# Summary of Ph.D. Dissertation
# Evaluation of the plant biostimulant effects of selected eukaryotic green microalgae


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## 1. Background

Using chemical fertilizers and pesticides in agriculture has been causing pollution and eutrophication in water bodies. It is, therefore, imperative to find eco-friendly alternatives to increase crop production as the global food demand continues to soar.

Biostimulants, substances that promote plant growth regardless of their chemical composition, have been proposed as a solution. Seaweeds, plants, and microorganisms could be used as biostimulants. However, seaweeds might get depleted, plants compete for land with crops, and microorganisms may require special facilities and media for cultivation. On the contrary, microalgae (MA) can proliferate in wastewater and in the open to accumulate huge biomass quickly. Besides, their incorporation into existing systems like wastewater treatment or hydroponic plant cultivation contributes towards sustainable water use.

Microalgae are ubiquitous photosynthetic microorganisms that produce many bioactive compounds with a broad biotechnological application. Despite the

promising prospects of MA application in agriculture, their use is still in its infancy.

Agricultural application of microalgae is limited because of the absence of a universal extract preparation procedure, lack of knowledge of the best application time and method, and lack of knowledge of algal strain-specific effects. Cutting down the steps of algae processing before application to plants may enhance the scalability of microalgae application as biostimulants. Thus, applying living cells could be one of the convenient options. If cell disruption must be done, the crude extracts should be effective without requiring downstream purification steps. In our research, we applied unprocessed algal biomass to soil and plants. We applied whole cultures (cells and growth media), cells only, and crushed cells.

## 2. Research objectives

The primary objective of this study was to assess the biostimulant effect of unprocessed Chlorophyta MA species on plant growth. We conducted the experiments with two model plants; *Medicago truncatula* and *Solanum lycopersicum*.

We conducted the first experiments with *M. truncatula* to screen for strains with biostimulant properties. We then selected and tested promising strains on *S. lycopersicum*.

We hypothesized that applying different portions of algae culture using different application modes would influence plant growth, crop yield, and quality. The objective of this study was to answer the following:

- Does the portion of algae used as a biostimulant matter? Does the age of plants matter? Is there a strain-specific effect on S. lycopersicum?
- Since MA promoted flowering, which processes were upregulated or downregulated?
- Since MA soil drenches affected plant growth, how did it affect the soil microbial community?

## 3. Resources and Methods

We selected the three algae species for the studies due to their rapid biomass accumulation. These were two microalgal species from the Mosonmagyaróvár Algal Culture Collection (MACC) belonging to the Chlorella genus (*Chlorella* sp. MACC-360 and *Chlorella* sp. MACC-38) and Chlamydomonas genus (*C. reinhardtii* cc124) from the Institute of plant biology, Biological Research Centre, Szeged. We analyzed the potential differences between the strains to determine if they had strain-specific effects on plants. Their ability to produce hormones and exopolysaccharides (EPS) was the most scrutinized characteristic.

We tested the three selected species on *M. truncatula*, A17 ecotype, in the first phase of our investigation. First, using growth curves and microscopy, the growth patterns and cell size microalga strains were determined. 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. We applied living algae cells to the plants using the soil drench method. The

physiological reactions of plants to the addition of algal biomass were comprehensively studied. We then analyzed the MA morphology with microscopy investigations and evaluated their ability to synthesize auxins.

In the second phase of the investigation, we dropped *Chlorella* sp. MACC-38 from further investigation. The biostimulant effects of *Chlorella* sp. MACC-360 and *C. reinhardtii* cc124 on S. lycopersicum (tomato) were studied. Tomatoes were grown in pots with a clay layer at the bottom and a mixture of soil and vermiculate (2:1). In two trials, we applied living algae and algal extract and living algae with growth media plus extracts to the soil and plant leaves, respectively. In the first group, we centrifuged the culture suspension (algal culture), removed the growth media, and suspended the algae pellet in water (treatment B). This treatment was applied weekly to the soil while the algae extract (cell disrupted algae suspension – treatment C) was sprayed on the leaves bi-weekly.

We tested this mode of application with two regimes; the week 1 regime and the week 5 regime. Under

week 1 regime, we initiated algal soil drenching on one-week-old plants and foliar spray later on five-week-old plants. Under the week 5 regime, we applied soil drench and foliar spray on five-week-old plants. We then analyzed the blooming process, plant morphology, fruit characteristics, and pigment content.

In the second set of tests, whole culture suspension (cells and growth media, treatment A) was administered weekly to the soil, and treatment C was sprayed bi-weekly on the leaves. We started soil drenching on the first week of plant growth and foliar spray on the fifth week. We then investigated the kinetics of flowering, reproductive capability, and photosynthetic characteristics. We also collected soil samples for soil DNA extraction for soil metagenomics studies.

Due to the early flowering phenotype observed in *Chlorella* sp. MACC-360-treated plants, we examined the whole transcriptome of unopened flower buds. This step was crucial in providing genomic details to explain the algal mode of action at the molecular level.

In summary, we used soil drench and foliar spray methods of application. We analyzed parameters relating to plant growth and performance. In contrast to previous research, we did not process the algal biomass. This approach proved simple, eco-friendly, and inexpensive at the laboratory scale. The algal biomass was applied together with the growth media or without the growth media. The algal biomass was homogenized under liquid nitrogen and then diluted with water for foliar spray.

In summary, we applied the following techniques:

1. Microbial techniques: Microalgae culturing, growth kinetics, size, and cell number determination.
2. Plant techniques: Seed sterilization, germination, transplantation, and proper maintenance in the greenhouse. Plant photosynthetic performance determination with an absorbance-based fluorescent device. Plant phenotyping using the nomenclature code for *M. truncatula* and fruit parameters for *S. lycopersicum*.

3. Biochemical techniques: Indole acetic acid determination via colorimetric assay. Leaf pigments (Chlorophyll and carotenoids) extraction and quantification with a spectrophotometer.
4. Molecular biology techniques: Plant DNA and RNA extraction, purification, quantification, and quality determination by visualization through agarose gel electrophoresis. Real-time qPCR to determine the expression of selected genes.
5. Microscopy techniques: Confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) of MA cells.
6. Bioinformatics: (a) Transcriptomic/ RNA sequencing analysis using tools validated with Galaxy. Gene ontology and functional annotation of differentially expressed genes via several tools including Shiny GO, g: Profiler, Database for Annotation, Visualization and Integrated Discovery (DAVID) cite.

   (b) Soil metagenomics: Data was curated and analyzed in Metagenomics rapid annotation using subsystems technology (MG-RAST) server.

## 4. Results

Only one *Chlorella* strain produced detectable EPS, while all strains produced auxins. The different strains also displayed different cell wall compositions. They also produced different concentrations of the representative hormone (IAA).

In *M. truncatula*, MA 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 larger leaves and more flowers/pods than the control, which received the same total nutrients.

*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 pigments and marginally increased leaf thickness and leaf

temperature differential. Both algal strains enhanced crucial agronomical tomato features such as fruit weight.

The whole unopened flower bud transcriptomic studies demonstrated the induction of genes involved in systemic resistance and response to abiotic stress, as opposed to the known/reported induction of flowering genes.

Soil metagenomics results revealed that algae influenced the construction of the tomato rhizosphere microbiome. In soils saturated with MA, the number of Ascomycota fungus, *Limnobacter*, *Brevundimonas*, and other helpful bacteria known for providing plants with nutrients and defense against pathogenic microbes increased. In contrast, the control soils lacked Ascomycota and contained many pathogenic bacteria, including *Streptomyces* sp. LBUM 1475 strain is responsible for potato scub disease.

In a nutshell, we found that:

- Algal live cells with their growth media had greater biostimulant effects than live cells alone.

- Most MA cultures may contain hormones, but not all have EPS material.
- The age of plants during treatment may affect the overall results of MA biostimulants.
- Different algal strains elicit different responses from plants.
- Although the effects of algal biostimulants on photosynthetic performance, leaf thickness, and temperature differential were not significant, they could additively improve crop performance.
- MA influences the transcription of genes in carbohydrate metabolism, sugar transport, and hormone signaling pathways in unopened flower buds.
- Most affected genes participate in the defense and abiotic stress tolerance, indicating induced systemic resistance.
- MA soil-drench treatment increases the abundance of plant-beneficial bacteria and fungi.

# 5. List of Publications

**MTMT ID**: 10074887

Cumulative impact factor: 23.396

5.1 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**

5.2 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**

## 6. Conferences

- Biostimulant Effect of Selected Eukaryotic Microalgae on *Solanum lycopersicum* L. 2021.The 2nd International Electronic Conference on Plant Sciences—10th Anniversary of Journal Plants (Poster presentation).

- "The promising future of microalgae as biostimulants". Biostimulants Europe 2022 Summit, Seville, Spain, November 30-December 1 (Oral presentation).

- "Microalgae promotes plant growth and primes plants for response to abiotic stress" Alga Europe 2022, Rome, Italy, December 13-15 (Oral presentation).

## 7. Co-author waiver

I hereby certify that I am familiar with the thesis of the Ph.D. candidate, Margaret Mukami Gitau. Regarding our jointly published results, I declare the applicant's contribution was prominent. The main articles used in this dissertation have not and will not be used for a Ph.D. dissertation in the future.

| Name | Signature |
|---|---|
| Gergely Maróti |  |