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Circular beer production: brewery wastewater-derived microalgal biomass for cereal biofertilisation and agricultural soil remediation

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Abstract: The valorisation of microalgal biomass cultivated in agro-industrial effluents represents a promising strategy for sustainable agriculture, water and nutrients circularity. This study evaluated the potential of *Chlorococcum amblystomatis* biomass, cultivated in brewery wastewater, as a biostimulant and biofertiliser for cereals used in beer production, namely barley, wheat, and rye, while also exploring its potential for environmental remediation. Germination responses were cereal-dependent, with the most favourable results observed at intermediate biomass concentrations (0.2-0.5 $g \cdot L^{-1}$). Although no significant differences were observed compared to the control, germination index, values above 100% were obtained for barley and rye, suggesting a potential biostimulant effect worth for further investigation at these concentrations. In contrast, the culture supernatant inhibited germination. In greenhouse, biomass application at 0.5 $g \cdot L^{-1}$ sustained plant growth at levels comparable to the Hoagland (synthetic fertiliser), with no significant differences in plant height, chlorophyll content, or vegetative fresh and dry weight. Importantly, biomass application enhanced reproductive performance, promoting earlier transition and increasing spike number (by 75.7%), length (by 24.5%), and fresh weight (by 85.3%), resulting in yields per unit area comparable to the synthetic fertiliser and significantly higher than the supernatant treatment. Additionally, the biomass exhibited strong hydrophobicity and emulsification capacity towards agricultural diesel and used cooking oil, highlighting its potential for interaction with hydrophobic pollutants. Overall, this study demonstrated a circular bioeconomy approach in which brewery wastewater, generating biomass with potential applications in cereal production, sustainable fertilisation, and soil remediation, thereby promoting nutrient recycling and greater sustainability within the beer production chain.

Keywords: Brewery wastewater; Microalgae; *Chlorococcum amblystomatis*; Biostimulants; Bioremediation; Biosurfactants.

Introduction

The increasing instability of global synthetic fertiliser supply chains, driven by geopolitical conflicts and rising energy costs, has led to significant price volatility and reduced availability, placing considerable pressure on agricultural systems worldwide (Argus; FAO). Moreover, overuse and improper disposal of synthetic fertilisers often result in contamination of the crops, soils and waters. Recent conflicts have further exacerbated this situation, with fertiliser prices increasing by up to 30–65% in some regions due

to disruptions in production, trade routes, and raw material supply (CRU; FAO). This context has reinforced the urgent need to develop sustainable and locally available alternatives, such as biofertilisers and biostimulants, to reduce dependency on conventional inputs and enhance the resilience of agricultural production systems.

Biofertilisers and biostimulants have emerged as sustainable, cost-effective, and environmentally friendly alternatives to synthetic agrochemicals, as they can enhance agricultural productivity while reducing environmental pollution (Ferreira et al., 2023, 2024; Miranda et al., 2024). Biofertilisers contribute to improved soil fertility and biological activity, promote plant growth, and facilitate the transformation of nutrients into forms readily assimilable by plants (Ferreira et al., 2019; Alling et al., 2023). In contrast, biostimulants act by stimulating plant physiological processes, improving nutrient use efficiency, enhancing tolerance to abiotic stress, or supporting overall crop performance (Han et al., 2018; Deolu-Ajayi et al., 2022).

Microalgal biomass has gained increasing attention as both a biofertiliser and biostimulant due to its rich composition in bioactive compounds, such as amino acids, vitamins, polysaccharides, or phytohormone compounds such as auxins, cytokinins, and gibberellins, which can regulate key plant processes, including root development and stress responses (Navarro-López et al., 2020). In addition to these agricultural benefits, microalgae produce metabolites with antimicrobial and antioxidant properties, further supporting plant health and soil quality (Ferreira et al., 2023). Their high biodegradability, low phytotoxicity, need for lower concentrations and versatility in application make them particularly attractive for sustainable agricultural practices (Han et al., 2018; Wang et al., 2021).

Beyond their role in plant growth, the biochemical complexity of microalgal biomass also underpins its potential for environmental remediation. Microalgae are known to produce surface-active compounds, including biosurfactants and emulsifying agents, which can enhance the interaction between microorganisms and hydrophobic pollutants. This is especially relevant in contaminated soils, where poorly water-soluble compounds such as hydrocarbons exhibit low bioavailability, limiting the efficiency of bioremediation processes (Abdelfattah et al., 2022; Filho et al., 2017). Soil is a natural resource with a slow regeneration rate, and its sustainable use is essential to prevent long-term degradation. Strategies that enhance the interaction between microorganisms and hydrophobic contaminants, such as emulsification, are therefore crucial (Ferreira et al., 2024).

Biosurfactants are amphiphilic compounds produced by microorganisms that reduce surface and interfacial tension, promoting the interaction between cells and contaminants. Due to their low toxicity, high biodegradability, and environmental compatibility, they are considered sustainable alternatives to synthetic surfactants (Ferreira et al., 2024; Lima e Silva et al., 2024). In this context, microalgae have gained attention as potential producers of biosurfactant-like compounds. Their ability to exhibit cell surface hydrophobicity is a key parameter influencing microbial interaction with hydrophobic compounds that is widely used to evaluate biotechnological potential. These properties support the use of microalgae as promising agents for the remediation of hydrocarbon-contaminated environments (Sachdev & Cameotra, 2013; Ferreira et al., 2024). Among the various microalgal species, *Chlorococcum amblystomatis* has shown particular potential due to its tolerance to fluctuating environmental conditions, rapid growth rate, and ability to synthesise valuable biocompounds (Conde et al., 2021; Correia et al., 2023).

This work is built upon previous research on microalgal cultivation in brewery wastewater, advancing from biomass production towards its practical application in agriculture and environmental remediation. Accordingly, this study investigated the potential of *C. amblystomatis* biomass grown in brewery wastewater as a biostimulant for cereal crops commonly used in beer production (barley, wheat, and rye), while also assessing its applicability in soil bioremediation, in line with circular bioeconomy principles.

Despite the increasing interest in microalgae-based systems, only a limited number of studies had explored their integrated use for both wastewater valorisation and environmental remediation. In this context, *C. amblystomatis* emerged as a particularly promising yet still underexplored species.

Materials and Methods

Preparation of irrigation treatments

The biomass used in this study was produced under the cultivation conditions described in a parallel study. Briefly, the microalga was cultivated in 50-L flat-panel photobioreactors using brewery wastewater supplies by Sociedade Central de Cervejas e Bebidas (SCC) (Vialonga, Portugal) The wastewater was supplemented with 4 mM nitrogen as urea. After 7 days, biomass was harvested by centrifugation, washed to remove residual medium, and resuspended in distilled water to obtain final concentrations of 0.2 $g \cdot L^{-1}$ (B0.2), 0.5 $g \cdot L^{-1}$ (B0.5), and 1.0 $g \cdot L^{-1}$ (B1).

The culture supernatant (S), obtained after biomass removal by sedimentation, was used without further dilution. Electrical conductivity (κ) of the irrigation treatments was measured with a conductivity meter to estimate the concentration of dissolved ions and to indirectly assess osmotic load.

Germination trials

Germination assays were conducted using barley (*Hordeum vulgare*), wheat (*Triticum* spp.), and rye (*Secale cereale*). Ten seeds per species were placed in Petri dishes lined with Whatman No. 1 filter paper and moistened with 6 mL of each irrigation treatment (B0.2, B0.5, B1, or S). Each treatment was performed in triplicate (n=3), totalling 30 seeds per condition. Distilled water was used as the negative control (CTR).

Dishes were incubated for 5 days at 25 °C in the dark in a controlled growth chamber (FITOCLIMA S600 PL, Aralab, Portugal). Number of germinated seeds was recorded, and root length was measured using ImageJ (v1.53t, National Institutes of Health, USA). The germination index (GI), given in %, was calculated according to Equation (1) (Zucconi et al., 1981):

$$GI = \frac{G \times L}{G_W \times L_W} \times 100 \quad (1)$$

where G and L correspond to germination percentage and mean root length of the treatment, and G_w and L_w represent the negative control values (distilled water). GI > 100% was interpreted as positive biostimulant activity.

Greenhouse trial

Experimental setup

A greenhouse experiment was conducted to evaluate the biostimulant and biofertiliser potential of *C. amblystomatis* biomass. Barley was selected based on its superior performance in the preliminary germination assays. Cylindrical pots (34 cm height, 16 cm diameter) were filled with seedling substrate (SIRO Germinação Biológica) mixed with perlite (2:1, v/v). Seven seeds were sown per pot and thinned to five plants after germination (25 plants per treatment; n=5 pots). Treatments included microalgal biomass at 0.5 $g \cdot L^{-1}$ (B0.5), culture supernatant (S), and Hoagland nutrient solution as control (HOAG). The Hoagland solution consisted of 5 mM $Ca(NO_3)_2 \cdot 4H_2O$, 5 mM KNO_3, 1 mM KH_2PO_4, 2 mM $MgSO_4 \cdot 7H_2O$, 60 µM H_3BO_3, 9 µM $MnCl_2 \cdot 4H_2O$, 0.8 µM $ZnSO_4 \cdot 7H_2O$, 0.4 µM

$CuSO_4 \cdot 5H_2O$, 2.0 µM MoO_3, and 10 µM Fe-EDDHA (Vieira et al., 2023). The treatments were applied via irrigation every two weeks by applying 200 mL of the respective solution per pot. Additional watering with tap water was provided depending on environmental conditions and plant demand. The experiment was carried out for 162 days (November 2024 to May 2025) in a greenhouse (FCT, Universidade do Algarve, Faro, Portugal) under natural photoperiod.

Vegetative and physiologic measurements

To assess the potential effects of the treatments on vegetative biomass accumulation, plant growth was monitored throughout the experiment. Every two weeks, one representative plant per pot was selected for evaluation. Plant height, number of leaves, and number of tillers were recorded. Physiologic status was evaluated through measurement of the chlorophyll content using a SPAD-502 meter (Soil and Plant Analysis Development, Konica Minolta, Inc., Japan). SPAD readings were obtained from two young, fully expanded green leaves per plant to ensure consistency across treatments.

At harvest, the vegetative tissues (shoots) were collected, and both fresh and dry biomass were determined. Dry weight was measured after oven-drying (MMM Medcenter Venticell, Germany) at 103 °C for 48 h. Representative images of the experimental procedures are shown in Fig. 1.

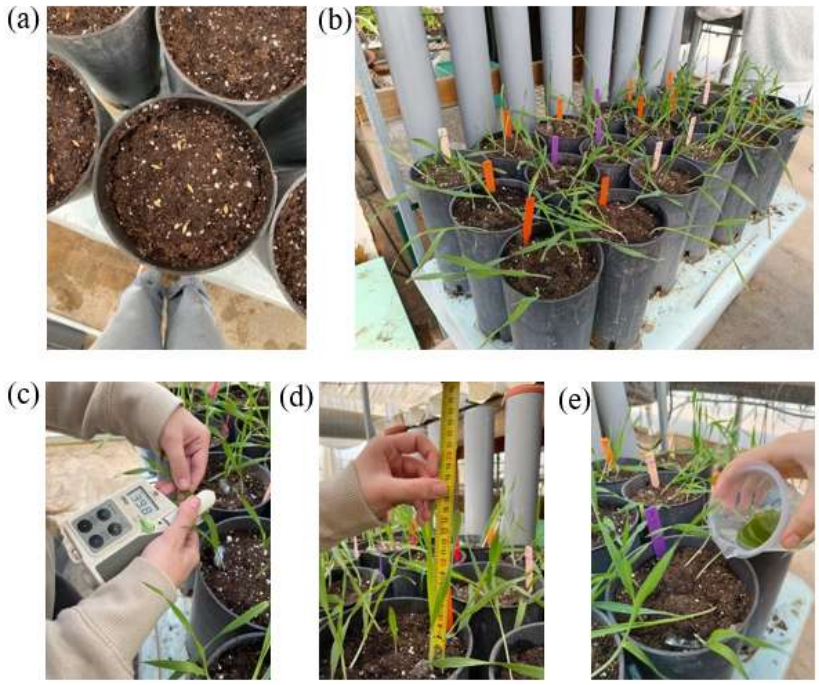


Fig. 1 – Overview of the greenhouse trial. (a) seed arrangement in the pot; (b) experimental layout of treatments; (c) stem length measurements; (d) SPAD measurements; (e) application of irrigation solutions.

Reproductive measurements

Reproductive development was assessed at the final harvest (day 162). The total number of spikes per pot and per plant was recorded. Spike length and fresh weight were measured immediately after harvest, and dry weight was determined after oven-drying at 103 °C for 48 h.

To provide an agronomically relevant yield proxy, total spike dry biomass was normalised to ground area ($g \cdot m^{-2}$). These parameters were used to evaluate the effects of the different irrigation treatments on reproductive performance.

Biosurfactants and Relative Hydrophobicity

C. amblystomatis biomass was evaluated for its potential in hydrocarbon bioremediation using the microbial adhesion to hydrocarbons (MATH) assay (Rosenberg et al., 1980). Samples were resuspended in PUM buffer (phosphate–urea–$MgSO_4$) to standardize the initial absorbance at 680 nm to approximately 0.5 $g \cdot L^{-1}$, using PUM buffer as the blank. The standardized suspension was transferred to test tubes, and hydrocarbons (hydraulic, engine, and gear oils from agricultural machinery, and agricultural diesel) were added at a 1:1 (v/v) ratio. The mixture was incubated in a water bath at 30 °C for 10 min, vortexed for 2 min, and allowed to stand for 15 min at room temperature to promote phase separation. The aqueous phase was then collected, and the residual absorbance at 680 nm was measured. Relative hydrophobicity (RH), given in %, was calculated according to Equation (2):

$$HR = \frac{Abs_0 - Abs_f}{Abs_0} \times 100 \quad (2)$$

The partition coefficient (*Kp*) is a measure of the differential solubility of a compound between two immiscible solvents, reflecting its distribution between the aqueous and organic phases. The logarithmic form, $\log_{10}(Kp)$, is widely used as an indicator of hydrophobicity: negative values indicate a higher affinity for the aqueous phase (hydrophilic behaviour), while positive values indicate a greater affinity for the organic phase (hydrophobic behaviour). A value close to zero indicates an equal distribution between both phases. The coefficient was calculated according to Equation (3):

$$\log_{10}(Kp) = \log_{10}\left(\frac{Abs_0 - Abs_f}{Abs_f}\right) \quad (3)$$

For Equations (2) and (3), Abs_o corresponds to the initial absorbance of the standardized microalgal biomass suspensions measured at 680 nm, while Abs_f represents the final absorbance at the same wavelength of the aqueous phase (lower layer) after phase separation.

Emulsifying Index

Emulsifying index was evaluated by mixing biomass resuspended in distilled water (approximately 0.5 $g \cdot L^{-1}$) with equal volumes (1:1, v:v) of different hydrocarbons, including agricultural diesel, hydraulic, engine, and gear oils from agricultural machinery, as well as used cooking oil and sweet almond oil.

Two controls were included: a negative control (distilled water) and a positive control consisting of sodium dodecyl sulphate (SDS, 0.5 $g \cdot L^{-1}$), both prepared under the same conditions and mixed with the tested oils.

The mixtures were vortexed for 2 min and allowed to stand at room temperature. Emulsion formation was evaluated after 5 min by measuring the length of the emulsion layer relative to the total length of the mixture. After 24 h, measurements were repeated to assess emulsion stability. The emulsification index (EI), given in %, was calculated according to Equation (4):

$$EI_t = \frac{L_e}{L_t} \times 100 \quad (4)$$

where L_e and L_t correspond to the length of the emulsion layer and the total length of the mixture, respectively.

Statistical analysis

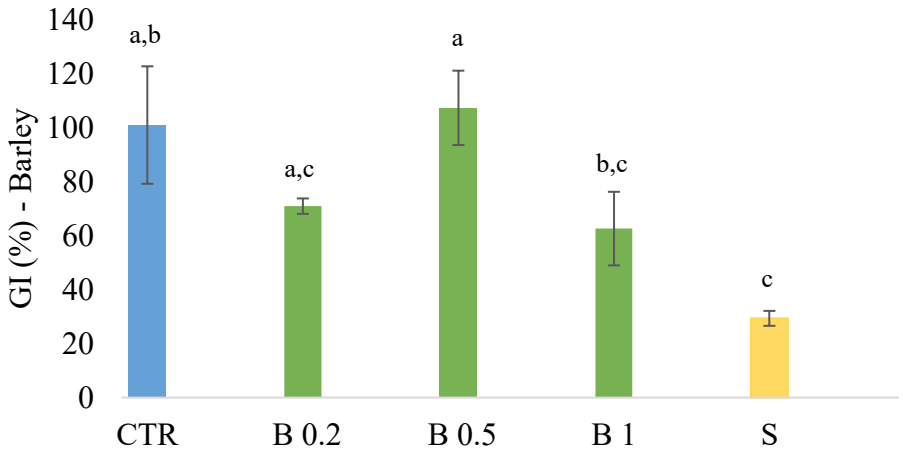
All germination data are presented as mean ± standard deviation, whereas final greenhouse measurements are expressed as mean ± standard error. Statistical comparisons among treatments were assessed using one-way ANOVA followed by Tukey's post hoc test (GraphPad Prism v10.4.2), with significance set at $p < 0.05$.

Results

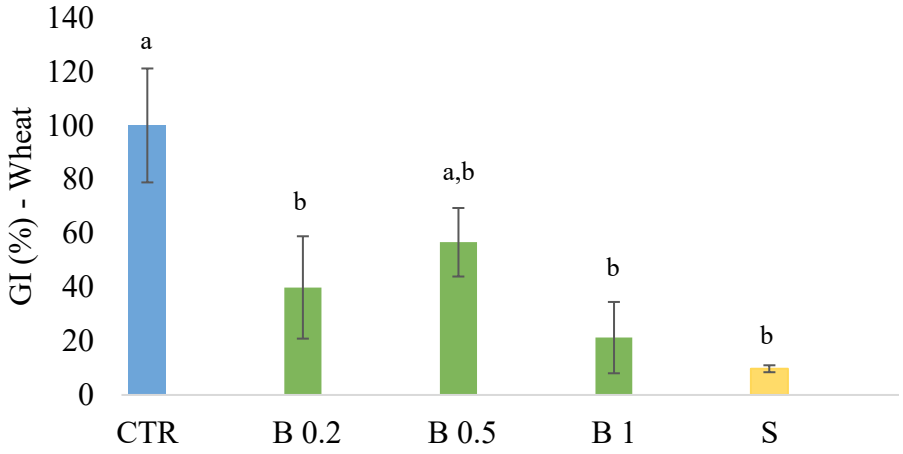
Biostimulant effect on plant germination

The biostimulant activity of *Chlorococcum amblystomatis* biomass cultivated in brewery wastewater, as well as the culture supernatant, was evaluated based on the germination index (GI) of barley, wheat, and rye seeds, and the results are presented in Fig. 2.

(a)



(b)



(c)

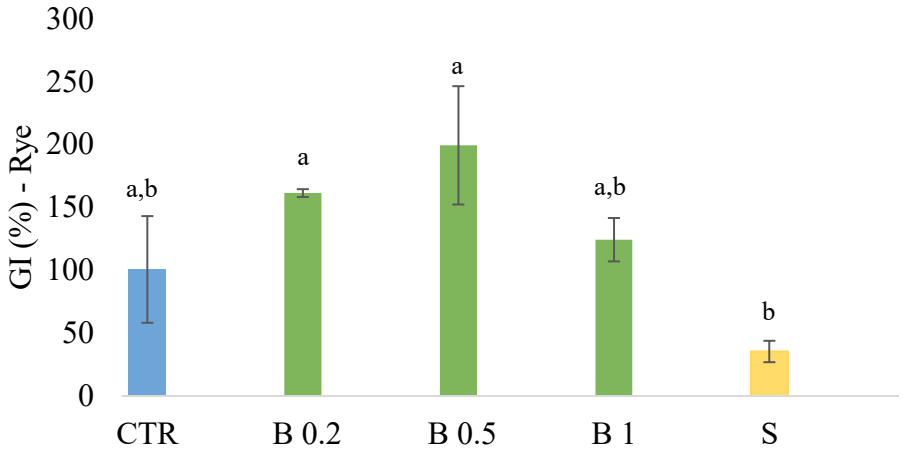


Fig. 2 – Influence of *Chlorococcum amblystomatis* cultivated in brewery wastewater 0.2 g·L^{-1} (B0.2), 0.5 g·L^{-1} (B0.5), and 1 g·L^{-1} (B1), culture supernatant (S), and distilled water as control (CTR) on Germination index (GI, %) of: (a) barley seeds; (b) wheat seeds; (c) rye seeds. Different letters indicate statistically significant differences among treatments according to Tukey's post hoc test ($p < 0.05$). Results are expressed as mean ± standard deviation ($n = 3$).

To interpret the GI, distilled water was used as the negative control (CTR; GI = 100%), and only values above 100% were considered indicative of a biostimulant effect.

For barley, the highest GI value was observed at B0.5 (105.26%), indicating a favourable response to this biomass concentration. Although no significant differences were detected compared to the control ($p \geq 0.05$), this treatment consistently showed the most promising performance among the tested conditions. In contrast, the culture supernatant significantly reduced germination ($p < 0.05$), likely due to its higher nutrient concentration, which may have imposed constraints during the early germination stage.

In wheat, biomass treatments did not promote germination, with all concentrations (0.2, 0.5, and 1.0 g·L^{-1}) resulting in GI values below 100%. No significant differences were observed relative to the control ($p \geq 0.05$), indicating a limited response of this cereal to the tested conditions. Similarly, the culture supernatant negatively affected germination ($p < 0.05$).

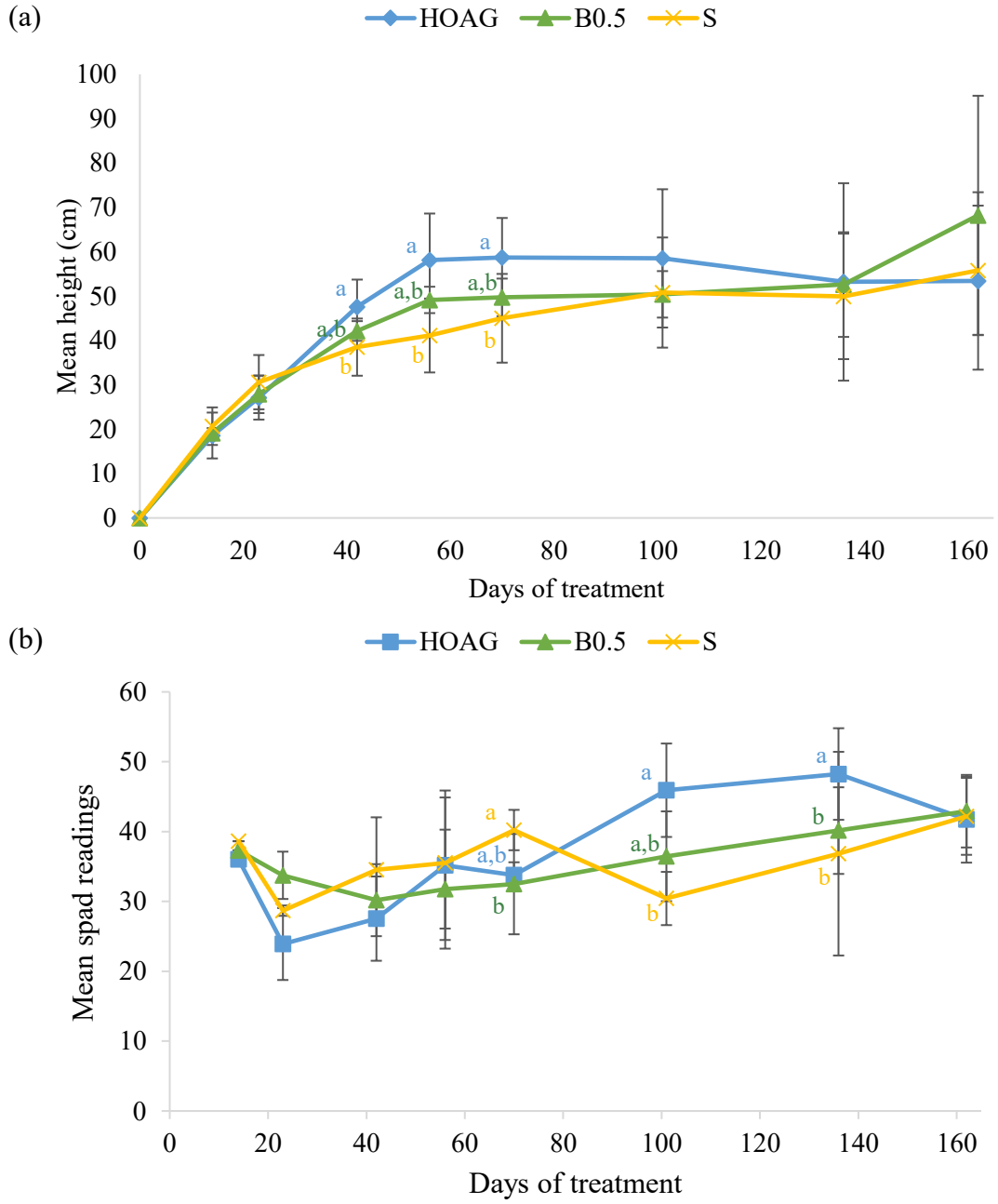
Rye exhibited the most responsive pattern, with biomass treatments at 0.2 and 0.5 g·L^{-1} showing the highest GI values (128.53% and 156.89%, respectively), suggesting a positive effect on germination. Although these differences were not statistically significant ($p \geq 0.05$), all biomass concentrations, as well as the supernatant, outperformed comparably to the control when considering the overall dataset.

Germination responses were cereal-dependent, with the most favourable trends observed at intermediate biomass concentrations (0.2-0.5 g·L^{-1}). In particular, B0.5 showed the most consistent performance, especially in barley, while both B0.2 and B0.5 promoted higher germination in rye. Although not always statistically significant, these patterns suggest a positive effect of microalgal biomass on early development. In contrast, the culture supernatant generally inhibited germination, likely due to its higher nutrient concentration.

Pot trials in Greenhouse

Intermediate measurements

The present assay aimed to evaluate the potential of *C. amblystomatis* biomass, cultivated in brewery wastewater, as well as the culture supernatant, as biostimulant and biofertiliser agents in barley cultivation, contributing to nutrient circularity. The results of the intermediate measurements performed throughout the experiment for Hoagland solution (HOAG), biomass at 0.5 $g \cdot L^{-1}$ (B0.5), and culture supernatant (S) are presented in Fig. 3, highlighting the temporal evolution of plant growth under the different treatments.



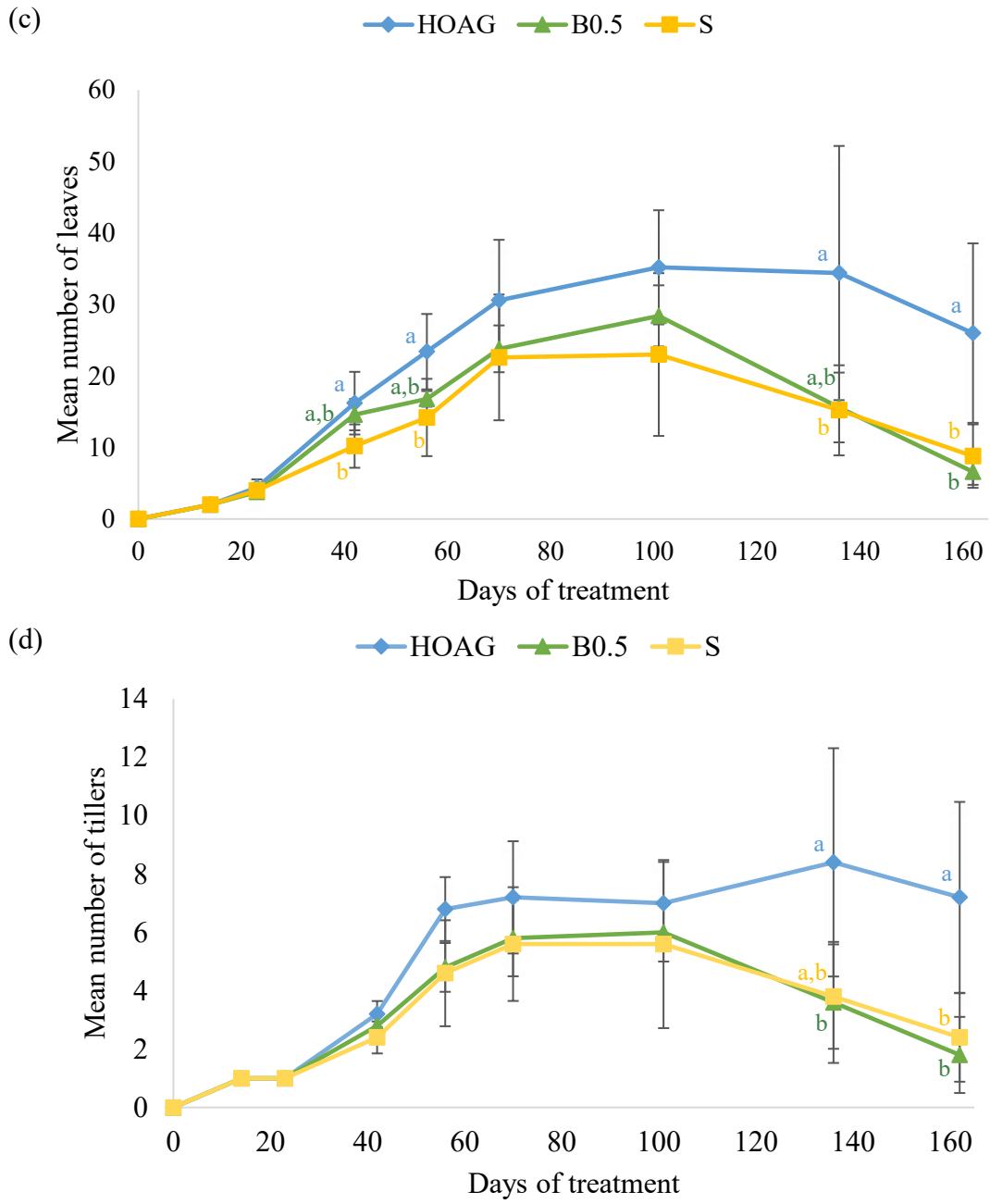


Fig. 3 – Values of (a) height of plants (cm), (b) SPAD readings, (c) number of leaves, (d) and number of tillers of barley during the greenhouse experiment under different irrigation treatments: biomass of *Chloro-coccum amblystomatis* at 0.5 $g \cdot L^{-1}$ (B0.5), cultivation supernatant (S), and Hoagland solution (HOAG). Different letters indicate significant differences among treatments according to Tukey's post hoc test ($p < 0.05$). Results are expressed as mean ± standard deviation ($n = 5$).

Plant height evolution throughout the cultivation period (Fig. 3a) showed a similar growth pattern across treatments, with a marked increase up to approximately day 70, followed by a steadying phase. Although significant differences ($p < 0.05$) were observed between the HOAG and the S treatment at days 42, 56, and 70, these differences were not maintained until the end of the experiment. At the final harvest (day 162), all treatments reached comparable plant heights, with mean values of 68.22 cm, 55.80 cm, and 44.78 cm for B0.5, S, and HOAG, respectively.

A similar trend was observed for SPAD values measured in young leaves (Fig. 3b). Although differences between treatments were detected at specific time points during the experiment, these variations diminished over time and were no longer evident at the final measurement. After 162 days, all treatments exhibited comparable SPAD values, indicating similar chlorophyll content in newly developed leaves.

Regarding leaf number, the HOAG exhibited a significantly higher number of leaves than the S treatment at days 42 and 56 ($p < 0.05$) (Fig. 3c). This pattern is consistent with the plant height shown in Fig. 3a. Electrical conductivity was measured, yielding values of 146 $\mu S \cdot cm^{-1}$ for B0.5, 5470 $\mu S \cdot cm^{-1}$ for S, and 2353 $\mu S \cdot cm^{-1}$ for HOAG. The higher electrical conductivity and nutrient load in the S treatment may have imposed an initial osmotic constraint on plant development. However, as observed over time in the same figure, plants were able to acclimate, restoring normal comparable growth patterns at later stages. At day 136, a significant difference ($p < 0.05$) between S and the HOAG was again observed, and at the final measurement, both B0.5 and S treatments showed significantly lower values compared to the HOAG ($p < 0.05$). Similar pattern was observed for tiller number (Fig. 3d). No significant differences were observed among treatments (HOAG, B0.5, and S) during the vegetative phase. However, at the final stage, plants grown under HOAG exhibited a significantly higher number of tillers ($p < 0.05$).

This interpretation is further supported by the visual evidence in Fig. 4, showing the pots at the end of the experimental period. In the HOAG (Fig. 4a), plants exhibited a higher abundance of green leaves, indicating a prolonged investment in vegetative growth. In contrast, plants treated with *C. amblystomatis* biomass (Fig. 4b) and supernatant (Fig. 4c) displayed fewer green leaves and tillers, but more developed stems and visible spikes, consistent with a more advanced reproductive stage.

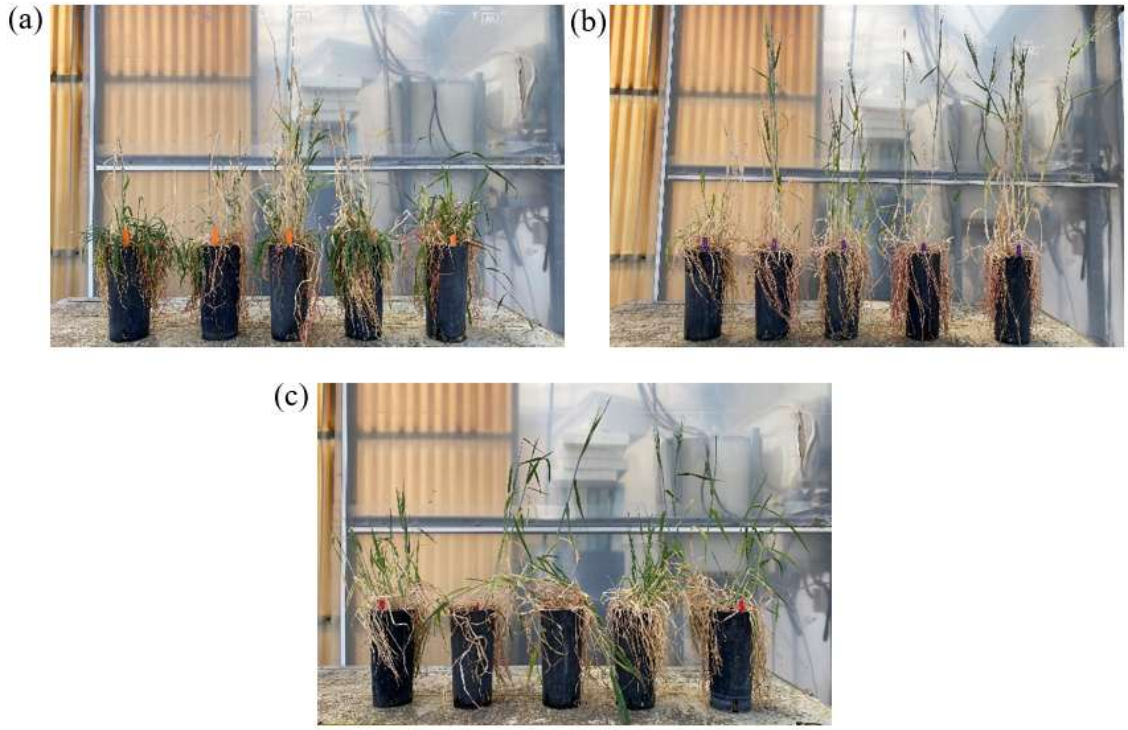


Fig. 4 - Representative photographs of the five pots per treatment after 162 days of barley cultivation. (a) Hoagland solution (HOAG); (b) *Chlorococcum amblystomatis* biomass cultivated in brewery wastewater applied at 0.5 $g \cdot L^{-1}$ (B0.5); and (c) corresponding culture supernatant (S).

In these findings, the application of *C. amblystomatis* biomass (B0.5) resulted in plant growth performance comparable to that obtained with the synthetic Hoagland

fertiliser (HOAG) across all monitored parameters. Although the supernatant treatment (S) initially showed reduced performance, plants progressively acclimated over time, reaching values similar to the other treatments at later stages.

Final measurements

At the final harvest, in addition to the previously reported measurements, the fresh and dry weight of the vegetative biomass were determined (Fig. 5). Reproductive parameters were also evaluated, including the mean number of spikes per plant, total spike number per treatment, spike length, and spike fresh and dry weight (Fig. 6). To estimate treatment yield, spike dry weight was further normalised to ground area. The results were as follows:

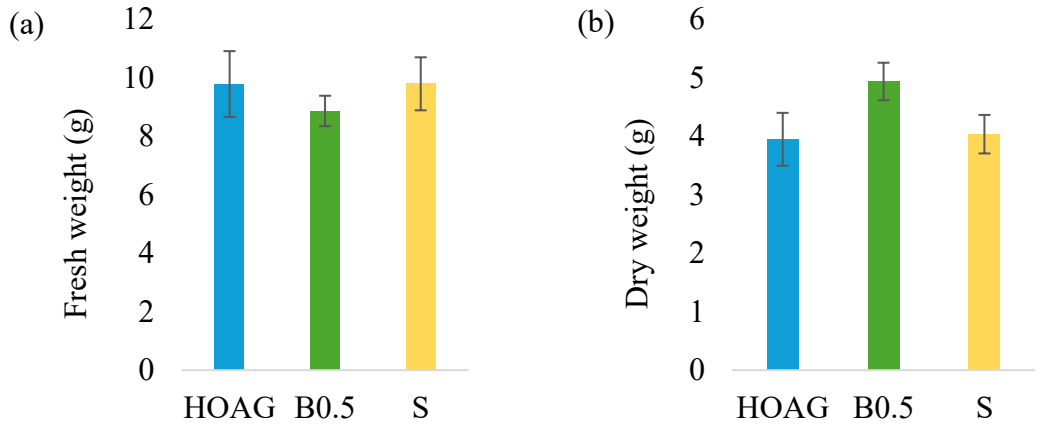
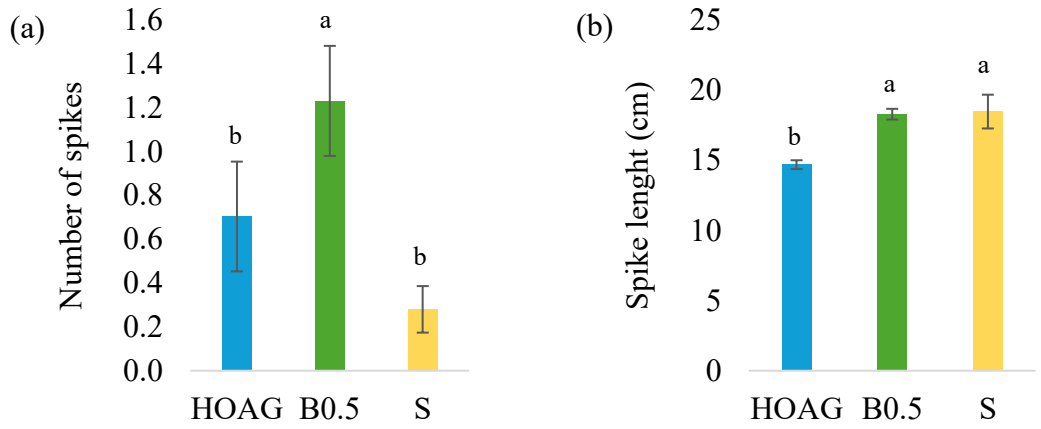


Fig. 5 - Fresh and dry shoot biomass of barley after 162 days of cultivation under different treatments: Hoagland nutrient solution (HOAG), *Chlorococcum amblystomatis* biomass applied at 0.5 g·L^{-1} (B0.5), and culture supernatant (S). Panels show (a) shoot fresh weight and (b) shoot dry weight. No significant differences were detected among treatments according to Tukey's post hoc test ($p \geq 0.05$). Data are presented as mean ± standard error.



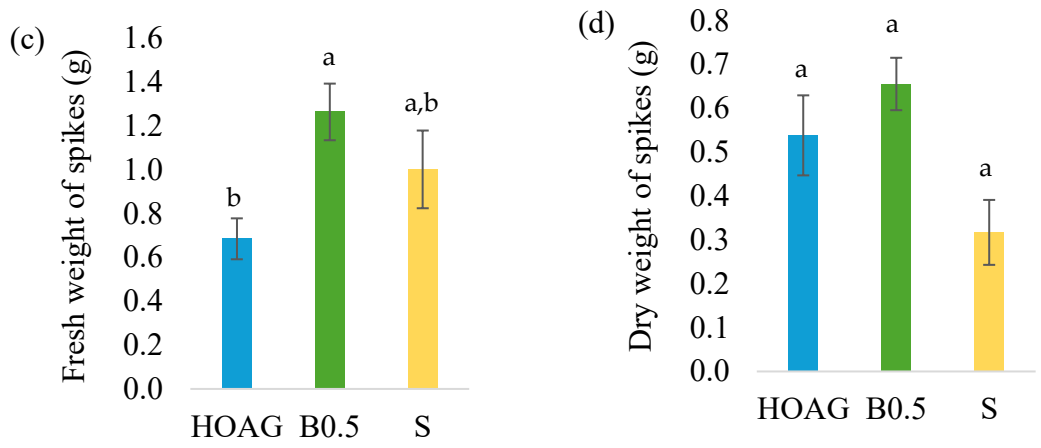


Fig. 6 - Reproductive characteristics of barley spikes after 162 days of cultivation under different treatments: *Chlorococcum amblystomatis* biomass applied at 0.5 $g \cdot L^{-1}$ (B0.5), culture supernatant (S), and Hoagland nutrient solution (HOAG). Panels show (a) mean number of spikes per plant, (b) spike length, (c) spike fresh weight, and (d) spike dry weight. Different letters indicate significant differences among treatments according to Tukey's post hoc test ($p < 0.05$). Data are presented as mean ± standard error.

No significant differences were observed in vegetative fresh (Fig. 5a) and dry (Fig. 5b) biomass among treatments B0.5, S and the HOAG ($p \geq 0.05$). These results indicate that the application of *C. amblystomatis* biomass and culture supernatant did not significantly affect vegetative biomass accumulation under the tested conditions.

Regarding reproductive parameters (Fig. 6), the treatment B0.5 resulted in a significantly higher number of spikes per plant compared to both the HOAG and the S treatment ($p < 0.05$) (Fig. 6a). In terms of spike length (Fig. 6b), both B0.5 and S treatments produced significantly longer spikes than the HOAG ($p < 0.05$).

Similarly, spike fresh weight (Fig. 6c) was significantly higher under B0.5 compared to the HOAG ($p < 0.05$), while no significant differences were observed among treatments for spike dry weight (Fig. 6d).

At the end of the experiment, all spikes produced in each treatment were counted, providing a relevant indicator of agronomic performance under practical field conditions. The HOAG presented 19 spikes from a total of 27 plants, while the B0.5 treatment produced 32 spikes from 26 plants. In contrast, the S treatment resulted in only 7 spikes from 25 plants. These results indicate a higher total spike production under the B0.5 treatment compared to both the HOAG and S treatment.

To estimate the final yield of each treatment, spike dry weight was normalised to ground area. The results are presented in Fig. 7.

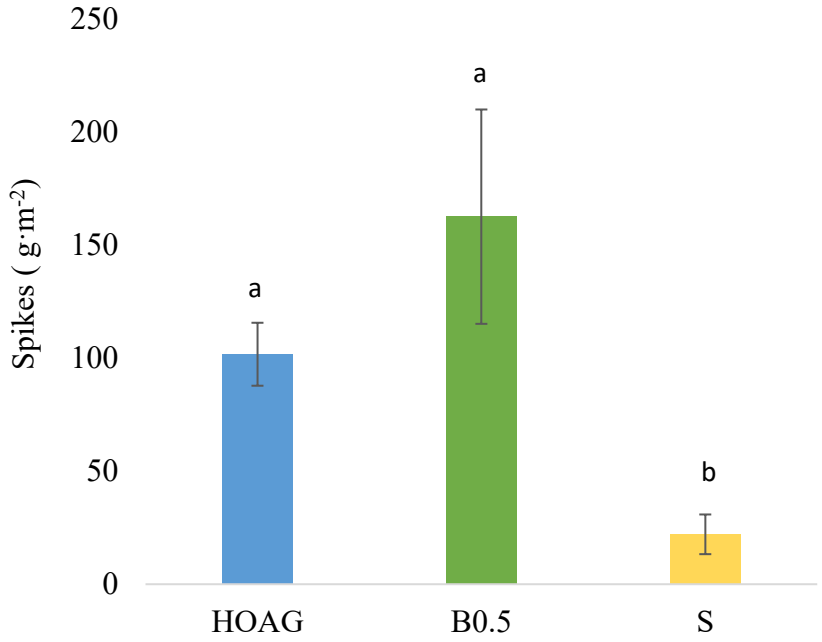


Fig. 7 - Total spike production expressed as dry biomass per unit area (g·m⁻²) after 162 days of barley cultivation under different irrigation treatments: *Chlorococcum amblystomatis* biomass applied at 0.5 g·L⁻¹ (B0.5), culture supernatant (S), and Hoagland nutrient solution (HOAG). Different letters indicate significant differences among treatments according to Tukey's post hoc test ($p < 0.05$). Data are presented as mean ± standard error.

The B0.5 treatment showed a yield per unit area comparable to the HOAG ($p \geq 0.05$), but significantly higher than the S treatment ($p < 0.05$) (Fig. 7). This suggests that the higher number of spikes in B0.5 contributed to greater biomass accumulation per area, an important parameter for estimating field productivity.

These results indicate a consistent effect of *C. amblystomatis* biomass grown in brewery wastewater on spike development, particularly in terms of spike number and fresh biomass, as well as spike length, which was higher in both the B0.5 and S treatments compared to the synthetic fertiliser control (HOAG). In this context, both the biomass and the culture supernatant showed advantageous performance relative to the conventional fertilisation treatment, depending on the evaluated parameter. These findings further support the potential of *C. amblystomatis* biomass as a promising alternative to synthetic nutrient solutions, particularly for enhancing reproductive development.

Soil bioremediation

Considering the increasing occurrence of environmental contamination by agricultural oils and other hydrophobic residues derived from farming activities, the hydrophobicity and emulsifying activity of the biomass obtained from wastewater cultivation were evaluated. These parameters were analysed to assess the feasibility of using *C. amblystomatis* biomass for bioremediation purposes, particularly for the removal of oil-based contaminants commonly found in accidental spills during farming activities.

Hydrophobicity

The ability of *C. amblystomatis* cultivated in brewery wastewater to produce biosurfactant-like molecules was evaluated using the microbial adhesion to hydrocarbons (MATH) assay. This test was performed to assess the potential application of this

microalga in the bioremediation of soils contaminated by agricultural fuels and oils, which may occur during accidental spills or improper handling of farming machinery. The hydrocarbons selected for the assay were representative contaminants commonly associated with agricultural activities, including agricultural diesel fuel and used tractor oils, such as hydraulic, gear, and engine oils. Used cooking oil (sunflower) was included as a representative waste edible oil to allow comparison with agricultural hydrocarbon-based oils, thereby assessing the versatility of the system in interacting with both biodegradable and petroleum-derived contaminants. The results are presented in Table 1.

Table 1 – Relative Hydrophobicity (RH) and logarithmic base 10 of the partition coefficient (log_{10} *Kp*) of *Chlorococcum amblystomatis* cultivated in brewery wastewater for the different oils studied. Values are given as mean ± standard deviation (*n*=3).

Oil compound	RH (%)	log_{10} *Kp*
Hydraulic oil	70.43 ± 5.05	0.79 ± 0.49
Engine oil	44.83 ± 11.49	-0.09 ± 0.21
Agricultural diesel	88.91 ± 1.14	0.91 ± 0.05
Cooking oil	83.12 ± 1.14	0.85 ± 0.47

C. amblystomatis biomass showed high relative hydrophobicity (RH) values towards some of the tested hydrocarbons, with higher values for agricultural diesel (88.91±1.14%), cooking oil (sunflower) (83.12±1.14%), and tractor hydraulic oil (70.43±5.05%). In contrast, a low RH value was observed for tractor engine oil (44.83±11.49%) (Table 1). These results indicate a higher affinity of the microalgal biomass for agricultural diesel, hydraulic oil, and cooking oil, while a lower interaction was observed for engine oil.

The partition coefficient (log_{10} *Kp*) was also determined to evaluate the distribution of the biomass between the aqueous and organic phases. Positive values of log_{10} *Kp* indicate higher affinity for the organic phase (hydrophobic behaviour), whereas negative values indicate preference for the aqueous phase (hydrophilic behaviour). *C. amblystomatis* biomass showed hydrophobic characteristics in the presence of agricultural diesel and tractor hydraulic oil, as well as used cooking oil, with log_{10} *Kp* values of 0.91±0.05, 0.79±0.49 and 0.85±0.47, respectively, while hydrophilic behaviour was observed for tractor engine oil, with log_{10} *Kp* value of -0.09±0.21. These results are consistent with the RH data, confirming that diesel and hydraulic oil are the hydrocarbons with the highest adsorption affinity for microalgal biomass.

Emulsification

Emulsification plays a key role in hydrocarbon removal processes. Therefore, the emulsifying activity of the biomass was evaluated to further explore the ability of *C. amblystomatis* to produce biosurfactant-like compounds capable of emulsifying hydrocarbons present in the tested agricultural oils. The emulsion stability index (EI) values are presented in Table 2.

Table 2 - Emulsification index after 5 min (EI5) and after 24 hours (EI24), and stability ratio (EI24/EI5) of the emulsion produced by *Chlorococcum amblystomatis* cultivated in brewery wastewater

for the different oils studied. Distilled water and Sodium Dodecyl Sulfate (SDS) at 0.5 $g \cdot L^{-1}$ were used as negative and positive controls, respectively. Different letters indicate significant differences between water, SDS and *C. amblystomatis* biomass, according to Tukey's post hoc test ($p < 0.05$Values are given as mean ± standard deviation (n=3).

Oil compound	Parameter	Control		
		Water (-)	SDS (+)	*C. amblystomatis* biomass
Hydraulic oil	EI_5 (%)	69.71 ± 14.90[a]	72.35 ± 11.10[a]	60.62 ± 2.36[a]
	EI_{24} (%)	44.83 ± 34.98[a]	51.48 ± 22.87[a]	16.86 ± 6.33[a]
	EI_{24}/EI_5	0.64	0.71	0.28
Engine oil	EI_5 (%)	93.88 ± 2.26[a]	96.55 ± 1.50[a]	87.68 ± 11.47[a]
	EI_{24} (%)	76.29 ± 2.24[a]	80.86 ± 2.90[a]	72.99 ± 19.32[a]
	EI_{24}/EI_5	0.81	0.84	0.83
Agricultural diesel	EI_5 (%)	0.00 ± 0.00[b]	67.40 ± 3.15[a]	65.07 ± 0.66[a]
	EI_{24} (%)	0.00 ± 0.00[b]	54.96 ± 12.16[a]	59.80 ± 0.97[a]
	EI_{24}/EI_5	0	0.82	0.92
Cooking oil	EI_5 (%)	0.00 ± 0.00[c]	78.65 ± 10.83[a]	58.35 ± 2.65[b]
	EI_{24} (%)	0.00 ± 0.00[b]	62.49 ± 0.95[a]	50.73 ± 2.69[b]
	EI_{24}/EI_5	0	0.79	0.87

For hydraulic and engine oils from tractors, it was not possible to conclusively assess the emulsifying properties of the microalga. This limitation arises from the formation of stable emulsions in the negative control (distilled water), where emulsion layers were observed for both oils, with stability ratios (EI_{24}/EI_5) equal to or higher than those obtained with the emulsifier SDS and with the microalgal biomass. For the remaining oils, the emulsion layers formed between both phases in the presence of microalgal biomass after 5 min remained stable over 24 h. In agricultural diesel, the EI_{24}/EI_5 ratio reached 0.92, exceeding that observed for SDS (0.82). Similarly, for used cooking oil, the microalga exhibited higher emulsion stability after 24 h compared to SDS, with an EI_{24}/EI_5 ratio of 0.87 for *C. amblystomatis* and 0.79 for SDS (Table 2). Only for cooking oil were statistically significant differences observed between the use of *C. amblystomatis* and SDS, with larger emulsification layers obtained when the biomass was used ($p < 0.05$). For agricultural oil, no significant differences were observed between the use of biomass and SDS. The emulsification assays confirmed the ability of *C. amblystomatis* to promote the formation of stable emulsions with different oil types showing values similar to those obtained with SDS.

Discussion

The present study demonstrates that *Chlorococcum amblystomatis* biomass cultivated in brewery wastewater can be effectively valorised for agricultural applications. Germination assays revealed that the biostimulant effect of the biomass is dependent on both cereal type and applied concentration, with the most favourable responses observed at intermediate concentrations (0.2-0.5 $g \cdot L^{-1}$). In particular, the B0.5 treatment showed the most consistent performance, especially in barley, while both B0.2 and B0.5 promoted higher germination trends in rye (Fig. 1). Although these effects were not always statistically significant, the overall patterns suggest a positive influence of microalgal biomass on early plant development.

The effectiveness of biostimulants has been widely associated with the regulation of the balance between gibberellic acid (GA) and abscisic acid (ABA), key phytohormones involved in seed germination. Microalgae are known to produce or contain phytohormone-like compounds, including gibberellins, auxins, and cytokinins, as well as other bioactive metabolites capable of modulating plant physiological responses. An increase in the GA/ABA ratio promotes germination, either through the direct supply of GA, stimulation of its biosynthesis, or enhancement of ABA degradation (Han et al., 2018; Wang et al., 2021; Alling et al., 2023). In this context, lower biomass concentrations may provide optimal levels of bioactive compounds capable of favourably modulating this hormonal balance, whereas higher concentrations may lead to inhibitory effects due to hormonal imbalance or the accumulation of stress-related compounds (Deolu-Ajayi et al., 2022; Ferreira et al., 2024).

In addition to hormonal regulation, other microalgal-derived compounds may contribute to the observed effects. Lipids, particularly polyunsaturated fatty acids (PUFAs), have been associated with membrane modulation and stress signalling, potentially influencing seed germination and early development. Furthermore, extracellular polysaccharides and low-molecular-weight metabolites present in microalgal biomass may enhance water uptake, seed hydration, and metabolic activation during germination. These combined biochemical mechanisms may underlie the concentration-dependent responses observed in this study (Ferreira et al., 2019; Navarro-López et al., 2020; Deolu-Ajayi et al., 2022).

Consistent with this, the application of *C. amblystomatis* biomass at 0.5 $g \cdot L^{-1}$ resulted in a germination index (GI) above 100%, suggesting that this concentration may represent a threshold for enhanced performance. This highlights the sensitivity of the biostimulant response to dosage, where small variations can lead to distinct physiological outcomes. Future studies should therefore focus on testing narrower concentration ranges around this threshold to better define optimal application conditions (Han et al., 2018; Navarro-López et al., 2020).

In contrast, the culture supernatant generally exerted an inhibitory effect on germination, particularly in barley and wheat, where significantly lower GI values were observed compared to the control. This response is consistent with previous findings (Ferreira et al., 2019) and suggests that the biostimulant effect is primarily associated with the microalgal biomass rather than residual nutrients present in the culture medium. The inhibitory effect observed may be related to the higher nutrient concentration or organic load of the supernatant, which can be detrimental during early germination stages. At this phase, seeds rely primarily on internal reserves for energy, and external nutrient supply may not only be unnecessary but potentially disruptive (Ferreira et al., 2023). These findings indicate that the application of the supernatant may be more suitable at later stages of plant development.

The absence of a biostimulant effect in wheat further highlights the variability of responses across different crops. While *C. amblystomatis* biomass did not enhance germination in this cereal, other studies have reported contrasting results, such as the stimulatory effect of *Tetradesmus obliquus* cultivated in swine wastewater (Ferreira et al., 2023). These discrepancies emphasise that biostimulant performance depends on multiple factors, including microalgal species, cultivation conditions, and the resulting profile of bioactive compounds (Wang et al., 2021; Alling et al., 2023). Additionally, differences in seed cultivar may contribute to the observed variability, as distinct genotypes exhibit different physiological sensitivities during germination (Duvnjak et al., 2024; Pasa et al., 2025).

Overall, the results indicate that the biostimulant potential of *C. amblystomatis* biomass is influenced by a combination of factors, including concentration, cereal type, and the characteristics of the cultivation system. These findings reinforce the need for case-

specific optimisation when applying microalgal-based biostimulants in agriculture, to maximise their effectiveness and ensure consistent performance in agricultural systems.

Following the germination assays, the greenhouse experiment provided further insight into the performance of *C. amblystomatis* biomass under more advanced growth conditions. Barley represents the main raw material used in beer production (Jaeger et al., 2021), and the cultivation of brewing ingredients is associated with a significant environmental footprint, largely due to the use of synthetic fertilisers (Lima e Silva et al., 2024). In this context, the present study aimed to evaluate the potential of *C. amblystomatis* biomass cultivated in brewery wastewater, as well as its corresponding supernatant, as a biostimulant or biofertiliser in barley cultivation, contributing to nutrient circularity within the brewing value chain.

The application of microalgal biomass (B0.5) supported plant development at levels comparable to those obtained with the synthetic Hoagland nutrient solution (HOAG), across key vegetative parameters, including plant height, leaf production, chlorophyll content, and tillering (Fig. 3). The absence of significant differences in fresh and dry biomass further indicates that microalgal biomass provides sufficient nutrients to support overall plant development, reinforcing its potential as an alternative fertilisation strategy.

In contrast, the initial reduction in performance observed under the supernatant treatment (S) suggests that its higher electrical conductivity and nutrient composition may impose transient constraints on early plant development (Zhang et al., 2025). However, the progressive recovery observed over time indicates a clear capacity for plant acclimation, ultimately leading to comparable growth outcomes at later stages. This adaptive response suggests that early limitations did not translate into long-term restrictions in biomass accumulation, supporting the potential of both biomass and supernatant fractions as viable inputs in sustainable cultivation systems.

Importantly, both B0.5 and S treatments appeared to favour the transition from vegetative to reproductive development. This was reflected in the reduced investment in vegetative structures, such as leaf number and tillering, at later growth stages, without compromising overall plant performance (Fig. 3). Interestingly, the higher number of leaves and tillers observed in the synthetic fertiliser (HOAG) at the end of the experiment does not necessarily represent an agronomically favourable outcome, as plants were already in the reproductive phase. During grain filling, it is expected that resources are redirected towards spike development, with a reduction in vegetative structures such as tillers, as observed in the B0.5 and S treatments (García del Moral et al., 2024). In line with this, the higher number of spikes per plant observed under B0.5 compared to the supernatant highlights the greater effectiveness of microalgal biomass in promoting reproductive development. Visual observations (Fig. 4) further support this interpretation, with microalgal-treated plants exhibiting fewer green leaves but more developed stems and spikes, consistent with a more advanced reproductive stage. This response suggests that these treatments did not delay, and may even have accelerated, the progression towards grain formation, potentially contributing to a more efficient cultivation cycle. The absence of significant differences in both fresh and dry biomass across treatments indicates that *C. amblystomatis*-based applications can sustain plant productivity at levels comparable to the conventional Hoagland solution (Fig. 5). This suggests that microalgal biomass can provide sufficient nutrients to maintain plant growth without compromising overall biomass accumulation. Moreover, the similar performance observed under the supernatant treatment, despite its initially reduced vegetative growth, indicates that early-stage constraints did not result in long-term limitations to plant development.

These patterns were further reflected at the reproductive level. The B0.5 treatment resulted in a higher number of spikes per plant, representing an increase of 75.7%, together with increased spike length (24.5%) and fresh weight compared to the Hoagland

synthetic fertiliser. Although no significant differences were observed in individual spike dry weight among treatments, the greater spike number under B0.5 translated into biomass production per unit area comparable to the control and significantly higher than the supernatant treatment, representing an increasing of 85.3%. In contrast, the reduced spike formation observed under S limited its overall production, despite similar dry biomass at the individual spike level. This contrasts with the findings of Ferreira et al., (2023), in which the application of *Tetradesmus obliquus* biomass cultivated in swine wastewater did not provide advantages over the Hoagland solution for wheat growth. In that study, positive effects were only observed when either the whole culture (biomass plus supernatant) or the supernatant alone was applied, particularly in terms of tiller number and length, as well as leaf development.

Collectively, these findings indicate that *C. amblystomatis* biomass can sustain plant growth while promoting efficient reproductive development, highlighting its potential as a viable alternative to synthetic fertilisation strategies within circular agricultural systems.

Moreover, the growing intensification of agricultural activities has led to a higher risk of soil contamination due to accidental spills of fuels and lubricants, while the improper disposal of used cooking oil remains a relevant environmental concern, contributing to soil and water pollution (Filho et al., 2017).

Hydrophobicity reflects the ability of the microalgal cells to adhere to hydrophobic substrates, facilitating direct contact with the contaminants, whereas emulsification capacity is associated with the production of extracellular biosurfactants that promote the dispersion of these compounds in the aqueous phase (Lima e Silva et al., 2024). *C. amblystomatis* biomass exhibited predominantly hydrophobic behaviour towards most of the tested hydrocarbons, showing high relative hydrophobicity (RH) towards agricultural diesel (88.91%), used cooking oil (sunflower) (83.12±1.14%), and tractor hydraulic oil (70.43±5.05%), indicating a strong affinity for these substrates. In a previous study by Ferreira et al., (2024), the potential of the microalga *Tetradesmus obliquus* cultivated in both synthetic medium and swine wastewater was evaluated. Biomass grown in synthetic medium exhibited a higher affinity for all tested compounds, with average hydrophobicity (HR) values of 84.4% for toluene, 72.5% for diesel oil, and 65.3% for used motor oil. In contrast, biomass cultivated in swine wastewater showed a predominantly hydrophilic to slightly hydrophobic character, indicating a lower capacity for interaction with the tested oils. In the present study, the HR values obtained for *C. amblystomatis*, particularly for agricultural diesel, were higher than those reported for the oils tested with *T. obliquus*. This enhanced hydrophobicity may be associated with differences in cell surface properties and biochemical composition, including lipid and protein fractions, which are known to influence cell–hydrocarbon interactions and adsorption capacity. Such characteristics can promote stronger adhesion to hydrophobic contaminants, facilitating their removal from contaminated matrices (Sachdev & Cameotra, 2013; Abdelfattah et al., 2022).

The spontaneous emulsion formation in water may be explained by the nature of these samples, as they consist of used lubricating oils from different tractor components, which can contain additives and degradation products that enhance their intrinsic emulsification capacity. The emulsification assays showed the best performance with agricultural diesel and used cooking oil, with EI_{24}/EI_5 ratios of 0.92 and 0.87, respectively, both exceeding those obtained with SDS, demonstrating that *C. amblystomatis* can enhance the dispersion of hydrophobic compounds in aqueous systems, supporting its potential application in the treatment of both agricultural and organic oil-contaminated environments (Alfatih et al., 2022). Interestingly, the good performance observed for used cooking oil in both assays highlights the versatility of this microalga, demonstrating its ability to interact with both petroleum-derived hydrocarbons and biodegradable, food-derived oils (Roy et al., 2024).

These findings highlight the potential of *C. amblystomatis* cultivated in brewery wastewater as an effective and sustainable agent for the bioremediation of hydrocarbon-contaminated soils, reinforcing its applicability within integrated wastewater valorisation and environmental remediation strategies (Dini et al., 2024; Roy et al., 2024).

Conclusions

This study demonstrated that biomass application, particularly at 0.5 $g \cdot L^{-1}$, was able to sustain plant growth at levels comparable to the synthetic Hoagland nutrient solution, while enhancing key reproductive parameters, including spike number, length, and fresh weight. Although germination responses were cereal-dependent and not always statistically significant, the observed patterns, especially in barley and rye, suggest a potential biostimulant effect at 0.5 $g \cdot L^{-1}$ biomass concentrations. The observed responses highlight the importance of optimising application parameters, particularly biomass concentration and crop specificity, to ensure consistent biostimulant performance. From an applied perspective, the direct use of microalgal biomass generated during wastewater treatment offers a practical approach for nutrient recovery and reuse, reducing reliance on synthetic fertilisers while contributing to the valorisation of agro-industrial effluents. In parallel, the biomass exhibited strong hydrophobicity and emulsification capacity, indicating that *C. amblystomatis* can interact with a broad range of hydrophobic substrates, including agricultural oils and used cooking oil, suggesting the involvement of complementary mechanisms in hydrocarbon binding and dispersion, and reinforcing its potential in bioremediation applications. Although still underexplored, microalgae-based remediation of hydrocarbon-contaminated environments emerges as a promising and scalable strategy. Overall, this work supports the implementation of circular bioeconomy principles within the brewing industry, where microalgal biomass produced from brewery wastewater can be reutilised for the cultivation of cereals used in beer production, while simultaneously contributing to environmental remediation. This integrated strategy highlights the potential of microalgae-based systems as scalable solutions for sustainable agriculture and pollution mitigation.

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