



Harnessing the power of microalgae consortia for sustainable crop production: case study on lettuce (*Lactuca sativa* L.)

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Abstract

Due to global food demand and need to protect the environment, improving crop productivity while reducing agrochemical use has become crucial. Microalgae offer a potential solution as they exhibit biostimulant and biofertilizer effects, improving both crop and soil quality. This study aimed to assess the impact of suspensions of freeze-dried *Chlorella vulgaris* (UTEX 265), *Scenedesmus obliquus* (UTEX 393), and *Haematococcus pluvialis* (UTEX 2505) biomass in distilled water on lettuce (*Lactuca sativa* L.) germination and development. Different microalgae treatments, individually and mixtures, were applied. The study evaluated various plant growth parameters and the concentration of nutritional constituents, including reducing sugars, total polyphenols, antioxidant capacity, and chlorophyll *a*. In the first stage (germination), microalgae treatments (*C. vulgaris* 2 g (CV2),; *C. vulgaris* 0.66 g, *S. obliquus* 0.66 g and *H. pluvialis* 0.7 g (CV0.7:SO0.7:HP0.7) and *C. vulgaris* 1.5 g and *S. obliquus* 1.5 g (CV1.5:SO1.5) increased *L. sativa* seed germination rate and the number of germinated seeds. Significant changes were observed in growth-related variables such as height (CV0.7:SO9.7:HP0.7), stem diameter (*C. vulgaris* 3 g (CV3)), root length (*S. obliquus* 3 g (SO3)), and leaf area (CV1.5:SO1.5). In the second stage (plant development), microalgae treatments significantly increased dry weight from 2.71% (CV1.5:SO1.5) to 48.52% (SO3), reduced sugars from 3.20% (CV3) to 59.05% (CV1.5:SO1.5), total polyphenols from 7.99% (CV3) to 40.15% (CV1.5:SO1.5), antioxidant capacity from 0.77% (CV0.7:SO9.7:HP0.7) to 17.40% (CV1:HP1), and chlorophyll *a* from 23.01% (CV1.5:SO1.5) ($p \leq 0.05$). *C. vulgaris* and *S. obliquus* in blending were identified as the main microalgae promoting positive effects on *L. sativa* development. This study confirms that microalgae, particularly *C. vulgaris* and *S. obliquus*, can enhance crop quality, specifically in *L. sativa*. These findings demonstrate the potential of microalgae as biostimulants, supporting their application to improve crop productivity and emphasizing their role in sustainable agriculture.

Keywords Lettuce · Microalgae · Germination · Growth · Nutritional constituents · Biostimulant

Introduction

Microalgae are photosynthetic eukaryotic unicellular organisms. Due to their capacity to produce bioactive metabolites such as polysaccharides, proteins, phytohormones, antioxidant compounds, lipids, reducing sugars, etc., their use has increased in the production of biofuels, human food, biofertilization, among others (Ronga et al. 2019; Gonçalves 2021). Microalgae also produce other compounds, such as

abscisic acid, auxins, gibberellins, ethylene and cytokinins (Lu & Xu 2015; Do et al. 2021), as well as nitrogen, phosphorus (Yaakob et al. 2021; Gao et al. 2023; Maltsev et al. 2023) and carbon among other macroelements, that following cell death can be released and used as fertilizer in farming (Ng & Chew 2022).

Regarding their use as biofertilizers, microalgae have been employed in wheat, rice, garlic, and tomato crops, due to their contribution in proteins (40–60%), elements such as P (1–3% dw), which are available like inorganic fertilizers, and phytohormones and antioxidants (Schreiber et al. 2018; Ronga et al. 2019). Additionally, these microorganisms capture a large amount of CO₂ per unit area, which helps mitigate the greenhouse effect (Prasad et al. 2021). These characteristics make microalgae a sustainable alternative to the excessive application of agrochemicals, particularly

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in high-value organic crops (Coppens et al. 2016). Consequently, agricultural studies have been conducted using algal biomass in wheat (Abd et al. 2008; Kholssi et al. 2019), sunflowers (Lăcătușu et al. 2017), grapevines (Abd et al. 2008), roses, tomatoes (Coppens et al. 2016; Barone et al. 2019a; Cordeiro et al. 2022; Alling et al. 2023), and other crops, showing positive effects on growth and increased production of secondary metabolites in these plants (Chiaiese et al. 2018; Ronga et al. 2019).

Haematococcus pluvialis is a highly studied freshwater microalga due to its production of astaxanthin, a red pigment with high antioxidant capacity (Oslan et al. 2021). *Haematococcus pluvialis* contains amino acids that serve as biostimulants for plant growth, crop yield, and reduction of abiotic stress (Khan et al. 2019), high levels of polysaccharides biostimulants for plant growth and its biomass can be a biofertilizer increasing plant growth and soil texture (Elarroussi et al. 2016; Delattre et al. 2016) and phytohormones involved in processes of development, growth and stress resistance in plants, among others. *Chlorella vulgaris* extracts showed a gibberellin-like effect improving the germination index of watercress seeds, an auxin-like effect showing an enhanced development of soybean roots, and a cytokinin-like activity in the cucumber cotyledon expansion test, which showed an increase in weight (Amaya-Santos, et al. 2022). Similar bioestimulant results were found by Navarro-López et al., (2020) with extracts of *Scenedesmus obliquus* in watercress seed, mung bean and cucumber. These effects were associated with phytohormone-like activities. In particular, the role of phytohormones from microalgae in plants is well understood, but the role of phytohormones in plant-microalgae interactions is not yet clear (Lu and Xu 2015; Tan, et al. 2021).

This study examines the effect on germination and plant growth *Lactuca sativa* using three microalgae, *Chlorella vulgaris* (UTEX 265), *Scenedesmus obliquus* (UTEX 393) and *Haematococcus pluvialis* (UTEX 2505), applied as a suspension of freeze-dried algal biomass in distilled water individually, in binary and ternary mixtures.

Materials and methods

Microalgae cultivation

Chlorella vulgaris (UTEX 265) and *Scenedesmus obliquus* (UTEX 393) were grown on Bold 3N medium while *Haematococcus pluvialis* (UTEX 2505) was grown on MES-Volvox medium, in 10-L photobioreactors (PBRs) aerated for 30 days. Bold 3N (<https://utex.org/products/bold-3n-medium?variant=30991784345690>) and MES-Volvox (<https://utex.org/products/mes-volvox-medium?variant=30991761047642>) media were prepared according to the protocol

described in the UTEX database. The cultures were maintained with an inoculum of 1.0×10^4 cell mL^{-1} and an average temperature of 22 ± 2 °C at an irradiance of $95 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (QSL-2100, Biospherical Instruments Inc.), with a 16/8 h light/dark photoperiod using cold white fluorescent tubes (P01426-3, Sylvania).

Characterization of microalgae

For each microalgae species, three independent cultures were prepared. One sample of dried biomass of each culture was analyzed to determine the levels of proteins, carbohydrates, and lipids. The cultures of each microalga were distributed in 50 mL plastic tubes and centrifuged at 2362 RCF at 4 °C for 20 min. The biomass was then lyophilized (Labconco Frezone Plus) for 12 h.

Lipid content was determined using a modified Bligh and Dyer (1959) method as described in Pereira et al. (2011). Prior to analysis, the samples were sonicated (Buch & Holm A/S, Herlev, Denmark) for 15 min at 15 °C to enhance cell rupture. Microalgal biomass (500 mg) was then homogenized with a mixture of methanol, chloroform, and water (2:2:1), at 25,000 rpm for 5 min using an Ultra-Turrax disperser (IKA-Werke GmbH, Germany). The homogenized samples were centrifuged at 2362 RCF for 10 min at 4 °C. The organic phase was carefully collected and transferred into new glass tubes. 5.0 mL of lipid extract was transferred into pre-weighed tubes and placed in a dry bath at 60 °C for 12 h. Afterward the tubes were weighed and total lipid content was determined gravimetrically. The defatted biomass was used for protein quantification.

For the determination of total protein, a micro-Kjeldahl technique was employed following the method described by Ma and Zuazaga (1942). A total of 500 mg of dry biomass was used for each microalgae species. Total proteins were calculated as total nitrogen (T.N) % multiplied by a conversion factor of 6.25.

Carbohydrate concentration was determined using a phenol-sulfuric acid method as described by Rao and Patibaraman (1989). The dried biomass was reconstituted in water to prepare a sample concentration of 1 mg mL^{-1} for each sample. 1 mL of the sample was mixed with 5 mL of sulfuric acid (96%) and 1 mL of phenol (5%). The mixtures were then incubated for 10 min at 90 °C in a water bath. Following incubation, the absorbance at 490 nm was measured. The absorbance measurements were compared to a standard curve based on glucose. Each experiment was carried in triplicate.

The concentration of carotenoids was determined as follows: 1 mL of acetone was added to 50 mg of dried microalgal biomass and the mixture was vortexed for 30 s. The mixture was then centrifuged at 2362 RCF for 10 min at 4 °C. The supernatant was carefully transferred to an amber

glass tube. The extraction process was repeated once and the supernatants were combined. The absorbance of the pooled supernatants was read at 470 nm, 645 nm, and 662 nm (Thermo Scientific Evolution 200, Germany), after blanking with acetone. The concentrations of total carotenoids (TC), and chlorophyll *a* (Chl-*a*) were determined in $\mu\text{g mL}^{-1}$ using the formulas provided by Lichtenthaler (1987).

Preparation and application of treatments

First stage

To see the effect of microalgae and their mixtures on *Lactuca sativa* seeds, 18 treatments with microalgae and four controls were prepared; microalgae biomass were freeze-dried and resuspended in ultrafiltered water for a total biomass concentration of 1.00, 2.00, or 3.00 g L^{-1} for each treatment according to Table 1. Three commercial fertilizer (14% N, 7% P and 15% K) solutions (1.00, 2.00, or 3.00 g L^{-1}) were prepared as positive control, and ultrafiltered water was used as negative control (Table 1).

Once resuspended, a Luria–Bertani (LB) agar culture was performed to verify that there was no contamination.

A total of 60 seeds of lettuce (*Lactuca sativa* L. Blanca Boston variety (Oro Verde®)), were sown in seedling trays of 200 cells for each treatment, two seedling trays per treatment. The germination substrate employed was a mixture of coconut fiber and soil in a 1:1 ratio, which underwent sterilization (Sterilof, Bogotá, Colombia) at 121 °C for 20 min under a pressure of 15 psi prior to its utilization. This stage encompassed the time span from seed sowing (day 0) until day 19, in accordance with the recommendations outlined by the Cámara de Comercio de Bogotá-Colombia (2015). The number of germinated seeds was recorded daily, while every third day the seeds were treated with irrigation water (0.5 mL) applied to the substrate. On day 19, half of the *L. sativa* samples (60 plants) were collected to conduct the following measurements: height (H), stem diameter (SD), root length (R), and leaf area (LD). A Vernier caliper was employed for these measurements, which were recorded in centimeters (cm). Before the measurements were taken, all plant material underwent three washes with ultrafiltered water.

Table 1 Experimental treatments and controls in the effect of microalgae and their mixtures on lettuce (*L. sativa*) seeds (biomass algal or fertilizer dry weight)

Treatments	Concentration				Total concentration (g L^{-1})
	<i>C. vulgaris</i> (g L^{-1})	<i>S. obliquus</i> (g L^{-1})	<i>H. pluvialis</i> (g L^{-1})	Fertilizer (g L^{-1})	
CV1	1.00	-	-	-	1.00
SO1	-	1.00	-	-	1.00
HP1	-	-	1.00	-	1.00
CV2	2.00	-	-	-	2.00
SO2	-	2.00	-	-	2.00
HP2	-	-	2.00	-	2.00
CV3	3.00	-	-	-	3.00
SO3	-	3.00	-	-	3.00
HP3	-	-	3.00	-	3.00
CV0.5:SO0.5	0.50	0.50	-	-	1.00
CV0.5:HP0.5	0.50	-	0.50	-	1.00
SO0.5:HP0.5	-	0.50	0.50	-	1.00
CV0.3:SO0.3:HP0.3	0.33	0.33	0.33	-	1.00
CV1:SO1	1.00	1.00	-	-	2.00
CV1:HP1	1.00	-	1.00	-	2.00
SO1:HP1	-	1.00	1.00	-	2.00
CV0.7:SO0.7:0.7T17	0.67	0.67	0.67	-	2.00
CV1.5:SO1.5	1.50	1.50	-	-	3.00
CV1.5:HP1.5	1.50	-	1.50	-	3.00
SO1.5:HP1.5	-	1.50	1.50	-	3.00
CV1:SO1:HP1	1.00	1.00	1.00	-	3.00
F1	-	-	-	1.00	1.00
F2	-	-	-	2.00	2.00
F3	-	-	-	3.00	3.00
B	-	-	-	-	-

The total chlorophyll concentration (T-Chl) in the seedlings was assessed using a digital chlorophyll meter (atLEAF, Wilmington, USA), which enabled non-destructive in situ measurements on the leaves.

Following the analysis of the collected samples, the five treatments (CV3, SO3, CV1:HP1, CV0.7:SO0.7:HP0.7, and CV1.5:SO1.5) that exhibited the most significant positive effects on seedling growth were selected for the second stage.

To facilitate the visualization of the results of the growth variables and chlorophyll quantity a heat map was done with the means. The color guide represents in a normalized form and on a scale from 0 to 100, the effect of each treatment on each variable, being 100% for the highest value mean and 0% for the lowest value mean for each of the variable measured.

Second stage

The second stage of the experiment spanned from day 20 to day 49 of cultivation. During this period, half of the seedlings from the first stage (60 plants) were transplanted into agricultural bags (Protek, 071025FL) containing 2.0 kg of sterilized soil (Sterilof), following the same sterilization conditions as in the first stage. Irrigation water treatments were applied to the soil of the plants every third day. The amount of water treatments was controlled at each irrigation through a 10 mL graduated pipette.

At the end of this stage, the samples were collected and immediately frozen at -80°C . Subsequently, each plant was freeze-dried at 30°C , 0.05 mbar for 24 h to determine the dry weight (dw) of each plant. After weighing, the samples were placed in 15 mL centrifuge tubes (Biologix) with the addition of glass beads, the tubes were protected from the light. The dry material was then pulverized by vortexing using the V1 plus vortex mixer (Boeco, Germany). Next, 70% v/v ethanol was added to the pulverized material to prepare the extracts, which were concentrated by rotaevaporation and resuspended in 70% v/v ethanol at a concentration of 1.0 mg mL^{-1} . These extracts were used for the measurement of the following parameters: chlorophyll *a* (Chl-*a*), reducing sugars (RS), total polyphenols (TP), and antioxidant capacity (AC).

Chl-*a* concentration was determined using UV–VIS spectrophotometry by measuring the absorbance of 100 μL of the extract at 655 nm in a plate reader (iMark, Bio-Rad Laboratories Inc.). A calibration curve ($2.0\text{--}20.0\text{ }\mu\text{g mL}^{-1}$) was prepared using Chl-*a* analytical standard (CAS number: 479–61-8, Sigma-Aldrich, USA).

Total antioxidant capacity was quantified using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) degradation method (Sharopov et al. 2014). Absorbance was measured at 515 nm in a plate reader (iMark, Bio-Rad Laboratories Inc.).

A Trolox calibration curve (CAS number: 135806–59-6, Sigma-Aldrich, USA) was prepared in the concentration range of 0.01–0.10 mM. For the assay, 100 μL of each extract or standard and 100 μL of DPPH (0.20 mM) were combined, allowed to react for 30 min in the dark, and then the absorbance was measured.

Total polyphenols were quantified using a modified version of the Follin-Ciocalteu method developed by Magalhães et al. (2010). A calibration curve for gallic acid was prepared in the range of $0.02\text{--}0.40\text{ mg mL}^{-1}$. In each well of a microplate, 50 μL of each extract or standard, 50 μL of Follin-Ciocalteu reagent (Sigma-Aldrich, USA), and 100 μL of 0.35 M NaOH were combined. The mixture was allowed to react for 3 min in the dark, and the absorbance was measured at 750 nm in a plate reader (iMark, Bio-Rad Laboratories Inc.).

The total concentration of reducing sugars was determined using a microplate adaptation of the dinitrosalicylic acid (DNS) method as described by Gonçalves et al. (2010). A D-glucose calibration curve (Merck Millipore, USA) was prepared in the concentration range of $0.25\text{--}4.00\text{ mg mL}^{-1}$. For the assay, 25 μL of each extract or standard and 25 μL of DNS were added to each well of a microplate. The microplate was placed in a water bath at 90°C for 5 min and then cooled in an ice bath. Subsequently, 250 μL of distilled water was added to each well. The absorbance was finally measured at 570 nm using a plate reader (iMark, Bio-Rad Laboratories Inc.).

Statistical analysis

The results of the characterization of microalgae biomass are presented as the arithmetic mean with its respective standard deviation ($\bar{x} \pm \text{SD}$).

The germination assays were conducted in duplicate. Each treatment was applied to 60 seeds. The obtained results for each treatment are presented as the arithmetic mean with its respective standard deviation ($\bar{x} \pm \text{SD}$). The results of growth variables for each treatment at the end of the first stage are represented by the mean $\pm$ standard deviation ($\bar{x} \pm \text{SD}$), $n=60$. After testing the assumptions of normality by the Shapiro–Wilk test, homoscedasticity by the Sharlett test and independence by the Durbin–Watson test, an analysis of variance of the means was carried out by ANOVA. Then, a Tuckey analysis was done, different letters in the same column indicate significant differences ($p \leq 0.05$) between means.

To analyze the data and reduce the dimensionality of the experiment in the first stage, principal components analysis was conducted using Minitab software version 21.2.0.0.0. This analysis included the variables height (H), stem diameter (SD), root length (R), leaf area (LD), and total chlorophyll (T-Chl). The principal components analysis resulted

in the creation of a single variable named "growth". To determine which treatments exhibited a significantly greater increase in the variable growth, after testing the assumptions of normality by the Shapiro–Wilk test, homoscedasticity by the Sharlett test and independence by the Durbin-Watson test, an Analysis of Variance (ANOVA) was performed with a significance level of 5% ($p \leq 0.05$), follow by a Dunnett test to compare de means of the treatments with the mean of the control (B).

The results of the variables evaluated for each treatment at the second stage are represented by the mean $\pm$ standard deviation ($\bar{x} \pm SD$), $n = 30$. After testing the assumptions of normality by the Shapiro–Wilk test, homoscedasticity by the Sharlett test and independence by the Durbin-Watson test, an analysis of variance of the means was carried out by ANOVA. Then, a Tuckey analysis was done, different letters in the same column indicate significant differences ($p \leq 0.05$) between means.

Results

Table 2 presents the partial characterization of the dried biomass from *H. pluvialis*, *S. obliquus*, and *C. vulgaris* grown under specific conditions. This table provides the protein, lipid, carbohydrate, and carotenoid contents in g per 100 g of dry weight (dw) for each microalgae species.

In summary, *S. obliquus* and *C. vulgaris* exhibited a preference for accumulating proteins, representing approximately 50% of the dry weight, and this protein content was twice as high as that found in *H. pluvialis*. The lipid content *H. pluvialis* was higher (6 to 7 g more per 100 g in dry weight) in comparison with *S. obliquus* and *C. vulgaris*, which presented similar levels. *Haematococcus pluvialis* showed the highest carbohydrate (approximately 38% in dry weight) and carotenoids content (5% in dry weight) among the three microalgae. The content carotenoids in *S. obliquus* and *C. vulgaris* was lower compared with *H. pluvialis*, ranging from 0.3 to 0.5 g (100 g)⁻¹ dw, compared to 5.3 g (100 g)⁻¹ dw.

Table 2 Characterization of microalgae biomass from *H. pluvialis* (UTEX 2505), *S. obliquus* (UTEX 393), and *C. vulgaris* (UTEX 265). These microalgae were grown in 10 L photobioreactors for 30 days. The values are expressed as mean $\pm$ standard deviation (SD)

Microalgae	Protein (g (100 g) ⁻¹ dw)	Lipids	Carbohydrates	Carotenoids
<i>Haematococcus pluvialis</i>	25.2 $\pm$ 1.5 ^a	22.3 $\pm$ 2.1 ^a	38.2 $\pm$ 1.2 ^a	5.2 $\pm$ 0.3 ^a
<i>Scenedemus obliquus</i>	52.3 $\pm$ 0.9 ^b	16.4 $\pm$ 1.3 ^b	24.6 $\pm$ 1.4 ^b	0.3 $\pm$ 0.1 ^b
<i>Chlorella vulgaris</i>	49.1 $\pm$ 1.8 ^b	15.2 $\pm$ 1.6 ^b	18.6 $\pm$ 1.6 ^c	0.5 $\pm$ 0.2 ^b

First stage

As a result of the daily monitoring, it was observed that the CV2 treatment (2.00 g L⁻¹ of *C. vulgaris*) presented a significant ($p \leq 0.049$) higher germination percentage of *L. sativa* seeds than all the treatments (including B), but a similar germination rate as B (Table 3). It is important to highlight that the treatments CV0.7:SO0.7:HP0.7 (mixture of 0.66 g L⁻¹ of *C. vulgaris*, 0.66 g L⁻¹ of *S. obliquus* and 0.66 g L⁻¹ of *H. pluvialis*) and CV1.5:SO1.5 (mixture of 1.50 g L⁻¹ of *C. vulgaris* with 1.50 g L⁻¹ of *S. obliquus*) were the most effective treatments, showing a significative positive effect, for both, germination percentage and rate compared to the treatment with ultrafiltered water (B).

In contrast, treatment CV0.5:HP0.5, which consisted of a mixture of 0.50 g L⁻¹ of *C. vulgaris* with 0.50 g L⁻¹ of *H. pluvialis*, exhibited a germination percentage equal to that of B (ultrafiltered water) but the highest germination rate. Notably, *C. vulgaris* was applied at a lower concentration in CV0.5:HP0.5 compared to CV2, CV0.7:SO0.7:HP0.7, and CV1.5:SO1.5. It is interesting that the other treatments inhibited or reduced the germination percentage and rate compared to B. The results indicate that, at the individual level, *H. pluvialis* (HP1, HP2, and HP3 with 1.00, 2.00, and 3.00 g L⁻¹ of *H. pluvialis*, respectively) did not contribute to the germination process, as longer times were observed compared to the target (B) (see Table 3).

At the end of the first 20 days of each treatment, some growth variables were measured in the plants, with the treatment using ultrafiltered water (B) serving as the reference (Fig. 1). The results showed that the treatments containing microalgae, either individually or in binary and ternary mixtures, as well as the treatments with fertilizer (F) at different concentrations, stimulated the five growth variables that were studied: height (H), stem diameter (SD), root length (R), leaf area (LD), and total chlorophyll (T-Chl). However, it was observed that when the total concentration of microalgae reached 1.00 g L⁻¹ (CV1, SO1, HP1, CV0.5:SO0.5, CV0.5:HP0.5, SO0.5:HP0.5, CV0.3:SO0.3:HP0.3) or the concentration of the fertilizer reached F1, the effect on these growth variables was like that of B, indicating that the

based on three replicates. Statistical analysis using ANOVA ($p \leq 0.05$) revealed significant differences between the microalgae species in terms of their biomass composition. Means followed by the same letter do not differ by Tukey test at 5% probability

Table 3 Maximum germination percentage (%) and germination rate (% germination per day) of *L. sativa* discriminated by treatments during the first stage of the study. Means followed by the same letter do not differ by Tukey test at 5% probability. The results of each treatment are represented by the mean $\pm$ standard deviation ($\bar{x} \pm SD$), $n = 2$

Treatment	Maximum germination percentage (%)	GR (% germination per day)
CV1	77.4 $\pm$ 1.97 ^b	31.6 $\pm$ 1.45 ^b
SO1	77.9 $\pm$ 1.26 ^b	29.6 $\pm$ 2.98 ^c
HP1	76.9 $\pm$ 2.68 ^b	31.1 $\pm$ 1.66 ^{b,d}
CV2	99.5 $\pm$ 0.71 ^c	37.2 $\pm$ 4.34 ^a
SO2	80.9 $\pm$ 1.28 ^d	33.7 $\pm$ 1.06 ^e
HP2	50.7 $\pm$ 1.07 ^e	21.4 $\pm$ 0.619 ^j
CV3	78.4 $\pm$ 0.56 ^b	28.8 $\pm$ 4.28 ^{c,f}
SO3	80.9 $\pm$ 1.28 ^d	33.7 $\pm$ 1.06 ^e
HP3	83.4 $\pm$ 2.01 ^f	30.7 $\pm$ 5.39 ^{b,c,d}
CV0.5:SO0.5	61.8 $\pm$ 2.57 ^g	23.0 $\pm$ 4.02 ^g
CV0.5:HP0.5	92.0 $\pm$ 2.78 ^a	48.6 $\pm$ 0.0712 ^k
SO0.5:HP0.5	68.8 $\pm$ 1.20 ^h	25.0 $\pm$ 5.14 ^l
CV0.3:SO0.3:HP0.3	83.4 $\pm$ 2.01 ^f	3.39 $\pm$ 1.54 ^e
CV1:SO1	82.9 $\pm$ 2.72 ^f	31.3 $\pm$ 4.09 ^{b,d}
CV1:HP1	73.9 $\pm$ 2.66 ⁱ	28.9 $\pm$ 2.37 ^c
SO1:HP1	79.4 $\pm$ 0.86 ^b	2.96 $\pm$ 2.98 ^c
CV0.7:SO0.7:HP0.7	97.5 $\pm$ 0.73 ^j	39.8 $\pm$ 1.31 ^h
CV1.5:SO1.5	98.5 $\pm$ 2.14 ^j	39.8 $\pm$ 1.31 ^h
CV1.5:HP1.5	77.4 $\pm$ 1.97 ^b	29.6 $\pm$ 2.98 ^c
SO1.5:HP1.5	77.9 $\pm$ 1.26 ^b	36.4 $\pm$ 0.266 ^a
CV1:SO1:HP1	76.9 $\pm$ 1.58 ⁱ	27.4 $\pm$ 4.98 ^f
F1	61.8 $\pm$ 1.69 ^k	27.4 $\pm$ 0.272 ^f
F2	64.3 $\pm$ 0.96 ^g	23.0 $\pm$ 4.02 ^g
F3	64.8 $\pm$ 1.67 ^g	23.0 $\pm$ 4.02 ^g
B	92.0 $\pm$ 2.78 ^a	37.3 $\pm$ 1.89 ^a

stimulation reached a plateau at this concentration. In other words, up to a concentration of 1.00 g L⁻¹ for microalgae or the specified concentration for the fertilizer, the growth variables continued to be stimulated. Below this concentration, no additional stimulation was observed, and the effect was comparable to that of the treatment with ultrafiltered water (B). The treatments CV3 (3.00 g L⁻¹ *C. vulgaris*), SO3 (3.00 g L⁻¹ *S. obliquus*), CV1:HP1 (mixture of 1.00 *C. vulgaris* with 1.00 g L⁻¹ *H. pluvialis*), CV0.7:SO0.7:HP0.7, and CV1.5:SO1.5 had a greater effect on the height of *L. sativa* plants, as shown in Fig. 1a.

Treatments CV1 (1.00 g L⁻¹ *C. vulgaris*), CV3, SO3, CV1:SO1 (mixture of 1.00 *S. obliquus* with 1.00 g L⁻¹ of *C. vulgaris*) and CV1:HP1, generated on average a greater thickness in the stem diameter of *L. sativa* plants (Fig. 1b).

Treatments CV1, SO2 (2.00 g L⁻¹ *S. obliquus*), CV3, SO3, CV0.5:HP0.5, SO0.5:HP0.5 (mixture of 0.50 *S. obliquus* and 0.50 g L⁻¹ *H. pluvialis*), CV0.3:SO0.3:HP0.3

(mixture of 0.67 *S. obliquus*, 0.67 of *H. pluvialis* and 0.67 g L⁻¹ of *C. vulgaris*), CV1:SO1 and CV1:HP1 increased, on average, root length as shown in Fig. 1c.

Treatments CV1, CV3, SO3, SO0.5:HP0.5, CV0.3:SO0.3:HP0.3, CV1:SO1, CV1:HP1, CV0.7:SO0.7:HP0.7, CV1.5:SO1.5 and F3 (3.00 g L⁻¹ of fertilizer) resulted in an increased the leaf area of the plants, as shown in Fig. 1d.

All treatments, except F1 (1.00 g L⁻¹ fertilizer) and F2 (2.00 g L⁻¹ fertilizer), increased total chlorophyll concentration, as depicted in Fig. 1e.

In general, the results of the first stage show the potential of microalgae (*C. vulgaris* and *S. obliquus*) as biostimulants during the early stages of plant growth. These microalgae showed a promoting effect on several variables such as plant height, stem diameter, root length, and leaf area of *L. sativa*. Additionally, the individual treatments of *H. pluvialis* exhibited similar results to B. This outcome can be attributed to the protein content per dry weight (dw) of the microalgae.

From the results of the Dunnett pairwise comparison test (Fig. 2c) the biggest differences between means and control mean were for CV3 (difference of means (DM) was 4.75), SO3 (DM of 4.84), CV1:HP1 (DM of 4.694), CV0.7:SO0.7:HP0.7 (DM of 4.288), and CV1.5:SO1.5 (DM of 4.186).

Thus, the treatments that resulted in the greatest change in growth variables were CV3, SO3, CV1:HP1, CV0.7:SO0.7:HP0.7 and CV1.5:SO1.5 (see Fig. 1f). These treatments consisted of individual microalgae (CV3 and SO3), binary mixtures (CV1:HP1 and CV1.5:SO1.5) and ternary mixture (CV0.7:SO0.7:HP0.7), with total concentrations of 2.00 and 3.00 g L⁻¹ (Table 1). This is visually evident in the heatmap shown in Fig. 1f. It is noteworthy that the treatments with microalgae had a greater impact on the growth variables in *L. sativa*, even when compared to the treatments with commercial fertilizer (F1, F2 and F3) in this case, *L. sativa*. Therefore, these five treatments were considered for the second stage and only F2 (2.00 g L⁻¹ of commercial fertilizer) and F3 (3.00 g L⁻¹ of commercial fertilizer) were considered as positive controls in the stage.

Second stage

When analyzing the treatments in terms of average dry weight, it was observed that CV3, SO3, CV1:HP1 and CV0.7:SO0.7:HP0.7 increased this variable in *L. sativa* plants by 16.62, 48.52, 12.70 and 24.21%, respectively, compared to B (Fig. 3). However, the treatments with fertilizer (F2 and F3) exhibited the greatest effect in terms of average dry weight in this study, tripling the value of this variable compared to B, as depicted in the heat map (Fig. 3).

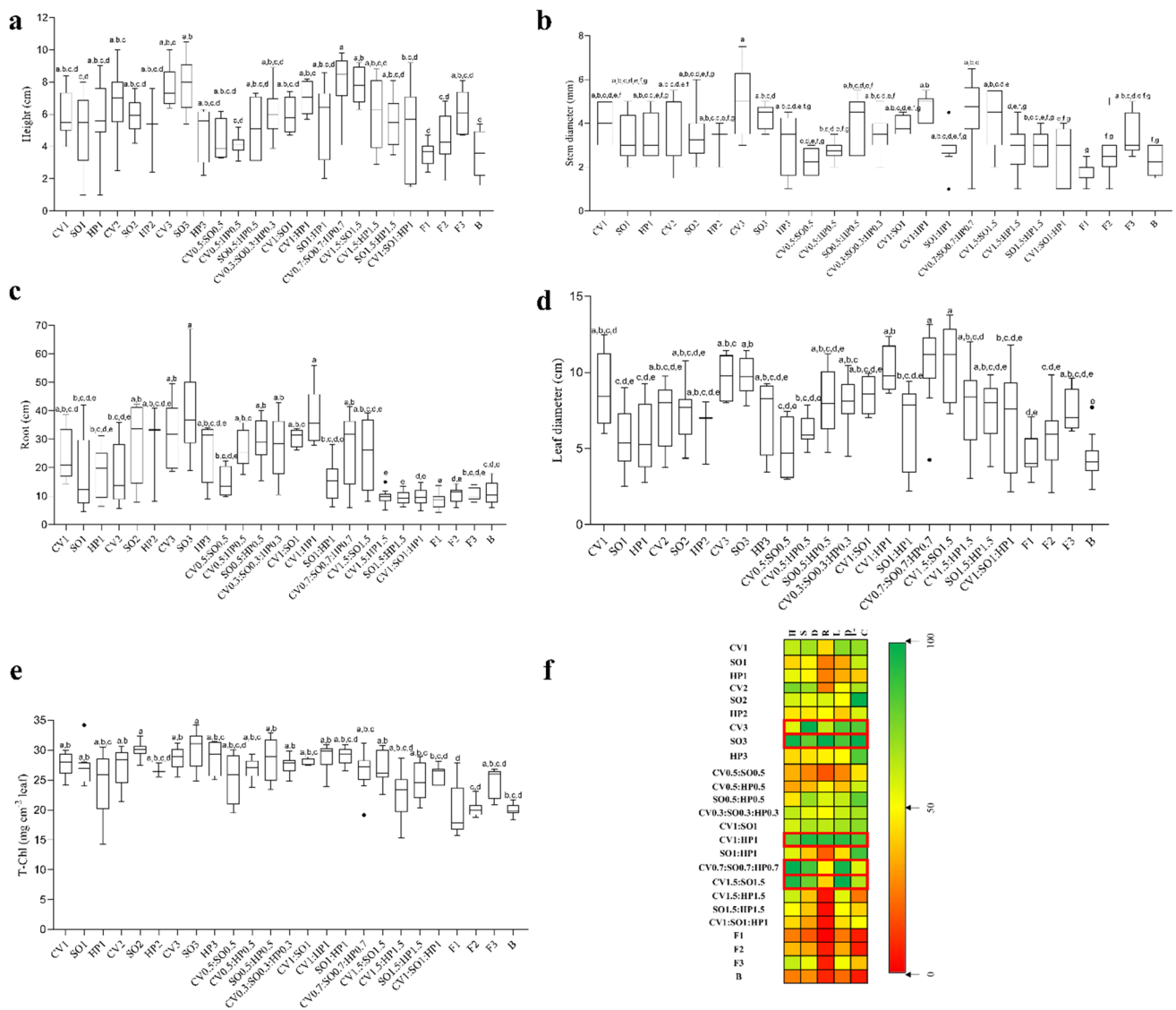


Fig. 1 Growth variables and chlorophyll quantity measured at the conclusion of the first stage (day 19) of *L. sativa* crop: **a**) height (cm), **b**) stem diameter (mm), **c**) root length (cm), **d**) leaf area (cm²), **e**) total chlorophyll (mg cm⁻² leaf) and **f**) heatmap of the growth variables and chlorophyll. The color guide represents in a normal-

ized form and on a scale from 0 to 100, the effect of each treatment on each variable. The purple boxes indicate the five treatments that showed the greatest effect on the variables measured in *L. sativa*. The results of each treatment are represented by the mean $\pm$ standard deviation ($\bar{x} \pm SD$), $n = 60$

Initially, it was hypothesized that the presence of *S. obliquus* was responsible for this effect since it was present in both SO3 and CV0.7:SO0.7:HP0.7. However, it is worth noting that CV1.5:SO1.5 contained 1.50 g L⁻¹ of *S. obliquus* but did not result in an increase in the average dry weight of *L. sativa* plants. This discrepancy suggests that factors other than the presence of *S. obliquus* alone may contribute to the observed effect on dry weight. It is possible that the specific combination of microalgae and their concentrations in SO3 and CV0.7:SO0.7:HP0.7, along with the presence of other components in the treatments, may have influenced the outcome. Further investigation is needed to elucidate

the specific factors responsible for the differential effects on average dry weight in the different treatments.

Regarding reducing sugar (RS) concentration, all microalgae treatments in this study promoted the accumulation of these metabolites in *L. sativa* plants, ranging from 3.20 to 59.05% compared to the control (B). Significant increases ($p < 0.05$) in RS concentration were observed in treatments SO3, CV1:HP1, CV0.7:SO0.7:HP0.7, and CV1.5:SO1.5, with increases of 12.06, 11.78, 9.71, and 59.05%, respectively. In contrast, the fertilizer treatments at both concentrations (2.00 g L⁻¹ and 3.00 g L⁻¹) resulted in a decrease of 39.29 and 17.34% in RS accumulation, respectively,

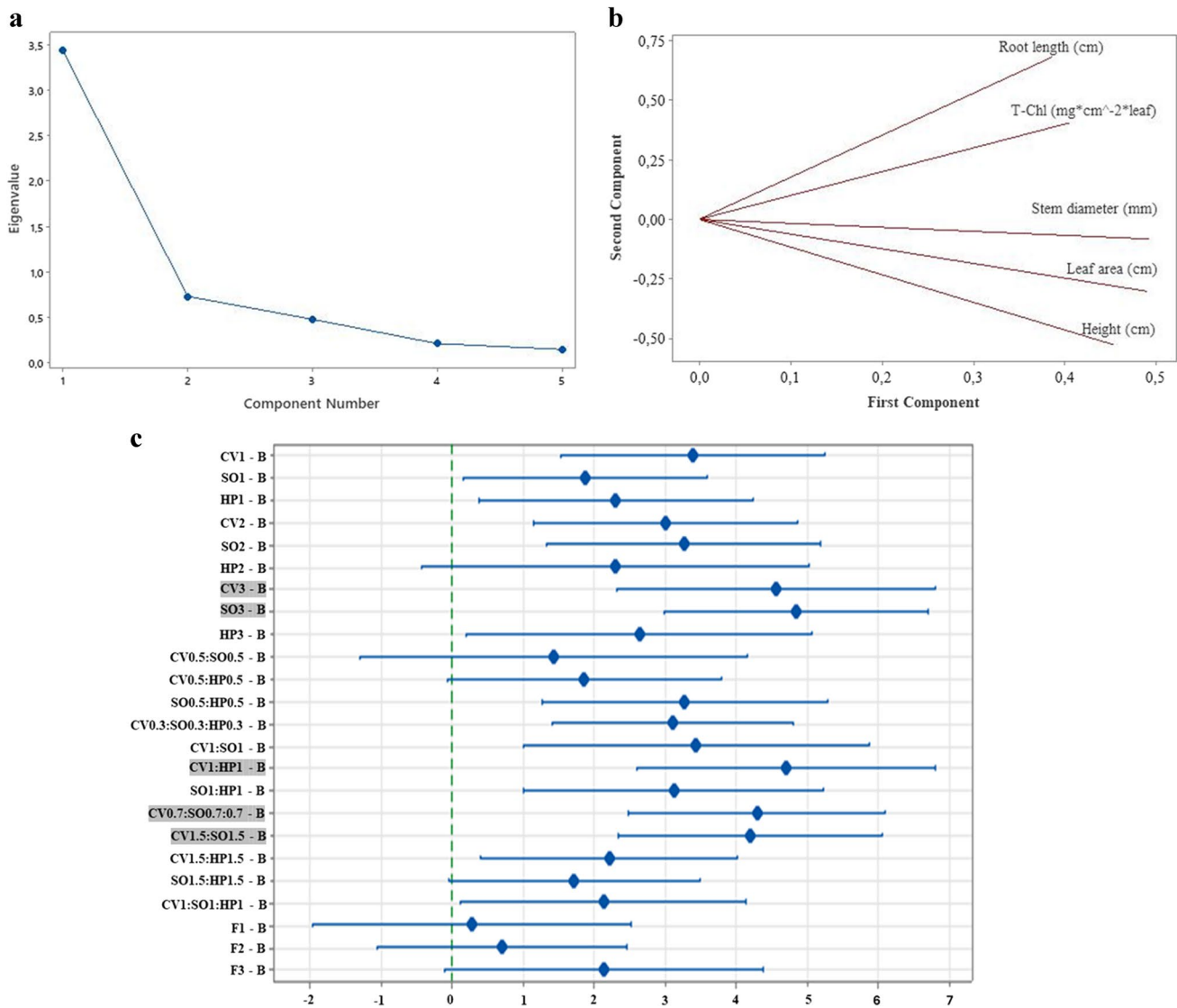


Fig. 2 Principal Component Analysis: a) Scree Plot. Selection of the number of main components to take in account for the analysis. b) Loading Plot c) Dunnett's 95% simultaneous CIs. If an interval

does not contain 0 the corresponding mean is significantly different from the treatment B mean. The treatments highlighted in grey were selected for stage two

compared to B. These results indicate the potential of microalgae as biostimulants in enhancing RS production in *L. sativa* plants.

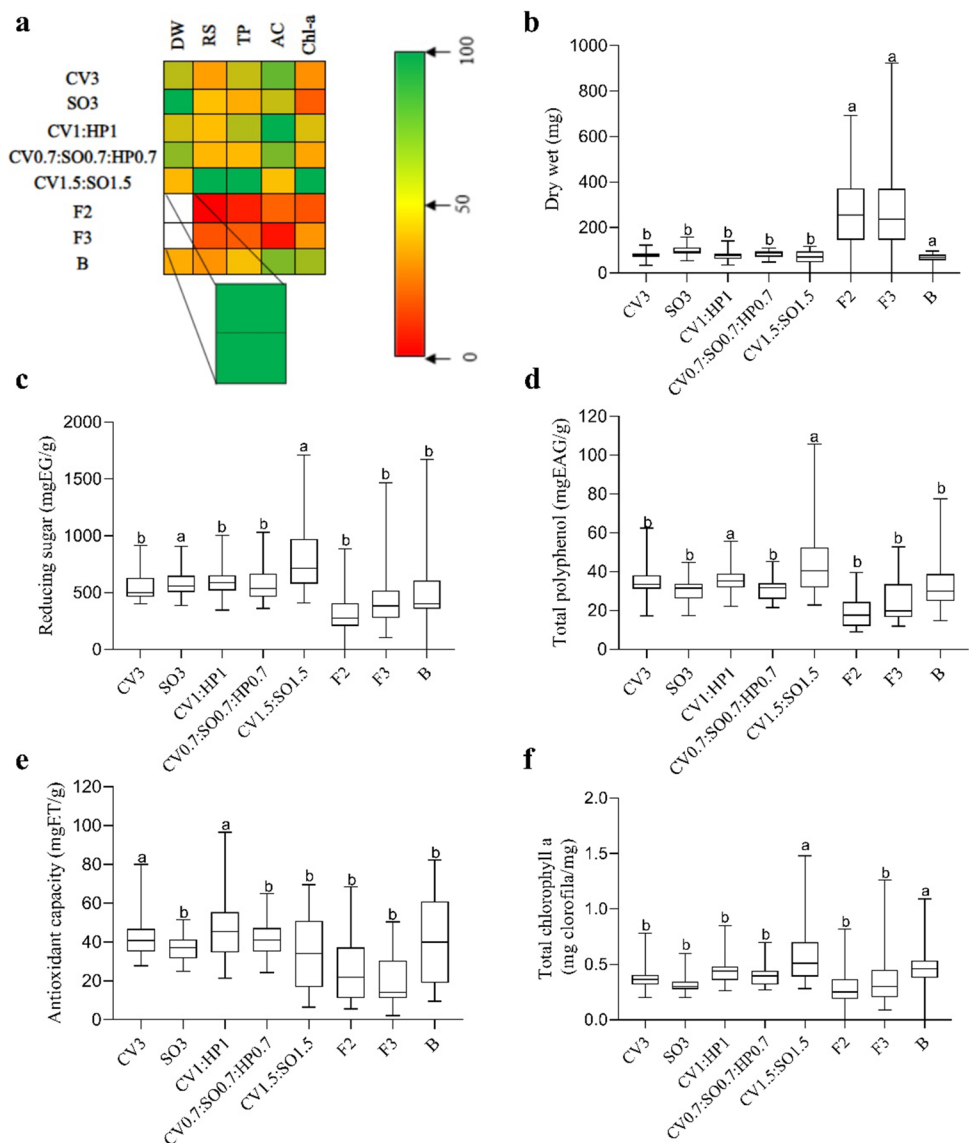
In terms of total polyphenols (TP) concentration, treatments CV3, CV1:HP1, and CV1.5:SO1.5 in this study resulted in increases of 7.99, 11.42, and 40.15%, respectively. However, treatments SO3, CV0.7:SO0.7:HP0.7, F2, and F3 showed a decrease in TP accumulation compared to B.

Regarding antioxidant capacity (AC), treatments CV3 and CV1:HP1 resulted in increases of 3.72 and 17.40%, respectively, in *L. sativa* plants. However, treatments SO3, CV1.5:SO1.5, F2 and F3 did not stimulate AC and even result in lower values compared to B.

In terms of Chl-*a* concentration, treatment CV1.5:SO1.5 resulted in a 23.01% increase compared to B. However, other treatments, including F2 and F3, caused a decrease in Chl-*a* concentration compared to B.

The findings of our study indicate that the application of mixtures containing *S. obliquus* and/or *C. vulgaris* microalgae resulted in improved development of *L. sativa* plants. The use of suspensions freeze-dried biomass from these microalgae species had positive effects on the biochemical functioning of the soil, leading to reduced germination time and an increased number of germinated seeds. Furthermore, these treatments stimulated the production of reducing sugars (RS), total polyphenols (TP), and chlorophyll-*a* (Chl-*a*) by more than 15%.

Fig. 3 a) Heat map of the effect of the treatments with microalgae or fertilizer in the second stage (plant development) on the measured variables: DW: dry weight, RS: reducing sugars, TP: total polyphenols, AC: antioxidant capacity and Chl-*a*: chlorophyll *a*. The color guide represents in a normalized form and on a scale from 0 to 100, the effect of each treatment on each variable. The zoom of the two green boxes was to highlight the positive effect of F2 and F3 on the DW b) dry weight (mg), c) reducing sugars (mgEG g⁻¹), d) total polyphenols (mgEAG g⁻¹), e) antioxidant capacity (mgET g⁻¹) and f) total chlorophyll *a* (μg mg⁻¹). The results of each treatment are represented by the mean ± standard deviation ($\bar{x} \pm SD$), n=30. Means followed by the same letter do not differ by Tukey test at 5% probability



Discussion

As part of this study a partial characterization of the three microalgae species involved was conducted. The biomass of *S. obliquus* and *C. vulgaris*, both eukaryotic unicellular Chlorophyta known for their ability to accumulate starch, proteins, and lipids (Becker 2013), exhibited a high protein content, as supported by previous findings by Ajala and Alexander (2020). These findings align with the research by Venkataraman and Becker (1985), who identified *S. obliquus* and *C. vulgaris* as microalgae species of significant nutritional value due to their high protein production and the bioaccumulation of essential amino acids. These microalgae species hold promise as potential alternatives to vegetable and animal proteins in future applications.

In contrast, *H. pluvialis* displayed higher lipid content (approximately 22% in dry weight) among the three

microalgae. However, a content of 32 to 37% was expected for this microalga in its red phase as has been established by others (Shah, et al. 2016; Mularczyk, et al. 2020). This result may be due to the fact that *H. pluvialis* cells were in transition to the red (aplanospore) phase and the culture conditions tested probably did not generate sufficient stress conditions to increase the lipid content, mainly polyunsaturated fatty acids (stored in chloroplasts in the green phase), which have been reported to be the most abundant in this fraction (Mularczyk, et al. 2020).

In the first phase (germination), treatments that included *C. vulgaris* biomass promoted the germination of a greater number of *L. sativa* seeds in shorter times. Similar results on sugar beet crops using aqueous extracts of *C. vulgaris* at concentrations of 1 and 2 mg C_{org} L⁻¹ reported an increase in germination percentage up to 1.8 and 2.0 times the control (without *C. vulgaris*), respectively. In comparison, *S.*

quadricauda only increased the germination percentage by 1.3 times compared to the control from the fourth day after germination (Puglisi et al. 2020a). The promoting effect of *C. vulgaris* on germination is believed to be attributed to its production of phytohormones, particularly cytokinins, such as iso-pentenyladenine zeatin and its conjugated ribosides. These phytohormones influence cell division and differentiation, contributing to the germination process (Kholssi et al. 2019; Ortiz-Moreno et al. 2019).

Regarding *H. pluvialis* no germination-promoting effects have been reported, it has only been reported that this microalga induces the production of the phytohormone ethylene, which plays an important role in the development and senescence processes of higher plants and in stress responses (Maillard et al., 1993).

The germination-promoting effect of *C. vulgaris* and *S. obliquus* may be due to the high protein content (51–58 and 63%, respectively) of these microalgae compared to *H. pluvialis* in its red stage (17–25%) (Grewe and Griehl 2012; Becker 2013). Among the functions of these proteins and amino acids are the mobilization of nutrients in plants through the complexation and chelation of essential minerals (Du Jardin 2015). The amino acids, glycine, betaine and proline, can mitigate abiotic stress by acting as osmoprotectants and antioxidants (Bulgari et al. 2015). Similarly, amino acids and proteins from microalgae can act as a source of reduced nitrogen for the promotion of the soil microbiome (Lee and Ryu 2021). Amino acids such as arginine and tryptophan have been found to mediate its biostimulant effect as precursors of polyamines (play an important role in plant growth and development processes) such as putrescine, spermidine and spermine; and auxins respectively (Mustafavi et al. 2018; Bulgari et al. 2019).

At the end of the first stage, a positive effect on the height of *L. sativa* seedlings was evidenced with treatments that included biomass of *C. vulgaris* or *S. obliquus* at the highest concentration (3 g L^{-1}) or in binary or ternary mixtures. This is consistent with reports by Huo et al. (2020), who conducted hydroponic cultivation experiments with lettuce (*L. sativa*), arugula (*Eruca vesicaria*) and purple kohlrabi (*Brassica oleracea*) applied treatments with *C. vulgaris*, finding that *L. sativa* plants that received the microalgae treatment, before the fourth week, were the highest (12 g plant^{-1}) compared to plants without microalgae treatment. However, it should be noted that in the fifth week, the biomass of microalgae-free *L. sativa* was higher ($13.9 \text{ g plant}^{-1}$) than the biomass of microalgae-treated plants. In the case of *E. vesicaria* and *B. oleracea*, the presence of microalgae did not promote growth. After 35 days, the cultures of *E. vesicaria* and *B. oleracea* had the highest biomass of 13.9 and $28.0 \text{ g plant}^{-1}$ respectively, in the condition without microalgae. On the other hand, in maize plant (*Zea mays* L.) crops treated with *Spirulina platensis* and *C. vulgaris*, a height of

17.1 and $16.9 \text{ cm plant}^{-1}$, respectively, was obtained after 20 days, compared to $13.6 \text{ cm plant}^{-1}$ without microalgae treatment (Dineshkumar et al. 2019).

A stimulation was observed in the other growth variables: stem diameter, root length, leaf area and total chlorophyll in the microalgae treatments compared to the control (B), mainly those containing *C. vulgaris* and *S. obliquus* (Fig. 2). Similar results on root growth were reported in sugar beet plants (20–30%) applying *C. vulgaris* (Barone et al. 2018) and in plants of *L. sativa* ($13.10 \pm 1.07 \text{ cm}$) when applying extracts of *Scenedesmus quadricauda* ($1.00 \text{ gC}_{\text{org}} \text{ L}^{-1}$) compared to the control ($10.00 \pm 1.41 \text{ cm}$) (Puglisi et al. 2020a). In *Zea mays* crops treated with *C. vulgaris*, a root length of $18.80 \text{ cm plant}^{-1}$, was obtained after 75 days, compared to $14.30 \text{ cm plant}^{-1}$ of root length without using microalgae (Dineshkumar et al. 2019). Positive effects on the root length of Swiss Chard (*Beta vulgaris* subsp. *cicla*) treated with *C. vulgaris* S45 when applied via foliar at a concentration of 10% (7.60 cm) and in soil (8.46 cm) compared to the control (6.75 cm), were reported by Hajnal-Jafari et al. (2020). Barone et al. (2019a, b) reported the co-cultivation of tomato (*Solanum lycopersicum* cultivar Missouri) with 50 mL aliquot suspension of *C. vulgaris* and *S. quadricauda*, during 46 days, resulting in an increase in root length of 18.50 and 15.50 cm respectively, front to the control (50 mL distilled water) that generated a root of 7.15 cm. Similar behavior was reported in the cucumber crop to which 2 mL of *C. vulgaris* suspension was applied for 12 days, resulting in a root length of 55.00 mm versus 21.60 mm with the control (sterilized culture medium) (Bumandalai and Tserennadmid 2019).

The results on total chlorophyll content are in accordance with the findings of Park et al. (2022), who found that treatments with *C. vulgaris* suspension increased the total chlorophyll content in "Red Russian" Kale (*Brassica napus* var. *Pabularia*) crops by 1.57 times, 42 days after transplantation. In general, through the PCA analysis, a high correlation was found between all the growth variables for *L. sativa*, finding that the treatments that most positively affected growth were: CV3, SO3, CV1:HP1, CV0.7:SO0.7:HP0.7, and CV1.5:SO1.5, where the presence of *C. vulgaris* and/or *S. obliquus* is highlighted. These treatments were selected for the second phase, to evaluate their continuity in the biostimulant effect on *L. sativa* plants. For the plant development the measured variables were dry weight, reducing sugars, total polyphenols, antioxidant capacity and chlorophyll *a*. Regarding dry weight, similar results were reported by Faheed & Abd-el Fattah (2008) who found an increase in the dry weight of *L. sativa* plants in treatments of 2 and 3 g of dry *C. vulgaris* per kg of soil. Puglisi et al. (2020a) observed an effect on leaf dry weights in *L. sativa* crops treated with *S. quadricauda* (0.37 g dw) compared to the control (0.32 g dw). Similarly, treatment of tomato plants with *S. quadricauda* ($1.00 \text{ mg organic carbon (C}_{\text{org}}) \text{ L}^{-1}$) led to a 25%

increase in leaf dry weight compared to the control (Barone et al. 2019b). Studies by the same group of researchers reported an increase in leaf dry weight when treating the soil of tomato plants with different concentrations of *C. vulgaris* (ranging from 2.22 ± 0.17 to 2.48 ± 0.23 g) and *S. quadricauda* (ranging from 2.19 ± 0.17 to 2.34 ± 0.21 g) for 18 days, compared to untreated plants (1.87 ± 0.14 g) (Barone et al. 2019b).

As for RS, an increase in content of 59.05% was obtained with CV1.5:SO1.5 (*C. vulgaris* 1.5 g L⁻¹ and *S. obliquus* 1.5 g L⁻¹). These finding align with previous reports of increased sugar in grapes treated with *C. vulgaris* (Abd et al. 2008). It is important to note that different results were observed in maize (*Z. mays*) crops, where the levels of reducing sugars decreased when treated with *S. platensis* (0.28 mg g⁻¹) and *C. vulgaris* (0.22 mg g⁻¹) compared to the control without microalgae (0.49 mg g⁻¹) (Dineshkumar et al. 2019). This suggests that the effects of microalgae on RS accumulation may vary depending on the plant species and specific experimental conditions.

An increase in total polyphenol (TP) production was found in three of the five treatments, CV3 (*C. vulgaris* 3 g L⁻¹), CV1:HP1 (*C. vulgaris* 1 g L⁻¹ and *H. pluvialis* 1 g L⁻¹) and CV1.5:SO1.5 (*C. vulgaris* 1.5 g L⁻¹ and *S. obliquus* 1.5 g L⁻¹) that could be attributed to the presence of *C. vulgaris*. These findings are consistent with previous studies that have shown an increase in polyphenol production when treating different plant species with microalgae. For example, treating *Cyanopsis tetragonoloba* with *C. vulgaris* resulted in increased polyphenols (Kusvuran and Can 2020), and treating *Allium cepa* with *C. vulgaris* and *S. platensis* led to increased polyphenol content (Dineshkumar et al. 2020). Similarly, Park et al. (2022), found that treatments with *C. vulgaris* supernatant increased polyphenol content in “Red Russian” Kale (*Brassica napus* var. *Pabularia*) by 1.30 times.

Of the five treatments evaluated, only two, CV3 and CV1:HP1, increased the antioxidant capacity of *L. sativa* plants. It should be noted that no studies were found in the literature that evaluated this variable using *C. vulgaris*. However, similar results have been reported in other studies, such as wheat plants treated with *Chlorella ellipsoidea* and *Spirulina maxima* (Abd et al. 2008), tomato plants treated with *Nannochloropsis oculata* (Coppens et al. 2016), and bell pepper (*Capsicum annuum*) treated with extracts of *Roholt-iella* sp. (Bello et al. 2021). Regarding the accumulation of chlorophyll *a*, only one treatment, CV1.5:SO1.5, stimulated this variable. Abd et al. (2008), found that the application of seawater with extracts from *C. ellipsoidea* and *S. maxima* a wheat plants generated an increase in Chl-*a* production between 0.53–0.61 and 0.53–0.84 mg g⁻¹ fw, compared to plants irrigated only with seawater, which generated 0.32 mg g⁻¹ fw of Chl-*a*. In crops of *Zea mays*, an increase in the production of Chl-*a* was obtained in the treatments with *S.*

platensis (1.23 mg g⁻¹) as well as *C. vulgaris* (1.21 mg g⁻¹), while the least concentrations were noticed in control (0.31 mg g⁻¹) (Dineshkumar et al. 2019). In crops of onion plants, the Chl-*a* content (0.90 and 0.93 mg g⁻¹) were observed in treatments with *S. platensis* (3.00 g kg⁻¹ soil) and *C. vulgaris* (3.00 g kg⁻¹ soil), front to control (0.83 mg g⁻¹) (Dineshkumar et al. 2020). In crops of *L. sativa* an increase in the level of Chl-*a* was found when treated with extract at a concentration of 1.00 mgC_{org} L⁻¹ of *S. quadricauda* (0.83 ± 0.042 mg g⁻¹) compared to the control (0.54 ± 0.025 mg g⁻¹) (Puglisi et al. 2020b). An increase in Chl-*a* production has also been reported when Swiss chard was treated with *C. vulgaris* S45 via foliar at a concentration of 10% (0.033 mg g⁻¹) and in soil (0.013 mg g⁻¹) compared to the control (0.001 mg g⁻¹) (Hajnal-Jafari et al. 2020). Similar results were reported by Barone et al. (2019a, b), who cultivated for 18 day tomato plants, treating the soil with different concentrations of *C. vulgaris* (39.40 ± 2.84 to 41.20 ± 3.14) and *S. quadricauda* (39.10 ± 1.82 to 42.30 ± 3.73), finding an increase in Chl-*a* compared to untreated (35.1 ± 2.14).

The promotion of germination and seedling growth can be attributed to the bioactive compounds present in the microalgae biomass, such as proteins, carbohydrates, vitamins, and phytohormones. These compounds can enhance seed germination, root development, and overall plant growth. The increased accumulation of RS indicates that microalgae treatments enhanced the synthesis or availability of these important metabolites, which play a crucial role in plant metabolism and growth. The significant increase in TP suggests that microalgae treatments promoted the biosynthesis of polyphenols in *L. sativa* plants. Polyphenols are known for their antioxidant properties and their involvement in various physiological processes, including defense against oxidative stress and modulation of plant growth and development. The stimulation of Chl-*a* production by microalgae treatments is indicative of enhanced chlorophyll synthesis, which is essential for photosynthesis and overall plant productivity.

Overall, the results highlight the potential of *C. vulgaris* and *S. obliquus* as beneficial biostimulants for promoting the growth and biochemical functioning of *L. sativa* plants. The observed improvements in germination, RS, TP, and Chl-*a* suggest that these microalgae treatments can positively impact plant performance and contribute to sustainable agriculture practices.

This research showed that individually applied microalgae have positive effects on the development of *L. sativa*. However, in the first stage, the mixture of these microorganisms, such as *C. vulgaris* and *S. obliquus*, presented synergy in the germination rate of *L. sativa* seeds and a greater number of germinated seeds. In the second stage, the use of these consortia increased the content of nutritional constituents in *L. sativa*, including reducing sugars

and total polyphenols. Additionally, it stimulated antioxidant capacity and chlorophyll a production.

Using microalgae cell suspensions enhances the growth of the seed and germination rate, and it could be by the presence of exopolysaccharides (EPS) that *S. obliquus* presented. A previous investigation of *S. obliquus* showed the production and secretion of these molecules (Córdoba-Castro et al. 2011). Likewise, the production of gibberellic acid (GA) in *C. vulgaris* can be the increase in germination as found by Alling et al. (2023) in tomato and barely. The mechanism by which this molecule can help is modulating physiological and biochemical processes of the plant, enhancement of the photosynthesis, and is seen in the root growth and nutrient availability (Moreira et al. 2022). Also, by increasing of the activity of defense enzymes such as lipoxygenase (LOX), phenylalanine ammonium lyase (PAL), and reactive oxygen species (ROS) scavenging antioxidant enzymes, that are related to the increase of polyphenol content. As shown in the results of treatments (CV3, SO3, CV1:HP1, CV0.7:SO0.7:HP0.7, and CV1.5:SO1.5) in the root diameter and the polyphenol content (Fig. 3). Similarly, Puglisi et al. (2022) found that the increase in phenolics of *C. vulgaris* and *S. quadricauda* impacts the PAL activity of lettuce crops.

Phytohormones like auxins, especially indole acetic acid (IAA), ethylene and jasmonic (JA) and salicylic acids (SA) have been found to be present in *Chlorella* and *Scenedesmus* species. These molecules are signaling molecules that help in growth processes such as root formation, defense, and tolerance to biotic and abiotic stresses (Castro et al. 2023). As shown by Gitau et al., (2023) the application of these microalgae can impact the regulation of genes and hormone signaling like the JA signaling pathways for flowering in tomato.

Thus, this synergic effect of the mixtures of microalgae could be attributed to the combined presence of phytohormones and metabolites, such as IAA, ethylene, exopolysaccharides, and polyphenols that impact the development of *L. sativa*. Unfortunately, the present study has the limitation that the presence of these metabolites was not determined either in the microalgae or in the resuspension water. It is recommended that studies be carried out to establish the presence of these metabolites and to establish the specific mechanism of their biostimulatory effect.

Conclusions

The use of microalgae consortia as biostimulants in agriculture shows promise in enhancing the growth and development of *L. sativa* and other plants while promoting

sustainability and reducing reliance on inorganic fertilizers. These findings highlight the potential of microalgae as viable alternatives to traditional commercial fertilizers in modern agricultural practices. However, further research is necessary to gain a deeper understanding of the effects of these consortia on different crops, explore their potential for sustainable food production, and assess their long-term impacts under various environmental conditions. In future research, it is of interest to establish the metabolic profile, macronutrients, and micronutrients of microalgae, as well as the production of phytohormones, polyamines, proteins, and vitamins to determine their role in plant growth, metabolism and development.

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Data availability The raw data can be obtained by request to the corresponding author (C.L. Garzón-Castro).

Declarations

Competing interests The authors declare no competing interests.

Conflicts of interest The authors declare they have no conflicts of interest concerning this article's research, authorship, and/or publication.

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