Evaluation of the plant biostimulant effects of selected eukaryotic green microalgae

Ph.D. Dissertation

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Abbreviations

ABA:	Abscisic acid
ANOVA:	Analysis of variance
AP2/ERF:	APETALA 2/Ethylene responsive factor
APX:	Ascorbate peroxidase
ATP:	Adenosine triphosphate
BP:	Biological process
Car:	Carotenoid
cc 124:	*Chlamydomonas reinhardtii* accession number cc 124
CC:	Cellular component
cDNA:	Complementary DNA
CFW:	Calcofluor-White
Chl:	Chlorophyll
CK:	Cytokinin
CLSM:	Confocal laser scanning microscopy
Con A:	Concanavalin A
DAP:	Days after planting
DEGs:	Differentially expressed genes
DNA:	Deoxyribonucleic acid
DW:	Distilled water
EPS:	Exopolysaccharides
ET:	Ethylene
FFAs:	Free fatty acids
Fv/Fm:	Maximum quantum yield
FW:	Forward
GAs:	Gibberellins
GO:	Gene ontology
IAA:	Indole acetic acid
JA:	Jasmonic acid
KEGG:	Kyoto Encyclopedia of Genes and Genomes

LEF:	Linear electron flow
LTD:	Leaf temperature differential
MA:	Microalgae
MACC 360:	Mosonmagyaróvár Algal Culture Collection number 360
MACC 38:	Mosonmagyaróvár Algal Culture Collection number 38
MACC:	Mosonmagyaróvár Algal Culture Collection
MF:	Molecular function
mRNA:	Messenger ribonucleic acid
NAC:	Nascent Polypeptide-Associated Complex
NAP:	NAC-domain containing protein
NGS:	Next-generation sequencing
NPQ:	Non-photochemical quenching
OD:	Optical density
OTU:	Operational taxonomic unit
PGPR:	Plant growth promoting rhizobacteria
Phi2:	PS II quantum yield
PhiNO:	Non-regulated energy loss/dissipation
PhiNPQ:	Regulated non-photochemical quenching
PS II:	Photosystem II
REV:	Reverse
RNA:	Ribonucleic acid
SEM:	Scanning electron microscopy
Sol 1:	Solution 1
SPAD:	Soil plant analysis development
TAP:	Tris-acetate-phosphate
TAS:	Tomato abscisic acid and environmental stress-inducible protein
TD2:	Threonine dehydrogenase 2
TPS:	Trehalose-6-phosphate synthase
VOCs:	Volatile organic compounds

1. Introduction

Population growth has dramatically increased the global demand for food at a time when agricultural land is scarce due to urbanization. Consequently, crop producers are under enormous pressure to increase food production at all costs. Using chemical fertilizers and pesticides in agriculture has increased food production in recent years. Unfortunately, it has had grave consequences, including environmental pollution, disruption of natural ecosystems, loss of biodiversity, and even severe effects on human health (Chiaiese et al., 2018; Cooper and Dobson, 2007; Fenner et al., 2013). Chemical fertilizers lead to the accumulation of harmful levels of elements such as nitrogen and phosphorus, which culminate in eutrophication in water bodies and land salinization. With a projected doubling of global food demand by 2050 (Singh, 2016), it is appalling that the current use of chemical fertilizers at low concentrations is ineffective at increasing crop yields, necessitating large volumes. Thus, the cost of using chemical fertilizers continues to rise. In the future, farmers will no longer find it cost-effective to apply chemical fertilizers when cultivating food and fodder crops. In what is known as "climate-smart agriculture," there is an urgent need to replace chemical fertilizers and pesticides with cheaper and environmentally friendly alternatives that will increase crop yields and nutritional value despite climate change (Campbell et al., 2014). In addition to eliminating chemical fertilizers, using natural resources such as saline land, saline water, and land contaminated with heavy metals could increase food production. Also, since most of the arable land already has accumulated chemical fertilizer that is not accessible to plants, biostimulants used alongside fewer amounts of chemical fertilizer could be a less costly approach to increasing crop production. Thus biostimulants could be an economically viable alternative.

Biostimulants are substances that promote growth without being nutrient-rich compounds, soil amendments, or pesticides. Kauffman emphasized that biostimulants are "non-fertilizer" substances that stimulate plant growth (Kauffman et al., 2007). As biostimulants, some authors use hormone-containing products and metabolic enhancers interchangeably (Zhang et al., 2003; Zhang and Schmidt, 2000). Others have defined biostimulants as substances of biological origin whose application to plants or rhizosphere stimulates the natural processes that improve nutrient uptake and efficiency, tolerance to stresses, crop quality, and yield (Woo and Pepe, 2018).

Although some scholars (du Jardin, 2015) consider biofertilizers to be a subcategory of biostimulants, many agree that the primary difference between the two is the quantity required to achieve the desired results. Small amounts of biostimulants are sufficient to cause growth promotion. In contrast, farmers need large quantities of biofertilizers to achieve the same outcome because the action of the biofertilizers strongly depends on their constituents. In contrast, the effect of a biostimulant does not depend on its nutritional/ mineral content; it mostly depends on the responses it elicits from the plants. Although du Jardin (2015) defined a biofertilizer as any microorganism applied to plants or soil to increase nutrient availability and use by plants, regardless of the nutrient content of the microorganism, this description better befits the biostimulants. If the soils have insufficient or plant-unavailable nutrients, biostimulants can solubilize some minerals, such as phosphates, making them available for plant uptake. Also, due to their influence on the soil microbial community, they can enhance nutrients present for example, if enrichment of nitrogen-fixing bacteria occurs, the plants will have access to nitrogen. Beneficial bacteria of the *Rhizobium* genus and arbuscular-mycorrhizal fungi (AMF) are two of the most well-known plant growth-promoting microorganisms (du Jardin, 2015).

The basis for classifying biostimulants depends on their origin and chemical makeup. Humic substances, amino acid-containing substances, and hormone-containing substances are the major groups (du Jardin, 2015). Biostimulants include protein hydrolysates and nitrogenous molecules such as betaines, polyamines, and non-protein amino acids. Consequently, a biostimulant may belong to multiple categories or contain multiple stimulating compounds. Some seaweed extracts, for instance, contain phytohormones and polysaccharides, but each component can be purified and used separately. A biostimulant's role or agricultural function can also influence the classification of biostimulants. In this instance, biostimulants can improve nutrient efficiency, abiotic stress tolerance, and crop quality characteristics. Botanicals are plant extracts representing another class of biostimulants known to ward off disease-causing pathogens hence their potential for application as biocontrol agents or biopesticides (du Jardin, 2015).

In recent years, scientists have investigated biostimulants derived from algae. Algae is a polyphyletic group comprising highly diverse independent evolutionary lines with the universal ability to perform oxygenic photosynthesis, whereby they convert

carbon dioxide into biomass and release oxygen (Vieira et al., 2022). Nevertheless, some algal species can grow in the dark and utilize organic compounds. The microscopic algae are the microalgae (MA), while the macroscopic ones are the macroalgae, commonly referred to as seaweeds. MA consists of prokaryotes (cyanobacteria) and eukaryotes (with five evolutionary lineages, including the green algae from which plants evolved). MA are photosynthetic microorganisms that grow in aquatic systems, including fresh, brackish, and saltwater, up to extreme salinities (Vieira et al., 2022). Many MA strains can proliferate in clean water, while Molazendah and colleagues noted that some had gained adaptation for growth in wastewater in association with other microorganisms (Molazadeh et al., 2019).

Biostimulants derived from macroalgae (Brain et al., 1973; Digruber et al., 2018; Fayzi et al., 2020; Kavipriya et al., 2011; Kocira et al., 2018; de Melo et al., 2020; Michalak et al., 2015b; Pomin, 2014) and plants (Francesca et al., 2020; Godlewska and Ciepiela, 2018; Gramss et al., 2003) improved plant growth and yield in several plant species under normal, as well as stressful conditions. However, preparing and purifying these biostimulants is laborious, energy-intensive, and costly. Despite the abundance of seaweed in the oceans, continuous harvesting alters the water ecosystems and may eventually deplete the seaweed population. In contrast to seaweed, plants require fertile land for cultivation and a considerable amount of time to produce enough biomass for biostimulant production. Microorganisms, including MA, proliferate rapidly, but mass cultivation and biomass processing may require specialized media and facilities. Kapoore and colleagues reported that the biostimulant effect of MA on plants is comparable to that of seaweed (Kapoore et al., 2021). Therefore, MA is an excellent candidate for biostimulant production among algae and land plants.

Studies have demonstrated that most green eukaryotic MA species produce many bioactive compounds with diverse applications (Ahmad et al., 2020; Hakim et al., 2016; Sassi et al., 2019; Skjånes et al., 2013; Suganya et al., 2016). Another advantage of MA is that they do not compete with food crops for cultivation space (Abdel-Raouf N, 2012; Chiaiese et al., 2018; Colla and Rouphael, 2020; Lee and Ryu, 2021; Renuka et al., 2018; Ronga et al., 2019). Furthermore, the production of biostimulants or biofertilizers from wastewater-cultivated-MA would provide a cost-effective and sustainable method of water recycling (Pavliukh et al., 2020; Renuka et al., 2016; Wuang et al., 2016). Although there are restrictions on the use of MA

cultivated in wastewater in the European Union (EU), especially for food and feed production, using such algae as biofertilizers or biostimulants in agriculture is permissible as long as the algae do not have chemicals, toxins or microbiological contaminants (Vieira et al., 2022). Incorporating MA into wastewater management or aquaculture/hydroponic production enables cyclic production and sustainable resource utilization.

Biostimulants and biofertilizers could enable the cultivation of crops in unfavorable conditions, such as extremely basic or acidic soils, arid, contaminated with heavy metals, and especially those contaminated with nitrogen and ammonia. MA can help utilize excess nitrogen and ammonia in contaminated soils. The latter kind of contamination usually arises from irrigation with wastewater or the continuous use of chemical fertilizers. For other types of heavy metal contamination, MA can bind the metal ions, making them unavailable for plant uptake. Thus, MA could be used solely for bioremediation before the toxic levels reduce to levels allowed for crop production. Therefore, in the long run, using plant biostimulants in land reclamation projects can simultaneously increase food production and restore the value of unproductive land. Additionally, some scholars have identified biostimulants as effective biofortification agents (Hawrylak-Nowak et al., 2019). This phenomenon, which is the foundation of "climate-smart agriculture," is the enrichment of specific nutrients in crop production to address the malnutrition problem by meeting the nutritional needs of humans.

Despite the demonstrated potential for MA biostimulants, their use is still in its infancy. The absence of a universal extract preparation procedure, the lack of knowledge regarding the optimal time and method of application, and the lack of knowledge regarding the strain-specific effects of MA hinder the agricultural application. It is imperative to overcome these obstacles, for example, by shortening algae processing steps before applying them to plants. Thus, using live cells may be one of the most suitable options. If disruption of cells is necessary, the preparation protocols should eliminate chemicals to guarantee sustainable production.

1.1 Microalgae as biostimulants

Multiple studies have established the potential of prokaryotic and eukaryotic algae to enhance crop production (Lee and Ryu, 2021; Priyadarshani and Rath, 2012). *Chlorella* (Agwa et al., 2017; Bumandalai and Tserennadmid, 2019; Dineshkumar et al., 2020; Ergun et al., 2020; Faheed and Fattah, 2008; Kholssi et al., 2019; Kim et al., 2018; Lima et al., 2020; Özdemir et al., 2016), *Scenedesmus* (Gatamaneni Loganathan et al., 2020; Navarro-López et al., 2020; Puglisi et al., 2020b, 2020a) and *Arthospira* (*Spirulina*) (Uddin et al., 2019; Wuang et al., 2016; Yanni et al., 2020) stimulated the growth of different plant species including corn, spinach, Chinese chives, onions, lettuce, and tomatoes.

A European survey authenticated that *Chlorella*, *Nannochloropsis* spp., *Haematococcus pluvialis*, and *Spirulina* were the most popular MA cultivated in 11 countries. *Chlorella* and *Spirulina* emerged as the most produced MA for dry biomass (Araújo et al., 2021). Species from the *Chlorella* genus were the first to be cultivated on an industrial scale in Japan in the 1960s (Mobin and Alam, 2017). The popularity of the *Chlorella* genus could be due to its fast growth and adaptability to a wide variety of environmental conditions, making it eligible for cultivation worldwide. Some strains from this genus are robust and can thrive in open ponds with low contamination risks. These properties allow large biomass production at a cheaper cost relative to closed or photoreactor-based cultivation. Another reason the genus is widespread is its vast industrial application; its species have high protein, carbohydrate, and B-Vitamin content, making them suitable for supplements in the food industry (Niccolai et al., 2019). Some *Chlorella* species help produce health-related tablets and capsules due to their bioactivity, including anti-fungal, anti-viral, anti-inflammatory, antioxidant, anti-carcinogenic, and immune system stimulation (Araújo et al., 2021). Araujo and colleagues (2021) further noted that *Chlorella* strains are also widely used in animal farming and aquaculture as feed. Furthermore, the cosmetic industry has used extracts from this genus to produce various products for skin care (Mobin and Alam, 2017). Thus, it is comprehensible why *Chlorella* species are also widely examined for biostimulant properties; about 75 % of literature concerning MA as biostimulants reports about *Chlorella*.

Despite being among the most abundant MA species in natural ecosystems, *Chlamydomonas* species still need to be studied and utilized in biotechnology and agriculture. This underutilization is despite their rapid biomass accumulation and ability to produce phytohormones such as auxins, ethylene (ET), brassinosteroids, cytokinin (CK), and trehalose (Carillo et al., 2020; Singh et al., 2017; Stirk et al., 2013, 2002). The quick biomass turnover and synthesis of phytohormones could make them appropriate for plant biostimulant application.

1.2 Preparation of microalgae-based biostimulants

Monocultures (Faheed and Fattah, 2008) and co-cultures of eukaryotic green algae with other green algae, bacteria, cyanobacteria, and fungi have consistently demonstrated plant growth stimulation (Gatamaneni Loganathan et al., 2020; Grzesik and Romanowska-Duda, 2015; Kopta et al., 2018; Renuka et al., 2018; Schwarz and Gross, 2004; Zayadan et al., 2014). Extracts (Navarro-López et al., 2020; Puglisi et al., 2020a), dry biomass (Agwa et al., 2017; Castro et al., 2020; Faheed and Fattah, 2008; Rasheed et al., 2020; Suleiman et al., 2020) spent medium/supernatant (Jo et al., 2020; Kholssi et al., 2019), whole cultures (cells plus supernatant) (Jo et al., 2020) as well as cell suspensions (cells only without their growth media) (Bumandalai and Tserennadmid, 2019; Kholssi et al., 2019) are other forms which have elicited positive responses in plants in the past.

Gomes and coworkers (2020) emphasized that the location of the microbial molecule of interest determines the most suitable method of extracting biomolecules from microbial cells. If the cells release molecules to the growth media, the downstream processes are cheaper, and the process can have more significant industrial applications. On the contrary, if the biomolecules accumulate inside the cells, using cell-disruption techniques followed by downstream purification steps to remove cellular debris is indispensable. For this reason, extracting intracellular biomolecules is costly and difficult for industrial up-scaling compared to extracting extracellular compounds (Gomes et al., 2020).

Extraction of bioactive compounds such as polysaccharides and hormone-like substances from the cells of algal biomass for plant or soil applications is typical (El-

Naggar et al., 2020; De Jesus Raposo et al., 2013; Rachidi et al., 2021). For intracellular-occurring biomolecules, cell destruction is essential. Gomes et al. (2020) classified cellular destruction methods into three categories: severe, gentle, or combination. Severe methods include non-specific mechanical methods such as ultrasonication, bead-milling, and high-pressure-homogenization. On the other hand, gentle methods have higher specificity and selectivity and are less aggressive than severe methods. Some gentle methods include osmotic shock, freeze-thawing, enzymatic lysis, and detergent or solvent treatments. The same authors noted that solvent and detergent treatments, ultrasonication, and glass beads are popular laboratory-scale methods (Gomes et al., 2020).

In past studies, the destruction of algal cell walls applied several methods. Mutale-joan et al. (2020) used a powerful acid (sulphuric acid) to hydrolyze the algal cell wall while Ördög and coworkers performed ultrasonication (Ördög et al., 2004b). Gomes et al. (2020) reported that enzymes are also efficient, especially when preserving the bioactivity of the molecules of interest. Microwave-assisted extraction, supercritical fluid, and pressurized liquid are contemporary techniques proposed for high-yield, high-quality extracts (Michalak et al., 2016, 2015a). All these methods appear to be laborious, time-consuming, and costly.

The methods highlighted above were influential on the laboratory scale, but their applicability at an industrial level is not feasible due to high operational costs. There is a need to identify methods that are cheap and scalable. Using cultures in their natural state could be easily scaled up to save energy and time. However, it is necessary first to characterize algae strains to establish where their biomolecules occur. If the cells release molecules to the media, the preparation method could be fast, cheap, and easy to scale up. The technique with minimal downstream processes could be affordable if the molecules accumulate in cells. Thus, the crude extract obtained from cell wall destruction should be effective as a biostimulant without requiring costly purification procedures.

1.3 Mode of microalgae biostimulants application

Typically, foliar treatments, soil drenches, or seed/sprout priming are the standard modes of algal product application. In some studies, plant tissues received injections of substances derived from algae (Rachidi et al., 2021). Brain and coworkers (1973) conveyed that the CK content of algal extracts is presumably lost when applied to the soil. They recommended spraying seaweed extracts to plant leaves. The same authors claimed the efficient absorption of CK through the leaf surface (Brain et al., 1973). Since CK is a well-known phytohormone, absorption of other hormones via the leaf might be possible, implying that foliar application might be the best method for phytohormones-containing biostimulants. In contrast, the soil drench method could be the most suitable application method for living algal cells supposing that they can multiply and alter soil properties and microbial communities via the continuous release of bioactive compounds and interaction with other microorganisms (Marks et al., 2019; Renuka et al., 2018; Ronga et al., 2019).

Applying the same MA strain's dry biomass, liquid fertilizer, and foliar spray resulted in positive outcomes for tomato plants. However, soil application of dry algae had the most significant impact (Özdemir et al., 2016). However, when Supraja and colleagues (2020b) compared the effect of microalgal extracts applied as seed treatment versus foliar spray on tomato plants, foliar spray proved to be the best application method (Supraja et al., 2020b). Observation of the desired results relative to the controls occurred regardless of the application method. This phenomenon indicates that MA did indeed have beneficial effects on plants, although the impact may depend on the mode of application (Ciepiela et al., 2013).

In the present study, we chose the most straightforward application methods: soil drenching and foliar spraying of greenhouse-grown plants. Soil drenching has the potential to increase soil microbial diversity. Nonetheless, Chiaiese and coworkers (2018) reported foliar spray to be more effective than soil drenches. They opined that foliar application during high humidity when the stomata are open increases the permeability and uptake of the applied substance (Chiaiese et al., 2018). Combining the two application techniques could increase the likelihood of achieving positive results. This method would also permit simultaneous evaluation of the effects of MA on plants and the soil microbiome.

1.4 Microalgae bioactive compounds with biostimulant properties

Microalgae produce metabolites, including phytohormones or hormone-like substances, exopolysaccharides (EPS), free fatty acids (FFAs), phenolic compounds, carotenoids (Cars), terpenoids, and a vast array of volatile organic compounds (VOCs). Phytohormones are small molecules that regulate fundamental cellular processes in plants via signal transduction. Lu and Xu (2015) noted that the five classical phytohormones, auxins, abscisic acid (ABA), CK, gibberellins (GAs), and ET, had been detected in different algal lineages, including species from the Chlorophyta phylum. However, their physiological roles remain elusive despite their resemblance to the plant phytohormones. The same authors pointed out that the difficulty in deciphering the functional roles of MA phytohormones is because the evidence has been partly drawn from the effects of plant phytohormones on MA and partly from correlation studies between environmental stimuli and microalga endogenous hormone levels. Nonetheless, in recent studies, a few molecular studies have supposedly revealed the function of microalgal phytohormones (Lu and Xu, 2015).

In higher plants, indole acetic acid (IAA), an auxin, regulates growth and development and participates in stress tolerance. Genetic manipulation resulting in overexpression of the iaaM gene soared IAA levels which caused parthenocarpic fruit development in eggplants and tobacco (Rotino et al., 1997). Rotino and colleagues (1997) also reported that overexpression of Pin-formed (PIN) 3 protein, involved in auxin transport, enhanced tolerance to drought. In a thorough review, Lu and Xu (2015) summarized that in MA, exogenous IAA improved cell growth of *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Chlorella sorokiana*, *H. pluvialis*, and *Nostoc* sp. It also increased the oil content in *C. reinhardtii* and *H. pluvialis*. In *C. vulgaris*, IAA causes tolerance to extreme salinity and heat (Lu and Xu, 2015). All these reports confirm the critical role of IAA in abiotic stress tolerance and growth.

Abscisic acid plays a role in plants' development, growth, and stress tolerance. Genetic manipulation to decrease ABA content led to rapid germination of seeds, whereas manipulation increasing ABA content inhibited seed germination (Frey et al., 1999). Past studies reported that increased ABA content conferred drought tolerance in transgenic wild tobacco (Qin and Zeevaart, 2002). In MA, exogenous ABA caused a decline in the growth of two diatoms, namely *Coscinodiscus granii* and

Nannochloropsis oceanica (Kentzer and Mazur, 1991; Lu et al., 2014). However, it increased the tolerance of various MA strains to desiccation, higher salinity, nitrogen starvation, oxidative stress, and osmotic stress (Lu and Xu, 2015). Applying MA-producing ABA on plants may confer tolerance to many abiotic stresses.

Cytokinins are involved in higher plants' development, growth, and stress tolerance. A drop in CK content caused rapid plant growth, while the opposite stunted growth and delayed senescence (Rupp et al., 1999; Werner et al., 2001). In rice, elevated CK content increased tolerance to water deprivation and drought stress (Qin and Zeevaart, 2002). Reports of exogenous CK increasing growth rate and oil content of *C. reinhardtii, Phaeodactylum tricornutum, and H. pluvialis* exist in the literature (Maor, 2010). Kentzer and Mazur (1991) also reported the role of CK on *N. oceanica's* growth rate and cell-cycle progression under different light regimes. In seaweeds, CK accumulated in heat-stressed *Ecklonian maxima* and *Macrocystis pyrifera,* suggesting its role in response to heat (Stirk et al., 2004). Thus, MA-released CKs might aid plants in withstanding hot weather and scarce water, the typical drought conditions.

In plants, GAs are essential for development and growth. Silencing of genes involved in GA biosynthesis in *Arabidopsis* caused a semi-dwarf phenotype, whereas increased levels elongated the hypocotyls and induced early flowering (Coles et al., 1999; Huang et al., 1998). In MA, exogenous GA increased the growth rate in *C. reinhardtii* (Park et al., 2013) and triggered *H. pluvialis* to synthesize more astaxanthin (Lu et al., 2010). Therefore, treating plants with MA species that produce GAs could cause tall and early flowering phenotypes.

Ethylene in lower plants is responsible for development and senescence. Ju and coworkers (2015) reported that ET regulated cell development in, *Spirogyra platensis*, a primitive organism relative to higher plants. In contrast, ET played a crucial role in the programmed cell death of *C. reinhardtii* (Yordanova et al., 2010). In higher plants, genetic manipulation of genes involved in ET biosynthesis reduced ET levels and interfered with fruit ripening in cantaloupe melon fruits (Ayub et al., 1996). These reports show that ET from MA applied to plants or soil could affect fruit formation and ripening.

Mazhar and colleagues (2013) noted that significant plant growth parameters positively correlated with cyanobacterial CK and auxin levels. Furthermore, the

interaction between plants and the MA increased plant hormone levels. Signal transduction between MA and plants altered endogenous and exogenous auxin levels, indicating that MA influences plant growth via phytohormones. Given that phytohormones from plants and algae have a similar structure (Mazhar et al., 2013), exogenous application of MA-released phytohormones to plants could elicit similar effects as the classical plant phytohormones.

Microalgae species also produce FFAs that indirectly promote plant growth by inhibiting other microorganisms' growth, thus reducing competition for available resources. For example, Ördög and coworkers reported the antimicrobial activity of some Chlorophyta strains against both gram-positive and gram-negative bacteria (Ördög et al., 2004b). *Chlorella* spp. mainly produce *Chlorellin* (a mixture of fatty acids), which is antibacterial and algicidal (Pratt et al., 1944). *C. reinhardtii,* on the other hand, produces algicidal fatty acids (Proctor, 1957). These substances may help eradicate pathogenic microbes hence enabling optimal plant growth.

On the other hand, EPS increased plant tolerance to stress and mitigated salt stress effects on growth by regulating sodium and potassium cation assimilation (EL Arroussi et al., 2018). Bacterial hydrolytic enzymes may break down MA-released EPS into small oligosaccharides. Plants perceive these small units via membrane receptors and adjust essential enzyme activities, including nitrate reductase and nicotinamide adenine dinucleotide-glutamate dehydrogenase (NAD-GDH) in nitrogen assimilation, thus affecting growth. Moreover, polysaccharides can increase NAD phosphate hydrogen (NADPH)-synthesizing enzymes, ascorbate peroxidase (APX), and ascorbate levels, which are essential in photosynthesis, cellular metabolism, and the cell cycle. Furthermore, Kang et al. (2021) reported that treating plants with polysaccharides triggered the upregulation of genes involved in signaling pathways such as salicylic acid (SA) and jasmonic acid (JA). Upregulation of these pathways consequently increased B-1-3 glucanase and phenylalanine-ammonia-lyase (PAL) activities which are central in plant defense systems (Kang et al., 2021).

Various algae produce VOCs such as terpenoids, esters, furans, alcohols, and ketones under different environmental conditions (Zuo, 2019). Typically, water bodies obtain their odor from the VOCs released by algae. According to Zuo, VOCs serve as communication signals; they increase stress tolerance in homogeneous algae but are

allelopathic to heterogeneous algae, macrophytes, and predators (Zuo, 2019). *C. reinhardtii* produces aldehydes, ketones, terpenoids, and alcohols under normal conditions, but when the cells encounter NaCl, $NaCO_3$, and acetic acid, they increase their VOC biosynthesis (Zuo et al., 2012a, 2012b). VOCs generally serve ecological functions in ecosystems where emitters are present (Zuo, 2019). In the soil, MA-released VOCs may attract microorganisms beneficial to plants, such as the plant growth-promoting rhizobacteria (PGPRs).

1.5 Microalgae effects on plants

Microalgae species induce cellular and molecular responses in plants, which may or may not manifest phenotypically, as summarized by Baltazar and colleagues (2021) in Figure 1.1. MA promoted plant growth by increasing both fresh and dry weight (Faheed and Fattah, 2008) as well as the chlorophyll (Chl) content of leaves (Barone et al., 2019a). Priming of *Phaseolus vulgaris* and *Triticum vulgaris* seeds with *Chlorella* EPS stimulated plant growth and increased fresh and dry weight, leaf area, shoot height, and root length (El-Naggar et al., 2020).

In a past study, algae-based biostimulants increased plant flowering and crop yields (Plaza et al., 2018). They improved the nutritional value and shelf life of fruits in several crops. Mannino and coworkers (2020) reported that treating tomato plants with biostimulants quickened fruit ripened and increased essential micronutrients and unsaturated fatty acids content (Mannino et al., 2020). Moreover, Cardarelli and coworkers observed improved fruit weight, Vitamin C, and lycopene content in biostimulant-treated tomato plants (Cardarelli et al., 2020).

Biostimulants enhanced the synthesis of photosynthetic pigments (Chls and Cars) at the cellular level, resulting in enhanced photosynthetic performance (Baltazar et al., 2021). Increased photosynthesis occurs with increased mineral uptake and transport, as well as improved nutrient uptake. Biostimulants also influenced stomatal conductance and relative water content, thus affecting the plant's stress-response mechanism. They also enhance antioxidant activity, thereby increasing plant tolerance to numerous types of stress.

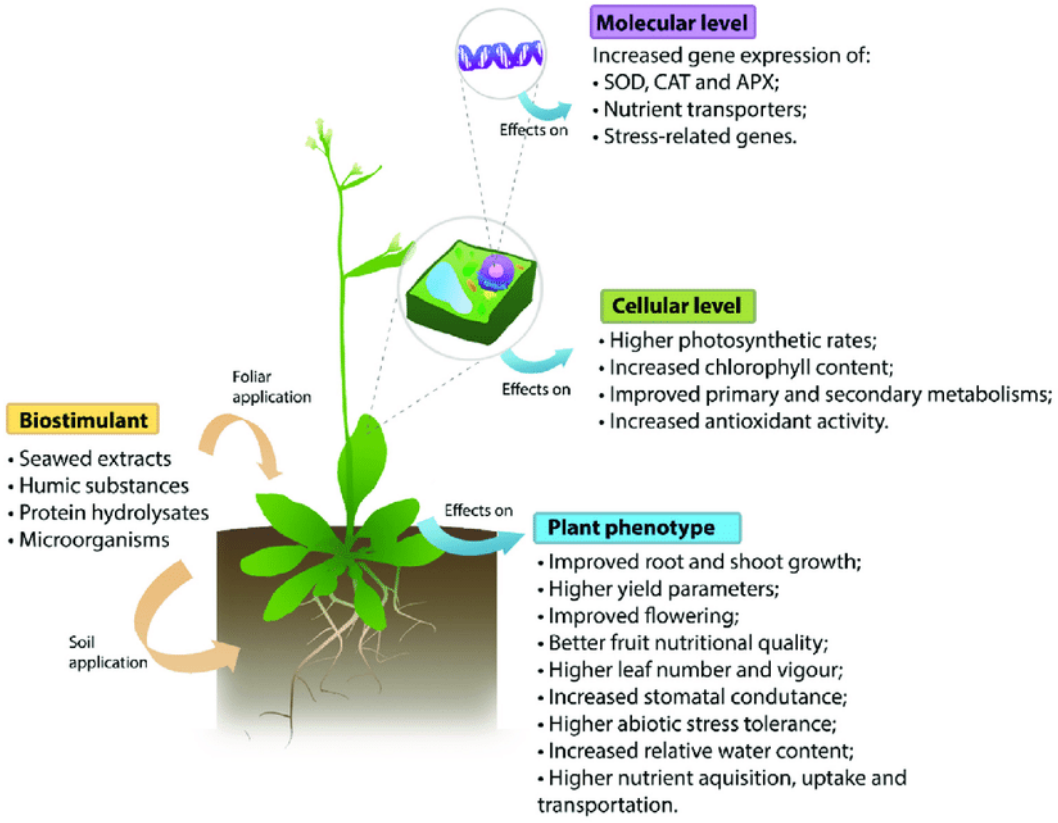

Figure 1.1. Summary of the effects of biostimulants at the cellular, molecular, and plant phenotype level (Baltazar et al., 2021).

At the molecular level, upregulation of genes responsible for antioxidant enzymes like APX, catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) occurs, as shown in Figure 1.1 (Baltazar et al., 2021). Applying biostimulants affected the expression of genes involved in nutrient transport and stress response. In tomato plants, the expression of genes involved in carbon and nitrogen metabolism and genes that control the synthesis of terpenes and phenols increased after biostimulant application (Ertani et al., 2017). Moreover, biostimulant application affected plant flowering (Plaza et al., 2018). Consequently, MA may influence the gene expression of flowering genes. These genes include the florigenic gene and its transcription factors. This phenomenon influences the transition from the vegetative phase to the reproductive state, which manifests in the architecture of plants.

In addition to promoting plant growth, biostimulants from microorganisms induce systemic resistance in plants. Yu and co-authors (2022) defined 'induced resistance' as the enhanced defensive capacity that plants develop in response to an appropriate stimuli application. Bacteria, fungi, and viruses can induce systemic

resistance in plants (Yu et al., 2022). Recent studies have demonstrated that MA and MA-derived substances can induce systemic resistance through various application methods. For example, spraying tomato leaves with polysaccharides extracted from different cyanobacteria strains induced systemic resistance (Rachidi et al., 2021). Moreover, sonicated extracts of various MA enhanced resistance to bacterial canker disease in tomato seedlings (Toribio et al., 2021). In Cucumber, leaves infected with *Colletotrichum orbiculare* exhibited cytological modifications, such as assemblage of vesicles and thickening of cell walls, due to a prior foliar spray of *Chlorella fuscus* suspensions (Kim et al., 2018).

Assessing plant height, diameter, leaf number, flower number, and fruit number, among other phenotypic parameters, is the easiest way of studying growth promotion. Challenging the plants with stress or pathogen infection followed by disease index assessment and determination of infection markers can aid in detecting induced resistance. Transcriptomics, proteomics, and metabolomics approaches are also valuable for assessing induced resistance. De novo data of transcriptome studies can inspire experimental designs to gain deeper insights into the signaling pathways underlying upregulated defense-related genes and pathways.

Determining the molecular effect of MA with transcriptomic studies is possible. All the genetic information in an organism is, in the form of deoxyribonucleic acid (DNA), in heredity units called genes in the cell nucleus. For growth, development, and responses to external stimuli, decoding this information must ensue in a process that follows the central dogma of molecular biology (Kukurba and Montgomery, 2015). DNA information decoding from the double-stranded code version to the single-stranded code version happens during transcription. Then the coded information from the ribonucleic acid (RNA) is used as instructions for assembling molecules necessary for different responses in a translation process. Transcription involves the transfer of DNA information into the messenger ribonucleic acid (mRNA), which passes from the nucleus to the ribosomes in the cytoplasm, where the translation of the message into proteins with the aid of transfer RNA (tRNA) transpires. Therefore, gene expression involves transcription and translation to convert DNA-coded instructions into functional products. Transcriptomic studies focus on the transcription process of gene expression. This process is highly regulated to control the synthesis of proteins; it can increase or decrease the level of proteins accordingly.

In transcriptomic studies, quantifying the mRNA levels allows the resolution of gene activity. A gene can either be downregulated or upregulated compared to a reference condition or treatment. High levels of mRNA show upregulation, while low levels show downregulation. Extraction of the total RNA of a sample followed by reverse transcription of mature mRNA only, using primers that bind to the Poly A tail of the mRNA, to make complementary DNA (cDNA) is the first process. Sequencing (reading the coded DNA information of the cDNA) to generate RNA sequence data follows. After obtaining the DNA sequences/information, they are mapped against the whole genome for identification and functional annotation. The more matches to a particular part of the genome, the higher the expression levels of that part of the genome, and the reverse is true. Comparison of transcripts from the control and treatment samples followed by statistical analysis to reveal if the overexpression or underexpression observed in a study occurred by chance or due to the treatment/condition is the final step.

1.6 Microalgae effects on soil

Microscopic algae are photosynthetic organisms responsible for nearly half of the photosynthesis occurring on Earth (Moroney and Ynalvez, 2009). They absorb carbon dioxide, thereby raising the carbon content of the soil. In addition, they integrate carbon into their organelles or in their cell surfaces in the form of EPS, thereby increasing the organic carbon content of the soil. Consequently, more microbes, microflora, and fauna colonize the soil. Eventually, the decomposition of these organisms further increases the soil's organic content. In a recent report, green algae biofertilizers improved microbial activity, microbial biomass, and the total soil organic carbon content (Guo et al., 2020).

In a review of algae application in agriculture, Renuka and colleagues (2018) noted that green MA species do not fix nitrogen like cyanobacteria but contribute towards the formation of biological soil crusts, preventing soil nitrogen leaching (Renuka et al., 2018). Algae affect soil fertility by mineralizing and solubilizing nutrients. Cyanobacteria are well-known to produce organic acids such as humic acids, whereas green algae produce EPS that can facilitate the bio-absorption of minerals such as phosphates. Green algae, including *Chlorella* and *Scenedesmus* species, can increase iron availability by producing metal chelators such as siderophores (Renuka et al., 2018). Siderophores are organic substances that assist in the chelation of ferric iron under iron-deficient conditions making iron available for uptake by microorganisms and plants (Ahmed and Holmström, 2014). Chelation is an ionic or molecular bonding to metal ions. Ahmed and Holmström (2014) emphasized that siderophores help weather soil minerals, enhance plant growth, and function as biocontrol, biosensors, and bioremediation compounds. Guo and coworkers also reported that green algae, bacteria, and cyanobacteria consortia augmented Mn, Cu, and Zn in plants (Guo et al., 2020). All the above reports show the multifarious roles of algae in modifying soil chemical properties to support plant growth.

The application of MA also impacts the physical properties of soil. Yilmaz and Sönmez documented that *Chlorella* species influenced micro soil aggregates by creating particles representing the minimal pore size required for root penetration and plant growth (Yilmaz and Sönmez, 2017). The formation of aggregated soil particles from algal EPS improves soil porosity and may also prevent soil erosion.

Barone and coworkers found that MA living cells and extracts modified soil enzyme activity and total soil biological activity (Barone et al., 2019b). They assessed the activity of fluorescein diacetate hydrolysis, dehydrogenase, urease, and acid and alkaline phosphomonoesters enzymes which changed upon MA treatment. De Caire and coworkers, on the other hand, reported that adding cyanobacteria and their EPS to soil increased the activity of soil-glycosidase, protease, phosphomonoesterase, arylsulphatase, and dehydrogenase (De Caire et al., 2000). Therefore, applying MA to soil affects the soil's chemical properties.

There are millions of microorganisms in the soil, including bacteria, fungi, algae, archaea, and viruses. In their work, Varma and coworkers (2019) put forward that plants interact with the microbes in the soil. Usually, plant roots exude some substances in the soil, which may attract or repel some microbes, ultimately creating a distinctive microbial community around the root biomass. Those microbes penetrating the root surface are the endosymbionts, while those occurring in the soil adjacent to the roots are said to be in the rhizosphere. A plant's rhizosphere is a biologically active interphase that varies from one species to another and is entirely distinct from the bulk soil (Varma et al., 2019). Studying a plant's rhizosphere can provide details about plant-microbe interaction, nutrient cycling, soil organic matter decomposition regulation, and root metabolism. In such studies, soil samples are collected and used as inoculants in microbial growth media in the laboratory. Then extracting and sequencing the DNA of the growing microbes allows their identification. However, this process is time-consuming and requires trials of different growth media because different microbes have different nutrient requirements. Furthermore, not all microbes present in the soil are culturable in the laboratory. A modern solution to this problem is soil metagenomics studies.

Metagenomics studies combine modern genomics techniques to reveal the operations of members of a microbial community and their interactions with their surroundings. It involves identifying and characterizing all the genomes present in a sample. With the invention of next-generation sequencing (NGS), it is possible to investigate the functional genetic diversity of various microbes without growing them in the laboratory. The invention of shotgun metagenomics sequencing has streamlined metagenomics studies while increasing the wealth of information gathered from a single study. All that is required is the DNA from the samples/soil of interest. Then a library

of clones is constructed and screened before sequencing and analysis of the isolated metagenomics DNA supplying information on the microbial life from the studied sample.

Innately, physical, chemical, and biological forces perturb microbial activity and population in the soil. Small shifts affecting plant growth, including nutrient levels, profoundly impact microbial communities. Therefore, adding microbes in the form of biostimulants affects the soil communities, and soil metagenomics studies are valuable for assessing these communities. Comparison of the control soils against the treated soils can allow discernment of the action of the added organism. Soil metagenomics studies enable us to answer these two questions: "Which microbe is present?" and "What role is it playing in the soil?".

1.7 *Medicago truncatula* as a model plant

Medicago truncatula is a model plant of the *Fabaceae* family, the third-largest angiosperm family, and the second most important to humans after the *Gramineae* (*Poaceae*) family (Ciepiela et al., 2013). This family contains several commercial crops, including soybean, garden pea, peanut, and alfalfa, the world's most cultivated and valuable forage plant. These plants are important sources of oil and protein for animals and humans. This nutritious property is because legumes can fix atmospheric nitrogen and assimilate it into crop produce, such as seeds for human consumption or forage for animal feed. Their ability to fix nitrogen also improves soil fertility enabling sustainable farming by eradicating the need to supply nitrogen fertilizers in soils. In addition, they are candidates for use as fuelwood because they sequester carbon. Fuelwood is wood subjected to combustion to release heat. After seed harvesting, the legume biomass from farms can serve in heating or cooking in homes or industrial processes. This substitution of trees with legume biomass can substantially reduce deforestation.

In addition, *M. truncatula* phenotyping has been standardized, allowing for an efficient method of assessing developmental milestones during plant growth (Bucciarelli et al., 2006). The nomenclature phenotyping coding system made *M. truncatula* an appropriate model for biostimulant-related plant growth experiments.

Based on this nomenclature, metamers are labeled along the central axis from bottom to top as M1, M2, M3, etcetera, as shown in Figure 1.2. A metamer is the section of a plant composed of an internode, a bud, and the associated leaf. A decimal code ranging from 0.1 to 0.9, beginning with the bud stage and ending with the fully expanded, blue-green leaf, depicts the developmental stage of the plant parts.

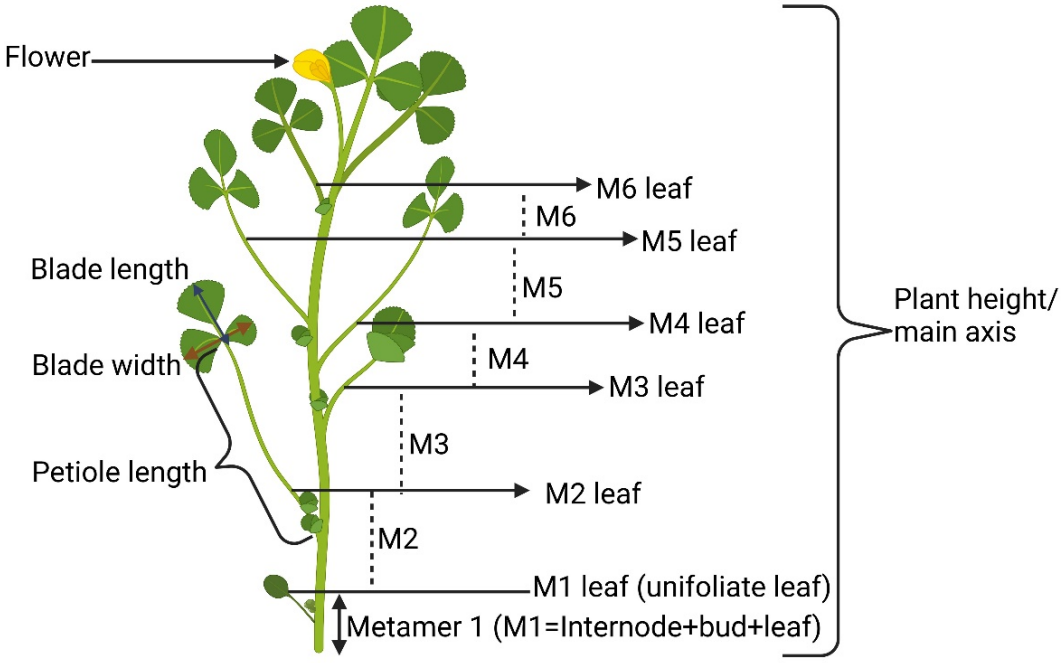

Figure 1.2. Illustrative diagram of *Medicago truncatula*, created with BioRender.com, showing the different measured phenotypic parameters. Metamers (internode, leaf, and bud) and their associated leaves are labeled from the bottom to the top along the central axis in ascending order. The red arrow on the second leaf depicts the blade width, the dark blue arrow depicts the blade length, and the brackets show the petiole length (Bucciarelli et al., 2006; Gitau et al., 2021).

Although most analyses on legumes focus on their interaction with PGPRs, a few investigations have evaluated the effects of seaweeds and MA biostimulants on these plants (Brahmbhatt, 2015; El-Sharkawy et al., 2017; Kavipriya et al., 2011; Kocira et al., 2018; Navarro-López et al., 2020; Paulert et al., 2009; Rengasamy et al., 2014; Sosnowski et al., 2017).

Most examinations on growth promotion in *M. truncatula* and its relatives, such as *M. sativa,* concentrate on the effects of growth-promoting bacteria (Bianco and Defez, 2009; Chinnaswamy et al., 2018; Kępczyńska and Karczyński, 2020; Viaene et al., 2016). In addition to focusing on the effects of these microorganisms on root

development and nodulation, these studies pay scant attention to plant architecture and leaf morphology. A few studies have reported the effect of algae biostimulants on legumes, and some are about seaweeds' effects on plants under normal or salt stress conditions (El-Sharkawy et al., 2017; Telekalo and Melnyk, 2020). To the best of our knowledge, however, no study has investigated the effects of axenic monocultures of Chlorophyta MA on *M. truncatula* plants.

Measuring leaf parameters of the compound leaf (blade length, width, and petiole length) based on the described nomenclature plus plant height is a simple method for comparing the growth of *M. truncatula* under different experimental conditions.

The other critical parameter analyzed during plant phenotyping is the pigment content. Chls and Cars are essential for absorbing light energy in photosynthetic organisms. In higher plants, two main Chls, *a* and *b,* occur in association with the integral proteins of the thylakoid membrane in the chloroplast (McDonald, 2003). When white light hits the leaf's surface, absorption of the red and blue wavelengths and reflection of the green wavelength occurs. This reflection gives plants the green color we see. Chl *a* and *b* are the green pigments in plants that absorb light at different wavelengths—their distribution in the chloroplast enhances light absorption. Chl *a* is the primary absorption pigment, while Chl *b* is an accessory pigment that absorbs light energy and then transfers it to Chl *a*. Chl *a* absorbs red, violet, and orange light (620-680nm) the most while Chl *b* absorbs blue and yellow light (420-450nm). The core reaction center primarily contains Chl *a*, while the light-harvesting complexes contain Chl *b*. The ratio between Chl *a* and *b* indicates how well a plant can respond to environmental stress or perturbations, exceptionally light stimuli. For example, a reduced Chl *a*: *b* ratio correlates to an increase in the size of the light-harvesting complex. Having a sizeable light-harvesting complex and a smaller reaction center in low light is profitable. In plants, Chl *a*: *b* and Chl: Car ratios change in response to various stresses.

On the other hand, Cars are the yellow, red and orange pigments responsible for the orange-colored leaves of plants during Autumn. They also capture light from the blue-green spectrum and pass it to the Chls, but their principal role is the protection of the photosynthetic apparatus. Young (1991) noted that the conversion of violaxanthin

Car into zeaxanthin was the chief pathway in the regulation of heat dissipation of photosystem II (PS II) when there was a surplus in energy and photochemistry could not consume all the energy (Young, 1991). Thus, Cars are essential because even in normal conditions, plants are overwhelmed by excess energy and need to protect their photosynthetic apparatus. In stressful conditions, this energy is even more destructive and necessitates the adjustment of Chl: Car ratios in favor of Car accumulation. The pigment content parameter in plants is, hence, essential in determining the onset of stress or predicting the ability of plants to respond to stress.

1.8 *Solanum lycopersicum* as a model plant

Tomatoes are among the most widely consumed vegetables in the world; salads, soups, purees, sauces, and pastes are some forms of ready-for-consumption tomato products. In addition to the delightful taste, they are devoured predominantly for their nutritional, nutraceutical, and antioxidant content (Giudice et al., 2017). Tomatoes are simple to propagate, making them indispensable for satisfying global food demand and assuring food security (Supraja et al., 2020a). The tomato plant, unlike other crops, can grow in all types of soil, but it has heightened nutritional requirements. Nutrition has a consequential impact on crop/fruit quality. Biostimulants could reduce tomato farming costs, particularly in low-fertility soils. The close relationship of tomato to numerous crops belonging to the Asterid clade, such as yerba mate, coffee, tea, tobacco, sunflower, and *Petunia,* justifies the preference of tomato over *Arabidopsis thaliana* for biostimulant studies. Thus, the knowledge from tomato biostimulant studies extends broadly to other crops.

The primary parameters dissected in tomato plants are those associated with the marketability of the fruit. These traits include fruit size (diameter and length), number per plant, firmness, weight, and mineral content, which includes total soluble sugars and lycopene content. Evaluation of the flowering stage is crucial because it correlates with crop harvest time.

In addition to these parameters, plant photosynthetic performance is essential because it correlates with each of the abovementioned parameters. Photosynthesis refers to the process by which plants use light energy, water, and carbon dioxide to

make sugars and give out oxygen as a byproduct. In the leaf, photosynthesis occurs in the chloroplast, which comprises two photosystems and the thylakoid membranes containing Chls responsible for absorbing light at different wavelengths. In brief, photosynthesis occurs in four steps; it begins with light energy absorption, electron transport, adenosine triphosphate (ATP) synthesis, and carbon fixation in that order. Determining photosynthetic performance includes measuring the proportion of light energy channeled to photosynthesis/photochemistry (maximum yield (Fv/Fm) or PS II yield (Phi2), the proportion of energy lost via regulated non-photochemical quenching (PhiNPQ), and the proportion of energy lost via unregulated non-photochemical quenching (PhiNO). To determine the activity of PS II, linear electron flow (LEF) is also measured.

Linear electron flow/transport is the photosynthetic electron transfer pathway that involves both PS I and PS II and supplies ATP and NADPH to the Calvin cycle (Joliot and Johnson, 2011). LEF represents the flow of electrons from the oxygen-evolving complex (OEC) to the NADP+, reducing it to NADPH after incoming light energy splits water molecules (Huang et al., 2018). This flow of electrons generates a proton motive force across the thylakoid membrane, which powers the production of ATP. Measuring LEF provides information about a plant's ATP generation capacity.

Other essential parameters include soil plant analysis development (SPAD). The SPAD value, the relative Chl content per leaf surface area, is a dimensionless parameter that strongly relates to the actual Chl content (Markwell et al., 1995; Monje and Bugbee, 1992; Parry et al., 2014). The nitrogen content strongly influences the Chl content (Cartelat et al., 2005; Samborski et al., 2009; Schepers et al., 1996). The chloroplast contains 80% of the leaf nitrogen, while about 50% is in photosynthetic proteins (Xiong et al., 2015). Thus, the SPAD value indicates a plant's nitrogen status (Culman et al., 2013; Xiong et al., 2015) and is valuable for cold acclimation and tolerance selection.

The leaf thickness and leaf temperature differential (LTD) are other essential parameters in plant phenotyping. Leaf thickness correlates to the leaf's internal morphology, and increased leaf thickness may indicate increased cell division or expansion. The LTD, on the other hand, is the ratio between the leaf surface and ambient temperature. The LTD indicates how well a plant performs under adverse conditions, such as extreme heat and drought. All parameters mentioned above are

simultaneously measured using a portable absorbance/fluorescence measuring device, as elaborated in the methodology section 3.3.2.

Contrary to the case of *M. truncatula*, there are numerous reports of the biostimulant effect of *Chlorella* strains on tomato plants. More than three-quarters of these studies apply cell extracts to plant/soil systems rather than algal suspensions containing living cells. In contrast, there are remarkably few reports of *C. reinhardtii* promoting plant growth and even these involve the maize crop (Martini et al., 2021). *Chlorella* strains are hardy and can withstand a broad range of environmental conditions, while the literature has the exhaustive characterization of *C. reinhardtii* as the benchmark green MA. Thus, members of these two genera (*Chlorella* and *Chlamydomonas*) are excellent representatives for MA biostimulant research.

2. Research objectives

The primary objective of this study was to assess the biostimulant effect of unprocessed Chlorophyta MA species on plant growth. In our studies, we performed experiments on two model plants; these were *M. truncatula* and *S. lycopersicum*.

The first part of the study was a comparative analysis of the growth-promoting effects of two *Chlorella* strains and one *Chlamydomonas* strain administered to *M. truncatula*. We delivered live algal biomass to plants via the soil drench technique. We tested the hypothesis that algal cells would affect yield and quality-determining parameters. These characteristics included the plant's structure and morphology, height, number of flowers, biomass, and pigment content. The goal was:

- To examine the strain-specific effects of selected green eukaryotic MA on *M. truncatula* grown under controlled conditions in a greenhouse.

In the second part of the study, we selected strains with promising biostimulant effects on *M. truncatula* and tested their effect on *S. lycopersicum* (tomato) plants. We screened the algal strains for their ability to produce auxins, polysaccharides, and form aggregates. The localization of the detected bioactive compounds would therefore affect the method of biostimulant preparation. The method with the growth media removal step would result in the loss of molecules released into the media. The preparation without further processing, i.e., using cultures straight from the incubator, would retain the bioactive compounds in the treatment. On the other hand, the destruction of cells by homogenization would release the bioactive compounds accumulated inside the algal cells.

The second series of experiments hypothesized that applying the different algae preparations would positively influence plant growth, crop yield, and quality. However, the magnitude of these effects could differ depending on the preparation method or the age of plants during the first biostimulant application. Therefore, we tested three algae treatments: A, B, and C.

Treatment A consisted of the cells and their growth media. Treatment B's preparation included the removal of the growth media and suspending the algae cells in sterile distilled water (DW). Treatment C's preparation involved removing the growth

media, freezing the resultant pellet, homogenizing, and suspending the slurry in water. We drenched soils with treatments A[1] and B[2] and sprayed plant leaves with treatment C. These were the objectives of this study:

1. Determine if soil treatment with algae cells (A or B) in conjunction with extract foliar spray (C[3]) promoted plant growth.
2. Determine whether there was a difference between early (one-week-old plants) and late applications of algae biostimulants to plants (five-week-old plants).
3. Determine any strain-specific effects of the MA on tomato plants based on morphological (plant height and diameter), reproductive (flower and fruit development), and physiological (photosynthesis) analyses.
4. Evaluate the effects of MA on the whole-tissue transcriptome of unopened flower buds.
5. Evaluate the effects of MA on the rhizosphere's microbial community.

[1] A=Live cells with growth media applied as a soil drench

[2] B=Live cells without growth media applied as a soil drench

[3] C= Destroyed cells suspended in water applied as a foliar spray

3. Resources and Methods

3.1 Selection and characterization of microalgae species

3.1.1 Comparative growth and cell number analysis

Based on their rapid biomass accumulation, we chose two MA species from the Mosonmagyaróvár Algal Culture Collection (MACC) belonging to the *Chlorella* genus (*Chlorella* sp. MACC-360 and *Chlorella* sp. MACC-38) and *C. reinhardtii* cc124 from the Institute of plant biology, Biological Research Centre, Szeged for plant biostimulant studies.

However, it was essential to compare their growth pattern and cell cycles to derive any species-specific effects. This step was crucial because secondary metabolite production depends on an organism's physiological state, which depends on the growth pattern—with the accumulation of most of the bioactive compounds usually occurring during the stationary phase. The cell number counts could also be necessary for standardizing the experiments to ensure the application of an equal number of cells every time for cell-number-sensitive assays.

The surface of a seven-day-old lawn algae culture on Tris-acetate-phosphate (TAP)-agar was scrubbed with a sterile rod and dipped into a 10 mL falcon tube containing 5 mL of TAP media under aseptic conditions (Harris, 1989). Before capping, the mouth of the falcon tube was sterilized by passing over the flame. The tubes were placed in an incubator with the following settings: 25 °C temperature, 16/8-hour light/dark cycle, white light, and 180 revolutions per minute (rpm) shaker speed. After three days, cultures' optical density (OD) was measured by spectrophotometer at 750 nm absorbance. The cultures were then used to inoculate 1500 µL TAP media in 24-well plates to produce cultures with a final OD of 0.2 at 750 nm. Each strain underwent six replications. Additionally, a blank was maintained and replicated six times. The plate was placed in the incubator, and the OD was measured daily using a Hidex Sense microplate reader (Hidex, Turku, Finland).

Three-day-old starter cultures were inoculated to an initial OD of 0.2 in 25 mL TAP medium. Two flasks were prepared for each strain. The flasks were incubated in the incubator described in the previous paragraph. For cell number determination, 100

µL was extracted from the flasks and diluted to 1 mL with water. Further dilution was made if necessary depending on the culture density. Then, 10 µL of the diluted culture was placed on Luna slides, and the cell count was determined using the fluorescent algae protocol on the Luna Automated cell counter (Luna FL-Logos Biosystems). Daily for five days, cell counts were determined at the same time. The means of daily cell counts from two replicates (two flasks) were plotted individually with GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA).

3.1.2 Confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM)

We conducted microscopy to visualize morphological differences between the algal strains to help understand their mode of action as biostimulants. We also followed their growth by conducting microscopy on different days after inoculation to establish the pattern of the biosynthesis of extracellular substances and aggregation.

For CLSM, 50 µL of algae culture was drawn into an Eppendorf tube and stained with Calcofluor-White (CFW) with 300/400 excitation-emission wavelength and CF®488A Conacavalin A (Con A) with 490/515 excitation-emission wavelength (Sigma-Aldrich). Bhavasar and colleagues (2010) described CFW as a disodium salt colorless dye usually used as a whitening agent in the textile and paper industries. It specifically binds to cellulose and chitin in microbes and is commonly used in fungal screening tests like the detection of candida (Bhavasar et al., 2010). Con A, on the other hand, is a conjugate lectin dye (Con A-Alexa Fluor 488 conjugate) used in microscopy and flow cytometry for the detection of glycoconjugates and glycoproteins, respectively. It binds to the glucose and mannose residues in cell wall polysaccharides to give off a green fluorescence (Chandra et al., 2001). Con A selectively stains the surfaces of live cells, although it can endure permeabilization and fixation. After penetrating the cell surface, Con A stains both the cell surface and secretory organelles. In our study, fixation was unnecessary as we aimed to visualize live cells; the two dyes were suitable for cell wall and surface analysis.

Each dye was prepared separately by dissolving the powders in water at 10g/L concentration. 0.5 µL CFW and 0.5 µL of Con A were added to the tube containing algae cells and incubated in the dark. After 30 minutes, 8 µL of cells were spotted on

the microscope slides, covered with 2% (w/v) agar slices, and observed using an Olympus Fluoview FV 1000 confocal laser microscope with a 60 × objective (Olympus Optical Co., Ltd., Tokyo, Japan). We chose the sequential scanning protocol to avoid crosstalk between the fluorescent dyes and Chl autofluorescence. CFW was first scanned at between 425-480nm, followed by Con A between 495-515nm, and finally, the chloroplast autofluorescence was captured at full range at 640-750nm. The scans were analyzed and merged into a final image comprising the three channels with Olympus FluoView version 4.2b viewer (Olympus).

For SEM investigations, 8 µL of algae from each strain were spotted on a silicon disc coated with 0.01% (w/v) poly-l-lysine (Merck Millipore, Billerica, MA, USA). Cells were fixed overnight at 4 °C with 2.5% (v/v) glutaraldehyde and 0.05 µM (pH 7.2) cacodylate buffer in potassium-buffered saline (PBS). The discs were washed twice with PBS and dehydrated with a series of increasing concentrations of ethanol (30%, 50%, 70%, 80%, and 100% ethanol (v/v) for 1.5 hours each at 4 °C). The samples were dried with a critical point dryer, then coated with 12 nm of gold (Quorum Technologies, Laughton, East Sussex, UK), and viewed with a JEOL JSM-7100F/LV scanning electron microscope (JEOL Ltd., Tokyo, Japan).

3.1.3 Detection of auxin content in tryptophan-enriched cultures

We analyzed the ability of the strains to produce auxins as a representative of the other phytohormones because a colorimetric procedure can easily measure the IAA auxin. Our goal was to confirm if the tested strains produced any auxins and evaluate if the auxin quantities differed between strains. These results would help in deciphering any species-specific growth promotion effects observed on plants.

Inoculated starter cultures of each strain were grown for five days in TAP media. Ten mL of five-day-old cultures were used to inoculate 25 mL of TAP containing 1 g/L tryptophan. Two replicates (flasks) were prepared for each strain and placed in the incubator under the previously described conditions. After seven days of growth, the presence of IAA was determined by colorimetric assay using Salkowski reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) early in the morning, just at the end of the dark cycle of the 16/8 light/dark cycle (Gang et al., 2019). Briefly, the cultures

were centrifuged, and 100 µL of the supernatant was transferred into two wells of a 96-well plate.

To prepare the standard curve, 1mg/mL of IAA solution was diluted with TAP media to make a 5 µg/mL concentration. This solution was serially diluted with TAP media at a dilution factor of 2 to make a series ranging between 5 to 0.005 µg/ml. A TAP media blank was also prepared (0 µg/mL IAA). 100 µL of each of these standards was transferred to 4 wells (as replicates) of a 96 well-plate.

A 100 µL of Salkowski reagent was transferred into each of the wells, and the plate was incubated in the dark at 30°C for 30 minutes to allow the development of pink color. In the Hidex plate reader, the samples and the standards were marked accordingly during the plate setup step. The absorbance at 536 nm was measured, and the output file contained the standard curve and the quantities of IAA present in each well. Manual calculations were executed using the linear equation of the standard curve to confirm the results, as shown in the supplementary (S) figure, Figure S3.1.

3.1.4 Preparation of the algae for plant treatment

We prepared different categories of algal biostimulants for use as treatments because the method of treatment preparation affects the contents and hence the performance of the biostimulant. Thus, it was essential to have the algae culture separated into various components, including the supernatant, the live cells only, and the destroyed cells (extracts). The supernatant would, therefore, contain substances released by the cells, which with time, could get depleted after plant use. Cells alone could continue to release substances in the soil after application, while the extracts immediately availed substances produced and retained within the cells. This assumption explains why we used cells, the whole culture (cells and growth media), and the water extracts.

Algae strains were grown in TAP media with a pH of 7 to prepare the treatments. Under aseptic conditions, a sterile rod was used to scrub the surface of a fully grown lawn algae culture from a TAP-agar plate. The rod was dipped in a 50 mL Erlenmeyer flask containing 15 mL TAP media. Before capping, the mouth of the flask was sterilized by passing over the flame. The flasks were then placed in an algal growth

chamber with the following conditions: 25 °C temperature, 16/8-hour light/dark cycle, white light, and 180 rpm shaker speed. 5 mL of the culture was transferred after five days into a 100 mL Erlenmeyer flask containing 50 mL TAP media and placed in the growth chamber with the aforementioned conditions. The cultures were allowed to develop for seven days. On the seventh day, 5 mL of the culture was transferred into a new conical flask containing 50 mL of TAP medium to initiate the next application's culture. 50 mL was used to prepare the algae treatment. The treatment fell into three categories, namely A[4], B[5], and C[6], as illustrated in Figure 3.1.

The seven-day-old cultures were transferred to 50 mL falcon tubes and centrifuged for 15 minutes at 4542 ×g (Multifuge 3L-R Heraeus, Hanau, Germany). The supernatant was discarded, and the cells were suspended in 50 mL sterile DW. The suspension was centrifuged at 4542 ×g for 15 minutes, and the supernatant was discarded. The fresh biomass of the harvested algae was calculated by weighing the pellet-containing tubes and subtracting the weight of the empty tubes. The pellet was resuspended in sterile DW at a concentration of approximately 1g/L to make the living cell treatment for the soil drench (B). The control consisted of sterile DW.

For experiments in which plants were treated with culture suspension (A=living cells with their growth media), 50 mL of suspension from a 100 mL Erlenmeyer flask was diluted to create a final volume of 1 L containing approximately 1 g/L of algal biomass. Each pot received 200 mL of treatment or DW for tomato experiments. For *M. truncatula* experiments, treatment was poured at the bottom of the boxes with potted plants and rose into the soil by capillarity.

In order to prepare the algae-water extracts (treatment C=cell disrupted algae suspension) for foliar application, 50 mL of culture was centrifuged for 15 minutes at 4542 ×g. The supernatant was removed, the fresh algal biomass was calculated, and the pellet was transferred to a mortar and frozen with liquid nitrogen. As the pellet began to thaw, it was crushed with a pestle to break up the cells and create a slurry. This slurry was then diluted with DW to a final volume of 300 mL with a concentration of approximately 3 g/L algal biomass for the B+C experiments. For A+C experiments, the

[4] A= Live cells with growth media applied as a soil drench

[5] B= Live cells without growth media applied as a soil drench

[6] C=Destroyed cells suspended in water applied as a foliar spray

slurry was brought to 5 mL with the recovered supernatant and then diluted to 300 mL with DW to produce a cell extract containing approximately 3 g/L of algal biomass. This cell extract was transferred into spray bottles, a separate bottle for each MA strain. For B+C experiments, 300 mL of DW served as the control. For A+C experiments, the controls were DW and TAP (5 mL), diluting the latter to 300 mL with water.

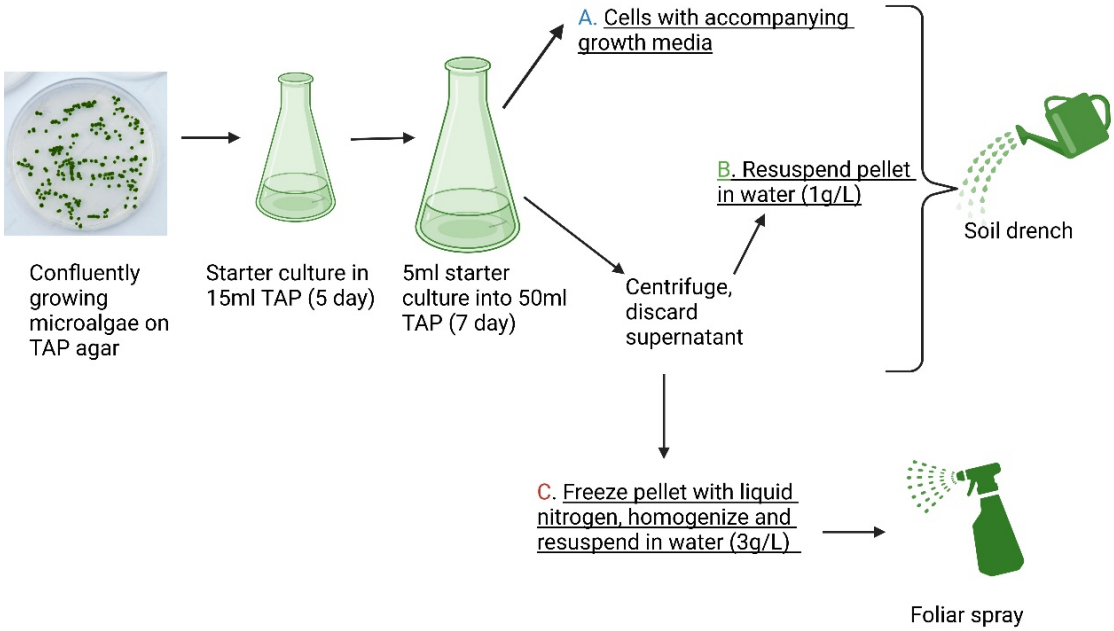

Figure 3.1. Preparation of algae culture into the three types of treatment; A= Culture (cells in their growth media), B= Cells only and C=Destroyed/homogenized cells. All the treatments were diluted in water before application to a concentration of 1g/L wet biomass for A and B and 3g/L wet biomass for C. Figure was created with Biorender.

Treatments A and B were applied as soil drenches, while treatment C was applied as a foliar spray. *M. truncatula* only received treatment B, while *S. lycopersicum* received all three treatments at different regimes explained in section 3.1.5.

3.1.5 Experimental design

In the first phase of the studies, *M. truncatula* received soil drench treatment from *C. reinhardtii cc124*, *Chlorella sp. MACC-38* and *Chlorella sp. MACC-360,* as shown in Figure 3.2. The treatment consisting of algae cells suspended in water was applied weekly for seven weeks. Experiments with *M. truncatula* were a screening process for identifying strains with potential biostimulant properties. After these experiments, *Chlorella sp. MACC-38* was dropped from further studies in the second phase of the project with *S. lycopersicum* because it did not demonstrate strong biostimulant effects on *M. truncatula* plants.

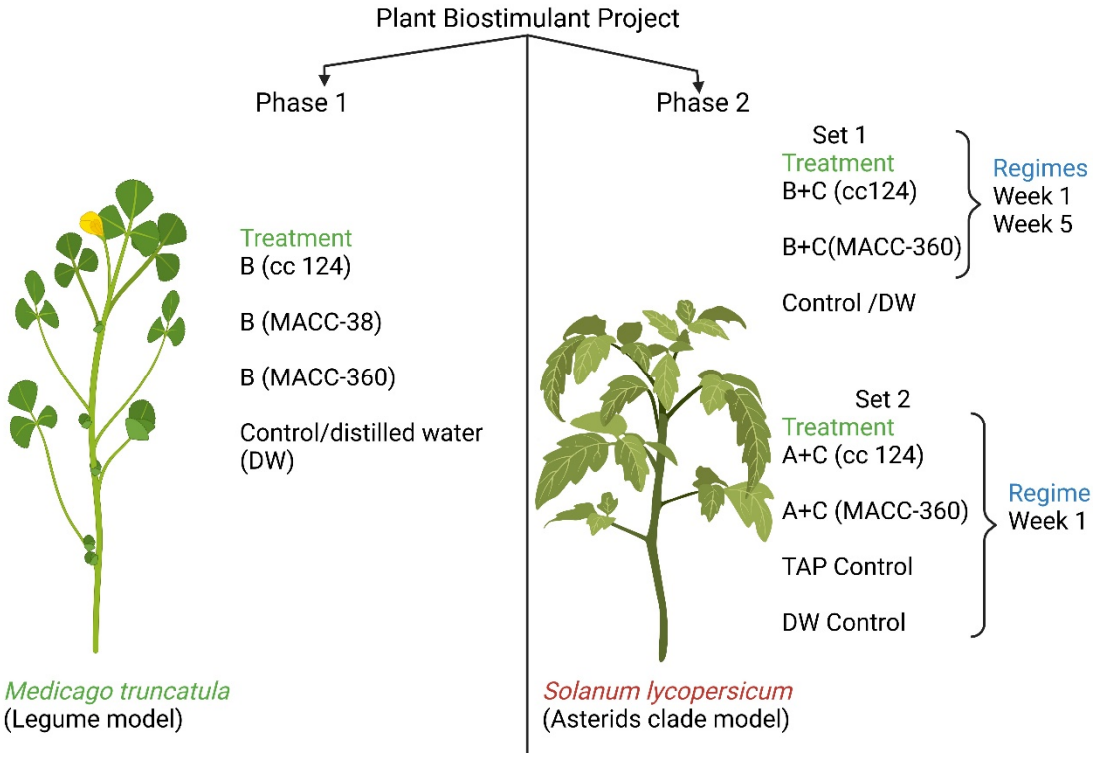

Figure 3.2. Experimental design for all experiments. For *Medicago truncatula*, cells of three algae strains (*C. reinhardtii* cc124, *Chlorella* sp. MACC-38, and *Chlorella* sp. MACC-360) suspended in distilled water (DW) were applied by soil drench method. The control treatment was DW. For *Solanum lycopersicum*, three treatments from two algae strains (*C. reinhardtti* cc 124 and *Chlorella* sp. MACC-360) were applied as A, B, and C treatment whereby; A= cells with their growth media, B= Cells only and C= destroyed/homogenized cells. Plants that received B+C treatment belonged to either Week 1 or Week 5 regime, while all those that received A+C treatment fell under the Week 1 regime. In the Week 1 regime, soil drench treatment was first applied to one-week-old plants, and foliar spray was initiated when the plants were five weeks old. In the Week 5 regime, soil drench and foliar spray were first applied to five-week-old plants. Figure was created with Biorender.

In *S. lycopersicum* studies, in addition to cells only, the whole culture was used as a treatment because microscopy pictures revealed that centrifugation stripped off the EPS from the cells. The auxin content in the culture supernatant also guided us on what portion of the algae culture to use for treatment. It, consequently, became apparent that the whole culture, including the growth media, contained more bioactive compounds than cells suspended in water. In addition, we decided to use foliar application guided by literature that this method could be effective for applying treatments containing phytohormones. Based on the literature, we also tested the effect of time of application/ age of plants on the plant biostimulant effect. Therefore, for later experiments, we used the whole culture for treatment and two application methods, soil drench and foliar spray, as shown in Figure 3.2.

Two groups of experiments were carried out (Table S3.1). The first set was conducted between February and July 2020. In these experiments, living cells (B) and cell extracts (C) were applied via soil drench and foliar spray. One-week-old plants received the first soil drench treatment in the first week, as shown in Figure 3.3 and five-week-old plants in the fifth week, as shown in Figure 3.4. Both groups of plants received foliar treatment beginning in the fifth week. Plants in the control group were treated with DW.

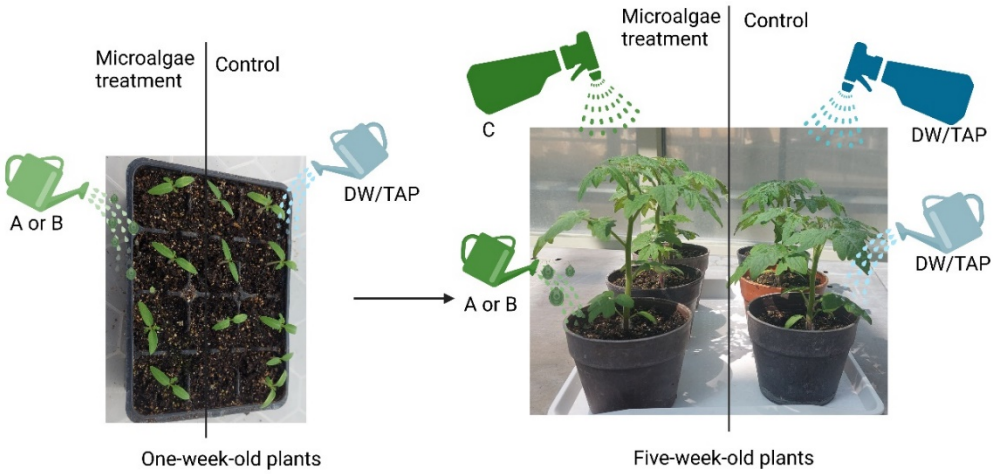

Figure 3.3. Week 1 regime where initial algae treatment was applied to one-week-old young plants via the soil drench method, while extracts (treatment C-foliar treatment) were initiated prior to flowering on five-week-old plants. Two controls were used if the treatment was A (cells and their growth media). These were the Tris-acetate-phosphate (TAP) media used for algal cultivation and distilled water (DW). If treatment B (cells only suspended in water) was used, only DW control was maintained. The figures were created with Biorender.

The second series of experiments were conducted from June to September 2021. Culture suspensions (A = living cells plus spent media) were applied as soil treatments. The first soil drench treatment was administered to one-week-old plants, while foliar treatment with extracts (C) was initiated five weeks later. This set of experiments falls under the week 1 regime. Since the whole culture was being used as a treatment, the medium used to cultivate the cells had to be included as a control treatment. Therefore, the two controls were TAP media and DW, as illustrated in Figure 3.3.

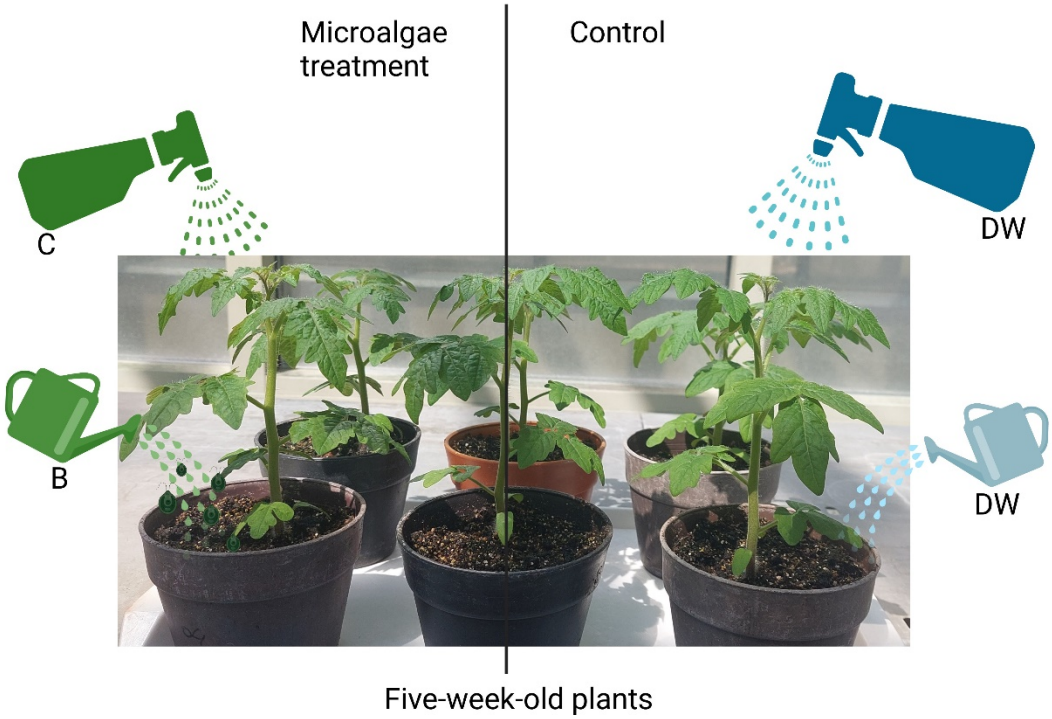

Figure 3.4. Week 5 regime where both soil drench (B=cells only suspended in water) and foliar treatment (C=destroyed cells suspended in water) were applied simultaneously to five-week-old plants. Only treatment B soil drench was tested on five-week-old plants; the control was distilled water (DW). The figure was created with Biorender.

In all experiments, soil drench treatments were applied once per week, while foliar sprays were applied after every two weeks (starting on the fifth week). The biostimulant application was terminated on the twelfth week (week 12).

3.2 Effect of microalgae on *Medicago truncatula*

3.2.1 Planting seeds and establishing plants

Wild-type *M. truncatula* seeds A17 Jemalong ecotype were used for these studies. The planting and phenotyping procedures followed a standard protocol (Bucciarelli et al., 2006). The seeds were surface-scarified with concentrated sulfuric acid for five minutes and then washed thoroughly with sterile ice-cold DW. The seeds were then surface-sterilized for three minutes with 0.01 % $HgCl_2$ and washed five times with sterile DW. The seeds were allowed to absorb water for two days at 4 °C before being transferred to Petri dishes lined with moistened filter paper and vernalized for twenty-one days at 4 °C. The plates were then placed in a growth chamber for two to three days.

Vernalized seeds with a radical length between 1 and 1.5 cm were treated for 20 minutes with DW or each of the algae suspensions (Control/DW, *Chlorella* sp. MACC-38, *C. reinhardtii* cc124, and *Chlorella* sp. MACC-360). The seedlings were then washed with water and planted in pots containing a 3:1 mixture of soil and vermiculite. The size of the pot was 10× 10 ×35 cm^3. Each pot contained four plants, and each treatment's box had five pots. During transplantation, plants were fertilized with 100 mL, per pot, of Solution I (Sol 1) diluted 40 times from the stock solution prepared as follows: Initially, the following macronutrient stock solutions were individually prepared: 20.2 g/L KNO_3, 73 g/L $CaCl_2 \times 2H_2O$, 24.6 g/L $MgSO_4$, 43.5 g/L K_2SO_4, 8.2 g/L Fe-Na-EDTA, 27.2 g/L KH_2PO_4, and 0.05 M H_3BO_3. Second, a microelement stock solution was prepared by adding 6.2 g of $MnSO_4$, 10 g of KCl, 1 g of $ZnSO_4$ $7H_2O$, 1 g of (NH_4) $Mo7O_2$ $4H_2O$, 0.5 g of $CuSO_4$, and 0.5 mL of H_2SO_4 to 1 L of water. The stock solutions and 800 mL of water distillate were autoclaved independently. Finally, 800 mL of sterile water was combined with 25 mL of each of the macronutrient solutions and 1.35 mL of the micronutrient stock solution to create the Sol 1 stock solution (Gitau et al., 2021). The temperature in the greenhouse ranged from 24 to 26 °C, and the photoperiod was 16 hours. On the seventh week of growth, plants were watered for the final time with water-based algae suspensions; from then on, plants were watered with regular tap water.

3.2.2 Phenotyping and harvesting

After 45 days (approximately 1.5 months), aerial photographs of the plants were taken to document the plant cover. The growth experiments were terminated five days later (50 days after planting (DAP)). Ten plants from each treatment were phenotyped. For the leaves associated with the first up to the fifth metamer (M1–M5), leaf petiole and blade length were measured. The leaf blade width was measured from the second metamer up to the sixth metamer, as indicated in Figure 1.2. The length of the leaf blade was measured along the midrib from the tip of the middle leaflet to the end of the leaf petiole. The width of the leaf blade was determined by measuring the distance between two opposite leaflets on a trifoliate leaf. Flower count and plant height (height of the main axis or one of the axes in bifurcated plants) were also recorded. All measurements were taken using a portable, flexible ruler (Gitau et al., 2021).

Plants were gently uprooted, and the roots were thoroughly washed with DW to remove all traces of soil. Five plants were arranged on a black background, and photographs were taken with a camera. The shoots and roots were separated, and their fresh biomass was determined with an analytical balance (Adventure pro AV 114C, Ohaus Corporation, NJ USA). The plants were then dried for 48 hours at 70 °C in an oven with dry air, and their dry weight was recorded. The average dry weight of the pooled sample for each treatment protocol was recorded (Gitau et al., 2021).

Another set of ten plants per treatment was collected and processed for plant pigment content determination as described in our previous report (Gitau et al., 2021). Two pooled samples from five plants were put into separate tubes for each treatment. About 0.1 g of this 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 minutes and cooled on ice. Then, 200 µL of the extract was transferred into two wells in a 96-well plate, and absorbance values were measured with a Hidex Sense microplate reader at 665nm, 649nm, and 470nm (Hidex, Turku, Finland). The content of Chls was calculated according to Arnon equations, and the formula for Cars was adopted from Lichtenthaler et al. equation specific to acetone extracts (Lichtenthaler, 1984; Manolopoulou et al., 2016).

3.3 Impact of microalgae on *Solanum lycopersicum*

3.3.1 Establishment and care of plants

The studies utilized *S. lycopersicum* seeds of the Vilma variety acquired from a retailer in Szeged, Hungary. The Vilma variety was chosen because it is dwarf and does not occupy ample space. The seed establishment procedure is according to the description in our recent report (Gitau et al., 2022). Five minutes of surface sterilization with a 10% hypochlorite solution was followed by thorough washing with sterile DW. The seeds were allowed to absorb water for approximately two hours to trigger germination. The seeds were then sown in a 12-well germination box with moist soil and vermiculate in a 2:1 ratio. After germination, the seedlings were kept on this platform in the greenhouse for two weeks.

The seedlings were transplanted into 3 L pots containing moist soil and vermiculate (2:1) moistened with Sol 1 diluted forty times. At this stage, the seedlings had well-developed root systems and required more space for root growth. The greenhouse had a 16-hr photoperiod and an ambient temperature ranging from 24°C to 26°C. Each treatment contained either twelve or ten plants, two per pot and five to six pots per treatment. Three pots were placed on a tray, and a treatment comprised two trays. The trays were arranged in a randomized block design, and their position on the bench was continuously rotated to ensure that they were exposed to environmental factors uniformly. The soil and plants were treated according to the experimental design shown in Figures 3.2, 3.3 and 3.4.

3.3.2 Harvesting and phenotyping

Plants treated with B+C

At the beginning of flowering, the daily number of open flowers per plant in each treatment was recorded. This information was utilized to illustrate the flowering kinetics. We took these measurements because we observed enhanced flowering in our previous studies with *M. truncatula*.

When the first batch of fruits had reached full maturity (ripening), they were harvested. The fruit number, diameter, weight, and plant yield were measured. The fruit

diameter was measured with Vernier calipers, while the weight was determined with a precision balance (Kern PLJ 2100-2M, Germany). The leaves from the plant's top, middle, and bottom were used to create a homogeneous sample for pigment extraction and determination, as described previously in section 3.2.2.

Plants treated with A+C

The number of open flowers per day was recorded during the first week of flowering. Only the freshly open flowers were counted, and withered flowers were ignored.

On the 50th, 60th, and 70th DAP, the number of trusses per plant, open flowers per truss, bearing trusses per plant, and number of fruits per truss were recorded. A truss is a group or cluster of small stems in which flowers and fruits develop. The trusses appear close to the junction between the primary stem, a secondary stem, or leaf petioles. Bearing trusses refer to trusses in which the flowers have transitioned into fruits. Plants that flowered earlier than others would have more bearing trusses than late bloomers at the initial stages of fruit development. Taking account of these parameters would reveal differences in the process of fruit development between treatments.

Photosynthetic parameters were taken weekly on plant leaves using the handheld device Multispeq (Kuhlgert et al., 2016). The Multispeq handheld device was used to take measurements analyzed in the open-access Photosynq app (https://photosynq.org) developed by David Kramer (Michigan State University Michigan, USA). Multispeq has a pulse-amplitude-modulated fluorimeter, a Chl meter, and a spectrometer. All the parameters are measured simultaneously in a single measurement providing information about photosynthetic performance and crop status. The protocols used for measurements come with inbuilt macros for immediate calculation of the photosynthetic performance based on the readings. For example, the leaf temperature differential was calculated as the difference between ambient and leaf temperature.

The Multispeq tool also captures environmental factors like temperature and humidity, which can be used to ensure plants are under uniform conditions, thus reducing variability. In brief, the device measures environmental parameters, including temperature, humidity, photosynthetically active radiation (PAR), and quality of light.

The light intensity in µmol photons m^{-2} s^{-1} at 400-700nm was determined for PAR measurements. This wavelength corresponds to the active intensity that plants utilize plants for photosynthesis. The PAR parameter was used for the calculation of LEF.

The Multispeq device has several light-emitting diodes (LEDs), which enable measuring the relative Chl (SPAD) shown in Figure 3.5. In the description of how the Multispeq works, Kuhlgert and colleagues mention that the recorded SPAD result accommodates measurements of thick and high-pigmented leaves (Kuhlgert et al., 2016) and was hence suitable for tomato leaves. The Chl content was obtained by measuring relative transmissions of the red (650nm) and the infrared (940nm) light.

Fluorescence-based photosynthetic parameters are also determined. The results returned by Chl fluorescence measurements allow an efficient non-destructive investigation of PS II of plants. When the Chls and Cars pigments capture light energy, it takes one of three competing routes. It can be used in photosynthesis, lost in the form of heat, or emitted as fluorescence. Analyzing how the Chl fluorescence changes in response to pulses, as shown in Figure 3.5, enables estimation of the fate of light energy in the PS II in terms of Phi2, PhiNPQ, or PhiNO, respectively.

Although traditional Chl-fluorescence-based methods require dark adaptation of leaves for analysis of fluorescence yield, the Multispeq reproduces the ambient PAR intensity inside the leaf chamber enabling high-throughput phenotyping, and dark adaptation is not necessary (Kuhlgert et al., 2016). The results gathered from Chl fluorescence measurements are informative of the status of the photosynthetic apparatus in response to fluctuations in environmental factors. They can therefore allow early detection of perturbations before the symptoms manifest in plants.

Our experiments measured Chl fluorescence, absorbance, and environmental variables with the Photosynthesis Ride 2.0 protocol of Photosynq. The measurements began after clamping a leaf, and the protocol gave specific light-emitting commands and measurement instructions, returning the following results. The saturated pulse-chlorophyll-fluorescence yield parameters (Fs, Fm', and Fo') were first recorded in light-adapted leaves. Then the steady-state fluorescence yield Fs was recorded during continuous actinic light. A saturating light pulse was then supplied to the leaf to estimate the maximum fluorescence under steady-state light Fm' with steady-state levels of non-photochemical quenching and with all PS II centers closed. The actinic

light was turned off, and a pulse of far-red light was supplied to the fully oxidized plastoquinone pool and quinone, enabling Fo' measurement in a steady state level of NPQ and with all PS II centers oxidized. Then the transmittance of red light (650nm, Chl absorbed) and infrared radiation (940nm, non-Chl absorbed) relative to a blank (ambient air) were determined. In the end, ambient light intensity in m^{-2} s^{-1}, ambient temperature (Ta), and leaf temperature (Tc) in ◦C were recorded. The above parameters were used for Phi2, PhiNPQ or PhiNO, SPAD, LEF, and LTD calculations.

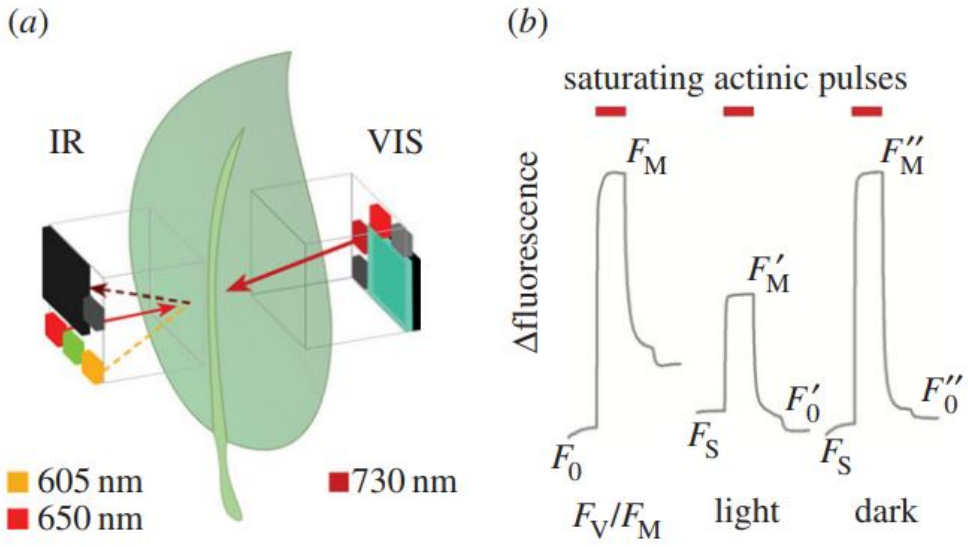

Figure 3.5. **Measurement with Multispeq** (a) Light emitting diodes (LED) and detector set-up to measure fluorescence-based kinetics. Shown are the light paths for the actinic illumination (650 nm, solid red arrow), fluorescence excitation (605 nm, orange dashed arrow), and far red (730 nm, dark red solid arrow) and chlorophyll (Chl) fluorescence (dark brown dashed arrow). (b) Representative fluorescence transients in an attached *Camelina sativa* leaf. Each sequence consists of 100 pulses of orange LED light (pulses with a duration of 10 µs, emission peak at 605 nm at 100 Hz). After 50 pulses, a 50-pulses long saturating flash using the 650 nm LED was given (approx. 10 000 µmol photons m−2 s −1) followed by far-red illumination (830 nm). From left to right, traces represent transient taken in the dark-adapted state, which can be used to calculate Fv/Fm; traces taken during steady-state illumination, which can be used to estimate ΦII (maximum quantum yield) and non-photochemical quenching NPQ parameters, and about 5 min after returning the leaf to the dark, which can be used to estimate qI (photosystem (PS) II open reaction centers according to lake model) (Kuhlgert et al., 2016).

3.3.3 Transcriptomic/gene expression studies

We conducted whole transcriptomic studies of the unopened flower buds after observing that algae treatment affected the blooming process. We were curious to observe which genes were up or downregulated in this tissue. The results could help us understand how algae could enhance or delay the flowering process relative to the control. The plants for these studies were established and maintained similarly, as explained in section 3.3.1. However, the plants did not receive foliar treatment as the flower bud formation began on the fourth week, and unopened buds were collected on the fifth week. Two independent experiments were conducted. RNA material was collected from both experiments, but only samples from the first experiment were sequenced. Samples from the two experiments were used in qPCR to validate the gene expression studies. In total, two samples were sequenced (pooled control sample comprising three biological replicates) and (pooled MA-treated sample comprising three biological replicates). For qPCR, 12 samples were analyzed (six biological replicates, three from each experiment for each treatment -control and MA-treated).

At the onset of flowering, unopened flower buds from A[7]-treated plants were collected in triplicates. Sterile forceps and scalpels were used to cut off the unopened flower buds from about five weeks old plants. The samples were immediately frozen in liquid nitrogen before RNA extraction.

RNA was extracted with the Qiagen RNeasy plant kit (Qiagen). The genomic DNA was removed with Thermo Fisher's DNase1 according to the manufacturer's protocol (Thermal Fisher Scientific). For RNA sequencing, an equal amount of RNA was added from each replicates to make a single pooled sample for each treatment. For each sample, 1 µg of total RNA was reverse-transcribed for 60 min at 42 °C and 10 min at 75 °C in a 20 µL reaction volume using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA products were diluted 1:10 in RNAse-free water (Lonza, Verviers, Belgium).

The quality of extracted RNA was assessed using an RNA ScreenTape on a TapeStation 4150, and the quantity of extracted RNA was determined with Qubit RNA Assay (Agilent Technologies, Santa Clara, CA, USA). In vitro fragment libraries were

[7] A=Live cells with the growth media applied as a soil drench

prepared using the TruSeq RNA Library Prep Kit v2 from Illumina; the libraries were sequenced on an Illumina NextSeq1000 NGS platform to generate 150 nucleotides (nt) paired-end reads. All reads were uploaded to National Center for Biotechnology Information (NCBI), Sequence Read Archive (SRA) database (PRJNA880331).

For differential gene expression analysis, the RNA sequencing data (RNAseq) data were first corrected with a kmer-based error corrector (https://github.com/mourisl/Rcorrector), which corrects random sequencing errors in Illumina RNA-seq using a De Bruijn graph to compactly represent all trusted k-mers in the input reads (Song and Florea, 2015). The error-corrected reads were subsequently trimmed utilizing Trimmomatic v0.39 (http://www.usadellab.org/cms/?page=trimmomatic) with adapter sequences and a quality score of 25 over a five base pair (bp) sliding window (Bolger et al., 2014). Reads that were shorter than 50 bp were omitted from further analysis. The trimmed reads were then mapped to reference *S. lycopersicum* transcripts v3.0 downloaded from ENSEMBL (https://plants.ensembl.org/*Solanum_lycopersicum*/Info/Index) with Kallisto v0.46.1 (https://pachterlab.github.io/kallisto/about) (Bray et al., 2016). Generalized fold change (GFOLD) was used to perform differential analysis on quantified Kallisto transcripts (https://pubmed.ncbi.nlm.nih.gov/22923299/) (Feng et al., 2012).

Functional and ontology enrichment studies investigated the gene functions of differentially expressed genes (DEGs) with a fold change of ±1.5. The lists of upregulated and downregulated genes were separately analyzed.

Gene enrichment studies were done in Shiny GO version 0.76.3 with default settings (Ge et al., 2020). The species selected was *S. lycopersicum*. We uploaded all the transcripts identified in our RNA sequencing data into Shiny GO to serve as the background. Other analysis platforms use all the genes in the *S. lycopersicum* genome as the background. Using only transcripts from our study was essential to uncover pathways of a gene list with a few genes (downregulated list).

Functional profiling analysis was conducted in g: Profiler with default settings (Raudvere et al., 2019). The Database for Annotation, Visualization and Integrated Discovery (DAVID) website (https://david.ncifcrf.gov/home.jsp) was used to produce

functional annotation clusters for both gene lists as well as identification of associated pathways (Huang et al., 2009; Sherman et al., 2022).

The results of the DEGs analysis were verified with real-time quantitative polymerase chain reaction (RT-qPCR). Transcripts from the downregulated gene list were randomly chosen to validate transcriptome data. Their random primers listed in Table 3 were designed with Primer Quest. The RT-qPCR reactions were carried out in the CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA). Three µL of a master mix containing 0.15 µL forward primer, 0.15 µL reverse primer, and 3µL Maxima SYBR Green/ROX qPCR Master Mix (2×) (Thermo Fisher Scientific) was aliquoted to Hard-Shell® 384-well plates (thin-wall, skirted, clear/white; Bio-Rad, Cat. no: HSP3805). Three µL cDNA of each sample was then added to make 6 µL PCR mixture per well. For amplification, a standard two-step thermal cycling profile was used (35 s at 95 °C and 1 min at 58 °C) during 40 cycles, after a 2 min preheating step at 50 °C and 7min at 95°C. Finally, a dissociation stage was added with 65 °C to 95 °C at an increment of 0.5 °C for 0.05 s. Data analysis was performed using Bio-Rad CFX Maestro (Bio-Rad) software and Microsoft Excel 2016.

Table 3.1. Primer name and sequences

Primer name	*Primer sequence*
Actin forward (FW)	TCCGATCTCCTCTCAGTTCC
Actin reverse (REV)	AGCCTTCACCATTCCAGTTC
Floral homeotic protein AGAMOUS (AG1) FW	CCAGAGAGATCTCACCACAAAG
AG1 REV	CAAACCATTGCGCCTCTTG
Carbonic anhydrase FW	*CTCAGCGAGAAAGCAGAACT*
Carbonic anhydrase REV	*TGCTCAACAGGGTCGAATG*
Lithium tolerant lipase 1 (LTL1) FW	TGGAGCAGCACAAACAGAG
LTL1 REV	CTTGCAGAGAAAGGGACCAA
Pectate lyase FW	TGAGGATGCAGCAGAAAGTG
Pectate lyase REV	AGCTCCAGCACCAGATCTTA
Threonine dehydrogenase 2 (TD2) FW	GGGTTAGGTTCAGGCAAAGA
TD2 REV	GCCCACAAGTCCAACAAATG
Beta galactosidase FW	CTGTTGCCGTCACCATGTA
Beta galactosidase REV	CAGACCAAGCTGGGACATT

The relative mRNA levels normalized to the average of Actin and mRNA expressions were calculated using the (2) –ΔΔ Ct method. The mRNA level of the untreated samples was used as control (relative mRNA level: 1). All tested amplification efficiencies were in a narrow range and were not used in the data normalization. Data were averaged from two independent biological experiments with three technical replicates for each gene/sample combination.

3.3.4 Soil metagenomics studies

We conducted soil metagenomics studies to evaluate the effect of algal biomass on the rhizosphere of the tomato plant after observing growth promotion with the soil drench MA application method. Even before the foliar application, initiated on the fifth week, there were apparent differences between control and algae-treated plants. The algae-treated plants were taller and more robust than their control counterparts.

Soil-microbial DNA was extracted using a modified Meta-G-Nome DNA isolation kit extraction protocol. After carefully uprooting the plants, they were shaken to remove excess soil. Several pieces were randomly cut from the plant root and placed in a falcon tube containing 10 mL of the extraction buffer containing 0.1% Tween 20. The tubes were vortexed at maximum speed for one minute to separate the soil from the root fragments. The soil suspension was centrifuged at 1600 × g for ten minutes. The supernatant was transferred to a fresh falcon tube. Twenty milliliters of extraction buffer were added to the root debris-containing pellet. For one minute, the mixture was vortexed at maximum speed. The tubes were centrifuged for ten minutes at 900 × g. The previous sample volume was adjusted to 30 mL by adding the new supernatant. The composite sample was centrifuged for 20 minutes at 4542 × g. The pellet served as the soil sample for DNA extraction, while the supernatant was discarded. Soil DNA was extracted with OMEGA-soil DNA Kit according to the manufacturer's instructions (Omega Bio-tek, USA).

The DNA content of samples was measured with nanodrop and confirmed with 1% sodium dodecyl sulfate (SDS)-agarose gel electrophoresis. Two µL of each DNA sample was diluted with 3 µL of DNAse free water, and 1 µL of 6x loading dye (Thermo Fisher Scientific). A control was prepared with 5 µL water and 1 µL 6x loading dye.

The samples and the Generuler 1 kb DNA ladder (Thermo Fisher Scientific) were loaded into 1% agarose gel containing 10 mg/mL Ethidium bromide at 210V for 20 minutes to allow separation of the bands in 10x SDS solution. The gel was visualized under ultraviolet (UV) light. The intensity and thickness of the genomic DNA bands correlated with the nanodrop measurements and were used as a criterion for confirming DNA quality. DNA degradation detected as smears on bands was not observed.

Extracted DNA was sequenced on the Illumina Nextseq550 platform at Seqomics Limited in Mórahalom, Hungary. Reads were sequenced in 150 bp format with paired ends. Adapters were trimmed from all reads using Bbduk v38.34 (https://benlangmead.github.io/aws-indexes/k2). Reads with a quality score of less than 20 were trimmed, and reads with a length of less than 36 bp were excluded from further analysis. Reads were also examined for PhiX contamination. PhiX is a sequencing control derived from a small bacteriophage genome. It is used to monitor Illumina sequencing error rates (Manley et al., 2016).

Reads were classified using Kraken2 against the standard Refseq database along with protozoa and fungal genomes (Wood et al., 2019). The kraken2 classifications were improved using Bracken with a threshold of 5 reads per classification (Lu et al., 2017). The operational taxonomy unit (OTU) classification table was imported into R and analyzed using Phyloseq (McMurdie and Holmes, 2013). Differentially abundant OTUs were identified using a negative binomial distribution-based differential expression analysis for sequence (Deseq2).

Alpha diversity estimates for measuring abundance and distribution were computed using Observed, Shannon, and Simpson indexes (Wagner et al., 2018). Richness is the number of distinct taxa present in a community without regard to their frequencies, whereas evenness is the distribution of taxa frequencies within the community. The Shannon index gives equal weight to richness and evenness, whereas the Simpson index emphasizes evenness. Species observed are equivalent to species richness (Jost, 2006). Thus, the Shannon index emphasizes rare species, while the Simpson index emphasizes relative abundance.

The beta diversity was depicted using a principal coordinate analysis (PCoA) based on a Bray-Curtis distance matrix. The permutational multivariate analysis of variance using distance matrices (ADONIS) and analysis of similarity (ANOSIM) tests

were used to determine whether the community composition was significantly dissimilar. All these statistics were done in Phyloseq (McMurdie and Holmes, 2013).

After obtaining the differentially abundant OTUs, a principal component analysis (PCA) based on a Euclidean distance matrix was used to show how the bacteria genera with differential abundances were distributed between the control and MA-treated samples. This plotting was achieved with PAleontological STatistics (PAST) version 4.03 for windows (Hammer et al., 2001).

Raw reads were also uploaded in Metagenomics Rapid Annotations using Subsystems Technology (MG-RAST) for data curation (Keegan et al., 2016). The server conducted quality control of the raw data. The quality control included the removal of the adapter and low sequence. Artificial sequences were removed, ambiguous bases were filtered out, and specification read size and length filtering were performed. Sequences that passed the quality check were annotated against the Refseq database. The taxonomy plots of the microbiomes were constructed at the phylum, class, and genus levels with the parameters set at e-value of 1e-30, 90% identity, 20 minimum lengths, and minimum abundance of 1000.

3.4 Statistical analysis

3.4.1 *Medicago truncatula* experiments

Data from three independent experiments (technical replicates) were used for statistical analysis; the data represented parameters measured from 30 plants (ten biological replicates per technical replicate) from each treatment and, in total, 120 plants. The collected datasets were tested for normality and homoscedasticity. Multiple groups or treatments were compared with a one-way analysis of variance (ANOVA) for plant height, flower number, biomass, and pigment parameters. Two-way ANOVA was applied to compare leaf parameters data, which were in the format of grouped data. Tukey's multiple comparison tests at $P < 0.05$ was used to analyze the significance of differences. All statistical analyses were executed in GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

3.4.2 *Solanum lycopersicum* experiments

The accumulated datasets from the three week 1 regime experiments and the three week 5 regime experiments, each with twelve biological replicates per treatment, were tested for normality and homoscedasticity (Table S3.1). Datasets that passed these tests were analyzed with Two-way ANOVA; the two factors were treatment and regime (age of plants during first MA application), respectively. Multiple t-tests were used for multiple comparisons. The Mann-Whitney test was used to analyze datasets that failed normality and homoscedasticity tests after transformation. The parameters analyzed in this set of experiments were the plant and fruit parameters.

The flowering data from the three week 1 (B+C experiments) was compared against the flowering data of the two A+C experiments (week 1 regime). Datasets in tables showing the dates and numbers of flowers/fruits (flowering and fruiting) were fitted to a non-linear model, and the results (mean, standard deviation, and degrees of freedom) were used in One-way ANOVA to infer significant differences. The $P < 0.05$ was used to indicate significant differences.

The reproductive parameters and photosynthetic performance data were only analyzed for the two A+C experiments. Every experiment conducted at different times was treated as a technical replicate and every plant as a biological replicate. All statistical analyses were executed in GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

4. Results and Discussion

4.1 Microalgae growth, morphology, and biomolecules biosynthesis

4.1.1 Microalgae growth

According to OD measurements, all investigated green algae strains had reached a stationary phase by the fifth day, as shown in Figure 4.1a. Nonetheless, the cell count indicated that *Chlorella* sp. MACC-360 had reached a plateau significantly earlier than the other two strains. *Chlorella* sp. MACC-360 had the most significant number of cells, eight times that of *C. reinhardtii* cc124 and three times that of *Chlorella* sp. MACC-38, as shown in Figure 4.1b. Due to its small cell size, *Chlorella* sp. MACC-360 had a higher initial cell number value for the same OD, which explains the differences in cell numbers. In addition, the cell division cycle of *Chlorella* sp. MACC-360 is shorter than that of other strains.

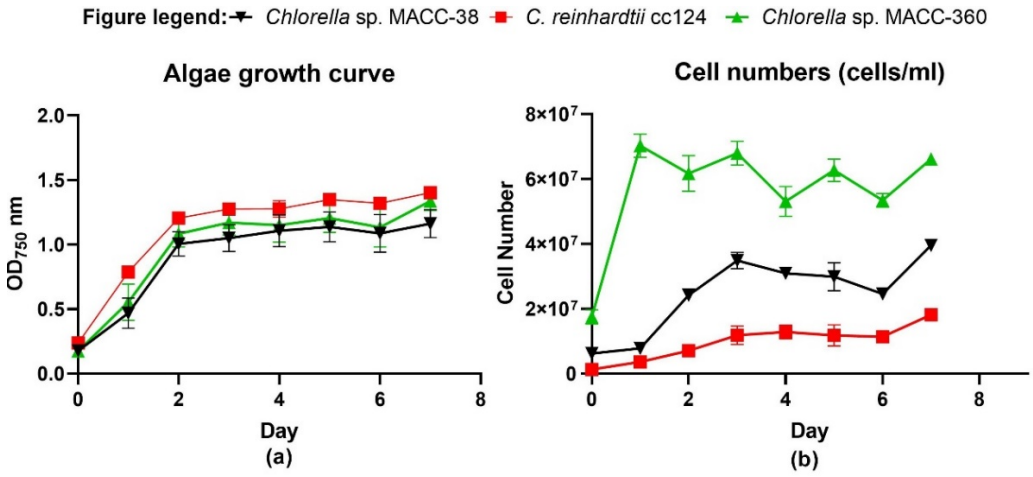

Figure 4.1. Growth in terms of optical density and cell numbers of the algae strains (*Chlorella* sp. MACC-38, *C. reinhardtii* cc124, and *Chlorella* sp. MACC-360) grown under light/dark conditions over seven days: (a) growth curve; (b) cell numbers. Error bars indicate the standard error of two replicates' mean (SEM) (Gitau et al., 2021).

4.1.2 Microalgae morphology and size

The morphology and size of the applied green MA cells were examined using SEM to reveal any observable differences. The images demonstrated that the three strains varied regarding surface texture—the surface of *Chlorella* sp. MACC-38, as shown in Figure 4.2a, was rough, whereas *C. reinhardtii* cc124 and *Chlorella* sp. MACC-360 were smooth in Figure 4.2b–c.

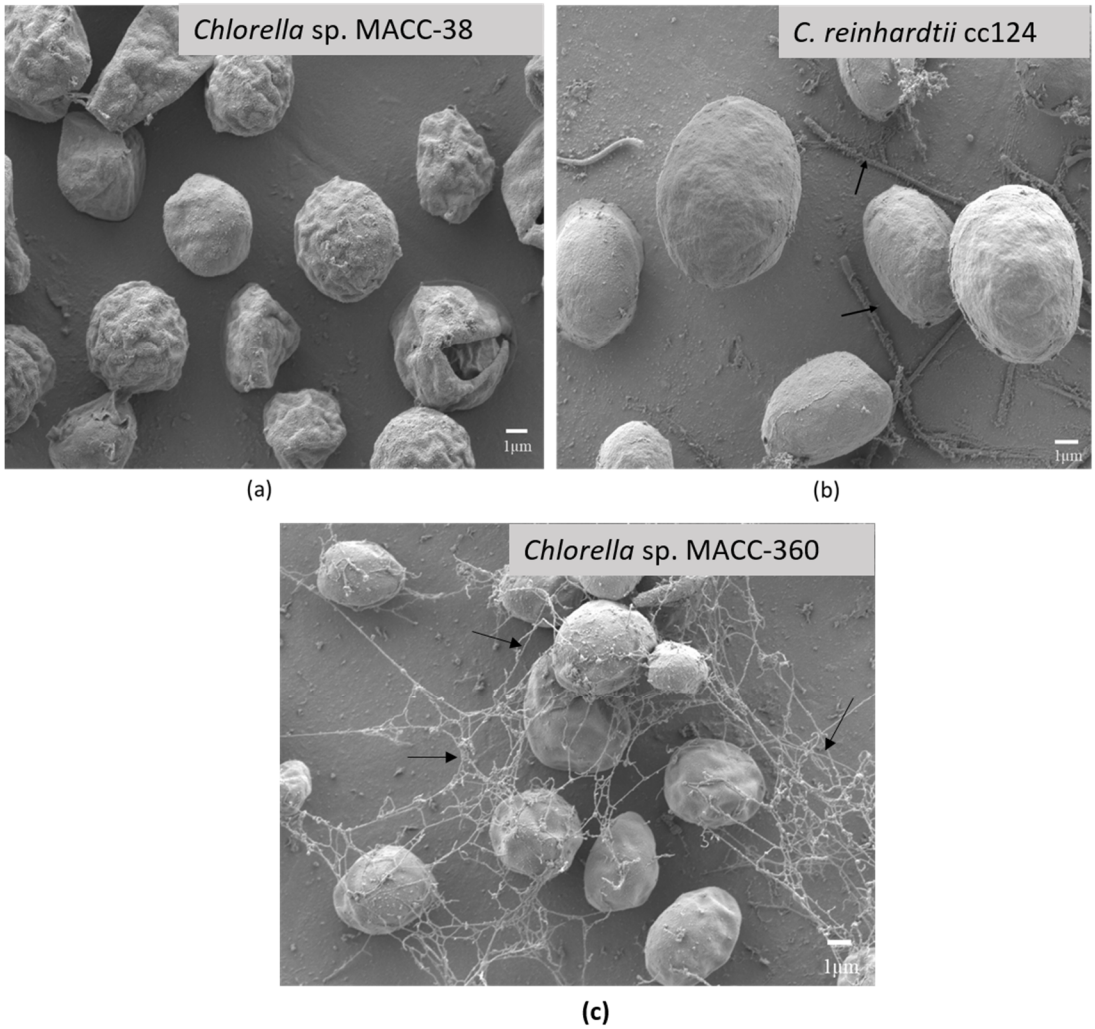

Figure 4.2. Scanning Electron Microscope (SEM) pictures of the three microalgae (MA) strains prepared from whole cultures containing live cells and the growth media (treatment A): (a) *Chlorella* sp. MACC-38; (b) *C. reinhardtii*, cc124; (c) *Chlorella* sp. MACC-360 at 5000× magnification. Black arrows on (b) indicate flagella; black arrows on (c) indicate the extracellular material connecting cells in the cell aggregations/matrix (Gitau et al., 2021).

Although the size distribution could not be observed with SEM because the preparation procedures (drying) tamper with the cell features, it appears that *C. reinhardtii* cc124 is more prominent than both *Chlorella* species. These results

correspond to what Lakatos et al. reported about these strains; *Chlorella* sp. MACC-360 size is 14.464 ± 3.925 μm^2, and *C. reinhartii's* size is 105.979 ± 42.727 μm^2 (Lakatos et al., 2017). In a recent study with exact algae strains, *Chlorella* sp. MACC-38 was 8 µm in diameter, while *Chlorella* sp. MACC-360 was only 6 µm wide (Hupp et al., 2022). These reports verify that *Chlorella* sp. MACC-360 cells are smaller than those of *C. reinhardtii* cc 124.

In *Chlorella* sp. MACC-38 and *C. reinhardtii* cc124 cultures, neither extracellular material nor cellular aggregations were present. In contrast, *Chlorella* sp. MACC-360 exhibited substantial aggregations; the cells in these aggregations were connected via filamentous extracellular material, as shown in Figure 4.2c.

At the root interphase, the microalga cell size may play an important role in interactions with other microbes and the plant surface. Smaller algal cells may occupy less space and interact with more microorganisms and a larger plant surface than more giant cells. Based on cell size, it is possible to hypothesize that *Chlorella* sp. MACC-360 interacts with soil microbes and plants more frequently than *Chlorella* sp. MACC-38 and *C. reinhardtii* cc124.

The ability of MA to form biofilms may also contribute to the strain-specificity of the biostimulant's effect. *Chlorella* sp. MACC-360 was capable of forming cellular aggregates. Beneficial interactions between various soil-dwelling bacteria and fungi promote the formation of biofilms through this phenomenon. We did not detect an intimate relationship between algal cells and roots with microscopy investigation. This observation is consistent with what Lee and Ryu reported in a recent review about MA being the new plant beneficial microbes: "Unlike prokaryotic algae, eukaryotic algae have not been reported to colonize on plant tissues" (Lee and Ryu, 2021). There are reports of synergistic growth-promoting effects of algae and bacteria on various plant species, with the relationship being interspecific and governed by specific metabolite patterns (Dukare et al., 2011; Nain et al., 2010; Sharma et al., 2020). Watson and coworkers reported that algae produce VOCs, such as terpenoids and nor-Cars, that serve as chemical signals for communication between cells (Watson, 2003). For example, algal-bacterial synergistic relationships are pervasive in natural ecosystems (Ramanan et al., 2016), and MA exposure to bacterial VOCs stimulated rapid growth of the MA (Achyuthan et al., 2017). These reports suggest that MA can send signals to

attract and accumulate microorganisms responsible for mineralizing and producing secondary metabolites such as antibiotics. Such interactions could increase algal growth, thereby enhancing colonization. These factors contribute to plant nourishment and protection from disease-causing pathogens, enhancing their overall health.

4.1.3 Microalgae aggregation and exopolysaccharide biosynthesis

Confocal laser scanning microscopy confirmed some size differences between algae strains. The largest was *C. reinhardtii* cc124, followed by *Chlorella* sp. MACC-38 and the smallest was *Chlorella* sp. MACC-360, as shown in Figure 4.3. Striking cell wall composition differences also became conspicuous. The two *Chlorella* strains, MACC-38 and MACC-360, were stained with blue dye on their cell walls, as shown on the top and bottom rows of Figure 4.3. In contrast, the blue dye did not appear in *C. reinhardtii* cell walls, as shown by the middle row of Figure 4.3. These results indicate different cell wall compositions between the *Chlorella* and the *Chlamydomonas* genera.

Chlorella sp. MACC-360 begins to aggregate on the third day following inoculation, as shown in the third row of Figure 4.3. On the third day, the green fluorescence is modest, but on the fifth day, it becomes stronger. It is the only strain among the three exhibiting aggregations and a green signal indicating EPS production. These findings suggest that a five to seven-day cultivation period was sufficient for forming bioactive chemicals, EPS, in algal cultures.

In *C. reinhardtii* cc124, the green fluorescence appears as a spot inside the cells or a weak halo around the cells during the first days after inoculation. The signal, however, gets weaker with time; by the fifth day, it is only present as dots. However, the green fluorescence appears in the extracellular matrix embedding the *Chlorella* sp. MACC-360 cells. This differential staining implies the different bioactive compound (EPS) locations in the different MA strains. In *C. reinhardtii*, the green spots could be secretory vesicles, indicating that Con A had penetrated the cell walls of this strain. This observation is per reports that Con A stains the secretory pathways if permeability occurs.

These results showed that applying B (living cells) to plants did not immediately deliver EPS. In contrast, application of the A (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 appeared inside the cells for *C. reinhardtii* cc124, destroying 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 *C. reinhardtii* cc124 is scanty" (Gitau et al., 2022).

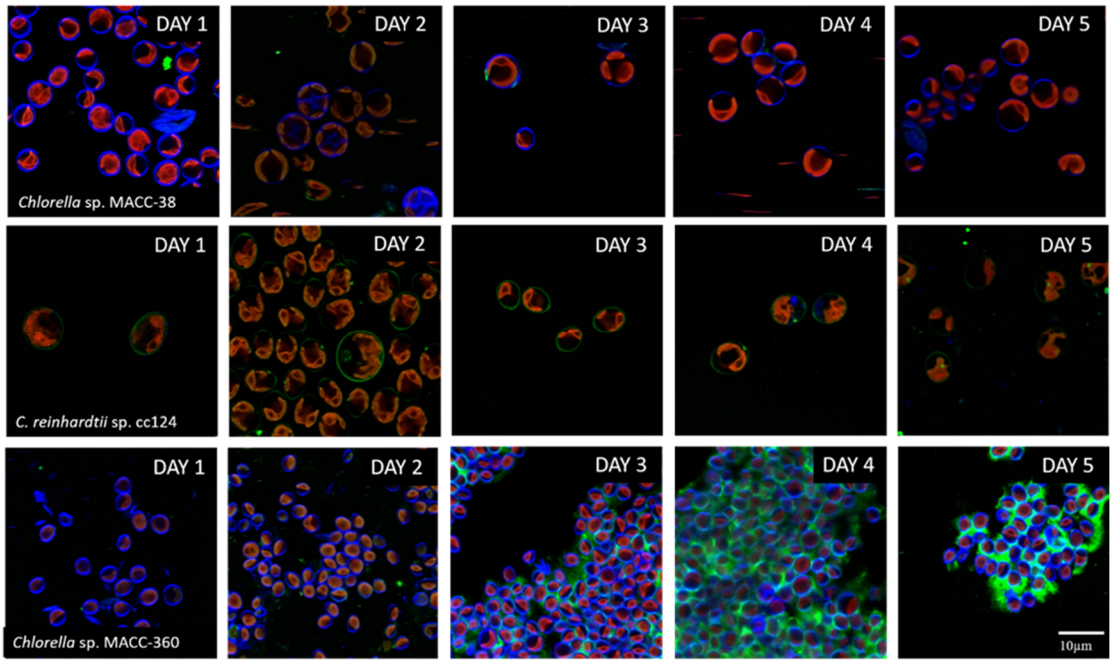

Figure 4.3. Confocal laser scanning microscopy (CLSM) images of *Chlorella* sp. MACC-38, *C. reinhardtii* cc124, and *Chlorella* sp. MACC-360 displays the progression of aggregation and matrix formation from the first day after inoculation to the fifth day after inoculation (DAY 1 to DAY 5 across). The blue fluorescence comes from the Cocafluor-White (CFW) dye, which stains the cell walls; the red fluorescence is the chloroplast autofluorescence of living cells; and the green fluorescence comes from the Conacavalin A (Con A) dye, which binds to the exopolysaccharides (EPS).

C. reinhardtii and *Chlorella* sp. MACC-38 did not aggregate nor produce extracellular EPS after seven days of cultivation, as no green fluorescence appeared in Figures 4.4a and b. *Chlorella* sp. MACC-360 displayed stronger aggregations and green fluorescence on the seventh day than on other days, as shown in Figure 4.4c, compared to the third row of Figure 4.3. These aggregations and green fluorescence were missing in *C. reinhardtii* cc 124 and *Chlorella* sp. MACC-38. These findings demonstrate that

only *Chlorella* sp. MACC-360 possessed both EPS and robust cellular aggregates when plants received the algae treatments.

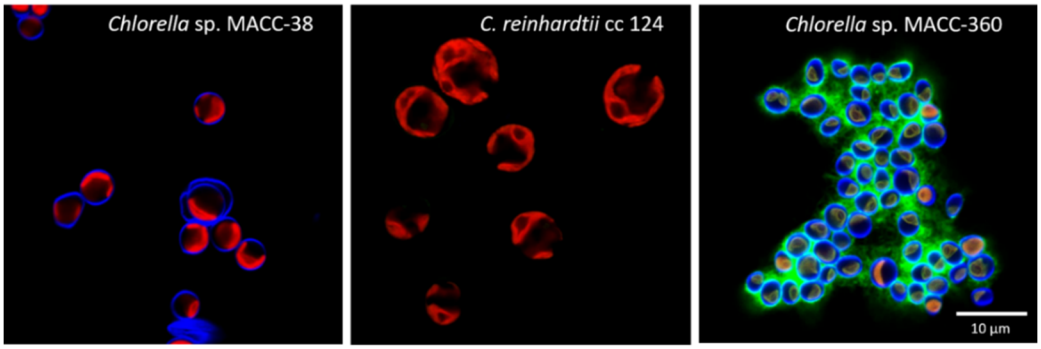

Figure 4.4. Confocal laser scanning microscopy (CLSM) images of microalgae (MA) cells (live cells in their growth media or treatment A) on the seventh day following inoculation; (a) *Chlorella* sp. MACC-38; (b) *C. reinhardtii* cc124; and (c) *Chlorella* sp. MACC-360 stained with Cocafluor-White (CFW) and Conacavalin (Con A). The blue fluorescence emission from the CFW dye, which stains the cell walls; the red fluorescence is the chloroplast autofluorescence of living cells; and the green fluorescence emission by the Con A dye, which binds to exopolysaccharides (EPS) (Gitau et al., 2021).

4.1.4 Influence of preparation method on the content of biostimulant

Figure 4.5b shows the cell suspension in water after centrifugation. It clearly shows that EPS from *Chlorella* sp. MACC-360 cultures detached from algal cells during centrifugation. Consequently, applying cells suspended in DW (treatment B) did not provide polysaccharides to plants. The application of the cells, along with their spent media (treatment A), as shown by Figure 4.5a, or the supernatant shown by Figure 4.5c, would provide plants with immediate access to substantial quantities of EPS. These results demonstrate the significance of the preparation method for the algal biostimulant component.

In fact, Müller and coworkers (1998) found EPS to be essential for rhizobium–legume symbiosis and, consequently, nitrogen fixation. The presence of algal EPS may aid in the recruitment of beneficial bacteria and fungi to the rhizosphere of plants. Ortiz-Moreno and colleagues (2019) reported that the presence of EPS in soil improved soil and nutrient availability by increasing the soil ionic content (Ortiz-Moreno et al., 2019). Yuan and co-authors (2020), on the other hand, confirmed the immunomodulatory and reactive oxygen species (ROS)-scavenging properties of EPS from *Chlorella* species

(Yuan et al., 2020). These properties suggest that EPS contributes to the design of microbial interactions and stress responses.

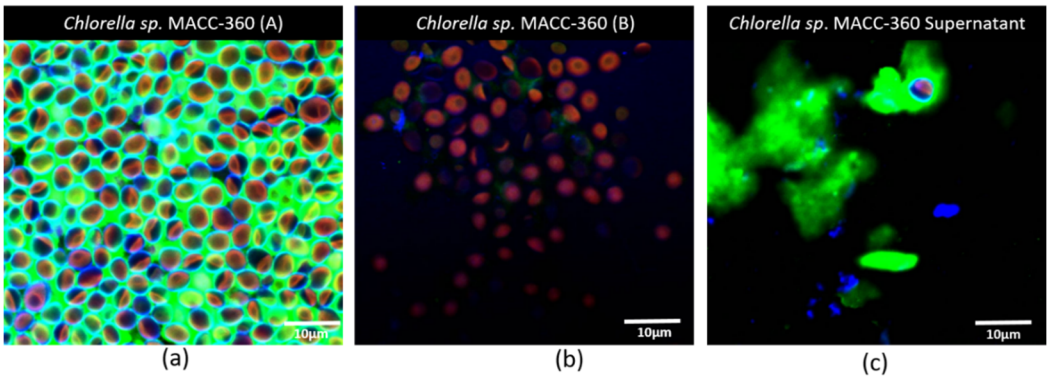

Figure 4.5. Confocal laser scanning microscopy (CLSM) images of *Chlorella* sp. MACC strain culture components: a), *Chlorella* sp. MACC-360 (A), b), *Chlorella* sp. MACC-360 (B), and c), *Chlorella* sp. MACC-360 supernatant/spent media. A refers to the living cells and their growth media; B refers to the living cells without spent media (the pellet of centrifuged culture resuspended in water); and spent media/supernatant is the growth media utilized by the cells. The red fluorescence represents chloroplast autofluorescence; the blue fluorescence represents the Cocafluor-White (CFW) dye staining the cell wall, and the green fluorescence represents the Conacavalin (Con A) dye staining the exopolysaccharides (EPS).

Exopolysaccharides also contain sugars that plants can directly absorb and utilize for growth. Evaluation of the biostimulant effect of *Chlorella*-derived polysaccharides revealed that they enhanced plant growth, pigmentation, and fresh biomass (El-Naggar et al., 2020).

Exopolysaccharides also significantly promote biofilm formation (cell aggregations). Colica and colleagues (2014) opined that biofilms enhance soil properties by absorbing atmospheric moisture and retaining it in the topsoil layers, making it more accessible to plants, particularly in sandy soils. Additionally, they reduce water infiltration, thereby preventing soil erosion (Colica et al., 2014). Thus, biofilms can contribute to plant growth promotion. Biofilmed biofertilizers (BFBFs) made with microbial consortiums have proven to be a sustainable method for boosting crop yield (Zakeel and Safeena, 2019). One study demonstrated that adding cyanobacteria to desertifying regions stimulated biocrust formation, which improved soil properties and triggered plant succession (Lan et al., 2014). Another study reported that biocrusts reduced the loss of soil organic carbon due to soil erosion (Chamizo et al., 2017). These studies revealed the role of biofilm-forming microorganisms in the maintenance of soil fertility as well as their potential as tools for the conservation of

soil resources and the restoration of soil fertility to dry land. The *Chlorella* sp. MACC-360 strain is a powerful EPS producer capable of forming biofilms, as shown in the micrographs. This evidence shows that it could be effective as a plant biostimulant.

A past study reported that polysaccharides interacted with leucine-rich repeat membrane receptors responsible for activating a mechanism that modulates the regulation of several genes that affect cell growth (Nardi et al., 2016). In addition, crude polysaccharides from the *Chlorella* and *Chlamydomonas* genera increased the expression of pathogenesis-related (PR) genes and genes encoding antioxidant enzymes such as POD, ascorbate peroxidase (APX), and -1-3 glucanase in tomato plants (Farid et al., 2019). Under normal and stressful conditions, MA-derived EPS applied via foliar spraying had biostimulant effects on tomato plants (EL Arroussi et al., 2018; Elarroussi et al., 2016). Altogether, the cited literature studies show the role of algal EPS as a biostimulant for plant growth promotion and a primer to increase stress tolerance.

4.1.5 Biosynthesis of indole acetic acid

The results showed that all three algae strains produced auxins. The color of their supernatants became pinkish after adding the Salkowski reagent. *C. reinhardtii* cc124 contained the most IAA, followed by *Chlorella* sp. MACC-38 while *Chlorella* sp. MACC-360 had the least amount, as shown in Figure 4.6.

C. reinhardtii cc124 produced two times as much IAA as *Chlorella* sp. MACC-360. These results indicated a difference in the biosynthesis of the quantified hormone. This phenomenon may hold for hormones we did not quantify in this study.

Microscopic algae produce growth-promoting exudates, including polyamines, vitamins, amino acids, betaines, auxins, and CK. Among these substances, many authors cite plant hormones and polysaccharides as responsible for biostimulation effects on plants (Gebser and Pohnert, 2013; Oancea et al., 2013; Spolaore et al., 2006; Stirk et al., 2002; Tate et al., 2013). As reported in a past study, all tested MA strains released auxins (Stirk et al., 2013).

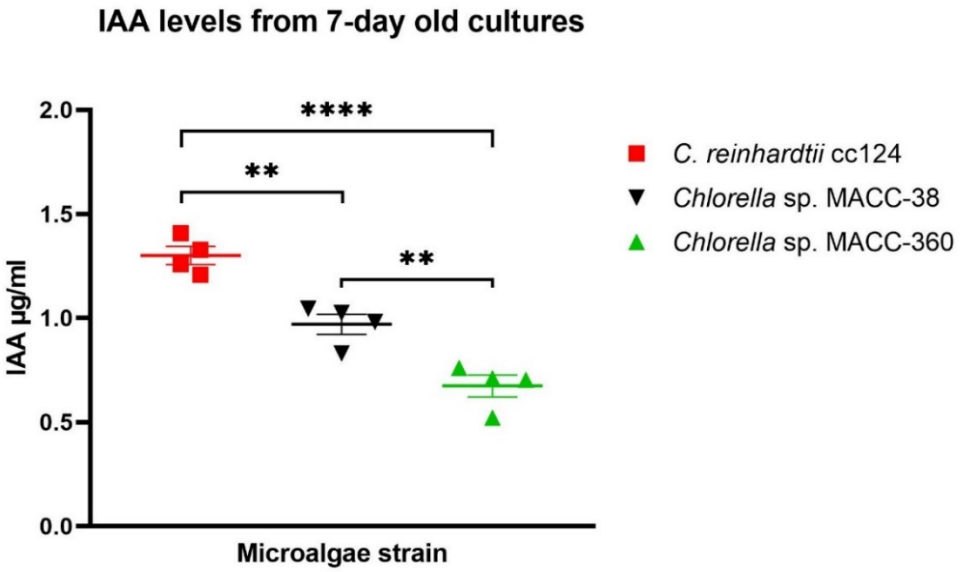

Figure 4.6. Auxin levels (indole acetic acid- IAA) released by *Chlorella* sp. MACC-38, *C. reinhardtii* cc124 and *Chlorella* sp. MACC-360 in L-tryptophan-supplemented Tris-acetate-phosphate (TAP) media. The graph is a scatter plot with all values from technical replicates of samples drawn from two distinct flasks per treatment. The horizontal line represents the mean, while the bars represent the standard deviation (SD). Ordinary one-way analysis of variance (ANOVA) with the Tukey test for multiple comparisons revealed significant differences between the three strains at $P < 0.05$, indicated by asterisks (the more the asterisks, the small the P value).

Since various strains had varying hormone concentrations, the exact amounts of MA-derived biostimulants should produce different plant-biostimulant effects in different plant species. 1g/L may be optimal for one strain but inhibitory for another. For example, roses and aubergines responded differently to seaweed and *Spirulina plantensis* extracts (Dias et al., 2016; Sumangala et al., 2019). In tomato plants, Kumari and coworkers found that low amounts of the same seaweed biostimulant had no effect, whereas high quantities were inhibitory, while Supraja and colleagues reported that MA extracts became growth-inhibiting above a specific limit/concentration (Kumari et al., 2011; Supraja et al., 2020b). In another study on tomato plants, Garcia-Gonzalez and Sommerfeld (2016) found that 3.75 g/L *Acutodesmus dimorphus* extract enhanced plant height, flower number, and branch number but lowered yields. Low doses of MA-based biofertilizer (10 g/L) boosted aubergine fruit output, while higher quantities (45 g/L) increased vegetative growth but decreased yield (Dias et al., 2016). These reports indicate the need for studies to identify the best concentration of biostimulants on plants. In the present study, we tested 1 g/L wet biomass for soil drench and 3 g/L (1%) wet biomass for foliar spray. These concentrations may not be optimal for tomatoes,

and identifying the most effective concentrations requires more experiments to test different concentrations.

4.2 Effects of microalgae on *Medicago truncatula*

4.2.1 Architecture and canopy cover of plants

Aerial photographs taken on the 45 DAP revealed the differences in canopy cover between treatments, in terms of the area covered by green plant material, as shown in the right pictures in Figure 4.7. In contrast, photographs of uprooted 50-day-old plants revealed the root structure differences on the left pictures in Figure 4.7 below. In the current study, we considered pots with densely packed plant tissue to have high biomass and pots with scant plant/leaf tissue to have lower biomass.

The 20 control plants shown in Figure 4.7a had less canopy cover than the 20 algae-treated plants from the MA-treated group shown in Figure 4.7b-d. *C. reinhardtii* cc124 and *Chlorella* sp. MACC-360-treated plants, as seen in Figures 4.7c and d, were more branched and leafy than control plants shown in Figure 4.7a. *C. reinhardtii* cc124 and *Chlorella* sp. MACC-360 treatments increased canopy cover. Uprooted *C. reinhardtii* cc124 and *Chlorella* sp. MACC-360-treated plants had more leaves and axillary branches than control plants. C. *reinhardtii cc124* and *Chlorella* sp. MACC-360 treatments also produced longer roots than the control. The *C. reinhardtii* cc124 regime had the longest roots, as shown in Figure 4.7c; *Chlorella* sp. MACC-360-treated samples in Figure 4.7d had moderately long roots; DW/Control and *Chlorella* sp. MACC-38-treated samples, shown in Figures 4.7a and b, had the least root biomass.

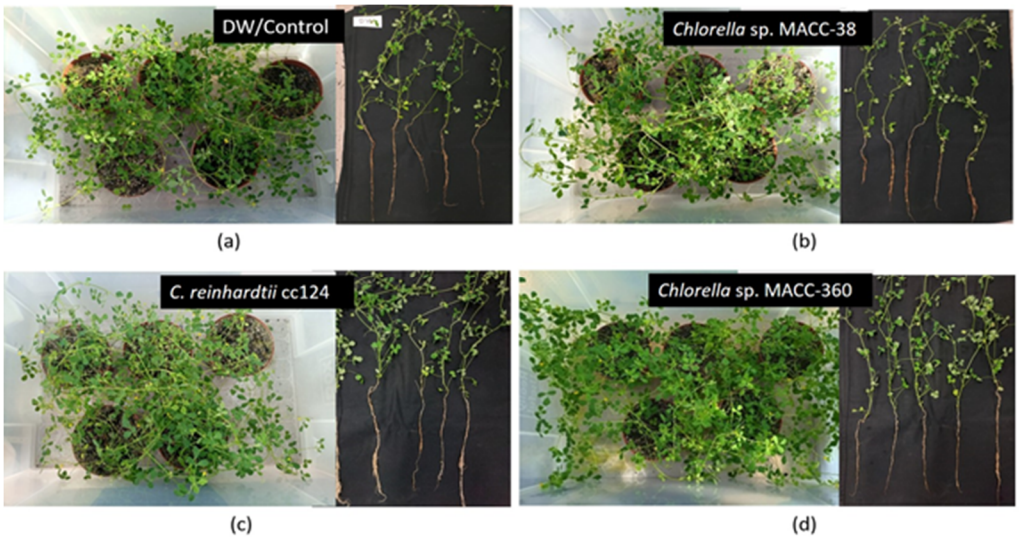

Figure 4.7. Aerial photographs of the plants 45 days after planting (DAP), five pots per treatment placed in a box: Distilled water (DW)/Control plants; *Chlorella* sp. MACC-38-treated plants; *C. reinhardtii* cc124-treated plants; *Chlorella* sp. MACC-360-treated plants. The left image in each panel depicts the aerial view, while the right image depicts the front view of uprooted plants (Gitau et al., 2021).

We observed that MA treatment affected *M. truncatula* development and shoot growth. The effect of MA on phyllotaxy explains the observed differences in biomass between treatments. *Chlorella* sp. MACC-38-treated plants displayed identical phyllotaxy to the control plants. In contrast, branches in *C. reinhardtii* cc124-treated plants bifurcated, while *Chlorella* sp. MACC-360-treated plants displayed improved axillary shoot growth. During the vegetative phase, *C. reinhardtii* cc124-treated plants lost the main shoot and formed two long branches. Most plants lacked unifoliate leaves and blossomed later. These characteristics resemble those of Headless (*hdl1*) mutants which have diminished axillary shoot development. This phenomenon in *C. reinhardtii* cc124-treated plants may be due to changes in the *Headless* (*HDL) 1* gene expression, which regulates shoot apical meristems (SAM) and leaf blade length. Heart-shaped leaves, short stems (dwarf plants), and poor flower production phenotypes were similar to *hdl* 1 mutants (Meng et al., 2019). *HDL1* gene is involved in auxin-dependent leaf morphogenesis (Ge et al., 2014); hence *C. reinhardtii* cc124 may have affected auxin homeostasis in plants.

4.2.2 Leaf dimensions

The application of algae altered the leaf size of plants, as demonstrated in Figure 4.8. Leaf petiole length, blade length, and blade width were measured (Figure 4.8a–c). Leaf parameters measurements were according to the previously given Figure 1.2 and the numerical nomenclature of *M. truncatula* (Bucciarelli et al., 2006).

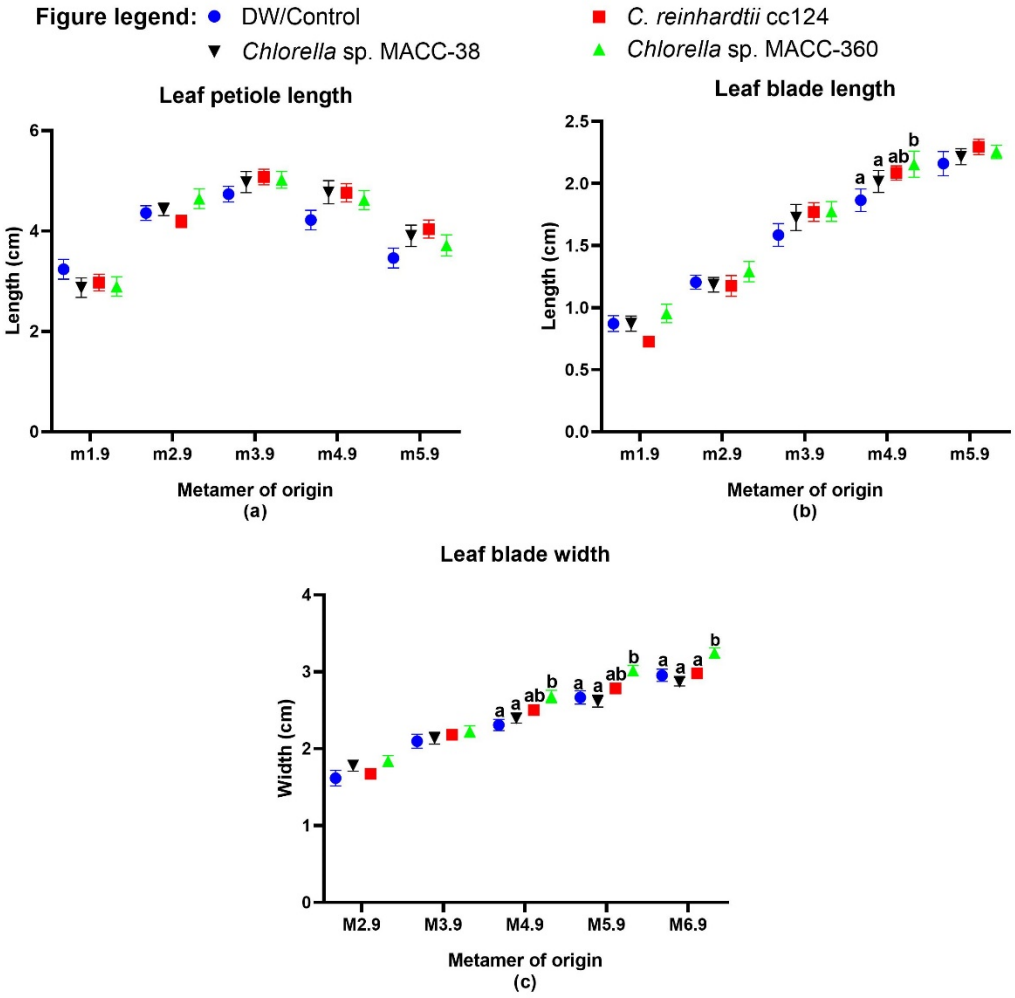

Figure 4.8. Effect of algae application on the leaf size of 50-day-old *M. truncatula* leaves; (a) Leaf petiole; (b) Leaf blade length; (c) Leaf blade width; 10 replicates were measured per experiment. Different letters on the bars indicate statistically significant differences ($P < 0.05$) between groups, as determined by Tukey's multiple comparison test. All parameters were analyzed using a two-way analysis of variance (ANOVA). The treatments were distilled water (DW) control, *C. reinhardtii* cc124, *Chlorella* sp. MACC-38 and *Chlorella* sp. MACC-360 (Gitau et al., 2021).

All strains diminished the petiole length of the first leaf, as shown in Figure 4.8a. On the second and third leaves, the effects were inconsequential. In contrast, MA increased the length of leaf petioles on younger leaves (M4.9 and M5.9). The effects of

Chlorella sp. MACC-38 and *C. reinhardtii* cc124 were greater than those of *Chlorella* sp. MACC-360. None of the strains significantly altered petiole length, as seen in Figure 4.8a.

During early development, both *Chlorella* sp. MACC-38 and *C. reinhardtii* cc124 decreased the blade length of plants. However, beginning with the third leaf, they lengthened the blade—*Chlorella* sp. MACC-360 increased leaf blade length throughout the growth period. In general, the effects of algae treatment on blade length were most outstanding between the third and fourth leaf developmental stages, as shown in Figure 4.8b. Altogether, only *Chlorella* sp. MACC-360 strain demonstrated statistically significant effects.

All algae treatments expanded leaf blade width during the growth period except for *Chlorella* sp. MACC-38 in Figure 4.8c. Overall, *Chlorella* sp. MACC-360 had the most significant impact on leaf width; it significantly increased the width of the fourth, fifth, and sixth leaves, as shown in Figure 4.8c.

Overall the additive effects of MA on leaf parameters suggest that they expanded leaf size/area. This observation could imply that MA-treated plants had a greater light-trapping capacity than untreated plants, which corresponded to the increased biomass in plant cover.

The phytohormones in the MA treatments may have increased cell division and cell elongation in the algae-treated plants, resulting in larger leaves. Since eukaryotic green MA produces both auxins and CK, applying MA will likely impact shoot and root elongation directly. Our findings are consistent with previous investigations that reported plant growth promotion by auxin-producing microorganisms such as bacteria and endophytic fungi (Bianco and Defez, 2010; Chinnaswamy et al., 2018; Defez et al., 2019; Kępczyńska and Karczyński, 2020; Maymon et al., 2015; Rey and Dumas, 2017; Varma et al., 2019; Viaene et al., 2016). Different ratios of auxins to CK or varying concentrations of specific phytohormones may account for the differences between the algae treatments. Even strains of the same genus can have vastly different CK production capabilities (Ördög et al., 2004a). In addition, different MA may produce distinct types of auxins, CK, and other hormones. For example, Stirk and coworkers demonstrated that most algal strains produce IAA auxin in higher proportions than indole-3-acetamide (IAM) (Stirk et al., 2013). Nonetheless, a previous study reported

that *Chlorella* sp. MACC-360 abundantly produces plant-growth-promoting phytohormones endogenously (Stirk et al., 2014).

Differential regulation of specific plant genes involved in leaf development in *M. truncatula* may account for the observed differences in leaf parameters in MA-treated plants compared to the control. For example, *Stenofolia* (*STF)* regulates leaf growth by regulating auxin levels (Tadege et al., 2011; Zhang et al., 2019, 2014). Recent research indicates that *STF* regulates auxin and CK homeostasis, and the hormonal crosstalk coordinates developmental signals at the adaxial–abaxial interface of primordial leaf cells (Niu et al., 2015). The change in leaf dimensions observed in the present study may be attributable to the MA effect on plants via their endogenous hormones, which affects the expression of genes associated with hormone homeostasis in plants.

4.2.3 Plant height, flowers, fresh weight, chlorophylls, and carotenoids

In addition to leaf dimensions, more phenotypic datasets, as shown in Figure 4.9, were evaluated to unravel physiological dissimilarities among treatments. These parameters included plant height, fresh biomass, dry biomass, and the number of flowers. We also determined the levels of Chl and Cars as biochemical parameters.

C. reinhardtii cc124 and *Chlorella* sp. MACC-360 raised plant height, while *Chlorella* sp. MACC-38 slightly lowered this parameter. Only *Chlorella* sp. MACC-360 affected plant height significantly, as shown in Figure 4.9a. All algae strains increased flower number but only *Chlorella* sp. MACC-360 did so significantly, as Figure 4.9b shows.

All algae treatments increased fresh shoot weight; however, only *Chlorella* sp. MACC-360 significantly outweighed the control, as demonstrated in Figure 4.9c. Both *C. reinhardtii* cc124 and *Chlorella* sp. MACC-360 strains increased fresh root weight relative to the control. On the contrary, plants treated with *Chlorella* sp. MACC-38 had less root fresh weight than the control, as shown in Figure 4.9c.

All algae strains increased total fresh weight but only the effect of *Chlorella* sp. MACC-360, shown in Figure 4.9c, was statistically significant. However, none of the

algae strains significantly affected dry weight, although *Chlorella* sp. MACC-360 increased shoot and total dry weight, as shown in Figure 4.9d.

Chlorella sp. MACC-360 and *Chlorella* sp. MACC-38 had negligible effects on Chl. In contrast, *C. reinhardtii* cc124, shown in Figure 4.9e, significantly increased Chl *b* and total Chl. All algae strains significantly increased plant Cars, but both *Chlorella* species outdid *C. reinhardtii* cc124, as shown in Figure 4.9f.

In summary, applying MA increased plant height and flower count, although only *Chlorella* sp. MACC-360 registered statistically significant effects for both parameters. Another striking phenomenon was the dramatic pigment increase (Chls and Cars). These findings are consistent with previous research regarding the effect of biostimulants on monocot and dicot plants (Faheed and Fattah, 2008; Fayzi et al., 2020; Kholssi et al., 2019; Kopta et al., 2018; Umamaheswari and Shanthakumar, 2021). In addition, *Chlorella* sp. MACC-360 induced early flowering, whereas *C. reinhardtii* cc124 slowed flowering. This early flowering accelerated pod development, resulting in smaller pods on control plants and larger pods on algae-treated plants at the scoring time, as shown in Figure S4.1.

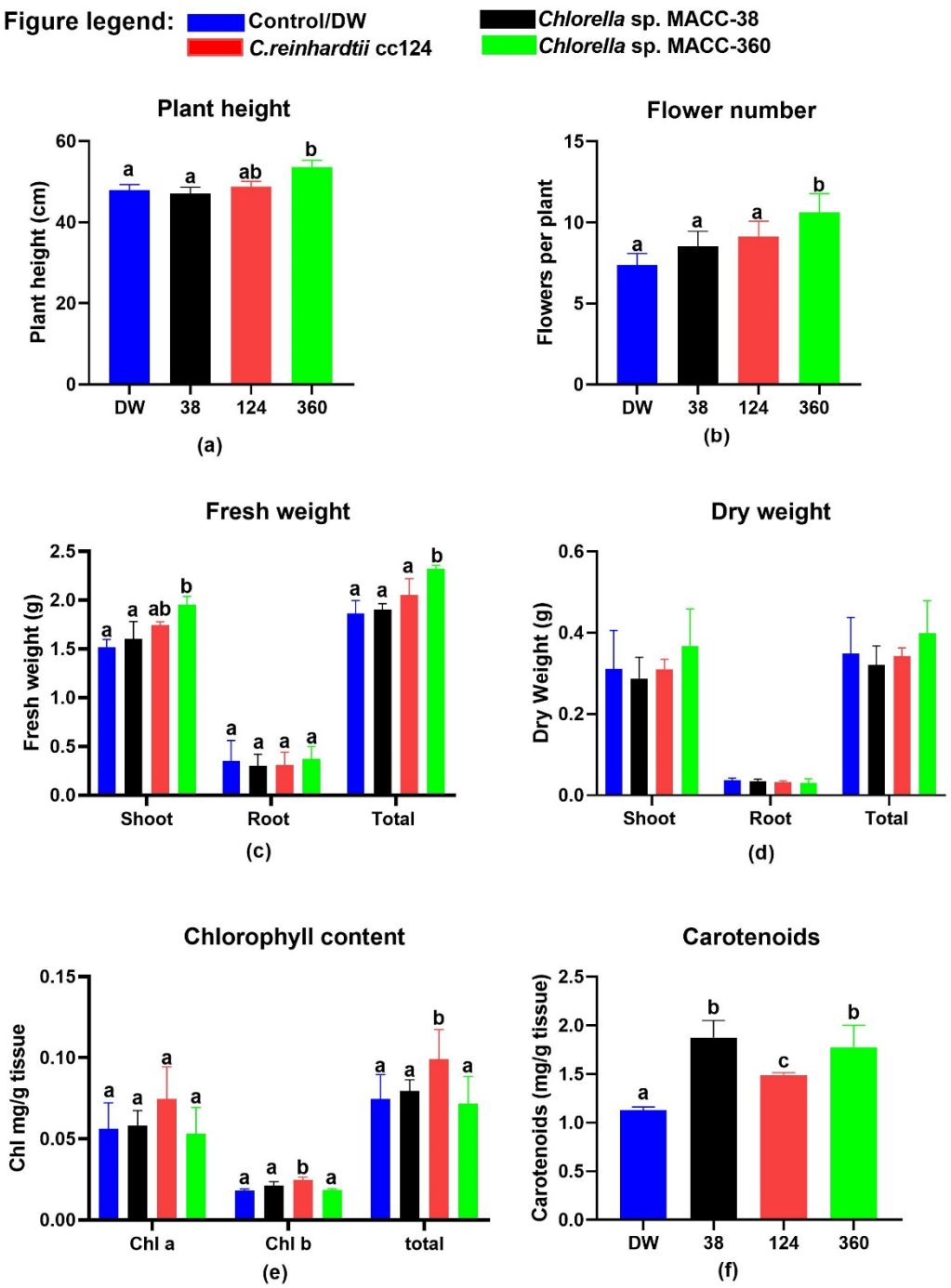

Figure 4.9. Effects of microalgae (MA) application on plants (50-day-old plants). Treatments are distilled water (DW) control, *Chlorella* sp. MACC-38, *C. reinhardtii* cc124 and *Chlorella* sp. MACC-360). Parameters are (a) plant height; (b) flower number; (c) fresh weight; (d) dry weight; (e) chlorophyll (Chl); (f) carotenoids (Cars). The data represent the means and standard error of means (error bars) of three technical experiments with ten biological replicates each. All parameters were analyzed using a one-way analysis of variance (ANOVA). Different letters on bars indicate significant differences between groups according to Tukey's test ($P < 0.05$) (Gitau et al., 2021).

4.3 Impact of microalgae on *Solanum lycopersicum*

4.3.1 Kinetics of flowering

When plants treated with the different treatments were compared (A[8]+C[9] versus B[10]+C), *Chlorella* sp. MACC-360 induced early flowering when applied in both forms and maintained the highest number of open flowers during the first week of flowering. In contrast, both Figure 4.10a and Figure S4.2 show that *C. reinhardtii* cc124 delayed flowering and recorded fewer open flowers than the control.

In Figure 4.10a, it is clear that *C. reinhardtii* significantly delayed flowering on the first week of blooming. *Chlorella sp. MACC-360* slightly enhanced flowering but remained similar to the control —however, the trend for *Chlorella sp. MACC-360* in the following weeks shows that it also stops flowering earlier. These results show the strain's influence on the crop cycle, which could affect harvest time. *C. reinhardtii* eventually caught up with the control. The flowering pattern observed may also influence yields, and it appears that *C. reinhardtii* reduced yields while *Chlorella* sp. MACC-360 hardly affected this parameter.

In the first week of flowering, *Chlorella* sp. MACC-360, TAP, and *C. reinhardtii* cc124-treated plants maintained a more significant number of open flowers than the control when MA was applied as A+C, as shown in Figure 4.10b. Figure 4.10b shows that *Chlorella* sp. MACC-360's difference from all treatments was statistically significant at $P < 0.05$. *C. reinhardtii* cc124-treated plants resembled TAP and DW-treated control plants (Figure 4.10b). The application of *Chlorella* sp. MACC-360 in any form stimulated flowering, although A+C application had the most excellent effect relative to the control. In contrast, *C. reinhardtii* cc124 application only promoted flowering when applied as A+C but delayed flowering when applied as B+C, as shown by Figure 4.10a and Figure 4.10b, respectively.

These findings demonstrate that the extra minerals in the growth media did not cause a significant biostimulant effect in our research in TAP-treated plants. Although TAP slightly enhanced flowering, *Chlorella* sp. MACC-360-treated plants

[8] A= whole culture (cells and their growth media) applied as a soil drench

[9] C= Destroyed cells suspended in water applied as a foliar spray

[10]B= Cells only suspended in water applied as a soil drench

outperformed TAP-treated plants significantly. In contrast, *C. reinhardtii* cc124 could only promote flowering in conjunction with TAP, indicating that it delayed flowering if applied without the growth media. Even so, it is worth noting that the TAP present in the MA treatment contained fewer minerals than the TAP used as a control treatment because the algae cells had utilized the minerals for growth during algae cultivation. These results indicate the importance of carefully selecting the portion of MA cultures for plant cultivation.

Various biostimulants have induced flowering in plants in the past (Plaza et al., 2018; Pohl et al., 2019). Although there are numerous reports of this phenomenon for *Chlorella* sp., no report about *Chlamydomonas* sp. exists. Our research indicates that *C. reinhardtii* cc124 slows the flowering process in *S. lycopersicum*.

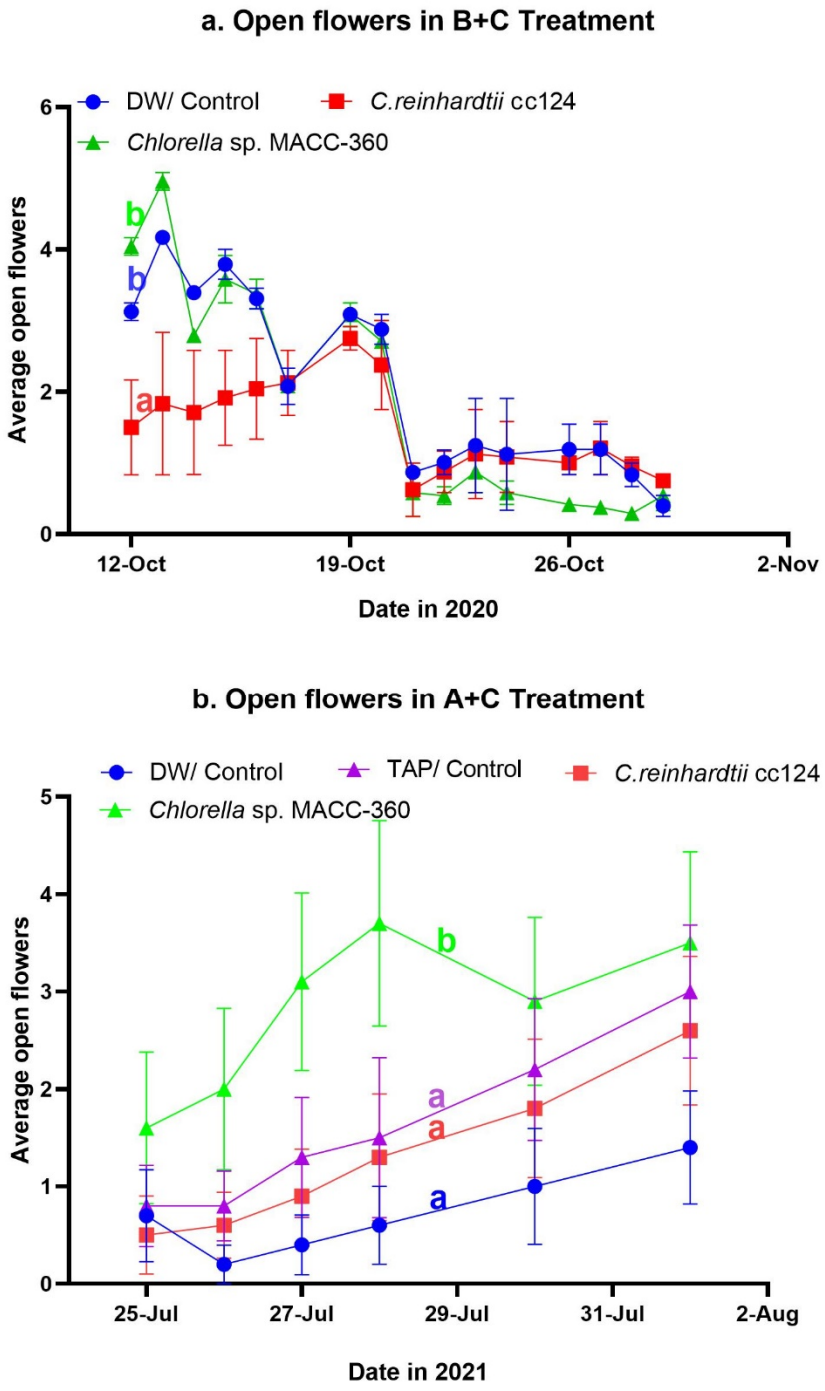

Figure 4.10. Flowering dynamics of tomato plants during the blooming season: a) Means of open flowers per plant for three experiments in set 1, Week 1 regime; plants treated with B (living cells) and C (water extracts) as a soil drench and foliar spray respectively; b) shows the means of open flowers per plant for the two experiments in set 2, Week 1 regime; plants treated with A (living cells with their growth media) and C (water extracts) as a soil drench and foliar spray respectively, during the first flowering phase; different letters show significant differences among treatments based on One-way ANOVA test. Error bars show standard error of means (SEM). The treatments are distilled water (DW)/Control, *Chlorella* sp. MACC-360, *C. reinhardtii* cc124, and Tris-acetate-phosphate (TAP) medium used for microalgae (MA) cultivation (Gitau et al., 2022).

4.3.2 Plant morphology, fruits, and pigments

Figure 4.11a shows that treatment of five-week-old plants with either MA strain's B+C treatment decreased plant height, while Figure 4.11b shows that this treatment increased plant diameter relative to the control. Figures 4.11a and b show that treatment of one-week-old seedlings with both strains slightly increased plant height but hardly affected plant diameter—only *Chlorella* sp. MACC-360 significantly decreased plant height when applied to five-week-old plants, as shown in Figure 4.11 a.

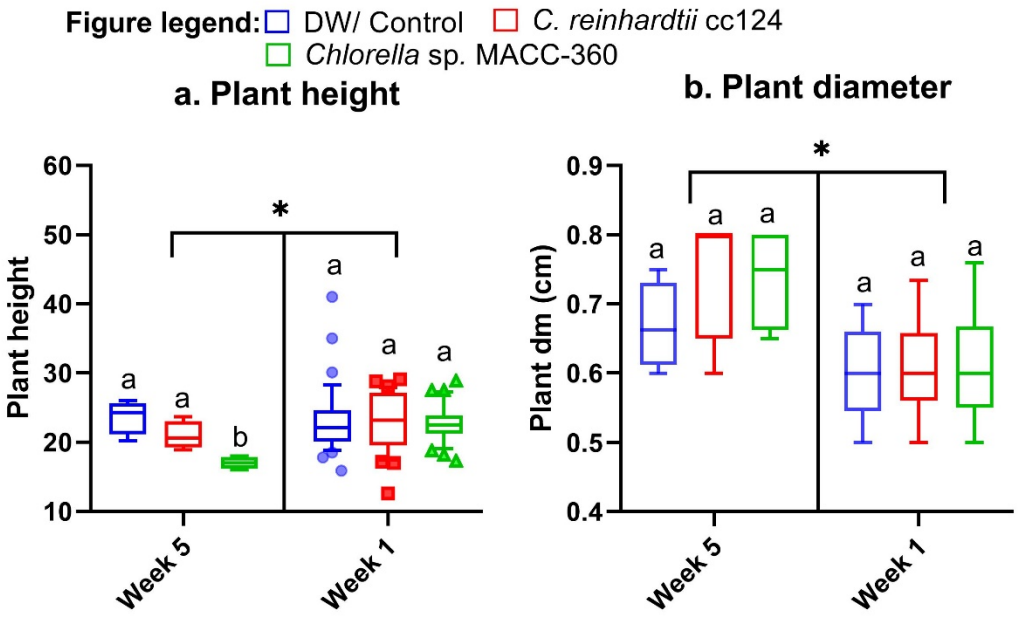

Figure 4.11. Comparison of the effects of algae treatment B+C (living cells and water extracts) on plants treated with algae at different ages (Week 1 and Week 5); a) plant height, b) plant diameter. The asterisk-marked lines across different treatment regimens indicate significant differences between regimens (Week 5 and Week 1). Asterisk-marked lines across different treatment regimens (Week 5 and Week 1) indicate significant differences between regimens at $P < 0.05$. Different letters within each regime indicate statistically significant differences between treatments within that regime at $P < 0.05$. Treatments include distilled water (DW)/Control, *C. reinhardtii* cc124, and *Chlorella* sp. MACC-360 (Gitau et al., 2022).

Except for *C. reinhardtii* cc 124-treated plants under the week 1 regime with fewer fruits than control, MA-treated plants had slightly higher fruit number, diameter, and weight in both regimes, as shown by Figure 4.12 a-c. Both MA did not significantly affect the yields on either regime, as shown by Figure 4.12d. Figure 4.12c shows that

only *Chlorella* sp. MACC-360 significantly increased fruit weight under the week 5 regime, but Figure 4.12b shows that both strains significantly increased fruit diameter under the week 1 regime.

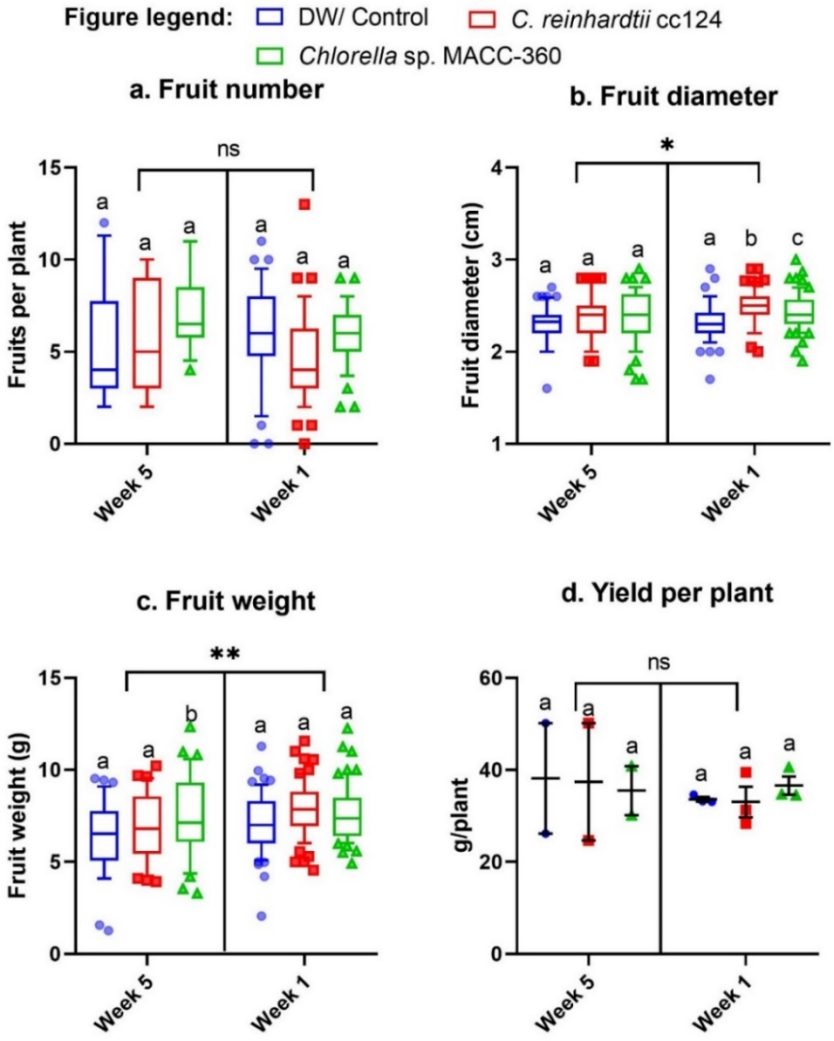

Figure 4.12. Comparison of the effects of algae treatment B+C (living cells as a soil drench and water extracts as a foliar spray) on fruit parameters of plants treated with algae at different ages (Week 1 and Week 5); a) fruit number, b) fruit diameter, c) fruit weight and d) yields. Asterisk-marked lines across different treatment regimens (Week 5 and Week 1) indicate significant differences between regimens at $P < 0.05$. Different letters within each regime indicate significant differences between treatments within that regime at $P < 0.05$. Treatments were distilled water (DW)/Control, *C. reinhardtii* cc124, and *Chlorella* sp. MACC-360 (Gitau et al., 2022).

Figure 4.13 shows that MA treatments of both strains increased leaf pigments, Chls, and Cars in all instances except for *C. reinhardtii* cc 124's slight drop of Cars during the week 5 regime. Significant effects occurred under the week 5 regime; Figure

4.13b and c show that *Chlorella* sp. MACC-360 significantly increased Chl *b* and Cars, respectively, relative to the control. Figure 4.13a shows that *C. reinhardtii* cc124 significantly increased Chl *a* only.

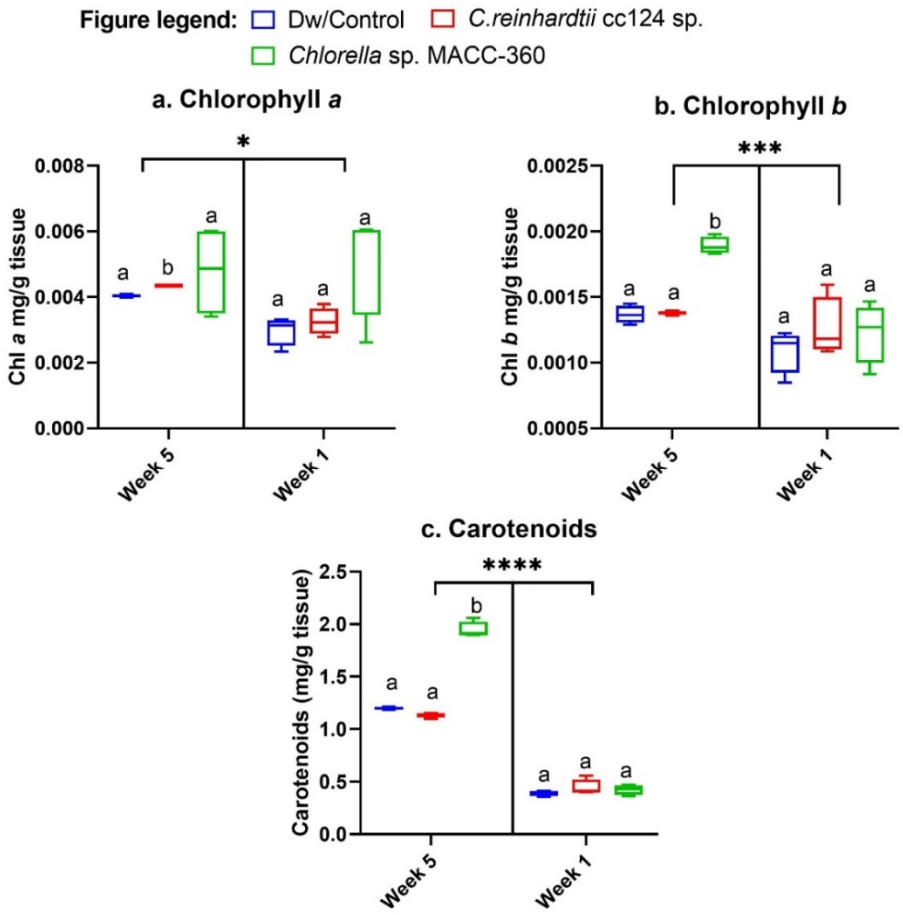

Figure 4.13. Comparison of the effects of algae treatment B+C (living cells as a soil drench and water extracts as a foliar spray) on plant pigments at different ages (Week 1 and Week 5); a) Chlorophyll (Chl) *a*, b) Chlorophyll (Chl) *b* and c) Carotenoids (Cars). Asterisk-marked lines across different treatment regimens (Week 5 and Week 1) indicate significant differences between regimens at $P < 0.05$. Different letters within each regime indicate statistically significant differences between treatments within that regime at $P < 0.05$. Treatments included distilled water (DW)/Control, *C. reinhardtii* cc124, and *Chlorella* sp. MACC-360 (Gitau et al., 2022).

Earlier, it was demonstrated that *S. lycopersicum* treated with various concentrations of biostimulants exhibited decreased fruit number, increased fruit weight and diameter, and decreased fruit yields (Mannino et al., 2020; Sutharsan et al., 2016). Reduced fruit production, particularly by *C. reinhardtii* cc124, may be attributed to harmful compounds such as 2,4-D auxin, as reported by Marth and coworkers (Marth

and Mitchelle, 1944). Past studies reported an increase in Chls and Cars in the leaves, flowers, and fruits of algae-treated plants (Coppens et al., 2016; Mutale-joan et al., 2020; Supraja et al., 2020a) which correlated with a decrease in Chl degradation and plant senescence (Blunden et al., 1996; Calvo et al., 2014).

Chlorophyll content correlates to SPAD values, which reflect plants' nitrogen status (Culman et al., 2013; Xiong et al., 2015). Thus, an increase in Chl indicates that plants received adequate or excess soil nutrients. Comparable results for algae-treated plants concerning nitrogen and phosphorus exist (Martini et al., 2021; Schreiber et al., 2018; Zhang et al., 2017).

Overall, treatment and age of plants at the first time of application had a significant impact on fruit diameter, with treatment accounting for a more significant proportion of variation than plant age or the interaction of the two factors. Nonetheless, only treatment alone significantly affected fruit number, whereas the age of plants during initiation of treatment only affected plant diameter. The only parameter significantly affected by the interaction between treatment and plant age during the initial application of algae was plant height. (Table 4.1).

The significant interaction between algae treatment and plant age at the commencement of algae treatment demonstrates that biostimulant action is time-dependent during plant development. The age of plants may influence plant height and diameter because juvenile plants prioritize vertical growth (height), while mature plants channel excess energy resources to girth extension (diameter) or reproduction/flowering. This observation occurred earlier when tomato plants that received *Scenedesmus sp*. biomass experienced a reduction in plant height (Ferreira et al., 2021). In addition, juvenile plants could have received most of the MA via soil drench during their active vegetative phase. Algae-treated plants may have had access to more nutrients than control plants. This assumption is because algae can affect the rhizosphere and, by extension, the entire metabolism of soil microbes, including the carbon and nitrogen cycles. Inoculating soil with cyanobacteria, for instance, resulted in a ten-fold increase in bacterial population diversity, while MA 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 are inaccessible to plants, application at a juvenile stage could provide soil microbes with sufficient time for mineralizing algal cells or developing various mutualistic relationships.

Table 4.1. Effect of treatment, plant age at initial application and their interaction on variation of measured plant and fruit parameters

	Treatment			Age at first application			Treatment x Age at first application		
Variable	% of variation	F	P value	% of variation	F	P value	% of variation	F	P value
Plant height	6.31	3.86	0.024	3.81	4.65	0.033	6.14	3.75	**0.027**
Plant diameter	1.59	1.92	0.151	21.8	31.7	**<0.0001**	2.64	1.16	0.318
Fruit No	4.53	3.45	**0.035**	0.06	0.92	0.340	2.16	1.64	0.197
Fruit DM	5.16	9.00	**0.0002**	1.14	3.97	**0.047**	0.59	1.04	0.356

Two-way analysis of variance (ANOVA) was conducted with two factors: Treatment (Distilled water (DW), *C. reinhardtii* cc124 and *Chlorella* sp. MACC-360) on the columns and Age of treatment (week 1 and week 5) on the rows. The parameters investigated were plant diameter, fruit number and fruit diameter (DM). The percentage of variation indicates the extent of a factor's contribution to the observed variation. F is the F statistic calculated from the degree of freedom from between the columns (DFn) and the degree of freedom from within the columns (DFd), the higher the F, the more significant the explained variation and vice versa. Bold *P* values are significant ($P < 0.05$) (Gitau et al., 2022).

The effect of MA on the soil microbial community may explain why week 1 regime plants exhibited more pronounced height effects than week 5 regime plants. This result is consistent with the findings of Garcia-Gonzalez and Sommerfeld, who observed that the timing and amount of application significantly impacted the agronomic performance of plants (Garcia-Gonzalez and Sommerfeld, 2016). Despite this, our overall findings indicate that the application of MA to tomato plants about to enter anthesis (Week 5) produces more desirable effects than application at a juvenile stage (Week 1). In addition to the above parameters, there was a significant difference between Week 1 and Week 5 regimes for all the pigments and fruit weights as shown in Figure S4.1. Other researchers have determined this age (approximately 35–40 DAP

or 1.5 months) to be optimal for initiating biostimulant application (Helaly et al., 2018; Plaza et al., 2018).

4.3.3 Reproductive capacity of tomato

Figure 4.14a shows that when plants received A+C treatments, *Chlorella* sp. MACC-360-treated plants maintained the highest number of trusses at all scoring times. This number was closely followed by TAP-treated and *C. reinhardtii* cc124-treated plants, while DW-treated plants produced the fewest trusses. At 50 DAP, *Chlorella* sp. MACC-360-treated plants produced the greatest number of flowers, while at 60 DAP and 70 DAP, they produced the least, as shown in Figure 4.14b. Figure 4.14b shows that the number of open flowers decreased from 50 DAP to 70 DAP for all plants, except for the DW-treated control plants, whose flowering remained constant between 60 DAP and 70 DAP.

Figure 4.14c shows that *Chlorella* sp. MACC-360-treated plants had the highest number of bearing trusses at 50 DAP and 60 DAP, whereas control plants had the lowest number of bearing trusses at all scoring times. In TAP/control and *C. reinhardtii* cc124-treated plants, the number of bearing trusses increased as scoring time progressed. The TAP control and *C. reinhardtii* cc124-treated plants significantly differed from the DW control on the 70 DAP. Figure 4.14c shows that even if *Chlorella* sp. MACC-360-treated plants had the highest number of bearing trusses; they did not differ significantly from the other treatments at 70 DAP.

Figure 4.14c shows that all treatments had more bearing trusses than the DW control on 50 DAP. However, this difference leveled off over the following 20 days (approximately three weeks), even though *Chlorella* sp. MACC-360-treated plants had slightly more bearing trusses than DW control plants at 70 DAP. This trend coincides with the early flowering phenomenon—all the flowers in *Chlorella* sp. MACC-360-treated plants could have transitioned into fruits, and at this point, there were no new flowers/trusses, while in control, the flowers were still transitioning. Figure 4.14d shows that TAP and *C. reinhardtii* cc124-treated plants produced fewer fruits per truss than DW control plants at 70 DAP.

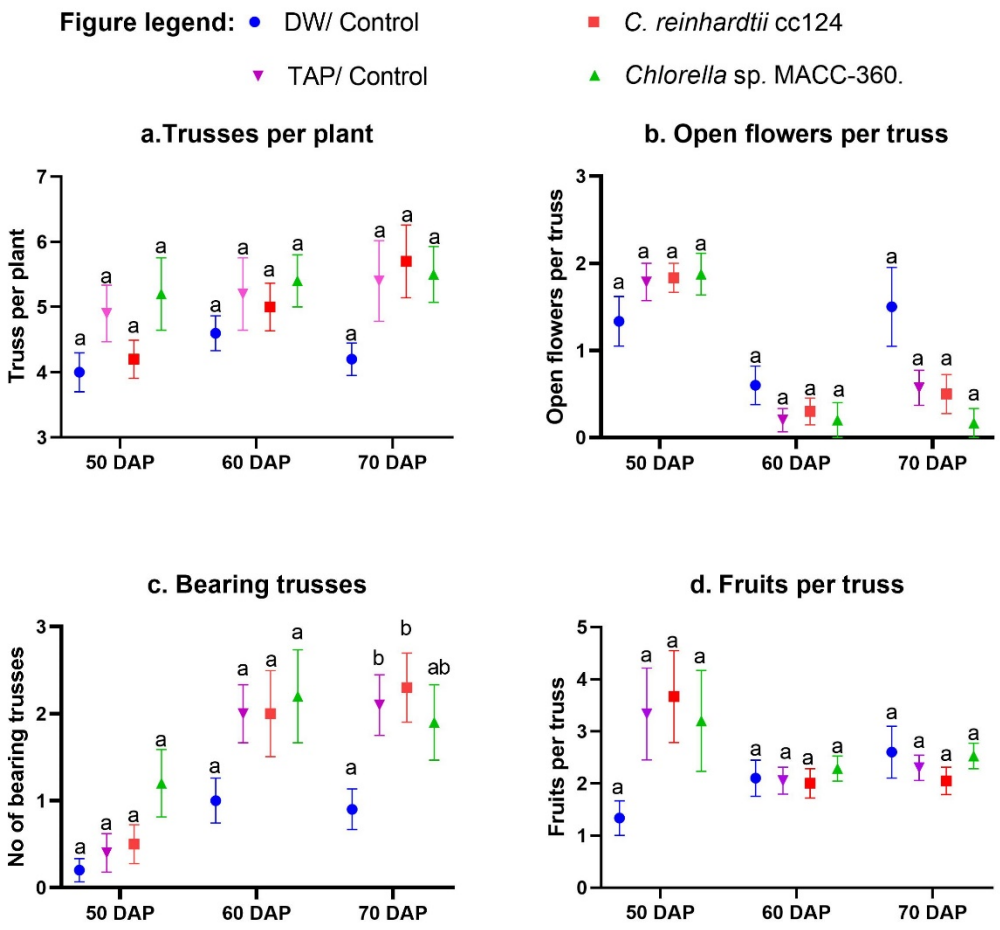

Figure 4.14. Reproductive parameters on the 50th, 60th, and 70th days after planting (DAP): a) Number of trusses, b) Number of open flowers per truss, c) Number of bearing trusses (trusses with fruits), and d) Number of fruits per truss. Alphabetical letters represent significant differences between groups; similar letters indicate no significant difference; different letters indicate $P < 0.05$ significant difference. The treatments were distilled water (DW)/Control, *C. reinhardtii* cc124, and *Chlorella* sp. MACC-360, and Tris-acetate-phosphate (TAP)/Control medium.

A Two-way ANOVA analysis revealed that, except for the number of bearing trusses, MA treatment explained the most significant proportion of observed variation for all parameters (Table 4.2). In this case, flowering and fruiting represent the daily data collected during the first two weeks of flowering. This information reveals the kinetics of flowering and fruit development rather than the number of flowers and fruits.

Overall, treatment substantially affected flowering and fruit development (Table 4.2). The scoring time significantly affected flowering and the number of bearing trusses because plants that bloomed earlier would produce fruits sooner than

those that bloomed later (Table 4.2). The number of open flowers per day included freshly opened flowers and previously existing but still to wither; this could be similar at any scoring time between treatments but the trend over the flowering period could be different. Examining the kinetics of flowering and fruit development captured this difference successfully, which this parameter (number of flowers) is incapable of revealing.

Table 4.2. Effect of treatment, time of scoring and their interaction on the variation of reproductive capacity parameters

	Treatment			Time of scoring			Treatment x Time of scoring		
Variable	% Variation	F	P value	% Variation	F	P value	% Variation	F	P value
FL	**11.7**	7.05	**0.0002**	6.98	4.19	**0.007**	1.45	0.29	0.977
FT	**9.39**	3.78	**0.013**	0.65	0.39	0.677	0.68	1.16	0.991
T	**8.54**	3.62	**0.016**	3.54	2.24	0.111	2.64	1.04	0.557
BT	**10.1**	5.41	**0.002**	20.0	16.1	**<0.0001**	2.92	0.78	0.585

Two-way analysis of variance (ANOVA) was conducted with two factors: Treatment (Distilled water (DW), *C. reinhardtii* cc124 and *Chlorella* sp. MACC-360) on the columns and time of scoring (50 days after planting (DAP), 60 DAP and 70 DAP) for all parameters and 4 time points for the flowering data) on the rows. The parameters investigated were FL=flowering, FT=fruiting, T= number of trusses and BT= Bearing trusses (trusses with fruits). The percentage of variation indicates the extent of a factor's contribution to the observed variation. F is the F statistic calculated from the degree of freedom from between the columns (DFn) and the degree of freedom from within the columns (DFd), the higher the F, the more significant the explained variation and vice versa. Bold *P* values are significant at ($P < 0.05$) (Gitau et al., 2022).

When plants have access to sufficient/excess nutrition or exposure to compounds that induce rapid cell division and enlargement, they experience rapid growth and early maturity. In past studies applying MA as a biofertilizer, growth promotion was already strongly associated with increased nutrient uptake, biomass accumulation, and crop yields (Kholssi et al., 2019; Shaaban, 2001). Mutale-joan and coworkers (2020) found out that MA extracts promoted shoot and root development in *S. lycopersicum*, resulting in enhanced absorption of nutrients and water from the soil, primarily due to an increase in root surface area for absorption. Other studies

demonstrated that MA extracts upregulated genes involved in biological pathways and processes, such as primary and secondary metabolisms and intracellular transports, primarily associated with root characteristics and nutrient uptake (Barone et al., 2018). These effects culminate in increased mineral absorption and, as a result, an increase in photosynthetic products. The fact that *Chlorella* sp. MACC-360 had a more significant impact on plant growth than TAP media suggests that it either contained more nutrients or bioactive compounds absent from other treatments. These findings are consistent with Ferreira and coworker's findings that *Synechocystis* sp. had a higher nitrogen content than *C. vulgaris*. Nevertheless, the latter had a more profound effect on plants (Ferreira et al., 2021), indicating MA triggered the growth-promoting action and not the extra nutrients.

4.3.4 Photosynthesis

When plants received A+C treatments, PhiNPQ and Fv/Fm differed significantly between plants treated with the MA strains, as shown in Figures 4.15c and 4.15d, respectively. *Chlorella* sp. MACC-360 promoted photochemistry, while *C. reinhardtii* cc 124 promoted the protection of PS II. Figure 4.15b shows that *C. reinhardtii* cc124 treatment significantly decreased PhiNO compared to the control. Figure 4.15h, on the other hand, shows that *Chlorella* sp. MACC-360-treated plants had significantly thicker leaves than all other treatments.

Leaf thickness in the wild tomato *Solanum pennellii* was associated with endopolyploidy-induced elongation of palisade mesophyll cells (Coneva et al., 2017; Coneva and Chitwood, 2018). Long palisade cells enhance carbon dioxide (CO2) uptake (Oguchi et al., 2005; Terashima et al., 2011) and light distribution (Brodersen et al., 2010, 2008), resulting in high photosynthetic efficiency. In addition, this trait helps leaves maintain a water potential during periods of low availability (Becker, 2007). This adaptive trait enables plants to increase performance by enhancing photosynthesis and water use efficiency. Poorter and colleagues (2009) concluded that under water-limiting conditions, plants might have to choose between rapid growth and leaf thickness (Poorter et al., 2009). It is plausible that plants treated with *Chlorella* sp. MACC-360 could withstand drought stress, as they already exhibit this trait without water restriction.

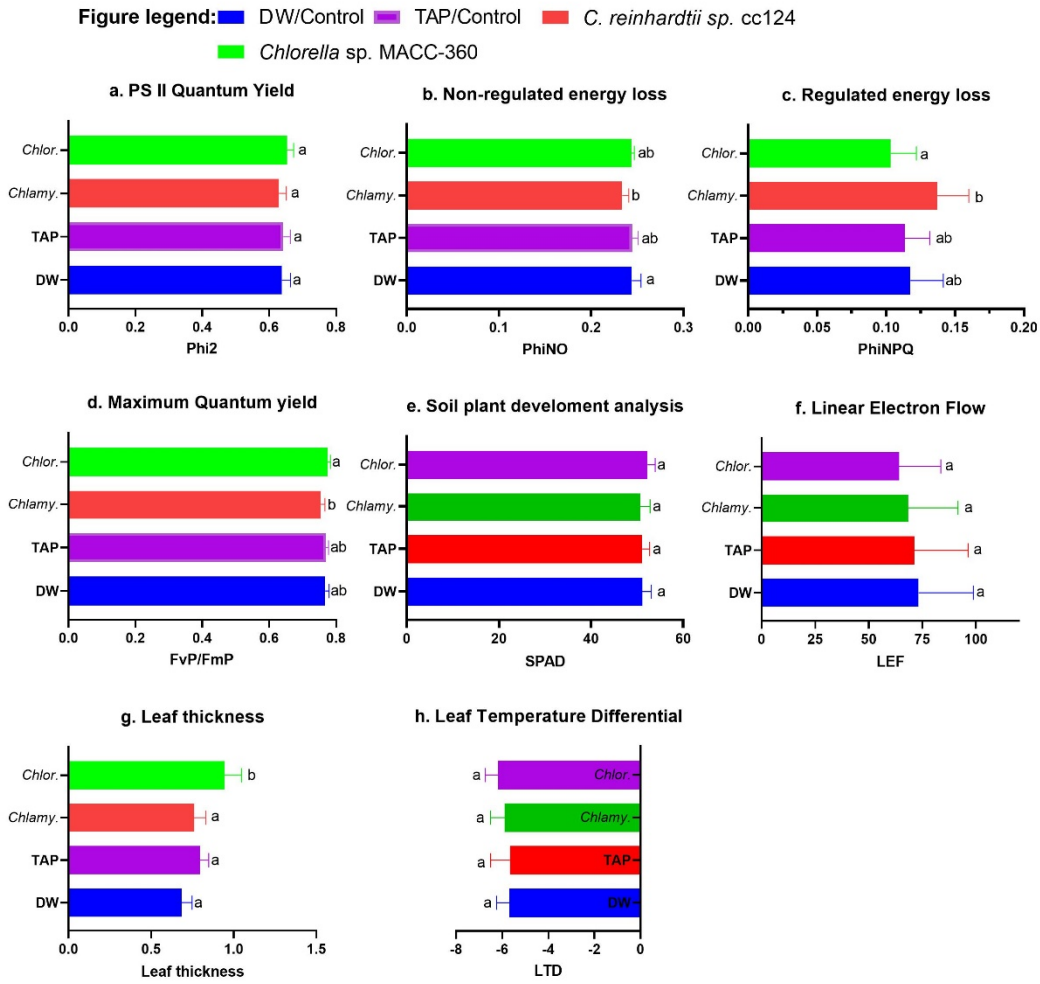

Figure 4.15. Parameters associated with the photosynthesis of plants belonging to the A+C treatment category where A is live cells and growth media and C the foliar water extracts; a. Phi2 is photosystem (PS) II quantum yield/ratio of incoming light (excited electrons) used in photochemistry/photosynthesis, b. PhiNO- the ratio of incoming light (excited electrons) lost in non-regulated processes and the products of which can be harmful/cause photodamage, c. PhiNPQ- the 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 indicates plant nitrogen status and relative chlorophyll (Chl), f. Leaf electron flow (LEF) is the linear electron flow and g. Leaf thickness is the thickness of the leaf section clamped by the Multispeq device, h. Leaf temperature differential (LTD) is the temperature difference between the leaf and its surroundings/environment. The bars represent the means, while the error bars represent the SE (Standard Error) measurements taken during the first five weeks of growth from 10 plants in each treatment group. Based on Tukey's analysis of variance (ANOVA) test, different letters at the ends of the bars indicate significant differences between groups at a $P < 0.05$. Distilled water (DW), Tris-acetate-phosphate (TAP) medium used for algae cultivation, *C. reinhardtii* cc124, and *Chlorella* sp. MACC-360 cultures in a TAP medium are the treatment regimes.

Both algal strains marginally increased LTD, indicating that the algae-treated plants could be more efficient at dissipating excess heat. These outcomes resemble those observed in maize treated with comparable MA (Martini et al., 2021). In *S.*

lycopersicum treated with algae, the increased LTD is associated with stomatal conductance and strongly correlates with improved root formation and water-stress tolerance (Martini et al., 2021; Oancea et al., 2013).

Both MA treatments caused an insignificant reduction of LEF, but only plants treated with *C. reinhardtii* cc124 showed a reduced PS II activity. The relationship between LEF and PS II activity is controversial. Although most scholars believe that reduced LEF suggests moderate photo-inhibition of PS II activity, others think that it could be due to increased acidity in the lumen due to the accumulation of photosynthetic products (Huang et al., 2018). 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.

Chls are very sensitive to organic nitrogen content (Padilla et al., 2018), and the slight increase in SPAD value in *Chlorella sp.* MACC-360-treated plants imply the presence of more Chls and accumulation of nitrogen in plants' chloroplast. Based on this fact, our results show that MA (*Chlorella sp.* MACC-360) treatment could enable the plant to take up more nitrogen. Zhang and coworkers observed this phenomenon when *Chlorella sp*. and tomato plants were co-cultured in a hydroponic system (Zhang et al., 2017). Martini and colleagues observed a strong growth-promotion effect by algae in maize grown in nitrogen-deficient conditions (Martini et al., 2021).

On the one hand, MA cells could increase the population of nitrogen-fixing organisms in the soil, increasing the nitrogen content available to plants. On the other hand, soil microbes could mineralize algal cells to become a source of nitrogen for plants. Either way, MA improves soil fertility and crop productivity (Kholssi et al., 2019). Overall, tomato plants treated with various MA extracts had increased levels of nitrogen and phosphorous relative to control. Schreiber et al. (2018) observed the transformation of phosphorous from algal biomass, supplied as wet or dry biomass, into a plant-available form. The author reports that nitrogen in *Chlorella sp.* cells was released for uptake by wheat roots which became longer and thicker than those of the unfertilized control plants for wheat grown in the sand (Schreiber et al., 2018). All thesestudies show that MA treatment increases nutrient use efficiency (NUE) in plants; however, in most cases, the exact mechanisms of action still need to be unfolded.

4.3.5 Transcription results

Only 103 transcripts of tomato genes had a fold change of +1.5 and above. Figure 4.16 shows the significantly enriched 12 GO biological process (GO: BP) terms associated with these transcripts; they clustered into two broad categories, cold-related and transcription-related terms. Response to cold was the most significant term. There were no significant enrichment terms of molecular function (MF) or cellular component (CC) categories.

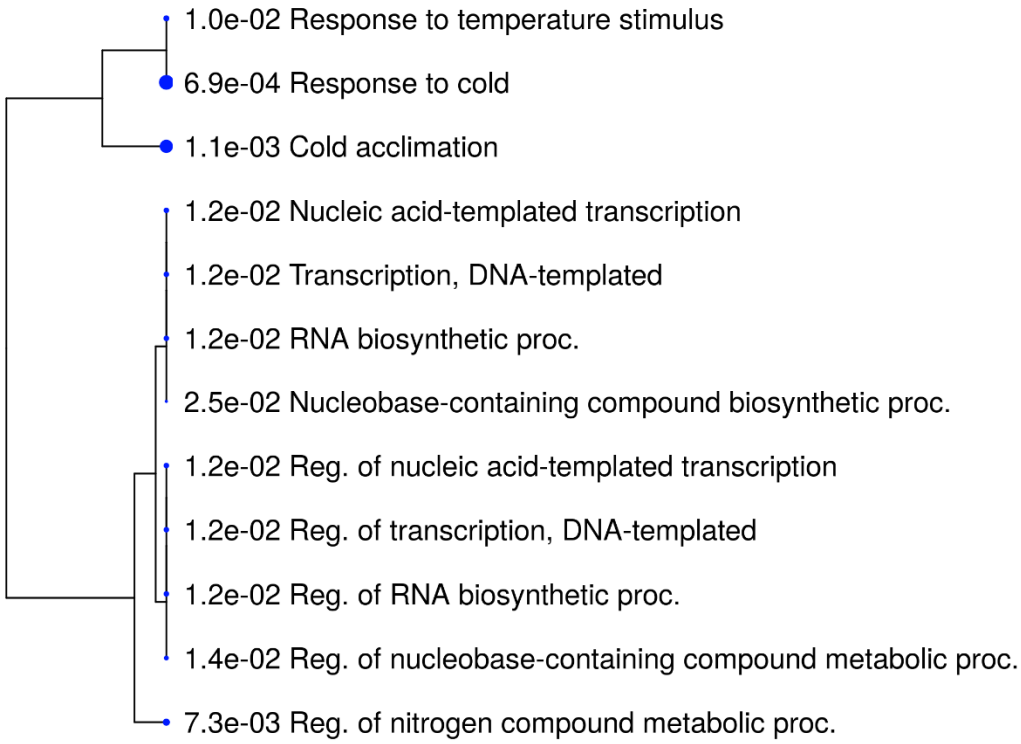

Figure 4.16. A hierarchical clustering tree from Shiny GO summarizing the correlation among significant Biological process (BP) pathways in the enrichment list of the gene transcripts overrepresented in the unopened flower buds of MA-treated plants relative to the control. Pathways with many shared genes clustered together. Bigger dots indicate more significant *P*-values than small dots.

In g: Profiler, one Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway term and some reproduction-related terms were identified in addition to the cold stress-related terms listed in Table 4.3. Response to cold and cold acclimation remained the most important terms.

Dehydrins play different roles in plants. In seed formation, they are involved in seed desiccation, while in vegetative organs, they accumulate during water scarcity. According to Interpro's description, all dehydrins have a central region composed of five to nine serines flanked by charged residues. They also have two copies of conserved lysine-rich octapeptides. One copy appears behind the charged residues next to the poly-serine region and the other at the C-terminal end (Blum et al., 2021; Paysan-Lafosse et al., 2022). This structure enables these proteins to dissolve at high temperatures and bind to proteins and acidic phospholipids, thereby protecting membranes from peroxidation (Godoy et al., 1990). Some authors suggest dehydrins act as chaperons or emulsifiers (Kosová et al., 2007). Tomato abscisic acid and environmental stress-inducible protein (TAS14), in particular, was found to accumulate in response to environmental stress (Del Mar Parra et al., 1996) or the presence of ABA (Godoy et al., 1990). Overexpression of TAS14 in drought-stressed plants revealed improvement in plant biomass and more significant and earlier accumulation of ABA (Muñoz-Mayor et al., 2012). Previous studies reported an accumulation of dehydrin proteins in seeds. However, in tomato plants, conditions in mature pollen were shown to trigger the expression of a late embryogenesis (LEA) protein involved in water stress responses (Taylor, 1997).

Moreover, González-Morales and colleagues reported an increase in transcripts that encode TAS14 and Ras-related protein (RAB) 18 in tomato and *A. thaliana* plants treated with *Ascophyllum nodosum* extracts (González-Morales et al., 2021). In another study, Puhakainen and colleagues overexpressed dehydrins in *A. thaliana* and observed increased freeze tolerance (Puhakainen et al., 2004). The upregulation of multiple dehydrins in MA-treated plants in the present study explains why 'cold acclimation' and 'response to cold' were the most significant terms.

Table 4.3. Functional profiles of upregulated genes from g; Profiler

Gene ID	Gene description	Pathway/mechanism
Solyc02g084850.3.1 [a, b]	TAS14 peptide (AA 1-130) (TAS14)	Dehydrin located in the cytosol; Binds to membranes phospholipids protecting membranes during stress
Solyc03g112440.1.1 [a]	Oleosin 18.2 kDa (LOC101263398)	Oleosin located in the membranes; Oil-body biogenesis; Oil body coalescence prevention
Solyc01g109920.2.1 [a, b]	Embryogenic cell protein 40 (LOC101259487)	LEA protein; Dehydrin located in the cytosol; Protein chaperons and emulsifiers
Solyc02g084840.3.1 [a, b]	Dehydrin (A0A3Q7F9F5_SOLLC)	Dehydrin located in the cytosol; Binds to membrane phospholipids protecting membranes during stress
Solyc02g085090.1.1 [a]	Syntaxin-112 (LOC104645739)	soluble N-ethylmaleimide attachment protein receptor (SNARE)vesicular transport; Intracellular protein transport, exocytosis
Solyc08g013830.1.1 [c]	Fatty alcohol: caffeoyl-CoA acyltransferase (LOC101244975)	Transferase; Cutin, suberin and wax biosynthesis
Solyc06g076800.3.1 [c]	Cytochrome P450 86A1 (LOC101259447)	Cytochrome P450 family protein; Located in the membrane; Fatty acid degradation; Cutin, suberin and wax biosynthesis
Solyc04g005610.3.1 [d-g]	Nascent Polypeptide-Associated Complex (NAC) domain-containing protein 2 (NAP2)	NAM protein; Located in the nucleus; Regulation of transcription; Developmental process, defense, plant hormonal control
Solyc05g007770.3.1 [d-g]	NAC domain-containing protein 1 (NAP1)	Regulation of transcription; Developmental process, defense, plant hormonal control

Superscripts on gene IDs indicate the GO term ID, name, and *P*-value reflecting significant association between the gene and the Go term where;

a= GO:0009409; Response to cold (*P* value= 0.0010)

b= GO:000963; Cold acclimation (*P* value= 0.0019)

c= KEGG:00073; Cutin, suberin and wax biosynthesis (*P* value=0.0288)

d= GO:0030582; Reproductive fruiting body development (*P* value=0.0051)

e= GO:0031155; Regulation of reproductive fruiting body development (*P* value=0.0051)

f= GO:0075259; Regulation of spore-bearing organ development (*P* value=0.0051)

g= GO:0075260; Regulation of spore-bearing organ development (*P* value=0.0051)

Conversely, oleosins are amphipathic structural proteins occurring in seeds and florets. They are 16kd to 24kd proteins bearing three domains; an N-terminal hydrophilic region, a central hydrophobic region, and a C-terminal amphipathic region (Murphy et al., 1991). The hydrophobic region consists of a β-strand structure which facilitates interaction with lipids. Due to this structure, they encompass triacylglycerols to make tiny droplets of oil bodies (Murphy et al., 1991). Thus, oleosins stabilize lipid bodies during colds by preventing oil body coalescence during desiccation. They also occur in the pollen surface in the lipoidal substance referred to as the tryphine or pollen coat (Murphy and Ross, 1998). Here, they enable pollen to stick together and on the surface of pollinators, thus promoting pollen dispersal. In addition, they aid in pollen attachment to the stigma surface and enable the uptake of water required for pollen germination (Kim et al., 2002). These reports demonstrate that the upregulation of the oleosin genes, in the algae-treated plants relative to the control, in the present study could have three explanations:

1. It could imply enhanced pollen transmission and successful germination.
2. It could indicate advanced pollen development in the floral buds of MA-treated plants relative to those of control plants.
3. It could indicate enhanced membrane stability due to the prevention of oil body coalescence during cold stress.

Applying MA to soils caused the upregulation of eight Nascent Polypeptide-Associated Complex (NAC)-domain-containing proteins in tomato flower buds, including the two in Table 4.3 and Table S4.2. The NAC-domain is an N-terminal module of about 160 amino acids in the No apical meristem (NAM) family proteins which are plant-specific transcriptional regulators (Aida et al., 1997). NAM proteins are involved in developmental processes such as shoot apical meristem, floral organ, and lateral shoot development. Furthermore, they control plant hormones and mediate defense responses. The NAC domain acts as a DNA-binding domain (DBD) and a dimerization domain (Duval et al., 2002; Xie et al., 2000). Ernest and colleagues (2004) emphasized that the universal structure of the NAC-domain has an extensively twisted antiparallel β-sheet with an N-terminal α-helix on one side and one shorter helix on the other side with helical elements surrounding it. This structure supposedly enables the

NAC domain to cause dimerization through the formation of salt bridges and to bind to DNA through the multiple positive charges in the NAC dimer face (Ernst et al., 2004).

The upregulation of genes encoding NAP in conjunction with enriched terms for ABA homeostasis may indicate increased senescence in algae-treated plants. The clustering of NAP genes with JA and its involvement in response to ABA suggests plant hormone regulation functions. Kim and colleagues reported an increase in rice leaf senescence mediated by a NAC and ABA biosynthesis pathway (Kim et al., 2019). Upregulation of ABA homeostasis results in enhanced tolerance to adverse environmental conditions such as water deprivation. Surprisingly, ABA-responding genes and dehydrin genes expressions are high in mature ovaries, with the levels dropping drastically after pollination (Vriezen et al., 2008). MA application may induce genes associated with flower development and abiotic stress. These results correspond to reports of flowering, abiotic stress, and herbivory-related genes sharing similar regulatory networks (Kazan and Lyons, 2016; Rasmann et al., 2018; Shavrukov et al., 2017).

Cytochrome P450 86A1 (CYP86A1) and Fatty alcohol: caffeoyl-CoA acyltransferase (LOC101244975) were associated with cutin, suberin, and wax biosynthesis KEGG pathway term (Table 4.3). According to Interpro's description (https://www.ebi.ac.uk/interpro/entry/InterPro/IPR001128/), the cytochrome P450 superfamily comprises heme-containing mono-oxygenases enzymes occurring in all kingdoms. These proteins use the heme to oxidize their targets; with protons donated from NADH or NADPH, they split the oxygen and add a single atom to the target. Such reactions also require electrons from several redox partners. In a comprehensive review, Kandel and colleagues (2006) reported their role in plants; they are critical enzymes in the biosynthesis of hormones, fatty acids, and defensive compounds. For example, the CYP86A1 participates in fatty acid degradation by hydrolyzing long fatty acids at the terminal methyl (ω-position) (Kandel et al., 2006). ω-hydrolyses participate in the synthesis of cutin and suberin. The thickening leaves in MA-treated plants could result from an elevated cutin synthesis. Increased cutin, suberin, or wax production enables the fortification of the cell walls to act as the first line of defense against biotrophic pathogens (Lewandowska et al., 2020). In a past study, upregulation of the wax biosynthesis occurred with the overexpression of APETALA 2/ Ethylene responsive factor (AP2/ERF) type transcription factors from *M. truncatula* in *M. sativa* whereby

the plants became tolerant to drought stress (Zhang et al., 2005). In our study, we observed the upregulation of similar transcription factors. In addition to cutin synthesis, cytochrome P450 proteins participate in plant development, reproduction, and detoxification. For example, in *A. thaliana*, CYP86A2, which shares 73 % amino acid sequence with CYP86A1, repressed bacterial type III genes enabling plants to withstand *Pseudomonas syringae* infestation (Xiao et al., 2004). CYP86A1 might play a similar role in plant-pathogen interactions (Kandel et al., 2006), implying that MA-treated plants could launch a more robust defense against bacteria pathogens than control plants.

Some authors cited carbohydrate metabolism as essential in stress and defense response (Keunen et al., 2013; Rojas et al., 2014). Keunen and colleagues (2013) mentioned that different sugar molecules control ionic balance, act as signaling molecules, detoxify ROS, and maintain turgor pressure in plants during stress. On the other hand, Roja and coworkers (2014) claimed that sugar metabolism positively regulates the expression of defense genes. In the present study, we identified the *Trehalose-6-phosphate synthase (TPS 1)* gene as an upregulated carbohydrate metabolism gene clustered with genes that respond to ABA and cold (Table S4.2). This gene is responsible for trehalose biosynthesis. In plants, trehalose plays essential roles in embryo formation and flowering, regulation of carbon metabolism, photosynthesis, and plant-microorganism interactions (Iturriaga et al., 2009). In *A. thaliana*, *TPS* promoted the signal for flowering pathway genes, and a defective gene resulted in the late flowering phenotype (Cho et al., 2018; Ponnu et al., 2011). Lyu and colleagues (2013) overexpressed a yeast *TPS 1* gene in a tomato leading to a transgenic tomato plant with enhanced abiotic stress tolerance and showing a greater photosynthetic rate under salt stress. Thus, in addition to flowering, *TPS 1* participates in stress amelioration due to its role in starch accumulation (Lyu et al., 2013). In the present study, upregulation of this gene in MA-treated plants correlates with the early flowering phenotype reported recently (Gitau et al., 2022). Biostimulants have promoted flowering in several studies, although the flowering genes reported in these studies, such as the single flowering truss (*SFT*) and the Falsifloras (*FA*) (Dookie et al., 2021; Lifschitz et al., 2006), were not detected in our studies. These differences might indicate the importance of sampling time or the actions of various biostimulants.

Only 50 transcripts showed differential downregulation with a fold change of minus 1.5 and below. Figures 4.17 to 4.20 show the significantly enriched terms associated with the transcripts underrepresented in MA-treated unopened flower buds relative to those of the control; they belong to the BP, MF, CC, and KEGG categories. Functional analysis in g: Profiler identified similar terms, as shown in Table 4.4.

Figure 4.17 shows the transcripts of the downregulated genes associated with the catabolic process and carbohydrate transmembrane transport terms. Sugar transmembrane transporter activity, polygalacturonase activity, and intramolecular activity were the most significant functions at the MF level, as shown in Figure 4.18. Figure 4.19 shows that transcripts of genes expressing proteins associated with the plasma membrane were the most downregulated, with a fold enrichment of 15. At the same time, those in the cell periphery were less affected. In general, most downregulated genes were components of the plasma membrane or cell wall. Some of these genes belong to the flavonoid biosynthesis pathway (two), metabolic pathways (ten), and the biosynthesis of secondary metabolites (eight), as shown in Figure 4.20.

The proteins whose transcripts were under-expressed in MA-treated plants relative to the control associated with carbohydrate metabolism are glycosidase/hydrolase enzymes, as shown in Figure 4.18 and Table 4.4. Glycosyl hydrolase enzymes disintegrate the glycosidic bonds between two or more carbohydrates and between a carbohydrate and a non-carbohydrate moiety. The activities of glycosyl enzymes are known, including those of Glycoside hydrolase family 28 (G28), to which most of the downregulated transcripts in the present study belonged. For example, polygalacturonase randomly hydrolysis 1,4-alpha-D-galactosiduronic linkages in pectate and other galacturonans (Huang and Schell, 1990; Ruttkowski et al., 1990). It is involved in the fruit ripening process that belongs to the cell wall metabolic processes. In microbes, these enzymes play a critical role in plant-pathogen interactions by causing maceration and soft-rotting of plant tissue in plants infected with *Ralstonia solanacearum*, *Erwinia carotovora* and *Aspergillus niger* (Huang and Schell, 1990; Ruttkowski et al., 1990).

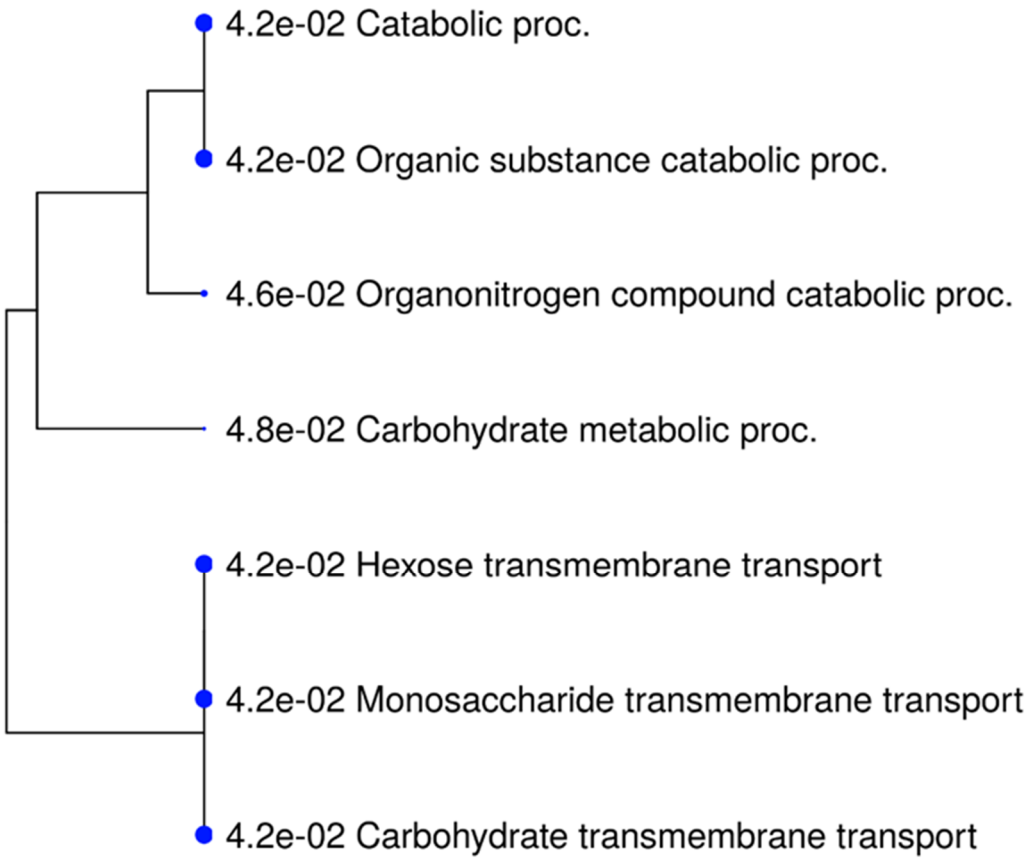

Figure 4.17. A hierarchical clustering tree summarizing the correlation among significant Biological process (BP) pathways in the enrichment list associated with the underrepresented transcripts in the unopened flower buds of MA-treated plants relative to the control. Pathways with many shared genes clustered together. Bigger dots indicate more significant *P*-values than smaller dots.

Generally, enzymes with the pectate lyase fold are virulence factors. They act on pectin or pectate (demethylated pectin) cell wall components. Accordingly, the downregulation of this category of genes implies a decline in the hydrolysis of oligosaccharides and the assemblage of large sugar molecules, specifically in the cell walls. Together with lowered catabolic processes, these processes facilitate the maintenance of cell wall integrity, which is critical as the first line of defense against microbial and herbivore attacks.

Table 4.4. Downregulated genes associated with significant GO terms in g:Profiler

Gene ID	Gene description	Pathway/mechanism
Solyc12g099070.1.1 [a]	Inositol transporter 4-like(LOC101246700)	Sugar/inositol transporter; Membrane protein; Phosphate transport
Solyc09g074530.3.1 [a]	Bidirectional sugar transporter NEC1(LOC101259076)	SWEET sugar transporter; Glycoside hydrolase; Membrane protein, sugar transport
Solyc08g080300.1.1 [a]	Sugar transport protein 1-like(LOC101266251)	Sugar/inositol transporter; Membrane protein; Phosphate transport; sugar transport
Solyc01g087280.1.1 [b]	Polygalacturonase-like(LOC101263946)	Glycoside hydrolase Family 28 (GH28); Cell wall component, secreted; Pentose and glucuronate interconversions; Metabolic pathways
Solyc07g044870.3.1 [b]	Polygalacturonase (A0A3Q7I6W1_SOLLC)	Glycoside hydrolase, GH28; Cell wall, secreted; glycosidase, hydrolase
Solyc07g056290.2.1 [b]	Exopolygalacturonase-like (A0A3Q7HDN8_SOLLC) CYTH domain-containing protein (A0A3Q7HT71_SOLLC)	Glycoside hydrolase, GH28; Pectate-lyase fold, cell wall component

Superscripts on gene IDs indicate the GO term ID, name, and *P* value reflecting significant association between the gene and the Go term where;

a= GO:0051119; Sugar transmembrane transporter activity (*P* value= 0.0318)

b= GO:0004650; Polygalacturonase activity (*P* value= 0.0353)

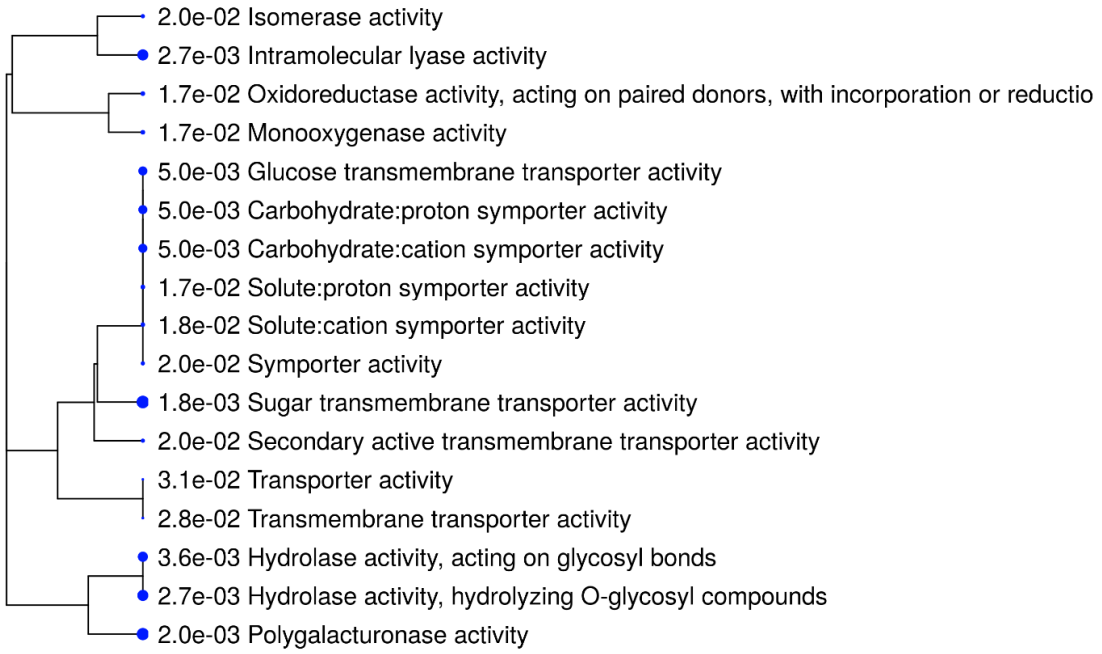

Figure 4.18. A hierarchical clustering tree summarizing the correlation among significant Molecular function (MF) pathways listed in the enrichment list associated with the underrepresented transcripts in the unopened flower buds of MA-treated plants relative to the control. Pathways with many shared genes clustered together. Bigger dots indicate more significant *P*-values than smaller dots.

A considerable portion of the downregulated genes belongs to the sugar transporters group (Table 4.4). Mueckler and colleagues (1985) opined that sugar transporters are membrane proteins in both prokaryotes and eukaryotes. They transport various compounds, including carbohydrates, acids, and organic alcohols (Mueckler et al., 1985). In particular, the SWEET sugar transporters function in plants' nectar production, pollen, and seed development. Moreover, Chen and coworkers (2010) reported that bacteria pathogens exploit rice sugar transporters for virulence. This phenomenon occurs when bacterial effectors bind directly to the SWEET promoter (Chen et al., 2010). In *Arabidopsis*, SWEETs are essential for pollen viability, whereas, in *M. truncatula*, they are essential for nodulation (Gamas et al., 1996). Thus, the downregulation of SWEET in plants treated with MA could offer protection against bacterial species whose effectors bind to the SWEET promoter. Downregulation of sugar transporters could also be a strategy to increase sugar molecules in the cytosol. Here, they may directly act as osmolytes, signaling molecules to trigger the transcription of defense molecules, or as the building blocks for synthesizing these defense molecules (Jeandet et al., 2022).

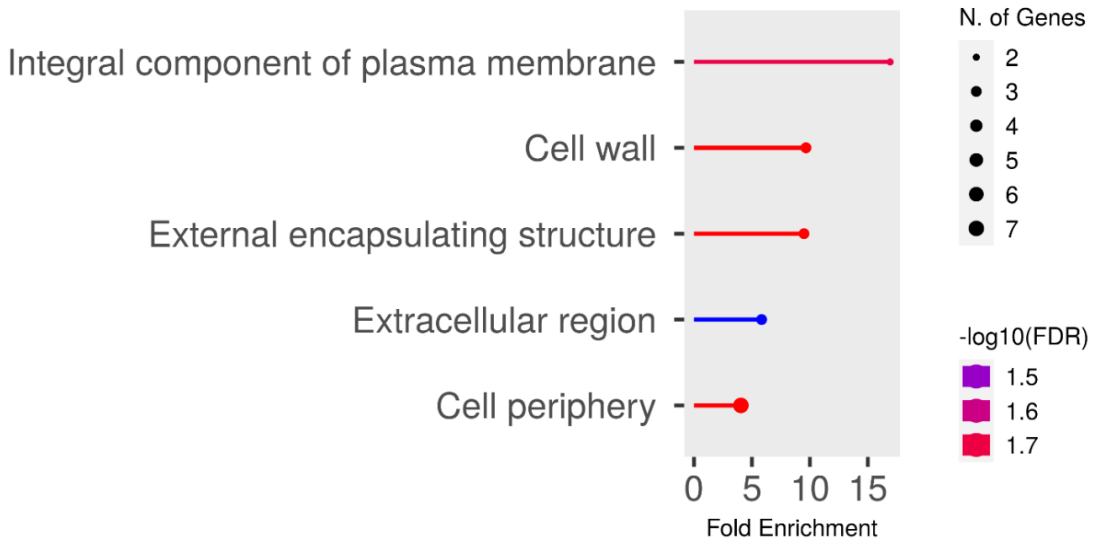

Figure 4.19. Cell Component (CC) terms associated with the underrepresented transcripts in the unopened flower buds of microalgae (MA)-treated plants relative to the control on the y-axis and Fold enrichment on the x-axis: Circle size indicates the number of genes involved, the bigger the circle, the more genes. The color indicates significance; light color shows high significance, while dark color shows low significance.

Figure 4.20 shows that among the downregulated DEGs, two belonged to the flavonoid biosynthesis pathway, while about ten belonged to the metabolic pathways and biosynthesis of secondary metabolite. Chalcone synthase (CHS1) and Chalcone flavanone isomerase (CHI2) are the two genes in the flavonoid biosynthesis. CHS1 catalyzes the reactions converting cinnamoyl-CoA, p-Coumaroyl-CoA, and Caffeoyl-CoA into their subsequent products, which end up in the flavanol or flavanone biosynthesis or anthocyanin biosynthesis sub-pathways of the flavonoid biosynthesis pathway. Downregulation of these genes restricts the pathway to the direction of synthesis of lignin precursors at the expense of anthocyanin production. Lignin is essential in fortifying the cell walls to make them impenetrable by pathogens and resistant to digestion by pathogen-related enzymes.

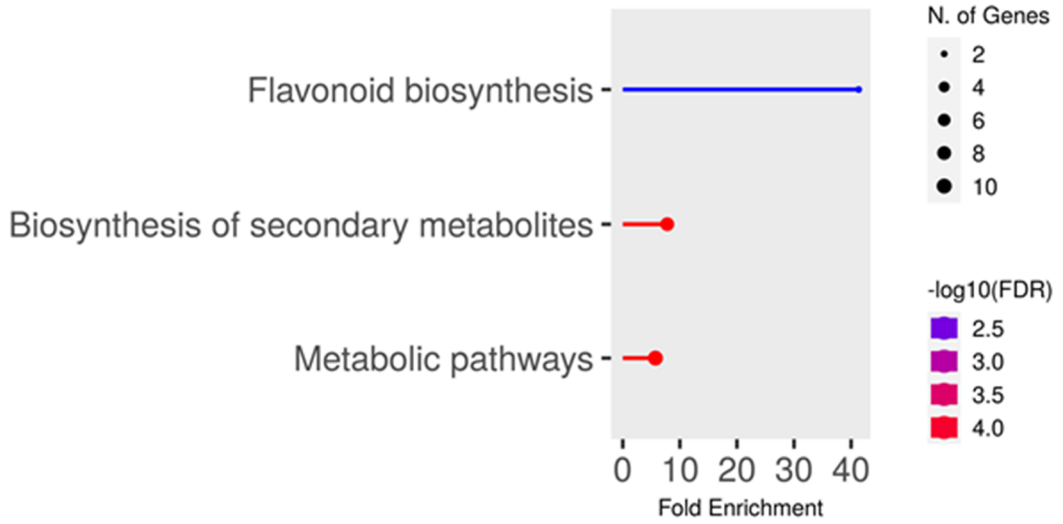

Figure 4.20. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway terms associated with the underrepresented transcripts in the unopened flower buds of microalgae (MA)-treated plants relative to the control on the y-axis and Fold enrichment on the x-axis: Circle size indicates the number of genes involved in the pathway, the bigger the circle, the more the genes. The color indicates significance; light color shows high significance, while dark color shows low significance.

Figure 4.21 shows the expression of randomly selected transcripts for validating transcriptomic results in the unopened flower buds of MA-treated plants relative to those of control plants. Among these genes was the one that codes for threonine dehydrogenase 2 (TD2) protein, involved in dehydrating threonine into 2-amino-3-ketobutyrate. This gene's downregulation leads to an accumulation of threonine and other amino acids synthesized upstream of threonine. TD2 plays a role in response to lepidopteran larvae attacks (Chen et al., 2007). Nonetheless, downregulation of this gene could have occurred alongside that of genes involved in floral development since MA-treated plants were ahead in the floral development process. This observation corresponds to recent reports that herbivory-induced defense responses and flower development share a common phytohormonal, metabolic, and molecular regulatory machinery (Ke et al., 2021).

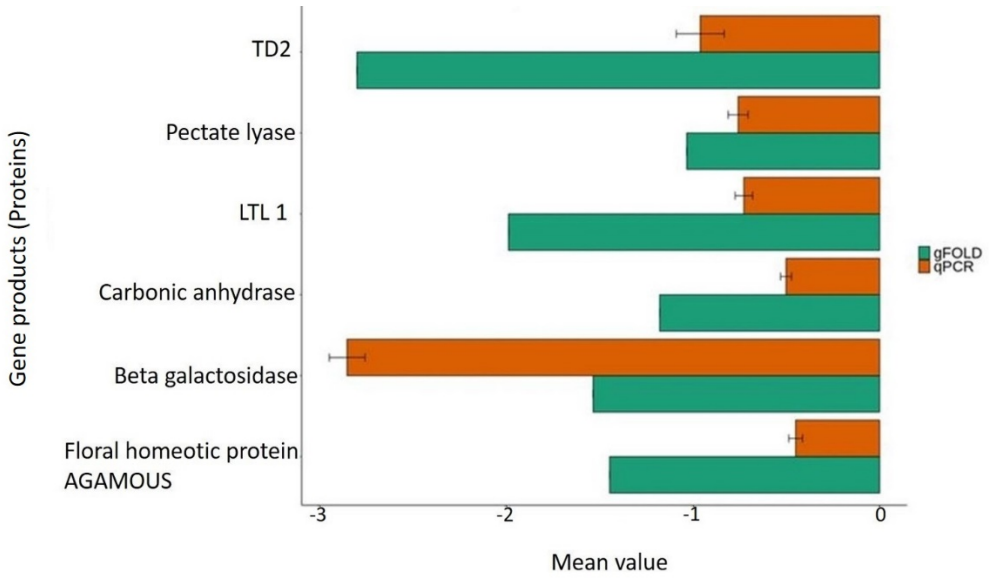

Figure 4.21. Validation of transcriptomic data with quantitative polymerase chain reaction (qPCR) of some randomly chosen underrepresented transcripts in the unopened flower buds of microalgae (MA)-treated plants relative to the control downregulated genes. The x-axis shows the mean relative expression of genes from two independent biological replicates relative to the control (green-colored bars represent g FOLD values based on transcription data from the first experiment, while orange-colored bars represent relative expression values based on qPCR analysis with samples from two independent experiments).

Beta-galactosidases and pectate lyase enzymes belong to the glycosidase hydrolase protein group aforementioned. A past study reported their diverse roles in early flowering, fruit development, and ripening (Kalamaki et al., 2007). In *S. lycopersicum*, there is limited literature about the function of these enzymes in flower buds. Nevertheless, silencing some beta-galactosidase (Smith et al., 2002) and pectate lyase genes (Yang et al., 2017) enhanced fruit firmness with no significant effects on the ripening process. Downregulation of these genes, which begins as early as the flower bud stage, could indicate increased fruit firmness in MA-treated plants. This trait prolongs the fruit shelf-life and thus improves fruit marketability. Further studies must confirm if the MA application will enhance fruit firmness. Some authors have even reported a reduction of tomato fruit susceptibility to the grey mold upon silencing a beta-galactosidase gene (Yang et al., 2017). All these pieces of evidence indicate that MA biostimulants increase desirable fruit characteristics.

4.3.6 Soil metagenomics results

A total of 2089 OTUs were identified taxonomically, most belonging to bacteria. Figure 4.22 shows that soil samples treated with *Chlorella* sp. MACC-360 had greater diversity across all metrics compared to control samples. Nevertheless, these differences were not statistically significant, as shown in Table 4.6. These results indicated that MA-treated samples contained a few different species which were absent from the control.

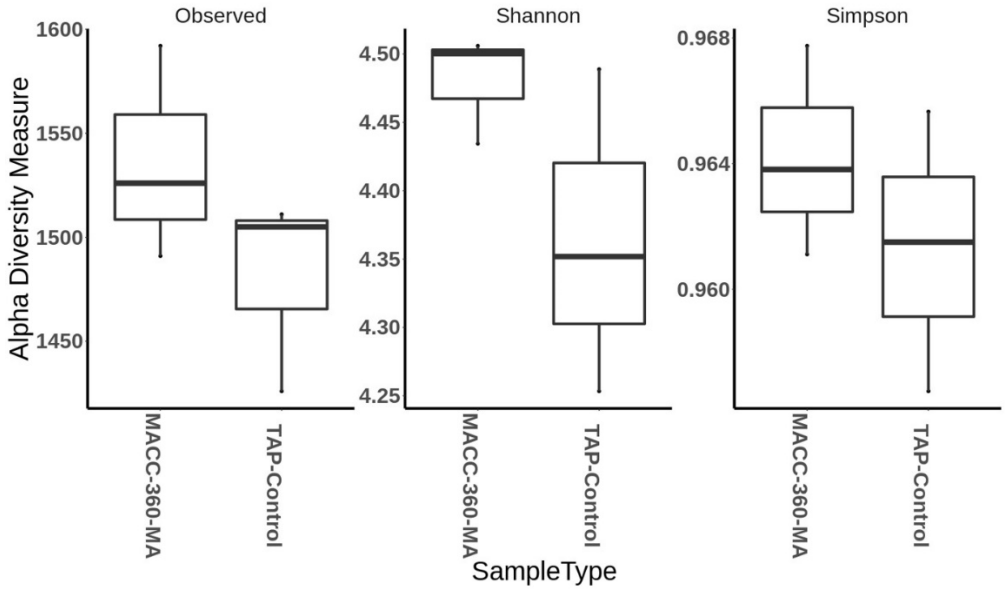

Figure 4.22. **Alpha diversity measures across samples**. Box plots showing alpha diversity of the soil samples on the y-axis and the treatment on the x-axis. The two lines extending from each side of the box show the minimum and the maximum values, the boxes show the interquartile region where most of the values fall and the central lines indicate the median. Tris-acetate-phosphate (TAP) Control and *Chlorella* sp. MACC-360 is the microalgae (MA) treatment. All diversity estimates were higher in samples treated with *Chlorella* sp. MACC-360 compared to control samples.

Table 4.6. Alpha diversity statistics

Diversity metric	Control	MA-treated	p.adj
Observed	TAP	MACC-360	0.4
Shannon	TAP	MACC-360	0.2
Simpson	TAP	MACC-360	0.7

Adjusted *p* values for multiple comparison at alpha $P < 0.05$.

Principal component analysis (PCA) with euclidean distances illustrated sample similarities for the list of differentially abundant OTUs at the genus level. Figure 4.23 shows the distribution of samples along the two components; Component 1 explained 90% of the variation along the X-axis, whereas Component 2 explained 6% of the variation along the Y-axis. MA-treated samples clustered together, while TAP-treated samples were scattered, indicating higher variability among these samples. The *Pseudonorcardia, Stigmatella*, *Limnobacter,* and *Halamonas* genera were associated with MA treatment, while *Streptomyces, Aromateleum*, and *Azoarcus* genera were abundant in control samples. However, the two treatments separated from each other, and variation due to treatment was high. Nonetheless, Table 4.7 shows that the differences between the samples were not statistically significant ($P = 0.1$).

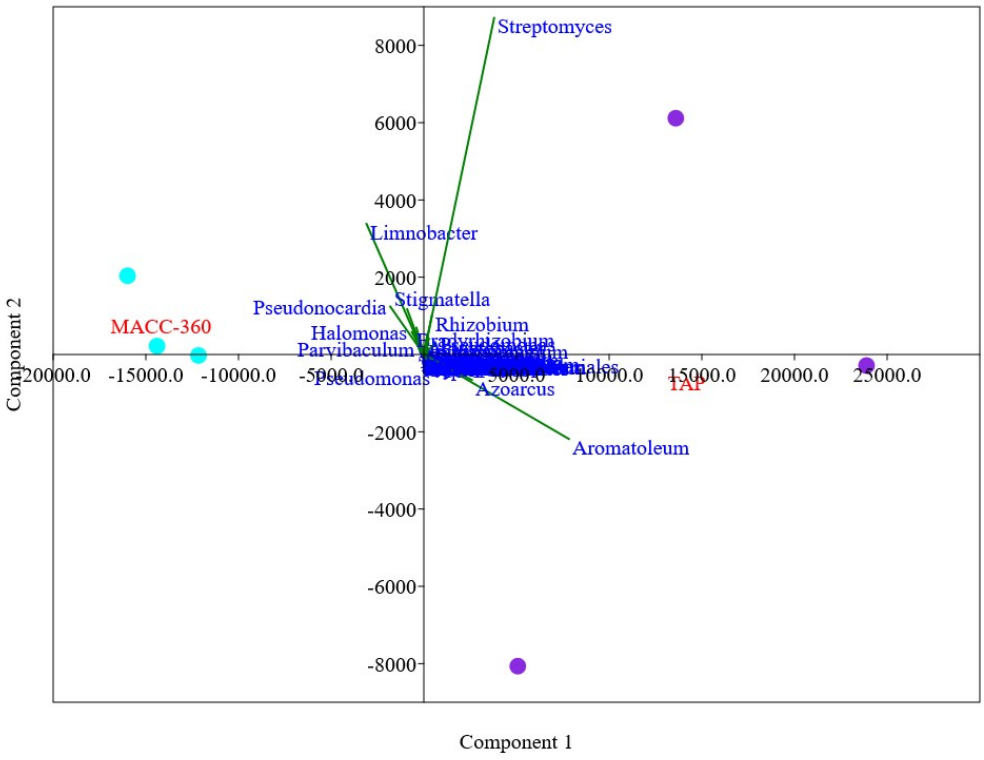

Figure 4.23. Clustering of the different soil samples. Aqua-colored dots represent the three replicates from the algae-treated samples (*Chlorella* sp. MACC-360 microalgae (MA)). Purple colored dots represent the three replicates from the control samples (Tris-acetate-phosphate (TAP) treated soils). The x-axis represents Component 1, which explains 90% of the variation, while the Y-axis represents Component 2, which explains 6% of the variation between the treatments.

Table 4.7. Beta diversity tests

	R value	p value	
ANOSIM	0.33	0.1	barely significant
ADONIS	0.26	0.1	barely significant

Adjusted *p* values for multiple comparison at alpha $P < 0.05$.

Only one of the technical sequencing replicates for each treatment was illustrated in the plotting of the donut charts. These were the samples from the technical replicate ending with the extension L001. The sequences uploaded ranged between 3, 768, 054 to 1, 875, 416 in TAP 1 and 3, respectively, as shown in Table 4.8. After quality control (QC), the sequence number ranged between 3, 483, 691 and 1, 742, 931 TAP 1 and 3, respectively. All the sequences had a guanine-cytosine (GC) content of about 61%. They had a mean length ranging between 244 ± 34 and 252 ± 34 base pairs. Plotting of the taxonomy charts used sequences that passed the quality control from the six metagenomes (Table 4.8).

Table 4.8. Summary of metagenome sequence data before and after quality control (QC)

Sample	Uploaded sequences	Post QC sequences	Mean GC (%)	Post QC Mean length base pairs (bp)
TAP 1	3768054	3483691	61 ± 11	244 ± 34
TAP 2	2446176	2273215	62 ± 11	250 ± 33
TAP 3	1875416	1742931	61 ± 11	251 ± 33
360 1	3244243	2890934	62 ± 10	250 ± 33
360 2	1927651	1796469	61 ± 11	252 ± 34
360 3	3022253	2739970	61 ± 11	251 ± 33

Tris-acetate-phosphate (TAP) samples are the control samples drenched with media used for algae cultivation. 360 refers to *Chlorella* sp. MACC-360, the microalgae (MA) strain used as algae treatment. QC refers to quality control executed in the Metagenomics Rapid Annotations using Subsystems Technology (MG-RAST) and GC is the guanine-cytosine content.

Figure 4.24 shows that Actinobacteria, Proteobacteria, and Firmicutes were the predominant phyla in all samples. Chlorophyta and Ascomycota were absent from the control samples but abundant in the MA-treated samples. Thus, the principal Chlorophyta species is what we applied as treatment which consequently caused the increase in fungal species.

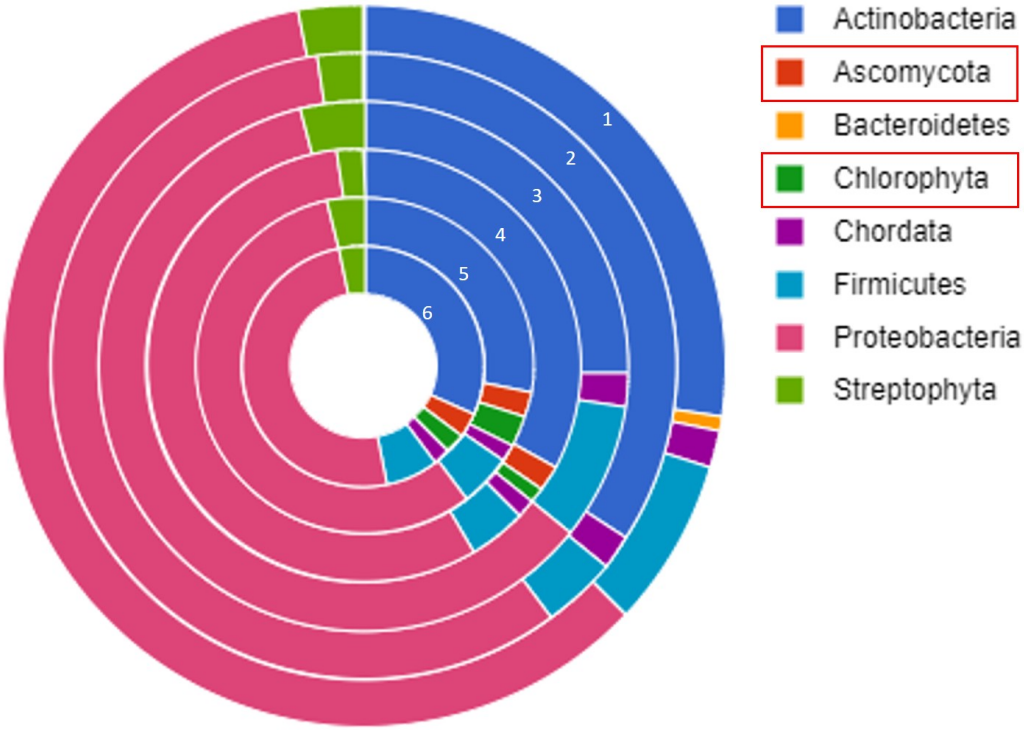

Figure 4.24. Donut taxonomy charts of the six metagenomes from three control and three microalgae (MA)-treated samples at the phylum level. Samples labeled 1 to 3 in the outer circles are from control soils, while samples labeled 4 to 6 in the inner circles are from MA-treated soils. Red rectangles highlight phyla with high abundances in algae treated samples relative to the control.

Figure 4.25 shows the distribution of families; *Rhodocyclaceae* was more abundant in the control samples, while *Trichomaceae* and *Caulobacteraceae* were prevalent in the MA-treated samples. These results clearly reveal that MA treatment influenced soil microbial structure by enriching these soils with microbial groups that play significant roles in soil ecosystems. For instance, *Caulobacteraceae* includes *Brevundimonas* species, a PGPR, while *Trichomaceae* includes *Aspergillus* species,

which play a central role in nitrogen and carbon cycling (Lee and Ryu, 2021; Nayak et al., 2020).

Figure 4.25. **Donut taxonomy charts of the six metagenomes from three control and three microalgae (MA)-treated samples at the Family level**. Samples labeled 1 to 3 in the outer circles are from the control soils, while samples labeled 4 to 6 in the inner circles are from MA-treated soils. Red rectangles highlight the families with high (*Caulobacteraceae* and *Trichomaceae*) and low (*Rhodocyclaceae*) abundances in algae treated soils relative to the control.

Overall, greater numbers of eukaryotic fungi appeared in samples treated with *Chlorella* sp. MACC-360. *Betaproteobacteria*, besides *Limnobacter* sp. SAORIC-580, are present in *Chlorella* sp. MACC-360-treated samples with lower abundance. In

addition, the abundance of specific *Deltaproteobacteria* increased in *Chlorella* sp. MACC-360-treated samples, as shown in Figure S4.3.

Limnobacter, *Pseudonocardia*, *Stigmatella*, and *Archangium* were more abundant in samples treated with MA, as shown in Figure 4.26a. *Limnobacter* belonging to the *Burkholderiaceae* family are heterotrophic, dicarboxylate-utilizing, Betaproteobacteria capable of amino acid and nitrate/nitrite assimilation, and sulfur oxidation (Chen et al., 2016). The increase in these bacteria in the presence of MA may indicate a symbiotic relationship in which the bacteria use the organic carbon or amino acids supplied by algae or plants while providing sulfur to the plants. In addition, they have MqsR (motility quorum-sensing regulator), which affects quorum sensing, biofilm formation, and stress response (Chen et al., 2016).

Pseudonocardia strains are oligotrophic, and numerous strains are plant endophytes. Several strains have demonstrated bioactivity that is effective against bacteria, fungi, and even algae (Park et al., 2008). Given that these strains are typically aerobic and utilize carbon monoxide (CO) as a carbon source, the increase in *Pseudonocadia* abundance in algal-treated soils could indicate a beneficial relationship with the applied algae (Park et al., 2008). Their increased growth could be a result of the oxygen released by algae. Due to its extensive array of bioactivities, this type of bacteria genera contributes to the protection of plants from pathogenic organisms.

Stigmatella and *Archagium* are myxobacteria that produce EPS and form cellular aggregates (Kunze et al., 2005). In this study, both *S. aurantiaca* and *A. gephyra* were abundant in MA-treated soils. These strains produce antimicrobial substances that are effective against a broad spectrum of fungi, yeasts, and bacteria (Kunze et al., 2005, 1984). The increased abundance of these bacteria in MA-treated soils indicates that the plants had greater access to nutrients due to the presence of EPS and lipopolysaccharides, and benefited from protection against potential bacterial and fungal pathogens.

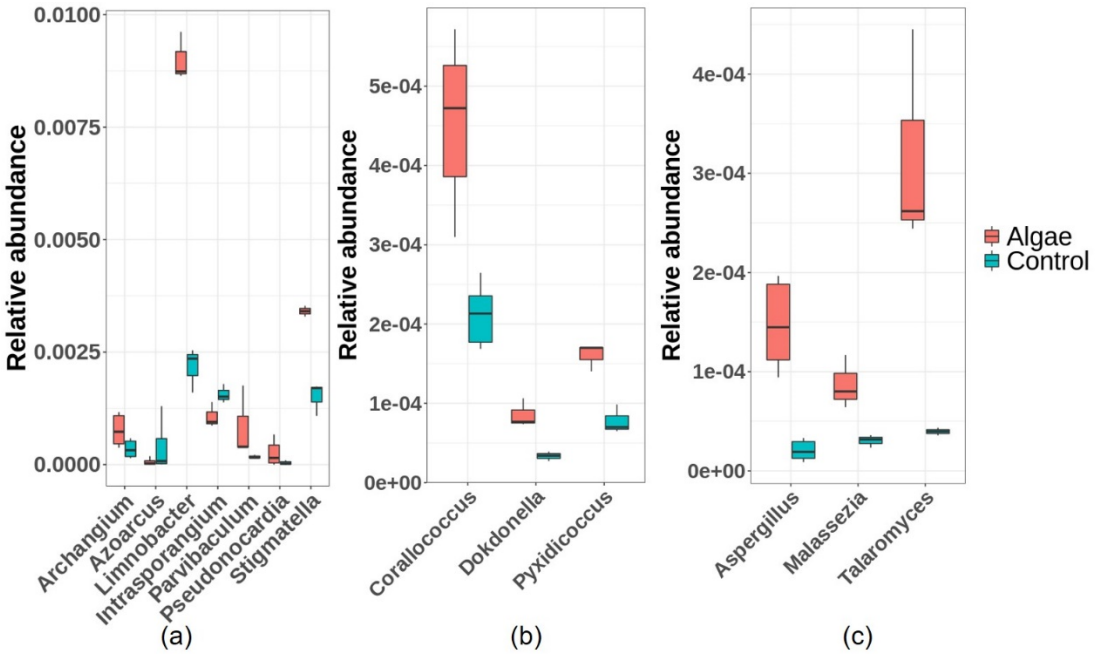

Figure 4.26. Box plots showing the distribution of the species in some genera of (a) Highly abundant bacteria, b) Low abundant bacteria, and c) Fungi—samples from soil drenched with *Chlorella* sp. MACC-360 are labeled orange (Algae), while control/TAP samples are labeled blue (Control).

Other myxobacteria enriched in MA-treated soils relative to the control were *Corallococcus* and *Pyxidicoccus,* as shown in Figure 4.26b. Members of these genera are predators of microorganisms, and some members even control soil-borne *Fusarium* wilt (Ye et al., 2020). *Dokdonella* species are strictly aerobic, and some can perform nitrate reduction. They are indicators of nitrogen fertilization and play a role in the nitrogen biogeochemical cycle in soil (Villamil et al., 2021). Accumulation of myxobacteria in algae-treated soils could be due to the increase in microorganisms, while the increase in *Dokdonella* could be due to the presence of oxygen released by the algae.

Additionally, *Ascomycota* fungi went up in the rhizosphere of MA-treated plants. *Eurotimycetes*-class fungi (genera *Malassezia*, *Aspergillus*, and *Talaromyces*) were more abundant in MA-treated samples than in control samples, as shown in Figure 4.26c. *Malassezia* yeasts depend on lipids because they lack fatty acid synthase (Ramírez et al., 2020). Their proliferation in MA-treated soils may have been due to algal and bacterial EPS, which increases lipid availability. *Aspergillus* and *Talaromyces* are both phosphate-solubilizing fungi (Doilom et al., 2020); they make the growth-limiting mineral phosphate available for plant uptake. Some strains within these groups,

such as *A. niger,* produce siderophores and growth-promoting substances like IAA (Li et al., 2016). *Talaromyces* species also produce bioactive compounds antagonistic to tomato and human pathogenic fungi and *Bacillus subtilis* (Manoch and Dethoup, 2011). *Talaromyces flavus*, in particular, has been shown to reduce the incidence of *Verticillium* wilt in tomato plants (Naraghi et al., 2010).

Some species of the *Rhodocyclaceae* family belonging to the *Azoarcus* were enriched in the control samples but diminished in the MA-treated samples. These bacteria, including the most abundant in this study, *Azoarcus* sp. CIB are known to fix nitrogen and produce IAA, promoting plant growth (Fernández et al., 2014). There are claims that the genus is endophytic to the rice roots, but its relationship to the tomato plant is unknown. *Aromateleum petrolei*, a closely related species, was ten times more prevalent in control samples than in MA-treated samples. This species and its relatives can cause anaerobic degradation of resistant organic compounds, such as aromatic and terpenoid compounds (Weiten et al., 2021). *Azoarcus* and *Aromateleum* are both anaerobic degradation specialists that degrade organic molecules that are typically conversion products in anaerobic conditions. Their decrease in the MA samples may result from oxygen released by the MA, which makes conditions unfavorable for accumulating aromatic compounds, rendering these bacteria ineffective. Therefore, the few abundant species in control may be strict anaerobes.

In addition, some strains were hardly present in the control samples but appeared in the MA-treated samples and vice versa, as shown in Figure 4.27. *Brevundimonas* sp. Bb-A, *Streptomyces* sp. S063, *Streptomyces* sp. RLB3-17, and *S. violaceoruber*, for example, were only found in samples treated with algae. *Brevundimonas* sp. Bb-A is a growth-promoting algal symbiont, while the *Streptomyces* genus consists of salt-tolerant and bioactive compound-producing strains (Blifernez-Klassen et al., 2021; Chen et al., 2019; Naragani et al., 2014). *Streptomyces* sp. LBUM 1475 was utterly absent from MA-treated samples, whereas it was abundant in control samples (Figure 4.27). Unfortunately, this strain is pathogenic and the agent responsible for potato scab disease (Naragani et al., 2014).

OTU_ids	pvalue	padj	Genus	Species	TAP1	TAP2	TAP3	MACC-3601	MACC-3602	MACC-3603
445576	2.557E-34	4.06E-31	Pseudonocardia	sp. AL041005-10	638	757	843	7008	5472	7570
2560058	1.311E-20	1.04E-17	Brevundimonas	sp. Bb-A	0	0	0	498	482	722
2306583	5.449E-18	2.88E-15	Halomonas	sp. JS92-SW72	66	143	64	1212	2364	2912
198107	6.505E-17	2.58E-14	Azoarcus	sp. CIB	8362	11051	7079	1903	895	914
76116	3.613E-16	1.15E-13	Aromatoleum	petrolei	22997	35529	19982	5858	2949	3243
1096868	7.262E-16	1.92E-13	Pseudonocardia	sp. EC080625-04	0	0	0	595	188	403
2074	2.086E-15	4.73E-13	Pseudonocardia	autotrophica	69	102	40	632	476	714
1047171	3.763E-14	7.46E-12	Zymoseptoria	tritici	9	4	7	112	220	241
76115	9.846E-14	1.74E-11	Aromatoleum	bremense	2057	3140	1791	610	307	364
2609807	1.673E-13	2.65E-11	Streptomyces	sp. LBUM 1475	15926	13194	0	0	0	0
37931	1.998E-12	2.88E-10	Paenarthrobacter	ureafaciens	637	640	574	171	233	238
2005885	1.057E-11	1.4E-09	Streptomyces	sp. S063	0	0	0	86	76	89
2732163	1.339E-11	1.63E-09	Limnobacter	sp. SAORIC-580	4318	2176	2891	12236	11215	15558
121627	2.029E-11	2.3E-09	Talaromyces	rugulosus	74	54	44	342	340	720
2735316	6.054E-11	6.4E-09	Paenarthrobacter	sp. YJN-5	85	134	55	0	0	0
1641402	6.68E-10	6.62E-08	Pseudonocardia	sp. HH130629-09	41	58	67	397	238	395
356837	2.024E-09	1.89E-07	Azoarcus	sp. DN11	856	1137	674	262	126	103
184914	2.666E-09	2.35E-07	Corallococcus	coralloides	407	305	325	801	653	864
335406	3.873E-08	3.23E-06	Sphingosinicella	microcystinivorans	221	221	142	454	503	609
83263	4.436E-08	3.52E-06	Aminobacter	aminovorans	922	691	748	472	378	336
261302	2.025E-07	1.53E-05	Paraburkholderia	phytofirmans	27	60	36	0	0	0
746128	3.556E-07	2.56E-05	Aspergillus	fumigatus	39	45	19	177	212	493
930166	5.148E-07	3.55E-05	Pseudomonas	brassicacearum	1119	1180	586	431	393	354
43263	5.457E-07	3.61E-05	Pseudomonas	alcaligenes	1546	1438	1651	187	149	511
2294034	6.695E-07	4.25E-05	Micromonospora	craniellae	0	0	0	36	31	28

Figure 4.27. The ranking of the top 25 differentially abundant operational taxonomic units (OTUs) at the species level from most significant to least significant. Indicated by highlighted columns are the species that were present in one group but absent in the other. Column 1 displays the OTU id, column 2 displays the p value, column 3 displays the adjusted p, column 4 displays the genus, and column 5 displays the species. The final six columns display the read counts which matched the corresponding species for each treatment group.

Soil drenching with MA effected the soil microbial community in the tomato rhizosphere. This effect results from MA's ability to alter soil's physical and chemical properties, resulting in the proliferation of microorganisms that prefer these conditions. There have been reports of an increase in the number of *Ascomycota* in the rhizosphere of tomato plants treated with a combination of microorganisms (Nuzzo et al., 2020). Higher species richness was associated with greater plant biomass and a more significant number of leaves and flowers (Lau et al., 2011). In reality, soil microbial status influenced the selection of plant traits, with rich-diversity communities favoring the early flowering phenotype (Chaney and Baucom, 2020). The sampling time is crucial, as selection occurs over time, in determining the effect of added inoculum on the soil community. In one month, for instance, the soil microbial community composition varied from one week to another (Chaney and Baucom, 2020). In our studies, the effect of the soil microbe community during the fifth week may not be significant, but it would be interesting to observe the community at the end of the growth period. Nonetheless, the increased species diversity observed in MA-treated samples may have contributed to the reported early flowering phenotype.

Conclusion and Perspectives

The examined eukaryotic green MA stimulated the growth of both *M. truncatula* and *S. lycopersicum*, a phenomenon due to the generation of phytohormones, algal EPS, and influence on the rhizosphere microbial community. The application of algae to plants through soil drenching affected leaf size, pigment content, and pod/flower output.

The *Chlorella* sp. MACC-360 strain had the most significant effect on *M. truncatula* plants. However, treatment with *C. reinhardtii* cc124 consistently enhanced the Chl and Car contents of the plants, unlike treatment with *Chlorella* species.

Based on the microscope evaluation, we concluded that defining the condition-dependent development characteristics of a particular MA strain is essential. This characterization influences the decision of the fraction of algae to use as a biostimulant as well as the method of administration to plants. In plant biostimulant investigations, living algal cells (B) and living algal cells with their growth medium (A) were evaluated. Application of A could be the best option for algae strains producing high amounts of EPS if the objective is to boost bioactive chemicals' availability to plants rapidly. Cell wall destruction may be necessary for certain strains like *C. reinhardtii* cc124 to create an effective biostimulant treatment.

Microalgae from *Chlorella* and *Chlamydomonas* genera demonstrated biostimulant effects on tomato plants, regardless of the contents of algae cultures supplied. Regardless of plant age, *Chlorella* sp. MACC-360 treatment increased fruit diameter, weight, Chl *b*, and Cars content compared to the controls. In contrast, *C. reinhardtii* cc124 dramatically increased fruit diameter and Chl *a* concentration regardless of plant age but reduced fruit number relative to the control.

The study on tomato plants demonstrated that the biostimulant effect of MA modulated photosynthetic performance and was strain-dependent. Even though the differences between MA-treated plants and control plants were not statistically significant for several parameters, the differences between the two algal treatments were statistically significant for some critical parameters, including maximum quantum yield and regulated energy loss. To determine the effect of MA on photosynthesis, we intend to expose the plants to adverse conditions. To this end, future research will

incorporate stress situations such as cold and water deprivation. We will include stress because this study's transcription analysis results suggest that MA is involved in responses to these stresses.

Although we detected auxins and EPS in our studied algal strains, at this stage, we cannot precisely determine which components stimulate plant growth, given that both cells and cells with their exudates (released in growth media) had positive impacts on plants. We removed secreted hormones and EPS for the B-treated experiments using centrifugation and washing with water. Unless living cells continue to create bioactive substances in soil, we cannot confirm that the two components we studied solely contribute to growth promotion. We sought to identify a physical interface between the algae cells and the root surface, but we could not. Studies in which the treatment comprises cell-free supernatants might answer these questions.

Therefore, we intend to monitor auxin and EPS levels in the soil before and after algae application. In addition, we plan to characterize the composition of the MA fractions (cells, supernatant/spent medium, separated EPS, and whole cell extract). The strain-specific biostimulant action of the examined algal strains could be better understood if the phytohormone content of each strain was analyzed. In the future, we also intend to test various doses of MA on nutrient-deficient plants to determine the ideal amounts that improve crop quality and output.

According to our unopened-flower bud-transcriptome analyses, MA affected the expression of genes involved in carbohydrate metabolism, amino acid metabolism, hormone signaling, and abiotic stress response. These results indicate that MA treatment interacts with the metabolic processes of plants and primes them for abiotic stress resistance. The MA effect on the expression of defense-related and flowering genes may be because these genes share the same regulatory pathways. In this study, the only flowering-related gene which also plays a crucial function in sugar metabolism was *TPS 1*. We did not notice overexpression of any other flowering gene, although the upregulation of several NAC genes and JA-related genes occurred. Since the JA signaling pathway participates in flowering, we hypothesize that the overexpression of JA pathway genes in MA-treated plants contributes to the phenotype of early blooming. Further research utilizing mutants for some affected genes could enhance understanding of this phenomenon.

The application of MA to the soil affected the microbial population of *S. lycopersicum*'s rhizosphere. The most notable distinction was the presence of *Ascomycota* and *Limnobacter* in samples treated with MA. In addition, enrichment of pathogenic species occurred in the control samples, while that of antibacterial and antifungal strains occurred in the MA-treated samples. Although growth-promoting bacteria occurred in all treatments, the MA-treated samples contained additional strains, such as *Brevundimona* sp. and *Bradyrhizobium* sp. Future comparisons of soil samples obtained at different times will shed light on whether or not selection occurs when plants reach maturity.

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Összefoglalás

A mikroalgákból bioaktív vegyületek széles skálája nyerhető ki, melyek különféle iparágakban alkalmazhatók. Bár felmerült, hogy a zöldalga biomassza a mezőgazdaságban zöldtrágyaként is használható lehet, a biofertilizálószerként való alkalmazás gazdaságossága erősen kétséges. Tanulmányom célja annak felmérése volt, hogy a mikroalgák mint természetes biostimuláns szerek felhasználhatóak-e a növények növekedésének elősegítésére.

A munka során az eukarióta zöldalga referenciatörzs *C. reinhardtii*-t és két *Chlorella* törzset választottam. Korábbi publikációk alapján a *Chlorella* extraktumok elősegítik a növények növekedését, viszont keveset tudunk a *Chlamydomonas* algák növényi növekedésserkentő hatásairól. Elemeztem az algatörzsek közötti különbségeket, célom volt megérteni, hogy van-e törzsspecifikus növekedésserkentő hatásuk a kiválasztott növényekre. Megvizsgáltam az algák növényi hormon- és EPS (extrapoliszacharid) termelő képességét. Mindegyik alkalmazott törzs termelt auxint (bár eltérő mennyiségben), de csak az egyik *Chlorella* törzs termelt kimutatható mennyiségű EPS-t.

Vizsgálataim során a mikroalgákat talajba juttatva és közvetlenül a növényekre permetezve is alkalmaztam, majd részletesen elemeztem és számszerűsítettem a növények növekedésére gyakorolt hatásukat. Két növényt használtam két filogenetikailag távoli családból. A *Medicago truncatula* egy modellnövény a hüvelyesek családjából, míg a másik a paradicsom haszonnövény (*Solanum lycopersicum*), a burgonyafélék családjának a képviselője.

A korábbi ismert vizsgálatoktól eltérően az algák biomasszájából nem készítettem kivonatot. A biomasszát begyűjtöttem, víztelenítettem, illetve egyes kísérletekben a tápközeggel együtt is alkalmaztam. Ez az előkészítési mód egyszerűnek és környezetbarátnak, mindemellett olcsónak is bizonyult. Lombpermetezéshez az alga biomasszát folyékony nitrogén alatt homogenizáltam, majd vízben oldottam.

Megfigyeltem, hogy a *Chlorella* sp. MACC-38 nem gyakorolt szignifikáns hatást a *M. truncatula* növényre, míg a *Chlorella* sp. MACC-360 és a *C. reinhardtii* egyértelmű biostimuláns hatást mutattak. Ezért a továbbiakban a fent említett két törzzsel folytattam a vizsgálatokat paradicsomon is. A *Chlorella* sp. MACC-360 kezelés a paradicsom virágzását a kontrollhoz képest szignifikánsan előrébb hozta

időben, és mindkét mikroalga növelte a levél pigmenttartalmát mindkét modellnövény esetében. A mikroalgák hatására megnövekedett a levélméret *M. truncatula* növényeken. A paradicsom terméseinek átmérője megnőtt, bár az összes terméshozam nem változott.

A *Chlorella* sp. MACC-360-nal kezelt paradicsom növényeken szignifikáns korai virágzási fenotípust azonosítottam. Ennek hátterének megértéséhez teljes transzkriptom analízist végeztem a növények virágbimbóin, a virágok nyílása előtti állapotban. Az eredmények alapján az algakezelés a növények szisztémás rezisztenciájában szerepet játszó gének indukcióját okozta. Érdekes módon az ismert virágzásért felelős gének nem mutattak specifikus expresszióváltozást.

A paradicsom rizoszféra minták metagenom elemzése megnövekedett fajdiverzitást mutatott a mikroalgákkal kezelt mintákban a kontrollokhoz képest, bár ez a különbség nem volt szignifikáns. A mikroalga kezelés specifikusan növelte a *Limnobacter* és további ismert növekedést serkentő baktériumok relatív abundanciáját a rizoszférában. Továbbá az *Ascomycota* gombák esetében is relatív abundancia növekedés volt megfigyelhető algakezelés hatására. A kontroll mintákban hiányzott az *Ascomycota*, és nagy mennyiségben tartalmaztak olyan patogén baktériumokat, mint például a burgonyavarasodás betegségért felelős *Streptomyces* sp. LBUM 1475.

Eredményeinkből a következő főbb konklúziókat vontam le:

- Az alga biomassza és a növesztési tápközeg együttes alkalmazásával érhető el a legnagyobb mértékű növényi biostimuláció
- Törzsfüggő az algák hormon és EPS tartalma
- A növények életkora (a mikroalga kezelés időpontjában) befolyásolja az eredményt
- A különböző algatörzsek különböző fenotípusos változásokat idéznek el a növényeken
- Az algák biostimuláns hatása befolyásolja a növényi fotoszintézist, változik a fotoszintetikus teljesítmény, a levél vastagsága és egyéb paraméterei is
- Molekuláris szinten a mikroalgák befolyásolják a növényi szénhidrát anyagcserét, a cukortranszportban és a hormonjelátvitelben részt vevő gének génexpresszióját
- Az érintett gének többsége az abiotikus stresszel szembeni védekezéssel és toleranciával kapcsolatos

- A hatékony mikroalgák szisztémás rezisztenciát indukálnak a növényekben
- A mikroalgák alkalmazása növényi biostimulánsként hatással van a talaj mikrobiális közösségére

Summary

Microalgae are ubiquitous photosynthetic microorganisms found in nature. They produce a vast array of bioactive compounds, some of which have been purified and used in various industries. It has been suggested that they can be used as green fertilizers in agriculture, but the preparation process can be laborious, time-consuming, and costly. Our study's main objective was to determine whether MA can be used in its natural state to stimulate plant growth. Our experiments aimed to examine the effects of three selected strains of eukaryotic green microalgae on plant growth. We selected a benchmark strain of the *Chlamydomonas* genus and two species of the *Chlorella* genus. Although it has been reported that *Chlorella* extracts stimulate plant growth, little is known about *Chlamydomonas* sp. We analyzed the potential differences between the strains to determine if they had strain-specific effects on plants. Their ability to produce hormones and EPS was the most scrutinized characteristic. Only one *Chlorella* strain produced detectable EPS, while all strains produced auxins. Each tested strain produced different concentrations of the representative hormone (IAA).

Two *Chlorella* species (Mosonmagyaróvár Algal Culture Collection (MACC)-360 and MACC-38) and a *C. reinhardtii* strain (cc124) were examined in *Medicago truncatula*, A17 ecotype, in the first part of our investigation. First, using growth curves and microscopy, the growth patterns, cell size, and morphology of the microalgal strains were determined. In addition, their ability to synthesize auxins was evaluated. In the greenhouse, *M. truncatula* was grown in pots containing a mixture of vermiculite and soil (1:3) with a clay layer at the bottom. Using the soil drench method, living cells of algae were applied to the plants. The physiological reactions of plants to the addition of algal biomass were comprehensively studied. Microalgae substantially boosted the plant's stem length, leaf size, fresh weight, number of flowers, and pigment content. For most of the investigated factors, there was a strain-specific effect. Overall, the application of *Chlorella* sp. MACC-360 resulted in more robust plants with greater fresh biomass, larger leaves, and more flowers/pods than the control, which received the same total nutrients.

In the second phase of the investigation, the biostimulant effects of *Chlorella* sp. MACC-360 and *C. reinhardtii* cc124 on *Solanum lycopersicum* (tomato) were

studied. This study's first purpose was to determine whether the two strains had biostimulant effects on tomato plants.

The significance of application mode and timing (plant age) was also studied. Thirdly, the strain-specific effects of the two algal strains were evaluated. Finally, transcriptomic and metagenomics analyses were conducted to identify the effect of algae on plants and the soil microbial community. Tomatoes were grown in pots with a clay layer at the bottom and a mixture of soil and vermiculate (2:1). In two sets of trials, living algae and algal extract and living algae with their growth media plus extracts were applied to the soil, and plant leaves, respectively. In the first group (B[11]+C[12] experiments), the culture suspension was centrifuged, and the algae pellet was re-suspended in water, which was applied weekly to the soil (treatment B). In contrast, the algae extract (cell disrupted algae suspension – treatment C) was sprayed on the leaves bi-weekly. Analyses were conducted on the blooming process, plant morphology, fruit characteristics, and pigment content.

In the second set of tests (A[13]+C experiments), the whole culture consisting of cells and their growth media (treatment A) was administered weekly to the soil, and C was sprayed bi-weekly on the leaves. The kinetics of flowering, reproductive capability, and photosynthetic characteristics were investigated. Both algal strains enhanced the leaf pigments, fruit weight, and diameter but barely affected yields. The age of the plant at the onset of treatment was a significant determinant of the outcome; treatments initiated later (week 5) produced superior results than those initiated at a juvenile level (week 1). *Chlorella* sp. MACC-360 stimulated early blooming and fruit development, whereas *C. reinhardtii* cc124 considerably slowed these processes. *Chlorella* facilitated the transformation of light energy into chemical energy, whereas *Chlamydomonas* boosted the protection of photosynthetic parameters. Both strains increased leaf thickness and temperature differential. Both algal strains enhanced crucial agronomically functional tomato processes.

Due to the early flowering phenotype observed in *Chlorella* sp. MACC-360-treated plants, we examined the unopened flower buds' whole transcriptome. The

[11] B=Living cells applied as a soil drench

[12] C=Destroyed cells applied as a foliar spray

[13] A=Cells and their growth media applied as a soil drench

results demonstrated the induction of genes involved in systemic resistance and response to abiotic stress, as opposed to the known/reported induction of flowering genes.

According to soil metagenomics research results, algae influenced the construction of the tomato rhizosphere microbiome. There was a marginally significant increase in species diversity between MA-treated and untreated samples. In soils saturated with microalgae, the number of Ascomycota fungus, *Limnobacter*, *Brevundimonas*, and other helpful bacteria that provided plant nutrition and defense against dangerous microbes increased. In contrast, the control lacked Ascomycota and was rife with pathogenic bacteria, including *Streptomyces* sp. LBUM 1475 strain which is responsible for potato scab disease.

I have to emphasize that in our research, we applied MA to both soil and plants and analyzed the effects on plant growth and performance, and in contrast to previous research, we did not process the algal biomass. The algal biomass was harvested, dehydrated, and applied alone or alongside the growth media. The algal biomass was homogenized under liquid nitrogen and then diluted with water for foliar spray. This approach proved to be simple, eco-friendly, and inexpensive at the laboratory scale.

From our findings, we concluded the following:

- Algal biomass with accompanying growth media had more beneficial effects than algal biomass alone.
- Not all MA cultures contain hormones and EPS; this characteristic varies by strain.
- Plant age at the time of treatment influences the effects of MA.
- Different algal strains elicit different responses from plants.
- The effects of algal biostimulants on photosynthetic performance, leaf thickness, and leaf temperature differential were not significant but might synergistically improve plant growth.
- At the molecular level, MA modulates the transcription of genes involved in carbohydrate metabolism, sugar transport, and hormone signaling in unopened flower buds.

- The majority of affected genes are associated with defense and abiotic stress tolerance.
- MA induces systemic resistance in plants.
- Application of MA via soil drench influences the soil microbial community.

Appendices

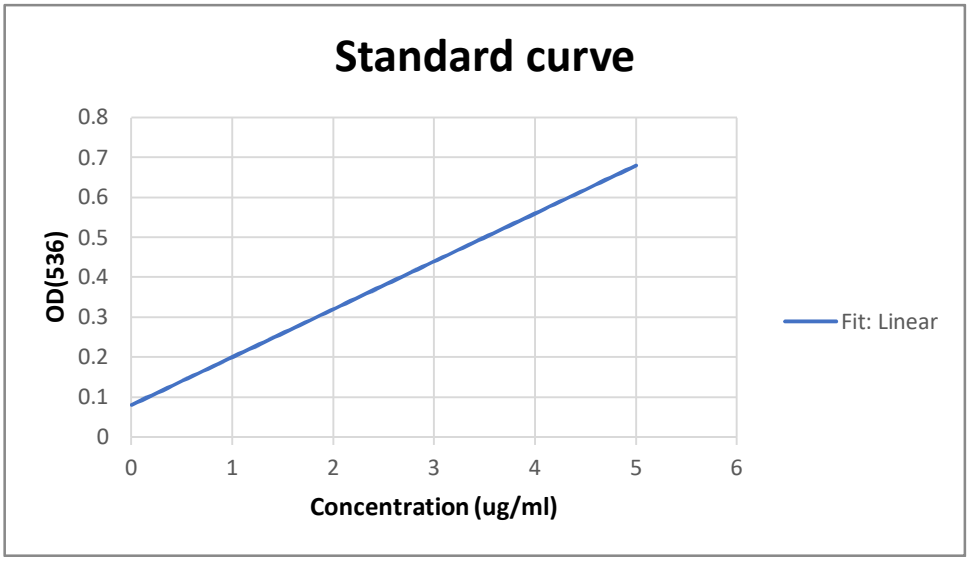

Figure S3.1. The indole acetic acid (IAA) standard curve generated by the Hidex after measuring the absorbance of a series of IAA concentrations at 536 nm. The curve was fitted to the linear equation y=Ax+B where A=0.11993, B=0.08049 and R^2=0.9986.

Table S3.1. Experiments and Time when conducted

Living cells + Extracts (B+C) Experiments	Time of the year	Experiment Regime
1a	19 February-28 May 2020	Week 5
1b	19 February-01 June 2020	Week 5
2	28 March- 15 July 2020	Week 5
3	18 August-7 December 2020	Week 1
4	19 August-8 December 2020	Week 1
5	20 August-9 December 2020	Week 1

Living cells with growth media + Extracts (A+C) Experiments	**Time of the year**	**Experiment Regime**
A	7 June-7 September 2021	Week 1
B	14 June-14 September 2021	Week 1

B+C experiments were conducted in 2020 while A+C were conducted in 2021. B refers to lives cells and C to destroyed cells while A refers to cells and the growth media. A and B were soil drenches while C was a folia spray. Week 5 regime means plants received both foliar and soil drench treatment at the age of 5 weeks. Week 1 regime means plants received soil drench treatment from week 1 and foliar treatment from week 5 onwards

Table S4.1. Mann Whitney test to compare the difference between plants treated with MA at different ages (One-week-old versus five-week-old)

Table Analyzed/ Variable	Fruit weight	Chl *a*	Chl *b*	Carotenoids	Yields
Column B	Week 1	Week 1	Week 1	Week 1	Week 1
vs.	vs.	vs.	vs.	vs.	vs.
Column A	Week 5	Week 5	Week 5	Week 5	Week 5
Mann Whitney test					
P value	**0.0033**	**0.0397**	**0.0007**	**<0.0001**	0.9265
Exact or approximate P value?	Approximate	Exact	Exact	Exact	Exact
P value summary	**	*	***	****	ns
Significantly different ($P < .05$)?	Yes	Yes	Yes	Yes	No
One- or two-tailed P value?	Two-tailed	Two-tailed	Two-tailed	Two-tailed	Two-tailed
Sum of ranks in column A, B	13509 , 27533	185.5 , 114.5	206 , 94	222 , 78	49 , 71
Mann-Whitney U	7623	36.5	16	0	26
Difference between medians					
Median of column A	6.700, n=108	0.004214, n=12	0.001390, n=12	1.198, n=12	35.50, n=6
Median of column B	7.365, n=178	0.003234, n=12	0.001184, n=12	0.4100, n=12	34.53, n=9
Difference: Actual	0.665	-0.0009805	-0.0002055	-0.788	-0.97
Difference: Hodges-Lehmann	0.62	-0.0008615	-0.0002565	-0.7903	-0.8133

Week 5 regime means plants received both foliar and soil drench treatment at the age of 5 weeks. Week 1 regime means plants received soil drench treatment from week 1 and foliar treatment from week 5 onwards.

Table S4.2: Functionally annotated clusters of the upregulated genes from DAVID

Cluster 1, Enrichment score =3.51, Significant term= NAC domain

Transcript ID	Gene Name
Solyc03g119580.1.1	AP2/ERF domain-containing protein (A0A3Q7FWB6_SOLLC)
Solyc06g063380.1.1	NAC domain-containing protein 52-like (LOC112941642)
Solyc03g114260.1.1	NAC domain-containing protein (A0A3Q7GFY9_SOLLC)
Solyc08g028850.1.1	NAC domain-containing protein (A0A3Q7IH21_SOLLC)
Solyc05g007770.3.1	NAC domain-containing protein 1 (NAP1)
Solyc04g005610.3.1	NAC domain-containing protein 2 (NAP2)
Solyc07g066330.3.1	NAC domain-containing protein 21/22 (LOC101261342)
Solyc02g088180.3.1	NAC2-domain-containing protein (NAC2)
Solyc12g013620.2.1	jasmonic acid 2 (JA2)

Cluster 2, Enrichment score =2.75, Significant term= Response to abscicic acid and cold acclimation

Transcript ID	Gene Name
Solyc02g084840.3.1	Dehydrin (A0A3Q7F9F5_SOLLC)
Solyc04g005610.3.1	NAC domain-containing protein 2 (NAP2)
Solyc09g008620.2.1	Polyadenylate-binding protein (A0A3Q7HYP3_SOLLC)
Solyc02g084850.3.1	TAS14 peptide (AA 1-130) (TAS14)
Solyc02g030105.1.1	Vps54 domain-containing protein (A0A3Q7EWI9_SOLLC)
Solyc01g109920.2.1	embryogenic cell protein 40 (LOC101259487)
Solyc04g005380.3.1	ninja-family protein AFP3 (LOC101268860)
Solyc07g006500.3.1	trehalose-6-phosphate synthase (TPS1)

Cluster 3, Enrichment score = 2.19, Significant term= Sequence-specific DNA binding

Transcript ID	Gene Name
Solyc09g005610.3.1	DOG1 domain-containing protein (A0A3Q7HXN2_SOLLC)
Solyc05g007770.3.1	NAC domain-containing protein 1 (NAP1)
Solyc04g005610.3.1	NAC domain-containing protein 2 (NAP2)
Solyc01g096320.3.1	homeobox-leucine zipper protein ATHB-12 (LOC101262661)
Solyc06g053220.3.1	homeobox-leucine zipper protein ATHB-12 (LOC101264731)
Solyc02g085630.3.1	homeobox-leucine zipper protein ATHB-40 (LOC101251349)
Solyc03g082550.3.1	homeobox-leucine zipper protein ATHB-7 (LOC101245037)

Cluster 4, Enrichment score = 0.11, No significant term

Transcript ID	Gene Name
Solyc07g062630.1.1	ABC transporter G family member 19 (ABCG19)
Solyc04g072580.1.1	ATP-dependent 6-phosphofructokinase 6-like (LOC101259840)
Solyc03g121780.1.1	Protein kinase domain-containing protein (K4BMX7_SOLLC)
Solyc12g098560.2.1	X8 domain-containing protein (A0A3Q7JEE6_SOLLC)

Four clusters labelled from the most enriched to the least enriched. The enrichment score is based on the EASE scores/P-value at 0.05 of each term member; the higher the score the more enriched the cluster.

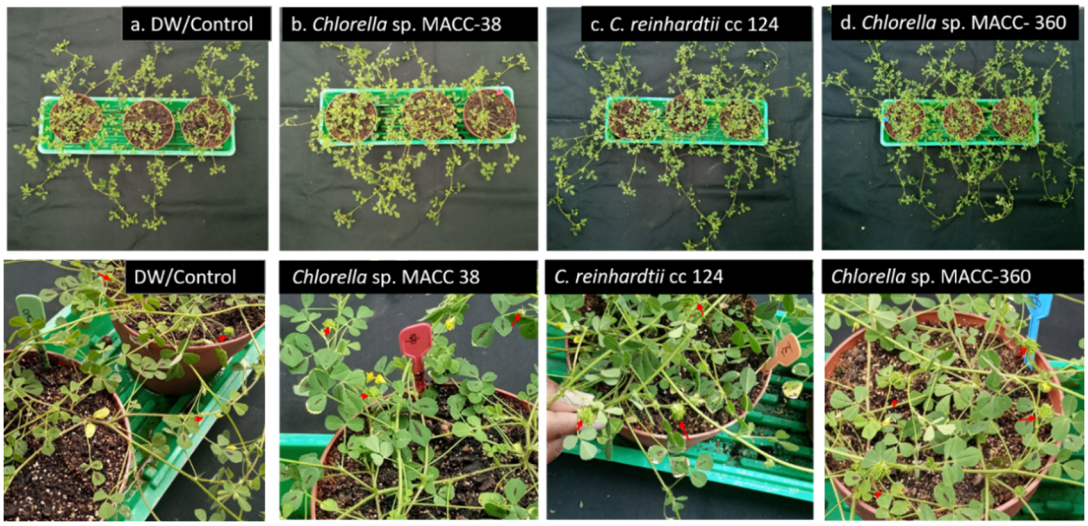

Figure S4.1. Aerial view of *M. truncatula* plants a) Distilled water (DW)/Control, b) *Chlorella* sp. MACC-38 c) *C. reinhardtii* cc 124 and *Chlorella* sp. MACC-360. Figures on the top row show the extent of branching and height of plants. Red labels on the bottom row show the pod size.

Figure S4.2. Aerial view of plants treated with living cells plus the spent media cultures at 2 weeks of flowering; a) Distilled water (DW)-treated plants/control, b *Chlorella sp.* MACC-360 -treated plants, and c) *C. reinhardtii* cc124 -treated plants. Red arrows point to clusters of flowers. The red circle in b. shows the tiny fruits. There are three pots per row with two plants per pot for each treatment.

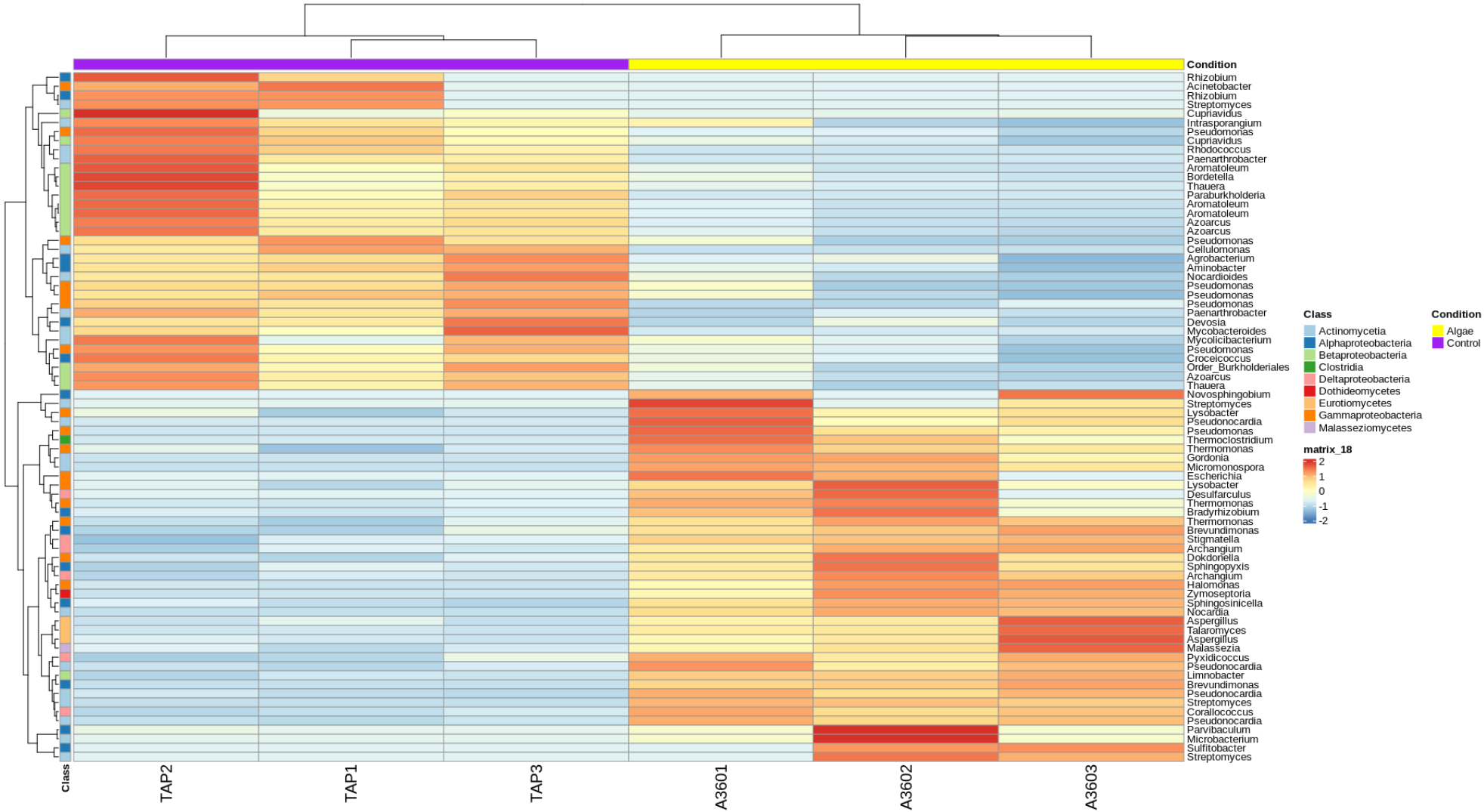
Condition
Rhizobium
Acinetobacter
Rhizobium
Streptomyces
Cupriavidus
Intrasporangium
Pseudomonas
Cupriavidus
Rhodococcus
Paenarthrobacter
Aromatoleum
Bordetella
Thauera
Paraburkholderia
Aromatoleum
Aromatoleum
Azoarcus
Azoarcus
Pseudomonas
Cellulomonas
Agrobacterium
Aminobacter
Nocardioides
Pseudomonas
Pseudomonas
Pseudomonas
Paenarthrobacter
Devosia
Mycobacteroides
Mycolicibacterium
Pseudomonas
Croceicoccus
Order_Burkholderiales
Azoarcus
Thauera
Novosphingobium
Streptomyces
Lysobacter
Pseudonocardia
Pseudomonas
Thermoclostridium
Thermomonas
Gordonia
Micromonospora
Escherichia
Lysobacter
Desulfarculus
Thermomonas
Bradyrhizobium
Thermomonas
Brevundimonas
Stigmatella
Archangium
Dokdonella
Sphingopyxis
Archangium
Halomonas
Zymoseptoria
Sphingosinicella
Nocardia
Aspergillus
Talaromyces
Aspergillus
Malassezia
Pyxidicoccus
Pseudonocardia
Limnobacter
Brevundimonas
Pseudonocardia
Streptomyces
Corallococcus
Pseudonocardia
Parvibaculum
Microbacterium
Sulfitobacter
Streptomyces
Class
TAP2
TAP1
TAP3
A3601
A3602
A3603
Class
Actinomycetia
Alphaproteobacteria
Betaproteobacteria
Clostridia
Deltaproteobacteria
Dothideomycetes
Eurotiomycetes
Gammaproteobacteria
Malasseziomycetes
Condition
Algae
Control
matrix_18
2
1
0
-1
-2

Figure S4.3. A heatmap of the genera with differential abundances in control/TAP and *Chlorella* sp. MACC-360 (A360) treated samples.

Publications

Primary sources of thesis

Gitau, M.M., Farkas, A., Ördög, V. and Maróti, G., 2022. Evaluation of the biostimulant effects of two Chlorophyta microalgae on tomato (*Solanum lycopersicum*). *Journal of Cleaner Production*, *364*, p.132689. **Impact Factor:11.072**

Gitau, M.M., Farkas, A., Balla, B., Ördög, V., Futó, Z. and Maróti, G., 2021. Strain-Specific Biostimulant Effects of *Chlorella* and *Chlamydomonas* Green Microalgae on *Medicago truncatula*. *Plants*, 10(6), p.1060. **Impact Factor: 4.658**

Other publications

Shetty, P., **Gitau, M.M**. and Maróti, G., 2019. Salinity stress responses and adaptation mechanisms in eukaryotic green microalgae. *Cells*, *8*(12), p.1657. **Impact Factor: 7.666**