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Cashew Rootstock Production Using *Spirulina platensis* Biomass

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HIGHLIGHTS

- The use of *Spirulina platensis* increased the growth of cashew seedlings.
- *S. platensis* increased the accumulation of plant biomass of cashew seedlings.
- *S. platensis* can be used as a biostimulant for plant production.

Abstract: Microalgae are considered a rich source of biostimulants for plants, as an excellent business opportunity for the agricultural sector, as they can activate plant metabolism, contributing to the improvement in physiological performance, phytomass and, subsequently, vigor and quality of seedlings. The objective of this work was to evaluate the potential of *Spirulina platensis* biomass, in enabling improvements in physiological performance, phytomass and, subsequently, vigor and quality of cashew rootstock seedlings. The experiment was carried out in a greenhouse in a completely randomized design, in a 4 x 2 factorial scheme (concentrations x rootstock), with three replications. The concentrations used were 0%, 0.04%, 0.08%, and 0.12% and the FAGA and CCP 76 genotypes. The seeds were immersed in the solutions for 12

hours. Applications were made via the leaves at 30 days after sowing (DAS), with an interval of seven days between each application and at 90 DAS evaluations were made. The FAGA and CCP 76 genotypes showed a positive response to the supply of *S. platensis*, with concentrations 0.04% and 0.08% which provided greater root length, aerial part length, number of leaves, diameter and leaf area. Concentrations 0.04% and 0.08% also promoted better percentages in the physiology of the CCP 76 genotype, while the FAGA genotype showed opposite action, which may be related to the mode of action of the microalgae on the different genetic and phenotypic characteristics of each genotype. The 0.12% concentration provided greater accumulations of aerial part and total dry mass to the genotypes.

Keywords: *Anacardium occidentale*; biotechnology; microalgae; propagation; quality; vigor.

INTRODUCTION

The cashew tree (*Anacardium occidentale* L.) is a plant native to Brazil, typical of regions with a tropical climate. The production of this fruit tree is concentrated in the Northeast region of country Brazil, responsible for almost all national production (99.26%) in 2019. Distributed among the states of Ceará, Piauí and Rio Grande do Norte, respectively [1,2].

The models of cashew agribusiness exploration are diversified throughout the world, also known as cashew farming. In the Northeast, small areas of crops prevail, usually in intercropping with other crops of local interest. Its diffusion has increased in recent years, not only because it is an agricultural activity more favorable to regions with hot and dry climates, but also because it is a fruit supplier of raw material for the manufacture of various by-products, such as juices, pulp, sweets, wood from pruning, chestnut almonds, among other products [3]. It is noteworthy that the exploitation of cashew in monocultures presents itself as one of the main difficulties for the production of the crop [4,5].

In the period from 2012 to 2016, the Northeast region, the main producing region, went through a long period of drought, resulting in decline and loss in cashew production areas. After this period, with an increase in the volume of rain in the region, there has been a growing increase in its production and, consequently, an increase in the supply of Brazil nuts, the main product produced [2].

However, Brazil is still far from the world's main producers, with low productivity per hectare, mainly due to the part of the orchards in a declining phase and factors related to low production technology [6]. Given these circumstances, there is a concern to develop technologies aimed at improving the cultivation of the crop and optimizing its production in a sustainable way [7].

Considering that the production of good quality seedlings is essential for the future establishment and management of an orchard with greater productive, phytosanitary and production quality performance, the use of microalgae has the capacity to enhance plant growth through bioactive components present in the biomass of microalgae that can act through gene expression, signaling and hormonal interactions [8].

Microalgae, especially those produced under Brazilian climatic conditions, have significant potential for use as a biostimulant [9], given that they have high levels of free amino acids, proteins, carbohydrates, lipids, and mineral nutrients [10]. *S. platensis* has several applications in the agronomic area and many studies have focused on its use for biofertilization and biostimulation of plants in the cultivation phase [11,12]. In this aspect, researches involving technology for the use of *Spirulina platensis* microalgae biomass may innovate seedling production technology, and mainly, optimize the productive performance of important species for semiarid regions, such as *A. occidentale*.

In this context, the objective was to evaluate the potential of *Spirulina platensis* biomass, in enabling improvements in physiological performance, phytomass and, subsequently, vigor and quality of cashew rootstock seedlings.

MATERIAL AND METHODS

Materials and experimental conduct

The biomass of *S. platensis* used was provided by the private company J. H. de Lima, Paraíba, Brazil, whose chemical composition is shown in Table 1.

Table 1. Composition of the *Spirulina platensis* biomass applied to cashew genotypes (FAGA and CCP 76).

Physical-chemical characterization	Value	Organic composition (g 100 g^{-1})	Value
Humidity and volatiles (g 100 g^{-1})	12.10	Aspartic acid	4.85
Ashes (g 100 g^{-1})	9.00	Glutamic acid	7.99
Proteins (g 100 g^{-1})	51.82	Serine	2.38
Nitrogen (g 100 g^{-1})	8.29	Glycine	2.63
Carbohydrates (g 100 g^{-1})	14.20	Histidine	0.75
Arsenic (mg 100 g^{-1})	0.0044	Arginine	3.45
Cadmium (mg 100 g^{-1})	0.004	Threonine	2.63
Calcium (g 100 g^{-1})	0.198	Alanine	3.72
Copper (mg 100 g^{-1})	0.111	Proline	1.85
Chromium (mg 100 g^{-1})	0.052	Tyrosine	2.50
Iron (mg 100 g^{-1})	20.10	Valine	3.23
Phosphorus (g 100 g^{-1})	1.58	Methionine	0.90
Magnesium (g 100 g^{-1})	0.39	Cysteine	0.21
Manganese (mg 100 g^{-1})	3.26	Isoleucine	3.60
Potassium (mg 100 g^{-1})	2.29	Leucine	4.47
Sodium (mg 100 g^{-1})	1.297	Phenylalanine	2.29
Zinc (mg 100 g^{-1})	0.84	Lysine	2.43
-	-	Tryptophan	0.67
-	-	Total lipids	6.90
-	-	Carbohydrates	14.20

The rootstocks used were the FAGA and CCP 76 cashew genotypes, obtained from municipality Serra do Mel, Rio Grande do Norte, Brazil.

The substrates for the production of seedlings were prepared from the surface layer (horizon A) of chronic Luvissol, passed through a sieve with a 6 mm mesh, and later autoclaved for sterilization, and sampled for analysis in the laboratory.

Black bags measuring 28 cm x 15 cm x 0.15 mm were used.

The work was carried out in a greenhouse at the Federal University of Campina Grande (UFCG), Pombal, Paraíba, Brazil, (6º48'16"S and 37º49'15"W), at an altitude of 175 m. The predominant the climate in this region is hot semi-arid, with low rainfall, between 250 and 800 mm per year [13].

Before planting the seeds, a selection was carried out to eliminate the lack of formations, malformed and with phytosanitary problems. For this, the density technique was used, eliminating the seeds that remain floating in the water deposited in a shallow container.

Planting was done directly in the containers, using one seed per container, of good quality. The substrate was moistened before planting to facilitate penetration of the nuts, which were set to germinate in a vertical position, with the chestnut/peduncle incision point facing upwards and at a depth of 3 cm from the substrate surface.

Experimental Design

The experimental design used was completely randomized, in a factorial scheme (4x2), with 3 replications, totaling 24 experimental units. The biomass concentrations of *Spirulina platensis* were 0%, 0.04%, 0.08% and 0.12% and the rootstocks used were the genotypes FAGA and CCP 76.

Biomass application

The microalgae biomass was diluted and homogenized in distilled water, using a heating plate, under constant agitation for 15 minutes at 40 °C. After homogenizing the solution, the electrical conductivity and pH of the solutions were analyzed (Table 2).

Table 2. Electrical conductivity and pH of the solutions applied to cashew genotypes (FAGA and CCP 76).

Doses (%)	Electric conductivity (µS)	pH
0.00	4.81	6.89
0.04	54.27	6.20
0.08	92.32	6.07
0.12	135.7	5.94

The seeds were immersed in the solutions for 12 hours before planting and applying to the plants after 30 days after sowing, following an interval of 7 days for each application, making a total of 8 applications which were made via spraying with use of a manual spray sprayer (Plasart, Brazil), in the leaf area, with a volume of 25 mL per plant in the first 4 applications and 50 mL in the other applications.

Growth Analysis

At the end of 90 days after sowing were evaluated: The number of leaves, measured from leaves that are greater than 3 cm in size; Length of the aerial part (LAP), measured from the plant's neck to the apical bud; Average diameter of the rootstock, determined through measurements in the median portion of the main stake, using a digital caliper (150 mm, Mtx); Length of the root, measured from the distance from the neck to the apex of the main root, with a ruler graduated in cm; Fresh mass from the aerial part and Fresh mass from the root, by weighing in an analytical balance (M214AIH, Bel); Aerial part dry mass (ADM) and Root dry mass (RDM), determined after drying for 48 hours in a forced air circulation oven at 60 ºC (SSDCR-64L, SolidSteel), until reaching constant weight, weighing in analytical balance accurate to 0.01 g (S2202H, Bel); Total dry mass (TDM), obtained from the sum of aerial part and root dry matter; and Dickson Quality Index [14], obtained through the formula:

$$DQI = \frac{TDM\ (g)}{\frac{LAP\ (cm)}{DR\ (mm)} + \frac{ADM\ (g)}{RDM\ (g)}} \quad (1)$$

The leaf area and the root/aerial part ratio (R/AP = Root phytomass / Phytomass of aerial part, in g g^{-1}) [16], and the leaf area ratio (LAR = Leaf area / Total dry phytomass, in cm^2 g^{-1}) with the aid of a Ceptometer (ACCUPAR, LP-80, Decagon Devices, Inc., Germany).

Gas exchange Analysis

Gas exchange, determined in fully developed leaves, was evaluated from 7:00 am to 9:00 am, using portable photosynthesis measurement equipment (LCPro T, ADC BioScientific Ltda., United Kingdom). The following were determined: CO_2 assimilation rate (A) (µmol m^{-2} s^{-1}); transpiration (E) (mmol H_2O m^{-2} s^{-1}); stomatal conductance (gs) (mol of H_2O m^{-2} s^{-1}); internal concentration of CO_2 (Ci) (µmol mol-1); water use efficiency (USA) (A/T) [(µmol m^{-2} s^{-1}) (mol H_2O m^{-2} s^{-1})$^{-1}$] and the instantaneous carboxylation efficiency Φc (A/Ci) [15,16].

Microscopy Analysis

Scanning electron microscopy was performed to evaluate the different types of structures formed on the surface of the microalgae. The photomicrography was carried out with the aid of a Scanning Electron Microscope (SEM / LEO – Model 1430VP, England) with a magnification of 1800x.

An amount of biomass was used to fill the area of 0.5 cm² and excess loose powder was removed. Then, they were fixed on the microscope sample holder support (stubs) and covered with a layer of gold (between 20 nm and 30 nm) with the aid of a Sputter Coater metallizer (Balzers - Model SCD 010), to increase the conductivity of the surface of the samples [17].

Statistical analysis

The results were submitted to analysis of variance by the F test and, in cases of significance, a polynomial regression analysis was performed. The computer program System for Analysis of Variance – SISVAR 5.6 [18].

RESULTS AND DISCUSSION

The mean values obtained for root length, aerial part growth, number of leaves and rootstock diameter, at 90 DAS, showed growth the maximum and subsequent decline trend in response to *S. platensis* extract concentrations (Figure 1).

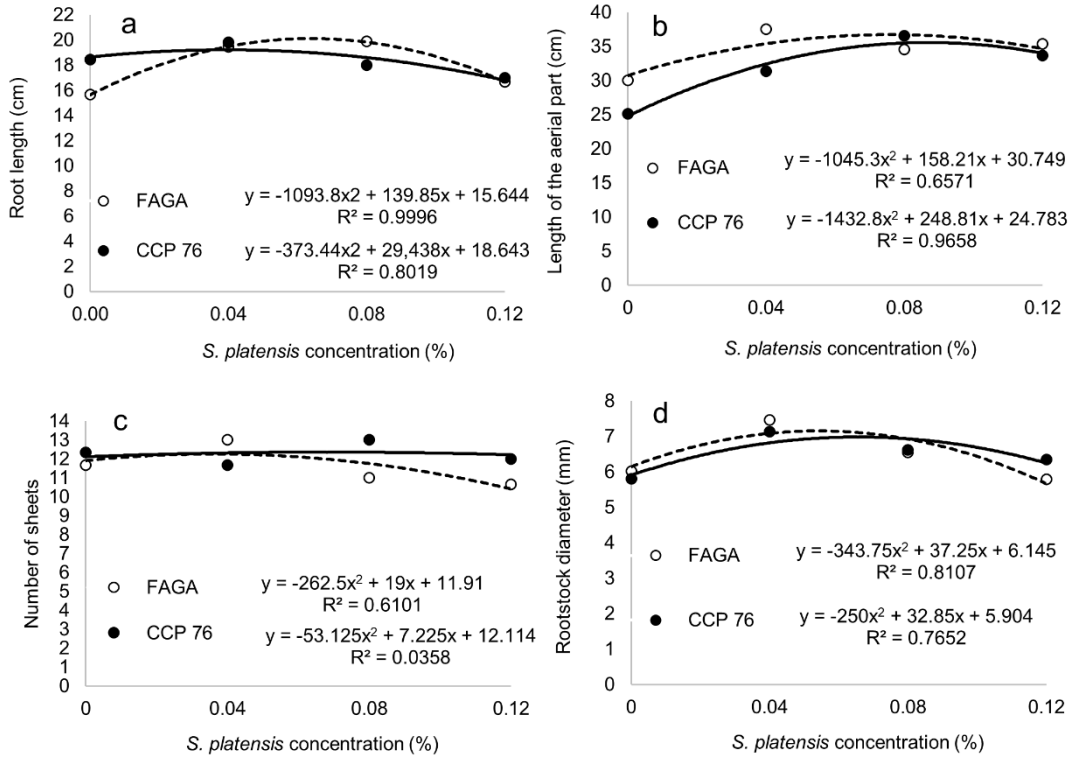

Figure 1. Root length (RL) (a), length of the aerial part (LAP) (b), number of leaves (NL) (c), rootstock diameter (RD) (d) of cashew seedlings of FAGA and CCP 76 genotypes, at 90 DAS, as a function of *Spirulina platensis* concentrations through foliar application.

The largest root lengths were obtained using the 0.08% concentration for the FAGA genotype, with an increment of 21.64%. The CCP 76 genotype had better development of the root system with the concentration 0.04%, providing an increase of (3.02%), when compared to the control. However, with the increase in concentration (0.08%), there was a reduction in its root system, and the maximum decrease (10.95%) occurred when the maximum biomass dosage (0.12%) was applied (Figure1A).

The increase in the root system may be related to the ability of the biostimulant to promote the hormonal balance of plants, favoring the expression of their genetic potential, thus stimulating the development of the root system [9].

Elarroussi and coauthors (2016) [19] when testing foliar applications of *S. platensis* in tomato and pepper plants, they observed root system growth, unlike Guedes and coauthors (2018) [10], studying the production of papaya seedlings with application of *S. platensis* concentrations, observed that the foliar application of the microalgae did not provide growth of the root system of the seedlings.

The 0.08% concentration provided greater growth in aerial part length (36.72 cm) to the FAGA genotype and (35.52 cm) to the CCP 76 genotype, with increments of 16.26% and 30.23%, respectively, compared to the control (Figure 1B). Then there is a reduction in growth with increasing concentration. A fact that can probably be related to the excess of cytokinin present in the microalgae biomass causing a hormonal imbalance [20].

The highest number of leaves were obtained using the concentrations 0.04% and 0.08% for the FAGA (12.25) and CCP 76 (12.35) genotypes, equivalent to increments of 2.77% and 1.94% respectively, compared to the control (Figure 1C). Unlike Garcia and coauthors (2014) [21] evaluating the effect of the microalgae extract Ascophyllum nodosum on cashew seedlings, found that the concentrations of the extract did not influence the number of leaves of the seedlings, as well as Guimarães and coauthors (2012) [22] testing the same microalgae on papaya seedlings.

The largest diameters were obtained at concentrations of 0.04% (7.09 mm) and 0.08% (6.93 mm), for the FAGA genotype with an increment of 13.25%, and for the CCP 76 genotype (14.86%) (Figure 1D). The increase in the diameter of the rootstocks can be attributed to the presence of cytokinin in the microalgae biomass. Which, even in small concentrations, can stimulate cell division and influence the increase in stem diameter [23]. Sá and coauthors (2013) [24], they also point out that the diameter is one of the characteristics that expresses a greater quality and resistance of the seedling to be introduced in the field.

The highest concentration of 0.12% biomass provided greater accumulation of fresh phytomass from the aerial part in the FAGA genotype, equivalent to an increase of 13.16% compared to the control (Figure 2A). However, *S. platensis* concentrations negatively affected the fresh root phytomass of this genotype, resulting in a decrease of up to 17.39% at the 0.08% concentration (Figure 2B). Results corroborate those of Guedes and coauthors (2018) [10] working on the production of papaya seedlings under doses of *S. platensis*, report greater accumulation of fresh mass of the aerial part, while the fresh mass of the root suffered a reduction with the concentrations used.

The effect of *S. platensis* concentrations on the CCP 76 genotype was completely reversed. There was a reduction in the fresh mass of the aerial part with the concentrations of *S. platensis*, with a decrease of up to 13.57% with the concentration of 0.08%. And an increase in root fresh mass of 6.89% with the highest concentration (0.12%), which was probably due to the different genetic and phenotypic characteristics of the genotypes (Fig. 2 A, B).

The 0.12% concentration promoted higher aerial part dry mass accumulation (5.80 g plant^{-1}) to the FAGA genotype and (5.77 g plant^{-1}) to the CCP 76 genotype, with increments of 7.76% and 25.48%, respectively (Figure 2C). Likewise, this concentration allowed for greater accumulation of total dry mass, with an increase of 5.78% for the FAGA genotype and 16.54% for the CCP 76 genotype, compared to the control (Figure 2D).

The increase in aerial part and total dry mass was possibly due to the increase in leaf area (Figure 4C), thus reflecting a greater plant production, which is related to the photosynthetic capacity of plants, thus promoting greater light assimilation and photosynthesis , consequently greater accumulation of dry matter [25].

On the other hand, in relation to root dry mass (Figure 2E), all concentrations of *S. platensis* had a negative effect on the two genotypes studied, the concentration 0.08% provided the largest decreases 82.19% and 80%, respectively FAGA and CCP 76. Rocha and coauthors (2017) [26], working with foliar application of Spirufert® in the production of papaya seedlings, observed that there was no significant effect on root dry mass, as well as Silva and coauthors (2017) [27], evaluating the effect of the microalgae extract *S. platensis* on the lettuce crop, found that the concentrations tested did not promote an effect to this variable in the lettuce plants.

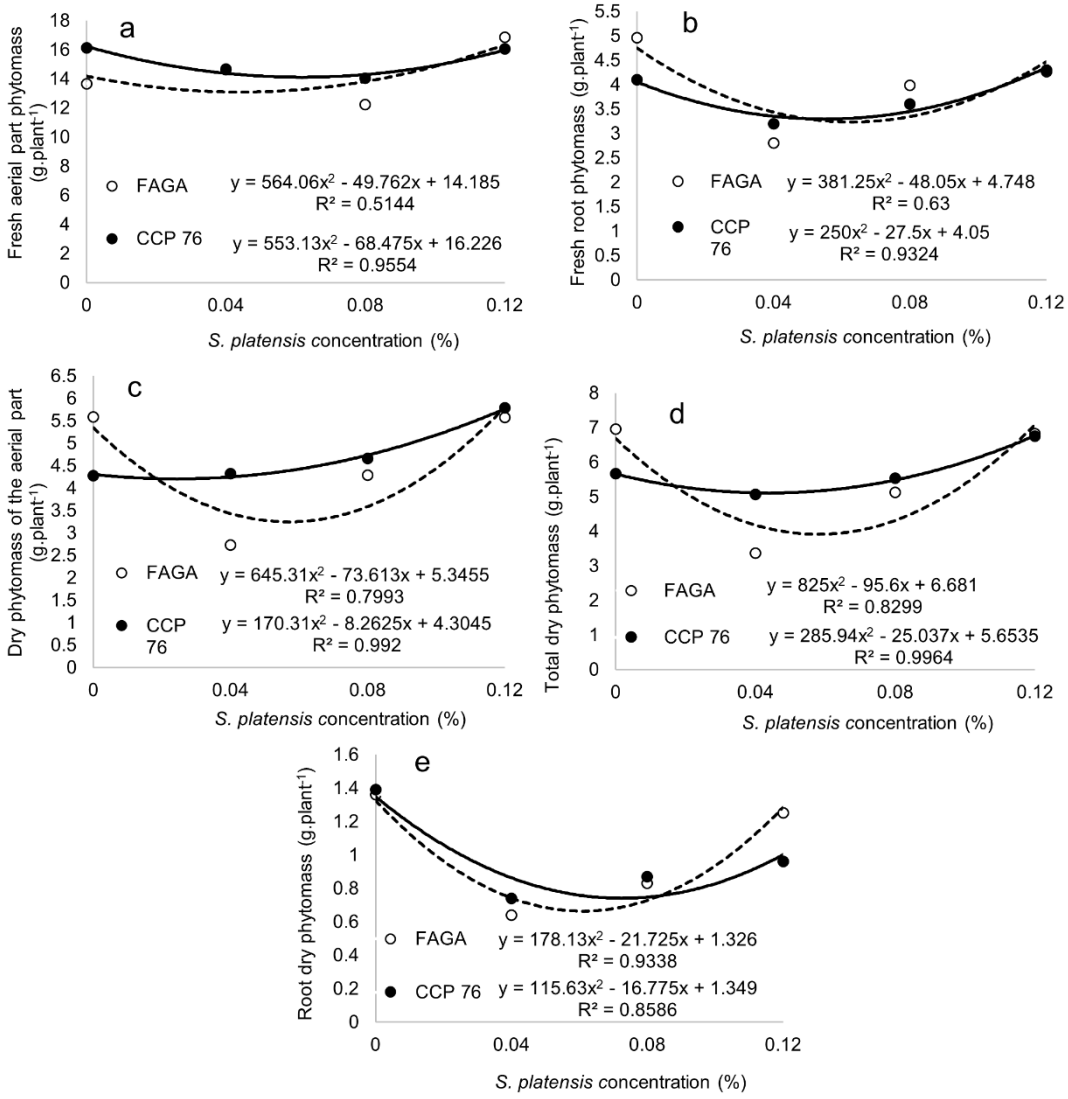

Figure 2. Fresh phytomass of the aerial part (a), fresh root phytomass (b), dry aerial part phytomass (c), total dry phytomass (d) and root dry phytomass (e) of cashew seedlings of FAGA and CCP 76 genotypes, at 90 DAS, as a function of *Spirulina platensis* concentrations through foliar application.

The 0.04% concentration of *S. platensis* provided higher internal CO_2 concentration (247.93 μmol m^{-2} s^{-1}) to the CCP 76 genotype, equivalent to an increase of 3.54%, compared to the control. However, higher concentrations caused a decrease in the internal CO_2 concentration of this genotype. The FAGA genotype had a similar behavior to CCP 76, in which the increase in the doses of *S. platensis* resulted in a reduction in the internal concentration of CO_2, representing a reduction of 17.12% in the highest concentration of *S. platensis* (0.12%) (Figure 3A). Results that corroborate the stomatal conductance (Figure 3B). Because stomatal closure is directly related to decreases in internal CO_2 concentration [28-30].

The CCP 76 genotype had maximum stomatal conductance with concentrations 0.04% and 0.08%, with the same increase of 35.71%. While the FAGA genotype had decreases in its stomatal conductance with doses with *S. platensis*, however, the concentration 0.08% caused the greatest decrease, reaching 70% compared to the control (Figure 3B). This effect can be attributed to the action of amino acids contained in the extract's biomass, since they act as stomatal opening modulators, thus stimulating photosynthesis and controlling the plant's stomatal conductance [31].

The 0.08% concentration of *S. platensis* provided maximum transpiration to the CCP 76 genotype (3.56 mmol of H_2O m^{-2} s^{-1}) equivalent to an increase of 33.99% in relation to the control. However, this concentration was not beneficial to the FAGA genotype, resulting in the greatest decrease of 23.37% with transpiration of 2.31 mmol H_2O m^{-2} s^{-1} (Figure 3C). These results corroborate stomatal conductance (Figure 3B), since the greater the stomatal conductance, the more the plant will have functioning open stomata, consequently the

plant will have greater transpiration, a fact that may not be favorable to the CCP 76 genotype, as the greater the greater perspiration will be the loss of water.

The 0.04% concentration of *S. platensis* provided maximum CO_2 assimilation rate (10.17 μmol m^{-2} s^{-1}), an increase of 16.91% for the CCP6 genotype. While the 0.08% concentration caused a greater decrease of 67.60% in the CO_2 assimilation rate to the FAGA genotype (Figure 3D). Possibly, this may have occurred because the CCP 76 genotype had a higher internal concentration of CO_2, since the influx of CO_2 into the leaf mesophyll is directly associated with the stomatal opening, enabling greater CO_2 assimilation [32].

The instantaneous efficiency of carboxylation was positively influenced by all concentrations of *S. platensis* applied to the CCP 76 genotype. The maximum efficiency was obtained at the concentration of 0.08%, providing 0.041 [(μmol m^{-2} s^{-1}) (mmol of CO_2 m^{-2})$^{-1}$], with an increase of 34.14%. However, none of the studied *S. platensis* doses favored the carboxylation efficiency of the FAGA genotype. Furthermore, the concentration of 0.08% was responsible for the lower instantaneous efficiency of carboxylation, representing a decrease of 55.17% compared to the control (Figure 3E).

The instantaneous carboxylation efficiency is related to the plant's internal concentration and assimilation rate of CO_2 [33]. According to Ferraz and coauthors (2012) [34], observing the gas exchange and photosynthetic efficiency of common bean, reported an increase in the instantaneous efficiency of carboxylation with the gain of internal CO_2 concentration and the assimilation rate.

The CCP 76 genotype had better water use efficiency, with concentrations 0.04% and 0.08% of 0.268 [(μmol $m^{-2}s^{-1}$) (mmol H_2O $m^{-2}s^{-1}$)$^{-1}$], resulting in an increase of 32.84% in relation to the control. The FAGA genotype, however, was negatively influenced with a concentration of 0.08% resulting in a maximum decrease of 76.56% (Figure 3F). This fact occurs due to transpiration and CO_2 assimilation of the genotypes, since the instantaneous efficiency of water use is directly related to the amount of carbon that is fixed by each unit of water that the plant loses [35]. The efficiency of water use, as well as the instantaneous efficiency of carboxylation, are determining variables in the use of absorbed water and the extent to which the plant is able to convert this water into plant biomass [36].

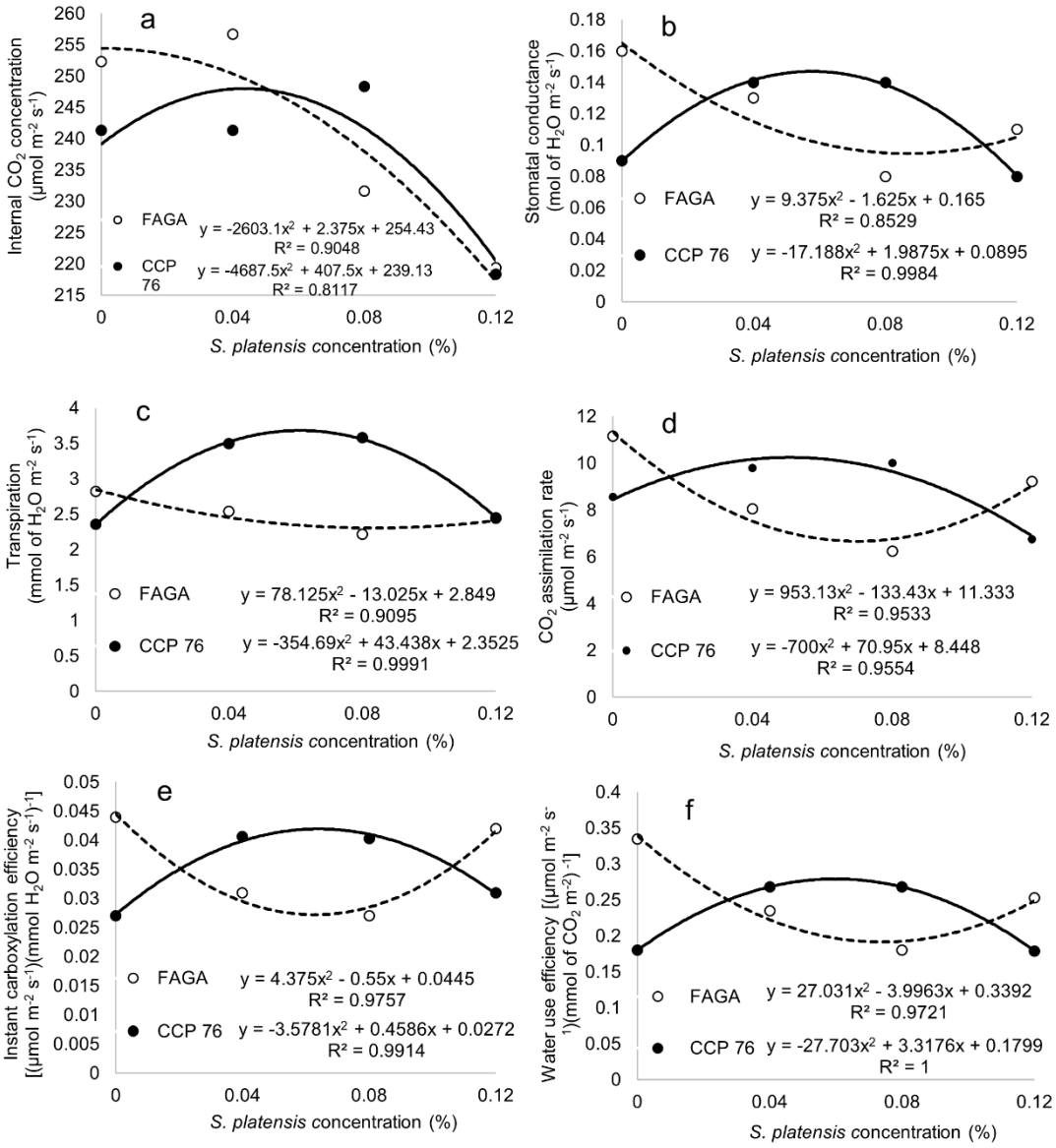

Figure 3. Internal CO_2 concentration (a), stomatal conductance (b), transpiration (c), CO_2 assimilation rate (d), instant carboxylation efficiency (e), water use efficiency (f) of the FAGA and CCP 76 cashew genotypes at 90 DAS, as a function of concentrations of *Spirulina platensis* through foliar application.

The root/areial part ratio and leaf area ratio were negatively influenced by the concentrations of *S. platensis* for the two cashew genotypes (Fig. 4 A, B). The 0.08% concentration resulted in the lowest root/aerial part ratio for the two genotypes, representing a reduction of (19.04%, FAGA) and (106.67%, CCP 76) (Figure 4A). The leaf area ratio was also negatively influenced with the application of concentrations 0.04% and 0.08% for the two studied cashew genotypes (Figure 4B).

The leaf area of the two genotypes showed a positive response to the application of concentrations 0.04% and 0.08%, with emphasis on the concentration 0.08% which provided maximum leaf area (1.28 cm²) to the FAGA genotype and (1.29 cm²) to the CCP 76 genotype, with an increase of 36.72% and 55.81%, respectively, compared to the control (Figure 4C).

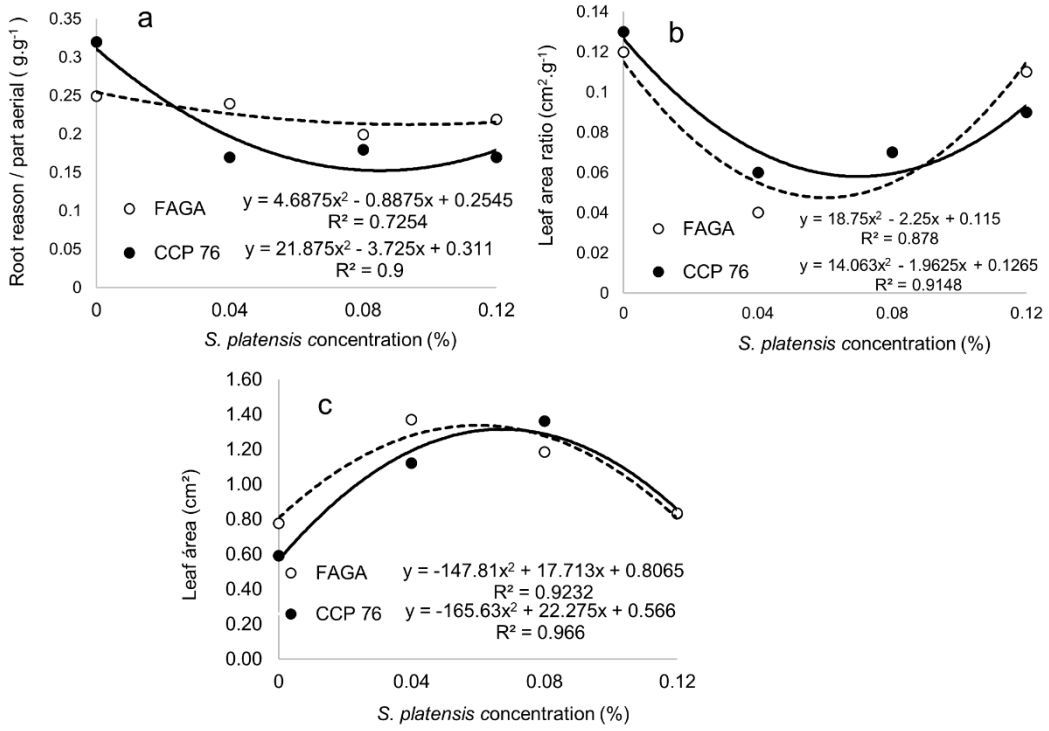

Figure 4. Root reason/part aerial (RR/PA) (a), leaf area ratio (LAR) (b) and leaf area (LA) (c) of FAGA and CCP 76 cashew genotypes at 90 DAS, as a function to apply *Spirulina platensis* through foliar application.

The Dickson quality index was negatively influenced by the concentrations of *S. platensis.* The 0.08% concentration provided a maximum decrease of 85.36% and 59.57% for the FAGA and CCP 76 genotypes respectively (Figure 5). Results may be related to the electrical conductivity of the solution, since with increasing doses of S. platensis, its electrical conductivity also increases [10]. This fact may explain the increase in the IQD when the highest concentration (0.12%) was used, a concentration that had the highest electrical conductivity of 135.7 µS (Table 2).

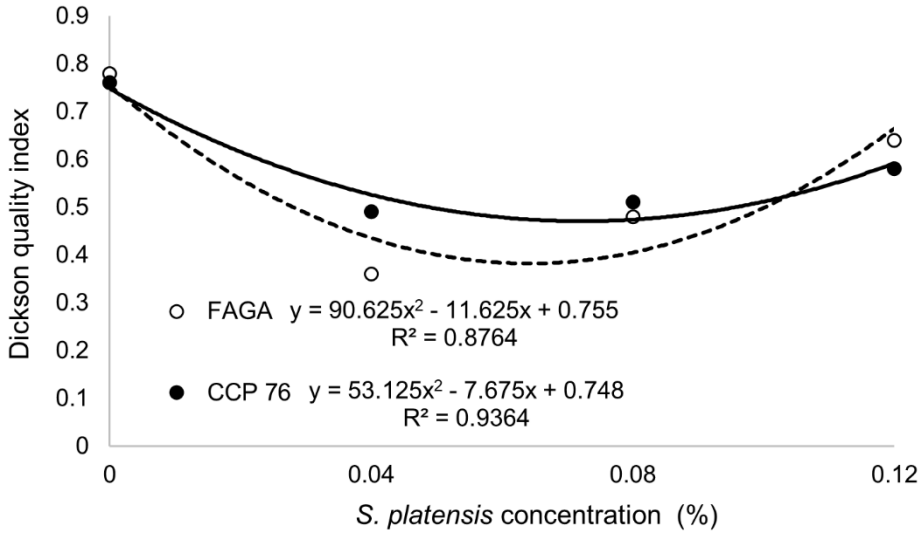

Figure 5. Dickson quality index (IQD) of FAGA and CCP 76 genotypes of cashew at 90 DAS, as a function of *Spirulina platensis* concentrations through foliar application.

The particle size distribution of *S. platensis* ranged from 50 nm to 4 µm (Figure 6). This indicates that the particles are still at a size considered to be large for agricultural applications such as plant applications. It is necessary to perform a fractionation in order to reach the nano size. Because it is believed that the smaller

the particle, the better the homogenization of microalgae in solution, since microalgae have a great capacity for agglomeration of particles.

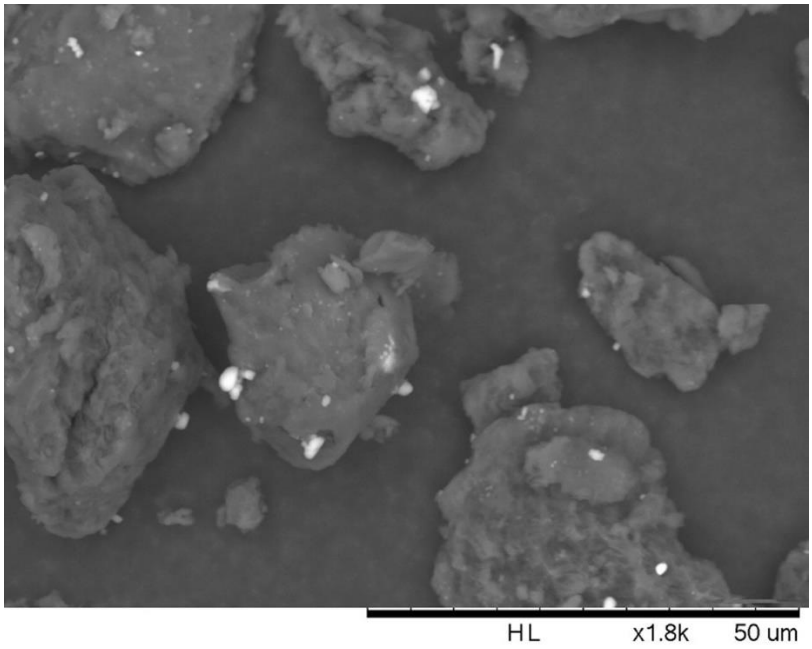

Figure 6. Surface photomicrographs from the biomass of *Spirulina platensis*, after high energy wet grinding, obtained in scanning electron microscopy (SEM).

The agglomeration of *S. platensis* bioparticles with plasticized surfaces may have been caused by the shear process at the time of production [37].

It is noteworthy that the interactivities exerted on dry mass do not occur in the same way in a liquid medium, especially in pH changes, both for acidic and alkaline characteristics. Bioparticles tend to dissociate and form colloidal gradients with exposure of functional groups, which are responsible for the antimicrobial effect [38].

CONCLUSION

Foliar application with *Spirulina platensis* biomass provided growth of vegetative parts of cashew seedlings of FAGA and CCP 76 genotypes, with concentrations of 0.04% and 0.08%. And the 0.12% concentration provided greater accumulation of biomass to the genotypes. The CCP 76 genotype showed better reaction the concentrations 0.04% and 0.08% with regard to physiological parameters. While the FAGA gwnotype did not have the same reaction. Concentrations with *Spirulina platensis* extract had a negative effect on the leaf area ratio, root/part area ratio and dickson quality index, and a positive effect on the leaf area of the genotypes.

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Conflicts of Interest: The authors declare no conflict of interest.

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