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Utilization of *Chlorella* sp. as biostimulant in the germination of melon seeds (*Cucumis melo* L.)

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Abstract

Bioactive microalgae compounds have been shown to be excellent stimulants for seed germination. This study aimed at the production of biomass of *Chlorella* sp. through the modulation of the culture medium and its further use as bioestimulant in melon seed germination (*Cucumis melo* L.). *Chlorella* sp. cells were grown in medium containing distinctive levels of nitrogen (N), phosphorus (P), potassium (K) and carbon (C), and analyzes of yield, hygroscopic behavior and the amino acid profile of the biomass were carried out for all treatments. In addition, mechanical hydrolysis of the biomass through high-energy wet grinding was performed, obtaining suitable particle size. After biomass characterization, an aqueous solution of the hydrolyzate was used for biostimulation of melon seeds. The different growth medium composition affected biomass yield and amino acid profile. The most significant effects for seed biostimulation were obtained during growth in medium containing intermediary or low levels of NPK and C, with germination index (GI) of 92 and 95%, and germination speed index (GSI) of 8.00000 and 8,26087 seedlings.day^{-1}, respectively. It is concluded that the modulation of the cultivation of *Chlorella* sp. is a viable strategy for the production of active compounds. The hydrolysates of *Chlorella* sp. demonstrated a biostimulant potential and can be a source of molecules for many applications in agriculture.

Keywords: microalgae, biostimulants, amino acids, *Cucumis melo* L

1. Introduction

Plant biostimulants are sets of natural molecules, which, when applied to plants or the rhizosphere in small amounts, act synergistically promoting plant growth, improving fruiting,

nutrient absorption by the roots, increasing resistance to abiotic stresses, and do not generate waste for the environment, different from synthetic chemical stimulants (Chojnacka et al., 2014; Patrick, 2015; Ricci et al., 2019).

Algae extracts are good sources of biostimulants (Michalak *et al*., 2016; Michalak *et al*., 2017), and have received special attention due to their functional and phytochemical properties, due to the bioactive compounds in their composition, including high levels of proteins, peptides, essential amino acids and plant hormones (cytokinins, auxins, gibberellins, abscisic acid and ethylene) (Khan et al., 2009; Stirk et al., 2013; Stirk et al., 2013). Some microalgae species such as *Scenedesmus obliquo*, *Chlorella* sp. and *Spirulina platensis*, have been presented as a good source of raw material for the production of plant biotimulants, and these have been used in all stages of agricultural production, including as seed treatment, as leaf sprays during growth and in harvested products (Guedes et al., 2018; Dias et al., 2019). The action of the mode / mechanisms of "biostimulants" is equally diverse and may include activation of nitrogen metabolism or release of phosphorus in soils, general stimulation of soil microbial activity or stimulation of plant root growth (Yakhin et al., 2017).

Chlorella sp. is a green microalga that inhabits salt and sweet waters, belonging to the Chlorophyceae class, and its biomass is mostly made up of 60% proteins, with lower concentrations of fats, fibers, carbohydrates, vitamins (folic acid lutein, B vitamins, E vitamins and beta carotene) and minerals (iron, calcium, potassium, zinc, manganese, selenium, magnesium). Approximately 2000 tons of biomass / year of this species are produced in over 70 countries (Zielinski et al., 2020) and the biomass of *Chlorella* sp. have in their composition an elementary amount of potentially active substances, capable of exerting biological effect in many plant species, as they contain some amino acids such as tryptophan and arginine, which improve the growth and yield of cultivated crops, and are metabolic precursors of the main phytohormones in plants (Colla et al., 2014; Colla et al., 2016; Colla and Rouphael, 2015; Rouphael and Colla, 2018). It is known that adequate concentrations of these amino acids promote stimulation of hormonal synthesis in plant pathways (Stirk *et al*., 2013).

The commercial preservation of microalgae is made possible by drying to moisture levels that prevent chemical, biochemical, and microbiological deterioration. After drying, the material remains stable if it does not adsorb water in the form of vapor present in the surrounding environment. Algae biomass is composed by numerous constituents that can influence its hygroscopicity, defined by the behavior of moisture adsorption isotherms. One of the main applications of equations describing isotherms refers to the energy of water bonds and their adsorption on the surface of materials. It is also necessary to consider the characteristics of the molecular monolayer of water, which are directly linked to the chemical reactions that occur in the deterioration of biological materials due to solid matrix response areas (Sanchez et al., 2008; Konig-Péter et al., 2015). Studies to predict the behavior of isotherms through model adjustments have sought to define the behavior of many materials of biological origin, which helps to explain the characteristics of each material and its reactivity over time (Rezaei *et al*., 2011; Jelínek *et al*., 2015). Most of these models are empirical, and due to the characteristics of each material, it is more difficult to find generalist models that can be used

in all matrices (Park et al., 2014). The biomass of *Chlorella* sp. is composed of a multivariate class of compounds whose nature determines their hygroscopic behavior, providing information for the proper conservation of the constituents of interest (Cui et al., 2018).

Here, we aimed to modulate the cultivation of the microalgae *Chlorella* sp. in different concentrations of nutrients. In addition, it was evaluated the hygroscopic behavior of the biomass, using isotherms of water adsorption at 25°C. The analysis of amino acid profile produced during cultivation, the mechanical hydrolysis of the biomass and its granulometry were also performed. Subsequently, the hydrolyzed biomass was used as a potential biostimulant for seed germination of melon (*Cucumis melo* L.).

2. Material and Methods

A flowchart of the stages of the cultivation and processing of microalgae C*hlorella* sp. and the stages of the analysis of biostimulation of seed germination is shown in Figure 1.

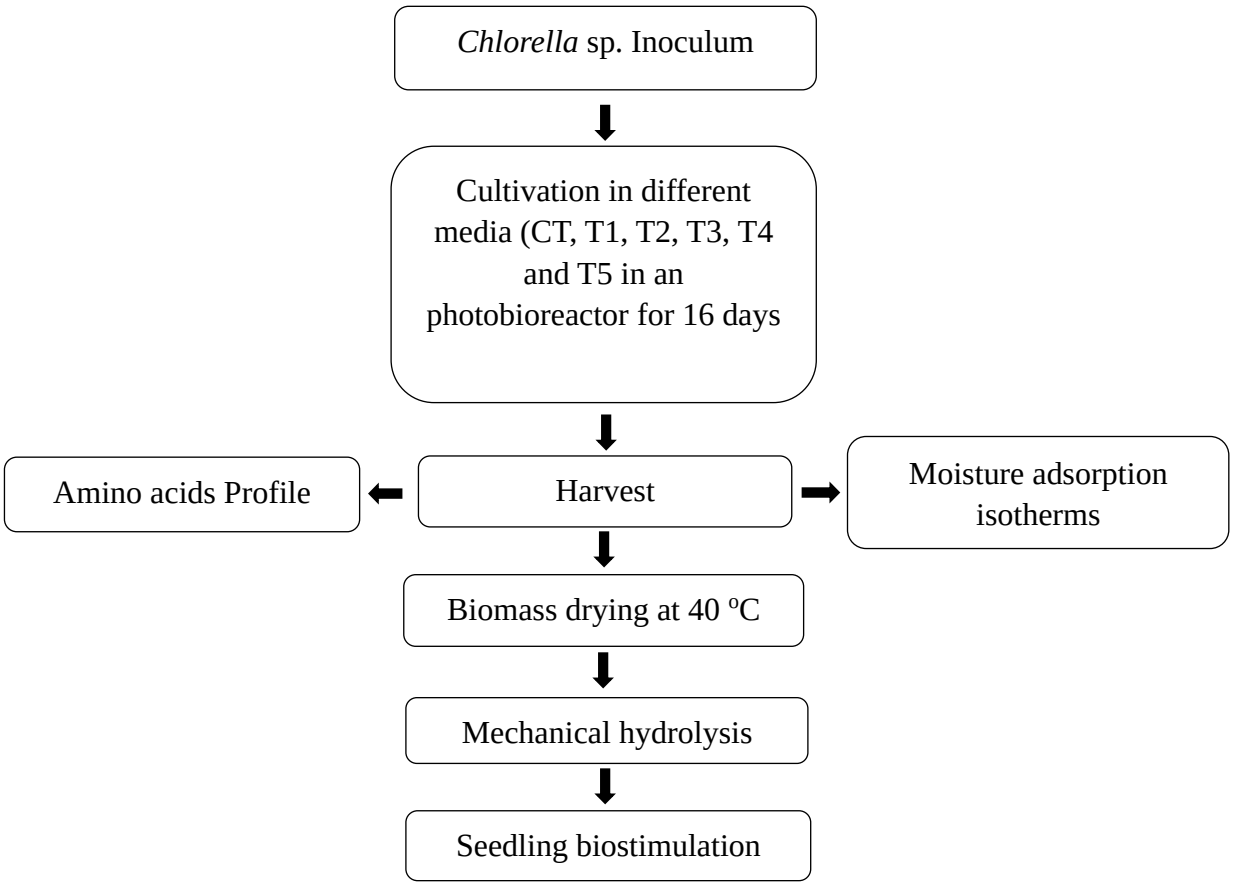

Figure 1. Flowchart of the cultivation and processing steps of the microalgae *Chlorella* sp

2.1 Microalgae Cultivation

The microalgae (*Chlorella* sp.) used in this work were grown in open photobioreactors, "raceway model", exposed to sunlight, with a volume of 10 m^3, with agitation performed with an axial aerator (Aeromack, CRE-01) with continuous flow of 1.4 m^3 / min, pressure of 1,200 mca, power of 0.5 HP. The culture medium was prepared according to (Watanabe, 1960;

Becker, 1994; Morais, 2007; Baumgartner, *et al*., 2013), called "Medium for *Chlorella ellipsoidea*", composed by KNO_3 nitrate potassium 1.25 g / L, KH_2PO_4 monopotassium phosphate 1.25 g / L, $MgSO_4$. 7 H_2O, $FeSO_4$ iron sulphate heptahydrate 0.02 g / L, A_5 (micronutrients 1mL / L solution composed of H_3BO_3 boric acid 2.90 g / L, $MnCl_2$. $4H_2O$ manganese chloride tetrahydrate 1.81 g / L, $ZnCl_2$ zinc chloride 0.11 g / L, $CuSO_4$. $5H_2O$ copper sulfate pentahydrate 0.08 g / L, $3(NH_4)_2O$. $7MoO_3$. $4H_2O$, ammonium selenate tetrahydrate 0.018 g / L, with changes in nutrient concentrations, corresponding to treatments with CT (control without changes in nutrient concentrations), T1, T2, T3, T4 and T5 as described in (Table 1).

Table 1. Concentration of chemical constituents for culture modulation *Chlorella* sp. for the different treatments

Treatments	KNO_3 (g/L)	KH_2PO_4 (g/L)	$FeSO_4$ 7H2O (mg/L)	$NaHCO_3$ (%)
CT	1.250	1.250	10.00	0.04
T1	0.625	0.625	5.00	0.04
T2	0.313	0.313	2.50	0.06
T3	0.157	0.157	1.25	0.08
T4	0.157	0.157	1.25	0.10
T5	0.157	0.157	1.25	0.12

Figure 1 shows the flowchart of the steps from cultivation to microalgae processing after 16 days of cultivation. Microalgae harvesting from different treatments was performed by flocculation with aluminum sulfate (Al_2 (SO_4) H_2O) with a concentration of 0.5 g / L for 30 minutes (Lira, 2011), followed by decantation. The decanted biomass of *Chlorella* sp. was placed on a 325 mesh nylon filter for 3 hours and then dried in a forced air circulation oven at 40 ºC and 2 m / s air velocity. After, the biomass was distributed in trays on slides with thicknesses of 0.3 cm and 0.5 cm in width and constant weight. After drying, the biomass was removed from the trays and ground in a hammer mill with a rotation speed of 8000 rpm and 0.5 mm sieve, obtaining the biomass powder from different treatments.

2.2 Moisture Adsorption Isotherms

The evaluation of the moisture adsorption isotherms at 25 ºC of *Chlorella* sp. The different treatments were determined in triplicate using the static-indirect method (Crapiste & Rotstein, 1982). Water activity readings were taken using a Decal Devices Aqualab model 3TE hygrometer and the equilibrium moisture content determined at 105 oC until constant weight. The GAB (Equation 1), Oswin (Equation 2) and Peleg (Equation 3) mathematical models

were adjusted to the moisture adsorption isotherms by nonlinear regression by the Quasi-Newton estimation method, using the Statistica software.

$$X_e = \frac{X_m C K a_w}{(1 - K a_w)(1 - K a_w + C K a_w)} \quad (1)$$

On what:

X_e - equilibrium moisture content;

a_w - water activity;

X_m - moisture in the molecular monolayer;

C e K - parameters that depend on the temperature of the nature of the product.

$$X_e = a\left(\frac{a_w}{(1 - a_w)}\right)^b \quad (2)$$

On what:

X_e - equilibrium moisture content

a_w - water activity

a e b - model tuning parameters

$$X_e = K_1 a_w^{n_1} + K_2 a_w^{n_2} \quad (3)$$

On what:

Xe - equilibrium moisture content

K_1 e K_2- constants of the equation

a_w - water activity

n_1 e n_2 - constants of the equation

The criteria used to determine the best fit of the models to the isotherms were the coefficient of determination (R^2) and the mean percentage deviation (P), calculated according to Equation 4.

$$P = \frac{100}{n} \cdot \sum_{i=1}^{n} \frac{\left|\left(X_{exp} - X_{teor}\right)\right|}{X_{exp}} \quad (4)$$

on what:

P - mean percentage deviation (%);

X_{exp}- experimentally obtained values;

X_{teor} - values predicted by the model;

n - number of experimental data

2.3 Amino Acid Profile Analysis

The determination of the amino acid profile in *Chlorella* sp. grown in different treatments was performed by the derivatization method using phenylisothiocyanate (PITC), after acid hydrolysis. A mixture of various amino acids was obtained, which were further subjected to analysis by high performance liquid chromatography (HPLC).

The proteins contained in the biomass powders were hydrolyzed at 110 °C with 6 N hydrochloric acid for 24 hours. PITC-reacted amino acids released in acid hydrolysis (Hagen et al., 1989) were separated by reverse phase HPLC using LUNA C18 column (100Å, 5 µm 250 x 4.6 mm; code 00G-4252-EQ), and quantified by UV detector at 254 nm.

Quantification was performed by multilevel internal calibration using α-aminobutyric acid (AAAB) as the internal standard (White et al., 1986). For the calculation of the content of each amino acid the molecular weight value of each amino acid was taken in condensed form, ie the full molecular weight of the amino acid, decreased by 18 a.m. (mass of a molecule of water) so as to consider the amino acid as being in its protein form. Tryptophan values were determined by spectrophotometry in alkaline medium.

The sample was subjected to enzymatic hydrolysis with pronase at 40 ºC for 24 h, then subjected to colorimetric reaction with *p*-dimethylene benzaldehyde (DAB) and read at 590 nm spectrophotometer (Spies, 1967). Tryptophan concentration was calculated by comparison with the standard curve.

2.4 Mechanical Hydrolysis and Analysis (SEM)

To perform mechanical hydrolysis, 50g of the biomass of each treatment was weighed in triplicate, and introduced in stainless steel milling stations, with a capacity of 400 mL, containing 20 mL of distilled water and 10 stainless steel balls of 10 mm in diameter. After, the biomass was processed in 0.4 L NOAH NQM planetary ball mill (China) with speed of 400 rpm, grinding times 5, 10, 15 and 20 min, based on Bello and co-workers (2015), with modifications. At the end of each grinding, the material was collected and dried under vacuum, and analyzed by scanning electron microscopy (SEM). For (SEM) analyses, the samples were fixed in a sample holder with the aid of carbon tape, and metallized with a conductive film, formed by a gold / palladium alloy and later analyzed in a scanning electron microscope (Shimadzu, SSX- 550), operating 15 KV, with an image increase of x2000. To determine the particle size ranges, the software Image-Pro Plus 7.0 (Media Cybernetics) was used.

2.5 Seed Germination

The seed germination was carried out with four replications of 50 yellow melon seeds, for each treatment, conducted in a greenhouse, at the Center for Science and Agri-Food

Technology - CCTA, Federal University of Campina Grande - UFCG, Campus Pombal, PB, at the geographical coordinates 6º 48' 16" south latitude and 37º49'15" longitude west, and altitude of 175 m. The climate of this region, according to Köppen, is of the Aw type, called semi-arid, hot and dry, with summer rains, with temperatures above 25 ° C and rainfall of 650 mm year irregularly distributed (Melo et al. 2008).

The substrate used for seed germination was Chromic Luvisol, autoclaved at 120ºC for 4 hours. Then, the substrate was arranged in 6 polystyrene trays of 200 cells, filling all cells in each tray evenly. For biostimulation of melon seeds. 6 aqueous solutions were prepared containing hydrolyzate from *Chlorella* sp. CT, T1, T2, T3, T4 and T5 treatments, with a final concentration of 1.5 mg / mL. Each solution was transferred to a 500 mL Erlenmeyer, and incubated at 70ºC for 15 minutes, with magnetic stirring at 300 rpm, in a magnetic heating shaker, based on (Navarro-Lopéz et al., 2020). Then, 10 g of yellow melon seeds were introduced for each of the six prepared solutions and left to rest for 6 hours before sowing. The sowing was performed in polystyrene trays of 200 cells, using seeds that were biostimulated, by sowing a seed in each cell of the tray. The irrigations throughout the experiment were carried out twice a day, at 9 am and at 4 pm.

The evaluation of seedling emergence was determined by reduced counts of seedlings emerging until stabilization, which occurred up to 10 days after sowing, considering that they emerged as seedlings with exposed cotyledons. To determine the emergency speed index (IVE), use an equation proposed by Maguire, (1962): IVE = E1 / N1 + E2 / N2 + ... + En / Nn; where: E1, E2, En = number of normal seedlings observed in the first, second and last count; N1, N2, Nn = number of sowing days in the first, second and last count. After stabilization, an emergency percentage was determined using the formula: % E = N / A, where N = number of seeds emerged; A = total number of seeds sown. Average germination time calculated according to the formula proposed by Labouriau (1983), with results expressed in days.

At 25 days after sowing, plants were harvested, washed to remove the substrate adhered to them and taken to the laboratory, where the following variables were considered: number of leaves (NL), length of the aerial parts, (LAP), length of the root (LR), diameter of the capsule measured with the aid of a caliper, fresh mass of the aerial part (FMAP), fresh root mass (FRM) dry mass of the aerial part (DMAP), dry mass of the root (DMR). To determine the dry mass, the plant material was placed in an oven with forced air circulation, at 95°C, until constant mass was obtained. An analysis of variance was used to verify the effect of bioestimulation, followed by the Tukey test with 5% probability, using the SISVAR computer program (Ferreira, 2011).

3. Results and Discussion

3.1 Microalgae Cultivation

Table 2 presents the average values of the data referring to dry weight of *Chlorella* sp. biomass in the CT, T1, T2, T3, T4 and T5 treatments. The most expressive results were obtained when the microalgae was grown in the control treatment, where the biomass reached 4.85 g/L of dry biomass on the 10th day, followed by T1 treatment, which yielded 4.75 g/L of

dry biomass on the 10th day. Cell growth in treatment T2 did not show significant difference between days 10, 12, 14 and 16, with average values of 3.63, 3.79, 3.57 and 3.58 g / L of dry biomass, respectively; in treatment T3 the best growth was achieved on the 12th day of cultivation with 2.63 g / L of dry biomass. Moreover, treatment T4 had the greatest growth on the 16th day with 2.86 g / L of dry biomass, and the T5 treatment showed the highest growth in the 14th with 1.77 g / L of dry biomass, but there were no significant differences between the averages at the times 10, 12, 14 and 16 days indicating that, statistically, the 10th day can be considered as the maximum growth.

Although each element in the medium has its relative importance for algae nutrition, there is no exact number of essential chemical elements, as certain elements are essential for certain species and are not essential for others (Baumgatner et al., 2013). Among the elements contained in the formulation of the culture medium, nitrogen (N) is considered to be the most important because it is the constituent element of many compounds of the primary metabolism, its abundance in the culture medium can favor the synthesis of proteins and chlorophyll and its deficiency tends to reduce cell growth rates (Lourenço, 2006; Bertoldi, 2008). Moreover, the deprivation of nitrogen, phosphorus and potassium might induce the cells to a growth route with priority in the intracellular reserve stock, to support the scarcity of these nutrients, showing that an adjustment in the ideal amount of NPK can result in the production of biomass in greater volume, along with other products of interest, such as carbohydrates and lipids (Martin et al., 2014).

Table 2. Average values of dry weight of *Chlorella* sp. (g / L) during the cultivation in different treatments

Time	Dry weight (g/L)					
(day)	CT	T1	T2	T3	T4	T5
1	0.34 gA	0.34 fA	0.33 fA	0.38 fA	0.36 gA	0.39 eA
2	1.03 fA	1.06 eA	0.90 eAB	0.63 eC	0.60 gC	0.77dBC
4	2.80 eA	2.61 dA	2.00 dB	1.34 dC	0.97 fD	1.08 cD
6	3.67 dA	3.73 cA	2.91 cB	1.82 cC	1.30 eD	1.13 cD
8	4.33 cA	4.05 bB	3.22 bC	2.00 cD	1.82 dD	1.42 bE
10	4.85 aA	4.75 aA	3.63 aB	2.33 bC	2.00 cdD	1.64 abE
12	4.69 abA	4.21 bB	3.79 aC	2.63 aD	2.18 cE	1.77 aF
14	4.61 abA	4.09 bB	3.57 aC	2.50 abD	2.53 bD	1.75 aE
16	4.54 bcA	4.01 bB	3.58 aC	2.43 abE	2.86 aD	1.66 abF

GM = 2.34; CV = 4.04%; SMD for columns = 0.24; SMD for lines = 0.22; GM - General average; CV - Coefficient of variation; SMD - Significant minimum deviation note: Averages followed by the same lowercase letter in the columns and uppercase in the lines do not differ statistically by the Tukey test, at 5% probability.

As observed, the increase of the carbon source in treatments T3, T4 and T5, did not favor the increase of the cellular concentration (Tables 1 and 2), suggesting that nitrogen was not the limiting factor for the reduction of cell growth, but the element phosphorus (P), as this element is necessary for the normal growth of algae. Potassium is the element of greatest

abundance in the culture medium; however, it was also not the limiting factor for cell growth in this study

The biomass production was, in all treatments, satisfactory, when compared with the production reported by Matos (2012), working with *Chlorella vulgaris* grown in BBM (Bold Basal Medium), producing 0.590 g / L of dry biomass; by Li *et al*. (2011) producing 2.83 g / L of dry biomass of *Chlorella vulgaris* using the SRH (hydroponic residual solution) and glucose as a carbon source; by Putri et al. (2011) with a production of *Chlorella vulgaris* of 0.118 g / L, *Chlorella pyrenoidosa* of 0.058 g / L and *Chlorella sorokiana* of 0.160 g / L by Niels et al. (2012) with a production of *Chlorella* sp. 0.410 g / L and very short 0.490 g / L *Chlorella* using the modified BG11 (blue green medium) culture medium and by Mokashi et al. (2016) with productivity of 0.160 g / L of dry biomass in the cultivation of *Chlorella vulgaris* with BG11 culture medium (blue green medium). It is concluded that the cell growth obtained in this work is in accordance with the literature.

3.2 Moisture Adsorption Isotherms

We analyzed moisture adsorption isotherms at 25ºC of *Chlorella* sp. submitted to different treatments. Table 3 shows the parameter values of the Oswin, GAB and Peleg models adjusted to the 25 ºC moisture adsorption isotherms of *Chlorella* sp. for CT, T1, T2, T3, T4 and T5 treatments, in addition to the determination coefficients (R^2) and the mean percentage deviations (P).

All determination coefficients (R^2) showed values above 0.97 and mean percentage deviations (P) below 10%, indicating that all models tested (GAB, Peleg and Oswin) for the prediction of moisture adsorption isotherms of biomass can be used accurately. Several researchers have found good estimates of isotherms with these models (Alexandre *et al*., 2007; Lima et al., 2008; Silva et al., 2015; Ribeiro et al., 2016; Trevisan, et al., 2019). The model that best fit the experimental isotherm data for the CT, T1, T2 and T4 treatments was Peleg, and for the T3 and T5 treatments, GAB. This behavior was expected due to the larger number of parameters of these models.

It is verified for Oswin's model that the constant 'a' had values ranging from 4.9262 (T4) to 6.1936 (T2) and for the 'b' constant from 0.5134 (T3) to 0.6295 (T4), being in agreement with Alexandre et al. (2007), which mentioned that for this model the values for the constant 'a' must be greater than zero and for the constant 'b' must be between zero and 1.0. Close values were found for the water adsorption isotherm at 20 ºC of dried mango residual fiber flour, with 'a' equal to 7.79 and 'b' equal to 0.88 (Silva et al., 2015).

The GAB model presented moisture values in the molecular monolayer (Xm) ranging from 2.8427 (CT) to 9.4789% (T4). The value of Xm indicates the amount of water that is strongly adsorbed to the active sites on the surface of the products and is also related to the stability of the product, being considered as the best value to guarantee stability (Gabas *et al*., 2009; Fabra *et al*., 2011). Moisture levels in the molecular monolayer (Xm) within this range were found for dried *spirulina*, with values of 6.9, 7.3 and 8.2%, at temperatures of 10, 20 and 30 ºC, respectively (Oliveira *et al*., 2009a).

The constant C determines the binding force of water molecules to the primary sites on the product surface (Bhusari *et al.*, 2016), and ranged from 0.3951 (T4) to 117.1637 (CT). Oliveira et al. (2009b) found for *Spirulina platensis* C values ranging from 36.14 to 37.41.

The constant K of the GAB model ranged from 0.8278 to 0.9499, with values close to that determined by Oliveira et al. (2009a) for the adsorption isotherms of dried *spirulina*, which ranged from 0.92 to 0.97. The K value provides a measure of the interactions between molecules in the multilayer and the adsorbent and tends to lie between the energy value of the molecules in the monolayer and liquid water (Cano-Higuita et al., 2015).

Analyzing the values of the parameters K and C of the GAB model and according to the Blahovec (2004) classification, the moisture adsorption isotherms of the powder biomass for the treatments CT, T1 and T5 were classified as type II ($0 < K \leq 1$, $C > 2$) and for T2, T3 and T4 as type III ($0 < K \leq 1$, $0 \leq C \leq 2$).

Table 3. Parameters, coefficients of determination (R^2) and mean percentage deviations (P) of the Oswin, GAB and Peleg models adjusted to the moisture adsorption isotherms at 25 ºC of *Chlorella* sp. for TC, T1, T2, T3, T4 and T5 treatments

Treatment / Model		Parameter				R^2	P (%)
		a	b				
Oswin	CT	5.3563	0.5237			0.9974	2.04
	T1	5.7243	0.5278			0.9868	5.56
	T2	6.1936	0.5313			0.9834	5.02
	T3	5.1677	0.5134			0.9842	7.42
	T4	4.9262	0.6295			0.9860	7.42
	T5	5.9119	0.6019			0.9980	1.36
GAB	Parameter	X_m	C	K		R^2	P (%)
	CT	2.8427	117.1637	0.9267		0.9941	2.86
	T1	3.1491	33.1764	0.9220		0.9909	4.69
	T2	4.4858	2.5589	0.8853		0.9918	4.84
	T3	4.4257	2.0589	0.9064		0.9933	5.20
	T4	9.4789	0.3951	0.8278		0.9960	3.81
	T5	3.2570	68.7968	0.9499		0.9987	2.33
Peleg	Parameter	K_1	n_1	K_2	n_2	R^2	P (%)
	CT	30.8481	17.749	13.7767	1.2627	0.9989	1.36
	T1	25.3102	6.7153	6.1091	0.0252	0.9958	2.52
	T2	6.0412	0.2670	25.5107	5.4444	0.9960	2.61
	T3	24.2719	4.1146	1.7216	-1.036	0.9871	6.49
	T4	29.5145	5.4416	3.4002	-0.054	0.9977	1.69
	T5	34.7553	10.0032	11.4451	0.79226	0.9979	2.66

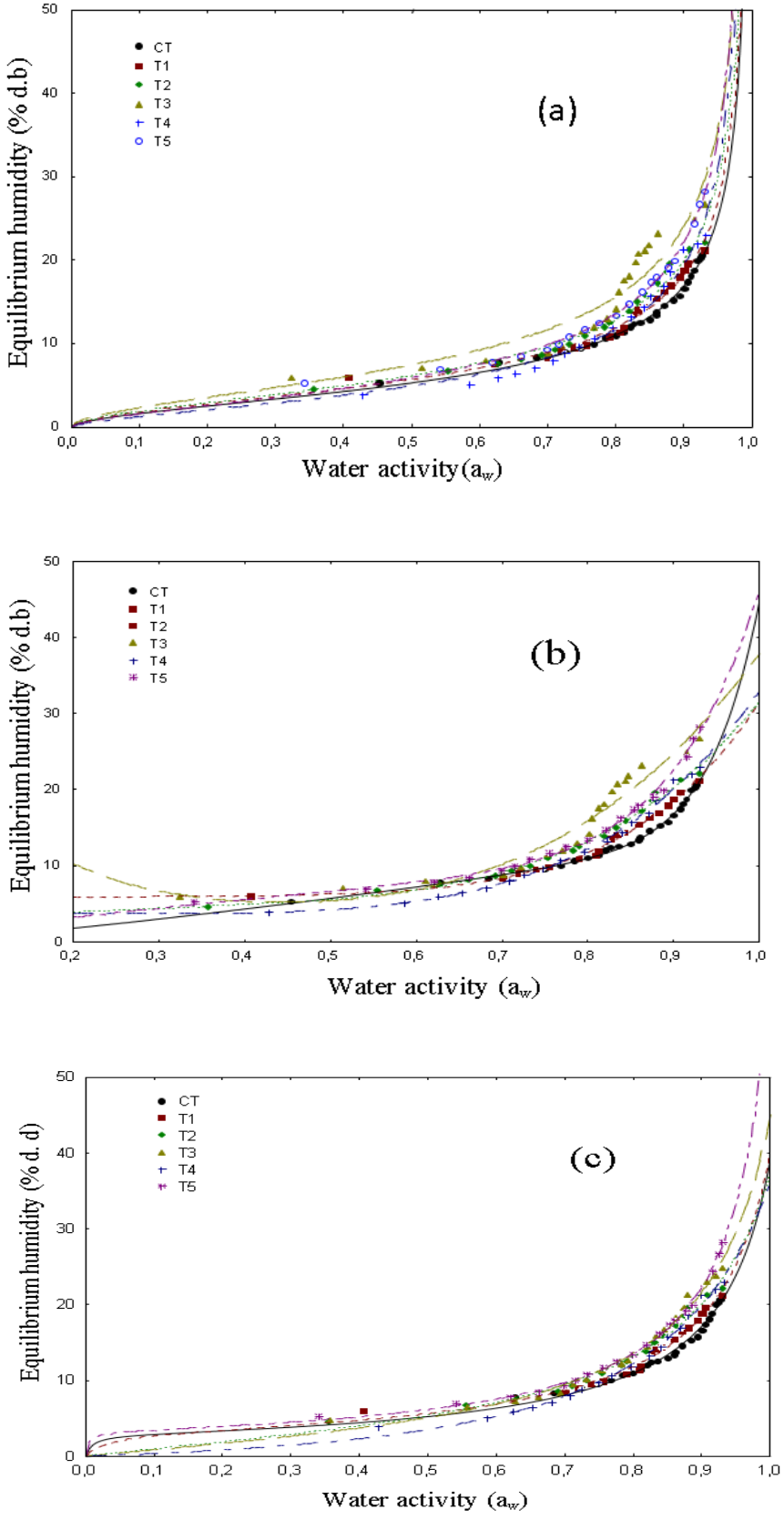

Figure 2. Moisture adsorption isotherms at 25ºC of *Chlorella* sp. for the different treatments with adjustments of Oswin (a), GAB (b) and Peleg (c)

Figure 2 shows the moisture adsorption isotherms at 25ºC of *Chlorella* sp. for CT, T1, T2, T3, T4 and T5 treatments adjusted by Oswin, GAB and Peleg models. Type III water adsorption isotherms are J-shaped and, according to the classification by Brunauer et al. (1940) are generally typical of foods rich in soluble compounds, such as foods high in sugars or salts; and Type II are S-shaped sigmoidal, typical of soluble products, with asymptotic tendency. Potato starch, amylopectin and amylose powder isotherms have also been classified as Type II (Al-Muhtaseb et al., 2004).

The equilibrium moisture content (Xeq) of the samples increased with increasing water activity (aw) and ranged from 3.84 to 28.30% b.s. All treatments had very similar equilibrium humidity, with few variations, justified by the small differences in the composition of the samples. Alcântara et al. (2009) also verified for the moisture adsorption isotherm at 25 ºC of the cashew dry peduncle that with the increase of aw there was an increase of Xeq and that in the aw of 0.86 to Xeq was 29.42% bs, results similar to that of *Chlorella* sp., which in a_w of 0.93 presented Xeq of 28.30% b.s., even having very different chemical compositions.The same trend was found for the Peleg model by several researchers (McMinn & Magee, 2003; Aguirre-Cruz *et al*., 2010; Claudera-Oliveira *et al*., 2011; Chisté et al., 2012; Spada et al., 2013). According to Goula et al. (2008), water adsorption in food is a complex phenomenon and the main constituents of food water adsorption are polymers such as proteins, starch, cellulose and hemicellulose.

3.3 Amino Acid Profile

Table 4 shows the results of the amino acid profile of *Chlorella* sp. submitted to different treatments.The total amino acid content ranged from 17.79 to 36.56 g / 100 g, with the lowest concentration for the T5 treatment and the highest concentration for CT, respectively. Higher amino acid values were quantified in the *Chlorella vulgaris* biomass grown in triple fertilizer 15 medium (Nutrimon 15-15-15®) yielding a total of 41.41 g / 100 g amino acids (Anastasakis & Ross, 2011) and in *Chlorella* biomass grown in BBM (Bold Basal Medium) medium which obtained 39.9 g / 100 g. Similar results of amino acid levels obtained from treatments T2 and T3 were verified by Guccione et al., 2014 for *Chlorella* sp. grown in BG11 medium (fresh water - based medium), with a content of 26.1 g / 100 g, and lower value was verified with a yield of 14.6 g / 100 g in the biomass of cultivated *Chlorella vulgaris*. in the medium 3NBBM + V (modified Bold Basal medium) (Slocombe et al., 2015).

It was found a total of 18 amino acids in the T3 treatment and 17 amino acids in the CT, T1, T2, T4 and T5 treatments. In the T3 treatment a tryptophan content of 0.16 g / 100 g was quantified. In other treatments, the tryptophan content was not observed. Similarly, amino acid production in the biomass of *Chlorella pyrenoidosa* species grown in F / 2 medium (Guillard) produced 18 amino acid types, including tryptophan, with a higher dry biomass concentration of 2.5 g / 100 g (Slocombe et al., 2015). In all treatments, the highest concentration was observed for the essential amino acids, followed by the conditionally essential and in lower concentration the non-essential amino acids. Among the essential amino acids, the highest concentration was L-leucine, the conditionally essential was L-arginine and non-essential L-glutamic acid.

Table 4. Amino Acid profile (g / 100 g) of *Chlorella* sp. submitted to different treatments

Amino acids	Treatments (g / 100 g)					
	CT	T1	T2	T3	T4	T5
L-Phenylalanine *	1.82	1.69	1.53	1.24	1.07	1.06
L – Lysine *	2.02	1.87	1.75	1.49	1.11	1.10
Tryptophan *	-	-	-	0.16	-	-
L – Threonine *	2.43	2.22	2.17	1.58	1.33	1.30
L – Isoleucine *	2.70	2.43	2.15	1.38	1.38	1.31
L – Leucine *	3.00	2.76	2.49	1.83	1.64	1.61
L – Arginine **	3.72	3.35	3.03	2.42	1.94	1.89
L – Proline **	1.55	1.65	1.61	1.62	1.02	1.03
L – Tyrosine **	1.81	1.60	1.39	0.97	0.91	0.87
L – Cystine **	0.33	0.31	0.16	0.13	0.22	0.20
L – Serine **	2.00	1.77	1.60	1.36	0.99	0.97
Glycine **	2.06	1.91	1.77	1.26	1.19	1.17
L – Histidine **	0.74	0.66	0.56	0.60	0.36	0.33
L-Alanine	2.80	2.58	2.34	1.44	1.60	1.56
L-Aspartic Acid	1.51	1.06	0.69	1.33	0.33	0.26
L-Glutamic Acid	4.79	3.97	3.21	2.37	1.44	1.33
Total	36.56	32.83	29	23	18.45	17.79

* Essential amino acids; ** Conditionally essential amino acids; too many are nonessential

3.4 Mechanical Hydrolysis and SEM of Biomass

Ultrafine grinding allows the production of ultrafine powders, with a significant increase in the specific surface area, as well as other desirable properties, such as amorphization and greater chemical reactivity due to the increase in free energy on the particle surfaces (Tavares et al., 2001). The increase in the grinding time can lead to fracture, change in the shape and agglomeration of particles. These consequences also lead to a change in the porosity of the components produced from this material (Tavares, 2005).

Analyzing the results of the high-energy wet milling of *Chlorella* spp. (Figure 3), the CT, T1, T2 and T3 treatments have surfaces with a greater amount of amorphous materials, “possibly plasticized starches” in relation to the T4 and T5 treatments, and in all treatments it is possible to check points of crystalline materials overlapping.

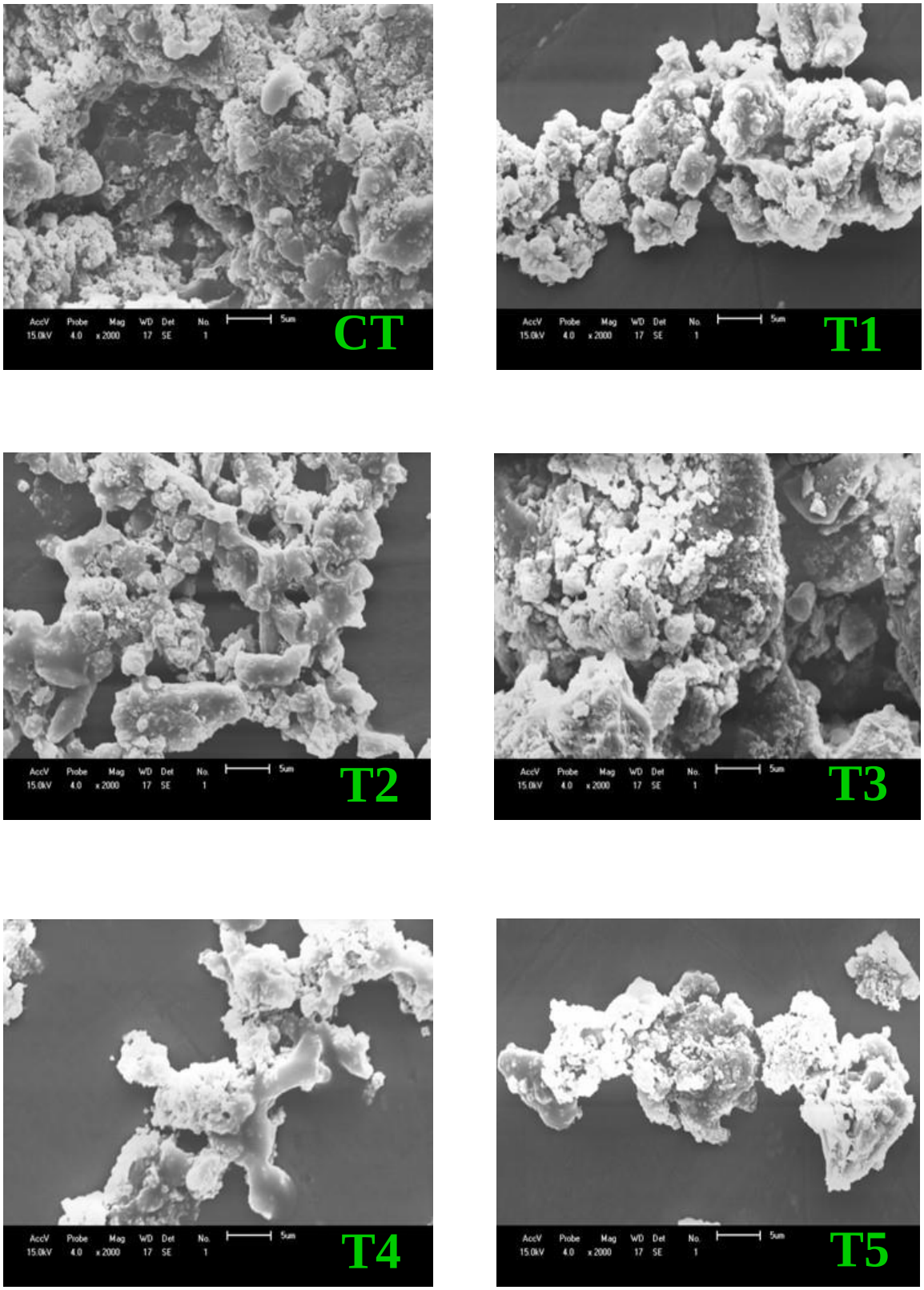

Figure 3. Micrograph of the mechanical hydrolysis of *Chlorella* sp biomass submitted to different treatments

It was found that the best grinding time was obtained at 15 min, speed 400 rpm, with

percentile sizes of 80 nm, (Table 5). The hydrolysis parameters presented different configurations, in relation to the grinding time, with the following averages: interval of 5 minutes 551 nm, 10 minutes 183 nm, 15 minutes 80 nm and 20 minutes 199 nm (Table 5). The results obtained show that the high energy grinding must be adjusted to the characteristics of each material. In this study, the times 5 and 10 minutes were insufficient to reach the ideal granulometry (~ 80 to 100 nm), while time of 20 minutes caused the increase in particle size. This phenomenon possibly occurred due to the release of polysaccharides contained in the cell walls causing plasticizing effects, molecular clusters and changes in hydrolysis results, as mentioned in Bello, et al., 2015.

Table 5. Analysis of micrographs of mechanical hydrolysis of *Chlorella* sp. CT, T1, T2, T3, T4 and T5, through Image-Pro Plus 7.0 software

	Average Particle Size (nm)					
Grinding time	CT	T1	T2	T3	T4	T5
5 min	571	549	554	528	562	541
10 min	182	176	193	171	185	190
15 min	87	74	83	94	65	77
20 min	207	201	198	185	211	194

In a case study, the reduction in the size of particles of bioative materials led to the high-energy grinding of a drug named “Efavirenz”, increasing its solubility, indicating a strategy to increase both the solubility and its effects. In another study with coconut fiber, it was evaluated the properties and the production of particles of coconut fiber, and it was found that after increasing the grinding time, structural changes occurred, as well as synthesis of new compounds during the procedure (Bello, et al., 2015). The standardization of wet milling might be strategic for the sector of production of plant biostimulants, mainly beacuse the costs with enzymatic hydrolysis and other methods may not be viable when applied at scale. Thus, our proposal here was to start a preliminary investigation, and future work is needed to deepen these investigations.

3.5 Seedling Biostimulation

Biostimulants are products that, when applied in low concentrations, help the twinning, development, and productivity of plants, regulating and improving their physiological processes (Kawalekar, 2013; Toscano, et al., 2018). It is pivotal to study the feasibility to produce biomass as bioestimulants, to analyze their composition and active ingredients. Currently, some studies have investigated the application of algae biomass as a raw material for use in agriculture (Elarroussi et al., 2016). Here, we analyzed the bio-stimulating potential of *Chlorella* sp. produced in different concentrations of nutrients (CT and T treatments). The results obtained

demonstrated that the extracts of *Chlorella* sp. produced by mechanical hydrolysis were able to biostimulate germination and the development of yellow melon seedlings (Tables 6 and 7).

As shown in Table 6, the best results were obtained of biomass from treatments T2, T4 and T5, for the germination index (IG) 92.00, 92.00 and 95.00%, germination speed index (GSI) 8.00000, 8.00000 and 8.26087 seedlings / day and the average germination time (AGT) 7.66667, 7.66667 and 7.91667 days, respectively. Analyzing the intercalations between the effects obtained in the germination and the modulation of the means of cultivation of the microalgae *Chlorella* sp. it was noticed that, even reducing the levels of macro elements in the treatments, the biostimulant effect was greater in the treatments modulated with lower concentrations of NPK, even though there was a reduction in protein synthesis in these treatments (Table 4). It is suspected that the production of peptides with the biostimulant function may have occurred in specific medium composition, a fact that we will investigate in future research.

The results obtained for Seedling Height (SH), Fresh Mass from the Aerial Part (FMAP) and Dry Mass from the Aerial Part (DMAP) were significant for the T2 treatment with values of 50.99 mm, 0.94 and 0.12 g, respectively, compared to other treatments. The number of leaves (NL) showed no significant difference between all treatments. Best Root Length (RL) was obtained in treatment T1, with a value of 91.80 mm. For Dry Root Mass (DRM), the best stimulation occurred in treatment T5 with a value of 0.6 g. For seedlings color there was no statistical difference between all treatments, as shown in Table 7.

Table 6. Values of analysis of variance of parameters of biostimulation of seedlings of (*Cucumis melo* L.) by the biomass of *Chlorella* sp. in different procedures

	Treatment averages		
Treatments	GP	GSI	AGT
CT	51.00^{ns}	4.47826^{ns}	4.29167^{ns}
T1	59.00 ^{ns}	5.13043^{ns}	4.91667^{ns}
T2	92.00**	8.00000**	7.66667**
T3	63.00*	5.52174*	5.29167*
T4	92.00**	8.00000**	7,66667**
T5	95.00**	8.26087**	7.91667**
CV (%)	1.63	0.26000	1.63170

GP germination percentage; GSI = germination speed index; AGT = Average germination time; ** significant at 1%; * significant at 5%; ns not significant.

Overall, our results suggest that the modulation of *Chlorella* sp. growth medium played a fundamental role in the biostimulation of seedlings of yellow melon, mainly in the increase of the mass gain and in the lengthening of the stem. Some recent studies have been demonstrated the biostimulant effects of microalgae biomass. For instance, Ronga *et a*l. (2019) studied a set of microalgae, including *Chlorella vulgaris*, which showed a high rate of stimulus to seed germination, with an increase in the germination rate around 144.51%. Barone *et al*. (2018a) tested extracts of microalgae of *C. vulgares*, *S. quadrilata* and *C. vulgares* on sugar beet, obtaining stimulation of root growth and increased plant growth and development. Moreover, Navarro-López et al. (2020) tested the effects of *Scenedesmus obliquo* extracts in mung bean and cucumber cultures, obtaining promising results, demonstrating stimulating effects both from phyto-endogenous hormones and amino acids contained in the extracts, corroborating with the results obtained in the present study.

Table 7. Summary of analysis of variance for the variables: seedling height (SH), number of leaves (NL), root length (RL), fresh mass from the aerial part (FMAP), fresh root mass (FRM), dry mass from the aerial part (DMAP), root dry mass (RDM), L*, a* and b*: color of yellow melon seedlings stimulated with microalgae biomass

TREATMENT	Averages of Treatments				
	SH	NL	RL	FMAP	FRM
CT	38.90 b	1.06 a	91.80 a	0.78 b	0.53 ab
T1	39.16 b	1.00 a	60.79 b	0.70 b	0.73 a
T2	50.99 a	1.00 a	58.37 b	0.94 a	0.67 ab
T3	37.92 b	1.06 a	59.90 b	0.77 b	0.59 ab
T4	43.34 b	1.00 a	52.67 b	0.81 b	0.69 ab
T5	41.28 b	1.00 a	68.93 b	0.78 b	0.51 b
	DMAP	DRM	L*	a*	b*
CT	0.11 ab	0.03 b	30.06 a	17.53 a	36.06 a
T1	0.09 c	0.04 ab	36.53 a	17.73 a	36.53 a
T2	0.12 a	0.05 ab	36.91 a	17.94 a	36.91 a
T3	0.10 bc	0.04 ab	35.20 a	17.52 a	35.20 a
T4	0.09 bc	0.05 ab	36.71 a	17.53 a	36.71 a
T5	0.08 c	0.06 a	36.34 a	17.54 a	36.34 a

Means followed by the same letter in the columns do not differ statistically by Tukey test at 5% probability level.

4. Conclusions

Modulation of the cultivation of *Chlorella* sp. in different concentrations of nutrients alters biomass yield, amino acids profile and bioestimulant properties. It was shown that intermediary levels of NPK (nitrogen, phosphate, potassium), and carbon source (T2 treatment) allowed strong bioestimulant effects on melon (*Cucumis melo* L.) germination, influencing stem elongation and

amount of fresh and dry matter of the aerial part of seedlings. In addition, low levels of NPK and increased levels of C had strong effects on bioestimulant germination properties of *Chlorella* biomass, as characterized by increased germination percentage (GP), germination speed index (GSI) and average germination time (AGT). These results indicate that the balance of nutrients in the cultivation medium is pivotal for the bioestimulant effects of *Chlorella* sp. biomass, despite the biomass yield. Future research is needed to characterize the peptide and other components in the biomass of *Chlorella* sp after the growth modulation, to elucidate the bioestimulant mechanisms of its biomass.

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