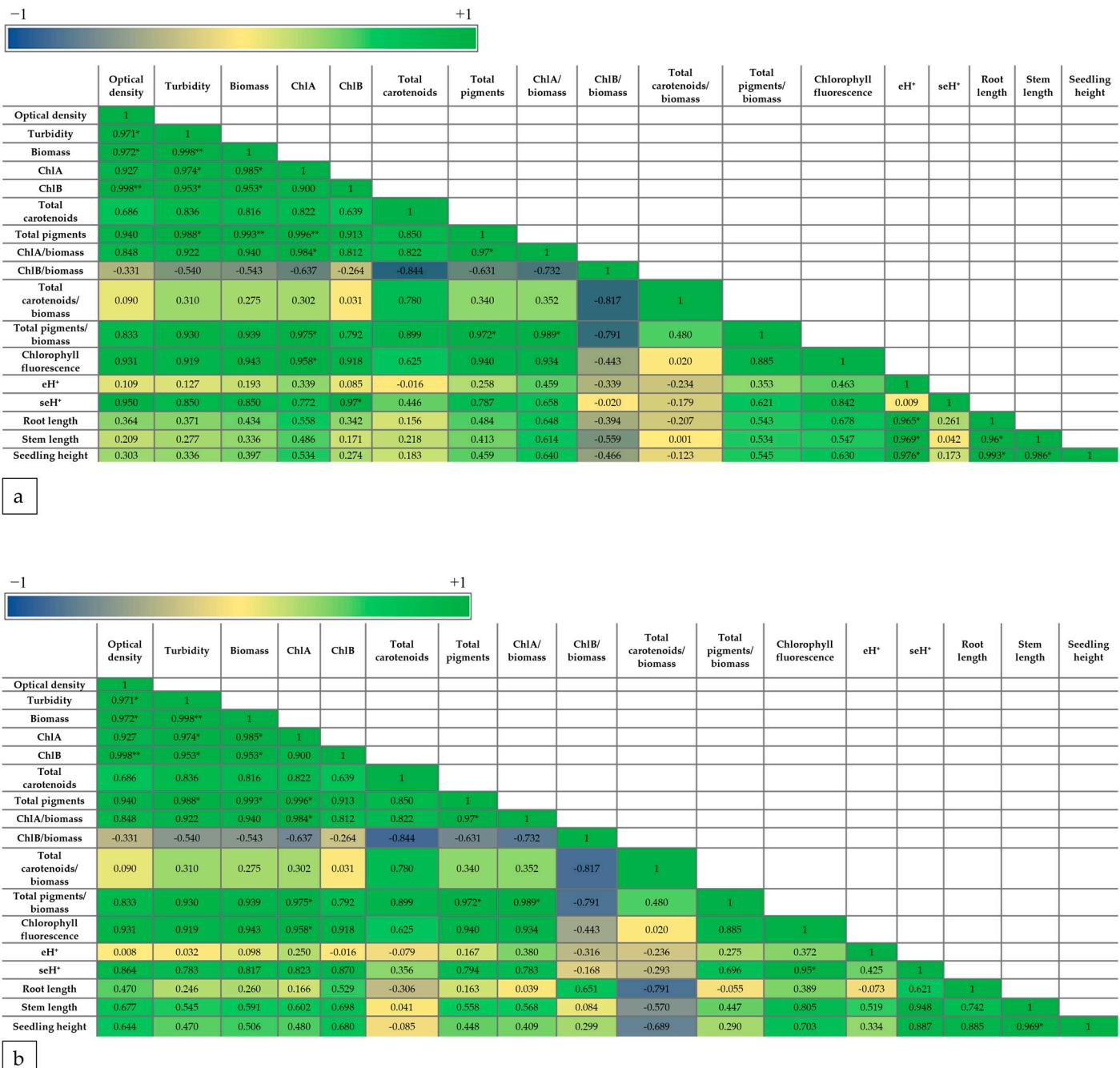


Figure 10. Pearson correlation between the mung seedling parameters and microalgae *C. sorokiniana* parameters in the absence (a) and presence (b) of salt stress on the mung seedlings; * Correlation is significant at the 0.05 level; ** Correlation is significant at the 0.01 level.

In both cases, eH^+ was in general positively correlated with the microalga parameters, except with ChlB/biomass and total carotenoids/biomass, i.e., with the level of expression of ChlB and total carotenoids.

The seedling parameters had a tendency towards a negative correlation with ChlB/biomass and total carotenoids/biomass, especially in the absence of salt. The seedling growth parameters were positively correlated with seH^+ just for the salt-stressed seedlings. The chlorophyll fluorescence parameter tends to be positively correlated with all parameters determined in mung bean seedlings, stressed or not-stressed. For the salt-stressed seedlings, seH^+ of mung roots was highly statistically significant (positively) correlated with the chlorophyll fluorescence of the microalgae.

3. Discussions

3.1. Lower Doses of Synthetic Strigolactones as Microalgal Biostimulant

Synthetic SLs enhance microalgae growth parameters due to an increased photosynthetic performance. The photosynthetic yield of the photosystem II, determined by recording the chlorophyll fluorescence induced by pulse amplitude modulated (PAM) saturating light, was reported to be improved by optimal concentrations of synthetic SLs [55,60]. A similar effect of exogenously applied synthetic strigolactone on PAM-induced chlorophyll fluorescence was reported for plants submitted to abiotic stress — synthetic SLs reduced photoinhibition and improved the photosynthetic yield in plants exposed to (low) light stress [69,70]. In our present study, the increased photosynthetic yield (chlorophyll fluorescence) positively correlates with biomass accumulation. The correlations of the chlorophyll fluorescence with optical density and turbidity are statistically significant. The correlation between total biomass and pigment accumulation, Chl *a*, Chl *b*, total carotenoids, and total pigments is (highly) statistically significant. Synthetic SLs applied at optimal concentrations (in our case, 10^{-8} M and 10^{-9} M) increased the growth rate and accumulation of photosynthetic pigments. However, the expression of the photosynthetic pigments, i.e., specific pigments per total biomass, tends to be influenced differently. Chl A/ biomass and ChlB/ biomass tend to correlate positively with biomass accumulation, and total carotenoids/biomass tends to be negatively correlated with microalgae biomass accumulation.

Applying synthetic SLs seems to compensate for light attenuation along the light path due to photosynthetic active radiation (PAR) absorption by the photosynthetic pigments of the microalgae cells from the upper layers [71,72]. Due to culture shaking, microalgal cells travel in the culture flask, from the high light at the flask transparent wall to the low light/absence of PAR in the center of the flask culture. This situation involves transitioning from high light, which leads to photoinhibition and photosynthesis pigment loss, to light-limited conditions that reduce growth [73]. An argument for such a compensating effect of the light gradient in the microalgae culture is growth stimulation, which is more significant in cultures with a high cell density and a higher light attenuation rate. From the fifth day of culturing, marginal significant differences emerged between the control groups and the experimental treatments with synthetic SLs.

Various solutions were proposed to compensate for this drawback of microalgae cultivation at high cell density, such as applying flashing light [74-76], photobioreactor design that improves homogenous light distribution, e.g., internally illuminated photobioreactor [77,78], or photobioreactor internally illuminated with mirror walls [79], fluorescent dyes [80] or quantum dots [81], etc. Photosynthesis efficiency (determined by chlorophyll fluorescence/photosystem II yield) is increased in microalgal culture grown in internally illuminated photobioreactor [82] or with optimal high flashing light application [75]. Applying microalgal biostimulants based on synthetic SLs seems to represent an alternative to compensate for the stress of light attenuation in the microalgae cultivation systems. More studies are needed in this direction, including combining synthetic SLs with other solutions for homogenous light distribution and compensation of the light stress resulting from light attenuation in high cell density culture.

The ratio of total carotenoid pigments per biomass tends to be negatively correlated with biomass production and Chl *b* accumulation. Carotenoid expression per microalgal cell tends to decrease in the presence of synthetic SLs. The microalgae adapt to different light regimes by adjusting their photosynthetic pigment accumulation [83]. The lower accumulation of carotenoids in the presence of synthetic strigolactones seems to be related to an adaptation of *C. sorokiniana* to light attenuation and an enhanced tolerance to higher reactive oxygen species (ROS). The carotenoid pigments are involved in the photoprotection of the thylakoids through the xanthophyll cycle [84]. In microalgal cells from the *Chlorella* genus (*C. vulgaris*), carotenoids were demonstrated to be involved in photoprotective quenching in both photosystem I and II [85] and antioxidant protection against ROS [86]. Lower accumulation of carotenoids tends to generate more reactive oxygen species due to reduced quenching and reduced antioxidant protection. The plant biostimulants induce molecular priming [87] and enhance plant cell tolerance to oxidative stress [88,89]. A similar effect seems to be involved in microalgae cell response to microalgal biostimulant application. The detailed mechanism involved in this effect of synthetic SLs on microalgae photosynthetic performance is not yet known.

The effects of synthetic SLs application on endo-signals networks from microalgal cells are not yet completely understood. In *Monoraphidium* sp. QLY-1 microalgae, the application of GR24 determines an increased level of Ca^{2+} and nitric oxide (NO) acting as intracellular signals [66]. The treatment with 1 μM GR24 to the macrozooid stage of *Haematococcus pluvialis* determined a significant increase in biomass production due to increased photosynthetic efficiency. In the haematocyst stage, the same concentration of GR24 induced a higher accumulation of astaxanthin due to increased antioxidant activities and modification of fatty acids metabolism [90]. In terrestrial plants, the strigolactone endo-signaling interplay with Ca^{2+} [91] and NO [92].

However, the endogenous SLs were not identified in microalgae [53] despite these similarities that suggest a phytohormonal role of SLs in microalgae. The investigations done till now have demonstrated a limited presence of the following genes coding for enzymes involved in strigolactone biosynthesis in microalgae, carotenoid cleavage dioxygenase 7 (CCD7), carotenoid cleavage dioxygenase 8 (CCD8) and 9-cis/all-trans- β -carotene isomerase (D27) [93]. Microalgal genomes do not include sequences coding for receptors specific for strigolactones perception and signaling, e.g., D14 (DWARF14), an α/β -hydrolase protein acting as a non-canonical strigolactone receptor, and the other proteins involved in D14-mediated SLs perception, the F-box protein D3 and the D53 repressor protein [53].

Exo-signals that include a butenolide ring were described in various biological systems, such as those isolated from lichen-derived bacteria [94] or the quorum-sensing quenchers and biofilm inhibitors produced by seaweeds/macroalgae [95]. Perception and response systems to such exo-signals, potentially developed in microalgae, could also detect the butenolide ring from synthetic SLs. The reported effects of SLs on microalgae are mainly related to the formation of association microalgae – bacteria, microalgae – fungi, or microalgae – bacteria – fungi in aquatic systems. GR24 was reported to induce the formation of different symbiotic associations that are efficient in biogas upgrading and pollutant removal. Applying 10^{-9}M GR24 increases the performance of *C. vulgaris* FACH-B – endophytic bacteria S395-2 in CO_2 removal and consumption of organic and mineral nutrients [96]. GR24 applied in the same 10^{-9} M concentration increased the efficiency of symbiosis between *C. vulgaris* FACHB-8 and *Pleurotus geesteranus* bio-32868 and improved pollutant and CO_2 removal [97]. 5-Deoxystrigol, applied at a concentration of 10^{-11} M , promotes the growth of symbiotic association *C. vulgaris* – *G. lucidum* – endophytic bacteria (S395-2), biogas upgrade, and nutrient removal [98].

The induction of the tolerance to the microalgae high-density cell culture could be related to a modulation of the quorum-sensing. Strigolactones modulate quorum sensing in different systems, leading to various effects. The initial function of strigolactone in the terrestrial plants seems to be a quorum-sensing (QS) signals. The *Physcomitrella patens* moss regulates its colony extension and protonema branching by producing strigolactones [99]. In flowering plants, SLs are also involved in

detecting neighboring plants [100]. Besides these QS functions in land plants, similar QS activities for SLs in other biological systems. The SL analog 2'-Epi-GR24 stimulated swarming motility in rhizobia [101]. SL-6 promoted biofilm formation in *P. graminis*, a nodulation-helper bacteria [62]. Natural and synthetic strigolactones modulated quorum sensing in *Vibrio cholerae*, promoting biofilm formation and reducing toxin production [102]. The marine *V. cholerae* bacteria developed its quorum sensing coordinated biosynthesis of human toxin during shrimp chitin hydrolysis [103]. Hydrolyzed (shrimp) chitin was reported to promote microalgae growth [104].

Using synthetic SLs as microalgal biostimulants has implications for applied research. Increased microalgae tolerance to abiotic stress, including light (attenuation) stress, is important for higher yield and practical applications for microalgae. Elucidation of the systems from microalgae involved in perception and response to butenolide exo-signals is a fundamental research direction, which will lead to a better understanding of microalgae/phytoplankton ecology or lichen formation.

3.2. Biostimulant Effects of Microalgal Extracts Towards Mung Seedlings

The plant biostimulant functions of microalgae extracts have undoubtedly been demonstrated in the last decade [23,24,105,106]. The seed treatment with microalgae extract improved the response of resulting seedlings and/or plants to salt stress in wheat [107], bell pepper [108] and leafy lettuce [109]. Among the mechanisms involved in salt stress mitigation by microalgae extract are enhanced root growth and improved nutrient uptake [110]. In our study, the extracts from *C. sorokiniana* which were stimulated with different doses of SL-6 strigolactone mimic positively influenced mung seedlings development and physiology in the presence or the absence of salt stress. The effects were higher than those of the non-stimulated microalgal culture. The control treatments C-7, C-8, and C-9 were included to evaluate the baseline effects without microalgal extract. The results indicated that these controls did not significantly impact the measured parameters, including radicle length, hypocotyl length, plant height, and proton pump activity.

The same observations can also be done when salt stress was applied. This result means that the observed enhanced roots and shoots are due to the active ingredients from the microalga extracts and not from the residual effect of SL mimic. Regarding seedling measurements (Figure 8), the treatments with the microalgal extracts from the biomass grown in the presence of synthetic SL increased both radicle and hypocotyl compared to water control (C). In the absence of salt, for Sk-7 treatment, the radicle growth compared to control C was approximately 17.36% higher, from 2.4077 ± 0.133 cm to 2.8259 ± 0.108 cm, and the hypocotyl length increased by 19.22%. The treatment Sk-9 increased the root length to 2.812 ± 0.11 cm, 16.80% longer than the control and the shoot length with approximately 18.04% more than control. The Sk-8 recorded the best results, showing an increase of approx. 26.47% compared to the control C. Similar results were obtained in the presence of salt, but the highest stimulation was for Sk-9, a little higher than Sk-8.

Roots proton pump enhances nutrient bioavailability, especially phosphorus, and nutrient uptake [111] and is a marker for biostimulant action [112]. Results obtained in our study for proton pump activity showed that the treatments with the microalgal extracts enhanced the extracellular H^+ level observed and measured by the medium acidification (and the resulting yellow color). The same happened for the specific extracellular H^+ level (se H^+), wherein the total area of the root (radicle) was considered. The chlorophyll fluorescence of microalga culture tends to be positively correlated with the root se H^+ under normal conditions, and it is correlated at a highly significant statistical level with the root se H^+ in salt-stressed seedlings.

This correlation suggests an involvement of the strigolactone exo-signals as a mediator of the associative interactions between edaphic (soil) benefit microalgae and plants (roots). SL-6 is a strigolactone mimic acting as exogenous signals for rhizosphere organisms [61]. Studies from the last decade spotlight the importance of microalgae as a component of the soil plant beneficial microbiome [113-115]. The microalgae from genus *Chlorella* (*Trebouxiophyceae*) have a great ecological plasticity, being adapted to both aquatic and soil environment. The strain ASIB BB67, isolated from soil alpine habitat, re-assessed as a *C. vulgaris* strain, has a tolerance to dessication greater than the type strain

C. vulgaris SAG 211-11b, isolated from an aquatic environment [116]. *C. sorokiniana* strains were isolated edaphic environment, irrigated soils [117] and arid soils [118]. Populations / strain from this *Chlorella* genus acts as plant biostimulant / biofertilizer. The filtrate of the *C. sorokiniana* strain isolated from irrigated soil promote wheat plant growth [117]. The *C. vulgaris* strain MACC-GA0056, isolated from a sub-tropical soil, applied as crude culture, algal extract, and culture filtrate, improve wheat germination and growth [119].

Strigolactones are a “cry for help” in the rhizosphere [120], shaping plant beneficial microbiome [121,122] and recruiting microorganisms that promote plant tolerance to abiotic stress [123]. Strigolactones are released as exo-signals in the rhizosphere by salt-stressed plants in the presence of microorganisms that contribute to salt stress mitigation [124]. Chlorophyll fluorescence is a biomarker of microalgae cell health and chloroplast integrity [125]. As discussed above, the synthetic SLs (exo-signals) improved the photosynthetic performance of *C. sorokiniana* (measured as chlorophyll fluorescence). The extracts of microalgae (healthier) cells, from (synthetic) strigolactone challenged cultures, promote seedling development and enhance seedling tolerance to salt stress. The microalgae benefits from the presence of healthier plants – lower light stress at the soil surface due to the shadow effect, and root exudates as an alternative carbon source for mixotrophic/heterotrophic growth. Such a potential benefic interactions between the plant roots and microalgae suggest request further investigations on the role of strigolactones as exo-signals for the recruitment of beneficial microalgae by stressed plants.

4. Materials and Methods

4.1. Materials

The strigolactone analog (\pm) GR24 was supplied by StrigoLab (Turin, Italy). The strigolactone mimic SL-6, (2-4 –methyl – 5 – oxo - 2,5 – dihydro – furan – 2 - yloxy) – benzo [de] isoquinoline - 1,3-dione), with molecular mass of 309.28 da, was synthesized as previously described [126], starting from 1,8-naphthalic anhydride.

The chemicals for microalga cultivation were: Iron(III) chloride (FeCl_3), sodium nitrate (NaNO_3), calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), cobalt(II) nitrate ($\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) were obtained from Merck (Darmstadt, Germany). Citric acid, Potassium dihydrogen phosphate (KH_2PO_4), zinc sulfate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), boric acid (H_3BO_3), sodium molybdate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$), were supplied by Scharlau (Barcelona, Spain). Manganese chloride ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$) was taken from Carl Roth (Karlsruhe, Germany) and EDTA disodium salt ($\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$) was purchased from Fluka (Honeywell, Morris Plains, NJ, USA). Copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) was procured from Chimopar (Bucharest, Romania). The reagent for chlorophyll extraction Dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany).

Agar and bromocresol purple, used for mung seedlings bioassay, were purchased from Scharlau (Barcelona, Spain).

The strain *C. sorokiniana* NIVA-CHL 17 was supplied by Norwegian Culture Collection of Algae, NORCCA, Norwegian Institute for Water Research (NIVA). Mung bean seeds (*Vigna radiata* (L.) R. Wilczek var. *radiata*) were supplied by a Romanian distributor of Vilmorin (Vilmorin, La Ménitré, France).

4.2. Testing the Effect of SL-6 on Microalgal Growth

Both GR24 and SL-6 were first solubilized in dimethyl sulfoxide (DMSO). Two stock solutions of 1 mM were prepared, considering the molar mass of each strigolactone: for GR24, MW_t = 298.29 g/mol and for SL-6, MW_t = 309.28 g/mol. The stock solutions were used to obtain the final tested concentrations, by serial dilutions with BG11 medium. The treatments applied were assessed in triplicate and were 1×10^{-7} M (c1), 1×10^{-8} M (c2) and, respectively 1×10^{-9} M (c3), together with a control and a solvent control (SC) with 10^{-7} M DMSO.

4.2.1. Microalgae Cultivation

Microalgae *C. sorokiniana* NIVA-CHL 17 were grown in sterilized 100 ml Erlenmeyer flasks, which contained 50 ml BG-11 growth media [127]. The medium was sterilized in an autoclave (Panasonic) at 121 °C for 15 min. The final chemical concentrations were the following: 0.24 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.23 mM KH_2PO_4 , 0.3 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 31 mM $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$, 0.0027 mM EDTA disodium salt, 0.021 mM ammonium iron(III) citrate, 0.19 mM Na_2CO_3 , BG-11 Trace Metals Solution 0.1% (0.046 mM H_3BO_3 , 9 mM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.77 mM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.17 mM $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 1.6 mM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.3 mM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$).

Fresh cultures were used for the test, with the final concentration of the inoculum 1% (500 $\mu\text{L}/50\text{ mL}$) of *C. sorokiniana* culture at the exponential phase. The sterile media distribution and flask inoculations were done in a microbiological hood Bio 2 Advantage Plus (Telstar, Barcelona, Spain), to keep axenic conditions.

The experimental treatments were incubated in a growth chamber AlgaeTron AG-230-Eco (Photon Systems Instruments, Drásov, Czech Republic) under controlled conditions of light (white, fluorescent lamp at 130 $\mu\text{mol}/\text{m}^2 \cdot \text{s}$) and temperature ($25 \pm 1^\circ\text{C}$), with a photoperiod of 14/10 h of light/dark. For 15 days the vessels were continuously shaken by an orbital shaker, Unimax 1010 (Heidolph, Schwabach, Germany) set at 140 rpm.

4.2.2. Measurement of Growth Parameters

The effect of GR24 / SL-6 on *C. sorokiniana* was evaluated by measuring the main growth parameters such as optical density, turbidity, biomass production, and pigment concentrations for 15 days.

Optical Density: To assess the microalgal growth, the absorbance at 750 nm of a ten-fold dilution sample was measured with a UV-Vis spectrophotometer USB4000-UV-VIS Ocean Insight (Orlando, Florida, United States). This wavelength was selected to minimize measurement errors caused by chlorophyll pigment interference [128]. The parameter was measured after 3 days, 5 days, 7 days, 11 days and at the end of the experiment, after 15 days respectively.

Turbidity: The cell density of the cultures was determined by measuring turbidity using a turbidimeter Grant-Bio DEN-1B (Grant Instruments, Shepreth, Cambridgeshire United Kingdom), expressed in McFarland measurement units. The analysis was conducted with a working volume of 2 ml culture, harvested under sterile conditions.

Biomass: For biomass determination, a volume of 5 ml from each variant was aseptically piped in pre-weighed Falcon tube and centrifuged at 9000 $\times g$ for 10 min using a Universal 32 centrifuge (Hettich, Tuttlingen, Germany). After removing the supernatant, samples were dried to a constant weight in an oven Memmert UN 75 (Memmert, Schwabach, Germany), at 50°C [129].

Pigment concentration: 2 ml of each sample was centrifuged at 8000 $\times g$ for 10 min at 20°C, to separate the cellular biomass from the supernatant. 2 ml of DMSO pre-heated at 60°C were added over the remaining pellet, and vortexed for 10 min. Next, the eppendorf tubes were centrifuged at 8000 $\times g$ for 10 min. The absorbance was measured at three different wavelengths, 480 nm, 649 nm and 665 nm, necessary to calculate the content of chlorophyll *a*, chlorophyll *b*, total carotenoids and total pigments in the samples (mg/L), according to the following formulas [129]:

$$\text{Chlorophyll } a (\text{ChlA}) = 12.47 \times (\text{OD}_{665}) - 3.62 \times (\text{OD}_{649}) \quad (1)$$

$$\text{Chlorophyll } b (\text{ChlB}) = 25.06 \times (\text{OD}_{649}) - 6.5 \times (\text{OD}_{665}) \quad (2)$$

$$\text{Total carotenoid} = [1000 \times (\text{OD}_{480}) - 1.29 \times (\text{ChlA}) - 53.78 \times (\text{ChlB})]/220 \quad (3)$$

$$\text{Total pigments} = (1) + (2) + (3) \quad (4)$$

Additionally, the expression of pigments per gram of biomass was determined by normalizing the concentration to the microalgal biomass.

Chlorophyll fluorescence. The chlorophyll fluorescence was measured after 7 and 15 days of culturing, using a PAM fluorometer (PSI AquaPen AP 110/P, Photon Systems Instruments, Drásov).

The manufacturer instructions were followed, to let the microalgal cultures adapt to dark for 10 min. The maximum quantum yield of PSII photochemistry $\phi P0$ was calculated according to the following formula :

$$\phi P0 = Fv/Fm \quad (5)$$

where Fm —maximum chlorophyll a fluorescence (after actinic flash), Fv —maximum variable fluorescence, $Fm-Fo$; Fo —minimum chlorophyll a fluorescence (after dark adaptation).

4.3. Preparation of Microalgal Biomass Extract

For microalgal extracts, we selected the *C. sorokiniana* biomass from the treatments stimulated by the SL-6 mimic, which is more advantageous than GR24 from the economic point of view.

The microalgal biomass was concentrated by centrifugation at $9000 \times g$ for 10 min, and the suspension was brought to an average concentration of 0.8 g/L. The equation used to calculate the volume of supernatant that needed to be removed (ΔV) in order to achieve the final concentration was the following:

$$\Delta V = V_1 - \frac{C_1 \times V_1}{C_2} \quad (6)$$

where: C_1 = the concentration of microalgal biomass in each treatment (g/L); V_1 = the microalga culture volume used for centrifugation (L); C_2 = final concentration of microalgal biomass (g/L); V_2 = final volume of microalgae culture after removing the excess supernatant (L). V_2 was calculated from the equation, and the difference V_2-V_1 was the volume of the supernatant removed from the sample.

The suspensions were subjected to ultrasonication treatment for cell rupture and extraction of the intracellular active ingredients. Each suspension was ultrasonicated for 10 min, with 20 sec ON and 20 sec OFF, with an 80% amplitude, using a 400W ultrasonic processor UP400 st (Hielscher, Teltow, Germany) [130]. The extracts were centrifuged at $4^\circ C$ and $9000 \times g$ for 10 min, and the supernatant was collected. Solutions were filtered first by $1.2 \mu m$ cellulose acetate filter (Sartorius Stedim Biotech GmbH) using a vacuum pump, and for sterility with a $0.2 \mu m$ PES sterile syringe filter. The final extract solutions combined both the extracellular microalgal biochemicals and the extracted intracellular active ingredients.

4.4. Plant Biostimulant Biotests on Seedlings

Mung bean seeds (*Vigna radiata*) were selected to test the microalgal extracts. The seeds were des-infected according to the following procedure: 3 min 90% EtOH immersion, water rinse, followed by 3 min 7% sodium hypochlorite immersion and 10 times rinsing with sterile water [131]

The biotests were carried out in 90 mm sterile petri dishes, each one containing two sterile gauze patches. On top 9 ml of each extract were aseptically pipetted and 10 sterile seeds were evenly placed using a sterilized tweezer. The experimental variants included controls with water, controls with SL-6 solutions (1×10^{-7} M, 1×10^{-8} M and 1×10^{-9} M, respectively), and treatments with the microalgal extracts, both without and with salt stress with a final molar concentration of 50 mM NaCl. The mung seeds were incubated in a growth chamber Micro Clima-Series™ Economic Lux Chamber (Snijders Labs, Tilburg, The Netherlands) with a temperature of $26^\circ C$ for a 16 h light cycle, $240 \mu mol m^{-2} s^{-1}$ constant led light and $22^\circ C$ for an 8 h dark cycle and were monitored for 5 days of growth.

4.4.1. Mung Bean Seedling Measurement

At the end of the growth period, the mung bean seedlings were photographed using a professional camera. Using the ImageJ software [132] the root (radicle) and shoot (hypocotyl) lengths were measured.

4.4.2. Medium Acidification Assay

The method applied was conducted based on [133]. On the second day of seedling growth, a solution of 0.04 g/L bromocresol purple in ddH₂O was prepared and adjusted to pH 6.7 with 1 N NaOH and supplemented with agar at a final concentration of 0.75%, autoclaved at 121°C, and poured into sterile 90 mm Petri dishes. Briefly, one mung seedling of each triplicate was placed on the partially hardened agar at approximately 30°C, pushing the roots into the agar until being partially embedded into the gel. After 24 h the plates were photographed and the yellow area, the color intensity of the yellow area and the root area were measured with ImageJ. The data were used to semi-quantify the specific extracellular H⁺ level (seH⁺) and the total H⁺ level (eH⁺), using the following equations: [133]

$$seH^+ = \frac{A_y \times I}{A_r} \quad (7)$$

$$eH^+ = A_y \times I \quad (8)$$

where A_y is the yellow area, I is the intensity of the yellow area, and A_r is the root area.

4.5. Statistical Analysis and Figure Design

The data underwent one-way ANOVA analysis (with a significance level set at $p \leq 0.05$), followed by a comparison of mean differences using the Tukey HSD post hoc test. Each experiment was conducted in triplicate, presenting the results as mean \pm standard error. The graphs from the figures were designed using Origin Pro 2018 software (OriginLab, Northampton, MA, USA). IBM SPSS Statistics for Windows, software version 26.0.0.0 (IBM, Armonk, NY, USA) was used for statistical analysis (One-Way ANOVA) and Pearson correlation analysis (Bivariate correlations).

5. Conclusions

The study investigated the effects of a strigolactone analog (GR24) and a mimic (SL-6) on the growth and biomass production of *Chlorella sorokiniana*. Further studies were conducted on the effects of microalgal culture extracts on mung bean development and growth. SL-6, a cost-effective alternative to GR24, promoted microalgae growth and enhanced photosynthetic performance. Treatment with SL-6 consistently resulted in comparable or superior outcomes in biomass production, pigment synthesis, and photosystem II yield (determined as chlorophyll fluorescence) compared to GR24 treatments. These findings highlight the potential of SL-6 as a cost-efficient yet highly effective solution for enhancing microalgal cultivation and biomass yield.

The higher dose of 10⁻⁷ M inhibited microalgal growth determined as cell density, biomass production, or pigment synthesis for both tested synthetic strigolactones, GR24 and SL-6. Lower doses stimulated the growth parameters of *C. sorokiniana*, enhancing up to 24.00% the total pigment accumulation when SL-6 at 10⁻⁹ M was applied. These variations demonstrate a hormetic effect, with higher doses causing inhibition and lower doses have a stimulant activity.

The treatments with synthetic strigolactones, especially with SL-6, improved microalgae tolerance to the light attenuation stress in high-density culture. These results have practical implications for microalgae cultivation, offering an alternative to the solutions for compensating light stress in microalgae cultivated in agitated media.

Microalgal extracts were proved to have plant biostimulant functions, enhancing radicle elongation and overall seedling length in normal conditions and increasing mung bean tolerance to salt stress. The activity of the root proton pump, a marker for biostimulant action [112], is amplified by treatment with microalgae extracts. The extract from microalgae challenged with SL-6, especially the treatment with SK-8 (extract of *C. sorokiniana* culture stimulated with 10⁻⁸ M SL-6), demonstrated a more pronounced plant biostimulant activity than non-challenged culture. The results suggest a potential involvement of strigolactone exo-signals in establishing the mutual benefit associations between edaphic microalgae and plant roots.

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