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ORIGINAL PAPER

Time of application and cultivar influence on the effectiveness of microalgae biomass upon winter wheat (*Triticum aestivum* L.)

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Abstract
The capability of microalgae had been studied for a long time; however, some basics of using microalgae as a biostimulant are still in question. In the present work, experiments were conducted to reply to questions such as (a) how does the application time affect the effects of microalgae treatments and (b) does variety or genetic variation cause differences in the effect of microalgae biomass application on the plants? The different times of application had different weightage on different parameters; however, when applied at the early reproductive stage the yield as well as the nitrogen % in grain was significantly affected. As per the comparison, the result suggested that varietal differences had negligible differences in biological yield, hexose content, and total phenol content. Furthermore, microalgae biomass treatment irrespective of the strain species or genus influences the biological photosynthate accumulation and nitrogen uptake or in short, the efficiency of uptake. Finally, the metabolomic analyses suggested the influence of the microalgae strains on the biochemical composition of the plants.

Keywords Microalgae · Biostimulant · Nitrogen · Metabolomic · Photosynthate

Introduction

The use of natural sources to promote growth and increase agricultural yield has been a way of bringing agricultural sustainability. Due to the diverse bioactive molecules in their composition, algae has been one of these potential sources. In the agricultural sector, researchers have been exploring ways of utilizing algae, especially microalgae. Besides application as a nutrient supplement, evaluating the effects on growth at low doses has also been experimented to monitor the potentiality of microalgae as biostimulants. To be able to use as a biostimulant, every product should possess some characteristics at the European level such as influencing nutrient uptake efficiency and improving abiotic stress tolerance and finally should improve the quality of the product. This has been proven in horticultural crops like tomato (Supraja et al. 2020), *Petunia x Hybrida* (Plaza et al. 2018), etc., by accelerated plant development or earlier flowering. The growth improvement can be dependent on various aspects. In the case of nitrogen-fixing cyanobacterium, as per the report of Kulk (1995) and Adam (1999), the nitrogenase and nitrate reductase activities of algae associated with the plant surfaces could have induced the plant growth.

Findings on the use of total polysaccharide extract in tomato and pepper had enough evidence of the potentialities of microalgae (Flo et al. 2002; Iwamoto et al. 2005). It has been assumed that the interaction between oligosaccharides and plants' toll-like receptors (TLRs) induced cytokine production which is responsible for several cell signalling pathways and cellular communication. It was further added that LRR receptors (leucine-rich repeat) available widely in the plasma membrane of the plant have similar interaction with polysaccharides.

The timing of the application of biostimulants like microalgae has mostly been at the critical stages of growth. And in many cases, two–three times low-dose applications are done. But the number of applications is based on a calendar approach, covering most of the growing season. For instance, if it is intended for morphological growth, it is usually applied at the early stages, and when intended to

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enhance crop tolerance to abiotic stresses, it is preferably applied before the stressful event to prime plant physiological defences (Plaza et al. 2018). There have been findings that application was also done in two stages in sunflower crops with the first application at the 4–6 leaf stage and the second treatment at the rosette stage (Pőthe et al. 2014). Tóth et al. (2014) applied *Nostoc enthophytum* microalgae in three stages in pepper, viz. 10–15 leaves, green bud stage, and early fruiting stage.

It has been found by Deepika and Mubarak Ali (2020) that plants application of 20% liquid algal extract (fertilizer) could increase protein content, lipid content, carbohydrate content and total phenol content in *Vigna radiata.* Increased biochemical parameters were also observed in *Cucumis sativus*, *Capsicum annuum,* and *Solanum lycopersicum.* The effect of microalgae on cereals like maize (Ördög et al. 2021) and winter wheat (Takács et al. 2019) had been experimented with using cyanobacterium, *Nostoc piscinale*. In both cases, foliar application was done in the vegetative stage and 1.0 g L^{-1} sprayed at 400 L ha^{-1} was the most effective dose rate. In 2019, Dineshkumar and colleagues conducted a study to evaluate the impact of two microalgae species, namely *Spirulina platensis* and *Chlorella vulgaris*, on maize yield. Although the weight per cob exhibited a minimal disparity between these two species, a noticeable distinction of 13–15 g per corn was observed when compared to the control group. This suggests the promising influence of microalgae on yield enhancement. Furthermore, a similar investigation by Dineshkumar et al. (2018) demonstrated a substantial boost in rice yield, ranging from 7 to 20.9%, using the same microalgae species.

However, the interaction between plants and biostimulants is very complex. It has been shown that the effectiveness may differ based on plant genotype (species, cultivar, clone) and the growing conditions, including seasonal climatic variability (Andreotti 2020; Ördög et al. (2021)). Such complexity has yet to be unravelled in microalgae application. Though researchers have been conducted on winter wheat (Ördög et al. 2021), many gaps are yet to be filled such as the application critical tillering and critical flowering stages.

Field experiments of winter wheat have usually been conducted by applying at the vegetative stage and reproductive stage, in short, two-time applications. However, many researchers have also given the view that a one-time application would have been better to reduce labour. In this paper, three different pot experiments will be shown, in order to: (1) assess the yield attributes by applying only to the early reproductive stage or (2) the critical flowering stage and (3) assess the vegetative state trial in different varieties to monitor any effect of genetical variation on the influence. Since there are proven experiments that show an improvement in plant productivity and tolerance to abiotic stressors by modifying and regulating the activities of carbon and nitrogen metabolism enzymes, hormonal activities, and secondary metabolism (Rouphael et al. 2018; Calvo et al. 2014; Colla et al. 2017; Rouphael et al., 2017a, b), here, the parameters will also be related to carbon and nitrogen metabolism. A proper quantification of the secondary metabolites was performed in the treated plants to monitor the differences created in their level of content after the application of microalgae.

Materials and methods

Experiment setup and treatment application

Germinated winter wheat plants (Mv Nádor and Mv Béres varieties bred in Agricultural Institute, Centre for Agricultural Research, Martonvásár, Hungary) were vernalized at 2 °C for 6 weeks, and then, they 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 the time of application or the variety used. In Trial 1, the application timing was at critical flowering stage or early reproductive stage, and in Trials 2 and 3, algae treatments were applied at early vegetative stage. Completely randomized experimental design was used.

Morphological parameters and yield attributes

Plant height at the flowering stage, number of tillers, and spike length were measured at harvest. Biological yield refers to the total biomass accumulation in the plant system. It is the sum of grain yield as well the straw yield and was calculated in g/plant.

Hexose sugar

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. In 2 mL of sample extract, 0.05 mL (50 µl) of 80% phenol (20 mL distilled water in 80 mL phenol) was added. Then, 5 ml. of concentrated sulfuric acid was added rapidly, the stream of acid being directed against the liquid surface rather than against the side of the test tube to obtain good mixing.

The tubes were allowed to stand for 10 min, and then, they were shaken and placed for 10 to 20 min in a water bath

at 25° to 30 °C before readings are taken. The absorbance of the characteristic yellow-orange colour was measured with CARY 50 SCAN UV–visible spectrophotometer (Varian) at 490 nm for hexose sugar.

Total phenol content

0.5 ml (100 µg mL^{-1}) of the sample extract was mixed with 2 ml of Folin–Ciocalteu reagent (Singleton et al. 1999) (diluted 10 times with de-ionized water, 1:10). This was then neutralized with 4 ml of 7.5% sodium carbonate solution. The reaction mixture was then incubated for 30 min at room temperature with intermittent shaking for colour development. The absorbance of the resulting blue colour was at 765 nm using a CARY 50 SCAN UV–visible spectrophotometer (Varian).

Nitrogen content

Elementar Analysensysteme GmbH-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 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.

Metabolomics

Sample preparation for untargeted GCxGC TOFMS analyses was based on Gondor et al. (2021) with modifications. Plant samples were extracted after the internal standard was added (Adonitol 30 µL of 1 mg mL^{-1} solution). Metabolites were extracted twice with 60% concentration methanol and water solution and then 90% of methanol. The homogenized samples were mixed for 30 s, then kept in ultrasonic bath for 5 min, then mixed again for 15 s, and centrifuged before the supernatant was collected. After the supernatant collection, the higher concentration of methanol was added twice to the pellet and the supernatant is collected again. 200 µL extract was dried under vacuum, and derivative is formed with methoxyamine hydrochloride-dissolved pyridine (20 mg ml^{-1}) (Merck-Sigma group, Darmstadt, Germany) at 37 °C for 90 min and with N-Trimethylsilyl-N-methyl trifluoroacetamide (Merck-Sigma group, Darmstadt, Germany) at 37 °C for 30 min. The derivatized samples were transferred into the 30 m column (HP-5MS ui 30 m, 0.25 mm, 0.25 µm) and the 1.5 m column (Rxi-17Sil MS phase 0.18 mm, 0.18 µm). The carrier gas (He) was used at constant flow rate (1 ml min^{-1}) through the injector in split mode to the LECO Pegasus 4D GCxGC TOFMS (LECO, St Joseph, MI, USA) at 230 °C. Thermal programme started with 70 °C for 3 min and increased to 320 °C for 5 min in 7 °C min^{-1} rate. Modulation period was 5 s in 2D GC mode. Data evaluation and GC analyses was performed LECO ChromaTOF version 4.72 programme, for identification analytical standards, Kovats retention, and Finn and Nist version 2.3 databases.

Statistical analysis

Data obtained 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 & 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).

Results

In order to test the effects of microalgae on agronomically important parameters, the total biological yield, hexose content, and total phenol content, nitrogen % in grain and in straw were determined first. The results were inconsistent. First, there were no significant differences in most of the parameters taken. However, in Trial 1, using Mv Nádor with microalgae application at early reproductive stage, the biological yield after MACC-612 application was significantly higher than in control or other algal treatments (Table 1).

On the other hand, in Trial 2 (Table 2), where algae were applied 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.

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

The morphological data of the trials with the same type of application but different varieties were compared (Table 4). Morphological data for Trial 1 were not included in the comparison as it was irrelevant in this case (application done after the vegetative growth stage). We found no significant influences of microalgal treatment in

Table 1 Trial 1 with microalgae applied at early reproductive stage using Mv Nádor

	Treatment	Total biological yield (g/plant)	Hexose content (mg/g)	Total phenol content (mg GAE/g dry extract)	Nitrogen % in grain	Nitrogen % in straw
1	Control	5.17	24.00	2.62	3.38	1.17
2	MACC-430	4.90	24.45	2.62	3.54	1.09
3	MACC-922	4.92	24.41	2.63	3.63	0.93
4	MACC-612	6.36	24.22	2.63	3.55	1.05
	SEd	0.49	0.68	0.19	0.39	0.15
	Sig level	**	Nsig	N sig	Nsig	Nsig
	CD 1%	1.50	1.57	0.57	1.13	0.45
	CD 5%	2.38	1.14	0.41	0.82	0.33

Table 2 Trial 2 with microalgae applied at early vegetative stage using Mv Nádor

	Treatment	Total biological yield (g/plant)	Hexose content (mg/g)	Total phenol content (mg GAE/g dry extract)	Nitrogen % in grain	Nitrogen % in straw
1	Control	4.64	38.90	1.52	2.8	0.81
2	MACC-430	4.47	45.77	1.71	3.2	0.64
3	MACC-922	4.77	43.16	1.75	2.96	0.72
4	MACC-612	4.78	40.17	1.60	3.21	0.71
	SEd	1.38	3.718	0.13	0.08	0.11
	Sig	Nsig	Nsig	Nsig	**	N sig
	CD 1%	1.29	11.35	0.41	0.25	0.34
	CD 5%	0.93	8.09	0.29	0.18	0.25

Table 3 Trial 3 with microalgae applied at the early reproductive stage using Mv Béres

	Treatment	Total biological yield (g/plant)	Hexose content (mg/g)	Total phenol content (mg GAE/g dry extract)	Nitrogen % in grain
1	Control	3.74	23.03	2.62	2.89
2	MACC-430	4.94	24.06	2.62	3.12
3	MACC-922	5.27	24.62	2.63	2.74
4	MACC-612	5.2	23.82	2.62	2.77
	SEd	0.5	0.518	0.003	0.1
	Sig	**	N sig	Nsig	**
	CD 1%	2.65	1.58	0.0104	0.31
	CD 5%	1.93	1.12	0.007	0.22

the two compared trials. The differences in the value were a result of the genetic characteristics of the respective varieties. It was also observed that certain algal treatments caused a delay in the 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.

Metabolomic analyses

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

Table 4 Morphological parameters (Trials 2 and 3)

		Trial 2 (Nádor)			Trial 3 (Béres)		
	Treatment	Plant Height (cm)	Number of leaves	Number of tillers	Plant Height (cm)	Number of leaves	Number of tillers
1	Control	26.1	10	1	60.3	8	2
2	MACC-430	26.4	10	1	55	9	3
3	MACC-922	26.2	11	1	55.6	8	3
4	MACC-612	26.2	11	1	53.6	9	2
	SEd	6.2	2.5	0.5	4.8	2.2	0.4
	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

18 organic acids, 8 carbohydrates, and 4 alcohols could be identified and quantified. Mean and standard deviation values can be seen in Supplementary material. The qualitative determination was based on Kovats retention index and Finn and Nist version 2.3 databases. The quantitative determination was based the internal standard quantities of the sample. Further compounds including 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 indicates that although the differences were not statistically significant in all the cases, they tended to decrease in the plants treated with algal strains (Fig. 1). 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.

Discussion

The slightly higher biological accumulation may reflect on their photosynthesis efficiency and the source-sink relations. Higher biological yield in Trial 1 with MACC-612, i.e. Nostoc sp., could mean a higher accumulation of photosynthates in the plants. Since in this trial microalgae were applied later, then the speed of accumulation accelerated at the reproductive stage. It confers with the report of Tandon and Dubey (2015) where they found biozyme biostimulants affecting the biological yield in soybean. Shirkhodaei et al. (2014) also reported an increase in biomass yield by application of biostimulant in coriander. A study on two microalga types of *Chlorella vulgaris* and *Spirulina platensis* shows that both influenced the yield parameters of rice (Dineshkumar et al. 2018). However, the results seem inconsistent and require further confirmation and field experiments. The lower value could be because of the external factor, time of application, or variety.

While in Trial 2, the results on the total biological yields were not significant, in Trial 3, using another variety, we found a significant increase in biological yield, and unlike in Trial 1, the value was all higher than the control and photosynthates accumulation has improved with the application of microalgae. Overall, the result is in accordance with the result of KV et al. (2020) who found that foliar spraying of 60% algal extracts resulted in increased dry biomass content.

Thus, microalgae can affect biological accumulation depending on the time of application, and variety. Even if Trials 2 and 3 are applied at the same time, the degree of effectiveness is different, so neither we cannot say that time of application is affecting nor could we conclude that variety influenced photosynthates accumulation, as the significant results are found in Trial 1, where Mv Nádor, and Trial 3 where Mv Béres was used.

Hexose sugar content was also studied as they are the primary product of photosynthesis and the origin of most organic matter. Hexose to be utilized is first phosphorylated with phosphorylated enzymes like hexokinases, and fructokinases, and these enzymes play an important role in plant metabolism and development. Hexokinases are discovered to be dual-function enzymes such as in sensing sugar levels independent of their catalytic activity, controlling gene expression and major developmental pathways, and hormonal interactions, while fructokinases have been reported to play a role in controlling the allocation of sugar for vascular development (Granot et al. 2013). Here in these three trials, we observed non-significant differences in hexose content. However, when the microalgae biomass is applied in the early phase, there could be a higher accumulation of hexose in the grains irrespective of a particular strain. We also observed the potentiality of *Chlorella vulgaris*, *Chlamydopodium fusiforme* slightly higher than *Nostoc linckia* in case of improvement in hexose content. The results are in conformity with the studies of Carillo et al. (2019) on

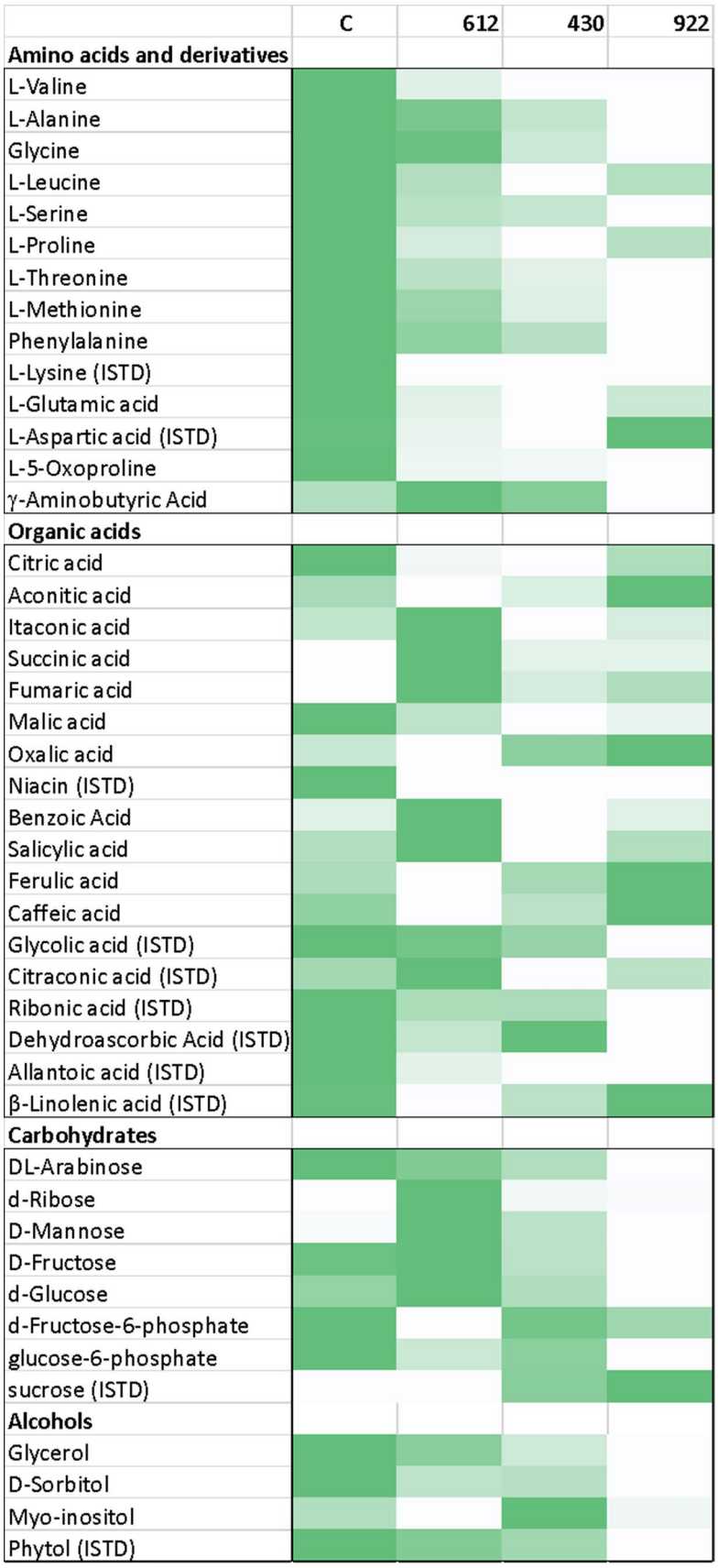

Fig. 1 Heat map demonstrating the changes in the amino acids

plant extract biostimulants that hexose was not significantly increased; however, we cannot conclude that all biostimulants would not have an influence on hexose content. The stable hexose content can be an indication of sufficient nitrogen content because more accumulation of fructan, sucrose, or starch in the sink and source is an indication of nitrogen deficiency (Wang and Tillberg 1996). In addition, hexose sugar concentrations followed a reverse pattern of increase in the later-order leaves, and these also increased as the nitrogen supply decreased.

Phenolic compounds or secondary metabolites reflect the defence mechanism of the plant. So, by analysing the total phenolic content we found an estimate of redox properties responsible for antioxidant activity (Soobrattee et al. 2005). Research conducted on Jute, *Corchorus olitorius* by applicating plant extract biostimulant (Carillo et al. 2019), found no increase in total phenol content. This result harmonized with our results where we found no differences between the control and the treatments and even within the treatments in all the three pot trials. However, in a study conducted on broccoli by applying commercial brown seaweed extract (Lola Luz et al., 2014) they found a tremendous increase in total phenol content. The composition of microalgae is closer to seaweed (Gomez-Zavaglia et al. 2019), so considering this regard, there should be an increase in total phenol content. Nevertheless, we cannot neglect the condition of growth as our experiments were conducted in pots with no abiotic and biotic stresses, while the mentioned experiment of treatment with commercial brown seaweed extract was in field conditions vulnerable to external stress conditions.

Furthermore, the nitrogen content, be it on the grains or stalk biomass, reflects nutrient uptake. The nitrogen content in the grain, as well as the stalk, was statistically insignificant when microalgae were applied in the flowering stage. This is in accordance with the findings of Szczepanek et al. (2018) who found no significant difference in nitrogen concentration in the grain. However, though negligible difference we found higher values of grain nitrogen(sink) in microalgae-treated plants than in control, indicating higher deposition of available nitrogen or a better uptake in the sink. While it was the opposite in the case of stalk biomass (source) nitrogen, the higher content of nitrogen in the control showed a lower efficiency of depositing nitrogen in the sink. The greater N content in the amino acid was due to greater amino acid source-to-sink allocation efficiency during various growth stages which also depended on the nitrogen use efficiency of the genotype (Liang et al. 2022). In these 3 independent trials, we can see a significant increase in nitrogen content in plants where microalgae biomass was treated at the early vegetative stage better than in the early flowering stage application. Similar results have been published with other biostimulants like humic acid where an increase in N-uptake rate was observed (Zanin et al. 2016).

In a pot experiment conducted by Chittapun et al. (2018) on rice, significant differences on due to the application of cyanobacteria were found in morphological parameters such as root length, shoot length, and the number of spikes. However, in our case, there were no significant differences due to the treatment even with changes in variety.

It seems to backfire on amino acids content after application of all the three types of microalgae. There are several functions of amino acids with some mentionable functions such as monomer unit in protein synthesis, a substrate for biosynthetic reactions, synthesis of several hormones, and derivation of neurotransmitters from amino acid (Gutiérrez-Preciado et al. 2010). So, as per the heat map in Fig. 1, there is a negative effect because of the application. For example, glutamine is a key metabolite for ammonia storage, as per metabolomic analysis, since we have lesser glutamine in treated plants as per the theory, those plants would have ammonia storage.

Organic acids such as succinic acid, fumaric acid, salicylic acid, and benzoic acid were in higher concentration when treated with MACC-612, *Nostoc linckia,* more than in untreated plants. In a study conducted by Knyazeva et al. (2021) on lettuce plants, the role of succinic acid was identified to increase the accumulation of dry matter, while fumaric acid is a component of the tricarboxylic acid cycle and may be involved in carbon transport (Chia et al. 2000). Hayat et al. (2007) mentioned the involvement of salicylic acid in ethylene biosynthesis, stomatal movement, reversing the effects of abscisic acid on leaf abscission, enhancing photosynthetic rate, flower induction, and uptake of ions, and Lee et al. (1995) added that benzoic acid was needed in the salicylic acid biosynthesis and metabolism. Thereby, the application of microalgae especially MACC-612 may improve the plant in a similar manner as mentioned above but this is yet to be proven. Since the glucose, mannose, and fructose ribose are more in treated plants, it had an advantage in sugar metabolism over the control plants. This is in conformity with the finding of Coppens et al. (2016) where an increase in sugar content was observed with the application of microalgal fertilizers.

Conclusion

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 influences 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 or 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 on the biochemical composition of the plants.

Finally, the potential impact of integrating microalgae into larger-scale agricultural practices becomes evident. Microalgae possess the ability to enhance biomass and grain yield, while their capacity to improve quality is underscored by significant fluctuations in grain nitrogen content, directly affecting protein levels. Considering these findings, exploring the application of microalgae in boosting biomass yield, particularly in silage or fodder crops, warrants further investigation.

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Author contributions LM was involved in the compilation of the literature, conducting the research, and drafting into a research paper; TJ participated in the supervision and incorporation of scientific views; and WK aided in supervision. ZM was involved in the supervision and rectification of the research paper. The manuscript has been read, reviewed, and approved by all authors.

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Declarations

Conflict of interest The authors declare there is no conflict of interest.

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