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Microalga as biofertilizer improves yield, sugars and amino acids content in red beets

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Abstract

The growing uncertainty of future changes in our global climate may pose a threat to some traditional farming methods, stimulating the development of nature friendly technologies. Microalgae are a group of micro photosynthetic organisms, which have immense potential as a renewable and ecofriendly bioresource for various industries. The present study was developed to evaluate foliar sprays of a green microalga *Asterarcys quadricellulare* (CCAP 294/1) on organically grown red beets (*Beta vulgaris L*) A field experiment was implemented to evaluate microalgal biomass bioactivity and its effects in leaves and hypocotyl growth, comparing fresh and dry weight, yield; and biochemical alterations, comparing pigments, sugars and free amino acids. The spray dried biomass of the *A. quadricellulare* was applied to leaves at concentrations 0.05g, 0.1 g, 0.15g and 0.25 g L^{-1}. The sprays with the microalga biomass increased yield, and improved free amino acids, sugars, dry weight and beets commercial diameter. This study shows that the foliar sprays presented a biofertilizer effect, emphasizing the solution at 0.25 g L^{-1}, which resulted in a 52% increase in yield. The results indicate that the microalga is a nature friendly, renewable and economic resource for red beet production.

Keywords: *Asterarcys quadricellulare*, *Beta vulgaris L.*, biofertilizer, renewable agriculture

Introduction

The world's future food security depends on achieving an enduring agricultural sustainability. Modern agriculture must be more efficient, but the intensification of agriculture has caused impacts on the environment (Pingali, 2012; Muller et al., 2017). Therefore, sustainable inputs acquired importance as alternatives to traditional farming methods. Their use has shown importance, enhancing yield and environment quality (Singh et al., 2011; Renuka et al., 2018; Alvarez et al, 2021). One important vegetable, the red beet (*Beta vulgaris* L.), belongs to the Chenopodiaceae family (Pethybridge et al., 2018) and is rich in vitamins and minerals, characterized by its red color and sweet flavor. World production is over 240 tons per year (Neelwarne et al., 2013, Enock, 2022).

Some algae are considered renewable sources of biofertilizers (Mógor et al., 2022). According to Brazilian regulations, natural sources with plant growth promotion capacity are part of a class of products called biofertilizers, products that can act directly or indirectly on all or parts of the cultivated plants (Brasil, 2020). In that regard, protein rich solutions, composed mainly by signaling peptides and free amino acids have shown various of such results (Colla et al., 2014). These have been usually associated with their bioactive molecules (Calvo et al., 2014). Besides the range of free L-amino acids, compounds such as polyamines are incorporated by plants, improving their performance (Mógor et al. 2018).

Microalgae are photosynthetic microscopic organisms that grow in a large range of aquatic habitats, including wastewaters. These organisms present high growth rates, adaptability and exhibit a pool of traits that make it an economical and sustainable bioresource, that has been successfully applied in different fields. In agriculture, microalgae have shown plant growth promotion (Prasanna et al. 2016, Azaman et al. 2017,

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Mógor et al., 2017, Chanda et al., 2019).

Among thousands of microalgal species, the agricultural potential of some poorly studied, and previously overlooked algal biotypes can be of importance. This includes *Asterarcys quadricellulare*, a green eukaryotic microalga from the Chlorophyta class (Hong et al. 2012). *A. quadricellulare* shows a high content of proteins, carbohydrates and lipids, indicating it might be an adequate source for agricultural inputs (Varhneya et al. 2018; Singh et al. 2019). Recent works indicated the capacity of *A. quadricellulare* (CCAP 294/1) strain on promoting plant growth, yield gains, bud sprouting, and polyamine metabolism in different crops (Cordeiro et al. 2022, Mógor et al. 2022, Lara et al. 2022).

With limited resources, studies must be conducted to meet a 60% demand increase in agricultural yield for the next 30 years. Therefore, the aim of this work was to evaluate the effect of *A. quadricellulare* (CCAP 294/1) biomass on growth, yield and biochemical variables of red beet plants grown in organic system.

Material and methods

Cultivation conditions

The experiment was conducted in the area of organic vegetable studies, at the Experimental Station of Canguiri, Federal University of Paraná, located in the city of Pinhais, Brazil, at 25°23'30'' S and 49°07' 30'' W, and average altitude of 920 meters, of temperate climate, Cfb-type according to the Köppen classification. Chemical analysis of the 0-20 cm soil layer at the experiment site indicated the mean values: 6.30 pH (H2O), 33.30 g.dm-³ of organic matter; 0.1331 g.dm-³ P; 0.563 g.dm-³ K; 1.86 g.dm-³ Ca; 0.523 g.dm-³ Mg; 0 g.dm-³ Al; 0.332 g.dm-³ Al + H; and 80% base saturation. Seven days before sowing, the soil was prepared to incorporate 8 t. Ha^{-1} of organic compost with the following mean values: N= 25 $g.kg^{-1}$, C = 30.3 $g.kg^{-1}$, P = 8.5 $g.kg^{-1}$, K = 6.6 g.kg-¹, Ca = 8.1 $g.kg^{-1}$, Mg = 4.1 g.kg-¹.

The cultivar used, Early Wonder Tall Top (TopSeed ®), is considered a plant with large, upright foliage, smooth roots – which are, morphologically, the expanded hypocotyls of the plant (Goldman & Janick, 2021), and an intense red color, reaching an average weight of 170 grams. The average cycle after sowing is 65-75 days. The sowing took place directly on soil beds,at a depth of 2 cm, using a spacing 0.25 m between rows and 0.10 m between plants. Thinning was performed 30 days after seedlings emergence (DAE).

Asterarcys quadricellulare biomass

The microalgae biomass *Asterarcys quadricellulare* (CCAP 294/1) supplied by Alltech® Crop Sciences – Brazil, produced in a mixotrophic type cultivation, then atomized using spray drying method, resulting in a fine greenish powder. Following cell disruption (Show et al. 2015; Stirk et al. 2020), the free amino acids were extracted (Magné and Larher 1992; Winters et al. 2002), indicating a concentration of 90.94 mg g^{-1}, which corresponds to 9% w/w of the microalgae biomass. Determinations of the amino acid profile of *A. quadricellulare* (CCAP 294/1) and protein content were performed according to the methodologies of Lucas and Sotelo (1980), White et al. (1986), and Hagen et al. (1989). The contents of the amino acid were determined using an SPC1000 amino acid analyzer adapted to the pre-column derivatization method with phenylisothiocyanate (PITC) and quantification by reverse-phase high performance liquid chromatography (HPLC) using UV detection at 254 nm. The set consisted of a degasser, a quaternary pump module, a Rheodyne injection valve, an oven module, and a UV detection module, equipped with a LUNA C18 100 Å 5µ column, 250 × 4.6 mm 00G-4252-EQ. The amino acid score was calculated through the ratio between the values of essential amino acids in the samples (mg g^{-1}) and the standard values (FAO/WHO 1991) indicating the content of: 4.24% glutamic acid, 3.32 % aspartic acid, 2.41 % alanine, 2.17 % arginine, 2.36 % leucine, 2.11 % lysine, 1.66 % serine, 1.54 % glycine, 0,71 % histidine, 1.45 % threonine, 1.6 % proline, 0.95 % tyrosine, 1.81 % valine, 0.51 % methionine, 0.29 % cysteine, 1.41% isoleucine, 1.37 % phenylalanine, 0.37 % triptophan.

Treatments

The *A. quadricellulare* (CCAP 294/1) (AQ) biomass was suspended at the following concentrations: 50, 150, 250, and 400 g L^{-1}. From each suspension, a 1 mL L^{-1} aliquot was taken and diluted with distilled water, resulting in solutions equivalent to the biomass concentrations of: 0.05 g L^{-1} (AQ 5); 0.1 g L^{-1} (AQ 10); 0.15 g L^{-1} (AQ 15); and 0.25 g L^{-1} (AQ 25). Foliar sprays of these solutions containing a fraction of the microalgae biomass were applied weekly to the plants, plus a spray of water control. Treatments were arranged in a completely randomized design applied, in four repetitions each, once a week.

The beds were divided into 20 plots of 1.3 m² each, containing 52 plants per plot. Weekly applications began 30 days after emergence (DAE), immediately after thinning, and ended one week before harvest, totaling 11 applications. These were carried out with a pressurized CO_2 sprayer at constant pressure (40 psi) and consumption of 50 ml spray per plot, at a rate of 394 L ha^{-1}. The area was irrigated through sprinklers, maintaining soil

moisture at 80% established with aid of tensiometer.

Biometric Assessments

Harvest took place 80 days after sowing (DAS), for biometric and biochemical analysis of the plants. Eight central plants were collected from each plot, and the border plants were excluded. Both aerial part and roots (hypocotyls) were analyzed. With the aid of a ruler, the individual root-hypocotyl were weighed and classified, into the following classes: I: diameters up to 35 mm, II: > 35 to 50 mm, III: > 50 to 70 mm, and IV: > 70 mm. Mass, leaf area, and biochemical composition were evaluated. Fresh masses (g) were measured on a precision scale. Dry mass (g) was quantified, after drying in an oven at 65 °C with forced air circulation until constant weight, measured on a precision scale. The leaf area (cm^2) was obtained using the computer program WinRhizo®, coupled to an LA1600 Scanner (Regent Instruments Inc., Canada).

Biochemical analysis

At 80 DAS, 4 central plants were collected from plots, between 9 and 10 am, to perform biochemical analyses of the leaves and root-hypocotyls. From the collected plants, two fully expanded leaves of the middle-third part of plants and their root-hypocotyls were selected for samples. Leaves and roots samples were then frozen and macerated in liquid nitrogen immediately after harvest, macerated until obtained a fine powder.

Total sugars were determined with a standard curve obtained with glucose at 1 mg mL^{-1} (5.5 mM), with values between 50 and 800 µg mL^{-1}. Readings were performed at 540 nm (Maldonade et al. 2013) and the values were expressed in micrograms of sugars per gram of fresh plant material.

For pigments, two analyzes took place. The first, at 60 DAE, determined the relative levels of chlorophyll using the portable chlorophyll meter (N-Tester). For the second, chlorophyll and carotenoids were extracted with 80% acetone in distilled water and the addition of 0.1% CaCO3 (w/v) and readings were performed on a UV–visible spectrophotometer at 663, 647, and 470 nm (Lichtenthaler and Buschmann, 2001), and the values expressed in micrograms of chlorophyll per gram of fresh plant material used.

Total free amino acids were extracted, and the colorimetric reaction was performed with 1 mL of the sample plus 0.5 mL of 0.2 M pH 4.6 citrate bufer and 1 mL with ninhydrin solution (1% ninhydrin, 3% ascorbic acid in 2-methoxy ethanol). Readings were made at 570 nm. A standard curve was made with glutamine and asparagine (2 mM) with values ranging from 28 and 140 µg mL^{-1} (Magné and Larher 1992) and the results are expressed in micrograms of total free amino acids per gram of plant material.

Statistical analysis

Data normality was tested using the Shapiro-Wilk test, and homoscedasticity using the Bartley test. For the ANOVA, the Duncan Test was applied when $p<0.05$. Variables with significant differences were submitted to regression analysis or comparison of means by Duncan's test for coefficients of determination (R2) < 0.7. Data that did not show normality and/or homoscedasticity were transformed by Box-Cox, to meet the ANOVA criteria. The non-parametric Kruskal-Wallis test was used for the percentage test. Statistical analyzes were performed using Assistat software (Silva e Azevedo 2016).

Results and Discussion

The AQ biomass applications had an observed effect of promoting plant growth on both root-hypocotyls and leaves. The fresh mass of leaves was influenced, and the three highest concentrations resulted in increments (**Figure 1** – a), reaching an increase of 52.5% in compared to the control, for AQ 15. Root-hypocotyl's fresh mass showed changes in plants treated with the microalga. Application of AQ 25 interfered significantly, resulting in an increased mass of 53.6% compared to the control (Figure 1 – b). As for root-hypocotyl's dry mass, AQ 25 applications promoted an increase of 27.4%, compared to the control (Figure 1 – c).

In biochemical evaluations of sugars in plant tissues, there were significant changes in both leaves and root-hypocotyls. This may indicate that there was an improved fixation of carbon onto sugars by the plants that received the microalga application. The leaves that received the AQ 25 sprays showed, on average, a 31% increase in the total sugars of leaf tissues (**Figure 2** - a).

Chlorophyll content was determined two times during the experiment. In none of the analyzes were there significant differences in the pigment content of the plants. They varied from 118 µg/g for the control, to 123 µg/g for AQ25, with an avarage of 108 µg/g, and a variation of 19%.

The application of AQ showed a considerable increase in free amino acids in leaf tissues. Biochemical evaluations showed a linear effect on increasing amino acid concentrations (**Figure 3**). This increase reached 154.6% for AQ 25 compared to control.

Treatments with AQ 25 increased the yield of treated plants compared to the control. This proved to be possible due to the increase in masses with the

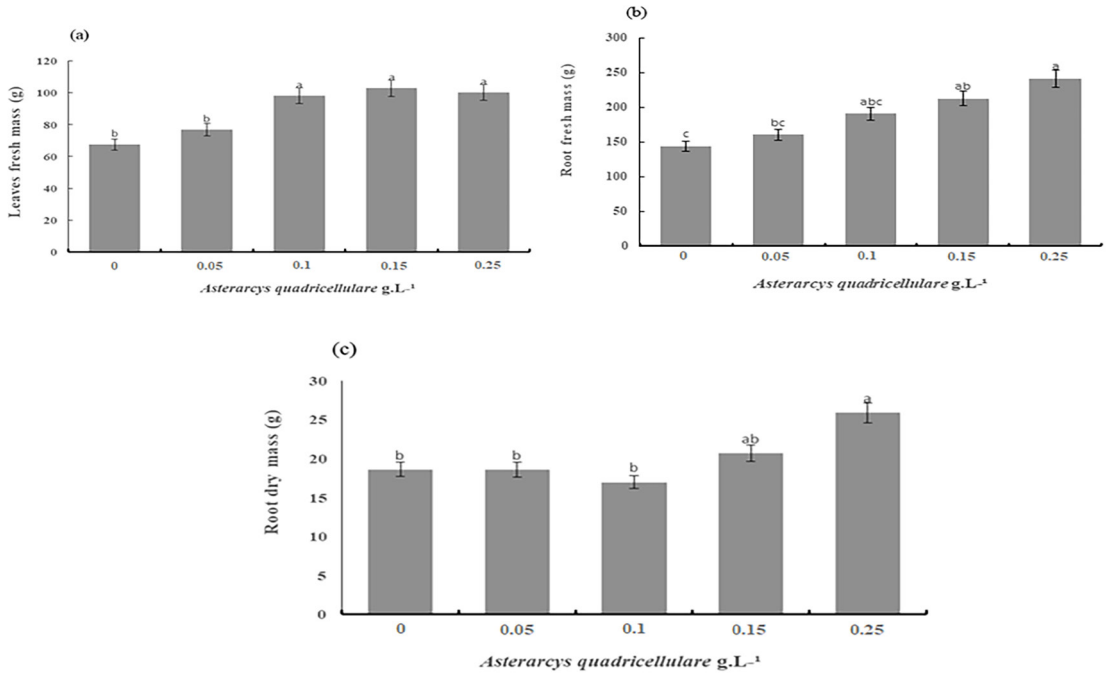

Figure 1. Fresh leaf mass (a), hypocotyls fresh mass (b), and dry mass (c) of beets submitted to foliar applications of *Asterarcys quadricellulare*.

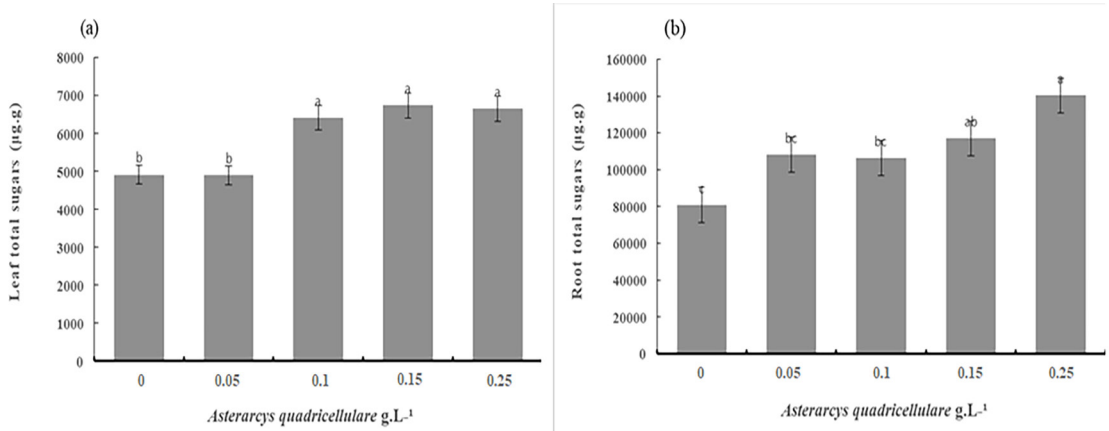

Figure 2. Total sugars in leaves (a) and in hypocotyls (b) of beets subjected to foliar applications of *Asterarcys quadricellulare*.

application of microalga, larger masses translated into greater yield per hectare. The data (**Figure 4**) for yield presented a linear behavior, adjusting to the regression model.

The increase in yield is related to the expansion of root-hypocotyls, as shown in their classification by diameter. There was an increase in the percentage of vegetables in class IV (Ø>70 mm) for the AQ 25 compared to the control (**Figure 5**). In the commercialization of beet, the diameters in classes III and IV are of greatest commercial interest and, therefore, of greatest value. There was an increase in yield with AQ 25, in mass, and in percentage of class IV root-hypocotyls, compared to the control. Thus, the gains were both in yield per hectare and in the commercial value per vegetable, resulting in a more profitable production.

The red beet is a traditional and popular vegetable in many parts of the world. It is eaten from roots to leaves, both being a source of innumerous health benefits (Ceclu et al. 2020). *Asterarcys quadricellulare* exibits an important concentration of protein and carbohydrate (Varhneya et al. 2018). It also shows high production of pigments and has been evaluated safe and nontoxic (Singh et a. 2019, Mustafa et al. 2022), indicating it as potential biofertilizer source for horticulture use. The effect of such other microalgae was observed in lettuce (*Lactuca sativa*), where its application increased the fresh and dry weight of plants (Faheed et al. 2008). In comparison, the results here obtained show that the accumulation of biomass was significantly higher in plants

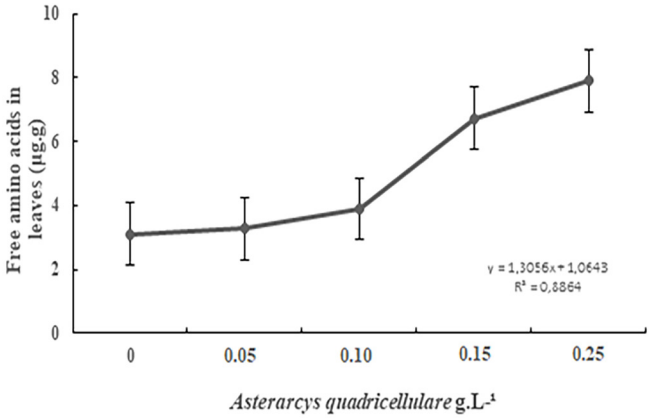

Figure 3. Free amino acids in leaves of red beets subjected to foliar applications of *Asterarcys quadricellulare*.

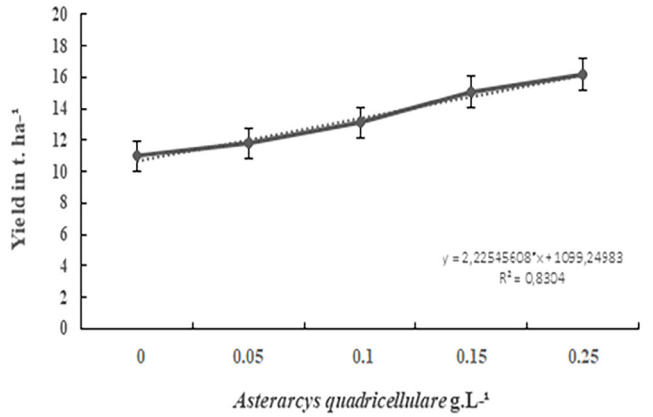

Figure 4. Yield on red beets subjected to foliar applications of *Asterarcys quadricellulare*.

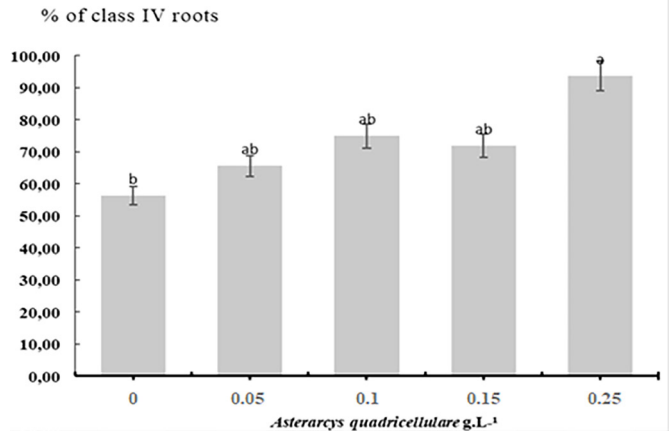

Figure 5. Values of the sum of ratings (ASR) in Percentage of hypocotyl calibers in beets treated with different concentrations of *Asterarcys quadricellulare*.

treated with AQ 25. Red beets responded in a study using *Arthrospira platensis*, where there was an increase in root-hypocotyl growth, biofertilizing effect related to the free L-amino acid content in cyanobacteria biomass (Mógor et al. 2018).

The growth and yield of drain organs, such as an expanding beet root-hypocotyl, depends heavily on the amount of carbohydrates (sugars) and nitrogen (N) compounds transported from the source leaves to such drain tissues (Thomas et al. 2009). While the leaves presented a 31% addition, this increase was around 74% when it came to sugars in same plant's root-hypocotyls, compared to the controls. This indicates that these sugars were produced, then translocated from the treated source leaves, towards the drains, which in the red beet cycle, are the roots (Sediyama et al., 2011).

One can argue that there was amino acid supply, as fertilizing input, providing nitrogenous compounds for plant development. But as the microalga's concentration in the applied solution was so small, it cannot have had a fertilizing effect. Therefore, the results must be explained by an amino acid metabolic stimulation through transporters in plant cell membranes, regulating plant metabolism and promoting growth (Dinkeloo et al. 2018; Yang et al. 2020). Effects on the expression of genes related to nutrient acquisition and root traits have been reported with microalgae before (Barone et al. 2018).

In the recent past decade, studies identified many amino acid transporters active in cell membranes and some genes with a proposed role in source to sink translocation of amino acids (Tegeder, 2012). It has been reported that treatment with protein hydrolysates increased the expression of genes encoding for amino acid permeases and transporters of amino acids and nitrogen in cucumber (Wilson et al., 2018). Treatment with a free L- amino acid rich solution, even at a small concentration as the one used in this experiment, may have triggered similar results, stimulating plant growth, as previously reported by Röder et al. (20018), using free L-amino acid obtained by bacterial fermentation.

Some studies with foliar applications of free L-amino acids (L-aa) in various forms have also improved plant biochemical variables, demonstrating the direct effects of L-aa leaf supply (Khan et al. 2012; Tsouvaltzis et al. 2014; Yu et al. 2015, Teixeira et al. 2017).

Note that in the aminogram for the AQ biomass, the main amino acid present is glutamic acid/ glutamate (Glu). The first molecule produced by plants from N is Glutamine (Gln), followed by Glu. They are transformed to synthesize all protein compounds of the cell (Pratelli et al., 2014). Biosynthesis pathways for these L-aa are regulated and communicate to other plant development pathways (Okumoto, 2016). These L-aa (Gln/Glu) also act as precursor molecules or as plant signalers. It is well established that the addition of Gln/Glu induces a transcriptional change in plants (Gutiérrez et al. 2008),

and the imbalance of these amino acids induces stress responses (Yu et al. 2015).

As the N metabolic process continues, glutamine is transformed, by a single decarboxylation reaction, into amino acid GABA (γ-aminobutyric acid), which exerts its effects in plants via transporters. These effects include the regulation of root growth, nutrition and is considered a legitimate signaling molecule (Ramesh et al. 2015).

As the addition in amino acid leaf concentration was considerable (Figure 3), and fixation of carbon was improved and translocated into root-hypocotyl sugars (Figure 2-c), there was a clear nitrogen metabolism enhancement. One important part of the N metabolism is the nitrate reductase enzyme, one responsible for the assimilation of N in plants (Röder et al, 2018). AQ showed to increase the activity of the nitrate reductase enzyme in potatoes (Cordeiro et al. 2022). It also improved spermidine content in sugarcane sprouts, indicating the polyamines pathway that was triggered by microalga biomass (Mógor et al., 2022). Therefore, the stimulating properties of this microalga may be in the way of increasing the plants own endogenous synthesis of amino acids and its concentration in plant tissue, as was found in red beet leaves (Figure 4), effect also reported using L-aa from bacterial fermentation (Röder et al, 2018), and microalgal hydrolyzed biomass (Mógor et al 2017).

An increase in sugars is desirable for many reasons. Plants can turn them into cellulose, use them as transporters or transform them into yield (Moghaddam et al. 2010). Carbohydrates generated by photosynthesis are the building units and energy providers to produce and support plant biomass. In addition, they tightly control transcriptional processes in planta acting as signaling molecules (Muller et al. 2011, Keunen et al. 2013). As the main product of photosynthesis and its intimate involvement in growth, development, storage, signaling and stress acclimation, sugar is regarded as the key in plant life (Salerno & Curatti, 2003). Previous reports stated that red beetroot is among the top ten vegetable species with the strongest antioxidant properties because of betalain (Baião et al., 2020). Betalains are secondary metabolites derived from the amino acid L-tyrosine, the major advantages of betalains as dietary antioxidants are their bioavailability, which is greater than that of most flavanoids, and their superior stability in comparison to anthocyanin. Therefore, an increase in sugars and amino acids in red beet content, besides its economic advantage, can be of great value to consumers.

The remarkable increase in amino acids in the red beet leaves obtained with the application of AQ 25, may have stimulated the assimilation and translocation of carbohydrates to drain tissues of expanding roots-hypocotyls. This improvement translated into a significant increase in yield, of approximately 53%. The results showed the effect of *Asterarcys quadricellulare* as a promising renewable source of bioactive free L-aa, for use as a biofertilizer in agriculture. New studies must be conducted to better elucidate the physiological and especially molecular mechanisms, related to the results obtained in this research.

Conclusion

The application of microalgae *Asterarcys quadricellulare* (CCAP 294/1) biomass had a significant influence on growth and yield parameters of red beets grown in an organic system. The suspension concentrating 0.25 g. L^{-1} of microalga biomass was highlighted. These findings can be attributed to the biologically active free L-amino acids present in the biomass, that in low concentration promoted an increase in red beet leaf and root-hypocotyl sugars, and a remarkable increase in leaf amino acid concentration. The sprays also performed an increase in yield and improved beets marketable classification. The results indicate that the *A. quadricellulare* (CCAP 294/1) is an effective bioactive nature friendly source for biofetilizer production.

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