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Microalgae from Nordic collections demonstrate biostimulant effect by enhancing plant growth and photosynthetic performance

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Abstract

We investigated the biostimulant potential of six microalgal species from Nordic collections extracted with two different procedures: thermal hydrolysis with a weak solution of sulfuric acid accompanied by ultrasonication and bead-milling with aqueous extraction followed by centrifugation. To this aim, we designed a phenotyping pipeline consisting of a root growth assay in the model plant *Arabidopsis thaliana*, complemented with greenhouse experiments to evaluate lettuce yield (*Lactuca sativa* L. cv. Finstar) and photosynthetic performance. The best-performing hydrolyzed extracts stimulated *Arabidopsis* root elongation by 8%–13% and lettuce yield by 12%–15%. The in situ measured photosynthetic performance of lettuce was upregulated in the efficient extracts: PSII quantum yield increased by 26%–34%, and thylakoid proton flux increase was in the range of 34%–60%. In contrast, aqueous extracts acquired by bead-milling showed high dependence on biomass concentration in the extract and an overall plant growth enhancement was not attained in any of the applied dosages. Our results indicate that hydrolysis of the biomass can be a decisive factor for rendering effective plant biostimulants from microalgae.

1 | INTRODUCTION

The genetic diversity of microalgae is an unfathomed source of bioactive compounds for multivarious products, such as pharmaceuticals, nutraceuticals, biopesticides, and biostimulants (González-Pérez et al., 2021; Silva et al., 2022). Plant biostimulants facilitate nutrient uptake, improve crop performance and tolerance to abiotic stress, and therefore can have a crucial role in reducing dependency on synthetic fertilizers and plant protection products, and thus address agronomic sustainability challenges (Chiaiese et al., 2018). Though macroalgae have long been known for their beneficial influence on crop production, the interest in microalgae is on the rise due to recent technological development in microalgae cultivation, enabling nutrient-looped circular economies, and refineries rendering biomass with standard composition (Allahverdiyeva et al., 2021; Lacroux et al., 2023).

Several microalgal and cyanobacterial species have been reported to stimulate germination, seedling growth, shoot, and root biomass of various crops (Bulgari et al., 2019; Gonçalves, 2021; Ronga et al., 2019). Microalgae with plant biostimulant effects have often been reported based on so called hormone-like effects that focus on hormonal evaluation (Garcia-Gonzalez & Sommerfeld, 2016; Navarro-López et al., 2020). However, aside from hormones, many other components in microalgae with bioactive potential might remain unknown. Microalgal crude extracts (MCEs) can induce nutrient uptake and plant growth by triggering accumulation of metabolites in primary and secondary biosynthetic pathways, such as lipid and jasmonate synthesis (Mutale-Joan

TABLE 1 List of microalgae screened for plant-biostimulant activity.

Microalga species	Strain ID	Growth medium	Culture collection
Chlorella sorokiniana	NIVA-CHL176	BG11	NORCCA
Coelastrum sp.	K-0559	BG11 + vitamins[a]	NORCCA
Porphyridum purpureum	NIVA-1/92	50% BG11 in seawater[b]	NORCCA
Scenedesmus sp.	UHCC0027	BG11	HAMBI
Tetradesmus obliquus	NIVA-CHL107	BG11	NORCCA
Tetraselmis subcordiformis	NIVA-2/94	50% BG11 in seawater[b]	NORCCA

[a] https://norcca.scrol.net/norcca-algal-culture-medium.

[b] Instant Ocean, Aquarium Systems, France.

et al., 2020). Microalgal polysaccharides have also improved the metabolite profile of azelaic acids, phytosterol, and other precursors in plant defense mechanisms (Rachidi et al., 2021).

Although a wide range of bioactive compounds has been identified in microalgae, including phytohormones, amino acids, polyamines, glycosides, fatty acids, vitamins, terpenoids, and flavonoids (Mazepa et al., 2021; Mógor et al., 2018; Plaza et al., 2018; Puglisi et al., 2018; Refaay et al., 2021), the extract composition varies depending not only on microalgal strain, but also on cultivation method, harvesting time, and extraction technique (Aremu et al., 2015; Gitau et al., 2022; Mironiuk & Chojnacka, 2018). While extraction should be inexpensive and environmentally sustainable, bioactive compounds must be preserved (Kapoore et al., 2021). In this context, mechanical methods seem more favorable than chemical or thermal methods. Among mechanical methods, bead milling is considered one of the most effective methods for cell disruption (Kapoore et al., 2018), and bead-milling has previously shown an effective increase in the antioxidant activity of *Chlorella vulgaris* and *Scenedesmus acutus* (Stirk et al., 2020). For the processing of macroalgal biomass, one of the most utilized extraction processes on the industrial level is chemical hydrolysis (El Boukhari et al., 2020). Microalgal extracts acquired by acid hydrolysis on a laboratory scale stimulated tomato shoot and root growth and increased nutrient efficiency uptake of the seedlings (Mutale-Joan et al., 2020). Though several studies have shown lettuce growth enhancement by microalgal extracts (Kopta et al., 2018; Puglisi et al., 2020; Rouphael et al., 2017), the underlying physiological processes have not been studied thoroughly.

In this study, we hypothesized that biomass of intensively grown microalgae could be utilized to produce efficient plant biostimulants on the condition that effective strains and extraction procedures are employed. We developed a cost-effective phenotyping pipeline, consisting of a rapid root growth assay and medium-term greenhouse experiments, that was used to compare two relatively inexpensive and scalable extraction methods—bead-milling and thermal hydrolysis. We also attempted to identify physiological processes responsible for observed effects by in situ evaluation of photosynthetic performance. Our research aims to further the understanding of microalgal plant growth-promoting activity and thus contribute to the development of effective plant biostimulants acquired from sustainably acquired microalgal biomass.

2 | MATERIALS AND METHODS

2.1 | Microalgae cultivation and harvesting

Six microalgal species were acquired from the Norwegian Culture Collection of Algae (NORCCA) and the Microbial Domain Biological Resource Centre (HAMBI). The strains were used to inoculate 100 mL of BG11 medium (pH = 7.5) (Stanier et al., 1971), with or without the addition of seawater and vitamins if required by guidelines (see Table 1), and maintained at room temperature and continuous light (50 μmol m^{-2} s^{-1} photosynthetic photon flux density; PPFD). After 4 weeks, cultures were used for inoculation of glass cultivation bottles containing 4 L of BG11 (with or without the addition of seawater and vitamins) in duplicate batches. Each culture was cultivated in a growth chamber (Sanyo) with continuous light (130 μmol m^{-2} s^{-1} PPFD), at 25°C and aerated with 1.5% CO_2. Continuous mixing of CO_2 with growth media was secured with magnetic stirrers. The culture growth was recorded spectrophotometrically at OD_{750} (Genesys 10S UV–vis, Thermo Scientific), each culture commencing with an $OD_{750} = 0.1 \pm 0.05$. Cultures were harvested in the late exponential phase (Figure S1). Biomass was separated from the medium by centrifugation (6000*g*, 15 min, 18°C; Avanti JXN-26, Beckmann), freeze-dried overnight (Alpha 1-4LSC, Christ), and the dried biomass was stored at −20°C.

2.2 | Preparation of microalgal crude extracts

2.2.1 | Aqueous crude extracts

A sample of 67.5 mg of dried microalgal biomass was ground in a mortar and resuspended in 1.5 mL deionized water (Milli-Q; Merck). Cellular disruption was performed at 4°C with 0.5 mm ZrO beads filling the 2 mL Eppendorf tubes to one quarter (~1 g) using a bead beater for 5 min at maximum speed (Bullet Blender Storm 24, Next Advance). Subsequently, the suspension was centrifuged (16,000 *g*, 5 min, 4°C), and the supernatant was collected. The pellet was then again bead-milled with added MQ-water two more times to get 4.5 mL of concentrated suspension (15 g_{DW} L^{-1}). The resulting aqueous crude extracts (ACEs) were stored at −20°C and adjusted to the final concentration with distilled water shortly before the experiments.

2.2.2 | Hydrolyzed crude extracts

Thermal acid hydrolysis was performed according to Mutale-Joan et al. (2020) with modifications. In short, 0.75 g of freeze-dried microalgal biomass was ground in a mortar and hydrolyzed with 50 mL of 0.1 M H_2SO_4 (Scharlab, Spain) to have a biomass concentration of 15 g_{DW} L^{-1}. The mixture was vortexed and heated for 2 h at 95°C with constant stirring while interrupted every 30 min for 10 min bath sonication (Venus2; Polaris). After the hydrolysis, the suspension was cooled to room temperature and centrifuged (6000 *g*, 10 min, 4°C). The supernatant was collected, and the pH was adjusted to 5.8 (KOH). The hydrolyzed crude extracts (HCEs) were stored at −20°C and adjusted to experimental concentration by adding distilled water.

2.3 | *Arabidopsis* rooting assay

For the *Arabidopsis* rooting assay, we employed and modified the protocol described by Rayorath et al., 2008. Seeds of *A. thaliana* ecotype Columbia were sieved for size 280–300 μm to minimize heterogeneity, sterilized according to chlorine gas protocol (Lindsey et al., 2017), and transferred to square plates with autoclaved ½ MS media (Duchefa BV), 0.8% agar (Duchefa BV), 1% sucrose (Millipore) with pH adjusted to 5.8 (KOH). The seeds were placed in one line with ca., 3–4 mm apart and stratified in 4°C dark for 48 h for similar germination timing. Then, plates were placed vertically in a growth chamber (Fitotron–Weiss) with ambient light (120 μmol m^{-2} s^{-1} PPFD, 16/8 h light–dark cycle) and constant temperature (22°C). After 5 days, 15 seedlings with similar root lengths (17–20 mm) were carefully transferred in sterile conditions to another square plate with autoclaved ½ MS media, 5 mM MES (pH 5.8, Sigma-Aldrich), 0.8% agar. These plates were in the case of treatment groups supplemented with MCE in specific final doses (DW)—ACEs with 0.05, 0.1, 0.3, and 0.5 g L^{-1}, and HCEs with 0.1, 0.5, and 1 g L^{-1}. Seedlings were arranged in one line with 7 mm spaces, and the root tip position was marked with a fine tip permanent marker. Plates with the seedlings were placed vertically in the growth chamber with the previous settings. In one experimental batch, plates supplemented with extracts in different concentrations were tested. Batches were repeated for each microalga and concentration to validate the reproducibility of results ($n = 2 \times 15$). RGB images were taken on the 3rd, 5th, and 7th day after transfer with a Canon EOS 250 camera. Roots were analyzed with the ImageJ software (Schindelin et al., 2012). Primary root elongation was measured from the position on day 0, and lateral roots (length >2 mm) were counted on day 5 from the transfer. Statistical significance was evaluated within each day with the Kruskal–Wallis test ($p < 0.05$) followed by Dunn's multiple comparison tests adjusted according to Benjamini et al. (2006) for false discovery rate (FDR) control.

2.4 | Greenhouse lettuce experiments

To investigate the effects of MCE on yield and photosynthetic performance of a crop, we conducted experiments with lettuce in a semi-controlled glass greenhouse environment (Ruissalo Botanical Garden of the University of Turku, Finland) from May to October 2022. The environmental settings during the experiments were: 15–18°C, natural light supplemented with halogen light (∼300 μmol m^{-2} s^{-1} PPFD) in a 16/8 h diurnal cycle, 60% rel. humidity. Seeds of a crisp-type lettuce variety, *Lactuca sativa* L. cv. Finstar (Helle Oy) were sown into 15-cell seedling trays with moist sphagnum peat substrate (VHM620 pH 6.0 R8060; Kekkilä). The presence of pests was prevented by applying the *Steinernema* sp. nematodes (Helle Oy) at the beginning of experiments. MCE-treated and control plants (untreated) were arranged in a completely randomized block design ($n = 15$) with at least one control plant in each block. MCEs were applied by soil drenching on the 14th, 21st, and 28th day after germination (DAG) in specific doses—ACE with 0.1, 0.3, 0.5, and 1 g L^{-1}, and HCE with 0.5 and 1 g L^{-1}. Trays were irrigated abundantly, and individual plants were supplemented with equal amounts of nutrient solution (Ferticare 7-9-32; Yara) on the 18th, 25th, and 32nd DAG. Plants were harvested on the 35th DAG, and the fresh weight (FW) of the shoot parts was determined immediately. Statistically significant differences between the mean values were determined using one-way ANOVA followed by Fisher's least significant difference (LSD) post hoc test with a significance level of $p < 0.05$.

2.4.1 | Lettuce photosynthetic performance

Spectroscopic measurements of photosynthetic parameters were performed with MultispeQ V2.0 (PhotosynQ LLC) using Photosynthesis RIDES 2.0 protocol based on pulse-amplitude modulation (PAM) fluorometry. The measurements were taken on the 32nd DAG with seven replicates per treatment. Notable parameters, that is, the relative chlorophyll content (SPAD), the quantum yield of PSII (ΦII), the thylakoid membrane proton conductivity (gH^+), and proton flux (vH^+), were determined under light acclimation (Kononchuk et al., 2022; Kuhlgert et al., 2016). Treatments were considered significantly different from control if they had a $p < 0.05$, based on one-way ANOVA followed by Fisher's LSD test, or for non-parametric testing—based on Kruskal–Wallis rank test followed by Dunn's comparison adjusted for FDR control according to Benjamini et al., 2006. Normality of residuals was tested using the Shapiro–Wilk test and the homogeneity of variances using the Brown–Forsythe test.

3 | RESULTS

The workflow of the experiments is presented in Figure 1. Six microalgal species acquired from NORCCA and HAMBI algal collections (Table 1) were cultivated in a laboratory setup until the late

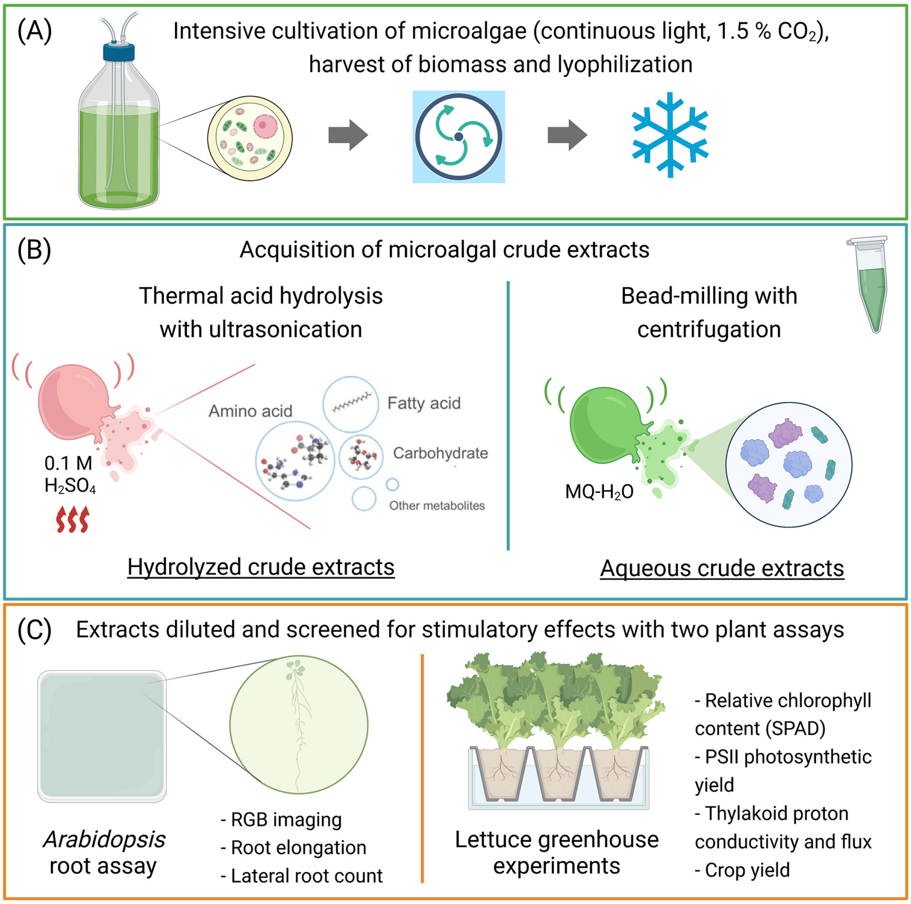


FIGURE 1 The workflow of screening microalgae for plant biostimulant effects. (A) Six microalgae were cultivated in bottles with growth media, continuous light, and 1.5% CO_2, harvested by centrifugation, and freeze-dried. (B) Microalgal crude extracts were acquired by either thermal acid hydrolysis with weak sulfuric acid (0.1 M) complemented with ultrasonication (hydrolyzed crude extracts) or by bead-milling followed by centrifugation (aqueous crude extracts). In both cases, the supernatant was collected and stored. (C) The phenotypic characterization for discerning growth-enhancing effects of the diluted microalgal crude extracts was performed via two plant bioassays: *Arabidopsis* rooting assay conducted on agar media and greenhouse experiments for evaluating relative chlorophyll content, photosynthetic performance, and crop yield of lettuce. The image was created with BioRender.com.

exponential phase (Figure S1). The resulting biomass was freeze-dried and then either bead-milled with MQ-water or hydrolyzed with a weak solution of sulfuric acid to acquire crude extracts. Our experimental design of screening the microalgae for plant growth stimulating effects consisted of two types of bioassays: a rapid rooting assay conducted on ½ MS agar media supplemented with MCE and greenhouse experiments for crop yield assessment.

3.1 | Hydrolyzed crude extracts from microalgae enhance *Arabidopsis* root growth

A. thaliana is a small terrestrial plant with a short vegetative cycle and, therefore, suitable for rapid and large-scale screenings (Pavicic et al., 2017). Most pronounced stimulation effects on the *Arabidopsis* root elongation were observed on day 7 from transfer (mean ± SE and *p*-values for all treatment groups and each analyzed day are listed in Tables S2 and S3). Primary root growth was stimulated with several HCEs, especially at the 0.5 g L^{-1} dose (Figure 2A). *Scenedesmus* sp. HCE stimulated root elongation in all applied concentrations (0.5 g L^{-1}: $p < 0.0001$, 0.1 g L^{-1}: $p = 4.9\text{E} - 03$, 1 g L^{-1}: $p = 6.2\text{E} - 03$). A significant increase was also attained with a 0.5 g L^{-1} dose HCE of *P. purpureum* and *Coelastrum* sp. ($p < 0.0001$). Interestingly, *C. sorokiniana* HCE increased primary root elongation at a lower dose (0.1 g L^{-1}; $p = 2.8\text{E} - 02$) but showed slight inhibition at 1 g L^{-1} ($p = 5.79\text{E} - 02$). *T. subcordiformis* HCE (0.5 g L^{-1}) enhanced root elongation on day 5 ($p = 1.18\text{E} - 02$), but the stimulation effect faded on day 7 ($p = 2.62\text{E} - 01$). The highest stimulation of the root elongation (13.35%) was observed with the *P. purpureum* HCE, 0.5 g L^{-1}. Effects of *T.* obliquus HCE were weak, and a 1 g L^{-1} dose inhibited *Arabidopsis* root elongation ($p = 1.33\text{E} - 02$). Though lateral root establishment is promoted by nutrient heterogeneity in the growth media, lateral roots are important for increasing the surface area of a healthy root system (Banda et al., 2019). Nevertheless, among hydrolyzed extracts, only *C. sorokiniana* (0.1 g L^{-1}) and *Coelastrum* sp. (0.5 g L^{-1}) demonstrated significant stimulation of lateral root establishment (Figure 2B; $p = 1.19\text{E} - 02$ and $p = 7.74\text{E} - 03$, respectively). Altogether, *Coelastrum* sp. HCE enhanced *Arabidopsis* root growth in horizontal and vertical directions.

3.2 | Effects of aqueous crude extracts are highly concentration-dependent

ACEs of *Chlorella* sp. and *Scenedesmus* sp. microalga were previously reported for high antioxidant activity and enhancement of root formation (Stirk et al., 2020). The rooting assay allowed us to determine

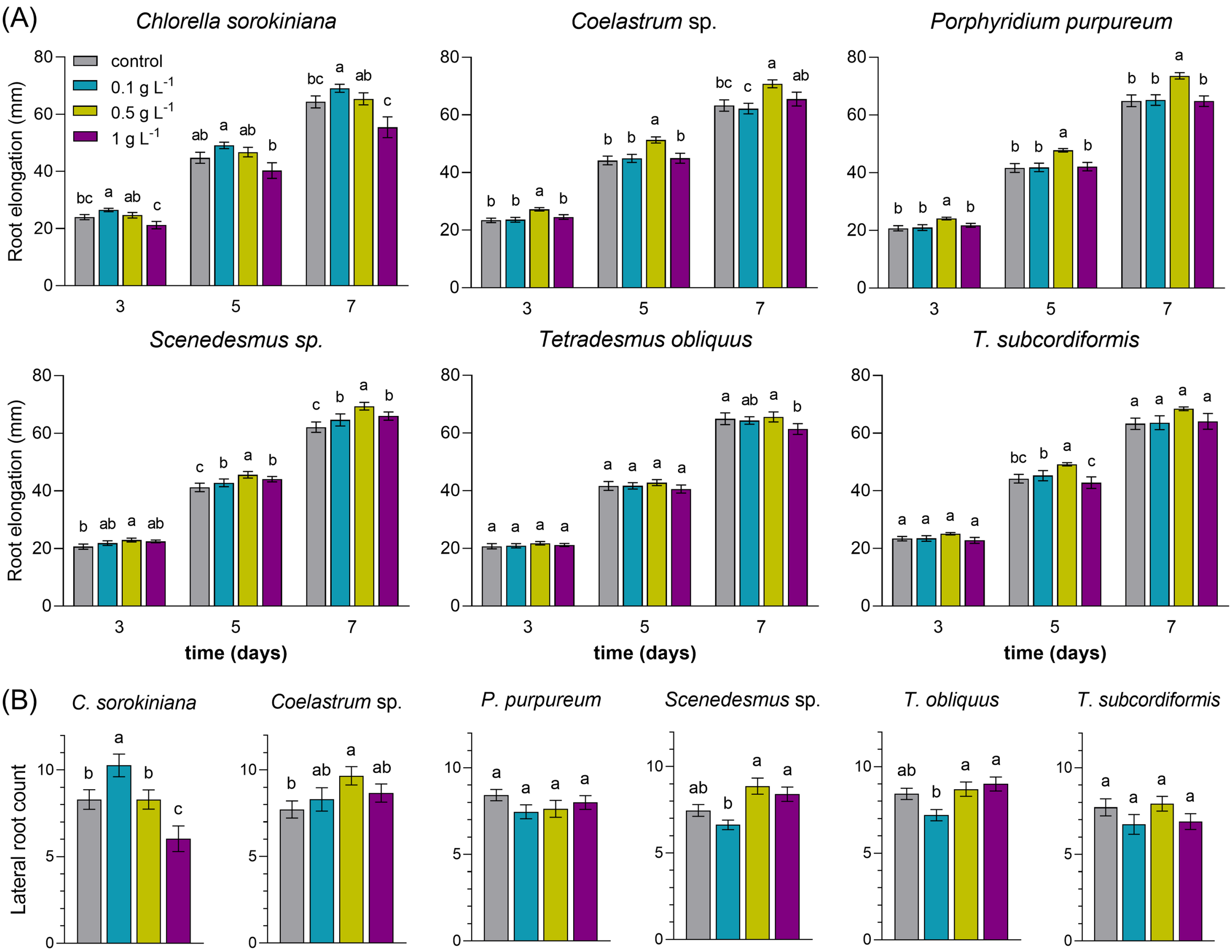


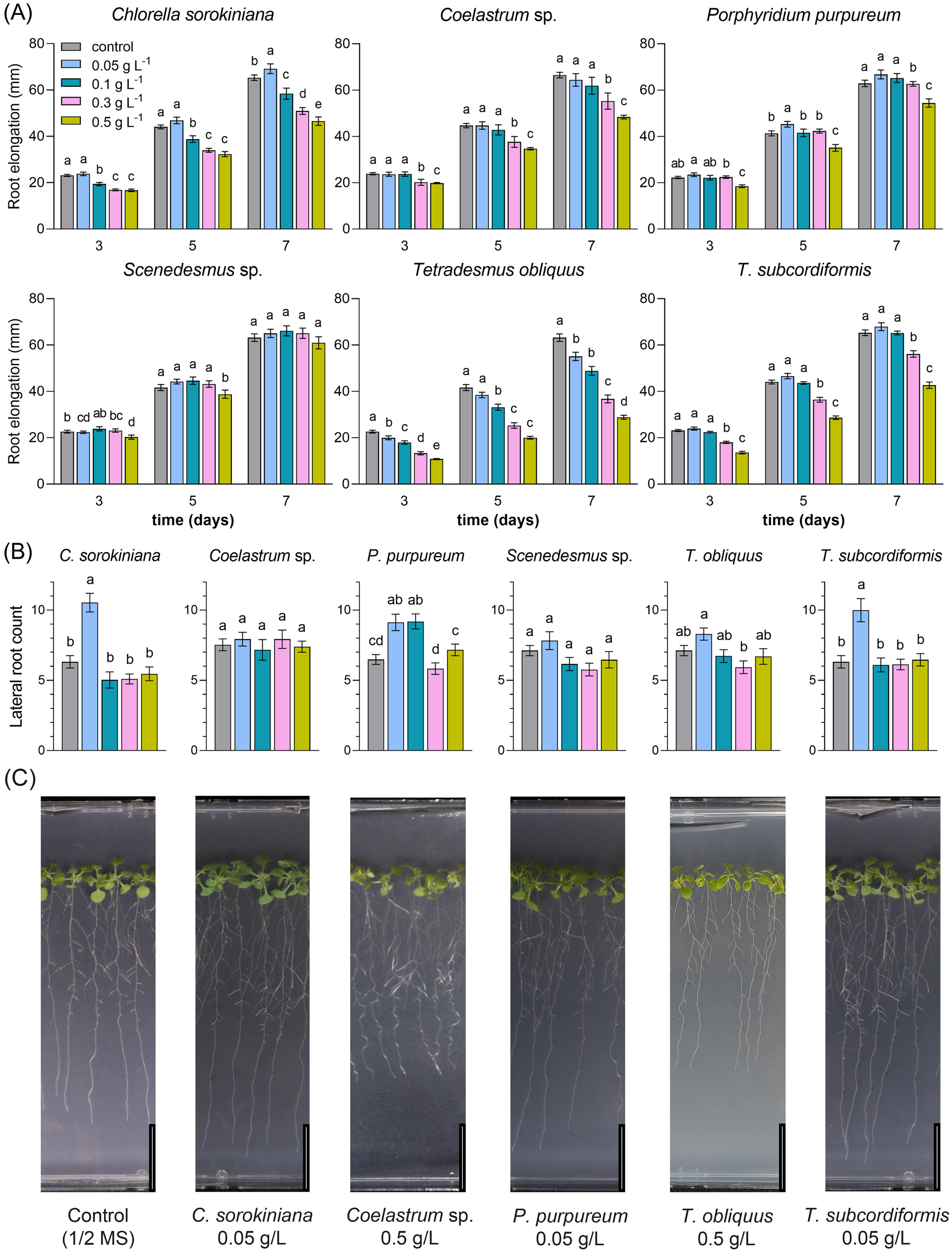
FIGURE 2 Several hydrolyzed microalgal crude extracts significantly increase root elongation and lateral root establishment in *Arabidopsis*. Five-days old *A. thaliana* seedlings were transferred to ½ MS agar media (control) supplemented with crude extracts acquired from six screened microalgae in different concentrations (0.1 g L^{-1}, 0.5 g L^{-1}, and 1 g L^{-1}). (A) Primary root elongation analyzed by ImageJ on days 3, 5, and 7 after transfer. (B) Lateral roots counted on day 5 from transfer. Different letters above bars indicate significant differences within 1 day according to Dunn's test (FDR adjusted; $p < 0.05$). Bars represent mean ± SE ($n = 28–30$).

in vivo responses to similar extracts. A dose of 0.05 g L^{-1} ACE of *C. sorokiniana* stimulated both primary root growth ($p = 3.82E - 02$) and lateral root establishment ($p = 5.2E - 06$; Figure 3A,B), while a higher concentration inhibited root elongation. Although similar trends emerged, none of the other extracts stimulated primary root formation. Lateral roots were further enhanced with a 0.05 g L^{-1} and 0.1 g L^{-1} dose of *P. purpureum* ($p = 6.83E - 05$ and $p = 3.01E - 05$, respectively) and a 0.05 g L^{-1} dose of *T. subcordiformis* ($p = 1.06E - 04$) ACE.

In comparing the effects on the root phenotype of ACE (Figure 3C), *C. sorokiniana*, *P. purpureum*, and *T. subcordiformis* ACE at a dose of 0.05 g L^{-1} promoted a healthy, branched root system. In contrast, 0.5 g L^{-1} ACE of *Coelastrum* sp. and *T. obliquus* caused shortening of the root complemented with distortion from normal growth similar to an overdose of ethylene or ABA (De Smet et al., 2003; Negi et al., 2008).

3.3 | Hydrolyzed microalgal crude extracts increase lettuce crop yield

To assess the stimulating potential of the MCEs on crop yield and green biomass of plants, we applied the hydrolyzed and aqueous extracts to the soil with lettuce seedlings (*L. sativa* L. cv. Finstar) three times throughout a shortened vegetative cycle (35 days). The FWs were determined at harvest to assess crop yield performance. The results indicate that lettuces treated with ACE showed negligible differences from control plants or even growth inhibition (Figure 4A)—the ACE of *Coelastrum* sp., *P. purpureum*, and *T. subcordiformis* (all with 1 g L^{-1} dose) displayed a decrease of green biomass FW (18.16%, 11.64%, and 13.20%, respectively). However, the yield significantly increased after application of the following HCE-dose (Figure 4B): *T. obliquus*: 1 g L^{-1} (15.97%), *Coelastrum*: 0.5 and 1 g L^{-1} (14.22% and 13.19%, respectively), *T. subcordiformis*: 0.5 g L^{-1} (13.34%), *P. purpureum*: 1 g L^{-1} (12.78%), and *Scenedesmus* sp.: 0.5 g L^{-1} was at the threshold of detectability ($p = 0.05$; 12.52%). Surprisingly, although a low dose of *C. sorokiniana* HCE stimulated *Arabidopsis* root growth, neither of the two applied doses enhanced lettuce yield significantly.



Control (1/2 MS)
C. sorokiniana 0.05 g/L
Coelastrum sp. 0.5 g/L
P. purpureum 0.05 g/L
T. obliquus 0.5 g/L
T. subcordiformis 0.05 g/L

FIGURE 3 Legend on next page.

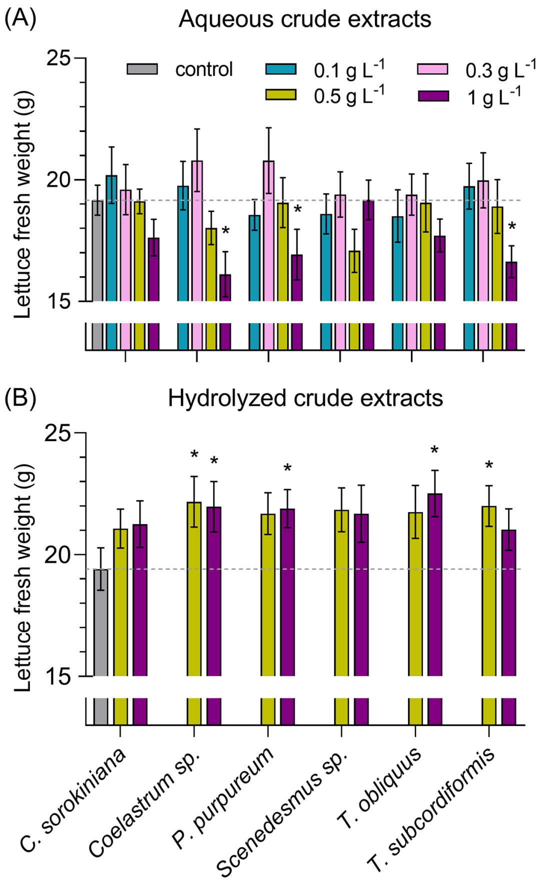


FIGURE 4 Best-performing microalgal crude extracts increase greenhouse-grown lettuce fresh weight. Lettuces treated with (A) aqueous crude extracts in 0.1 g L^{-1}, 0.3 g L^{-1}, 0.5 g L^{-1}, and 1 g L^{-1} dose; (B) hydrolyzed crude extracts in 0.5 g L^{-1} and 1 g L^{-1} dose; control = untreated plants. Extracts acquired from six microalgal species were applied three times throughout the vegetative cycle by soil drenching in different concentrations. Green biomass was harvested and weighed 35 days after germination. Bars represent mean ± SE ($n = 15$) and asterisks indicate significant differences to the control plants as determined by protected Fisher's LSD test (ANOVA; $p < 0.05$).

3.4 | Effective microalgal crude extracts upregulated photosynthesis in lettuce

Photosynthetic performance can delineate the general fitness, productivity, and the state of the underlying physiological processes (Kromdijk et al., 2016). On day 32 (DAG), we conducted spectrophotometric measurements of photosynthetic parameters on lettuce leaves. The results of the photosynthetic measurements on lettuces treated with HCE indicate no significant variation in the relative chlorophyll content (Figure 5A). However, several parameters assessing the photosynthetic performance were increased after the HCE treatments. PSII quantum yield measured on light-acclimated leaves was significantly upregulated after six treatments with relative increases ranging from 25.96% with *P. purpureum* (0.5 g L^{-1}) to 33.90% with *Scenedesmus* sp. (1 g L^{-1}; Figure 5B). The thylakoid proton conductivity in the lettuce leaves significantly increased after the HCE treatment of two microalga, *C. sorokiniana* ($p = 3.34E-03$; 1 g L^{-1}) and *P. purpureum* ($p = 7.34E-03$; 0.5 g L^{-1}; Figure 5C). Finally, the lettuce thylakoid proton flux has increased after treatment with most of the hydrolyzed microalgal extracts (Figure 5D)—ranging from 33.99% with up to 59.87% (*C. sorokiniana*—0.5 g L^{-1} and 1 g L^{-1}, respectively).

4 | DISCUSSION

New effective plant biostimulants ought to be acquired from natural resources following a bottom-up approach where biological screening represents the first step in the process (Povero, 2020). Despite abundant research on microalgal plant biostimulants, extensive screenings of the diverse microalga collections are largely underscored. The main reason is probably a lack of cheap and relatively fast available methods. Therefore, many current screenings rely on assays for assessment of the hormonal activity of microalgal extracts (Masojídek et al., 2022; Rupawalla et al., 2022). However, this diminishes the possibility of finding other bioactive compounds with plant-enhancing effects. In this work, we developed a rapid and affordable phenotyping pipeline for evaluating plant biostimulant effects of microalgae. Microalgae can be a feasible resource for producing high-value products if suitable strains, cultivation, and extraction methods are utilized (Carnovale et al., 2021; Kapoore et al., 2021). For industrial potential, advantageous microalgae should meet the minimum requirement of scalability (Allahverdiyeva et al., 2021; Barone et al., 2019). Therefore, species with robust biomass growth and suitable for intensive cultivation were selected for our experiments. For screening purposes, we designed the cultivation set-up to fit the laboratory scale and mimic intensive culturing. For further biomass processing, we followed a stepwise approach with freeze-drying in the first step and bead-milling or thermal acid hydrolysis in the second step. Bead-milling was followed by centrifugation, and hydrolysis was further accompanied by heating and ultra-sonication. Altogether, we tested 42 extracts from six microalgal strains acquired with two extraction methods and applied in

FIGURE 3 Aqueous microalgal crude extracts can inhibit *Arabidopsis* root growth. Five-days old *A. thaliana* seedlings were transferred to ½ MS agar media (control) supplemented with crude extracts acquired from six screened microalgae in four different concentrations (0.05 g L^{-1}, 0.1 g L^{-1}, 0.3 g L^{-1}, and 0.5 g L^{-1}). (A) Primary root elongation analyzed by ImageJ on days 3, 5, and 7 after transfer. (B) Lateral roots counted on day 5 from transfer. Different letters above bars indicate significant differences within 1 day according to Dunn's test (FDR adjusted; $p < 0.05$). Bars represent mean ± SE ($n = 28–30$). (C) Representative images of *A. thaliana* seedlings grown on agar media supplemented with aqueous crude extracts acquired from microalgae (g_{DW} L^{-1}). Scale bar = 20 mm.

different doses to identify optimal plant-enhancing effects by evaluating *Arabidopsis* rooting and lettuce crop performance.

The processes responsible for root elongation in *A. thaliana* are viewed as a result of an intricate interplay of environmental conditions and plant innate regulation mechanisms (Band et al., 2012). Microalgae biomass contains significant amounts of plant hormones, such as auxins, cytokinins, gibberellins, abscisic acid (ABA), ethylene, and so on (Gonçalves, 2021; Kapoore et al., 2021). ACEs acquired by bead-milling have often displayed hormonal activity assuming stimulation of tissue-specific processes, such as germination, cotyledon expansion, and rooting of excised shoots (Ferreira et al., 2021; Navarro-López et al., 2020; Stirk et al., 2020). Our results of the rooting assays indicate that 7 days of intensive ACE exposure can inhibit root growth (Figure 3). Only *C. sorokinana* ACE in 0.05 g L^{-1} dose significantly increased root elongation and lateral root development in *Arabidopsis*. High concentrations of ACE inhibited root growth and, in some cases, resulted in an altered root morphology (*Coelastrum* sp.; Figure 3C). The root development of *Arabidopsis* seedlings can be susceptible to increased concentrations of plant hormones, especially ABA or ethylene (De Smet et al., 2003; Negi et al., 2008). Interestingly, decreasing the concentration of ACE to 0.05 g L^{-1} did not have the expected root-stimulation outcome for most of the ACE. Also, application of ACEs did not increase the FW of lettuce in any of the applied concentrations (0.1–1 g L^{-1}; Figure 4A). Furthermore, treatment with *Coelastrum* sp., *P. purpureum*, and *T. subcordiformis* ACE resulted in a decrease in lettuce yield when applied at the highest concentration. Some studies point out the importance of microalgal circadian rhythms and harvesting time in relation to balanced hormonal composition of the extracts (Stirk et al., 2011; Xu et al., 2016), which was not considered in this study due to the focus on intensive cultivation.

Contrastingly, HCEs stimulated root elongation in four of the six HCE (Figure 2A). *C. sorokiniana* HCE enhanced both primary and lateral root development, while *P. purpureum* HCE rendered a distinctive root phenotype with increased primary root growth (Figure 2A) but slightly decreased lateral root development (Figure 2B). *Coelastrum* sp. and *Scenedesmus* sp. enhanced both observed aspects resulting in a healthy root system. The optimal concentration was 0.5 g L^{-1} for *Coelastrum* sp., *P. purpureum*, *Scenedesmus* sp., and 0.1 g L^{-1} for *C. sorokiniana*. HCE of *C. sorokiniana* displayed previously significant stimulation of tomato root and shoot growth, as well as metabolite stimulation (Mutale-Joan et al., 2020). Even though we observed a stimulation in lettuce photosynthetic performance, our analysis of green biomass did not show a significant increase with *C. sorokiniana*. However, all other HCE in this study improved lettuce crop yield by

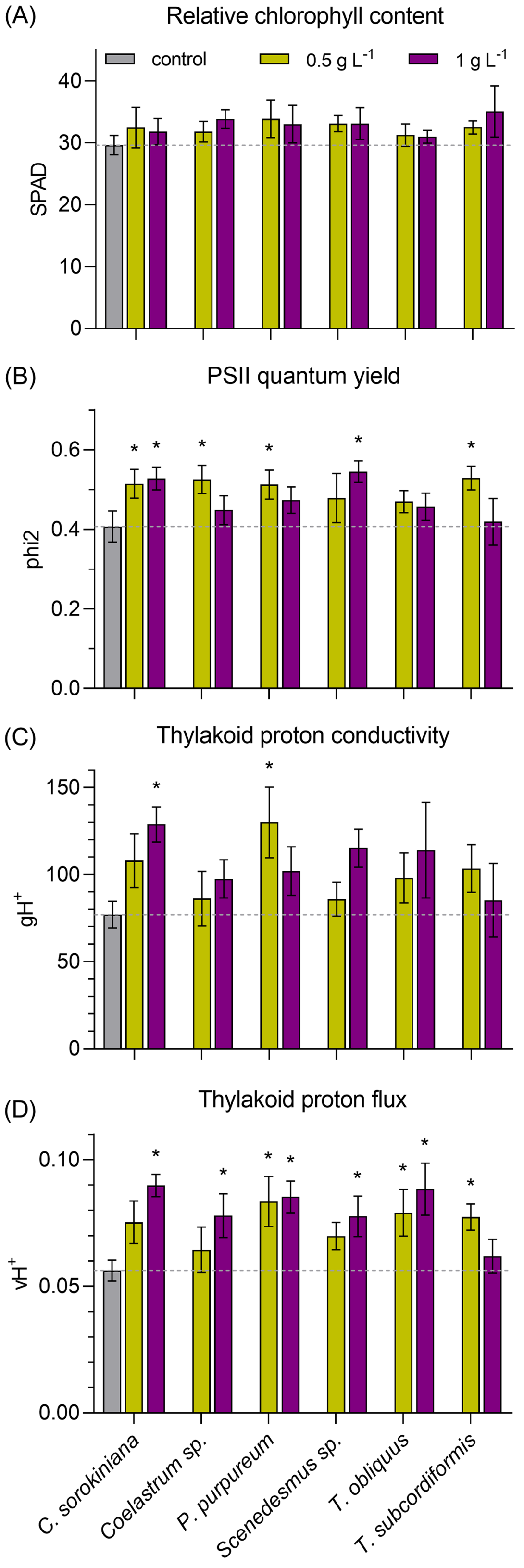


FIGURE 5 Hydrolyzed crude extracts acquired from six microalgae improved photosynthetic performance of lettuce leaves. Photosynthetic parameters were measured spectrophotometrically on the 32nd day after germination by MultispeQ device. (A) Relative chlorophyll content (SPAD value), (B) photosystem II quantum yield, (C) thylakoid proton conductivity, and (D) thylakoid proton flux. Control was acquired from untreated plants. Bars represent mean ± SE ($n = 5$–7) and asterisks indicate significant differences ($p < 0.05$) according to one-way ANOVA followed by Fisher's LSD test (B,D) or Kruskal–Wallis test followed by Dunn's multiple comparison test (FDR adjusted; A,C).

12%–16% (Figure 4B). Interestingly, *T. obliquus* HCE stimulated lettuce crop performance but failed to stimulate *Arabidopsis* root growth, indicating that extract effectivity can be plant- or tissue-specific. Regarding the photosynthetic performance of lettuce, a global enhancement was attained in the thylakoid proton flux parameter (Figure 5D), which can be attributed significantly to the ATP synthase activity. Congruently, in tomato plants treated with HCE, Mutale-Joan et al. (2020) observed an increase in pyridine-3-carboxamide, the primary precursor of adenine dinucleotide (NAD+) and the substrate for ADP polymerization.

Sulfuric acid, which was utilized in preparing our HCE, is in low concentration and is known to effectively solubilize and hydrolyze the microalgal biomass (Silva & Bertucco, 2017). The polysaccharide fraction of thermally hydrolyzed microalgal extracts has previously improved plant profiles of primary and secondary metabolites (Rachidi et al., 2020). Sugars and amino acids are common products of acid hydrolysis of organic biomass (Bin Hossain et al., 2015; Darragh & Moughan, 2005). Though plants are essentially primary producers, the uptake of amino acids and saccharides by roots is well-known in *Arabidopsis* (Tegeder & Rentsch, 2010; Yamada et al., 2011). Amino acids can be utilized in protein build-up or in regulatory pathways, while saccharides can contribute to metabolism bidirectionally. Still, regulatory pathways, effective metabolites, and molecular mechanisms partaking in the described effects of microalgal extracts are elusive. More fundamental knowledge about the mode of action of microalgal plant biostimulants is necessary to understand the underlying processes, synergies, and metabolic implications that can, in the end, help develop reliable formulations with described effects.

5 | CONCLUSIONS

Here, we described a cost-effective phenotyping pipeline for evaluating plant biostimulant potential of microalgal crude extracts that consists of *Arabidopsis* rooting assay and lettuce greenhouse experiments. Six screened microalgae showed potential to be utilized as future plant biostimulants on the condition of an effective biomass extraction procedure. Hydrolyzed crude extracts enhanced *Arabidopsis* root development and demonstrated a superior potential for lettuce crop improvement with five of the six evaluated microalgae. Lettuce fresh weight increase was concomitant with increased photosynthetic performance. More fundamental research of microalgal plant biostimulant effects is essential to understand specific *in planta* modes of action and explain the synergistic effects of microalgal bioactive compounds.

AUTHOR CONTRIBUTIONS

Yagut Allahverdiyeva conceived the research and acquired funding. Erik Chovanček, Sema Şirin, Yagut Allahverdiyeva conceptualized and designed research. Erik Chovanček performed experiments, curated data, and drafted the manuscript. João Salazar aided with experiments. All authors contributed to the discussion and revision of the manuscript draft. All authors read and approved of the published version of the manuscript.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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