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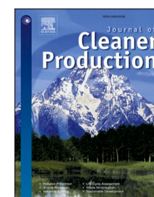

Evaluation of the biostimulant effects of two Chlorophyta microalgae on tomato (*Solanum lycopersicum*)

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ABSTRACT

Eukaryotic microalgae from the *Chlorophyta* division are used in various bio-industries due to their ability to produce high value compounds. Some of these compounds show plant biostimulant properties when applied to plants, soil or growth medium in hydroponic chambers. The first objective of this study was to evaluate if *Chlamydomonas reinhardtii* cc 124 and *Chlorella* sp. MACC-360 had biostimulant effect on *Solanum lycopersicum* L. The second objective was to investigate the importance of the application mode and time. The third goal was to reveal strain-specific actions of the two algae strains. Tomato plants were grown in pots layered with clay at the bottom and filled with the mixture of soil and vermiculate. In two sets of experiments the soil and plant leaves were treated with living algae and algal extract, respectively. In the first set, the culture suspension (CS) was centrifuged, the algae pellet was re-suspended in water (CCS), and this was applied weekly to soil, while algae extract (cell disrupted algae suspension – CDS) was sprayed on leaves bi-weekly. The flowering process, plant morphology, fruit features and pigment contents were analyzed. In the second set of experiments, the culture suspension per se (CS) was applied to the soil weekly and CDS was sprayed on leaves bi-weekly. Flowering kinetics, reproductive capacity and photosynthetic parameters were examined. Both algae strains increased pigment content, fruit weight and fruit diameter of tomato. Plants that received initial algae treatment at an advanced age performed better than those initially treated at a young age. *Chlorella* induced early flowering and fruit development while *Chlamydomonas* significantly delayed these milestone functions. *Chlorella* promoted conversion of light energy to chemical energy, while *Chlamydomonas* enhanced protection of photosynthetic parameters. Both strains increased leaf temperature differential as well as leaf thickness. Overall, both algae strains stimulated important agronomic-valuable functions in tomato.

1. Introduction

The ever-growing human population has tremendously increased global food demand. Thus, crop producers are under immense pressure to increase food production at all costs. In the past few years, use of chemical fertilizers and pesticides in agriculture has substantially increased the food supply. Unfortunately, it has come with grave repercussions such as environmental pollution, disruption of natural ecosystems, loss of diversity and even dire effects on human health (Chiaiese et al., 2018; Cooper and Dobson, 2007; Fenner et al., 2013). Thus, there is an urgent need to replace chemical fertilizers and pesticides with eco-friendly options which will improve crop yields and nutritional value amidst climate change in what is termed as 'climate-smart-agriculture' (Campbell et al., 2014).

Biofertilizers and biostimulants extracted from seaweeds and plants have been proposed as alternatives to chemical fertilizers because they enhance crop performance (Battacharyya et al., 2015; Calvo et al., 2014; Fayzi et al., 2020; Hamed et al., 2018; Kavipriya et al., 2011; Ronga

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et al., 2019). Nonetheless, seaweed sources may get depleted with continuous collection while plants require land for production and take a long time to harvest. Microalgae (MA) represent a better alternative because they grow rapidly, produce a whole range of bioactive compounds and do not compete with food crop for cultivation land/space (Abdel-Raouf, 2012; Chiaiese et al., 2018; Colla and Rouphael, 2020; Lee and Ryu, 2021; Renuka and Guldhe, 2018; Ronga et al., 2019). Besides, MA cultivation for biofertilizers using wastewater would offer a cheap sustainable way of water recycling (Pavliukh et al., 2020; Rana et al., 2016; Wuang et al., 2016). The use of microalgae as biostimulants/biofertilizers in farming is still at its infancy because strategies of processing and applying algal material are yet to be developed and standardized.

Numerous research studies have shown the potential of algae, both prokaryotic and eukaryotic, to improve crop production (Lee and Ryu, 2021; Priyadarshani and Rath, 2012). Mostly, the algal biomass is harvested and processed to liberate bioactive compounds such as polysaccharides and hormone-like substances from the cells for plant or soil applications (El-Naggar et al., 2020; Raposo et al., 2013; Rachidi et al., 2021). The cell disruption may require special technology such as the "cell-bursting" technique and chemicals such as formalin for conservation (Stirk and Van Staden, 2006). These requirements make the entire process laborious, time-consuming, and expensive. The algae products are usually applied as foliar treatment, soil drench or seed/cuttings priming. In some studies, the algal-derived substances are injected into the plant tissues (Rachidi et al., 2021). Foliar application is the most popular method for extract application as phytohormones are best absorbed via the stomata of leaves (Brain et al., 1973). In contrast, soil drench method is the most preferred for application of living algal cells because they can multiply and alter soil properties and microbial communities through continuous release of bioactive compounds. Thus, some bottleneck in application of MA in agriculture are the absence of a universal extract preparation procedure, lack of knowledge of the best time and method for application as well as lack of knowledge of strain-specific effects of MA. To make MA use in agriculture feasible, these obstacles must be overcome, for example by cutting down the steps of algae processing before application to plants. Thus, the application of living cells could be one of the convenient options. If cell disruption must be done, no chemicals should be added to maintain sustainable production.

Tomato is one of the most cultivated crop plants belonging to the *Solanaceae* family with a global economic and nutritional value. Tomato is among the world's most consumed vegetable; it can be consumed in salads, soups, purees, sauces, pastes etc. Apart from the pleasant flavor, it is mostly consumed for its nutritional, nutraceutical, and antioxidant content (Giudice et al., 2017). Tomatoes are easy to propagate which makes them indispensable in meeting the global food demand and ensuring food security (Supraja et al., 2020a). Unlike other crops, the tomato plant can grow in almost all soil types, but it has high nutritional demands. The quality of crop/fruit is highly dependent on nutrition. Biostimulants, substances which enhance plant growth, crop performance, yields and quality when applied at minute concentrations could reduce the costs of tomato farming, especially in low fertility soils.

Several accounts of the biostimulant action of *Chlorella* strains on the tomato plant exist (Barone et al., 2019a, 2019b; Coban et al., 2020; Zhang et al., 2017; Özdemir et al., 2016). More than three-quarters of these studies apply cell extracts rather than algal suspensions containing living cells to plants/soil-systems. On the contrary, very few reports of *Chlamydomonas reinhardtii*'s growth promotion exist and even the few accounts involve plants such as maize (Martini et al., 2021). *Chlorella* strains are robust and can withstand a whole range of environmental conditions while the latter is a benchmark strain which has been thoroughly characterized. Thus, these two strains are good *Chlorophyta* representatives for MA biostimulant studies.

This study aimed at evaluating the efficacy of two strains of green MA from two genera as biostimulants on tomato under controlled conditions (without stress). Living cells suspended in distilled water (CCS) as well as culture suspensions (CS = living cells plus spent media) were applied via soil drenching. Algal extracts (CDS) prepared by crushing the cells under liquid nitrogen and resuspension of the slurry in water were also applied by foliar spraying. The first objective of this study was to elucidate whether soil treatment with living cells (CCS and CS) coupled with extract foliar spray stimulated plant growth. The second objective was to identify if there was a difference between applications of algae to plants commencing at a young age or at a later stage (just about to flower). The third objective was to discover any strain-specific effects of the MA on tomato plants based on morphological (plant height, diameter), reproductive capacity (flower and fruit development) and physiological response (photosynthetic activity) investigations.

2. Materials and methodology

2.1. Strain selection and cultivation

Chlamydomonas reinhardtii cc 124 was obtained from the collection of the Institute of Plant Biology of Biological Research Center (Szeged, Hungary) and *Chlorella* sp. MACC-360 from the Mosonmagyaróvár Algal Culture Collection (MACC, Mosonmagyaróvár, Hungary). *Chlamydomonas reinhardtii* cc 124 is a well-studied model green algae, while *Chlorella* sp. MACC-360 strain was chosen based on its fast growth rate and high biomass accumulation capability.

Confocal and scanning microscopy was conducted to characterize the strains' ability to release extracellular polysaccharides (EPS) according to a previous protocol (Gitau et al., 2021). Algal fresh weight was determined by subtracting the weight of an empty falcon tube from the weight of the tube plus algae pellet retained after centrifugation and medium elimination.

The algae strains were cultivated in Tris-Acetate-Phosphate (TAP) media, at pH 7 (Harris, 1989). Algae biomass was scrapped off the surface of a TAP-Agar plate with a sterile rod and transferred into a 50 mL Erlenmeyer flask containing 15 mL of TAP media. The flasks were incubated in an algae growth chamber with the following conditions: 25 °C, 16:8 h light:dark cycle, 50 μmol photons m^{-2} s^{-1} light intensity, and continuous shaking at 180 rpm. After 5 days, 5 mL of the culture was transferred into a 100 mL Erlenmeyer flask containing 50 mL TAP media and placed in the growth chamber with the aforementioned conditions. The cultures of each strain were left to grow for 7 days. On the 7th day, 5 mL of the culture was transferred into a 250 mL Erlenmeyer flask containing 50 mL of TAP media to start a fresh culture for the next application. The remaining 50 mL was used to prepare the algae for plant treatment.

2.2. Preparation of the algae for plant treatment

Culture suspension from the 100 mL Erlenmeyer flasks were transferred into pre-weighed 50 mL tubes on day 7 and centrifuged at 4600 rpm for 15 min. The supernatant was discarded, and the pellets containing living cells were re-suspended in 50 mL of sterile distilled water (DW). The suspension was centrifuged again at 4600 rpm for 15 min and the supernatant was discarded. The fresh biomass of the harvested algae was determined by measuring the tubes containing the pellet and subtracting the weight of the empty tube. To prepare the soil drench treatment, the pellet was re-suspended again in sterile DW at a concentration of approximately 1 g/L to make the living cell treatment (CCS). The control was 1 L of sterile DW.

For experiments in which plants were treated with culture suspension (CS = living cells plus spent media), 50 mL suspension from the 100 mL Erlenmeyer flask was diluted to make a final volume of 1 L with approximately 1 g/L algal biomass.

To prepare the water extract of algae (CDS) for foliar treatment, 50 mL of culture was centrifuged at 4600 rpm for 15 min. The supernatant

was discarded, algal fresh biomass determined, and the pellet transferred into a mortar and frozen with liquid nitrogen. When thawing began, the pellet was crushed with a pestle to disrupt the cells and make a slurry. For the CCS experiments, this slurry was then diluted with DW to make a final volume of 300 mL with the concentration of approximately 3 g/L algal biomass. For CS experiments, the slurry was made up to 5 mL with the recovered supernatant then diluted to 300 mL with DW to make a cell extract with approximately 3 g/L algal biomass. This cell extract was transferred into spray bottles, a separate bottle for each MA strain. The control was 300 mL of DW for CCS experiments. The controls were DW and TAP (5 mL) for CS experiments, the latter was diluted with water to 300 mL.

2.3. Detection of auxin content in tryptophan-enriched cultures

Starter cultures of each strain were inoculated and grown for 5 days in TAP media. Ten mL of 5 day old cultures were used for inoculation into 25 mL TAP supplemented with 1 g/L tryptophan. Two replicates (flasks) were prepared for each strain and placed in the incubator with the aforementioned conditions. The cultures were grown for 7 days and indole acetic acid (IAA) presence was determined by colorimetry using Salkowski reagent (Gang et al., 2019). Briefly, the cultures were centrifuged and 1 mL of the supernatant mixed with 1 mL of Salkowski reagent and the mixture incubated for about 30 min in the dark at 30 °C to allow color development. The absorbance at 536 nm was then measured using a spectrophotometer. Quantification against a standard curve prepared with IAA was done.

2.4. Experimental design

Two sets of experiments were conducted (Supplementary Table 1). The first set was conducted between February and July 2020. In these experiments, living cells (CCS) were applied by soil drench method and cell extracts (CDS) by foliar spray. Under this category, young plants received the first soil drench treatment on the first week (Fig. 1. A, Left) and older plants on the fifth week (Fig. 1. A, Right) of growth. Both sets of plants received foliar treatment from the fifth week onwards. Control plants were treated with DW.

The second set of experiments was conducted between June and September 2021. Culture suspensions (CS = living cells plus spent media) were used for soil treatments. First soil drench treatment was applied on one-week-old plants while foliar treatment with extracts (CDS) was initiated on the fifth week of growth. Thus, this set of experiments falls under the week 1 regime but with the additional TAP media control (Fig. 1. B).

In both experiments, soil drench treatments were done weekly while foliar sprays were initiated on week 5, done bi-weekly and terminated on the 12th week for both experiments.

2.5. Plant establishment and treatment

Solanum lycopersicum L. seeds of Vilma variety purchased from a retailer in Szeged, Hungary, were used for the studies. Plants were surface sterilized with 10% hypochlorite solution for 5 min and then thoroughly washed with sterile DW. The seeds were allowed to imbibe

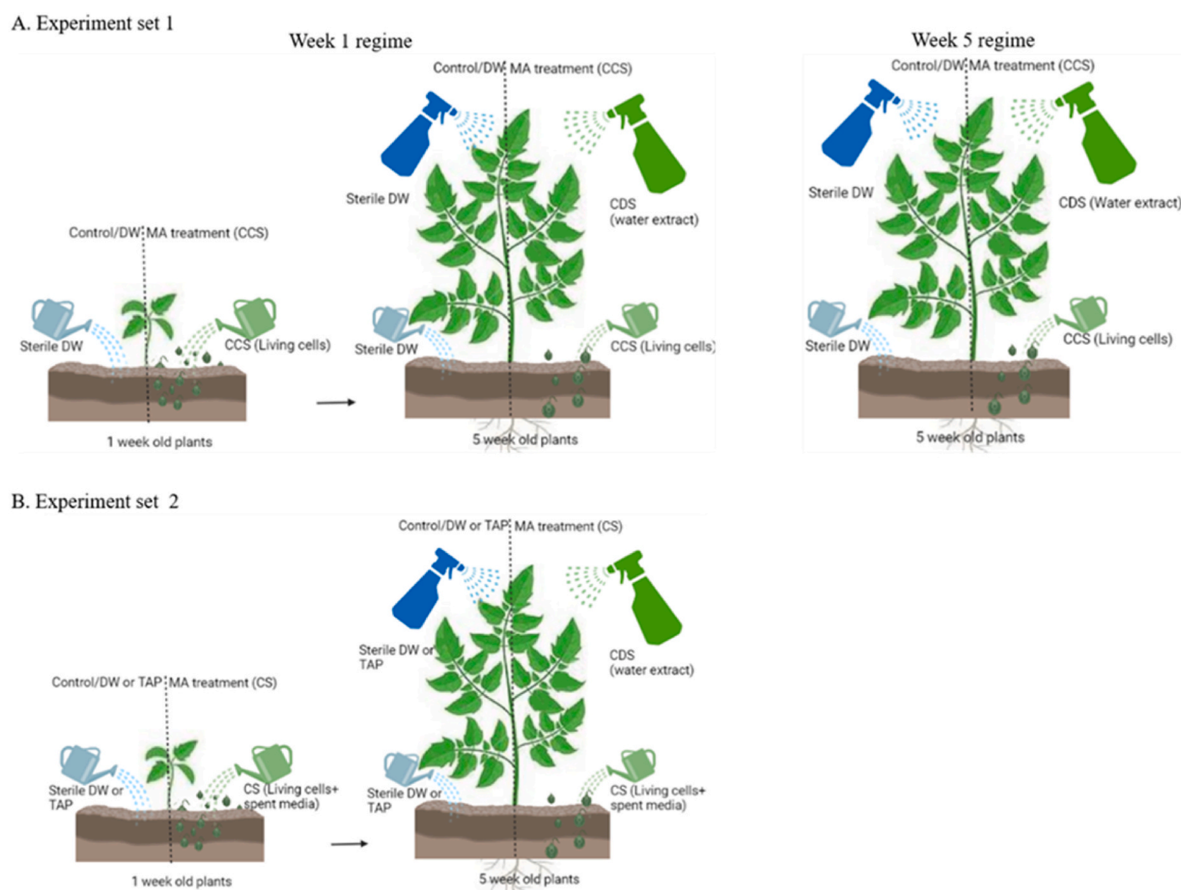

Fig. 1. Experiment sets; Panel A shows treatment of plants with algal living cells. Left figure shows week 1 regime where the initial application of algae (CCS) was given to 1 week old young plants in the form of soil drench method while extracts (foliar treatment) was initiated before flowering, when the plants were 5 weeks old. Right figure shows week 5 regime where both soil drench and foliar treatment were initiated at the same time to 5 week old plants. Panel B shows treatment of plants with living algal cells and the accompanying spent media (CS). Plants received the first soil drench treatment on the first week while foliar spray only started on the fifth week. Image was created with BioRender.com.

water for about 2 h. The seeds were then sown on a 12-well germination box containing moist soil and vermiculate in the ratio of 2:1. The seeds were germinated and maintained in the greenhouse in this platform for 2 weeks.

Seedlings were then transplanted into 3 L pots (2 seedlings per pot) containing moist soil and vermiculate (2:1) which was moistened with a Solution 1 diluted 40 times. Solution 1 was prepared from the stock solution with the following nutrient solutions: KNO_3, 15 mM; Ca $(NO_3)_2$ $4H_2O$, 12.5 mM; $Ca(H_2PO_4)_2$, 1 mM; $MgSO_4$ $7H_2O$, 1 mM; Fe EDTA, 0.01 mM; $MnCl_2$, 0.004 mM; H_3BO_3, 0.02 mM; $ZnSO_4$ $7H_2O$, 0.0004 mM; $NaMoO_4$, 0.0001 mM; and $CaSO_4$ $5H_2O$, 0.0001 mM. Plants were grown in the greenhouse at 24 °C–26 °C and with a 16-h photoperiod. Each treatment had a total of 12 plants: 2 plants per pot, 6 pots per treatment. There were 3 pots placed on a tray and a treatment included 2 trays. The trays were arranged in a randomized block design and the position on the bench constantly changed to ensure uniform exposure to environmental factors. The soil and plants were treated according to the experimental design.

2.6. Phenotyping

2.6.1. Plants treated with CCS (living cells without spent media as soil drench) and (CDS) extracts as foliar spray)

On the onset of flowering, the number of open flowers per plant in each treatment was recorded per day. This data was used to visualize the flowering kinetics.

When the first set of the crop had completely ripened, fruits were harvested. The fruit number, fruit diameter, fruit weight and total yields per plant were determined.

Leaves collected from the top, middle and bottom of the plants were used to make a homogenous sample for pigment extraction and determination. About 0.1 g of fresh leaf material was placed in a test tube and 10 mL of 80% acetone was added. The tubes were placed in a water bath set at 60 °C for 30 min and cooled in ice. 200 μl of the extract was transferred into 96-well plates and absorbance values were measured with a HIDEX plate reader. The amounts of chlorophylls were calculated according to Arnon equations and the formula for carotenoids was adopted from Lichtenthaler et al. equation specific for acetone extracts (Lichtenthaler, 1987; Manolopoulou, E., Varzakas, T. and Petsalaki, 2016).

2.6.2. Plants treated with CS (living cells plus spent media as soil drench) and CDS (extracts as foliar spray)

Flowering data (open flowers per day per plant) was recorded during the first week of flowering.

The number of trusses per plant, open flowers per truss, bearing trusses per plant and number of fruits per truss was recorded on the 50th, 60th and 70th day after planting (DAP).

Fluorescence-based measurement of photosynthetic parameters were taken on plant leaves on a weekly basis with the Multispeq handheld device (Kuhlgert et al., 2016). This data was recorded for 5 weeks beginning at the first week after transplantation.

2.7. Statistical analysis

The collected data were tested for normality and homoscedasticity. Data sets that passed these tests were analyzed with Two-way ANOVA. Multiple *t*-test was used for multiple comparisons. Mann-Whitney test was used to analyze data sets that failed normality and homoscedasticity tests. Data sets in tables showing the dates and numbers of flowers/fruits (Flowering and fruiting) were fitted to non-linear model and the results used in One-way ANOVA to infer significant differences. The alpha 0.05 was used to indicate significant differences. All statistical analyses were executed with GraphPad Prism 8.

3. Results

3.1. Composition of algae CS and CCS (cell suspension per se and living cells without spent media, respectively)

Microscopy of cells grown in liquid TAP media revealed interesting differences between the two green algae strains with respect to size and cellular composition. *Ch. reinhardtii* cc124 cells (Fig. 2 A and E) are about 3.5 x larger than *Chlorella* sp. MACC-360 cells (Fig. 2B and F) (Lakatos et al., 2017). *Ch. reinhardtii* cc124 cells also do not stain with Calcofluor White (CWF) while *Chlorella* sp. MACC-360 do. Moreover, the green fluorescence from Concanavalin A (Con A) is localized in a spot inside the *Ch. reinhardtii* cc124 cells while it appears in the extracellular matrix embedding the *Chlorella* sp. MACC-360 cells. This implies to the different cellular compositions of the applied MA strains.

Chlorella sp. MACC-360 also forms cellular aggregations while *Ch. reinhardtii* cc124 does not (Fig. 2 A versus B and E versus F). The culture (Fig. 2B) and supernatant (Fig. 2D) of *Chlorella* sp. MACC-360 are stained by Con-A showing that the cells are embedded in a matrix containing β-glucans which is lost upon centrifugation and resuspension in water. The supernatant showed a strong signal of Con-A implying the presence of EPS (Fig. 2D).

These results clearly showed that the application of CCS (living cells) to plants did not immediately avail EPS to plants. In contrast, application of the CS (living cells and their spent media/supernatant) would immediately supply EPS material to the soil for the case of *Chlorella* sp. MACC-360**.** Since green fluorescence was localized in the cells for *Ch. reinhardtii* cc124, destruction of the cells is necessary to release the polysaccharides into the media. Therefore, it is crucial to point out that foliar spraying delivered polysaccharides of both strains while only the soil-drench treatment delivered polysaccharides from *Chlorella* sp. MACC-360. Nevertheless, *Chlorella* sp. MACC-360 displayed a profuse polysaccharide biosynthesis while that of *Ch. reinhardtii* cc124 is scanty.

3.2. Auxin biosynthesis by the selected microalgae

Both strains tested positive for auxin production. The color of their supernatants turned pinkish on addition of the Salkowski reagent (Fig. 3). However, upon quantification, *Ch. reinhardtii* cc124 had higher IAA content than *Chlorella* sp. MACC-360.

Ch. reinhardtii cc124 produced approximately double the amount of IAA produced by *Chlorella* sp. MACC-360. This indicated that the two strains produced different hormone quantities and this phenomenon could be similar for other hormones not determined in our study.

3.3. Effect of MA living cells (CCS + CDS) versus cells plus spent media (CS + CDS) on flowering kinetics

When applied in the form of living cells suspended in water as soil drench and extracts as foliar spray, *Chlorella* sp. MACC-360 not only induced early flowering but maintained the highest number of open flowers during the first week of flowering. In contrast, *Ch. reinhardtii* cc124 delayed flowering and registered the least number of open flowers relative to the control (Fig. 4a and Supplementary Fig. 1). A significant difference between the effects of the two applied MA species was observed (Fig. 4b).

Chlorella sp. MACC-360, TAP and *Ch. reinhardtii* cc124 treated plants maintained a higher number of open flowers than control during the first week of flowering if MA was applied as CS (Fig. 4c). The difference between *Chlorella* sp. MACC-360 and either TAP or *Ch. reinhardtii* cc124 was significant at alpha $p = 0.05$. *Ch. reinhardtii* cc124 was not different from both TAP and DW control (Fig. 4d). The application of *Chlorella* sp. MACC-360 in all forms promoted flowering although the significant influence relative to control was observed with CCS application. In contrast, application of *Ch. reinhardtii* cc124 only promoted flowering when applied in the form of CS (living cells plus spent media) Fig. 4b but

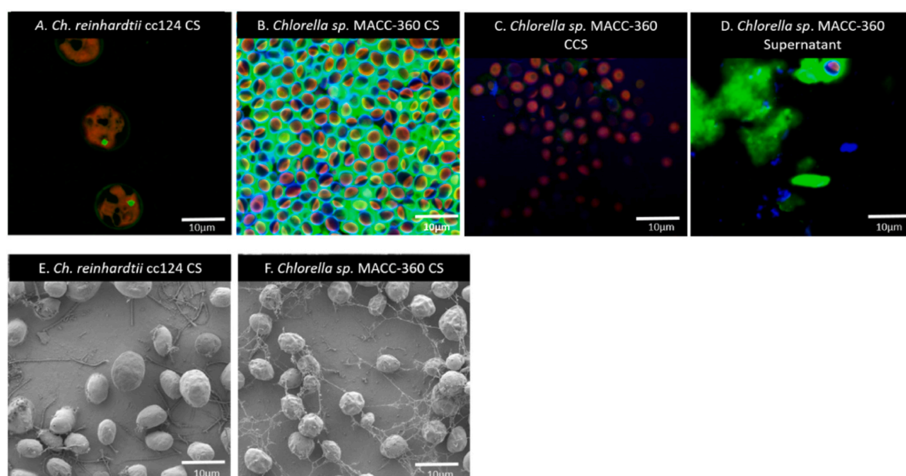

Fig. 2. Confocal microscopy (A–D) and scanning microscopy (E and F) pictures of the microalgae: A. *Ch. reinhardtii* cc124 CS, B. *Chlorella* sp. MACC-360 CS, C. *Chlorella* sp. MACC-360 CCS, D. *Chlorella* sp. MACC-360 supernatant/spent media, E. *Ch. reinhardtii* cc124 CS at 2000x magnification and F. *Chlorella* sp. MACC-360 CS at 5000x magnification. In all cases, CS refers to the living cells plus the growth media; CCS refers to the living cells without spent media (the pellet of centrifuged culture re-suspended in water) and spent media/supernatant is the media utilized by the cells for growth. The Red channel shows chloroplast autofluorescence, blue channel is Calcofluor White (CFW) dye staining the cell wall while the green channel is Concanavalin **(Con-A) staining the EPS.**

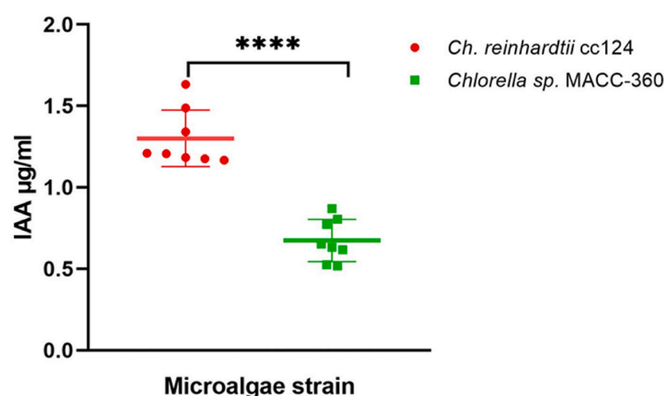

Fig. 3. Auxin levels released by the microalgae, *Ch. reinhardtii* cc124 and *Chlorella* sp. MACC-360 in TAP media supplemented with L-tryptophan. The graph is a scatter plot with all individual values from technical replicates of samples drawn from two different flasks per treatment. The long horizontal line shows the mean while the bars show standard deviation (SD). Asterisks show significant difference between the two strains at p = 0.005 based on a two-tailed unpaired *t*-test.

delayed flowering when applied as CCS (living cells) (Fig. 4 a).

3.4. *Effect of MA living cells (CSS as soil drench) and extracts (CDS as foliar spray) on plant morphology and fruits*

Treatment of well-grown plants (Week 5) with either of the microalgae strains reduced plant height, increased plant diameter, fruit number, fruit diameter, fruit weight and pigment content compared to the control treatment (Fig. 5 a-i). Algae treatment did not affect fruit yields. *Chlorella* sp. MACC-360 significantly reduced plant height and increased both fruit weight, chlorophyll-b and carotenoids relative to control (Fig. 5a, f, 5h and 5i). *Ch. reinhardtii* cc124 significantly increased only chlorophyll-a content relative to the control (Fig. 5g).

Treatment of juvenile plants (Week 1) with either *Chlorella* sp. MACC-360 or *Ch. reinhardtii* cc124 algae strains slightly increased plant height, did not affect plant diameter, and slightly increased both fruit diameter and fruit weight as well as all the pigment contents (Fig. 5 a, b, e, f, g-i). *Chlorella* sp. MACC-360 slightly increased yields and hardly affected fruit number (Fig. 5 c and d). In contrast, *Ch. reinhardtii* cc124 reduced the fruit number and yields (Fig. 5 c and d). Significant differences between groups were observed only for fruit diameter. Both MA strains significantly increased fruit diameter although *Ch. reinhardtii* cc124's effect was stronger than that of *Chlorella* sp. MACC-360 (Fig. 5e).

Overall, both treatment and age of plants at first application significantly affected fruit diameter ($p = 0.0002$ and 0.047 respectively) with treatment explaining a higher percentage of variation than the age or their interaction. However, only treatment alone affected fruit number ($p = 0.035$) significantly, even without significant differences between treatments within regimes, while time of first application alone affected plant diameter ($p < 0.0001$). Of all the parameters, the interaction between treatment and age of plants during the first algae application only significantly affected plant height ($p = 0.027$) (Table 1).

The age of plants at the time of application significantly affected fruit weight ($p = 0.003$) and pigment content; chlorophyll-a ($p = 0.0397$), chlorophyll-b ($p = 0.0007$) and carotenoids ($p = < 0.0001$). Effects on carotenoids and chlorophyll-b were the most evident (Supplementary Table 2).

3.5. *Effects of microalgae CS (living cells plus spent media as soil drench) and extracts CDS (foliar spray) on reproductive capacity of tomato*

Chlorella sp. MACC-360-treated plants maintained the highest number of trusses at any scoring time. This was closely followed by plants treated with TAP and *Ch. reinhardtii* cc124 while the DW-treated plants had the lowest number of trusses (Fig. 6a). *Chlorella* sp. MACC-360-treated plants had the highest number of flowers at 50DAP (day after planting) but the least at 60DAP and 70DAP (Fig. 6b). The number of open flowers declined from the 50DAP to the 70DAP for all plants except for the DW control where flowering continued steadily between 60 DAP and 70 DAP (Fig. 6b).

Chlorella sp. MACC-360-treated plants had the highest number of bearing trusses at the 50DAP and 60DAP while the control had the least, at all scoring times (Fig. 6c). TAP/control and *Ch. reinhardtii* cc124 treatments showed a similar trend where the number of bearing trusses increased throughout the scoring time. On the 70DAP, differences between groups were obvious with TAP and *Ch. reinhardtii* cc124-treated plants being significantly different from the control. *Chlorella* sp. MACC-360-treated plants did not significantly differ from any of the treatments at this stage despite having a higher number of trusses than control (Fig. 6c).

All treatments had a higher fruit number per truss in comparison to the DW control on 50DAP (Fig. 6d). However, this difference leveled off in the next 20 days although *Chlorella* sp. MACC-360-treated plants continued to have slightly more fruits than DW control plants. In contrast, TAP and *Ch. reinhardtii* cc124-treated plants ended up with fewer fruits per truss than DW control plants (Fig. 6d).

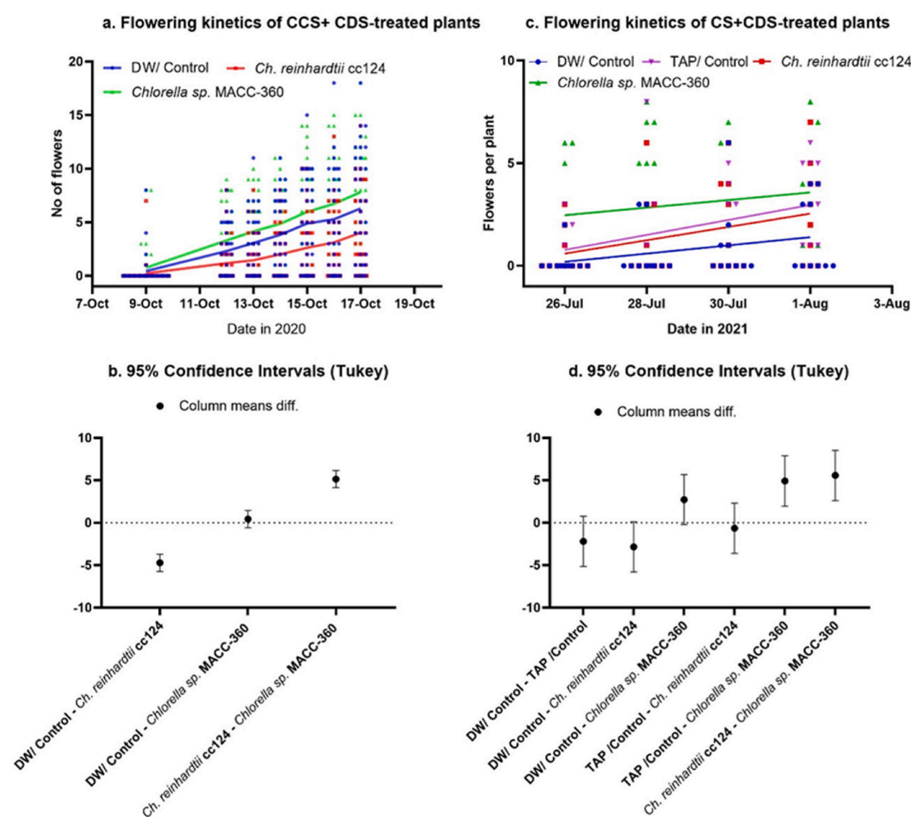

Fig. 4. Flowering kinetics of the tomato plants during the first and second weeks of flowering a) shows the scatter plots of individual counts per plant for Experiment set 1, Week 1 regime; plants treated with CSS (living cells) and CDS (water extracts) as soil drench and foliar spray respectively; b) shows a graph of 95% confidence intervals to show significant differences among treatments based on One way ANOVA test; c) shows the scatter plots of individual counts per plant for Experiment set 2; plants treated with CS (living cells plus spent media) and CDS (water extracts) as soil drench and foliar spray respectively, during the second week of flowering and d) shows a graph of 95% confidence intervals to show significant differences among treatments based on One way ANOVA test. The treatments are distilled water (DW)/ Control, *Ch. reinhardtii* cc124, *Chlorella* sp. MACC-360 and Tris-Acetate-Phosphate (TAP) media used for MA cultivation.

A Two-way ANOVA indicated that for all the parameters, except the number of bearing trusses, microalgae treatment explained the highest percentage of observed variation (Table 2). Flowering and fruiting in this case represent data collected on daily basis since the initiation of flowering for the first 2 weeks. This data, therefore, shows the kinetics of flowering and fruit development and not the actual number of flowers or fruits.

Overall, treatment had a strong impact on the flowering ($p = 0.0002$) and fruit development, processes (Table 2). The time of scoring significantly affected flowering ($p = 0.007$) and number of bearing trusses ($p = < 0.0001$), because -obviously - plants that flowered earlier would have fruits earlier than those showing delayed flowering (Table 2). Although the number of open flowers might have been the same, some flowers could be newly opened, and others open for even two days. This parameter (flower number) therefore fails to show any obvious difference, which was successfully captured by examining the kinetics of flowering and fruit development.

3.6. *Effect of microalgae CS (cells plus spent media as soil drench) and CDS (extracts foliar spray) on photosynthesis*

Chlorella sp. MACC-360 increased FvP/FmP (maximum quantum yield), Phi2 (light energy directed to photosynthesis/quantum yield of photosystem II (PS II) and soil plant analysis development (SPAD) but reduced PhiNPQ (regulated non-photochemical quenching), PhiNO and LEF, relative to the DW control. In contrast, *Ch. reinhardtii* cc124 increased PhiNPQ and PhiNO but reduced FvP/FmP, Phi2 and SPAD relative to the control/DW. Both strains increased ql (open PSII reaction centers which indicates the fraction of quinone A (Q_A) in oxidized state) (Kramer et al., 2004)), leaf thickness and leaf temperature differential (change in leaf temperature relative to ambient temperature). TAP's effect showed a more or less similar trend as *Chlorella* sp. MACC-360 but at a negligible capacity (Fig. 7 a-i).

Significant differences among treatments occurred between the two MA strains for maximum quantum yield (F_v/F_m) and for energy loss via non-photochemical quenching (PhiNPQ) (Fig. 7 c and d). *Ch. reinhardtii* cc124 treatment also significantly reduced non-regulated non-photochemical quenching (PhiNO) relative to the control (Fig. 7b). Plants treated with *Chlorella* sp. MACC-360 had significantly thicker leaves than all the other treatments (Fig. 7h).

Chlorella sp. MACC-360 promoted photosynthesis and reduced energy loss by both regulated and unregulated means while keeping a high fraction of the Q_A in an oxidized state. In contrast, *Ch. reinhardtii* cc124 reduced light energy channeled to photosynthesis, increased energy lost via regulated means but decreased energy loss by non-regulated means while keeping a lower Q_A than observed for *Chlorella* sp. MACC-360-treated plants. These results show differential effect of the two algal strains on photosynthetic performance in tomato plant. Nevertheless, the only significant effect relative to control was *Chlorella* sp. MACC-360 influence on leaf thickness.

4. Discussion

Application of MA to tomato plants caused growth stimulation which manifested in the form of early and enhanced flowering, increased fruit weight and diameter, increased leaf pigment, increased reproductive capacity and influence on photosynthesis especially on leaf thickness. The selected strains used in our study were found to produce bioactive compounds, auxins and exopolysaccharides, which could be the main contributors of plant biostimulation. The strain-specific effects of the two MA on plants as well as the influence of plant age on biostimulant effect were observed.

Induction of flowering in plants by various biostimulants has been reported before (Arthur et al., 2003; Ibrahim et al., 1970; Plaza et al., 2018; Pohl and Grabowska, 2019a, 2019b). Although this phenomenon has been reported for *Chlorella* sp., no record for *Chlamydomonas* sp. exists. Decreased fruit number, increased fruit weight and diameter plus reduced fruit yields have been reported in tomatoes treated with

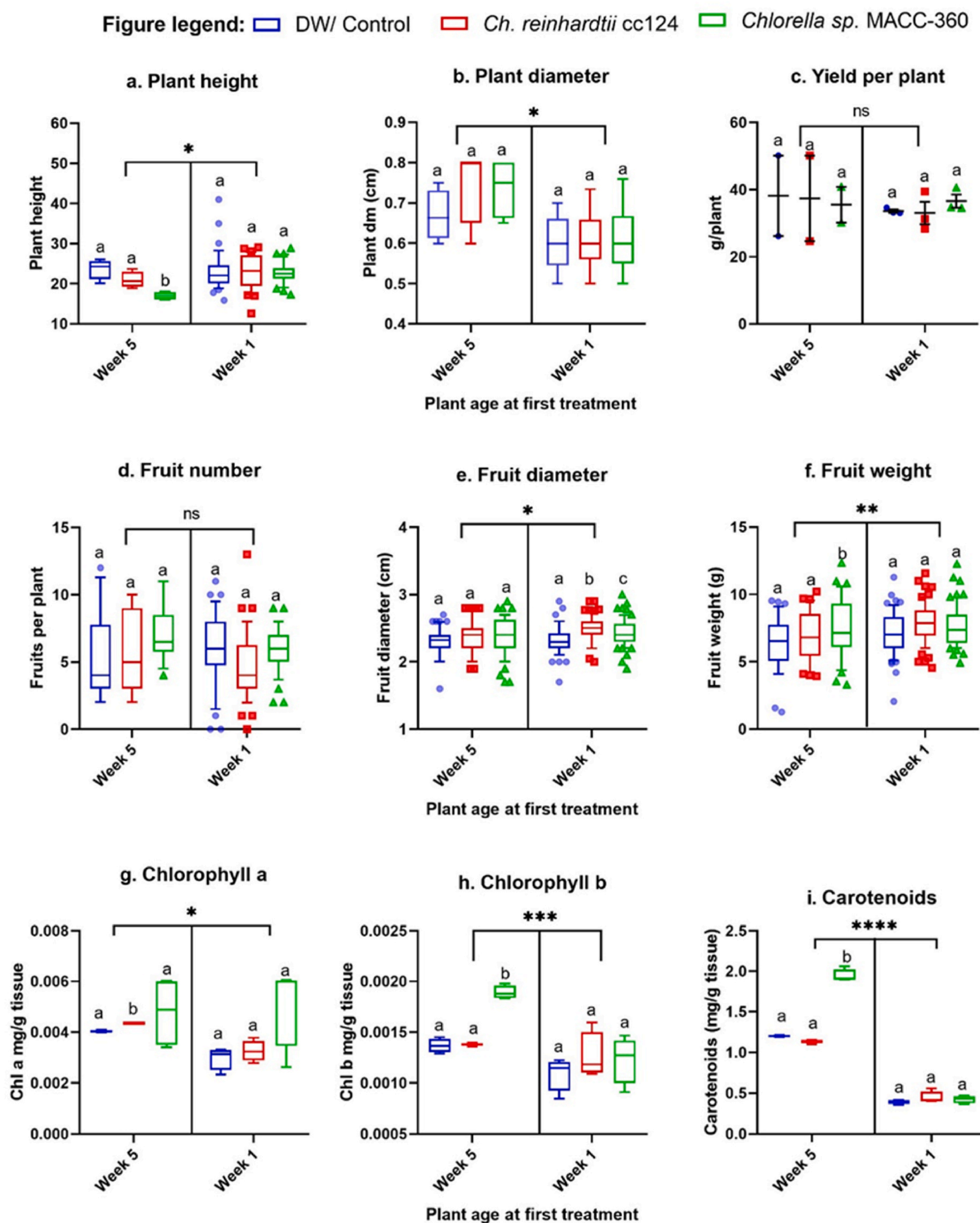

Fig. 5. Comparison of the effects of algae treatment (living cells as soil drench and extracts as foliar spray) on plants of different ages (Week 1 and Week 5); a) plant height, b) plant diameter, c) yield per plant, d) fruit number, e) fruit diameter, f) fruit weight, g) chlorophyll-a, h) chlorophyll-b and i) carotenoids. Lines with asterisks across different treatment regimes (Week 5 and Week 1) show significant differences between regimes at alpha = 0.05. Different letters on boxes within each regime show significant differences between treatments in that regime at $p = 0.05$. The treatments are distilled water (DW), *Ch. reinhardtii* cc124, *Chlorella* sp. MACC-360.

different concentrations of biostimulants (Dias et al., 2016; Sutharsan et al., 2014; Mannino et al., 2020). Reduction in fruit number especially by *Ch. reinhardtii* cc 124 could be due to the presence of harmful compounds such as the 2,4-D auxin reported by (Marth and Mitchelle, 1944). An increase in chlorophylls and carotenoids in leaves, flowers and fruits of algae-treated plants has been reported (Coppens et al., 2016; Mutale-Joan et al., 2020; Supraja et al., 2020a) and linked to reduced degradation of chlorophyll and decreased plant senescence (Blunden et al., 1996; Calvo et al., 2014). Chlorophyll content corresponds to SPAD values which correspond to the plants nitrogen (N) status (Culman et al., 2013; Xiong et al., 2015). The N content has a strong influence on the chlorophyll content (Cartelat et al., 2005; Samborski et al., 2009;

Table 1
Two-way ANOVA results showing percentage of variation explained by each factor.

Variable	Treatment			Age at first application			Treatment x Age at first application		
	% of total variation	F (DFn, DFd)	P	% of total variation	F (DFn, DFd)	P	% of total variation	F (DFn, DFd)	P
Plant height	6.31	F (2, 109) = 3.86	0.024	3.81	F (1, 109) = 4.65	0.033	6.14	F (2, 109) = 3.75	**0.027**
Plant diameter	1.59	F (2, 110) = 1.92	0.151	21.8	F (1, 110) = 31.7	< **0.0001**	2.64	F (2, 110) = 1.16	0.318
Fruit number	4.53	F (2, 141) = 3.45	**0.035**	0.06	F (1, 141) = 0.92	0.340	2.16	F (2, 141) = 1.64	0.197
Fruit diameter	5.16	F (2, 324) = 9.00	**0.0002**	1.14	F (1, 324) = 3.97	**0.047**	0.59	F (2, 324) = 1.04	0.356

Two-way ANOVA was conducted with two factors; Treatment (DW, 124 and 360) on the columns and Age of treatment (week 1 and week 5) on the rows. F is the F statistic, DFn is the degree of freedom from between the columns and DFd is the degree of freedom from within the columns. Bold P values are significant (*P < 0.05)*.

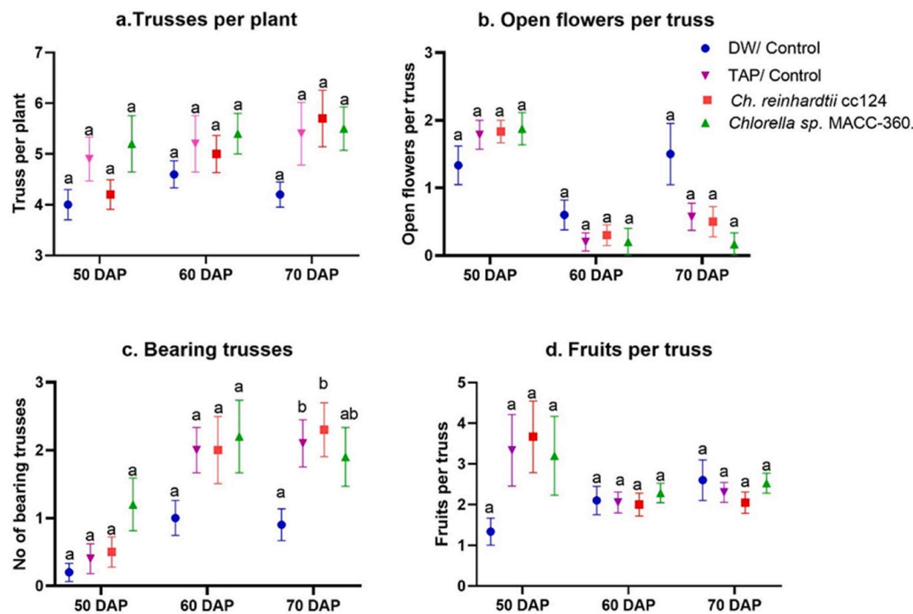

Fig. 6. Reproductive parameters on the 50th, 60th and 70th day after planting (DAP); a) Number of trusses, b) Number of open flowers per truss, c) Number of bearing trusses (trusses with fruits) and d) Number of fruits per truss. Alphabetical letters symbolize significant differences between groups; similar letters imply no significant difference; different letters show significant difference at p = 0.05. The treatments are distilled water (DW)/Control, *Ch. reinhardtii* cc124, *Chlorella* sp. MACC-360 and Tris Acetate Phosphate/Control medium used for MA cultivation.

Table 2
Two-way ANOVA results showing percentage of variation explained by each of the factor.

Variable	Treatment			Time of scoring			Treatment x Time of scoring		
	% Variation	F (DFn, DFd)	P	% Variation	F (DFn, DFd)	P	% Variation	F (DFn, DFd)	P
Flowering	**11.7**	F (3,144) = 7.05	**0.0002**	6.98	F (3,144) = 4.19	**0.007**	1.45	F (9,144) = 0.29	0.977
Fruiting	**9.39**	F (3,108) = 3.78	**0.013**	0.65	F (2,108) = 0.39	0.677	0.68	F (6,108) = 1.16	0.991
Trusses	**8.54**	F (3,108) = 3.62	**0.016**	3.54	F (2,108) = 2.24	0.111	2.64	F (6,108) = 1.04	0.557
Bearing trusses	**10.1**	F (3,108) = 5.41	**0.002**	20.0	F (2,108) = 16.1	< **0.0001**	2.92	F (6,108) = 0.78	0.585

Two-way ANOVA was conducted with two factors; Treatment (DW, TAP, 124 and 360) on the columns and Time of scoring (50DAP, 60DAP and 70DAP for all parameters and 4 time points for the flowering) on the rows. F is the F statistic, DFn is the degree of freedom from between the columns and DFd is the degree of freedom from within the columns. Bold P values are significant (*P < 0.05)*.

Schepers et al., 1996). Increase in chlorophyll thus indicates that plants received sufficient or surplus nutrition from soil. Similar results for algae-treated plants have been reported with respect to N and phosphorous (Schreiber et al., 2018; Martini et al., 2021; Zhang et al., 2017). Increase in leaf temperature differential (LTD), the difference between leaf temperature and the ambient temperature, implies that plants efficiently dissipate excess heat, a phenomenon that could assist plants to withstand abiotic stresses. Increased LTD was recorded in wheat treated with similar microalgae (Martini et al., 2021) and correlated with improved root formation and ability to withstand water stress in algae-treated tomatoes (Oancea and Fatu, 2013). Increased leaf thickness could suggest enhanced cell division or expansion in leaves. Leaf thickness in wild tomato, *Solanum pennellii*, was associated with elongation of palisade mesophyll cells which probably arose due to endopolyploidy (Coneva and Frank, 2017; Coneva and Chitwood, 2018). Leaves with long palisade cells have improved carbon dioxide (CO_2) uptake (Oguchi and Hirose, 2005; Terashima et al., 2011) and efficiently distribute light throughout the mesophyll cells (Brodersen and Vogelmann, 2010; 2008) resulting in high photosynthetic efficiency. In addition, this trait helps leaves maintain a water potential during low water supply (Becker, 2007), an adaptive feature, that enables plants to increase performance by increasing photosynthesis and water use efficiency. Poorter and colleagues concluded that plants might have to trade-off fast growth with leaf thickness in water limiting conditions (Poorter et al., 2009). Since plants treated with *Chlorella* sp. MACC-360 already portray this trait without water limitation, it is plausible that they could withstand drought stress. The treatment with both algae strains resulted in increased LTD, implying that algae-treated plants

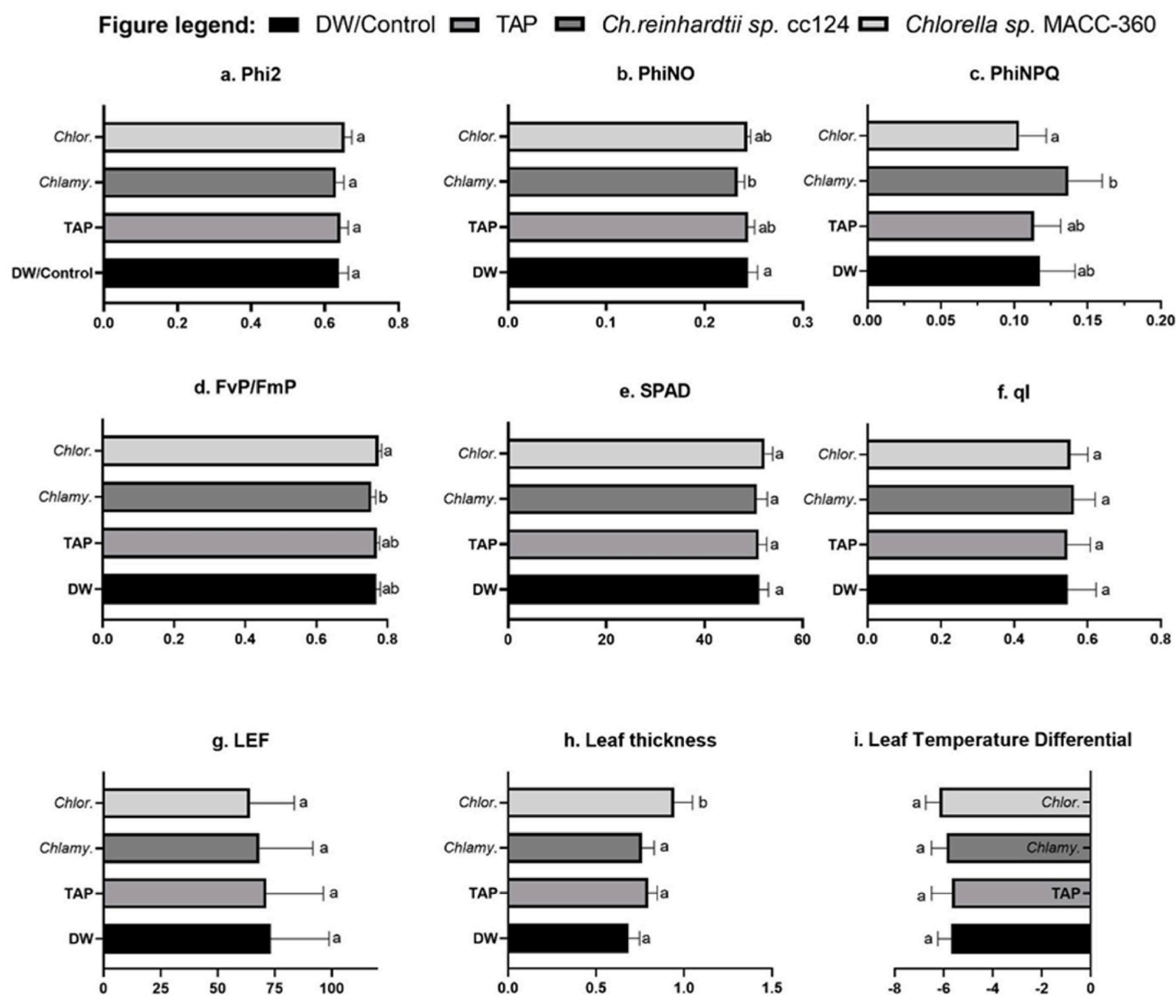

Fig. 7. Parameters related to photosynthesis; a. Phi2-PS II quantum yield/ratio of incoming light (excited electrons) used in photochemistry/photosynthesis, b. PhiNO- ratio of incoming light (excited electrons) that is lost in non-regulated processes and the products of which can be harmful/cause photodamage, c. PhiNPQ-ratio of incoming light (excited electrons) lost through regulated non-photochemical quenching d. Fv/Fm-maximum quantum yield, e. Soil plant analysis development (SPAD) value, an indicator of plant nitrogen status and relative chlorophyll, f. ql is the fraction of open PS II reaction centers, g. LEF is the linear electron flow and h. Leaf thickness is the thickness of the leaf section clamped by the Multispeq device and i) Leaf temperature differential (LTD), the temperature difference between the leaf and its surroundings/environment. Bars show the means; error bars show the SE (Standard Error) of measurements taken for the first five weeks of growth from 10 plants from each treatment regime. Different letters at the end of bars show significant differences between groups at alpha $p = 0.05$ based on Tukey's ANOVA test. The treatment regimens are DW = distilled water control, Tris-acetate-phosphate (TAP) medium = media control, Ch. reinhardtii cc124 culture in TAP medium and Chlorella sp. MACC-360 culture in TAP medium.

increased the efficiency of excess heat dissipation. These results mirror those of wheat treated with similar microalgae (Martini et al., 2021). The increased LTD is associated with stomatal conductance and strongly correlates with improved root formation and ability to withstand water-stress in algae-treated tomatoes (Martini et al., 2021; Oancea and Fatu, 2013).

LEF is the flow of electrons from the oxygen-evolving complex (OEC) to the NADP + reducing it to NADPH, after splitting of water by the incoming light energy (Huang et al., 2018). The relationship between LEF and PSII activity is controversial, although most scholars are of the view that reduced LEF suggest moderate photo-inhibition of PSII activity. Others opine that it could be due to increased acidity in the lumen due to accumulation of photosynthetic products (Huang et al., 2018). Both MA treatments caused an insignificant reduction of LEF, but only plants treated with *Ch. reinhardtii* cc124 showed a reduced PSII activity. However, it is unlikely that significant photo-inhibition occurred; the PhiNPQ was significantly high and PhiNO significantly low suggesting enhanced protection of the photosynthetic apparatus. This phenomenon is well in line with the observed increase in the antioxidant levels, especially carotenoids in the chloroplast (Farid et al., 2019).

Rapid growth occurs when plants have access to sufficient/surplus nutrition or exposure to compounds that induce fast multiplication of plant cells. In previous studies, growth promotion has been found to strongly associate with higher nutrient uptake, higher biomass accumulation and enhanced crop yields, when MA was applied as a biofertilizer (Shaaban, 2001; Kholssi et al., 2019). Microalgae extracts have been found to enhance shoot and root development in tomatoes (Mutale-Joan et al., 2020) which means enhanced absorption of nutrients and water from soil mostly due to an increased root surface area for absorption. Other studies highlighted that MA extracts upregulated some genes involved in biological pathways and processes such as primary and secondary metabolisms as well as intracellular transports mostly related to root traits and nutrient acquisition (Barone et al., 2018). These effects culminate in increased mineral uptake and consequently, increased photosynthetic products. The fact that *Chlorella* sp. had more profound effect on plants than TAP media further suggests that it contained more nutrients or had bioactive compounds absent in other treatments. These results resonate with Ferreira's report that *Synechocystis* sp. had higher nitrogen (N) content than *C. vulgaris,* yet the latter elicited a more profound effect on plants (Ferreira et al., 2021) implying

that the growth-promotion action was not from the extra nutrients but rather caused by MA biostimulation.

MA have been reported to produce growth promoting exudates including polyamines, vitamins, amino acids, betaines, auxins and cytokinins. Among these substances, plant hormones and polysaccharides are most frequently underlined as responsible compounds for plant biostimulating effects (Gebser and Pohnert, 2013; Oancea and Fatu, 2013; Spolaore et al., 2006; Stirk and Ördög, 2002; Tate et al., 2013). Similar to what Stirk et al. (2013) reported, both tested MA were found to release auxins, while *Chlorella* sp. released copious amounts of EPS as well. These two components could be the primary biostimulant agents in this study. Polysaccharides were reported to interact with leucin-rich repeat membrane receptors responsible for activating a mechanism that modulates the regulation of several genes that influence cell expansion (Nardi et al., 2016). Furthermore, crude polysaccharides from both *Chlorella* and *Chlamydomonas* genera were found to increase the expression of pathogenesis-related (PR) genes and genes responsible for antioxidants enzymes such as peroxidase (POD), ascorbate peroxidase (APX) and β-1-3 glucanase in tomatoes (Farid et al., 2019). In fact, MA-derived exopolysaccharides applied via foliar spraying method had biostimulant effects on tomatoes under normal and stressful conditions (El Arroussi et al., 2018; Elarroussi et al., 2016).

The strain-specific effect of the MA could be explained by the inherent differences in the biochemical and structural properties of their EPS, the capacity to produce phytohormones and the ability to form biofilms/cellular aggregations. Although both *Chlorella* sp. and *Chlamydomonas* sp. produce polysaccharides, they are not similar in structure and biochemical properties (Rossi and Philippis, 2016). Moreover, it is highly likely that *Chlorella* sp. MACC-360-treated plants had access to more polysaccharides than plants treated with *Ch. reinhardtii* cc124, since the former released copious amount of polysaccharides from the cells. In contrast the latter could only release the polysaccharides after cell disruption; if applied via soil-drench method, this could take a long time as it is dependent on the soil microbes.

Concentration plays a critical role in determining the biostimulant action considering that the MA extracts have varying concentrations of the bioactive compounds. The concentration-dependent effect of seaweed and *Spirulina plantensis* extracts applied to roses and aubergines, respectively was observed (Sumangala et al., 2019; Dias et al., 2016). Another study showed that exceptionally low concentrations had no effects, while extremely high concentrations had negative effects (Kumari et al., 2011). In yet another study on tomatoes, the growth-stimulating effect of MA extracts disappeared beyond a certain limit/concentration and extracts became growth-inhibiting (Supraja et al., 2020b). Garcia-Gonzalez and colleagues observed that the application of *Acutodesmus dimorphus* extract at a concentration of 3.75 g/L increased plant height, flower number and branch number but reduced yields in tomatoes (Garcia-Gonzalez and Sommerfeld, 2016). In aubergines, Dias et al. (2016) found that low concentrations of microalgae-based biofertilizer (10 g/L) increased fruit yield, while a higher concentration (45 g/L) increased vegetative growth but reduced yield. In the present study we applied algae concentrations of 1 g/L for soil drench and about 3 g/L (about 1%) for foliar spraying. Experiments to determine the algae strain-specific optimal concentrations are planned for various production plants.

The ability of MA to form biofilms could also be an underlying factor in the strain-specificity of the biostimulant action. *Chlorella* sp. MACC-360 displayed the ability to form cellular aggregations. This phenomenon promotes biofilm formation involving beneficial interactions between various bacteria and fungi present in the soil. No intimate relationship between the algal cells and roots were detected under the microscope. This is in line with what Lee and Ryu reported in a recent review about microalgae being the new plant beneficial microbes, "*Intriguingly, unlike prokaryotic algae, it is not reported that eukaryotic algae colonize on plant tissues*" (Lee and Ryu, 2021). Nevertheless, there are reports of synergistic growth-promoting effects of algae and bacteria on different plant species, whereby the relationship is interspecific and governed by specific metabolite patterns (Nain et al., 2010; Dukare et al., 2011; Rana et al., 2016; Sharma et al., 2020). Watson found that algae produced volatile compounds (VOCs) such as terpenoids and nor-carotenoids which act as chemical signals for communication with other cells (Watson, 2003). For instance, algal-bacterial synergistic relationships are ubiquitous in natural ecosystems (Ramanan et al., 2016) and exposure of MA to bacterial VOCs triggered fast growth of the MA (Achyuthan et al., 2017). This implies that MA can send signals to attract and accumulate beneficial microorganisms responsible for mineralization and production of secondary metabolites such as antibiotics. Such interactions could further enhance colonization by increasing algal growth. All these factors not only provide nutrition to plants, but also protect them from disease-causing pathogens, hence improving their overall health.

The significant interaction between algae treatment and the age of plants when algae treatment was initiated shows that biostimulant action is dependent on the selected time of application during plant growth. Plant height and plant diameter could be affected based on the age of plants because juvenile plants would prioritize vertical growth (height), while nearly mature plants would channel excess energy resources to girth extension (diameter) or reproduction/flowering. Reduction of plant height when tomatoes were treated with *Scenedesmus* sp. biomass has been reported (Ferreira et al., 2021). Besides, juvenile plants could have received most of the MA via soil drench method when they were in their active vegetative phase. Algae-treated-plants might have had access to a more diverse set of nutrients compared to the control plants. This is because algae can influence the rhizosphere and hence the whole metabolism of soil microbes including carbon and nitrogen cycles. For instance, inoculation of soil with cyanobacteria increased the bacterial population diversity by 10 fold while application of a microalgae suspension altered soil pH and increased the number and diversity of soil diatoms (Hastings et al., 2014; Priya et al., 2015). While algae cells are rigid and their contents cannot be readily available to plants, application at a juvenile stage could give soil microbes sufficient time to mineralize algal cells or to develop various mutualistic interactions. This can explain why week 1 regime plants showed more pronounced effects on height than their week 5 counterparts. This resonates with Garcia-Gonzalez and Sommerfeld's (2016) findings that the timing and dose of application significantly affected the agronomic performances of plants. Nevertheless, our overall results show that the application of MA to tomato plants which are about to enter anthesis (Week 5), results in more desirable effects than initiating the application at a juvenile stage (Week 1). This age (about 35–40 days after transplanting) has been found to be the ideal plant age for initiating biostimulant application by other scholars as well (Ibrahim et al., 1970; Plaza et al., 2018).

5. Conclusion

Microalgae from both *Chlorella* and *Chlamydomonas* genera had biostimulating effects on tomatoes irrespective of the selected portion of algae cultures administered to plants. When compared against the controls, *Chlorella* sp. MACC-360 treatment significantly affected fruit diameter, fruit weight, chlorophyll *b* and carotenoids irrespective of age of plants. In contrast, *Ch. reinhardtii* cc124 significantly affected fruit diameter and chlorophyll *a* content relative to control irrespective of age of plants.

Based on the microscopy studies, it is crucial to describe the condition-dependent growth characteristics of a specific microalgae strain to determine which portion of the algae to be used for application. Living algal cells (CCS), living algal cells plus spent media (CS) or supernatant/spent media were tested in the plant biostimulation studies. Application of CS could be the right choice for algal strains producing excessive amounts of EPS if the goal is to immediately increase bioactive compounds available to plants. For some strains, cell destruction might

be essential for preparation of the efficient biostimulant treatment.

This study proves that the biostimulant action of MA influences photosynthetic performance and is dependent on algal strain. Even though the differences between MA treated plants and the control plants were not significant for a number of parameters, the differences between the two algal treatments were significant for some of the important parameters such as maximum quantum yield and regulated energy loss. Although it is difficult to point out a single reason explaining the observed biostimulant action of MA in this study, the presence of EPS has a strong implication on MA biostimulant action. Nonetheless, a more thorough characterization of the composition of the MA portions (cells, supernatant/spent media, isolated EPS and total extract from destroyed cells) would provide more insight into the mode of action of the MA. Analysis of the phytohormone content in each strain would also offer a closer insight into the strain-specific biostimulant action of the two algal strains. Finally, testing of different concentrations of MA on nutrient-deprived plants is also planned in the future to establish the optimal levels that increase crop quality and yield.

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CRediT authorship contribution statement

Margaret Mukami Gitau: planned the research, wrote the manuscript and performed the experiments. **Attila Farkas:** Formal analysis, performed the microscopy analyses. **Vince Ördög:** provided useful practical hints and participated in the critical discussions. **Gergely Maróti:** Writing – review & editing, designed the study, reviewed the manuscript and discussed the relevant literature. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2022.132689.

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