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Microalgae as growth promotors (biostimulants) in *Triticum aestivum* L.

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ABBREVIATION

6-BAP	6-Benzylaminopurine
ABA	Abscisic acid
ANOVA	Analysis of Variance
ANU	Australian National University
AOB	Ammonia-Oxidizing Bacteria
BA	Benzoic acid
BBCH	Biologische Bundesanstalt, Bundessortenamt and CHemical industry
C	Carbon
$C_6H_{12}O_6$	Glucose
Ca	Calcium
$CaCO_3$	Calcium Carbonate
CD	Critical Difference
CEE	*Chlorella ellipsoida*
Ci	Intercellular CO_2 concentration
CK	Cytokinin
CO_2	Carbon dioxide
COD	Chemical Oxygen Demand
Cu	Copper
D	days of germination.
DAA	Days After Application
DDT	Dichlorodiphenyltrichloroethane
DFS	Days From Sowing
DNA	Deoxyribonucleic acid
DOMBW	De-oiled Microalgae Biomass Waste
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC	Electrical Conductivity
EPA	Eicosapentaenoic acid
EPS	Extracellular Polymeric Substances
EPS	Sulfated exopolysaccharides

ET	Ethylene
FAO	Food and Agriculture Organization
FC	Folin-Ciocalteau reagent
Fe	Iron
Fe^{2+}	Ferrous ion
FeNa-EDTA	Ferric-Sodium- Ethylenediaminetetraacetic Acid
FRAP	Ferric reducing antioxidants power
FRK	Fructokinase
GA	Gibberellin/Gibberellic acid
GA	Ground area
GI	Germination Index
Glu/Gli	Glutenins and the Gliadins ratio
gs	Stomatal conductivity
HCl	Hydrochloric Acid
HI	Harvest index
HPLC	High-Performance Liquid Chromatography
HPLC-DAD	High-Performance Liquid Chromatography with Diode-Array Detection
IAA	Indole-3-acetic acid
IAM	Indole-3-acetamide
IBA	Indole-3 butyric acid
IoT	Internet of Things
ISO	International Organization for Standardization
K	Potassium
K_2HPO_4	Dipotassium hydrogen phosphate
KNO_3	Potassium Nitrate
L:D cycle	Light:Dark cycle
LR	Lateral Root
LSD	Least Significant Differences
MACC	Mosonmagyaróvár Algae Culture Collection
MB	Microalgae Biomass
MDA	Malondialdehyde
Mg	Magnesium

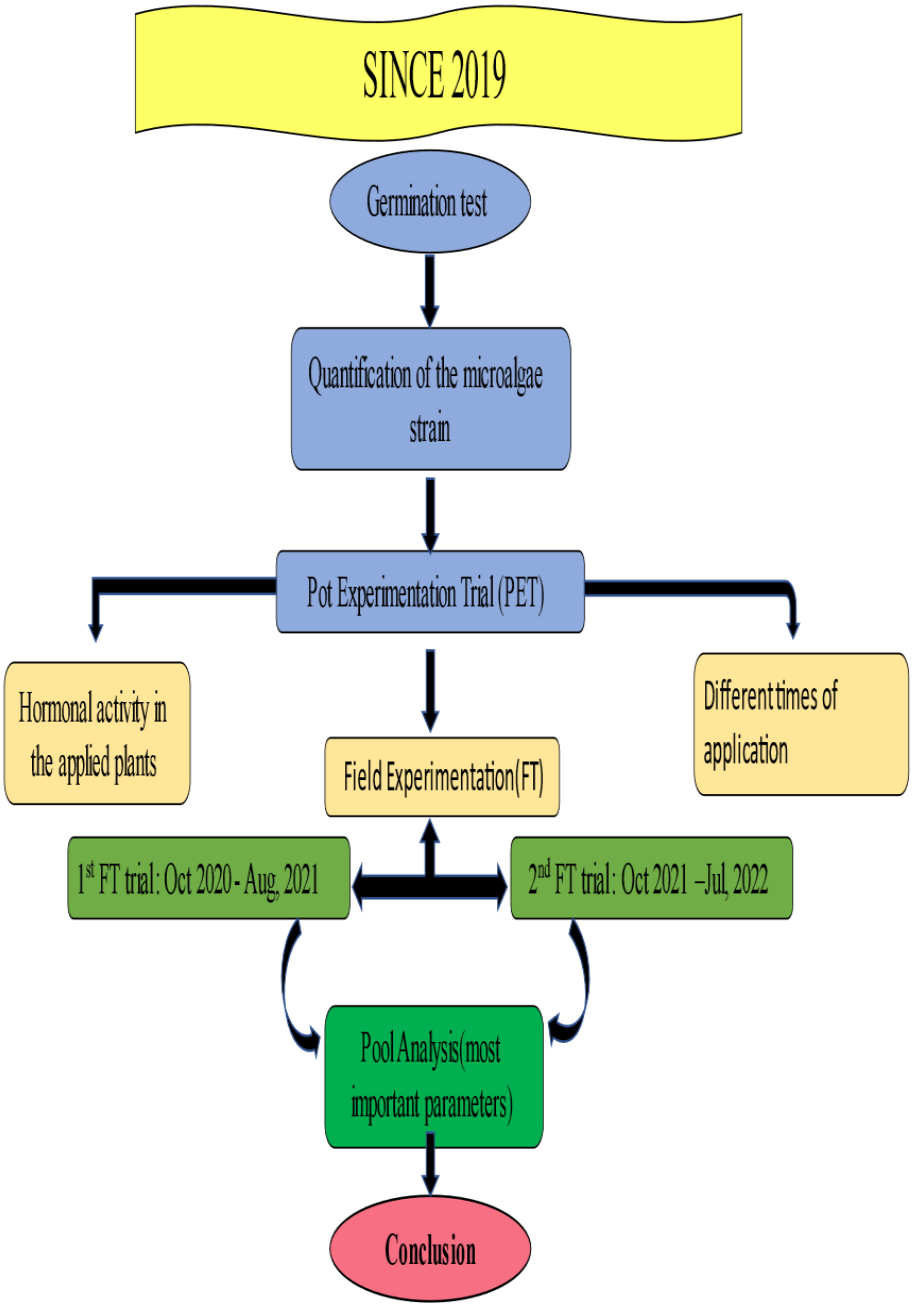
MG	Malachite Green
$MgSO_4$	Magnesium Sulphate
Mn	Manganese
N	Nitrogen
N	Number of germinated seeds
NAA	1-Naphthaleneacetic acid
NaCl	Sodium Chloride
$NaNO_3$	Sodium Nitrate
Nbiomass	Nitrogen in biomass
NED	N-(1-Naphthyl)-ethylenediamine
Ngrain	Nitrogen in grain
Nharvest	Nitrogen in soil after harvest
Ninitial	initial Nitrogen in soil
$N\text{-}NH_3$	Ammonical Nitrogen
NUE	Nitrogen Use Efficiency
ortho-P	Ortho-Phosphate
P	Phosphorus
PAA	2-Phenylacetic Acid
PC	Protein Content
pHBA	p-Hydroxybenzoic Acid
Pn	Net Photosynthesis
POF	Pelleted Organo-mineral Fertilizer
RLWC	Relative Leaf Water Content
RME	Rice Mill Effluent
S	Sulphur
SA	Salicylic Acid
SD	Standard Deviation
SEd	Standard Errors of difference
SE-HPLC	Size Exclusion High-Performance Liquid Chromatography
SG	Speed of Germination
SHF	Separate Hydrolysis and Fermentation
SPAD	Soil Plant Analysis Development
SUS	Sucrose Synthase

TAG	Triacylglycerol
tCA	t-Cinnamic Acid
TCAR	Total Carotenoid
TEP	Transparent Exopolymeric Particles
TOC	Tocopherol
TPC	Total Phenol Content
TPTZ	2,4,6-Tripyridyl-s-triazine
UPLC	Ultra-Performance Liquid Chromatography
UPP	Unextractable Polymeric Proteins
UV	Ultraviolet
WUE	Water Use Efficiency
Zn	Zinc
ZT	Zeatin
α-T	alpha-tocopherol

Units

%	percent
°C	degree Celcius
µgAEE /ml	micro gram ascorbic acid equivalent per milli litre
µL	microlitre
µmol m^{-2} s^{-1}	micro mole per metre square per second
CFU	Colony forming units
cm	centimetre
cm^3/100-liter	centimeter cube per hundred liter
g	gram
g/m^2/Day	gram per metre square per day
g/m^3/day	gram per metre cube per day
g/mL	gram per millilitre
g/L	gram/litre
Kg/ha	kilogram per hectare
L/h	liter per hour
M	molar

m^3/sec	cubic metre per second
mg	milligrams
mg C_{org}/L	milligrams of carbon (or organic carbon) per litre
mg/g	milligram per gram
mg/g	milligram per gram
Mg/g DW	milligram per gram Dry weight
mg GAE/g	milligrams of gallic acid equivalents per gram
mg/ml	milligram per millilitre
mL/min	milli Litre per minute
mm	millimetre
mM	milli Molar
ng/g DW	nanogram per gram dry weight
Ppm	parts-per-million
Rpm	revolutions per minute
t/ha	tons per hectare
V	volume
w/v	Weight per Volume
µE/m^2/s	microeinsteins per second per square meter
µg proline/mL	micro gram proline per milli litre
µg/µmol	microgram per micro mole
µmol CO_2/m^2/s	micro mole Carbon dioxide per meter square per second
µmol proline/g FW	micromole proline per gram fresh weight



GRAPHICAL ABSTRACT

ABSTRACT

The beneficial effects of microalgae extract on the growth and development of wheat (*Triticum aestivum* L.) have been demonstrated. Initially, eight algae strains from the Mosonmagyaróvár Algae Culture Collection (MACC) were used in a germination test showing substantial differences between the strains. According to the results of germination tests, some algae strains were selected to test their auxin-like effects and to determine their hormone levels. The mungbean rooting bioassay proves that microalgae biomass may exhibit auxin-like activity. The same strains used in mungbean bioassay were then quantified revealing the presence of secondary metabolites in the selected species; however, indole-3-acetic-acid was in the detectable range only in strain MACC-612 (*Nostoc sp.*). Foliar spray did not significantly alter the photosynthetic processes, but it influenced the secondary metabolite composition especially that of salicylic acid, abscisic acid, jasmonic acid-leucine/isoleucine conjugate composition in the plant. A decrease in indole-3-acetic acid was observed in the plants mostly in Mv Nádor cultivar.

As per the comparison between two varieties viz., Mv Nádor and Mv Béres in a pot experiment, the result suggested that varietal differences had negligible differences in biological yield, hexose content, and total phenol content. Furthermore, another two comparative pot experiment with different time of application suggested that application of microalgae biomass at early vegetative stage had an impact on the nitrogen content while application at reproductive stage had a

significant impact on both biological yield and nitrogen content. However, there was not huge differences because of variation in time of application.

To test further in field conditions, several parameters were included that can define whether the product is qualified to be used as a biostimulant. In the two successive trial, 2020-21 and 2021-22, three microalgae strains MACC-612, MACC-430 and MACC-922 were used. The application was done at the critical flowering stage (early reproductive stage). Some changes in the fertility %, chlorophyll content, proline content, relative leaf water content, FRAP content, total phenol content, etc. were observed. Moreover, the application had an overall positive impact on the yield attributes. The observations of the seed quality parameters such as protein, Zeleny sedimentation value, gluten % etc. had a mixed result as in the first trial, it had a smooth gradient after the application however in the second trial, no such positive impact of the application was observed. In conclusion, the type of microalgae strain applied created no great difference among the treatments; however, MACC-922, *Chlorella vulgaris* provided slightly superior results than the other two strains, *Nostoc linckia* (MACC-612) and *Chlamydopodium fusiforme* (MACC-430).

1. INTRODUCTION

Microalgae are present in most soils where moisture and sunlight are available. Their number in soil usually ranges from 100 to 10,000 cells per gram of soil. They are photoautotrophic often mixotrophic, aerobic organisms and obtain CO_2 from the atmosphere and nutrients from the soil, and energy from the sunlight. Green algae prefer acid soils while Cyanobacteria (blue-green algae) are commonly found in neutral and alkaline soils. The most common genera of green algae found in soil are *Chlorella*, and *Chlamydomonas,* dominant genera of *Cyanobacteria* in soil are *Chrococcus*, *Phormidium*, *Anabaena*, *Aphanocapra* and *Oscillatoria.*

Current applications of microalgae are as food, feed, cosmetics, etc., but in the future, researchers target to establish its applications in biofuel production, CO_2 mitigation, biofertilizers, bioremediation, chemical industry, etc. As agriculturists, many researchers have been trying to establish potential uses of microalgae, how, and in what way? Microalgae is assumed to have two action modes: plant-related and soil-related ones. In plants, application as a biofertilizer, biostimulant, or biocontrol agent changes the biochemical processes. While in the soil it can be used as a soil conditioner, fertilizer recycler, or soil remediator influencing mostly the physical or chemical properties of the soil (Verdelho, 2016). The use of organic fertilizers, biofertilizers,

and other microbial products are beneficial because it reduces chemical fertilizer application, which is harmful to the environment.

Since in algae, the number of natural substances is relatively smaller compared to synthetic mineral fertilizers, their foliar application seems to be the most appropriate way to increase the efficiency of biofertilization. During foliar fertilization, more than 90% of the compounds are utilized by a plant, while when they are supplied to the soil, only 10% of them are absorbed by crops. Thus, the foliar application can increase yields by 12-25% when compared to conventional fertilization (Ecochem, 2017).

Foliar application of algae extract has been noticed to increase photosynthetic pigments, crop growth, total biomass, yield, and yield components as well as quality, increase nutrient uptake, resistance to stress conditions, and growth-promoting hormones (Ghalab and Salem, 2001). It increases the functional activity of photosynthetic apparatus through raised chlorophyll content, total carbohydrates content, starch, amino acids, and protein (Yassen et al., 2007). Algae extracts are also important sources of potassium and contain considerable amounts of P, Cu, Ca, Fe, Mg, Zn, and Mn (Abd El-Mawgoud et al., 2010; Marrez et al., 2014). Iron may enhance photosynthetic activity and protein synthesis in leaves. Also, an iron important role in the biosynthesis of IAA, and it is required for prevention of the abscission layer formation (Hacisalihoglu et al., 2003).

The field application of microalgae was carried out as a biofertilizer to variable crops, tomato, *Solanum lycopersicum* (Garcia-Gonzalez and Sommerfeld, 2016; Özdemir et al., 2016), cucumber, *Cucumis sativus* and eggplant, *Solanum melongena* (Elhafiz et al., 2015), lettuce, *Lactuca sativa* (Elhafiz et al., 2015; Faheed and Abd El Fattah, 2008), okra, *Abelmoschus esculentus* (Agwa et al., 2017), spinach, *Spinacia oleracea* (Cassan et al., 1992; El-din and Hassan, 2016; Fan et al., 2013), rice, *Oryza sativa* (Elhafiz et al., 2015), wheat, *Triticum aestivum* (Renuka et al., 2016; Shaaban, 2001), maize, *Zea mays* (Dineshkumar et al., 2019), grape, *Vitis vinifera* (Abd El Moniem and Abd Allah, 2008), mango, *Mangifera indica* (El-Sharony et al., 2015), and orange, *Citrus sp*. (Amro, 2015). The application of algae has been shown to improve biomass, quality, and yield in overall described fruit and vegetable crops.

New regulation (EU) 2019/1009 (Rouphael and Colla, 2020) has defined Plant Biostimulants as follows: "A plant biostimulant shall be an EU fertilizing product the function of which is to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: i) nutrient use efficiency, ii) tolerance to abiotic stress, iii) quality traits, or iv) availability of confined nutrients in the soil or rhizosphere" (EU, 2019). The global market for biostimulants was valued at $2.19 billion in 2018 and is projected to reach a compound annual growth rate of 12.5% from 2019 to 2024. Although the largest market for

biostimulants is in Europe (approximately 40% of the market share), the North American market is estimated to reach $605.1 million in 2019. Based on the European Commission report (EU, 2016), algal extracts including both macroalgae and microalgae amount to up to 40% of the total biostimulant market.

Aim and objectives:

The main aim of the present work was to determine the possible mode of application of certain algal strains in wheat plants at different developmental stages and different growth conditions. Particularly, the following objectives were in focus:

1. To quantify the secondary metabolites, present in the selected microalgae.
2. To study the hormonal activities in the microalgae-applied wheat leaves.
3. To study the physiological and morphological parameters of the crop following the application of different microalgae at germination and seedling stages under greenhouse and field conditions.
4. To study the biochemical properties of the microalgae as well as the effect on biochemical properties of wheat.
5. To examine the effect of microalgae treatments on wheat quality.

2. REVIEW OF LITERATURE

One of the highly utilized microalgae is Cyanobacteria as researchers have experimented in many crops. Cyanobacteria is not the only microalgae experimented with. The following part discusses some of the findings before filling the gap with our new findings.

2.1 History of algae

When we connect algae to the theory of human and animal existence on earth, the biggest questions seem solved as per many researchers. A team from the Australian National University (ANU) led by Jochen Brocks (Brocks et al., 2017) published that as per their finding it was an algae explosion 650 million years ago that allowed human and animal life to evolve. To support the theory, he mentioned the presence of eukaryotic steroids through molecular fossil records and added that the only notable primary producers in the oceans before the Cryogenian period (720-635 million years ago) is bacteria. While observing the molecular fossil records in different time periods, they found a steady increase in steroid diversity which implied a rapid hike of marine planktonic alga (*Archaesplastida*). And thus, after it created a food web with enhanced efficiency in nutrient and energy transfers and here quoting their line 'the rise of algae' created food webs with more efficient nutrient and energy transfers, driving ecosystems towards larger and increasingly complex organisms". Furthermore, de Vries (2018) added that over millions of years, algae transform first

into non-vascular land plants, then into complex seed plants. History says the Chinese were the first to use microalgae, *Nostoc* to survive famine around 2000 years ago (Spolaore et al., 2006).

In the 18th century, Feigenbaum, (2016) wrote an essay that showed phycology (the study of algae) was in a neonatal stage. Algae came to the notice of many botanists but not quite remarkably noticed. During that period, only *Conferva fontinalis* (now identified as *Vaucheria fontinalis*, a species of yellow-green algae) was often known as it came along while looking for other species. These algae were found in clumps by the thousands into decumbent and individually were a half-inch length, filamentous light to dark green aquatic plants. These same algae species were mentioned by the Italian Botanist Pier Antonia Micheli in his '*Nova plantarum genera*' (1729) however named differently as *Byssus palustris subobscura, filamentis non ramosis, brevibus*. By the German-born English botanist, that same alga was illustrated in his '*Historia muscorum*' (1741) under a different name. Carl Linnaeus, the century's most famous botanist described the alga and gave a taxonomic position in his '*Species plantarum*' (1753), a book that stands at the beginning of modern botanical taxonomy. He gave the name *Conferva fontinalis* replacing the previous *Byssus palustris subobscura, filamentis non ramosis, brevibus*.

Later Joseph Priestley's experimentation on gases (1775) led to the discovery of the necessity of sunlight in oxygen production after accidental experimentation on an alga likely to be *C. fontinalis.* Another accidental encounter with alga is Blumenbach's experiment

where he showed the present-day asexual reproduction in his '*On an extraordinarily simple method of reproduction*' (1781) and inspired many new types of research.

The first research focused on algae began in the 19th century. Albrecht Wilhelm Roth (1757–1834) was one of the first few scientists that started intense research on algae to build guidelines, methods, and practices. His hard work yielded fruit and the then dismissals and confusion led to the recognition of economic potential and the vital role of algae in ecosystems. Thus, set the foundation of modern Phycology.

Later, in 1890 Beijerinck isolated alga for the first time in culture. The species he isolated was *Chlorella*. This *Chlorella* isolation was upgraded with the study of photosynthesis by Warburg in 1919. Gradually another important study conducted was the work on carbon dioxide assimilation in plants by Calvin and Benson in 1962 (Borowitzka, 2018).

2.2 Development of algal biotechnology

With increasing large-scale farming, chemical application has increased. This led to an increased chemical runoff, which deteriorated the aquatic ecosystem leading to an increase in algal bloom causing eutrophication. For example, in the Beibu Gulf of China between 2011-2015, a whooping ten folds increase in such algal blooms was observed (Xu, 2019). On one hand is the problem of eutrophication, on the other hand, is the issue of depleting natural resources. In this desperate state of finding a solution to these problems, researchers developed several

ways of utilization. A brief of different stages of research evolution leading to algal biotechnology is listed below.

With advancements in research, scientists began to believe that they could build a future algal-based bioeconomy by application of several modern techniques such as synthetic biology, high throughput phenomics, and the application of internet of things (IoT) to algal biotechnology for the elaborative study of algal biology. Even though algal biotechnology came to light because of the rising environmental and climatic changes, its commercial production started back in the Twentieth century. This could be traced from the article by (Mobin and Alam, 2017), which mentioned the first commercial cultivation of microalgae in Japan, in the 1960s. The species first commercially produced was *Chlorella vulgaris* mainly produced as a source of protein-rich food (Morimura and Tamiya, 1954). The lack of security for sufficient protein for the ever-increasing population in the early 1950s led to this commercial production as scientists found that *Chlorella* could be the ultimate alternative and unconventional protein source.

Within a few decades of the first commercial production of *Chlorella*, it went on with other species like *Spirulina*, *Dunaliella salina*, and *Haematococcus pluvialis* (Borowitzka, 1999). *Spirulina* like *Chlorella* was produced for health food. Its production began in the 1970s in lake Texcoco, Mexico by Sosa Texcoco S.A. (Durand-Chastel, 1980). This commercial production became popular in Asia, it spread from a plant in Thailand set in 1977 by Dai Nippon Ink and chemicals to 46 large-

scale plants within a span of 3 years. They had the capacity to produce more than 1000 kg of microalgae biomass per month focusing more on *Chlorella* production (Kawaguchi, 1980). After *Spirulina* the third microalgae industry that flourished was that of *Dunaliella salina* as a source of beta-carotene. The plant was established in 1986 by Australian companies called Western Biotechnological Ltd. and Betatene Ltd. At about the same time, commercial production of *Nostoc* (cyanobacteria) began in India (Venkatamaran,1987).

The use of algae was expanded to industrial sewage treatment. The quality of water can be improved by treating the grey water with microalgae and the biomass was then utilized to generate methane by fermentation. The first use in sewage treatment can be traced back to the 1900s. It has long been recognized as a critical microorganism in wastewater and sewage treatment. The underlying theory for utilizing it is that microalgae directly uptake organic and inorganic nutrients from the waste for growth and development. In due time the microalgae population multiplied while the nutrients present in the wastewater gets drained off from the water to the microalgae biomass. The biomass was then broken down indirectly through oxygenation by aerobic microbes or converting the biomass to methane through fermentation. It was in the 1950s, the effort to understand the microalgae-wastewater interactions on a large scale began after sizable investments (Paddock, 2019). The end of World War II triggered concerns about agricultural production to feed the population. Firstly, the announcement of the United Nations that half of the world was already hungry or

malnourished, and stating that to cope with the food scarcity issue, food production must be increased by 25-35% within a couple of decades raised high concern (FAO, 1947). Secondly, after proper water and sewage systems in the housing area, there was an increased effort to find more efficient methods of treating wastewater. Lastly, the growth of the environmentalist movement to opt for sustainable ways also plays a part in the advancement of research in microalgae wastewater treatment.

Another application in the early times was using microalgae as photosynthetic gas exchangers for space travel. Fear of another conflict, World War III, the engineers conducted research to survive in a closed environment such as space capsules to recycle waste products (Golueke et al., 1959; Myers, 1964). To maintain such closed systems, microalgae were used to constantly recycle waste to regenerate supplies for a long time. Their main role was in the air regeneration of oxygen by scrubbing carbon dioxide from the air. Besides the regeneration of oxygen in the cabin, the microalgae grown can be consumed for nutritional properties and for treating water.

Later with the rising energy crisis in the 1970s, the use of microalgae for generating renewable energy sources became very popular. Since then, scientists have been working on improving lipid biosynthesis to enhance product quality and efficiency (Spolaore et al., 2006).

In the 20th century, many scientists started realizing the importance of algae research or study. By then the small unicellular, green alga, *Chlamydomonas reinhardtii* had already emerged as a model system

for various studies like photosynthesis and other plant-specific metabolic processes. Also, it was known that this alga contained triacylglycerol (TAG) more than any other algae species. In that stage, Park et al., (2015) found that these algae, *Chlamydomonas reinhardtii*, when induced stress by depriving the nitrogen (N) supply in their medium of production, enhance nitrogen and lipid metabolism. As per their finding, they gave the explanation based on transcriptomic, proteomic, and metabolite changes in the alga when kept in N-deprived conditions. The combination of the different methods of analyzing, explains that when N was deprived (or stress was induced), it turns on a massive gluconeogenic metabolite state which then moves to a glycolytic state, This eventually affects the nitrogen and carbon responsive pathways reducing carbon assimilation and chlorophyll biosynthesis, thereby directing maximum energy in producing enzymes needed for nitrogen assimilation and lipid biosynthesis to compensate the reduced N from different available forms such as acetamide, nitrate, ammonia, amino acids, etc.

Singh and Singh (2014) highlighted the importance of algae besides its high potential for liquid fuel generation. The article mentions that algae absorb a large quantity of carbon dioxide during the process of photosynthesis under low water requirements. The water requirement can be reduced by 90% as compared to terrestrial plants.

The most recent applications are in the cosmetic and agricultural industries. In 1982, microalga *Chlorella* extract was first registered for a patent claiming its effect on hormone biosynthesis (Naohiko, 1982).

However as per Murata et al., (2021), after the first patent registration, there was a long gap of 15 years until the second patent registration. Later, in Japan, the *Chlorella* extract that was filed for patent registration was accepted and was sprayed on a wide range of fruit crops, vegetables, and rice (Yamaguchi, 1997). It was a slow development until 2015 after which booming research on the use of microalgae in agriculture, especially in the United States, China, and Europe. The registered patents and research were on the use of it as a plant growth promotor, biofertilizer, biopesticides, and for improving post-harvest quality. Besides the patent record, no confirm information could be gathered on when and how the first agricultural use began. However, Stirk and Van Staden (1996, 1997) found that seaweed, the macroalgae exhibit physiological reactions when plants were treated with their extract reported to have been evoked by plant growth regulators such as auxin or cytokinins, several researchers conducted experiments to study the stimulating effect of algal extracts on growth and development of plants. Series of progress since 2010 in the study of microalgae are as follows:

2010: Goh et al., (2010) tested the antioxidant capacity of *Chaetoceros sp.*, a diatom, and *Nannochloropsis sp.* Different antioxidant assay proved that the microalgae extracts can scavenge different types of free radicles through potential antioxidant compounds present in the microalgae biomass extract.

El-Baky et al., (2010) found that *Spirulina maxima* and *Chlorella ellipsoida* application to seawater stress conditions normalize the plant

metabolic activities. It was concluded that the biochemical activity was activated more when stress was induced by adding salt water and without the salt water or stress induction, the effect of microalgae was not significant.

Choi et al., (2010) reported high starch accumulation in *Chlamydomonas reinhardtii*. It contains as much as 44% on dry base. Here microalgae like *Chlamydomonas reinhardtii* were used to convert into ethanol by separate hydrolysis and fermentation (SHF) methods.

Abdo et al., (2010) studied the potentiality of primary products production in *Anabaena sphaerica*, *Chroococcus turgidus*, *Oscillatoria limnetica*, and *Spirulina platensis*. Parameters such as carotenoid, phycocyanin, were taken into consideration. In terms of carotenoid and phycocyanin production, *Spirulina platensis* had the highest potential with value of 1.4 and 4.5 mg/mL respectively while the lowest value observed was 0.8 and 0.18mg/mL of carotenoid and phycocyanin respectively in *Oscillatoria limnetica*. Quantification results showed the presence of various carbohydrate types such as glucose, galactose, mannose, fructose, xylose, glacturonic acid, sucrose and fucose in the strains studied while among the fatty acids worth mentionable was capric, lauric, myristic, palmitic and margaric. Oleic acid and linoleic acid were the unsaturated fatty acid identified in all studied strains with linolenic acid found additionally in *Anabaena sphaerica*.

2011: Goswami and Kalita (2011) worked on two freshwater microalgae strains, *Scenedesmus dimorphus*, and *Scenedesmus*

quadricauda. They changed the source of nitrogen to urea to check the right amount and concluded that the maximum increase in biomass production was at the concentration of 1.523 mg/L/day.

Dragone et al., (2011) studied the carbohydrate accumulation of *Chlorella vulgaris* to find the factors upon which the amount of accumulation depended. They concluded that careful maintenance of initial nitrogen supplementation in a culture medium can bring about increasing starch productivity and biomass productivity.

Freeze drying and storing are some steps which is often involved in microalgae biomass production. To monitor the effectiveness of such minor processes on biomass composition, Ryckebosch et al., (2011) conducted an experiment on *Phaeodactylum tricornutum*. The study confirms no effect of spray and freeze drying of fresh microalgal paste on total lipid content, fatty acid content, carotenoid content etc.

Vera et al., (2011) concluded that the polysaccharides collected from green, brown, and red seaweeds (marine macroalgae) contain elicitors that can bind to specific receptors located in the plasma membrane triggering enhance anti-pathogenic activities within the plant system. Such elicitors may be involved in the salicylic acid, jasmonic acid, or ethylene signaling pathways.

2012: Some fungi can be used for the benefit of another microorganism like microalgae, this had been proven by El-sheekh, et al., (2012). The lignocellulosic waste created by either *Pleurotus ostreatus* or *Trichoderma viride* had more reducing sugar as the carbohydrate upon

reacting with polysaccharide hydrolases released by the fungi, converted to compounds of lower molecular weight which was a readily available source of food for algal cells. So, when it was treated on *Chlorella vulgaris* and *Scenedesmus obliquus*, an increased in growth, carbohydrate, and protein content was observed.

Custódio et al., (2012) evaluated *Tetraselmis chuii*, *Nannochloropsis sp., Chlorella minutissima* and *Rhodomonas salina* for parameters like total phenolic contents, radical scavenging activity, metal chelating potential etc. Radical scavenging activity was more with methanol extract of 1mg/ml and highest in *C. minutissima* (20.8%) followed by *T. chuii* (20.0%), *N. oculata* (12.2%), *R. salina* (9.4 %).

Gol'din (2012) reported on the biocidal activity of cyanobacteria and microalgae. Like plant and microbial insecticides, it had deterrent and inhibitory effects on the insects, especially insects at the larval phase. Through various experiments, he showed that biocidal activity can be through inhibition of functions like fat synthesis, feeding, growth, etc.

Ratha et al., (2012) collected microalgae from different habitats in India and conducted biodiversity analyses and ultimately identification of them. In the study, certain characteristics were studied such as chlorophyll content, carbohydrate content, growth rate, etc. So, the team made a collection of germplasm beneficial for future use.

Moro et al., (2012) studied the effect of two herbicides, chlortoluron and mesotrione on *Pediastrum tetras*, *Ankistrodesmus fusiformis*, and *Amphora coffeaeformis*. Mesotrione showed the highest inhibition

against *A. coffeaeformis* while *A. fusiformis* was most susceptible to chlortoluron. The herbicides influenced carotenoid and chlorophyll content, however, the degree of the effect varied among the species studied depending on the number of days of exposure.

Research involving many species of microalgae such as *Isochrysis sp.*, *Phaeodactylum sp.*, *Chlorella sp.*, and *Tetraselmis suecica*, showed a potentiality of manipulating the total phenolic content by adjusting the favourable growing environment, similarly as in enhancing carotenoid content by manipulating the culture environment. *Chlorella sp.* had high antioxidant content while *Isochrysis* had relatively high phenolic content. The phenolic content of the species under study was 0.5 to 4.6 mg G.A.E./g biomass (Goiris et al., 2012).

There can be certain threats to the growth of microalgae. To investigate one such threat on microalgae, *Palmellococcus miniatus* isolated from desert soil, Yang et al., (2012) studied the effect of the volatile oil of *Artemisia ordosica* especially on their photosystem and antioxidant system. The oil containing several terpenoids, alcohols, esters, ketones etc., significantly affect photosynthesis, growth and caused oxidative damage in *Palmellococcus miniatus*.

García et al., (2012) performed enzymatic hydrolysis using alcalase and flavourzyme to produce L-amino acids concentrates from microalgae. The amino acid production depended on biomass concentration. As the biomass concentration increased, the enzymatic reaction decreased; hence, the free amino-acids concentration gets reduced despite the high protein concentration.

2013: Gonzalez et al., (2013) saw a great potentiality in seaweed marine algae. The work concluded that the cell walls containing polysaccharides and derived oligosaccharides were the magic compounds doing wonders for the growth of plants. There had been studies that proved the increasing nitrogen assimilation and basal metabolism. In tobacco (*Nicotiana tabacum*) plants application of red seaweed increased photosynthesis, cell division, and basal metabolism with improved anti-fungal and anti-bacterial characteristics.

Stirk et al., (2013) detected indole-3-acetic acid (IAA) and indole-3-acetamide (IAM) in all 24 axenic microalgae strains belonging to *Chlorophyceae, Trebouxiophyceae, Ulvophyceae*, and *Charophyceae* with their concentration ranging from 0.50 to 71.49 nmol IAA/g DW and 0.18 to 99.83 nmol IAM/g DW, respectively. Besides auxins, cytokinins were detected with a concentration range from 0.29 nmol/g DW to 21.40 nmol/g DW. Cis-zeatin was found most abundantly of all other cytokinins.

Chlorophyll pigment content in *Chlorella* was more than in plant leaves. Miazek and Ledakowicz (2013) proved it by extracting chlorophyll from *Chlorella sp.*, *Robinia pseudoacacia* leaves, *Pinus sylvestris* needles and *Sonchus arvensis* leaves and added that chlorophyll from *Chlorella* was easy to extract.

Ray et al., (2013) recognized the potentiality of cyanobacteria in supplementing phosphorous in phosphorus-deficient soil raising rice seedlings. They found a higher amount of plant available phosphorous released from super phosphate compared to cyanobacteria and

microalgal species within 30 days. The phosphorous released by superphosphate was 8 times higher than required by the rice seedlings. However, phosphate was released slower and reduced by using cyanobacteria and microalgae, lowering phosphorous toxicity or nutrient wastage in crop fields.

Bileva, (2013) proved that dry biomass of *Chlorella vulgaris* can be used against non-parasitic nematode like *Xiphinema index* that infests on the roots of grapes. Application of 1 g of *Chlorella vulgaris* on the ungrafted grape seedlings acted as a photoprotector and a strong growth stimulant.

2014: Deli et al., (2014) analysed carotenoid content of three non-toxic bloom-forming algae, namely *Dunaliella salina*, *Euglena sanguinea*, and a *Nostoc* strain. A high concentration of carotenoid was observed in all three strains; however, the major fractions of carotenoids differ among the strains. In *D. salina* the major fraction 52.1% was lutein, Echinenone was the major fraction of carotenoids in *Nostoc* strain, while in *Euglena sanguinea*, carotenoids comprised mostly of diatoxanthin fraction.

de Jesus Raposo et al., (2014) enriched the culture media of *Porphyridium cruentum* with Mg^{2+} supplementation and then use the sulphated exopolysaccharides (EPS) to test their efficacy against bacteria and viruses. The addition of Mg^{2+} in the form of $MgSO_4$ enhanced not only the production of biomass but also enhances the EPS yield. The protein content and sulphate content of *Porphyridium* improved. The EPS obtained possessed antimicrobial activity against

certain bacteria like *Staphylococcus aureus*, *Salmonella enteritidis* and viruses like *Herpes simplex*.

2015: Uysal et al., (2015) experimented the use of microalgae, *Chlorella vulgaris*, an alternative to biofertilizer in wheat and maize. However, after isolation, the multiplication of *C. vulgaris* was done in two different growing conditions-phototrophic and heterotrophic to evaluate if the growing conditions influence their potentiality. The germination height and germination rate increased after the application of microalgae at the dose rate of 1 litre liquid biofertilizer/400 L water and the performance of phototrophic algae performed better than heterotrophic algae.

Prasanna et al., (2015) prepared biofilms using cyanobacteria and tested them on various maize varieties. Parameters such as Soil Plant Analysis Development (SPAD) value, plant height andavailable nitrogen in the soil were assessed and concluded that Biofilms of different cyanobacteria elevated the result by 10-15%. This experiment showed the potentiality of combination formulation of *Anabaena – Providencia* and *Anabaena – Trichoderma* by increasing nitrogen fixation, enriching the soil with an additional 40-50 kg of nitrogen per ha.

2016: Mukherjee et al., (2016) showed the potentiality of utilizing the parboiled rice mill effluent (RME) in the production of cyanobacteria and microalgae and applying the so-produced algal consortium as slow-release phosphorous biofertilizers. The cyanobacteria and microalgae applied had high remediation capacity, removing 93.9%

phosphorous, 100% ammonia-nitrogen removal, 98.7% reduction in biological oxygen demand, and 91.6% reduction in chemical oxygen demand in the RME. There was an accumulation of polyphosphate with a higher release of phosphorous in the soil after the application of the algal consortium. When the algal-treated RME was added to rice seedlings, improvement in the shoot height and leaf width was observed.

Another remediation experiment was conducted by Renuka et al., (2016) highlighted the potentiality of microalgae produced at low cost to enhance nutrient use efficiency and supplement nutrient requirements in the soil by growing microalgae in wastewater. The nitrogen content increased as much as 3.56% in the sampled plant parts. A significant influence on plant dry weight, spike weight, 1000-grain weight was seen. It increased the dry weight of the plant up to 33% and 1000-grain weight improved by 5.6-8.4%.

Garcia-Gonzalez and Sommerfeld (2016) experimented on a green alga, *Acutodesmus dimorphus* by applying the cellular extracts and dry biomass in different forms such as seed primer, foliar spray, and biofertilizer. The treatment encouraged steady germination, faster plant growth, and better flowering in Roma tomato plants.

2017: Kholif et al., (2017) added *Chlorella vulgaris* to maize silage at different ratios to find influence on *C. vulgaris* in gas production. Parameters such as total gas, methane, and carbon dioxide were recorded. Higher *Chlorella vulgaris* % or higher % of silage in short imbalance in the constituents, recorded higher methane and carbon

dioxide. When the ratio of *Chlorella vulgaris* was balanced to 50:50 parts, the gas production decreased during the 48-h observation period.

Hamed et al., (2017) evaluated the tolerance of *Chlorella sorokiniana* and *Scenedesmus acuminatus* to sub-lethal doses of Cu after exposure for 7 days. A significant rise in proline, polyphenol, and tocopherol concentration despite low Cu uptake was found in *S. acuminatus*. Enzymes like superoxide dismutase enzymes, glutathione-S-transferase enzyme, glutathione reductase enzyme was also high in this microalga. Hence, copper toxicity was mitigated by inducing an antioxidant defence system in both microalgae.

Zhang et al., (2017) successfully cultivated *Chlorella infusionum* with tomato in hydroponic culture system. From the mix cultivation, biomass productivities were 32 $g/m^3/d$ of algae and 54.24 $g/m^3/d$ of tomato crops which was better than individual yield in monoculture production. Therefore, we can say that cultivation of algae with crops may have a synergistic effect on biomass productivity of both.

2018: Puglisi et al., (2018) investigated agro-industrial waste for biostimulant effects on *Chlorella vulgaris* and *Scenedesmus quadricauda* by supplementing in their growth medium. The treatment display a change in biomass and lipid production of *C. vulgaris* and *S. quadricauda* whereas addition of oil extraction residues from rape increases the saturated:unsaturated fatty acid ratio and an increase in carbohydrate and chlorophyll stimulated the sugar metabolism.

El-Arroussi et al., (2018) studied the potentiality of *Dunaliella salina* exopolysaccharides (EPS) to retaliate the salt stress effect on tomato (*Solanum lycopersicum*). On application of exopolysaccharides on tomato there was an elevated concentration of proline, phenolic compounds, Na+, and antioxidant enzymes which attenuated salt stress. So, the treatment reduced the negative impact of salt stress on the growth of plants and root systems.

Ertani et al., (2018) tested one extract from *Laminaria* and five extracts from *Ascophyllum nodosum* on maize (*Zea mays*) for their plant growth stimulation capacity. The extract enhanced the root growth, esterase activity and sugar content. The extracts supply nutrients required for plant growth.

2019: Figler et al., (2019) studied nine common freshwater microalgae to understand their salinity tolerance, salinity, and nutrient-reducing ability. The selected species belong to the genera *Chlorella*, *Chlorococcum*, *Desmodesmus*, *Scenedesmus*, and *Monoraphidium* which had been recognized as halotolerant. A significant amount of chloride and nutrient removal with reduced conductivity were observed. The potentiality of bioremediation and mitigation of salt stress conditions had been acknowledged.

Barone et al., (2019) monitored the complex soil–microorganisms–plant system after the application of *Chlorella vulgaris*, *Scenedesmus quadricauda*, or their extracts directly into the soil. It was a pot experiment and to study the complex interaction, a tomato plant was grown. The soil enzymatic activity was observed such as diacetate

hydrolysis, dehydrogenase, alkaline phosphor-monoestesterase, and urease enzyme activity. The treatments enhanced the enzymatic activities, thereby uplifting their soil biochemical index. In the tomato plants, a change in dry weight and chlorophyll index was seen accompanied by a significant increase in their growth as compared to untreated soil.

Ekinci et al., (2019) experimented on corn plants with three microalgae, *Chlorella sp.*, *Neochloris conjuncta*, and *Botryococcus braunii* having the potential to use as biofertilizers. They were applied at different doses (0, 5, 10 and 15 tons per ha.). Dose rates of 10 and 15 tons per ha containing *Botryococcus braunii* and *Neochloris conjuncta* were found to decrease plant growth and nutrients uptake, however, the lower dose of 5 tons per ha had no significant negative impact on the growth. Digestates of *Chlorella sp.* had no negative impact on growth and nutrient uptake.

2020: Kusvuran and Can (2020) tested the efficiency of *Chlorella vulgaris* in improving nutrient uptake, growth, and salt stress tolerance in guar plants. The treatment of the microalgae had a significant positive impact on plant morphological parameters such as shoot length, fresh weight, dry weight, leaf number, leaf area, and physiological parameters like photosynthetic pigments. Furthermore, as compared to plants grown in salt stress alone, microalgae-treated plants despite the salt stress condition had a higher concentration of total phenol, flavonoid, antioxidant enzymes such as ascorbate peroxidase, catalase, glutathione reductase, and superoxide dismutase,

and nutrient elements such as K^+ and Ca^{2+} ion while the concentration of Na^+ and Cl^- ion contents were decreased. Hence, they proposed the potentiality of administrating microalgae applications to mitigate salt stress.

Navarro-López et al., (2020) experimented with the potentiality of *Scenedesmus obliquus* as a plant biostimulant. Bioassays like germination index using watercress (*Lepidium sativum* L.) seeds, mungbean and cucumber (*Cucumis sativus* L.) bioassays for auxin-like activity, and cucumber bioassay for cytokinin-like activity were conducted. A 40% increase in germination index was obtained at 0.1 g/L as compared to untreated. In the mungbean, auxin-like activity was highest in 0.5 g/L. 187.5 % higher cytokinin-like activity was observed at 2 g/L concentration.

Mutale-Joan et al., (2020) studied bio extracts of *Aphanothece sp.* and *Chlorella ellipsoidea* for their bio-stimulating effect on tomato plants. A significant improvement in the tomato plant's morphological parameters like root, shoot length, root dry weight, and shoot dry weight were obtained after treatment with the extracts. There was an efficient uptake of nitrogen, phosphorus, and potassium in plants applied with an extract of *Aphanothece sp.* especially. Metabolomic analysis revealed a higher accumulation of palmitic acid, stearic acid, pyridine-3-carboxamide, and linolenic acid in the tomato plant samples treated with the extracts.

In the research conducted by Supraja et al., (2020), mixed algal consortia were used as a biofertilizer on tomato plants. Within 3 days,

a faster rate of germination with respect to control was observed. Higher chlorophyll content of 13.45 ± 0.307 mg/g was obtained with algal extract applications.

2021: Martini et al., (2021) used two green algae species, *Chlamydomonas reinhardtii* and *Chlorella sorokiniana* on maize. The treatment revealed the potentiality of using microalgae for mitigating abiotic stresses like nitrogen deficiency and drought stress. There was root growth promotion with an increased number of secondary roots as compared to the control. With an improved root system, nutrient uptake was enhanced and hence accumulation of micro-nutrients on roots and shoots was increased.

Mau et al., (2021) studied the plant growth responses to algal biomass by using *Chlorella vulgaris* and wheat plants. The algae were taken as dry and wet algae at two doses, 0.1% or 1% each. For the same set of doses, mineral fertilizer treatment was also done on the wheat plant and allowed to grow for 55 days. The nutrient content in the soil acquired from algal biomass was comparable to mineral fertilizer, nutrient supply in the soil. Moreover, it was discovered that the phosphorous from algae were more readily available to wheat plants.

2022: Gitau et al., (2022) investigated the bio stimulating capacity of *Chlamydomonas reinhardtii* and *Chlorella sp.* MACC-360 on tomato (*Solanum lycopersicum*). Algae pellet made after centrifugation were suspended in water and then applied to soil weekly and the algal extract was sprayed biweekly on the leaves. Both types of algae strain enhanced biochemical parameters like pigment content and yield

attributes such as fruit weight and fruit diameter of tomato. In this research application of algae at the advanced stage performed better than the early-stage application. Morphological parameters such as leaf temperature and leaf thickness were increased with the treatment.

Chlorella pyrenoidosa was the studied algae in the experiments of Ma et al., (2022). The alga was applied as seed primer and as biofertilizer on *Chenopodium quinoa*. Under saline-alkaline conditions, germination was reduced which was overcome in alga-primed seed. At 100 mM saline-alkaline stress level, the alga primed seed had 29.3% higher germination and 12.6% higher germination at 200 mM stress level as compared to the control. 75% dose of the biofertilizer exhibited a higher efficiency than the 100% dose. Better seedling growth was also recorded in the treated plants. When algae biofertilizer was used, root length, branch number, shoot length, leaf size, fresh weight, etc., were enhanced with respect to control. The availability of macronutrients, nitrogen, phosphorous, and potassium was improved.

2.3 Composition of microalgae

Blue-green algae extract contains macro and microelements, natural enzymes, auxins, and cytokinins in numerous amounts (Shaaban, 2001; Zhang and Ervin, 2004; Raupp and Oltmanns, 2006). Algae extracts are good sources of potassium and contain considerable amounts of P, Cu, Ca, Fe, Mg, Zn, and Mn (Abd El-Mawgoud et al., 2010 Marrez et al., 2014). Some contain macronutrients NPK @ 8.0%, 2.45% and 0.68% respectively and micronutrients such as Mg (20ppm), Ca (93ppm), Fe (1986ppm), Zn (31ppm), Mn (58ppm) and Cu (88ppm),

on an average (El-Moursey,2019). Modified algal extracts may also contain 0.3% boron as boric acid (17% B). Magnesium is found also in a high percentage in the green micro-algae (more than 1.0% on dry weight basis (Shaaban et al., 2010).

Chlorella is one kind of microalgae that contains a large amount of various functional materials such as crude protein 50-60%, carbohydrate 15-20%, crude lipid 12-18%, and chlorophyll (Kang et al., 2004; Wake et al., 1992; Elarroussia et al., 2016). Chlorophycean members including *Chlorella* were also being explored as biofertilizers as they are rich in carbohydrates, proteins, lipids, and growth hormones (Dineshkumar et al., 2019; Faheed and Abd El Fattah, 2008; Özdemir et al., 2016). Major constituents of alga *Scenedesmus* sp. include 50.56 % crude protein, 7.39 % ether extract, 9.83 % crude fiber, 9.18 % ash, 8.09 % N, 2.69% P, 0.65% K, 2057 Ppm Fe, 772 ppm Zn, 747 ppm Mn, 93 ppm Cu (Shaaban et al., 2010).

Kulk (1995) and Adam (1999) reported the growth promotion in response to the application of nitrogen fixer cyanobacterium *Nostoc muscorn* could be attributed to the nitrogenase as well as nitrate reductase activities of algae associated with the surface of plants, or the amino acids and peptides produced in the algal filtrate and/or other compounds that stimulated the growth of crop plants.

Also, algae produced some amino acids and polypeptides that improve plant growth, in addition to some substances that have antimicrobial properties and polymers (De Caire et al., 1993).

2.4 Effects of microalgae on morphological parameters and yield attributing parameters of different crops

Cyanobacteria have been shown to affect the morphological development of many crops. Renuka et al., (2016) used two formulations {(formulation with unicellular microalgae (MC1) and formulation with filamentous microalgae (MC2)} in wheat. Both the microalgal formulations significantly increased the N, P, and K content of roots, shoots, and grains, and the highest total N content of 3.56% in grains while 7.4-33% increase in plant dry weight and up to 10% in spike weight. Adam (1999) found that the algal filtrate of the cyanobacterium, *Nostoc muscorum* significantly increased the germination of wheat seeds as well as their growth parameters and nitrogen compounds, compared to controls. El-Moursy et al., (2019) showed that foliar spray of blue-green algae increases the shelling %, stalk diameter, and grain numbers in *Zea mays*.

In pulses like bean plants, fresh and dry weight/plant, plant height, number of leaves/plants, and leaf area/plant were enhanced by cyanobacteria. The combined application of cyanobacteria-method I with using 75% of the recommended chemical N fertilizer was found effective for enhancing plant growth (Hegazi et al., 2010).

Another most used microalgae have been *Chlorella sp.* Barone et al., (2018) found that the seedlings of sugar beets treated with *Chlorella vulgaris* highlighted higher values of total root length, fine root length, and the number of root tips than the untreated plants. In pulse crops like lablab bean (*Lablab purpureus*), green gram (*Vigna radiata*),

horsegram (*Macrotyloma uniflorum*), blackgram (*Vigna mungo*), pigeonpea (*Cajanus cajan*), the length of the radicle was longer in seeds treated with *Chlorella vulgaris* and *Scenedesmus sp.* but the length of plumule decreases in most of them (Vijayalakshmi et al, 2019). Application with *C. pyrenoidosa* improved a greater number of leaves with a bigger surface area in soybean seedlings (Dubey and Dubey, 2010).

Lettuce seedlings treated with *C. vulgaris* were positively affected by increased fresh and dry weight. An increase in germination, fresh and dry weights, and pigment content of *Lactuca sativa* seedlings treated with *Chlorella vulgaris* were observed (Faheed and Abd-El Fattah, 2008).

The thickness and number of spinach leaves treated with *Chlorella* were higher than that of the untreated. Also, the fresh weight and yield of the spinach treated with the chlorella were higher than that of the untreated (Kim et al., 2018). Cassan et al., (1992) reported that foliar sprays of the extracts of blue green algae increased the fresh weight of spinach (cv. Monstrueux De Viroflay and cv. Polka) leaves by 12-15%.

Some experiments involved many strains application for instance in a trial conducted by Hegazi et al., (2010) a mixture of *Nostoc muscorum*, *Nostoc humifusum*, *Anabaena oryzae*, *Wollea sp.*, *Phormedium*, and *Spirulina platensis* was used and found that addition of such algae can reduce chemical nitrogen by 50% (dry and drench) without affecting seed yield characters (Hegazi et al., 2010). Elarroussi et al., (2016) showed that polysaccharides extracted from *Spirulina platensis*

significantly promoted plant growth in *Capsicum annuum* and *Solanum lycopersicum*, which was demonstrated in terms of plant weight, plant size, and size/number of leaves.

2.5 Effects of microalgae on physiological and biochemical processes on different crops

Spirulina platensis also increased the zinc level (Anitha et al., 2016). Increments in chlorophyll content and dry weight of maize plants were obtained by Shaaban (2001) after soil application of *Chlorella vulgaris*. Higher chlorophyll content and net photosynthesis activity were also found after *Chlorella sp.* application in maize (Grzesik and Romanowska-Duda, 2015), while another study observed a pigment content increase in *Lactuca sativa* seedlings grown in fertilized soils with *C. vulgaris* (Faheed and Abd El Fattah 2008). Recently, a chlorophyll content increase was observed in *Salix viminalis*, after biofertilization using cyanobacteria and green algae (Grzesik and Romanowska-Duda 2015). Our results are also in accordance with those obtained by Barone et al., (2018), who found that extracts obtained from *C. vulgaris* and *S. quadricauda* were promising biostimulants in the early stages of plant growth in sugar beet. Moreover, Barone et al., (2019) showed that *C. vulgaris* and *S. quadricauda* enhanced the growth of tomato seedlings in hydroponic culture. The application of a low dosage of microalgae (max 68 mg of biomass kg^{-1} of soil) to the soil makes them a strong biostimulant for tomato plants.

Triple foliar biofertilization with intact cells of *Microcystis aeruginosa* MKR 0105, *Anabaena sp.* PCC 7120, and *Chlorella sp.* significantly enhanced the physiological performance and growth of plants fertilized with a synthetic fertilizer YaraMila Complex (1.0, 0.5, and 0.0 g per plant). This biofertilization increased the stability of cytomembranes, chlorophyll content, intensity of net photosynthesis, transpiration, stomatal conductance, and decreased intercellular CO_2 concentration. (Grzesik et al, 2017).

Ghallab and Salem (2001) studied the effect of some biofertilizer treatments, cerealin (*Azospirillum spp.*) and Nemales (*Serratia spp.*) on the wheat plant, found that the two biofertilizers increased growth characteristics and nutrients, sugar, amino acids, and growth regulators (IAA, GA, and cytokinin) and crude protein content in the plant. Monem et al., (2001) reported that fertilization with *Azospirillum brasilense* or commercial biofertilizer cerealin, improves the growth and yield of maize in rotation with wheat as affected by irrigation regime.

2.6 Effects of microalgae on crop quality parameters

Lozano et al., (1999) showed that an extract from algae (AlgaEnzims) applied in potatoes showed higher protein content than other commercial growth plant growth regulators. *Chlorella vulgaris* produces a range of high-value substances, and the biomass itself can be used in aquaculture for feeding purposes and as an additive for animal feed that is rich in vitamins. He also stated that the application

of an extract from algae to soil or foliage increased the ash, protein, and carbohydrate contents of potatoes (*Solanum tuberosum*).

The increases in leaf total chlorophyll content were reflected in increasing rate of photosynthesis rate and accumulation of carbohydrates reserves which led to a positive effect on fruit quality (Amro, 2015). El-Sheekh, (2000) noticed that all the crude extracts of seaweed increased protein content in root and shoot systems, total soluble sugars. A mixture of algae treatment enhanced antioxidant enzyme activities such as catalase, and ascorbate peroxidase enzyme of faba bean. Algal treatments improved the membrane stability and reduced MDA (Aldaby, 2020).

2.7 Effects of microalgae on soil properties

Soil pH is also known to be affected by the algal application. Saha and Mandal (1979) reported an initial increase in soil pH, whereas contradictory to it Subhashini and Kaushik (1981) reported a significant reduction not only in pH but also in hydraulic conductivity, electrical conductivity, and soil aggregation. Cyanobacteria are also known for their ability to release trace elements from insoluble materials. Fe, Mn, and Zn are known to be influenced in rice fields by cyanobacterial growth (Das et al., 1991). Lange (1976) reported the chelation of Fe, Cu, Mo, Zn, Co, and Mn through the gelatinous sheath of many cyanobacterial species. This sheath is also known to reduce particle erosion and may adsorb charged nutrient cations (Whitton, 2000). In summary, algal application influence soil properties through soil particle aggregation, phosphate and trace element release from

insoluble minerals, and N storage and its slow release. When grown in the inert substrate, the chlorella algae alone fixed 0.5 mg of CO_2-C over the test period, *Chlorella* has a greater effect on C fixation than native algae. There are several pieces of evidence that witnessed an increase in N content and organic matter of soils inoculated with cyanobacteria (Singh and Singh, 1989; Vaishampayan et al., 2001; Venkataraman G.S., 1993).

The effect of surface growth of inoculated cyanobacteria on subsurface properties of brown earth, silt loam soil was studied by Rao and Burns (1990). A significant increase in soil polysaccharides, dehydrogenase, urease, and phosphatase activities was recorded. Improvement in soil aggregation was also seen; stable soil aggregates are essential to soil fertility. layer of 0-0.7 cm depth.

Rao and Burns (1990 reported an eightfold increase in bacterial members in the cyanobacteria inoculated columns, whereas an increase in fungal population was not significant. Acea et al., (2001) reported greater than four logarithmic unit increases in heterotrophic bacteria, actinomycetes, algal, and fungal propagules and three logarithmic unit increases in fungal mycelia after inoculating burnt soils with cyanobacteria. Similarly, Rogers and Burns (1994) reported a significant difference in the heterotrophic microbial population after inoculation of soil with *Nostoc muscorum*. Nutrient status of soil specifically nitrogen and phosphorous determines the mineralization of available carbon and thus affects the microbial community (Anderson and Gray, 2003).

3. MATERIALS AND METHODS

3.1 Preliminary laboratory trials

3.1.1 Growth conditions

The microalgae strains MACC-755 (*Chlorella vulgaris*), MACC-922 (*Chlorella vulgaris*), MACC-612 (*Nostoc linckia*), MACC-683 (*Nostoc sp*.), MACC-430 (*Chlamydopodium fusiforme*), MACC- 677 (*Tetradesmus obliquus*), MACC-519 (*Chlorella sp*.), and MACC- 438 (*Chlorella sorokiniana*) are derived from Mosonmagyaróvár Algae Culture Collection (MACC), Hungary. The strains were incubated at 25 ±2 °C, in a 12:12 light/dark cycle. The microalgae biomass was produced in laboratory culture units. It was illuminated from below with a light intensity of 130 µmol m^{-2} s^{-1} and grown in Tamiya nutrient solution (Tamiya, 1957), with a starting concentration of 10 mg L^{-1} algal dry weight (dwt). 20 L h^{-1} of filtered compressed air enriched with 1.5% CO2 during the light period was used for aerating the culture strains (Ördög, 1982). The cultures grown in these conditions for 10 days were then centrifuged for 15 mins at 3000 rpm (Sigma 6 K15, Germany) and freeze-dried using Gamma 1–20 (Christ, Germany) and stored at -18 °C. Biomass samples were re-suspended in distilled water and sonicated (VirTis, VirSonic 600 Ultrasonic Cell Disruptor, US) 3 minutes just before plant treatments.

3.1.2 Germination Test

The experimental design used was Factorial Complete Randomized Design. For the Bioassay, 8 strains were taken with four different

concentrations from Mosonmagyaróvár Algae Culture Collection (MACC).

The strains MACC-922, MACC-612, MACC-683, MACC-755, MACC-430, MACC-677, MACC-519, and MACC-438 were taken as the main treatment at four different concentrations viz, 0.1g/L, 0.3g/L, 0.5g/L and 1g/L as the sub-treatments.

Winter wheat (*Triticum aestivum* L.) seeds were taken 10 seeds each were put in 9 cm Petri dish and placed between two filter papers. Each was replicated three times. Microalgae strains of different concentrations were put in each treatment. The solutions were used only once at the initial stage of seeding later the moisture requirement was supplied by deionized water. The germination recordings were carried out every morning at 9 am. The Petri plates were kept at 8 °C for 7 days (a total of 56-degree days). The radicle length was taken by the traditional method using thread and scale. The number of germinated seeds with emerged coleoptile was also recorded. Germination index, speed of germination, mean germination time and germination rate index were calculated using the recorded data. The formulas were as follows:

3.1.2.1 Germination Index (GI)

$GI = (7\times N1) + (6\times N2) + \ldots\ldots\ldots\ldots\ldots + (1\times N7)$

where N1, N2……N7 is the number of germinated seeds on the first, second, and subsequent days until 7^{th} day and the multipliers are the weights given to the days of germination.

3.1.2.2 Speed of germination (SG)

SG = N1/D1 + N2/D2+N3/D3……+N7/D7.

where N = no of germinated seeds and D= days of germination.

3.1.2.3 Mean germination time (MGT)

MGT: ∑(n × d) /N,

where n= number of seeds germinated on each day, d = the number of days from the beginning of the test, and N= the total number of seeds germinated at the termination of the experiment.

3.1.2.4 Germination rate index (GRI), %/day =G1/1 + G2/2 +……..+G7/7, where G1, G2……G7= Germination percentage on each day × 100

3.1.2.5 Mungbean rooting bioassay

One of the characteristic effects of auxins is their role in the induction of adventitious roots on stem cuttings. This effect was utilized in a bioassay developed by Hess (1961). The bioassay is simple to perform and largely insensitive to the presence of inhibitors. The bioassay was conducted with a control, three concentrations of indole butyric acid (IBA), MACC-430, MACC-612, MACC-922, and MACC-438, replicated 4 times. The concentrations of IBA are 0.3, 0.5, 0.7 mg/L and that of the microalgae biomass was 1g/L. Out of the 8 strains based

on radicle length and germination parameters, the 3 bet-ter strain from different genera, MACC-612, MACC-430, MACC-922 were selected, additionally, MACC-438 was used in the bioassay for better comparison of their per-formance was the worst of all treatments. The concentration 1 g/L was used for the strains in the bioassay as there was no huge difference between the concentrations in the germination test and to make it feasible for higher research. The mung bean (*Vigna radiata* (L.) Wilczek) seeds are soaked for 4 minutes in a 0.33% sodium hypochlorite so-lution, then removed and rinsed under running tap water for 24 hours. The seeds are planted at a depth of 1 cm in moistened perlite in plastic trays. The trays are placed in the growth chamber maintained at 27 °C and relative humidity of about 60 to 65%, illuminated with fluorescent lamps for 7 days. The seedlings should have fully expanded unifoliate and unexpanded (rolled) trifoliate leaves in the bud. The seedlings are then cut with a clean razor 3 cm below the cotyledons. Uniform seedling cuttings are selected for further use. They consist of a 3-cm hypocotyl, the epicotyl, the unifoliate leaves, and the trifoliate leaf bud. The cotyledons are carefully removed.

Five seedling cuttings are placed in vials of 25×90 mm (three vials per treatment) containing 10 mL of distilled water, algal nutrient solution, as controls, and algal suspension (generally 2 g/L dry matter), as treatment for 6 hours of soaking. For the comparison of the treatments a specific auxin, such as indole-3-butyric acid (IBA) can be used in concentrations of 0.3, 0.5, and 0.7 mg/L. After the soaking seedling

cuttings are rinsed with distilled water and placed back into the vials with 10 mL distilled water. They are placed back in the original growth conditions for 7 days. The solution level (lost by transpiration) is restored to its original state with distilled water daily. After the incubation period, the number of roots (longer than 1 mm) is counted on each hypocotyl. The number is directly proportional to the auxin concentration within the assay range. The mean number of roots, derived from each vial, must be compared to the controls; then it can be analyzed using the comparison concentrations made by a specific auxin (IBA).

3.1.2.6 Quantification

An Acquity I/Class UPLC system - Xevo TQ/XS (Waters, Milford, MA, USA) tandem mass spectrometer was used for the quantification of the four strains viz., MACC-612, MACC-430, MACC-922, MACC-438. The method was explained in Hrdlička et al., (2019). The freeze-dried samples were diluted with water in ratios of 1:250, 1:500, 1:1000, 1:1500, or 1:2000 (v/v), depending on the plant species, to maximize their stimulatory biological activity. Combinations of tandem mass spectrometry and gas or liquid chromatography are popular analytical techniques for the analysis of plant hormones and related compounds, providing the ultra-high sensitivity and selectivity of mass analyzers with excellent separation of analytes in samples with complex biological matrices (Novák et al., 2017). The same method was used for the quantification of leaf samples.

3.2 Pot experiments

3.2.1 Experiment setup and foliar treatment application

Vernalized winter wheat plants (Mv Nádor and Mv Béres varieties bred in Agricultural Institute, Centre for Agricultural Research, Martonvásár, Hungary) were grown under semi-controlled greenhouse conditions. Three independent pot experiments were conducted. In Trials 1 and 2 Mv Nádor, in Trial 3 Mv Béres variety was used. Three strains of algae were tested in each trial, MACC-612 (*Nostoc linckia*), MACC-430 (*Chlamydopodium fusiforme*), and MACC-922 (*Chlorella vulgaris*). The algal biomass solutions were homogenized before applying to the plant.

The difference between the three trials was either time of application or varietal difference. In Trial 1 the application timing was at critical flowering stage or early reproductive stage, in Trials 2 and 3 algae treatments were applied at early vegetative stage. Completely randomized experimental design was used.

In another pot experiment non-vernalized wheat plants were sprayed. These were mainly to check the hormonal activity.

3.2.2 Samplings

The following parameters were analysed:

3.2.2.1 Morphological parameters and yield attributes

Plant height at the flowering stage, number of tillers, and spike length were measured. Biological yield refers to the total biomass

accumulation in the plant system. It is the sum of grain yield as well the straw yield. We can calculate it by addition of the dry weight of grain and straw in tons per ha.

3.2.2.2 Hexose sugar content

To find the hexose content we used the phenol-sulphuric method (DuBois et al.,1956) The powdered sample was diluted 100 times with 80% ethanol.

The absorbance of the characteristic yellow orange colour was measured with CARY 50 SCAN UV-Visible spectrophotometer (Varian) at 490 nm for hexose sugar. The unit of measurement in mg/g of sample.

3.2.2.3 Total phenol content

Folin-Ciocalteau colorimetric method by Singleton and Rossi (1965) was the method employed to determine the total phenol content. The absorbance of the blue colour solution developed after cooling, was measured at 760nm with CARY 50 SCAN UV-Visible spectrophotometer (Varian). Using a standard curve of gallic acid, quantification was done. The units of the concentrations were expressed as gallic acid equivalents (GAE), milligrammes per gram of dry weight (DW). The calculation of total phenolic content in mg/g, in GAE (Gallic acid equivalent) are as follows:

$C = C1 \times V/m$

where C = total phenolic content in mg/g, in GAE (Gallic acid equivalent), C1 = concentration of Gallic acid established from the

calibration curve in mg/ml, V = volume of extract in ml, and m = the weight of the plant extract in g.

3.2.2.4 Nitrogen content

Elementar Analysensysteme Gm–H - Elementar-Straße–1 - 63505 Langenselbold (DE), simply the Rapid N cube was used for the analysis of nitrogen. It employs the principle of the Dumas method (dry combustion method) for nitrogen determination by quantitative combustion digestion of the sample at approx. 960 °C in excess oxygen. Tinted palettes were prepared by weighing 150 mg of powdered sample into tinfoil made into palettes. The palettes were then placed in the combustion chamber.

3.2.2.5 Metabolomics

Same method of analysis as described in **3.1.2.4** Using an Acquity I/Class UPLC system - Xevo TQ/XS tandem mass spectrometer (Waters, Milford, MA, USA) quantification of the four strains viz. MACC-612, MACC-430, MACC-922, MACC-438 was performed.

3.3 Field experiments

3.3.1 Trial setup

Experimental design: Randomized block design.

Area of one plot: 3m × 10 m.

Total number of treatments: 8.

Total number of replications: 4.

Crop: winter wheat, *Triticum aestivum* L.

Time of application: Critical flowering stage, Waddington 7.5 stage

Concentration of the microalgae biomass: 1 g/L

Table 3.1. Trial map treatment description

Trt	Code	Description
Trt 1	CHK	Untreated check
Trt 2	Standard CHK	6-BAP@1g/L conc. and spray volume of 300 L/ha
Trt 3		MACC-612 @1g/L conc. and spray volume of 300 L/ha
Trt 4		MACC-612 + Trend 90 @1g/L conc. and spray volume of 300 L/ha
Trt 5		MACC-430 @1g/L conc. and spray volume of 300 L/ha
Trt 6		MACC-430+ Trend 90@1g/L conc. and spray volume of 300 L/ha
Trt 7		MACC-922 @1g/L conc. and spray volume of 300 L/ha
Trt 8		MACC-922 + Trend 90 @1g/L conc. and spray volume of 300 L/ha

Figure 3.1 Trial map of field experiment. The same map used

Table 3.2. Growing and environmental conditions

	2020-21 growing season (02/10/2020-02/08/ 2021)	**2021-22 growing season (01/10/2020-11/07/ 2021)**
Location coordinates	Dunasziget 47.916267, 17.350554	Mosonmagyaróvár 47.9016780, 17.2547360
Initial soil status	pH: 7.12 Humus %: 2 Total N: 187.52 kg/ha	pH: 7.33 Humus % (m/m): 1.96 Total N: 237.51 kg/ha
Variety	GK Kunhalom	GK Kunhalom
Sowing date	Oct 2., 2020	Oct 1, 2021
Emergence date	Oct 4, 2020	Oct 2,2021

Contd. Table 3.2.

	2020-21 growing season (02/10/2020-02/08/ 2021)	**2021-22 growing season (01/10/2020-11/07/ 2021)**
Application date	May 28, 2021 BBCH 52-53	May 14, 2022 BBCH 52-53
Sampling 1 (Leaves)	June 12, 2021 **BBCH:65**	May 30, 2022 **BBCH:65**
Sampling 2 (leaves)	June 27, 2021 **BBCH 72-75**	June 15, 2022 **BBCH 72-75**
Sampling 3 (whole plant)At harvest	July 30, 2021 **BBCH 89/90**	July 8, 2022 **BBCH 89/90**
Maintenance	Fertilizer Fungicide (Elatus Era): April 25, 2021 No irrigation	Fertilizer Fungicide (Elatus Era): April 10, 2022 No irrigation
Harvesting date	August 2, 2021	July 11, 2022
Harvesting machine	Wintersteiger DELTAv2	Wintersteiger DELTAv2
Soil sampling 2	August 3, 2021	July 14, 2022

During the growing season, fertilizer was applied before sowing at the average dose of ca. 50 kg N/ha, 60 kg P_2O_5/ha and 60 kg K_2O/ha. In early February, an additional 50 kg N/ha of ammonium-nitrate (33% N) was top-dressed according to N-min analysis.

Table 3.3. Meteorological conditions in full growing season

	2020-21 growing season	**2021-22 growing season**
Growing days	304 days	283 days+
Cumulative precipitation	515.35 mm	408.6 mm
Min temperature	-10.4°C	-7.2°C
Max temperature	35.8°C	36.9 °C
Min. temperature after application	5.9°C	5.7°C
Max. temperature after application	38°C	36.9°C
Precipitation one week before and one week after application	20.7 mm	27.4 mm

(For detailed data see: Chapter 10. Appendices)

3.3.2 Soil microbial population count

3.3.2.1 Bacterial population

Nutrient agar media (The American Public Health Association, 1917) was used for bacteria culture for population count.

Further, the soil extract was prepared by serial dilution. Suitable dilution for bacterial population is 10^{-4} to 10^{-7}, but the exact dilution factor depends on the soil. To confirm we can check on to different dilution factors, in any Petri plates the colony should be less than 250

numbers. Inoculation of the extractant: 1 mL of extractant is added on the solidified agar medium (solification should be done in a sterilized condition to avoid contamination). They are then incubated at room temperature and can start assessing the culture after 24 hours.

3.3.2.2 Actinomycetes

Dextrose Nitrate Agar (Williams et al., 1983) was prepared. The sample was prepared by the serial dilution method (Johnson and Curl, 1972). Dilution factor of 10^{-4} to 10^{-8} achieved by serial dilution was inoculated on the agar media (1 mL each). The plates are then incubated at 28 ℃ for 5-25 days.

3.3.2.3 Fungal population

Rose Bengal agar media (Martin, 1950) supplemented with streptomycin sulphate was used to count the fungal colony in the soil. The sample was prepared by Serial dilution method (Johnson and Curl, 1972). The Petri plates with the media were inoculated with samples of different dilution factor (10^{-2} to 10^{-8}) and were incubated in front of a laminar airflow in a sterile room at 25°±1°C. The counting for the colony forming units (CFU) was estimated after 5 days.

A standard plate count of the colonies for all three, bacteria, actinomycetes and fungi were conducted from which the colony forming units (CFU) was calculated:

$$CFU/g = \sum c/(n_1+0.1\times n_2+0.01\times n_3) \times 10^i$$

where, ∑ c= (all the colonies in the Petri plates between 25-250); i = least diluted factor that has maximum colonies under 250; n1, n2, and n3 are the number of plates in each dilution.

3.3.3 Physiological parameters

3.3.3.1 Chlorophyll and carotenoid content

Chlorophyll and carotenoid content were measured in the laboratory by extracting the photosynthetic pigments themselves from a leaf sample (sampled 15 days after application of microalgae biomass) using acetone (Arnon, 1949). The chlorophyll and carotenoid concentrations were measured spectrophotometrically at 440.5, 645 and 663 nm wavelength, using CARY 50 SCAN UV-Visible spectrophotometer (Varian). From this measurement, we can find the total chlorophyll and carotenoid contents in fresh plant material. The ratio of chlorophyll-*a* to chlorophyll-*b* was also calculated from the result by simply dividing the chlorophyll-*a* value by chlorophyll-*b* value. The unit of measurement is mg/g fresh weight

3.3.4 Biochemical parameters

3.3.4.1 Estimation of hexose sugar

The Phenol-sulphuric acid method by DuBois et al., (1956) was used to determine hexose sugar in the plant and grain samples. The description is same as in **3.2.2.2**.

3.3.4.2 Estimation of total polyphenol content

Folin-Ciocalteau colorimetric method by Singleton and Rossi (1965) was the method employed to determine the total phenol content. Same procedure as mentioned in **3.2.2.3.**

3.3.4.3 Ferric reducing antioxidants power (FRAP) assay

Total antioxidant potential was measured for plant biomass sampled 15 days after application and the grains after harvest. The FRAP assay developed by Benzie and Strain (1996) was used to analyse the total antioxidant potential of the sample. The absorbance was measured at 593 nm (CARY 50 SCAN UV-Visible spectrophotometer, Varian) and the concentration was derived from the ascorbic acid standard curve. The result was then achieved by multiplying with a constant 155 (because of the solvents) and expressed as µg ascorbic acid equivalent/mL.

3.3.4.4 Estimation of proline content

The method developed by Bates (1973) was used for the estimation of proline concentration in plant samples. The leaves from two different

stages were analyzed viz. 15 days and 30 days after foliar treatment. 3% sulfosalicylic acid was used as an extractant. A random sampling of 5 plants/plot were conducted. Standard proline (Sigma Aldrich) was used for the preparation of the standard curve. Absorbance was measured at 520 nm using toluene for a blank (CARY 50 SCAN UV-Visible spectrophotometer, Varian). On a fresh weight basis, the proline content of the plant biomass (leaves) was calculated using the following:

[(μg proline/mL × 3 mL toluene)/115.5 μg/μmol]/[0.3 g sample/5] =μmol proline/g FW weight.

3.3.5 Determination of the floral development process

The crucial assessments to be made were fertility % and sterility %. Fertile floret number can be found from the differences between maximum floret primordia and the final floret that remains at anthesis. Final grain number can be counted before harvest. During each stage the main shoots of five plants for each plot were randomly selected to measure floret primordia and fertile floret number per spikelet. The main shoots of five plants were used for determining final grain number per spikelet. Maximum floret primordia number: it starts when the F1(the first floret from the spikelet base) in the central spikelet reached a stage of Waddington score 8 (stigmatic branches and hairs on ovary wall elongation.

Fertility % = No. of fertilized spikelets (seeds)/ total number of floral primordia × 100%.

Sterility % = No. of unfertilized spikelet/total number of floral primordia × 100%.

Number of unfertilized spikelets = Total number of floral primordia - No. of fertilized spikelets.

3.3.6 Crop growth rate (g/m^2/day)

Crop growth rate: it is measured as a mass increase in crop biomass per unit ground area per unit. One-meter-long row was selected in each subplot, harvested, sun dried and weighted. Data was recorded right before the treatment (one day before the application) and 30 days after application of the treatments. CGR will be measured by the following formula:

- CGR = –2 - W1/ –2 - T1 × 1/GA (g/m^2/day)
- W1 = Weight at T1 of the period
- W2 = Weight at T2 of the Period
- T1 = Time in date at the start of the period
- T2 = Time in date at the end of the period
- GA = Ground area

3.3.7 Relative leaf water content, RLWC (%)

We followed a simple procedure to measure relative leaf water content. The leaves after 15 days of application were sampled from each plot.

The fresh pluck leaves were cut into 5 cm length pieces, 3 such pieces form one sample. Every plot had three samples. First, the fresh weight of each sample was recorded, then the leaf pieces were soaked in deionized water overnight, to take the turgid weight. Finally, they were put in an oven of 60±5 °C for 48 hours, followed by weighing the samples, which was the dry weight.

Relative water content = [(fresh weight- dry weight)/ (turgid weight- dry weight)] × 100

3.3.8 Yield attributes

3.3.8.1 Number of tillers: Number of tillers in one hill was counted. A total of 5 hills were examined in each plot.

3.3.8.2 1000 kernel weight: Count 1000 seeds and the weight was taken in grams.

3.3.8.3 Number of grains per ear: The grain from one ear spike was removed and counted. 5 subsamples were taken from each plot.

3.3.8.4 Above-ground biological yield or straw yield: The dry matter weight of aboveground biomass of one-meter square area was measured. Then it was converted into kg/ha.

3.3.8.5 Grain yield: The yield of the grain per plot were determined through the machine harvester (Wintersteiger DELTAv2). From the per plot value, it was then converted to kg/ha.

3.3.8.6 Harvest index (HI): Harvest index is the ratio of economical

yield by biological yield (Donald and Hamblin, 1976) where economical yield is the total grain weight (dry weight) of the plant while biological yield is the above-ground biomass yield (grains + straw) of the plant.

Five subsamples were taken from each plot. The weight of the grains and biomass were taken separately. Here the biomass, denotes the total dry shoot matter or the above-ground biomass weight.

3.3.9 Estimation of quality parameters of the grain

3.3.9.1 Grain analysis: the Infratec™ 1241(Foss Tecator) introduced in 1987 was used as grain analyser to determine the protein, gluten and Zeleny sedimentation value. 500 g clean seeds from each plot were passed through the hopper for the analysis.

Sample preparation: fine wheat flour for sampling was obtained from clean grains using a FOC-109, model no.3525-002 (Metefém Hungary) grinder.

3.3.9.2 Protein subunits

Size exclusion high-performance liquid chromatography (SE-HPLC) was used to quantify the ratio of gluten proteins, the glutenins and the gliadins (Glu/Gli) and the amount of unextractable polymeric proteins (UPP).10 mg of floured sample were used in the analysis. The samples were sonified for 15 after suspending in 1 mL 0.5 % (w/v) SDS in phosphate buffer (pH 6.9). Sonification and shaking of samples were followed by centrifugation at 14,000 rpm. The filtrate after passing the

supernatant through 0.45 µm polyvinylidene fluoride filter was then analyzed using The Waters Alliance™ HPLC system (Waters Corporation Milford, MA, USA) with 2695 Separation Unit and 2996 Photodiode Array Detector (Waters Corporation Milford, MA, USA). The system used Phenomenex BIOSEP-SEC 4000 column (500 A, 5 µm, 7.8 × 300 mm) (Waters Corporation Milford, MA, USA) in an acetonitrile buffer (50% acetonitrile and 0.1% (w/v) trifluoroacetic acid) with a running time of 10 min (2 mL/min flow rate). The column temperature was maintained at 25 °C and the sample temperature at 15 °C. The injection volume was 50 µL, and UV-detection was done at 214 nm.

After determining the protein subunits, using Larroque and Békés (2000) the quantitative ratio of glutenins and gliadins was derived by dividing the total amount of soluble and insoluble glutenins by the total amount of soluble and insoluble gliadins.

3.3.10 Estimation of soil parameters

3.3.10.1 Total nitrogen content

The total nitrogen content was determined by the Dumas method according to AACC 46-30.01 method (2020), with Elementar Rapid N III Analyzer (Elementar, Langenselbold, Hesse, Germany). Same instrument was used for calculation of protein and total nitrogen.

3.3.10.2 Nitrate and nitrite content

The method of water analysis according to DIN EN ISO 13395(1995) was applied to determine the Nitrate-and-Nitrite nitrogen through Flow injection analysis. The samples were mixed with imidazole buffer. Copperized cadmium in the cadmium reductor (Cd reductor) reduced the nitrate further to nitrite. Diazonium salt form from sulfanilamide reacts with N-(1-Naphthyl)-ethylenediamine (NED) to form red azo dye. The dye concentration was then measured at 546 nm. The final result is a stoichiometric sum of nitrite and nitrate.

3.3.10.3 Nitrogen use efficiency, NUE

It is simply how much of the nitrogen available in the soil is utilized or taken up by the plant.

The nitrogen content in the soil before sowing was measured. The total nitrogen available to the plant for the season was the original nitrogen content in the soil (kg/ha) in addition to the fertilizer applied, which may be termed as $N_{initial}$ in kg/ha. After harvesting the nitrogen content in the grains (N_{grain}) and above-ground biomass ($N_{biomass}$) was measured. The same Dumas method using Elementar Rapid N III Analyzer was used to measure the nitrogen in the grain and the biomass. The nitrogen content in the soil after harvest ($N_{harvest}$) was also recorded.

Nitrogen use efficiency = $(N_{grain} + N_{biomass}/N_{initial} + N_{harvest}) \times 100\%$

3.3.10.4 Organic carbon %

The classic Walkley and Black chromic acid wet oxidation method (1934) method was used for determining the soil organic content**.** A series of standard concentrations (0, 100, 200, 300, 400, and 500ppm) of Glucosum anhydricum was used to prepare the standard curve. The densities at 660 nm were used to prepare the standard curve and to calculate the mean carbon % in the unit optical density.

The supernatant was measured for spectral reflectance at 660 nm wavelength using the SPECORD 210-PLUS (Analytik Jena, Germany) and the organic carbon (%) was determined using the Equation, Organic carbon = Optical density × mean carbon %.

3.4 Statistical analysis of data

Data obtained from **3.1** (preliminary trials) and **3.2** (pot experimentation) were subjected to analysis of variance (ANOVA) with a completely randomized design to determine the significance of differences among treatments. From the data presented for the mean of 6 replicates, standard errors (SE) were calculated. The least significant differences (LSD) method at $p \leq 0.05$ was used to compare all treatments (Snedecor and Cochran, 1989). Significant differences between the treatments and the genotypes were probed using the t-test method and ANOVA table (Microsoft 365 Apps for enterprise, Version 2112).

The data obtained from **3.3** (field trials) was processed using GDM

solutions' ARM 8 software (ARM is a recognized and respected standard throughout plant production, used by thousands of researchers around the world). ARM 2022.5(3rd) October 25, 2022 (final analysis version). Analysis method was Least square estimation and Student-Newman-Keuls was the mean comparison test at 5% significance or alpha level. Primary mean as mean descriptions and use adjusted mean as primary mean. To pool the data, pool analysis using the R package "PoolTestR" in R studio (version 4.1.2) while for the principle component analysis (PCA), the R package "Factoextra" was used in R studio (version 4.1.2).

4. RESULTS

4.1 Preliminary trials

4.1.1 Germination test

In the first experiment, the concentration-dependent effects of various types of algal strain biomass on the germination of a winter wheat Mv Nádor were tested. All parameters of germination are shown in **Table 4.1** MACC-430 at 1 g/L concentration presented the highest germination index (GI), while the lowest value occurred with MACC-438 at 1 g/L. Overall, every treatment is better compared to the control except for MACC-438 of all four concentrations and MACC-755 at 1 g/L. In some strains, the lower the concentration, the higher the GI observed, while some strains like MACC -430 and MACC-683 performed better at higher concentrations (**Table 4.1**). As for the speed of germination, the concentration of 0.3 g/L was at average in all the strains. In MACC-922, MACC-755, and MACC-612, the seeds germinated fastest in the 0.3 g/L concentration.

The radicle lengths on the 7th day were the highest in the case of MACC-612, MACC-430, and MACC-683 strains. Speaking of MACC-612, the lowest concentration 0.1 g/L had the longest radicle

length, while in MACC-430 1 g/L presented the longest radicle length. Also, in MACC-683 only the higher concentrations (0.5 and 1 g/L showed longer radicle lengths than the control (**Table 4.1**). The strain MACC-438 appeared to have a consistently lower value when compared with the control in all the parameters. It seems to affect germination, and in parameters like the speed of germination and the germination index, a considerable decline in the values was experienced with increasing germination. On the other hand, MACC-677 and MACC-755 also performed worse than the control, depending on the concentration.

Table 4.1. Different germination parameters under the influence of various concentrations and strains of microalgae. Germination index (GI), mean germination time (MGT), germination rate index (GRI), speed of germination (SG), radicle length (RL), of 8 strains at 3 different concentrations, 0.1, 0.3, 0.5, and 1 g/L. SEd between the different treatments were calculated.

Treatments	**GI**	**MGT**	**GRI**	**SG**	**RL7th day (cm)**
MACC-430@ 0.1 g/L	53	163	45.79	4.58	1.1
MACC-430@ 0.3g/L	78	194	62.29	6.23	1.1
MACC-430@ 0.5 g/L	65	175	53.79	5.38	1.2
MACC-430@ 1 g/L	87	201	68.12	6.81	1.2
MACC-683@ 0.1 g/L	48	160	42.95	4.30	1.0
MACC-683@ 0.3 g/L	61	179	51.45	5.15	0.9
MACC-683@ 0.5 g/L	74	182	59.62	5.96	1.2
MACC-683@ 1 g/L	77	195	61.79	6.18	1.2
MACC-922@ 0.1 g/L	68	180	55.79	5.58	1.2
MACC-922@ 0.3 g/L	74	182	59.62	5.96	0.9

Contd. Table 4.1

Treatments	**GI**	**MGT**	**GRI**	**SG**	**RL7th day (cm)**
MACC-922@ 0.5 g/L	62	178	51.95	5.20	0.9
MACC-922@ 1 g/L	70	186	57.29	5.73	0.8
MACC-519@ 0.1 g/L	66	174	54.12	5.41	0.8
MACC-519@ 0.3 g/L	62	178	51.95	5.20	0.8
MACC-519@ 0.5 g/L	54	170	46.95	4.70	0.6
MACC-519@ 1 g/L	57	175	48.95	4.90	0.7
MACC-612@ 0.1 g/L	75	189	60.29	6.03	1.2
MACC-612@ 0.3 g/L	78	194	62.29	6.23	1.1
MACC-612@ 0.5 g/L	77	195	61.45	6.15	1.0
MACC-612@ 1 g/L	62	178	51.95	5.20	1.1
MACC-677@ 0.1 g/L	57	167	48.29	4.83	0.7

Contd. Table 4.1

Treatments	**GI**	**MGT**	**GRI**	**SG**	**RL7th day (cm)**
MACC-677@ 0.5 g/L	56	168	47.79	4.78	0.6
MACC-677@ 1 g/L	55	169	47.45	4.75	0.7
MACC-755@ 0.1 g/L	61	179	51.45	5.15	0.5
MACC-755@ 0.3 g/L	58	174	49.45	4.95	0.5
MACC-755@ 0.5 g/L	64	184	53.45	5.35	0.6
MACC-755@ 1 g/L	39	145	36.95	3.70	0.5
MACC-438@ 0.1 g/L	36	140	34.95	3.50	0.3
MACC-438@ 0.3 g/L	34	134	33.29	3.33	0.2
MACC-438@ 0.5 g/L	32	128	31.62	3.16	0.2
MACC-438@ 1 g/L	29	123	29.62	2.96	0.3
CONTROL	45	147	40.12	4.01	0.8
CD @1%	4.5396	91.52	3.926	0.9246	0.1444
SEd	1.7115	34.47	0.987	0.3486	0.0544

After the germination test, the strains with overall better performance than the control viz., MACC-612, MACC-430, MACC-922, and the strain that shows a negative effect on the germination of wheat i.e., MACC-438 were used for further experiments with 1 g/L concentration.

4.1.2 Mungbean rooting bioassay

Mungbean rooting bioassay was conducted for the confirmation of auxin-like activity of the selected strains at 1g L^{-1} (**Fig. 4.1**).

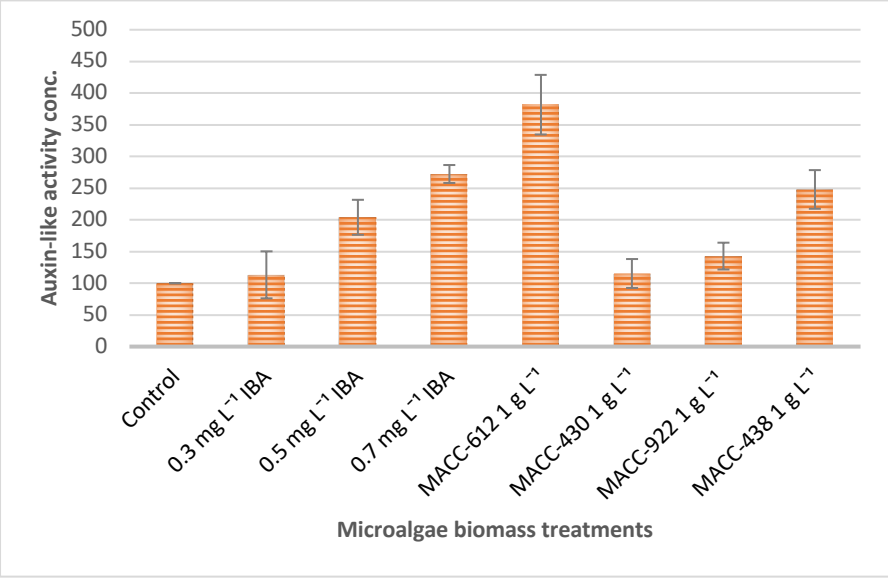


Figure 4.1. Mungbean hormonal activity bioassay – here number of rootlets is calculated as concentrations of auxin-like substance under the influence of auxin-like hormone present in the treatments. IBA was used as standards at 3 concentrations but the tested microalgae treatments in only one concentration (1 g/L or 1 gL^{-1}).

Mungbean plants treated with microalgae exhibited strain-dependent auxin-like activity, and the number of their rootlets was correlated to the concentration equivalent of IBA. **Figure 4.1** shows that MACC-

612 produced the highest level of root development in mungbean plants, while MACC-430 caused no significant effect.

4.1.3 Metabolite characterization of the microalgae strains

Metabolomic analyses focusing mainly on phenolic compounds in the algae strains were also carried out (**Table 4.2**). The secondary compounds detected are indole-3-acetic-acid, salicylic acid, para-hydroxybenzoic acid, benzoic acid, and trans-cinnamic acid. All strains contain salicylic acid, para-hydroxybenzoic acid, and benzoic acid, while strain MACC-612 has additional polyphenols such as the trans-cinnamic acid. The amount of all the polyphenols was higher in MACC-612 than in the others, while the differences between the other three strains were negligible. Interestingly, MACC-612 also showed a relatively high amount of IAA content, while in the other strains this hormone was under the detection limit.

Table 4.2. Metabolomic analysis of strains: Determination of indole-3-acetic-acid (IAA), salicylic acid (SA), *p*-hydroxybenzoic acid (pHBA), benzoic acid (BA), and *t*-cinnamic acid (tCA) from freeze-dried algae biomass samples using an Acquity I/Class UPLC syst–m - Xevo TQ/XS tandem mass spectrometer. Data represent mean ng g^{-1} DW ±SD; n=3. nd: not determined, under the detection limit. Different letters indicate significant differences at $p<0.05$.

Strains	**IAA**	**SA**	**pHBA**	**BA**	**tCA**
MACC-438	n.d.	21.1±1.3 bc	86.3±6.6 b	798.3±84.9 b	nd
MACC-430	n.d.	22.5±0.7 b	65.8±4.2 c	971.7±55.4 b	nd
MACC-612	59.3±2.6	35.1±1.9 a	698.3±27.8 a	2210.0±63.8a	38.2±1.0
MACC-922	n.d.	14.6±3.5 c	80.7±3.7 b	883.3±48.7 b	nd

4.1.4 Effects of algae strains on photosynthesis and metabolite contents of wheat

Based on the above-mentioned results, a pot experiment was designed to investigate the long-term effects of the physiological processes of microalgae treatments in the vegetative state of wheat plants. First, 15-day-old non-vernalized Mv Béres cultivar wheat plants were sprayed with the biomass of different algae strains, then 20 days after the spraying, photosynthetic parameters were measured, and leaf samples were collected for metabolome analyses. Previous studies indicate that salicylic acid may also influence the photosynthetic efficiency in plants

(Janda et al., 2014; Poór et al., 2019; Pál et al., 2020). This can be due to the modification of stress acclimation processes, leading to crosstalk between various signaling pathways (Filgueiras, et al., 2019; Poór et al., 2019; Pokotylo et al., 2019; Tajti et al., 2019; Saleem et al., 2020, Pál et al., 2020; Nadarajah, et al., 2021). Since all the algae strains contained salicylic acid together with its putative precursors benzoic acid and trans-cinnamic acid, we mainly focused on the photosynthesis-related processes. Different gas exchange parameters, such as net photosynthesis (Pn), stomatal conductivity (gs), or intercellular CO_2 concentration (Ci) and chlorophyll-*a* fluorescence induction values, such as Fv/Fm and ΔF/Fm', indicating the maximum and actual photochemical efficiency of Photosystem 2, respectively, or the Y(NPQ) and Y(NO), indicating the regulated and non-regulated non-photochemical quenching processes, respectively, suggested that neither the primary carbon assimilation nor the photosynthetic electron transport processes were significantly affected by the treatments with algae strains used in the present experiment (**Table 4.3**).

In contrast to the photosynthetic parameters, certain secondary metabolites showed significant differences between the control and the sprayed plants (**Table 4.4; Béres1**). While none of the algal treatments caused significant changes in indole-3-acetic acid, p-coumaric acid, abscisic acid, neochlorogenic acid, rutin, and naringenin, a slight, but statistically significant increase was discovered in the salicylic acid content after the treatment with MACC-922 or in *p*-hydroxybenzoic acid after the treatment with MACC-430 or MACC-438. All the algae

treatments significantly reduced the jasmonic acid and the jasmonic acid/isoleucine conjugate contents.

However, since the experiment was carried out under greenhouse conditions, where the environmental factors are only partly controlled; and most of the above-mentioned compounds are key components of stress signaling processes, we repeated the experiments with the same genotype Mv Béres and with another winter wheat cultivar, named Mv Nádor, at another time. The growth conditions were similar, but not the same. Plants were treated with algae 17 days after sowing, and samples were taken 28 days after the spray. Since the first sampling was carried out on 13th November 2020, and the 2nd sampling on 11th February 2021, light conditions could also slightly differ. Data from the 2nd set of experiments also showed that certain algae treatments significantly affected the hormonal contents of wheat plants. However, these results did not always correlate with the 1st set of experiments. These results suggest that treatment with microalgae may also influence the acclimation processes of wheat plants. Nevertheless, these effects are in interaction with other signaling processes induced by various environmental conditions.

Table 4.3. Chlorophyll-*a* fluorescence induction and gas exchange parameters. Pn: net photosynthesis (µmol $m^{-2}s^{-1}$); gs: stomatal conductivity (mmol $m^{-2}s^{-1}$); Ci: intercellular CO_2 concentration (µmol CO_2 mol^{-1}); E: transpiration (mmol $m^{-2}s^{-1}$), WUE: water use efficiency (Pn/E) in the control and the alga-treated Mv Béres wheat plants 28 days after spraying.

Para-meter	**Control**	**MACC-922**	**MACC-430**	**MACC-612**	**MACC-438**
Fv/Fm	0.784 ±0.015	0.788 ±0.006	0.786 ±0.004	0.780 ±0.011	0.778 ±0.013
Y (II)	0.541 ±0.042	0.556 ±0.022	0.550 ±0.020	0.530 ±0.029	0.535 ±0.039
Y(NO)	0.228 ±0.034	0.219 ±0.041	0.242 ±0.025	0.250 ±0.033	0.253 ±0.028
Pn	12.8 ±2.4	14.9 ±3.9	12.6 ±2.2	14.4 ±1.2	9.3 ±1.2
gs	337 ±157	525 ±183	358 ±98	438 ±54	246 ±51
Ci	195 ±33	210 ±17	202 ±16	200 ±17	197 ±8
E	3.85 ±1.09	5.05 ±0.90	4.05 ±0.67	4.68 ±0.37	3.20 ±0.49
WUE	3.42 ±0.49	2.95 ±0.64	3.11 ±0.21	3.09 ±0.29	3.20 ±0.49

Table 4.4. Metabolomic analysis of the leaves of wheat Mv Béres and Mv Nádor treated with different algal strains. Béres1 refers to data from the 1st experiment with Mv Béres; Béres2 and Nádor concern data from the 2nd experiments with Mv Béres and Mv Nádor, respectively. Data are in ng g^{-1} F.w, mean ± SD, n=5. *represents significant differences from the control at $p < 0.05$.

Mv Béres1					
	C	**MACC-430**	**MACC-438**	**MACC-612**	**MACC-922**
IAA	1.03 ±0.21	0.84 ±0.13	1.02 ±0.22	0.99 ±0.20	1.05 ±0.21
SA	47.2 ±6.5	50.3 ±11.9	49.1 ±10.1	45.8 ±12.8	64.2 ±12.3*
p-HBA	25.9 ±1.0	29.2 ±1.5*	29.3 ±2.5*	27.6 ±3.2	26.6 ±2.7
p-CA	19.7 ±5.0	16.4 ±4.1	29.0 ±11.4	22.9 ±5.8	18.8 ±3.5
JA	106.4 ±11.5	62.8 ±12.8*	72.8 ±15.4*	40.4 ±23.7*	57.1 ±11.3*
ABA	3.92 ±0.26	4.36 ±0.30	3.83 ±0.29	4.05 ±0.81	4.20 ±0.50
NCA	118 ±65	178 ±76	105 ±17	191 ±57	129 ±80
R	27.7 ±3.7	30.3 ±2.6	31.9 ±4.2	28.4 ±2.2	31.5 ±3.5
NG	1.28 ±0.20	1.27 ±0.19	1.61 ±0.36	1.97 ±1.28	1.71 ±0.31*
JAL/IC	50194 ±7023	21850 ±3644*	27942 ±5796*	15112 ±12476*	19417 ±5716*

Table 4.4 (Contd.)

Mv Nádor					
	C	**MACC-430**	**MACC-438**	**MACC-612**	**MACC-922**
IAA	1.04 ±0.20	0.78 ±0.13	0.75 ±0.04*	0.78 ±0.06*	0.69 ±0.06*
SA	55.8 ±8.7	44.8 ±5.0	115.4 ±29.3*	50.8 ±9.1	67.0 ±6.3
p-HBA	20.5 ±5.4	18.1 ±2.5	15.3 ±2.0	19.6 ±0.4	19.1 ±3.1
p-CA	32.7 ±5.1	27.4 ±8.2	25.8 ±1.5*	29.2 ±5.1	26.4 ±3.6
JA	18.1 ±2.9	22.9 ±4.9	59.5 ±20.1*	41.4 ±7.8*	20.9 ±3.2
ABA	15.24 ±0.73	8.61 ±2.31*	14.12 ±1.05	9.56 ±0.85*	7.34 ±0.30*
NCA	1271 ±405	1064 ±42	476 ±157*	522 ±277*	760 ±155*
R	3.4 ±0.8	3.3 ±0.8	3.4 ±0.5	3.4 ±0.3	3.5 ±0.5
NG	3.94 ±1.40	3.13 ±0.33	2.38 ±0.39	3.09 ±0.37	2.82 ±0.35
JAL/IC	9078 ±1267	7405 ±2070	17980 ±5702*	11842 ±2164	9934 ±1857

Table 4.4 (Contd.)

Mv Béres2					
	C	**MACC-430**	**MACC-438**	**MACC-612**	**MACC-922**
IAA	0.98 ±0.12	1.05 ±0.11	0.99 ±0.03	0.94 ±0.12	0.85 ±0.11
SA	142.2 ±8.7	114.3 ±29.3	98.7 ±14.6*	91.1 ±18.2	93.8 ±18.7*
p-HBA	19.8 ±1.1	25.1 ±5.4	19.4 ±1.2	23.5 ±2.6	18.3 ±1.1*
p-CA	14.0 ±1.4	16.8 ±2.6	14.6 ±5.1	15.0 ±3.1	12.3 ±1.8
JA	17.8 ±3.2	16.7 ±4.3	12.4 ±3.2*	16.5 ±5.2	17.1 ±5.9
ABA	6.51 ±0.42	7.28 ±0.22*	6.75 ±0.36	5.97 ±0.18	6.56 ±0.30
NCA	302 ±101	481 ±285	605 ±319	115 ±32*	399 ±107
R	22.7 ±5.3	24.4 ±8.6	19.9 ±6.8	16.7 ±2.8	21.4 ±2.6
NG	2.26 ±0.25	3.12 ±0.83	2.23 ±0.21	2.60 ±0.33	2.02 ±0.11
JAL/IC	5709 ±1081	4873 ±1436	3782 ±829*	4202 ±1266	7795 ±4907

Note: Indole-3-acetic-acid=IAA; Salicylic acid=SA; p-Hydroxybenzoic acid= pHBA; p-coumaric acid = p-CA; Jamonic acid =JA; Abscisic acid=ABA; Neochlorogenic acid =NCA; Rutin= R; Naringenin = NG; Jasmonic acid-leucine/isoleucine conjugate= JAL/IC.

4.2 Pot experiments

4.2.1 Effect on morphological, physiological, and biochemical characteristics under controlled conditions

First, total biological yield, hexose content, and total phenol content, nitrogen % in grain and in straw were determined. The results we found were inconsistent. First, there is no significant difference in most of the parameters taken. However, in Trial 1, the biological yield after MACC-612 application was significantly higher than in control or other treatments **(Table 4.5).**

On the other hand, in trial 2 (**Table 4.6**), where we applied algae in the vegetative stage in Mv Nádor, the nitrogen content in the grain had a significant difference, showing the highest values in MACC-430 and MACC-612. In contrast, the other parameters did not change significantly.

Table 4.5. Trial 1 with microalgae applied at early reproductive stage using Mv Nádor. Mean value from 5 replications is presented in the table at 1% and 5% CD. ** indicates significant difference between treatments.

Trt	TB Yield	HC	TPC	Ni(grain)	N (straw)
Control	5.17	24.004	2.622	3.38	1.172
MACC-430	4.908	24.449	2.622	3.537	1.0975
MACC-922	4.922	24.405	2.630	3.627	0.9325
MACC-612	6.358	24.219	2.626	3.555	1.0525
SEd	0.4912	0.677	0.186	0.385	0.1532
Sig level	**	Nsig	Nsig	Nsig	Nsig
CD 1 %	1.5004	1.566	0.569	1.1346	0.4515
CD 5 %	1.38	1.1371	0.4059	0.820	0.326

Note: Trt=Treatment; TB Yield: Total Biological yield, g/plant; HC: Hexose content=mg/g; TPC: Total phenol content =mg GAE/g dry extract; N (grain): Nitrogen in grain, %; N (straw)=Nitrogen % in straw.

In Trial 3, using another variety, Mv Béres, also with early vegetative stage application. The differences of total biological yield and nitrogen % in grain were statistically significant (**Table 4.7**). Untreated control had the lowest biological yield (g/plant) while the MACC-922 strain resulted the maximum influence on this parameter. The highest nitrogen % value in grain was in samples treated with MACC-430.

Table 4.6. Trial 2 with microalgae biomass applied at early vegetative stage using Mv Nádor. Mean value from 10 replications is presented in the table at 1% and 5% CD. ** indicates significant difference between treatments.

Trt	TB Yield	HC	TPC	N (grain)	N (straw)
Control	4.6455	38.90845	1.5266	2.8	0.812
MACC-430	4.477	45.774	1.716	3.205	0.64
MACC-922	4.7705	43.169	1.7591	2.962	0.72
MACC-612	4.7821	40.176	1.605	3.2175	0.716
SEd	1.3879	3.7185	0.1368	0.083	0.117
Sig	N.sig	Nsig	Nsig	**	N sig
CD 1 %	1.2912	11.3583	0.4177	0.254	0.3422
CD 5 %	0.9371	8.0934	0.2981	0.1815	0.248

Note: Trt=Treatment; TB Yield: Total Biological yield, g/plant; HC: Hexose content=mg/g; TPC: Total phenol content =mg GAE/g dry extract; N (grain): Nitrogen in grain, %; N (straw)=Nitrogen % in straw

In all three trials, the application of microalgae caused no significant changes in the total phenol and hexose contents even if the time of application and varieties have been changed, and they had strong influence on these parameters.

To better understand the mechanisms of the effects of microalgae in Trial 3, metabolomics analysis was conducted.

Table 4.7. Trial 3 with microalgae biomass applied at the early reproductive stage using Mv Béres. Mean value from 5 replications is presented in the table at 1% and 5% CD. ** indicates significant difference between treatments.

Trt	**TB yield**	**HC**	**TPC**	**N (grain)**
Control	3.744	23.038	2.622	2.892
MACC-430	4.94	24.065	2.622	3.12
MACC-922	5.27	24.626	2.630	2.747
MACC-612	5.200	23.824	2.626	2.77
SEd	0.506	0.5180	0.0034	0.105
Sig	**	N sig	N sig	**
CD 1 %	2.6598	1.5823	0.0104	0.3115
CD 5 %	1.9305	1.1291	0.0077	0.2253

Note: Trt=Treatment; TB Yield: Total Biological yield, g/plant; HC: Hexose content=mg/g; TPC: Total phenol content =mg GAE/g dry extract; N (grain): Nitrogen in grain, %; N (straw)=Nitrogen % in straw

Table 4.8. Morphological parameters (Trial 2 and 3) Mean value from multiple replications is presented in the table at 1% and 5% CD. ** indicates significant difference between treatments.

	Trial 2 (Mv Nádor)			**Trial 3 (Mv Béres)**		
Trt	**P Height**	**No. of leaves**	**No.of tillers**	**P Height**	**No.of leaves**	**No.of tillers**
Control	26.2	10	1	60.3	8	2
MACC-430	26.8	10	1	55	9	3
MACC-922	26.2	11	1	55.6	8	3
MACC-612	26.3	11	1	53.6	9	2
SEd	6.2	2.512	0.591	4.843	2.21	0.408
Sig	Nsig	Nsig	Nsig	Nsig	Nsig	Nsig
CD 1 %	18.109	7.339	1.72	16.25	6.46	1.369
CD 5 %	13.14	5.327	1.254	11.16	4.69	0.9414

Note: Trt=Treatment; P height= Plant height, cm; No. of leaves=Number of leaves

The morphological data of the trials with the same type of application but different varieties were compared **(Table 4.8)**. Morphological data for Trial 1 was not included in the comparison as it was irrelevant to include it (application done after the vegetative growth stage). We found no significant influences of microalgal biomass treatment in the two compared trials. The differences in the value were a result of the genetic characteristics of the respective varieties.

4.2.2 Metabolomic analysis

Data also showed that certain algal treatments caused a delay in the flowering time (appearance of the wheat ear) in Mv Béres. 127 days after germination, ears were visible in 67%, 67%, 43%, and 59% of

control, MACC-430, MACC-612, and MACC-922 treated plants, respectively. For the correct comparison, samples were taken for metabolomics analyses from the flag leaves with similar ear sizes. Using the GCxGC/TOF technique, 14 amino acids, 18 organic acids, 8 carbohydrates, and 4 alcohols could be identified and quantified.

Further compounds include 1-aminocyclopropanecarboxylic acid, isoleucine, β-alanine, cadaverine, cinnamic acid, putrescine, DL-ornithine, o-coumaric acid, shikimic acid, asparagine, tyrosine, D-mannitol, and pantothenic acid were also analysed; however, they were under the detection limit.

Heat map demonstrating the changes in the amino acids indicate that although the differences were not statistically significant in all the cases, they tended to decrease in the plants treated with algal strains **(Figure 4.2)**. In contrast to this, the different organic acids varied more in the different treatments. Interestingly, the carbohydrates and alcohols, including sorbitol, were also significantly lower, especially in MACC-922-treated plants, compared with the controls.

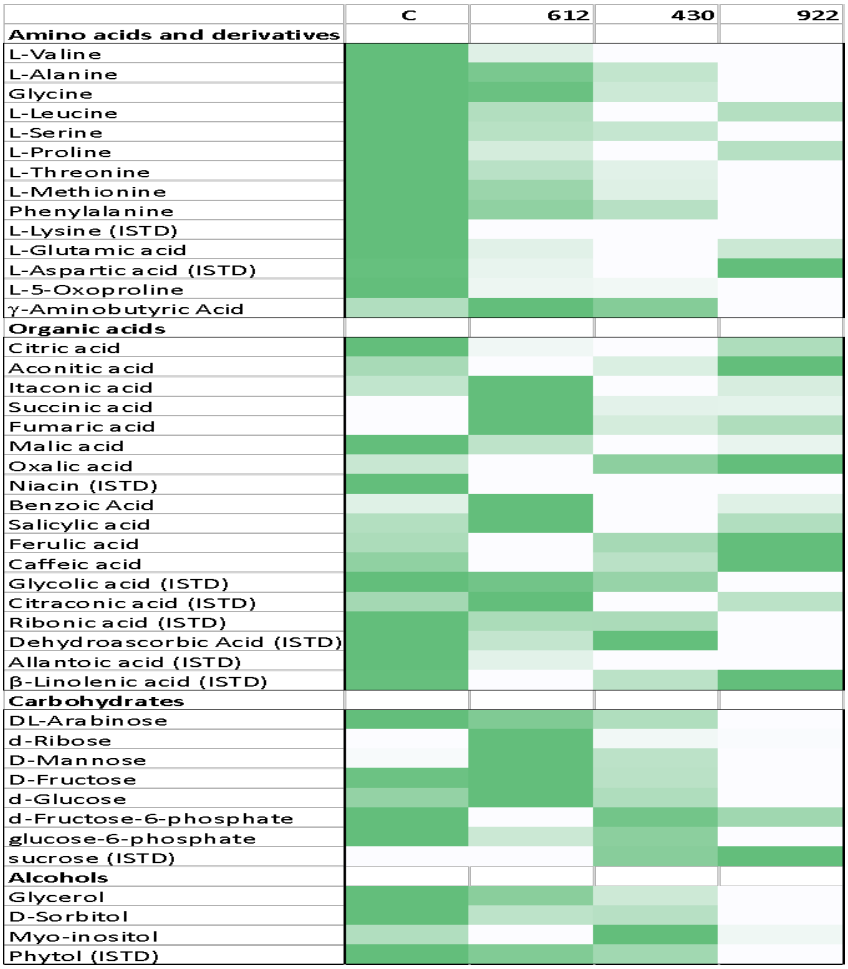


Figure 4.2. Heat map demonstrating the changes in the amino acids. The darker the green the more the concentration of the metabolites.

4.3 Field Experiments

4.3.1 Soil microbial population

To rule out the point of biochemical activity in the soil level, the microbial population was counted with results as same as before sowing.

Table 4.9. 2020-21 trial - soil microbial population. Mean value from 3 replications is presented in the table. The population was calculated as CFU/g for all the microbes viz. bacteria, actinomycetes and fungi.

Timing of soil sampling	**Samples**	**Bacteria CFU/g**	**Actinomycetes CFU/g**	**Fungi CFU/g**
Soil before planting		3.7×10^{-7}	1.6727×10^{-6}	2.22×10^{-4}
Soil after harvesting	Control	1.6×10^{-7}	4.56×10^{-8}	3.2×10^{-3}
	Standard	3.6×10^{-7}	1.2×10^{-8}	3.5×10^{-3}
	MACC-612	4.1×10^{-7}	1.1×10^{-7}	1.7×10^{-2}
	MACC- 612 + trend 90	1.2×10^{-7}	3.4×10^{-8}	2.6×10^{-2}
	MACC-430	2.1×10^{-7}	3.3×10^{-7}	4.1×10^{-2}
	MACC-430 + trend 90	3.1×10^{-7}	4.2×10^{-8}	2.2×10^{-3}
	MACC-922	1.1×10^{-7}	2.5×10^{-8}	1.8×10^{-3}
	MACC-922 + trend 90	1.4×10^{-7}	1.5×10^{-8}	1.7×10^{-3}

Table 4.10. 2021-22 trial - soil microbial population. Mean value from 3 replications is presented in the table. The population was calculated as CFU/g for all the microbes viz. bacteria, actinomycetes and fungi.

Timing of soil sampling	Samples	Bacteria CFU/g	Actinomycetes CFU/g	Fungi CFU/g
Soil before planting		3.1×10^{-5}	1.08×10^{-7}	1.36×10^{-4}
Soil after harvesting	Control	1.9×10^{-5}	3.21×10^{-7}	1.4×10^{-4}
	Standard	2.7×10^{-5}	4.4×10^{-7}	2.2×10^{-4}
	MACC-612	2.6×10^{-5}	3.7×10^{-7}	2.4×10^{-4}
	MACC- 612 + trend 90	1.8×10^{-5}	3.9×10^{-7}	2.5×10^{-4}
	MACC-430	4.3×10^{-5}	4.5×10^{-7}	3.1×10^{-4}
	MACC-430 + trend 90	3.5×10^{-5}	4.5×10^{-7}	3.2×10^{-4}
	MACC-922	4.2×10^{-5}	3.4×10^{-7}	2.6×10^{-4}
	MACC-922 + trend 90	9.4×10^{-5}	3.5×10^{-7}	2.8×10^{-4}

4.3.2 Sterility and fertility %

A significant increase in fertility % and a decrease in sterility % was seen in the first field trial of 2020-21. Among the treatments, there is no significant difference. Standard BAP-6 had maximum fertility, 92.74% with the least sterility % (7.25%) which was 10.15% more than the untreated.

Table 4.11. The effect of microalgal biomass on fertilization of flowers

	Fertility %		**Sterility %**	
	2020-21	**2021-22**	**2020-21**	**2021-22**
Trt 1	82.59c	90.37	17.40a	9.62
Trt 2	92.74a	90.29	7.25c	9.70
Trt 3	87.73b	93.25	12.26b	6.74
Trt 4	89.71ab	93.48	10.28bc	6.51
Trt 5	89.99ab	94.42	10.00bc	5.57
Trt 6	89.78ab	92.79	10.21bc	7.20
Trt 7	90.70ab	90.54	9.29bc	9.45
Trt 8	88.60ab	90.89	11.39bc	9.10
LSD	4.9182	3.962	4.91	3.96
Standard Deviation	3.34498	2.694	3.34	2.69

Means followed by same letter or symbol do not significantly differ (P=.05, LSD).
Standard deviation between treatments
Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

4.3.3 Pre-harvest (changes at 15 days after application and 30 days after application)

Among the physiological parameters, chlorophyll content was one of the most important. In 2020-21 Chlorophyll-*b*, carotenoids, and chlorophyll-*a* showed significant difference of the treated samples from untreated ones with highest chlorophyll-*b* content in treatment 4 (MACC-612 with Trend 90). The same treatment had highest total chlorophyll (though not significant) and also the carotenoid content. Treatment with MACC-430 had total chlorophyll content lower than untreated check. Chlorophyll-*a* content was higher in all microalgae biomass applied treatment irrespective of the strain used.

Effect of the treatment on total chlorophyll and chlorophyll-*a*:*b* were statistically non-significant. However, we could see the differences between MACC-612 + Trend 90 treatment (Trt 4) and the control, the former with total chlorophyll content of 1.29 mg/g fresh weight and the later with 0.99 mg/g fresh weight. Similarly, MACC-430 + Trend 90 treatment had a chlorophyll-*a*:*b* of 3.12 while 2.99 in control. It cannot be ruled out that there was a positive influence on the treated plants as some treated plots had lower total chlorophyll and chlorophyll-*a*:*b* as compared to the control.

Taking an account of 2021-22 trial, we show no statistically significant differences between treated and untreated plots. We found trivial chlorophyll-*a* differences, as small as 0.15 (control against the MACC-430, highest chlorophyll-*a*)., chlorophyll-*b* was highest in control with mark difference of 0.9 (mg/g fresh weight) from the lowest value, i.e., MACC-430 + Trend 90 (Trt 6).

Table 4.12. Effect of different microalgal biomass treatment on type of chlorophyll content, mg/g fresh weight. Chl a=Chlorophyll a; Chl b =Chlorophyll b; Car = Carotenoids; T Chlor =Total Chlorophyll;)

	Chl-*a*		**Chl-*b***		**Car**		**T chl**	
	2020-21	**2021-22**	**2020-21**	**2021-22**	**2020-21**	**2021-22**	**2020-21**	**2021-22**
Trt 1	0.35 c	1.12	0.34 d	0.50	1.52 bcd	1.40	0.99-	1.67
Trt 2	0.36 c	1.16	0.37 cd	0.44	1.54 bcd	1.43	1.04-	1.60
Trt 3	0.42 b	1.25	0.41 b	0.47	1.24 d	1.49	0.95-	1.73
Trt 4	0.51 a	1.22	0.50 a	0.46	1.92 a	1.47	1.29-	1.68
Trt 5	0.39 bc	1.27	0.40 bc	0.47	1.39 cd	1.54	0.93-	1.74
Trt 6	0.39 bc	1.12	0.39 bc	0.41	1.81 ab	1.35	1.22-	1.54
Trt 7	0.41 b	1.20	0.39 bc	0.46	1.74 abc	1.44	1.17-	1.66
Trt 8	0.38 bc	1.19	0.39 bc	0.42	1.58 bcd	1.34	1.13-	1.62
LSD, p=0.05	0.044	0.175	0.043	0.069	0.244	0.245	0.253	0.232
SD	0.030	0.119	0.029	0.047	0.166	0.166	0.172	0.158

Means followed by same letter or symbol do not significantly differ (P=.05, LSD). Standard deviation between treatments, Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

Table 4.13. Chlorophyll content derivates and relative leaf water content on the growing plants: Chl a:b; Chlorophyll-a:b; RLWC = Relative leaf water content (%)

	Chl a:b		**RLWC**	
	2020-21	**2021-22**	**2020-21**	**2021-22**
Trt 1	1.02-	2.25	72.28b	74.08
Trt 2	0.97-	2.61	77.68a	72.50
Trt 3	1.02-	2.61	78.50a	70.41
Trt 4	1.02-	2.62	78.57a	74.13
Trt 5	0.97-	2.66	70.30b	72.81
Trt 6	1.00-	2.68	77.82a	80.40
Trt 7	1.05-	2.58	74.02ab	73.93
Trt 8	0.97-	2.64	74.11ab	78.67
LSD	0.107	0.158	4.880	8.924
Standard Deviation	0.003	0.107	3.319	6.069

Means followed by same letter or symbol do not significantly differ (P=.05, LSD). Standard deviation between treatments, Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

The relative leaf water content in first season of field trial (2020-21) had a significant difference among the treatments. Standard, MACC-630, MACC-612 + Trend 90, MACC-430 had comparable values and not significantly different among themselves. But with control and Trt 5 (MACC-430), there was a difference of approximately, 8-10%.

In 2021-22 successive trials there was no marked differences of relative water content among the treatment. A notable point was that if we compare the control with MACC-430 + Trend 90, the treatment with microalgae (*Chlamydomonas sp.*) biomass application had an increase of 8.53% which was equivalent to the trial with significant differences. (**Table 4.13**).

Table 4.14 depicts the results of proline analysis. As we can see it was examined at two different stages. The samples collected from the treated plots, reveal no astonishing variations. In the samples collected on 15 DAA from the first field trial (2020-21), the performance of BAP-6 on uplifting the proline content was prominent as the other treatments showed a milder response with almost negligible differences. Nevertheless, similar significant influence of the standard was not seen in the successive trial where all treatments, the treated ones had more proline content than the untreated control.

Table 4.14. Effect of the microalgal biomass treatment on proline content, in μmol proline/g fresh weight, at two stages

	Proline 15 days,		**Proline, 30 days**	
	2020-21	**2021-22**	**2020-21**	**2021-22**
Trt 1	7.20-	4.77	11.52b	2.96
Trt 2	10.66-	4. 80	14.40ab	3.61
Trt 3	8.27-	7.16	18.90ab	6.84
Trt 4	6.93-	7.08	17.39ab	4.42
Trt 5	6.41-	5.16	12.46ab	5.27
Trt 6	7.97-	7.21	17.22ab	3.85
Trt 7	8.30-	7.07	18.01ab	2.24
Trt 8	8.64-	6.85	20.03a	4.41
LSD, p=0.05	2.43	3.09	5.13	3.75
Standard Deviation	1.65	2.10	3.49	2.55

Means followed by same letter or symbol do not significantly differ (P=.05, LSD).
Standard deviation between treatments
Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

In the samples collected 30 DAA in 2020-21, the results were statistically significant. A content of 20.034 µmol proline/g FW was observed in MACC-912 + Trend 90 in comparison with 11.52 µmol proline/g FW concentration in control. Like the observation on 15 DAA (2021-22), the samples collected bear no statistical differences, still a magnificent double time increase in concentration was observed in MACC-612 treatment.

In 2020-21, antioxidant potentially derived from the FRAP analysis, revealed similar pattern of influence of the treatment in both sampling stages, 15 DAA and 30 DAA. The performance of MACC-612 alone and MACC 612 + Trend 90 was significantly superior to other treatments in both 15 DAA sample and 30 DAA sample. The lowest FRAP value was 19.77 µg ascorbic acid equivalent/ml which was in Control. Unlike the 2020-21 trial, there was no distinguishing pattern observed in 2021-22. In 15 DAA samples, BAP-6 had highest FRAP value while in 30DAA samples, MACC-92 + Trend 90 (Trt 8) had the highest.

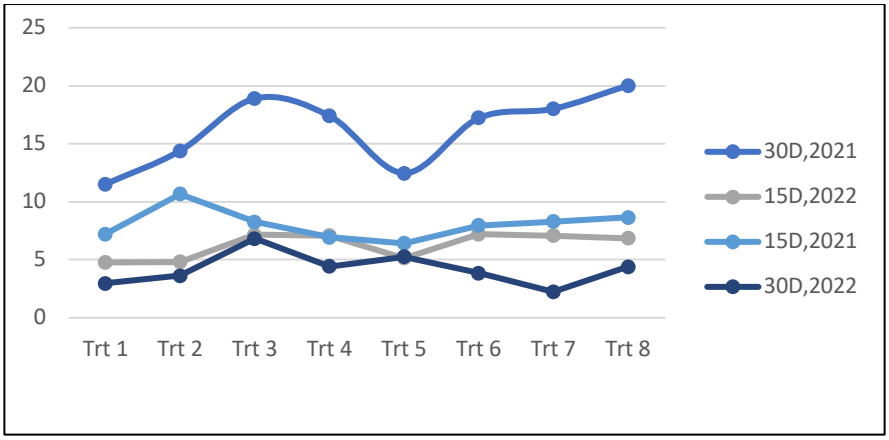


Fig.4.3. Comparative graph of Proline concentration in the two years trial after treating with microalgal biomass.

Table 4.15. Effect of the microalgal biomass treatment on antioxidant properties, FRAP assay, µg ascorbic acid equivalent/mL

	15 days FRAP		**FRAP, in harvested grain**	
	2020-21	**2021-22**	**2020-21**	**2021-22**
Trt 1	19.77b	66.33	17.57b	25.16
Trt 2	21.05b	73.57	23.18ab	28.89
Trt 3	25.59ab	64.87	21.09ab	26.31
Trt 4	27.51a	67.51	24.78a	35.79
Trt 5	27.27a	72.83	19.09b	34.75
Trt 6	25.52ab	65.82	25.79a	32.15
Trt 7	22.64ab	71.45	18.00b	33.75
Trt 8	20.01b	64.06	22.85ab	37.22
LSD, p=0.05	4.045	9.65	3.90	7.68
Standard Deviation	2.75	6.56	2.65	5.22

Means followed by same letter or symbol do not significantly differ (P=.05, LSD).
Standard deviation between treatments
Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

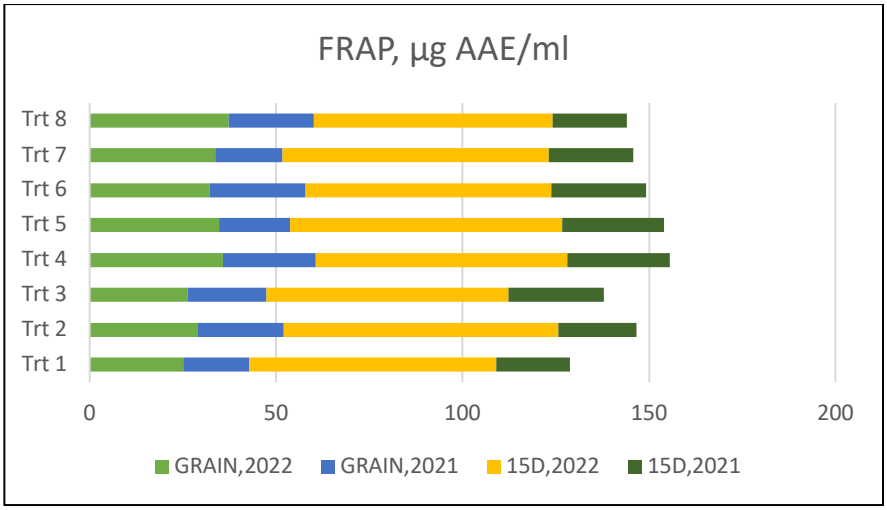


Fig 4.4 Comparative graph of FRAP in the two successive trials after the different treatments.

Table 4.16. Effect on microalgal biomass treatments phenolic contents, total phenol content assay, in mg/g, in GAE (Gallic Acid Equivalent).

	15 days total phenol content		**Total phenol content, in harvested grain**	
	2020-21	**2021-22**	**2020-21**	**2021-22**
Trt 1	4.52a	8.38	1.62a	3.58
Trt 2	3.97ab	9.30	1.42ab	3.17
Trt 3	4.72a	9.49	1.69a	3.66
Trt 4	4.65a	9.88	1.66a	4.12
Trt 5	4.72a	9.36	1.69a	3.66
Trt 6	4.88a	8.87	1.75a	3.49
Trt 7	3.31b	10.21	1.18b	3.65
Trt 8	4.57a	9.77	1.63a	3.92
LSD, p=0.05	0.85	1.44	0.30	0.88
Standard Deviation	0.58	0.98	0.20	0.59

Means followed by same letter or symbol do not significantly differ (P=.05, LSD).
Standard deviation between treatments
Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

The total phenol content was higher during growing stage in the leaves than in the harvested grains. TPC in standard and treatments such as Trt 7 (MACC-922) had milder concentration than in Control and all other treatments. On comparing TPC between the two successive trials, samples from 2021-22 trials revealed an overall increase in concentration irrespective of the type of treatment. The concentrations were doubled in the second year for the same treatment, same variety, and same fertilization amount except for the uncontrollable environmental condition.

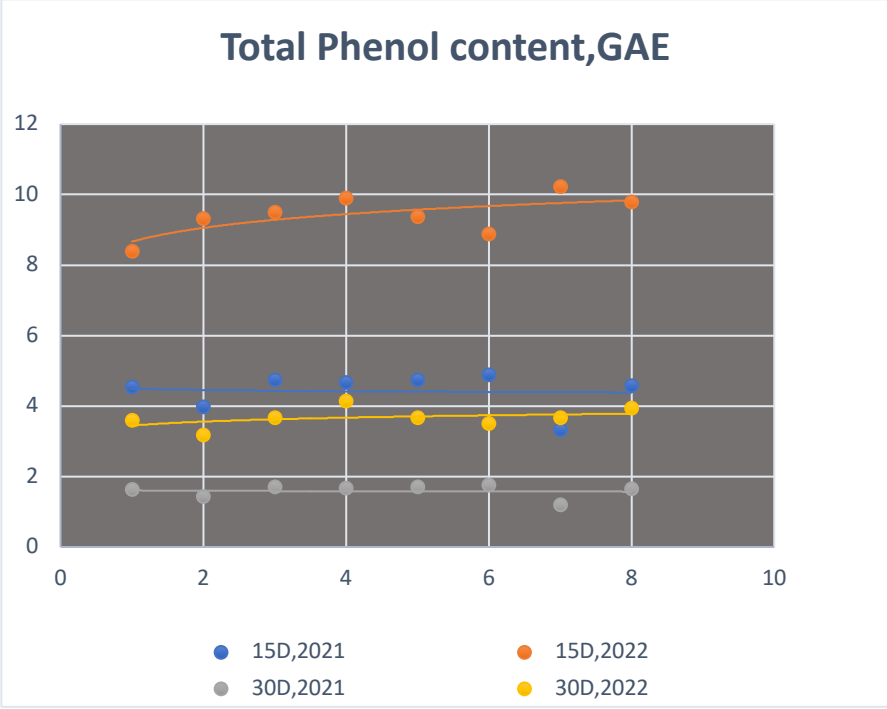


Fig 4.5. Comparative graph of TPC at different stages in the two years successive trial after the different treatment.

Though the concentration of the control was relatively lower than the samples from biomass-treated treatments, there was no statistical significance detected. Significant differences were shown in the 2020-21 trial, with a rather similar pattern of differences in the samples of 15 DAA and 30 DAA.

Carbohydrates in particular the hexose content tend to reduce as the plant approached physiological maturity. It was even lower in the harvested grain. The values in both the trials were close. The hexose concentration had no significant differences in the two trials at all the three stages of observation. An observation to be noted was seen between the treated and untreated treatments was observed in 30 DAA, in 2020-21 trial, hexose content in Trt 6 (MACC-612 + Trend 90 treatment) was relatively high as compared to control, bagging a difference of 17.80%. For the same stage of sampling, 11.60% increase in hexose concentration was obtained in Trt 7 (MACC-922 treatment) over the control. In the harvested grain differences in activity were observed but quite negligible.

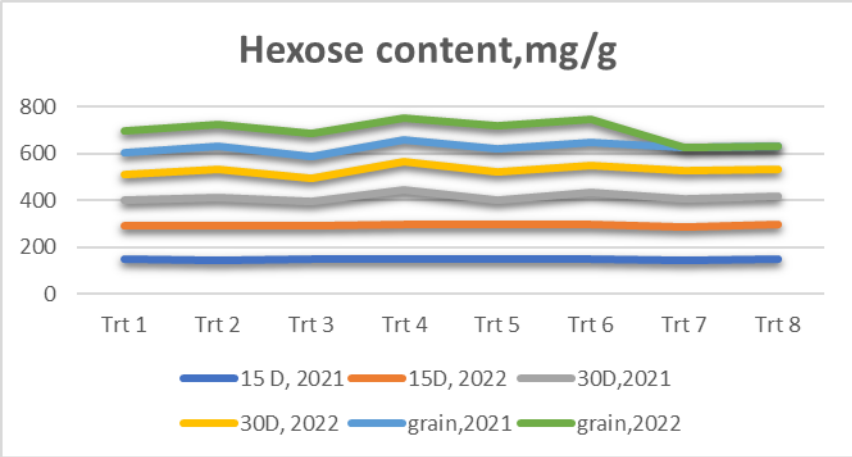


Fig.4.6. Hexose content at different stages in two consecutive seasons.

Table 4.17. Effect of the microalgal biomass treatments on hexose accumulation at different stages, esp. hexose content, mg/g

	Hexose content, 15 DAA		**Hexose content, 30 DAA**	
	2020-21	**2021-22**	**2020-21**	**2021-22**
Trt 1	147.14	143.93	111.95	106.95
Trt 2	144.44	145.14	123.08	121.08
Trt 3	146.72	145.72	101.05	100.05
Trt 4	148.33	147.23	148.05	120.07
Trt 5	149.98	148.87	104.51	118.61
Trt 6	149.42	148.33	136.20	116.20
Trt 7	142.42	143.54	120.87	120.99
Trt 8	150.53	148.03	118.27	115.19
LSD, p=0.05	9.192	9.140	53.256	52.658
Standard Deviation	6.250	6.216	36.216	35.218

(P=.05, LSD). Standard deviation between treatments
Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

Table 4.18. Hexose content in mg/g at harvest and crop growth rate (continuation)

	Hexose content, harvested grain		**Crop growth rate, g/m/day**	
	2020-21	**2021-22**	**2020-21**	**2021-22**
Trt 1	94.91	93.16-	12.92-	14.73
Trt 2	96.91	96.91-	20.27	16.34
Trt 3	96.22	96.22-	16.68	14.98
Trt 4	96.76	94.26-	18.33	16.51
Trt 5	98.45	98.45-	13.27	16.98
Trt 6	98.68	98.68-	18.38	19.36
Trt 7	97.65	95.15-	13.25	15.15
Trt 8	97.80	95.30-	20.31	15.91
LSD, p=0.05	4.659	4.918	7.575	6.540
Standard Deviation	3.168	3.344	5.992	4.447

(P=.05, LSD). Standard deviation between treatments, Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

There were not many irregularities in the crop growth rate. In 2020-21 and 2021-22, the values were very close. No statistically significant differences were exhibited by the treatment. However, in the first trial, the BAP-6 performance was superior to other treatments, especially from the control with CGR of 20.27 g m^{-1} day^{-1}. All other microalgae biomass treatments had an average CGR ranging from 13 to 20 g m^{-1} day^{-1}. The control had the lowest growth rate with 14.73 g m^{-1} day^{-1}, like the earlier trial it had no significant differences.

4.3.4 At harvest

Table 4.19. Influence of the microalgal biomass treatments on yield attributes

	No. of seeds per spike		**1000-kernel weight, g**	
	2020-21	2021-22	2020-21	2021-22
Trt 1	36a	38-	39.56-	39.78b
Trt 2	38a	34-	42.95-	43.85ab
Trt 3	35a	38-	44.65-	44.20ab
Trt 4	36a	34-	46.95-	44.60ab
Trt 5	35a	33-	40.84-	48.23a
Trt 6	29b	33-	42.72-	44.85ab
Trt 7	35a	31-	41.60-	41.53ab
Trt 8	35a	33-	41.84-	40.83b
LSD, p=0.05	3.9222	6.86	4.41	4.14
Standard Deviation	2.6672	4.67	3.00	2.81

Means followed by same letter or symbol do not significantly differ (P=.05, LSD). Standard deviation between treatments, Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

The ranges for the number of seeds per spike was 35-38 in 2020-21 and 31-38 in 2021-22.

No significant results of 1000-kernel weight were observed in 2020-21 while in 2021-22, distinguishing results were obtained. In the former trial, the control had a 1000-kernel weight of 39.565 g which was the lowest as compared to other treatments. The 1000 kernel weight in the later trial was approximately equal to the former i.e., 39.78 g on comparing the two trials of consecutive years, the value was greater in the later.

Table 4.20. Influence of the treatment on plant biological biomass, above ground parts and root weight.

	Average above ground biomass, g		**Root weight, g**	
	2020-21	**2021-22**	**2020-21**	**2021-22**
Trt 1	48.96-	55.17-	0.37-	0.28-
Trt 2	48.66-	57.61-	0.47-	0.33-
Trt 3	38.75-	55.03-	0.37-	0.27-
Trt 4	49.92-	51.79-	0.45-	0.46-
Trt 5	42.61-	55.57-	0.41-	0.32-
Trt 6	44.24-	56.53-	0.34-	0.41-
Trt 7	50.51-	57.12-	0.45-	0.26-
Trt 8	46.10-	51.89-	0.40-	0.28-
LSD, p=0.05	7.720	5.866	0.2057	0.193
Standard Deviation	5.250	3.989	0.1399	0.131

(P=.05, LSD). Standard deviation between treatments, Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

Above ground biomass and root weight were not significantly influenced by the treatments in both successive trials. In the first trial, the least biomass accumulation was observed in plants treated with MACC-612 (38.961 g) whereas the highest was in MACC-922 applied treatment.

As in above ground biomass weight, there were no differences between the treatments in both trials. Control or untreated had a value of 0.375 g and 0.285g in the two consecutive trials. The highest root weight was observed in treatment 4 i.e., MACC-612 +Ttrend 90 in both the trials, 0.458 g and 0.465 g respectively. However, a higher root weight was

observed in the first trial. The drastic changes in root weight were seen in the above-mentioned treatment.

Table 4.21. Influence of treatment on major yield parameter, yield, kg/ha and harvest index

	Yield, kg/ha		**Harvest index, %**	
	2020-21	**2021-22**	**2020-21**	**2021-22**
Trt 1	6845.7b	6810.0-	39.87	65.69-
Trt 2	8118.6a	7137.5-	58.87	60.24-
Trt 3	6922.3b	6995.0-	55.46	69.24-
Trt 4	7334.0b	7170.0-	51.30	65.08-
Trt 5	7067.3b	7057.5-	45.06	60.07-
Trt 6	7131.8b	7077.5-	56.07	64.38-
Trt 7	7028.9b	7467.5-	52.26	60.19-
Trt 8	7593.8ab	7015.0-	44.75	58.16-
LSD, p=0.05	606.21	739.59	13.97	11.45
Standard Deviation	412.25	502.95	9.50	7.78

Means followed by same letter or symbol do not significantly differ (P=.05, LSD).
Standard deviation between treatments
Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

The yield in kg/ha was significantly different in 2020-21. All microalgae biomass treatments influenced positively in the yield though the increased amount was not huge. An increase of approximately 2000 kg/ha or 2 t was observed as the maximum improvement contributed by the treatment of BAP-6 over the control. A difference of at least 50-100 kg/ha was noticed in all the treatments over the untreated.

In the 2021-22 trial, no statistically notable yield difference was measured. Even so, the yield in the control was less as compared to other treatments. As low as 50-100 kg yield increase was marked over the control. Within the trial results, the maximum output difference was noticed between MACC-922 and control, approximately 600 kg more than control. The yield though significantly different in 2020-21 trial had smaller standard deviation.

No significant differences in harvest index were noted in any of the trials. In the first trial, the harvest index was comparatively low as compared to other treatments. Still no influence of the treatment was observed in the second trial. The harvest index lies between, 0.58 or 58% and 0.65 or 65%.

4.3.5 Post harvest

4.3.5.1 Grain quality attributing parameters

The effect of the treatment on flour quality was determined by measuring protein, gluten and Zeleny sedimendation value. A remarkable difference was hard to observe in the first two parameters in both trials. Meanwhile, in the Zeleny sedimentation value, significant differences were observed in both the field trials, the least in control and the highest in MACC-612 + Trend 90 treatment.

Table 4.22. Effect of the microalgal biomass treatment on grain quality attributing parameters like Protein, wet Gluten, Zeleny sedimentation value.

	Protein (%)		**Wet Gluten (%)**		**Zeleny sedimentation value**	
	2020-21	**2021-22**	**2020-21**	**2021-22**	**2020-21**	**2021-22**
Trt 1	11.38-	14.03-	25.70-	30.53-	42.90b	47.65b
Trt 2	11.43-	13.75-	25.68-	30.96-	43.48b	50.75ab
Trt 3	11.50-	14.00-	25.83-	32.76-	44.13b	54.77ab
Trt 4	12.83-	14.23-	29.98-	31.92-	52.93a	55.85ab
Trt 5	11.70-	14.01-	26.48-	31.66-	45.50ab	51.50ab
Trt 6	12.55-	13.99-	28.98-	31.06-	49.55ab	53.18ab
Trt 7	12.10-	13.83-	27.88-	31.31-	48.40ab	55.32a
Trt 8	12.78-	13.89-	29.83-	31.94-	52.40a	50.49ab
LSD, p=0.05	0.97	0.39	2.83	1.89	5.62	4.83
Standard Deviation	0.66	0.26	1.93	1.29	3.82	3.28

Means followed by same letter or symbol do not significantly differ (P=.05, LSD).
Standard deviation between treatments
Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

In the second trial the Zeleny sedimentation value did lay above 50 except for the control while in the first trial all values were in 40 range except Trt-4 (MACC-612 + T rend 90) and Trt-8 (MACC-922 + Trend 90).

Protein content increased overall in all treatments in the second trial. The highest value in 2020-21 trial was 12.83% while in 2021-22, 14.235 % in MACC-612 + Trend 90 treatments. Among the treatment, there are negligible differences.

The nitrogen revived in the plants was estimated as N% (above-ground biomass) and N% (grain). First, there was no great variation among the treatments that could yield remarkably separate. Second, the negligible differences showed minimal activities as an almost similar pattern of the trivial changes was seen in the successive trials, in both trials control had a lower amount of N concentration in the above ground biomass even if it was by few decimals. The N% in the trend 90 applied treatments was always greater than microalgae biomass alone. And finally, MACC-922 treatments performed better than other strains in both trials.

Table 4.23. Changes in nitrogen accumulation in plants, in above ground biomass and in grain, % after application of Microalgal biomass.

	N %, above ground biomass		**N %, grain**	
	2020-21	**2021-22**	**2020-21**	**2021-22**
Trt 1	0.45-	0.58-	1.98-	2.24-
Trt 2	0.48-	0.59-	2.01-	2.20-
Trt 3	0.53-	0.62-	1.88-	2.27-
Trt 4	0.46-	0.67-	2.10-	2.24-
Trt 5	0.46-	0.58-	1.96-	2.24-
Trt 6	0.51-	0.60-	2.01-	2.24-
Trt 7	0.51-	0.59-	2.03-	2.21-
Trt 8	0.53-	0.67-	2.08-	2.22-
LSD, p=0.05	0.10	0.12	0.21	0.06
Standard Deviation	0.07	0.08	0.14	0.04

(P=.05, LSD). Standard deviation between treatments
Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

No marked difference or variability in N % (grain) was obtained in both trials. In first trial though not significant some fluctuations were observed in the results. But in the second trial an almost constant value was noticed.

4.3.5.2 Protein subunits

Table 4.24. The subunits of gluten under the influence of the treatment.

	Glutenin, mg/kg		**Gliadin, mg/kg**	
	2020-21	**2021-22**	**2020-21**	**2021-22**
Trt 1	4.68-	14.06	5.59-	14.89
Trt 2	5.01-	13.22	5.94-	13.31
Trt 3	4.62-	14.33	5.24-	15.64
Trt 4	5.15-	14.11	6.42-	14.89
Trt 5	4.88-	14.32	5.65-	15.02
Trt 6	4.81-	14.25	5.81-	14.95
Trt 7	5.08-	13.32	6.14-	14.46
Trt 8	5.41-	14.67	6.58-	15.09
LSD, p=0.05	0.86	1.29	1.48	1.51
Standard Deviation	0.58	0.87	1.01	1.03

(P=0.05, LSD). Standard deviation between treatments
Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

The quantification of gluten subunits yields did not prove positive result statistically. The individual comparison revealed some differences in the content of glutenin and gliadin. MACC-922 + Trend 90 was found to have higher value than the control and standard. Similarly, in gliadin counts, the same treatment was better than the control and standard (BAP-6) while Glu/Gli and UPP % were lowest in this treatment. A reverse pattern in the values was observed.

Table 4.25. Gluten subunits derived parameter.

	Glu/Gli		**UPP%**	
	2020-21	**2021-22**	**2020-21**	**2021-22**
Trt 1	0.94-	0.94	54.36-	48.96
Trt 2	0.94-	0.96	53.74-	48.65
Trt 3	0.95-	0.91	55.14-	48.84
Trt 4	0.90-	0.94	52.71-	48.18
Trt 5	0.94-	0.95	52.80-	49.90
Trt 6	0.93-	0.95	53.13-	48.17
Trt 7	0.93-	0.92	53.06-	48.52
Trt 8	0.91-	0.97	49.96-	49.33
LSD, p=0.05	0.04	0.06	3.77	3.14
Standard Deviation	0.03	0.04	2.56	2.13

(P=0.05, LSD). Standard deviation between treatments
Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

4.3.6 Soil assessment

The nitrate and nitrite content, kg/ha had statistically notable differences. Nevertheless, the significance can be overlooked as the treatments had the same alphabet ab or b in Mean comparision test. There were minimal variations in the second trial. On comparing the two trials, the nitrate and nitrite content in the soil was higher in the first trial location than in the second trial location. Similarly, the total nitrogen was almost equivalent within the treatments, and added there no significant differences between the two trials too. The carbon % in soil was around 1 % or >1.5 % in all the treatments in both trials.

Table 4.26. Soil status after harvest

	Nitrate and nitrite content, kg/ha		**Total nitrogen, in soil, kg/ha**		**Carbon % in soil**	
	2020-21	**2021-22**	**2020-21**	**2021-22**	**2020-21**	**2021-22**
Trt 1	35.56ab	21.15	7.41-	7.70	1.22-	1.30-
Trt 2	42.75a	23.40	7.89-	7.60	1.27-	1.26-
Trt 3	38.06ab	15.40	7.31-	7.41	1.19-	1.26-
Trt 4	29.00b	20.57	7.31-	7.31	1.27-	1.27-
Trt 5	36.43ab	15.79	7.21-	7.70	1.34-	1.31-
Trt 6	31.37b	20.37	7.50-	7.51	1.33-	1.37-
Trt 7	35.25ab	20.86	7.70-	7.41	1.18-	1.31-
Trt 8	27.67b	14.91	7.80-	7.41	1.20	1.26-
LSD, p=0.05	7.50	7.16	0.61	0.58	0.16	0.10
Standard Deviation	5.10	4.87	0.41	0.39	0.11	0.07

Means followed by same letter or symbol do not significantly differ (P=0.05, LSD).
Standard deviation between treatments
Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

Statistically, the treatments had an insignificant impact on nitrogen uptake and nitrogen use efficiency. Even so, if the treatments were compared individually, we saw a difference between the untreated and treated. The control or untreated checked had the lowest nitrogen uptake while treatment 8 (MACC-922 + Trend 90) had the highest value.

No observable differences in nitrogen use efficiency were exhibited by treatments. A slightly higher NUE was observed in the second trial.

Table 4.27. Secondary derivations to check the efficiency of the treatment esp. nitrogen.

	Nitrogen uptake, kg/ha		**Nitrogen use efficiency, %**	
	2020-21	**2021-22**	**2020-21**	**2021-22**
Trt 1	136.87-	154.20-	37.82-	39.77-
Trt 2	153.64-	156.35-	39.89-	40.78-
Trt 3	138.32-	159.04-	37.89-	40.97-
Trt 4	153.47-	160.77-	38.75-	41.95-
Trt 5	138.41-	158.05-	37.69-	40.02-
Trt 6	143.37-	158.50-	38.42-	40.93-
Trt 7	142.75-	165.35-	39.41-	41.55-
Trt 8	158.17-	155.66-	39.31-	40.86-
LSD, p=0.05	20.78	15.94	4.94	2.45
Standard Deviation	14.13	10.84	3.35	1.66

(P=0.05, LSD). Standard deviation between treatments
Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

4.4 Pool analysis

After pool analysis, fertility % and yield had a significant interaction effect. For the other parameters, there were no interaction effects. The selection of the parameters was based on significance level and based on the importance of the parameter in the evaluation of bio-stimulating or growth-promoting potentialities in a product.

Table 4.28. Pool Analysis of seven selected parameters based on which conclusion was drawn.

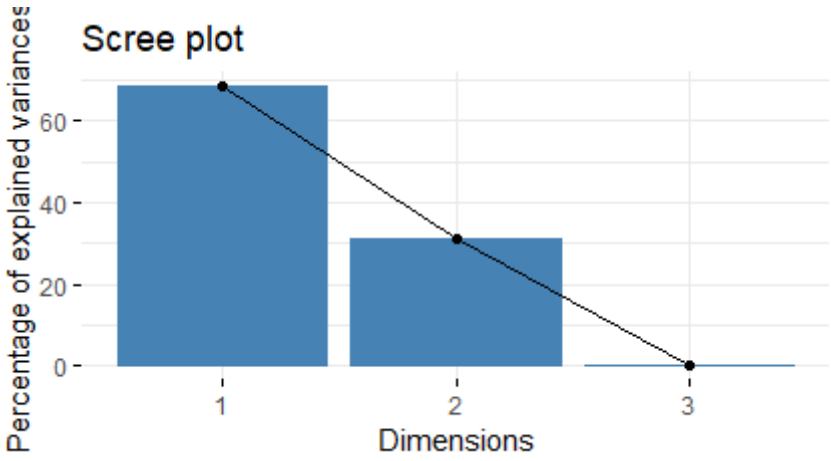
Trt	F	T.chl.	Prol	Y	Pro	Glu	NUE
Trt 1	86.00	1.26	5.37	6635.8	12.73	12.74	38.56
Trt 2	91.18	1.32	7.57	7791.3	12.46	12.47	40.41
Trt 3	91.60	1.32	6.32	6843.3	12.46	12.47	37.96
Trt 4	91.98	1.49	6.59	7360.5	13.17	13.18	39.80
Trt 5	92.16	1.32	5.87	7251.6	12.66	12.66	38.62
Trt 6	91.19	1.35	7.18	7353.3	13.12	13.12	38.93
Trt 7	90.45	1.44	7.59	7340.8	12.61	12.61	39.69
Trt 8	90.10	1.38	8.33	7418.3	12.93	12.93	39.88
Sig (Y× Trt)	0.001	0.714	0.298	0.0108	0.133	0.168	0.664
CD (Y)	4.022	0.276	1.58	537.13	1.386	2.971	3.512
CD (Trt)	6.335	0.151	2.24	1240.57	1.253	3.856	3.566
CD (Y× Trt)	4.033	0.266	2.805	965.293	1.332	4.274	5.986

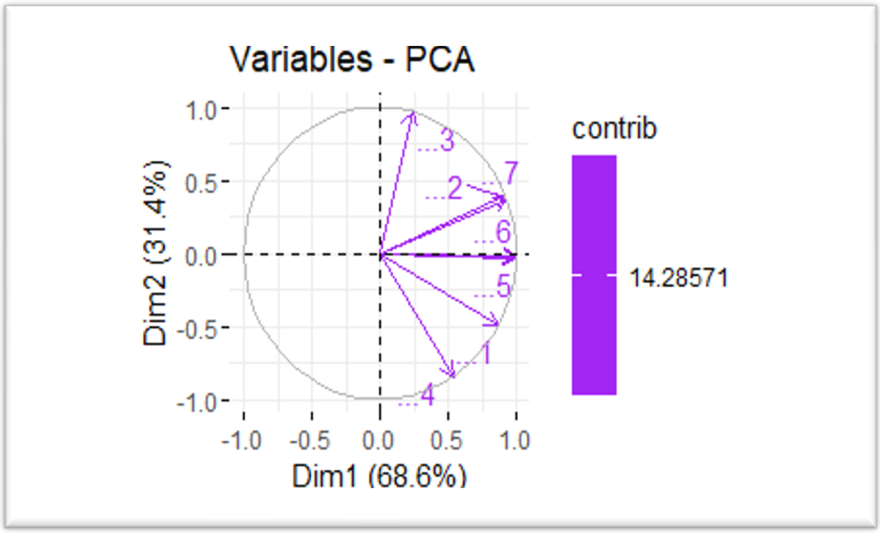
Note: Trt_= Treatment, F = Fertility %; T.chl = Total chlorophyll, mg g^{-1} fresh weight; Prol= Proline in µmol proline/g FW weight; Y= Yield, kg/ha; Prot= Protein, %; Glu= Gluten %; NUE= Nitrogen use efficiency, %., Sig = Significance; Y= Year.

Principal component analysis, or PCA, is a dimensionality reduction method that is often used to reduce the dimensionality of large data sets, by transforming a large set of variables into a smaller one that still contains most of the information in the large set. In our case to draw conclusion from the various parameters we will be reducing to smaller dimensions.

Table 4.29. Principal component analysis

	PC1	PC2	PC3
Fertility % (1)	0.39	0.33	0.62
Total Chlorophyll, mg g^{-1} fresh wt (2)	0.42	0.24	0.06
Proline, µmol proline/g FW weight. (3)	0.10	0.65	0.14
Yield, kg/ha (4)	0.24	0.57	0.63
Protein, %(5)	0.45	0.02	0.28
Gluten, %(6)	0.45	0.01	0.27
NUE, % (7)	0.41	0.26	0.13
Standard deviation	2.19	1.48	6.60e-15
Proportion of Variance	0.68	0.31	0.00e+00
Cumulative Proportion	0.68	1.00	1.00e+00
Eigen value	4.80e+00	2.19e+00	4.36e-29





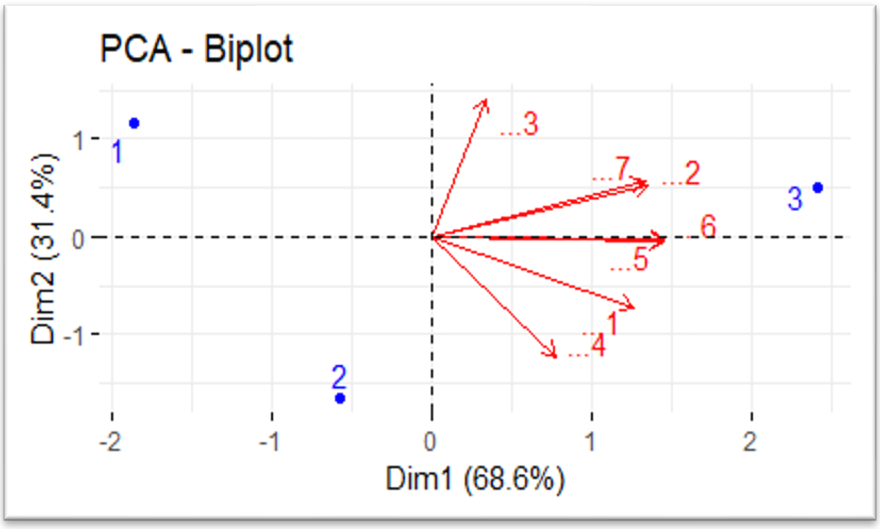


Figure 4.7. Scree graph, variable contribution and biplot of PCA on pooled data of three strains performance.

Based on the pooled data of the 3 strains, the largest variation was observed in Principle component 1(PC1) or Dimension 1. There was no correlation between the variables and PC1, however, proline and

yield were positively correlated in PC2. These can be observed in the fig. 4.7 where the contribution of variables 3 and 4 was highest in the variable contribution graph. So, our conclusion of the best strain can be based mostly on yield and proline concentration.

The order of strain performance based on yield was *Chlorella vulgaris* > *Chlamydopodium fusiforme* > *Nostoc linckia* while based on proline concentration the order was *Chlorella vulgaris* > *Nostoc linckia* > *Chlamydopodium fusiforme*.

5. DISCUSSION

5.1 Preliminary trial

Although various compounds were detected in MACC-612, it seems to have a negligible effect on germination, as the germination index was 62 at 1 g/L concentration, still higher than the germination index of the control, i.e., 45. According to Reigosa and P-Malvido (2007), trans-cinnamic acid inhibited *A. thaliana*'s total germination above 500 µM. This could be because the concentration of trans-cinnamic acid in the solution containing algae biomass was lower by several orders of magnitude (**Table 4.2**) than what was found critical in the case of *Arabidopsis*. Similarly, the phytotoxic concentration of the p-hydroxybenzoic acid was 750 µM but in the strains used in the present study, the concentrations were well below this critical value. In support of this result, no action of p-hydroxybenzoic acid on *Poa annua* L. germination was reported by Wu et al., (1998). Thus, with the above-compared studies, the secondary metabolites in the algae are in a safe range for the plants.

Mungbean rooting bioassays were conducted with numerous strains to evaluate their auxin content. These bioassays proved the presence of auxin without quantification. A similar bioassay was conducted in *Chlorella vulgaris* by Ranglova (2020) and was proven to have auxin-like activity. Navarro-López et al., (2020) used the same bioassay to prove auxin-like activity in *Scenedesmus obliquus*. Ulva extract <0.1% stimulated root growth. LR growth in concentrations that stimulate

primary root growth but do not affect germination (De Smet et al., 2003). There is no direct correlation between these components of a multivariate random variable. This means that these secondary metabolites do not affect directly the germination facilitating effects in wheat seeds. However, this does not eliminate the theory that the secondary metabolites may play their role in activating the enzymes or the hormones.

We detected some metabolites in the strains, which could explain why even though no IAA was detected in some strains, we found auxin-like activity. Besides in MACC-612, IAA was not detected (it may not be detected but does not prove its complete absence) which shows the differences in the composition of one strain from another. And that not all strains showing positive results in mungbean bioassay need not have high IAA content. There are some important hormone-like and growth-regulation substances that may be involved in various physiological activities such as brassinosteroids, jasmonic acid, polyamines, salicylates, and signal peptides (Du Jardin, 2015). Toribio et al., (2021) found that in 8 strains belonging to *Nostoc sp.*, *Chlorella sp.*, *Leptolyngbya sp.*, the salicylic acid (SA) content was in the range between 5 and 7 µg m/L. Whereas in the quantification we have conducted salicylic acid content was between 14 to 35 ng/g.

5.2 Pot experiments

In our findings, we found similar increases of net photosynthesis, stomatal conductivity, and intercellular CO_2 concentration and

transpiration in plants treated especially with MACC-922 and MACC-612. In research conducted by Khalvandi et al., (2021) it was found that the net photosynthesis was increased from 6.81 ± 0.44 to 8.75 ± 0.69 μmol $CO_2/m^2/s$ after the application of salicylic acid. Additionally, the internal CO_2 concentration, transpiration rate, and photosynthetic water use efficiency increased in the same manner after the application of salicylic acid. However, the water use efficiency was contrary to our results.

In the experiment conducted to observe the hormonal or metabolic activity, none of the treatments resulted in a long-term change in the ABA level. However, in the consecutive experiment considering two variety, some of the strains caused genotype-dependent changes such as in Mv Nádor, strains MACC-430 and MACC-612 decreased the ABA level, but in Mv Béres, MACC-430 induced a statistically significant increase. In stress conditions such as drought, extreme temperature, and high salinity, the ABA content in plants increases considerably, inspiring stress-tolerance effects that help plants to adapt and survive under these stressful situations (Ng et al., 2014).

Gorni et al., (2022) found that foliar application of rutin increases plant performance, fruit production, and the nutritional value of tomatoes. So, the presence of higher rutin is a positive trait. In our case, rutin was also deteced in wheat leaves but the concentrations differed in the different experiments. However, the rutin content was more dependent on the cultivar than on the treatment as Mv Nádor had lower rutin content Mv Béres.

In the first experiment, all the strains reduced their levels in the leaves; in the second experiment, these decreases were only significant in the case of MACC-438. However, in Mv Nádor, algae biomass treatments usually increased the jasmonic acid level, suggesting that although the physiological parameters indicated that plants are not exposed to severe stress factors, algae biomass treatments may modify their stress-related signaling processes. While spraying with the microalgae biomass caused trivial modifications in neochlorogenic acid, rutin, or naringenin contents in the wheat leaves, in several cases substantial changes occurred in the amount of jasmonic acid and jasmonic acid-leucine/isoleucine conjugate contents. Jasmonic acid is known as a key signaling compound involved in the suppression of necrotrophic pathogens and herbivorous insects, and it also plays a role in the responses to abiotic stressors (Wang et al., 2020; 2021). In addition, recent results show that jasmonic acid may also modify the diversity and functioning of the microbiome in wheat roots (Liu et al., 2017).

Furthermore, salicylic acid was detected in the freeze-dried strain, and it increased tremendously in the plants. The effect was seen clearly in trial Mv Béres1 and Mv Nádor. This could mean a direct effect on the salicylic content of the plant thereby benefitting the plant with its various roles in stress-related signaling processes, too. Another notable observation made was the increased concentration of p-hydroxybenzoic acid in the treated plants.

If quantification of the composition of all microalgae can be added to a database (a common worldwide database added by researchers in the

field) along with the bioassay results, then it will be an easier pick for a suitable strain to be applied as a biostimulant as per the composition. Also, the studies on physiological parameters give insight into the possible changes in plants depending on the type of strain used. Such studies identifying the suitable strain enable the producers to focus on a particular strain for mass production.

5.3 Field trials

Monitoring soil microbial populations gives an insight into the probability of revegetation prior to plant growth. Microbial populations in soil are determined by various factors such as soil depth, organic matter, porosity, oxygen and carbon dioxide concentration, soil pH, etc. In our experiments, it could be different as no live microbes were applied and only biomass was applied on the leaves. But to eliminate any chances this examination of the microbial population was conducted. We see no differences between the treatments, in short there was no effect of the treatment in the soil microbial population.

5.3.1 Sterility and fertility %

Biostimulant has a positive relationship with fertility in many crops. Pohl et al., (2019) found that fruit setting in eggplant was enhanced with the application of *Ascophylum nodosum* extract as biostimulant in field conditions in a temperate climatic zone. We found a similar result in our experiment. Maignan et al., (2022) also suggested that biostimulant effect of glutacetine formulations could increase wheat fertility during the flowering period.

5.3.2 Chlorophyll content

The chlorophyll content in winter wheat plants was 1.24 mg/g observed at end of flowering stage, 49-51 BBCH (Skudra and Ruza, 2017). This result conforms with our findings where it falls in the range of 1.54 to 1.74 mg/g.

In the findings of Liu et al., (2010), the chlorophyll-*a* content is larger than chlorophyll-*b* content, where he obtained 1.23 ± 0.01 chlorophyll-*a*, 0.42 ± 0.02 chlorophyll-*b* and the chlorophyll-*a*:*b* ratio 1.65 ± 0.01 larger than the two value in winter wheat at the early stage of growth. Compared with our result, we found a very low value of chlorophyll-*a* in the first trial, whilechlorophyll-*b* content was almost equivalent to chlorophyll-*b* content refered article. Lower chlorophyll content can be associated to leaf water potentials, which can be an indication of the pigment to increasing environmental stresses like drought (Moran et al., 1994; Younis et al., 2000).

Leaf water content has been one of the simplest ways to determine plant's drought stress response or treatment. Badr and Brüggemann (2020) showed that under drought stress, RLWC can be in the range of 83.3 % and 57.8% in *Zea mays*. Physiologically it means that with higher relative leaf water content, the plant can extract more water from the soil than the one with a lower RLWC. In short high RLWC is a sign of better stomatal control. Thus, in our results, we found differences in RWC significantly or non-significantly, in plant samples treated with microalgae biomass such as MACC-430, MACC-612 a higher RLWC

was recorded, which is a signal for the positive effect of the treatment on stomatal control.

5.3.3 Proline content

There have been reports on impact of growth promoters on proline content. As per the findings of Khan et al., (2020) proline accumulation was reduced after the growth promotor treatments which is quite contrary to our findings in which we found an increased proline concentration. Stress-induced proline accumulation in different plants may be contributed by disparate glutamate and ornithine pathways. The increased accumulation during stress may be a result of increased biosynthesis and inhibited degradation (Kavi Kishor et al., 2005).

5.3.4 Hexose content

According to Shanmugavelan et al., (2013), the sucrose content of glutinous barley, glutinous millet, and glutinous rice were 0.74, 0.31, and 0.24 g/100 g respectively.

The primary products of photosynthesis are hexose sugar. The triose-phosphate yielded via the Calvin cycle during photosynthesis may then be exported to the cytoplasm forming fructose1,6-bisphosphate, the first phosphorylated hexose (Dennis and Blakeley, 2000), and this triose-P is used for the formation of starch during the day. Vascular development or transportation to other sink tissues is done after cleaving sucrose by sucrose synthase (SUS). The release of hexose sugar is phosphorylated by hexose kinase enzymes or the fructokinase

enzyme. But if SUS cleaves too much sucrose, the concentration of fructose will increase and inhibit both SUS and FRK activity (sucrose allocation for vascular development may be affected). Upon arriving in the sink tissues, the sucrose sugar undergoes several enzymatic reactions converting into and storing as starch (Granot et al., 2013). From this, we can interpret that the hexose content in active photosynthesis will be more than in the plant maturation stage. In the grain, since it is the sink, the sucrose or hexose will be converted to starch, and the hexose content will reduce. In our research, we found similar changes in the hexose content. The hexose content in the grain after harvesting had the lowest hexose sugar content as compared to the other stages of analysis.

5.3.5 FRAP/Total phenol content

In our FRAP and total phenol analysis, we found a wide difference in FRAP content in the leaves between 2020-21 trial and 2021-22 trial. Effect of biostimulant on red peppers too had a weak increment of FRAP. Also overall FRAP and total phenol content enhanced more at the earlier plant development stages than at plant maturity (Witkovicz et al., 2020). Some studies suggested an increased phenol production in response to stress conditions after application of alfalfa-based hydrolizate effective microorganisms (EM)functioning as biostimulants (Ertani et al., 2011; Ertani et al., 2013). One theory of the increase was that biostimulants induced the phenylalanine ammonia lyase (PAL) enzyme activity which is the key regulator of phenolic compound biosynthesis. Regulation of the biosynthesis

enhanced phenolic compound production, and this was likely responsible for influences on FRAP activity (Oboh and Ademosun, 2012; Serrano et al., 2010; Witkovicz et al., 2020). By the FRAP analysis, the capacity to eliminating reactive oxygen species by non-enzymatic antioxidants with low-molecular-weight compounds such as ascorbic acids, carotenoids, tocopherol, polyphenols, polyamines, flavonoids, hormonal compounds, amino acids. etc., are detected (Xia et al., 2020; Lizcano et al., 2012; Galic et al., 2021). The total phenol content in winter wheat (grains) lies in conformity with the results of Li et al., (2015). The FRAP content follows the findings of Fogarasi et al., (2014).

5.3.6 Crop growth rate

Hidangmayum and Sharma (2017) found that seaweed liquid extract enhances the crop growth rate but not significantly when applied to onion (*Allium cepa* L.). This result is in accordance with our finding where we found negligible differences between treated and control. Furthermore, in a trial conducted on rapeseed (Jannin et al., 2013) and pea (Rana et al., 2007), seaweed extract was associated with a significant increase in CGR.

And the value of our results lies between the ranges published by several researchers with research conducted since very early times (Hunt,1974; Gul et al., 2013).

5.3.7 Yield attributes

There are many studies on the influence of biostimulants on the yield of the crop (Tejada et al., 2018; Jin et al., 2012). In these experiments, it had been concluded that the increase in the crop yields, number of corncobs per plant and grains per corncob may be because of the biostimulant in the form of N or as amino acids. Similar to our result, Maignan et al., (2020) also reported that there was no consistent change in the number of grains per spike in wheat following protein hydrolysate application. Several other research results on the effect of biostimulants on different crops such as rice, finger millet, cowpea, etc. have been published (Pretini et al., 2020). A little contradictory to our result on 1000-kernel weight, Maignan et al (2020) revealed a negative impact of glutacetine on the same parameter. However, the finding of Wang et al., (2020) and Yadav et al., (2020) supported our result. Taking our yield results in consideration we can see a potentiality of microalgae like any other biostimulants though not very significant.

The experiment conducted by Szczepanek et al., (2018) showed a positive relation with the harvest index, yet the degree of significance depended on the time/stage of application. Biological yield parameters were also in accordance with Macra and Sala (2022).

5.3.8 Grain analysis

The maximum temperature at the time of application (the early flowering stage) was higher with lesser precipitation in 2020-21 trial as compared to 2021-22 trial which could be the reason for the higher

protein %, gluten %, and Zeleny sedimentation value. However, in 2021-22 the protein % was in the ranges of 13-75-14.23% (11.38 – 12-89 % in 2020-21), the gluten % was between 30.53 - 34.94 % (25.68 – 29.93 % in 2020-21) and Zeleny sedimentation value was 47.65 – 55.85 (42.90 – 52.93 in 2020-21). Such a decrease in quality due to harsher environmental conditions has been depicted by other researchers too (Erekul and Köhn, 2006; Matysiak et al., 2018). The Zeleny sedimentation enhanced significantly. These enhanced qualities were in accordance with the studies of Popko et al., (2018). A high Zeleny sedimentation value could be linked to higher protein content and good baking quality (Eckert et al., 1993). So, with microalgae treatment, we found the potential to improve the flour or baking quality.

5.3.9 Protein subunits

Gliadins and glutenins are the grain storage proteins found in the starchy endosperm of the grain kernel and together they form gluten (Mazzeo et al., 2017; Shewry et al., 2009). The two protein subunits play an important role as it may affect the rheological properties of the dough on modifying the total amount of gliadins and glutenins and their ratio (Sissons, 2008; Barak et., 2013). The three parameters had a tremendous effect on the dough stability, dough development time, peak viscosity, breakdown viscosity, bread-specific volume, and crumb firmness. To these properties glutenins have strong negative relation while gliadins have a positive relation. However, glutenins are as necessary as gliadins. These had been proven in the findings of

Barak et al., (2013) where an effect on bread volume and crumb firmness was observed with a higher Gli/Glu ratio. A specific balance in the two subunits improved flour quality by stabilizing dough viscosity and elasticity/strength (Khatkar, Bell, & Schofield, 1995).

In our finding, a non-significant difference between treatments in the gliadins, glutenins and gliadins/glutenins ratio was observed. Another flour quality parameter such as unextractable polymeric protein (UPP %) was lower as compared to the control except for MACC-612 treatment and it was environment dependent. Like Gli/Glu ratio, UPP % contributes to dough strength Ciaffi et al., (1996b). The UPP % ranges of our results were in conformity with the findings of Zhang et al., (2008).

5.3.10 Nitrate -nitrite content in soil, N-uptake, and nitrogen use efficiency

Records on the effect of biostimulant on the prevention of increased N_2O emissions and NO_3 leaching had been found (Souza et al., 2019). This could be because of the increased uptake of mineralized nitrogen (Bardgett and Chan, 1999). In our research slight, non-significant differences in nitrate and nitrite content in the soil had been observed. So, the increased uptake of nitrogen with lower nitrate and nitrogen content could be suggestive of lower N_2O emissions and NO_3 leaching through high significance was not observed. Just like the suggestion of Souza et al., (2019) the exact mechanisms or effect cannot be deduced in this study and additional further study on biological processes need

to be studied. Many researchers had confirmed the use of algae like *Ascophyllum nodusum* to enhance the N-uptake in *Arabidopsis thaliana* and barley (Goni et al., 2021; Langowski et al., 2022). Other biostimulants such as AminoPrim and AminoHort (Calvo et al., 2014; Du Jardin, 2015; Paradikovic et al., 2013; Popko et al., 2018) showed the same impact on nitrogen uptake and hence also on nutrient use efficiency. Maignan et al., (2020) worked with glutacetine and found an increased in total grain N, reduction in root N concentration, etc. Similarly, reported increased total N in ears, a significantly higher N in grains in treated plants as compared to control.

All the results obtained with support from other studies suggest that the improvement in nitrogen uptake and other nitrogen concentrations suggest activation of nitrogen metabolism and that it could be the mode of action of the microalgae biomass. However, it needs further understanding and in-depth study of different crops in several seasons and laboratory conditions.

6. CONCLUSIONS

The biomass of algae strains like MACC-612 (*Nostoc linckia*), MACC-430 (*Chlamydopodium fusiforme*), MACC 922 (*Chlorella vulgaris*) showed significant differences with the control compared to other strains, so these three strains can be upgraded for the field experiment.

Certain algae strains improved, while others, e.g., MACC-438 (*Chlorella sorokiniana*), inhibited the germination processes. However, the way they affected germination may not work in the same way when they were used via leaves. What inhibits germination does not necessarily have an inhibitory effect on adult plants. A significant proportion of the strains was characterized by auxin-like activity. However, the auxin-like effect was not necessarily in direct relationship with the auxin content, but with their ability to influence secondary metabolism. Some of the metabolites detected could be the reason behind significant differences in the physiological parameters. Application of microalgae biomass tends to decrease IAA and enhance metabolites such as salicylic acid, jasmonic acid conjugates, and pHBA. However, the differences are unstable and need more trials involving different genotypes for confirmation.

Overall, there were no extreme significant differences between the treatment and the control in all the parameters however we found potential in the treatment. The microalgae biomass treatment irrespective of the strain species or genus influence the biological photosynthate accumulation and nitrogen uptake or in short, the

efficiency of uptake. After analysing the results of all three trials, we can conclude that microalgae biomass application affects certain physiological and biochemical properties but still works need to be done to improve the absorption or uptake of the microalgae biomass in the plants. One suggestion could be earlier spraying or increasing the number of sprays as we show potential differences in plants treated at an earlier stage, i.e., the vegetative stage. These results can differ in the field situation, so field trials of just one-time application at a later reproductive stage should also be done.

Furthermore, there appeared to be some negligible differences in biological yield, hexose content, or total phenol content because of the varietal differences, although the time of application remains constant. The morphological data comparison shows that differences in a variety had no influence on the effectiveness of the treatment. However, we cannot conclude that effectiveness is influenced by variety or genetic variation as further experiments with other varieties should also be performed.

Finally, the metabolomic analysis conducted independent of the time of application, suggested the influence of the microalgae strains in the biochemical composition of the plants.

Overall, no outstanding response was observed on many parameters, even so, we cannot ignore the fact that there was some effect of the treatment or the microalgae biomass. Our main aim was to conclude if any of the microalgae strains can be used as biostimulants or growth

promotors as per Regulation (EU) 2019/1009 on Fertilising Products (FPR). After considering some of the many parameters we can verify in the following way:

1) Proline concentration, which is a sign of abiotic stress tolerance potentiality, showed some increase in concentration after treatment of the biomass (esp. MACC-922) upon comparing with control.

2) Nitrogen use efficiency (NUE) or nitrogen uptake showed negligible difference with control. MACC-922 performed better than the other strains.

3) Quality traits such as the antioxidant potential (FRAP and TPC), protein %, gluten %, Zeleny sedimentation value, and the gluten subunits show some effect of the treatment esp. of MACC-612.

Based on the three points we can say that the microalgae strain, MACC-922, *Chlorella vulgaris* has the potential to promote or stimulate growth better than the other two, MACC-612, *Nostoc linckia* and MACC-430, *Chlamydopodium fusiforme*. However, to enhance their effectiveness it needs further improvement in the product by adding adjuvants as we found differences between treatments with or without Trend 90 (adjuvant).

As a future suggestion, we can say that by observing the hormonal activity, there lies great potential in microalgae biomass as a plant growth promotor or stimulant, yet some manipulation is needed in preparing a more homogeneous solution as the present method of ultra

sonifier fails to give a uniform solution and combination with other organic sources can also be trailed.

7. NOVEL SCIENTIFIC RESULTS OF DOCTORAL RESEARCH

- We determined the effect of the eight algae strains, i.e., MACC-922, MACC-612, MACC-683, MACC-755, MACC-430, MACC-677, MACC-519, and MACC-438 on germination and identified those that stimulate and inhibit germination processes in wheat plants. We found that the effect on germination is not necessarily the same as the effect on the development of the mature plant.
- We quantified secondary metabolites in the selected strains of microalgae biomass which revealed the presence of secondary metabolites such as salicylic acid, p-hydroxybenzoic acid, benzoic acid, etc.
- We have shown that the effect of algal treatments by foliar application on the secondary metabolite composition of winter wheat can be influenced by factors, like, the variety and the algal strain.
- We found variability in few metrics, such as biological yield, nitrogen content in the grain, because of the differences in the time of application i.e., early vegetative, and early reproductive stage.
- We concluded that genetic variability or variety played a negligible role in the effectiveness of microalgae biomass treatment on biological yield, hexose content and total phenol content.

- Observing the biochemical properties such as ferric reducing antioxidants power (FRAP) activity, total phenol content, and hexose content at different stages was the detailing added besides the common observations of proline content.
- Observations on nitrogen content in leaves, grain, and soil revealed the potentiality of microalgae in influencing the mobilization of nitrogen in the plant. Such potentiality has been reflected on nitrogen use efficiency and nitrogen uptake.
- We observed the effect of microalgae biomass application on the content of gliadins, glutenins, glutenins:gliadins ratio and unextractable polymeric protein (UPP) %.

8. PUBLICATIONS

Papers published:

Mutum, L.; Janda, T.; Darkó, É.; Szalai, G.; Hamow, K.Á.; Molnár, Z. (2023). Outcome of Microalgae Biomass Application on Seed Germination and Hormonal Activity in Winter Wheat Leaves. Agronomy. https://doi.org/10.3390/agronomy13041088

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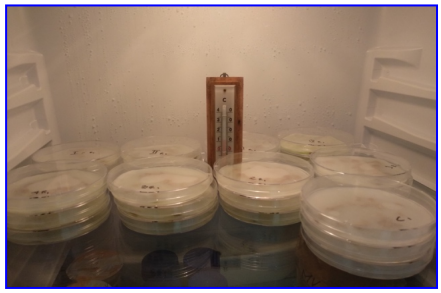
11. APPENDICES

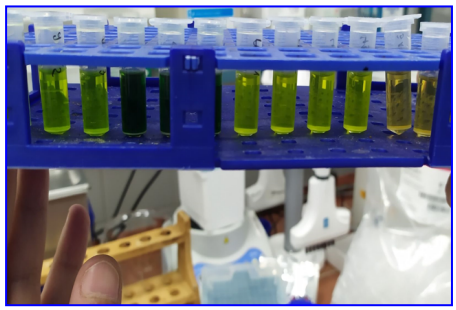
PHOTO PLATES

a. Microalgal production

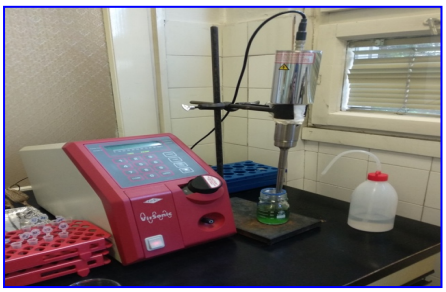


b. Germination bioassay

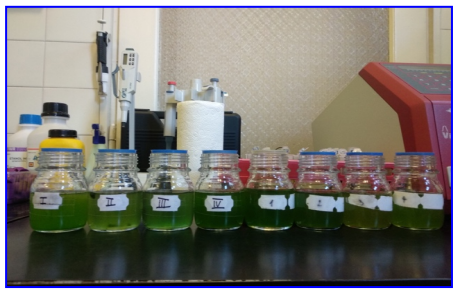
c. Mungbean bioassay



d. Quantification



e. Sonification



f. Treatment preparation

Photo Plate 1. Preliminary preparation of experimental materials (microalgae biomass, bioassays).

a. Non-vernalized plants

b. Vernalized plants

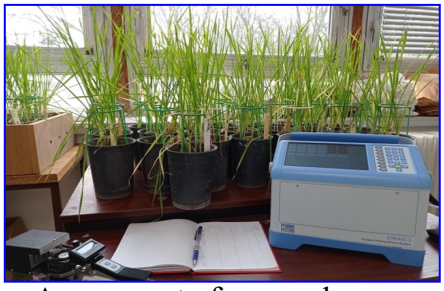
c. Application stage

d. 15 days after application

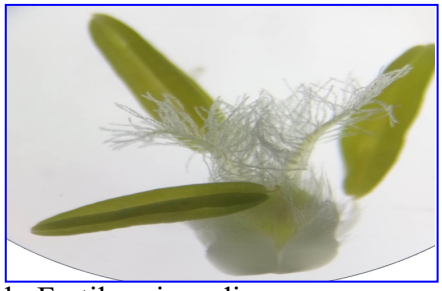
e. 30 days after application

f. At harvest

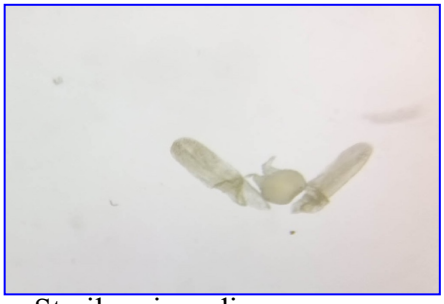
Photo Plate 2. Different experiments conducted along with important stages of field trials.



a. Assessment of gas exchange

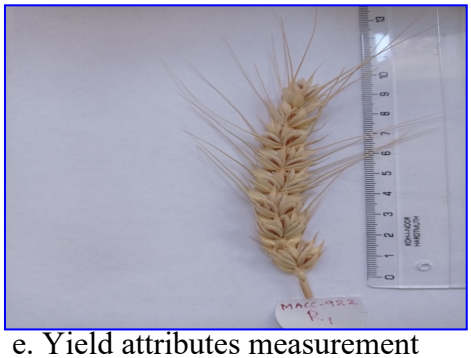


b. Fertile primordia (under microscope)



c. Sterile primordia (under microscope)

d. Plant sample preparation



e. Yield attributes measurement

Photo Plate 3. Assessment of physiological and morphological parameters.

a. Control

b. Standard

c. MACC-612

d. MACC-612 + Trend 90

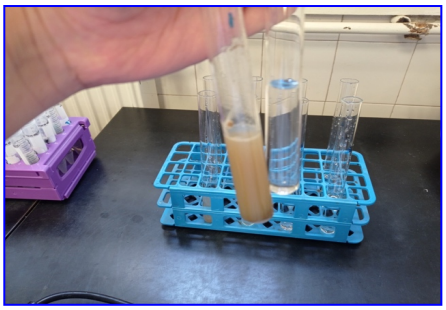
e. MACC-430

f. MACC-430 + Trend 90

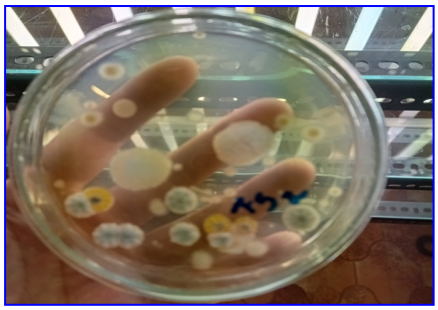
g. MACC-922

h. MACC-922 +Trend 90

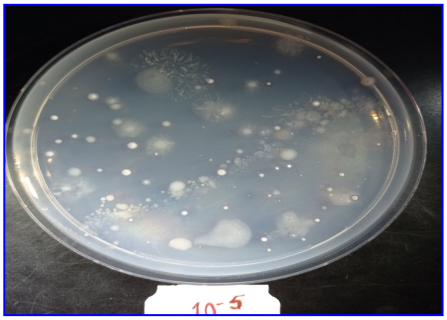
Photo Plate 4. Yield attributes comparison between different treatments of field trials.



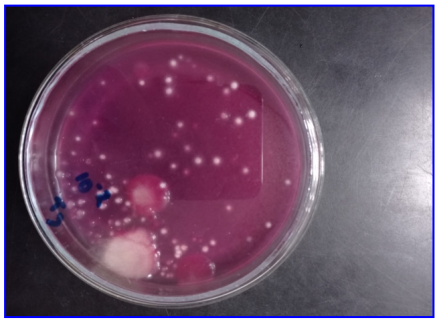
a. Extract preparation



b. Actinomycetes colony



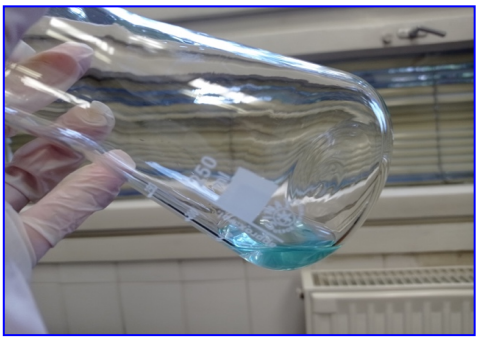
c. Bacterial colony



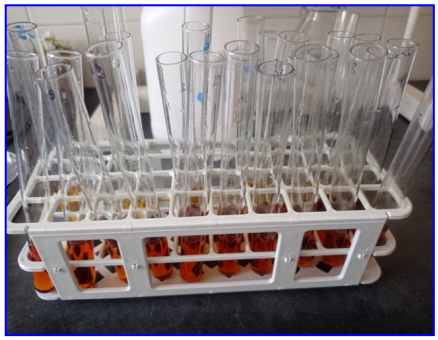
d. Fungal colony

e. Soil sample preparation (for soil chemical properties)

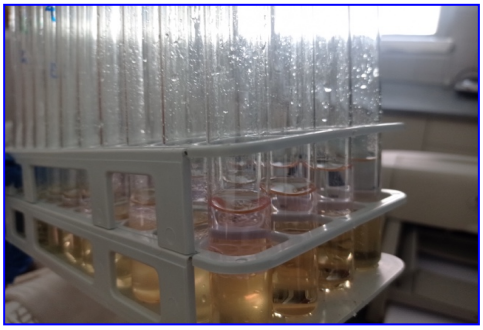
Photo Plate 5. Preparation and assessment of soil biological and chemical parameters.



a. FRAP analysis, end product



b. Hexose content analysis



c. Proline content analysis

d. Total phenol content analysis

Photo Plate 6. Biochemical analysis of plant samples (end products).

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Meteorological data, 2020-21 trial

October, 2020			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1	17.1	7.4	0.45
2	22.1	7.4	0
3	25.8	15.8	24.75
4	22.5	11.3	0
5	20.2	7.1	1.35
6	15.5	11	0
7	18.8	6.4	0
8	18.8	11	0
9	22.4	6.4	0
10	18.2	8.8	0.9
11	14.3	10.3	22.95
12	8.5	6.8	8.55
13	7	6.4	26.1
14	10	2.6	3.15
15	12.3	8.2	6.75
16	8.5	8.2	6.3
17	10.6	7.3	0
18	13.8	2.6	0
19	11.3	3.8	0
20	16.7	7.4	0
21	18.7	6.8	0
22	17	6.8	0
23	9.7	5.2	0
24	16.8	9.2	0.9
24	16.8	8.8	0
26	19.9	10.4	0
27	15	6.6	0.45
28	17.8	6.6	0
29	12.4	5.8	1.8
30	11.8	4.8	0
31	17	13.5	0
			104.4

November, 2020			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1	14.5	10.5	5.85
2	17.4	12.1	0
3	16.2	10.8	7.2
4	13.2	9.7	1.35
5	14.2	2.2	0
6	12	2.2	0
7	14.3	-0.1	0
8	14.6	3	0.45
9	7.4	4.7	0
10	9.4	7	0
11	8.2	7	0.45
12	8.3	4.9	0
13	7.9	4.9	0
14	8.7	7.3	0.45
15	10.1	7.3	0
16	9	3	0.45
17	13.5	2	0
18	8.9	5.8	0
19	8.9	5.8	4.05
20	8.5	6.9	0
21	6.3	-0.4	0
22	6.8	0.5	0
23	5	-2.3	0
24	2.7	-2.3	0
24	3.8	0.2	0
26	3.2	1.2	0
27	1.8	1.2	0.45
28	4.3	1	0
29	7.4	0.6	0
30	6	0.6	0
			20.7

Dec, 2020			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1	3.9	-1.4	0
2	0.1	-6.4	0
3	1.4	0.1	1.8
4	5.4	2.2	0
5	10.8	4.2	0
6	12	7.6	0.45
7	8.6	4.7	0
8	6.2	3	0
9	8.2	5.7	9.45
10	5.3	2.6	2.25
11	4.2	3.1	0
12	4.1	2.4	0
13	5	2.9	0
14	8.6	0.5	0
15	4.6	4.2	0
16	4.4	2.6	0
17	4.3	3.1	0
18	4.2	3.2	0.45
19	3.3	2.3	0.45
20	4.2	3	0.45
21	5	3.8	7.2
22	5.8	3.9	0
23	10.3	2.3	0
24	7.4	6.2	2.7
24	6.8	2.5	0
26	4	2	0
27	2.5	-6.7	0
28	7.5	1.4	4.95
29	9.6	2.5	2.25
30	6.8	5.4	0.9
31	5.3	-1.7	0
			33.3

Jan, 2021			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1	2.1	-3.3	0
2	6.3	-2.4	3.15
3	8.1	4.4	0
4	6.9	5.3	0
5	5.3	2.8	2.7
6	4.9	1.9	1.8
7	5.7	-0.8	0
8	2.3	-0.5	1.35
9	4.7	-0.5	1.8
10	0.5	-1.4	0
11	1.9	-8.9	0.45
12	0	-0.3	0
13	4.8	0.1	1.35
14	2.3	-0.8	1.35
15	3	-0.8	0
16	0	-1.3	0
17	-0.3	-10.7	0
18	-0.7	-2.9	0
19	5.1	-2	1.8
20	9	3.3	0
21	11.9	5.3	0
22	14.3	6.8	0
23	11.7	9	9.45
24	7.5	2.1	0
24	6	-0.2	0
26	4.7	1	0
27	5.4	-4.5	0.45
28	6.2	0.3	4.05
29	5.4	0.3	5.4
30	12.8	5	0
31	5.1	-5.8	0
			35.1

Feb, 2021			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1	4.9	-2.5	0
2	4.9	1.4	0.45
3	8.1	3.3	0.45
4	13.6	2	0
5	10.7	2.2	0
6	6.4	4.7	0.45
7	5.1	1.8	4.5
8	4.9	-0.2	0.45
9	2	-3.2	1.8
10	1.2	-5.2	6.3
11	-3.2	-10.5	0
12	-2.3	-10.5	0.45
13	-0.2	-9.4	0.45
14	1.9	-6.6	0.45
15	2.3	-4.9	0.45
16	1.4	0	0.45
17	8.3	3.7	1.35
18	13.1	0.9	0
19	8.8	0.9	0.9
20	5.2	0.2	0
21	6.5	3.4	0
22	14.8	-0.7	0
23	13.4	-1	0
24	17.2	2.2	0
24	20.3	0.9	0
26	19.1	0.9	0
27	8.9	5.1	0
28	10.5	0.5	0
			18.9

March, 2021			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1	12.8	0.5	0
2	15.2	0.8	0
3	15.2	-2.7	0
4	14.8	0.8	0
5	9.6	4.4	0
6	7.7	-3.9	0
7	10.7	-4.6	0
8	9	-2.8	0
9	6.4	-2.2	0
10	13.9	5	1.35
11	9.8	-5.3	0.9
12	14.3	5	0.45
13	14.6	0.6	0.45
14	10	5.8	0
15	11.4	1.4	0
16	10.2	4.3	0
17	9.4	3	0
18	9	2.4	0
19	7.8	-3.5	0
20	6.1	-4.4	0
21	4.3	-6.3	0.45
22	8.4	2.9	0.9
23	10.7	-3.2	0
24	10.8	4.8	0
24	15.5	-1.6	0
26	19.6	-0.1	0
27	20.1	2.6	0
28	14.3	6.5	0
29	19.6	1.2	0
30	24	9	0
31	24.5	5.9	0
			4.5

April, 2021			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1	25.9	7.2	0
2	18.3	13.2	0
3	11.3	6.6	0.45
4	11	2.8	0
5	17.5	-2.2	0.45
6	8	2.8	0
7	7.9	-0.7	0
8	8.5	-2.1	4.05
9	17.6	-3.1	0
10	22	5.6	0
11	18.5	8.3	0
12	20.8	7	1.35
13	6	4.3	18.45
14	9.3	2.9	0.9
15	8.9	3	0
16	10.3	2.3	0
17	12	4.5	0
18	13	5.7	7.2
19	12.5	7.2	9.9
20	16.9	7.6	0
21	19.7	5.7	0
22	18.1	3	0
23	16.4	3	0
24	18.3	-0.1	0
24	18	3.2	0
26	17.3	6.5	0
27	17.3	-0.7	0
28	19.8	6.5	0
29	24.6	11.3	0
30	22.5	11.3	0
			42.75

May, 2021			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1	25.2	11.4	0
2	16	7.9	0.45
3	16.3	3.4	0
4	22.1	7.1	0
5	24	6.4	3.6
6	15.2	9.4	0
7	15	9.4	0
8	17.9	5.1	0
9	24.8	9.3	0
10	28.5	15.3	0
11	31	13.9	0
12	20.6	12.3	4.75
13	15.9	12.3	4.2
14	18.6	11.4	77.2
15	18.8	8.3	4.76
16	22	13.5	5.33
17	14.5	10	10.9
18	19.6	8.4	0
19	17.7	10.2	17.6
20	20.2	6.2	0
21	22.5	6.2	0
22	18.2	8.8	0
23	15.3	8.4	13.95
24	18.8	5.1	3.6
24	16.1	3.6	0.45
26	21.8	8.1	0
27	20.5	10.7	0
28	21.2	10.7	0.45
29	20.8	9.2	0
30	19.6	7.1	0
31	19.6	7.5	0
			147.24

June, 2021			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1	20.9	5.9	2.25
2	23.2	6.3	0
3	27.2	9.7	0
4	29.2	10.8	0
5	30.8	12.4	0
6	27.7	13.4	0
7	29.6	13.1	0
8	30.8	16.2	0
9	30.1	17.7	0
10	28.8	13.9	0
11	28.9	13.9	0
12	30.5	16.8	0
13	22.7	11.6	0
14	24.4	8.3	0
15	28.8	12.2	0
16	31.5	14.8	0
17	32.9	14.8	0
18	32.2	17.1	0
19	33.9	15.8	0
20	33.9	17.9	0
21	33.9	18.9	0
22	34.6	20.5	0
23	33.1	20.1	0
24	32.2	20	0
24	35.8	18.8	0
26	29.4	18.8	0
27	29.1	17.4	0
28	34.5	17.7	0
29	33.8	16.9	0
30	28.9	13.4	6.3
			8.55

July, 2021			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1	24.7	15.7	0
2	22.7	15.7	0
3	27.6	15.8	0
4	29.3	12.3	0
5	28.8	13.5	0
6	33.8	17.9	0
7	38	20.8	0
8	38	19	0
9	27.5	20.8	0
10	30.6	14.5	0
11	22.9	16.9	30.6
12	28.9	18.6	0
13	34.2	19.2	0
14	29.9	15.4	0
15	29.1	14.3	0
16	31.5	14.3	17.1
17	28.5	19	18
18	33	18.5	11.7
19	28.3	15.8	0
20	24.8	11.1	0
21	26.1	10.6	0
22	28	11.5	0
23	28.4	11.5	0
24	31	12.3	3.15
24	30.2	17.9	0
26	32.7	16.9	0
27	30.8	19	0
28	33.9	16.6	1.8
29	30.3	15.9	0
30	33.1	15.9	0.9
31	30	18.3	18
			101.25

Meteorological data: 2021-22

October, 2021			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1.	19.9	4.2	0.0
2.	20.4	9.6	0.0
3.	24.0	9.6	0.0
4.	25.5	12.9	0.0
5.	25.4	14.8	0.0
6.	20.8	10.9	11.0
7.	14.5	10.6	13.7
8.	16.6	8.7	0.0
9.	14.8	5.9	0.0
10.	11.0	3.3	0.0
11.	15.5	8.1	0.0
12.	12.9	6.6	0.0
13.	11.5	6.0	1.5
14.	13.5	3.2	0.0
15.	14.9	6.2	0.0
16.	15.6	5.0	0.0
17.	15.3	0.1	0.0
18.	13.8	3.8	0.0
19.	17.3	2.8	0.0
20.	20.0	8.8	0.0
21.	20.6	9.5	0.0
22.	17.1	9.7	0.0
23.	13.2	6.5	0.0
24.	13.6	-0.9	0.0
25.	14.7	3.0	0.0
26.	13.0	0.3	0.0
27.	16.6	0.8	0.0
28.	15.4	1.1	0.0
29.	16.5	0.8	0.0
30.	15.9	2.2	0.0
31.	17.5	4.3	0.0
			26.2

November, 2021			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1.	17.0	3.8	0.0
2.	12.8	4.9	12.6
3.	15.5	3.7	1.0
4.	17.2	7.9	0.4
5.	12.4	2.6	0.0
6.	12.8	0.4	0.0
7.	13.3	4.2	0.0
8.	12.2	3.2	0.0
9.	13.2	1.3	0.0
10.	12.1	3.0	0.0
11.	8.0	4.3	0.0
12.	9.1	2.5	0.0
13.	5.4	-1.0	0.1
14.	6.9	4.8	0.0
15.	9.7	3.0	0.0
16.	11.5	2.9	0.0
17.	6.8	4.0	0.0
18.	10.3	5.5	0.0
19.	12.4	4.3	0.0
20.	12.8	4.5	0.0
21.	5.4	-0.4	0.0
22.	7.4	1.5	0.3
23.	6.2	0.0	0.0
24.	6.9	-3.7	0.0
25.	7.2	-0.8	0.0
26.	2.5	0.3	16.0
27.	3.5	-1.5	0.0
28.	4.8	1.3	4.1
29.	4.0	0.9	0.0
30.	5.8	0.0	11.1
			45.6

December, 2021			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1.	8.0	0.2	2.1
2.	7.8	5.0	3.8
3.	5.1	0.5	0.0
4.	4.0	-2.8	0.8
5.	1.3	0.0	6.1
6.	4.5	0.0	1.5
7.	2.8	-3.1	0.0
8.	5.8	-4.6	0.0
9.	1.5	0.1	15.1
10.	0.4	-1.8	0.0
11.	2.0	-2.0	0.0
12.	2.9	-1.0	0.0
13.	3.8	-1.0	0.0
14.	6.9	1.0	0.0
15.	7.0	4.2	0.0
16.	8.3	4.6	0.0
17.	7.1	4.2	0.0
18.	6.4	2.5	0.0
19.	6.3	3.3	0.0
20.	5.1	1.1	1.3
21.	3.2	-2.6	0.0
22.	1.4	-5.3	0.0
23.	0.9	-4.8	0.0
24.	4.6	-2.7	0.6
25.	5.1	-0.3	0.7
26.	0.2	-2.3	0.0
27.	2.8	-0.4	0.0
28.	1.7	-0.2	1.9
29.	3.2	-0.1	3.3
30.	8.1	2.3	1.9
31.	16.8	1.8	0.0
			39.1

January, 2022			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1.	15.4	4.3	0.0
2.	12.7	2.4	0.0
3.	14.3	5.8	0.0
4.	10.7	6.1	0.1
5.	10.5	5.3	0.6
6.	7.3	1.0	0.0
7.	2.6	-7.2	0.0
8.	0.2	-6.6	0.0
9.	1.7	-4.0	0.5
10.	3.3	-1.0	0.5
11.	1.2	-2.3	0.0
12.	-0.8	-4.9	0.0
13.	4.5	-3.3	0.0
14.	9.7	-2.4	0.0
15.	7.5	-0.5	0.0
16.	6.8	-5.4	0.0
17.	7.0	2.8	2.1
18.	4.8	-0.6	0.0
19.	5.0	-5.2	0.0
20.	6.8	-3.3	0.4
21.	2.3	-2.3	0.1
22.	3.4	-1.3	2.4
23.	2.2	-3.5	0.1
24.	1.8	-2.7	0.0
25.	3.3	-3.6	0.0
26.	4.0	1.7	0.0
27.	6.3	0.9	0.0
28.	7.8	0.9	0.0
29.	6.9	-0.8	4.6
30.	9.1	2.1	0.0
31.	6.5	2.0	0.2
			11.6

February, 2022			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1.	5.8	0.5	2.1
2.	8.5	0.4	0.8
3.	9.1	2.2	0.0
4.	10.9	0.6	0.0
5.	8.8	-1.0	0.0
6.	4.4	-0.3	0.0
7.	7.0	2.8	0.6
8.	8.7	1.7	0.0
9.	14.2	5.0	0.0
10.	13.5	-2.3	0.0
11.	10.3	-0.4	0.0
12.	8.4	0.5	0.0
13.	8.6	-2.5	0.0
14.	10.3	-1.5	0.0
15.	11.3	-3.6	2.2
16.	12.0	3.6	3.0
17.	16.4	5.8	6.6
18.	14.7	5.6	0.0
19.	12.2	7.9	0.0
20.	12.2	3.6	0.0
21.	8.7	4.4	0.4
22.	9.9	3.8	0.0
23.	11.7	3.2	2.0
24.	13.5	-1.6	0.0
25.	9.6	-1.3	0.0
26.	9.4	0.1	0.0
27.	10.0	-1.3	0.0
28.	6.4	-2.4	0.0
			17.7

March, 2022			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1.	7.6	-5.3	0.0
2.	7.4	-3.7	0.0
3.	9.2	-3.3	0.0
4.	6.9	-3.3	0.0
5.	5.7	-3.0	0.0
6.	6.6	-1.4	0.0
7.	3.7	-0.4	0.0
8.	8.4	-4.2	0.0
9.	10.8	-2.6	0.0
10.	8.1	-1.0	0.0
11.	4.8	-7.2	0.0
12.	7.7	-6.5	0.0
13.	12.6	-2.9	0.0
14.	14.6	1.2	0.0
15.	14.9	-0.8	0.1
16.	11.1	5.9	2.3
17.	12.7	-1.4	0.0
18.	12.3	-1.4	0.0
19.	11.8	-2.0	0.0
20.	10.8	-0.8	0.0
21.	12.9	-6.5	0.0
22.	16.6	-4.7	0.0
23.	20.5	-3.5	0.0
24.	20.9	-1.5	0.0
25.	19.3	0.7	0.0
26.	19.0	1.7	0.0
27.	18.3	6.2	0.0
28.	21.8	0.1	0.0
29.	21.6	2.2	0.1
30.	16.3	5.5	0.0
31.	14.3	7.8	8.2
			10.7

April, 2022			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1.	9.6	3.0	1.4
2.	4.5	0.7	1.8
3.	6.1	0.1	0.4
4.	9.0	-2.5	0.0
5.	11.1	2.7	0.9
6.	20.2	7.8	0.0
7.	20.8	7.8	0.0
8.	17.5	9.7	1.9
9.	17.1	6.8	0.2
10.	11.0	1.5	0.1
11.	12.7	-1.8	0.0
12.	17.6	0.4	0.0
13.	20.2	3.2	0.0
14.	22.5	4.8	0.0
15.	20.0	6.3	2.2
16.	14.6	7.9	0.0
17.	13.6	1.6	0.0
18.	13.6	-0.4	0.0
19.	10.2	4.6	1.4
20.	14.4	1.4	0.0
21.	17.9	1.1	0.0
22.	13.7	6.1	0.3
23.	15.8	7.9	1.3
24.	21.4	10.4	0.0
25.	19.0	6.9	0.0
26.	17.0	3.8	3.5
27.	14.4	8.5	3.5
28.	19.5	6.7	0.0
29.	20.1	3.8	0.0
30.	22.1	3.4	0.0
			18.9

May, 2022			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1.	20.6	8.6	0.2
2.	22.9	6.7	0.0
3.	24.3	5.5	0.1
4.	24.7	8.2	16.8
5.	24.1	10.6	0.0
6.	20.7	12.0	0.0
7.	21.2	13.2	16.0
8.	22.5	12.8	0.1
9.	23.7	12.1	0.1
10.	24.4	9.2	0.0
11.	27.7	9.2	0.0
12.	30.3	14.1	0.2
13.	25.8	16.3	1.8
14.	23.6	12.4	0.0
15.	26.4	9.2	0.0
16.	27.5	13.3	0.0
17.	22.5	16.7	0.6
18.	21.4	9.6	0.0
19.	25.9	5.7	0.0
20.	29.8	10.7	0.0
21.	26.6	17.6	8.6
22.	23.0	13.4	0.0
23.	23.8	10.8	0.0
24.	23.5	15.4	5.4
25.	22.9	15.3	1.3
26.	25.1	10.2	0.0
27.	28.4	14.2	0.0
28.	24.1	13.7	0.0
29.	19.0	7.0	0.0
30.	20.2	9.4	0.0
31.	25.0	8.8	0.0
			51.2

June, 2022			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1.	26.6	13.4	9.0
2.	26.5	14.3	1.0
3.	29.4	14.2	0.0
4.	31.5	19.4	0.1
5.	29.0	17.0	40.6
6.	26.3	16.2	21.0
7.	28.3	14.9	11.3
8.	24.1	15.8	4.8
9.	21.7	15.2	16.2
10.	22.8	15.5	0.9
11.	25.8	15.3	0.0
12.	27.8	13.7	0.0
13.	27.4	13.7	0.2
14.	23.1	13.2	0.0
15.	26.7	10.2	0.0
16.	28.8	13.5	0.0
17.	25.5	13.7	0.0
18.	28.3	12.1	0.0
19.	30.0	16.5	0.0
20.	31.5	17.3	0.2
21.	26.0	15.5	0.1
22.	27.5	12.6	0.5
23.	29.7	16.7	0.2
24.	30.3	14.0	7.9
25.	26.8	16.2	6.5
26.	30.2	15.6	0.0
27.	33.9	17.1	0.0
28.	31.5	20.6	3.5
29.	35.7	19.7	0.1
30.	35.8	18.9	0.0
			124.1

July, 2022			
Day	**Max°C**	**Min °C**	**Precipitation (mm)**
1.	35.4	21.4	0.0
2.	27.7	15.2	0.1
3.	32.0	12.8	0.0
4.	31.9	15.3	1.4
5.	26.7	18.6	25.1
6.	27.5	14.0	2.2
7.	25.6	12.7	0.0
8.	22.5	15.5	0.0
9.	26.1	12.5	0.0
10.	22.5	13.7	0.0
11.	23.8	12.2	0.0
12.	25.9	9.7	0.0
13.	31.7	13.1	0.0
14.	35.8	15.9	0.1
15.	31.5	17.2	0.5
16.	29.2	10.6	0.0
17.	27.7	12.4	0.0
18.	30.3	9.7	0.0
19.	33.0	11.2	0.0
20.	35.5	14.1	0.0
21.	36.7	15.9	0.0
22.	36.1	21.6	0.0
23.	36.9	19.5	0.7
24.	32.1	19.8	1.3
25.	34.6	14.5	0.0
26.	30.5	20.0	7.7
27.	28.9	14.6	2.3
28.	30.7	15.9	0.0
29.	34.1	16.0	0.0
30.	24.3	17.9	22.1
31.	28.5	17.9	0.0
			63.5