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Morphophysiology of Punica *Granatum L.* under microalgae biomass stimulation

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Abstract

The use of microalgae has been proeminent in agricultural scenario, because it is an alternative product considered extremelly productive, which contains essential elements for plants. Thus, this study aimed to evaluate the morphophysiology of *Punica granatum* L. seedlings treated with *Spirulina platensis* and *Scenedesmus* sp. nanoparticles in controlled environment. The research was carried out at the of Federal University of Campina Grande, Pombal-PB, using a completely randomized design with five replications. The factorial scheme (2 x 4) was adopted, represented by two types of microalgae (*Spirulina platensis*; *Scenedesmus* sp.) and four doses of application (0, 5, 10 and 15%). Growth evaluations happened at 135 days after planting, verifying the number of leaves and branches and shoot length. The physiological parameters evaluated were gas exchanges, chlorophyll '*a*' fluorescence, electron transport rate, stationary fluorescence, maximum fluorescence yield after light adaptation and quantum efficiency of PSII. Shoot length and number of branches had rises at doses of 6 and 15%. Number of leaves was induced in seedlings at dose of 8% with *Spirulina platensis*. Stomatal conductance and internal CO_2 concentration increased in seedlings sprayed with *Spirulina platensis*. extract of *Scenedesmus* sp. improved the stationary fluorescence and quantum efficiency of PSII in pomegranate seedlings.

Keywords: biostimulant, gas exchanges, pomegranate, seedling production

Introduction

Nowadays, the concern about quantity and quality to supply the global demand for food has grown, becoming fundamental to use sustainably techniques for higher productions. Pomegranate is a relevant fruit for tropical and subtropical regions, presenting great potential for exploitation due to the large amount of antioxydes contained in seeds, shell and fruits (Hmid et al., 2017). Most of these bioactive compounds have phenolic nature, such as flavonoids, phenolic acids and tannins, presenting excellent medicinal and nutritional properties (Singh et al., 2018).

In Brazil, the crop has aroused the interest of producers, especially in the Northeast region, due to its rapid growth and adaptability to the conditions of the Brazilian semi-arid region, as well as its high market value (Almeida et al., 2019).

The use of biostimulants is in evidence among techniques to increase agricultural production because they may induce and increase the uptake of nutrients, abiotic stress tolerance and quality of harvested products (Salvi et al., 2019). They are considered products containing alive microorganisms or natural substances that improve the quality of biological properties, promoting plant growth and restoring soil fertility (Ronga et al., 2019). Microalgae have attracted the interest of agrochemical producers and industries which intend to improve the sustainability of agricultural production (Elarroussia et al., 2016). These microalgae contain high levels of macronutrients and micronutrients, which are essential for a better plant development. In addition, microalgae showed potentially applicable as biostimulant (Garcia-Gonzalez and Sommerfeld, 2016; Soares et al., 2018; Abinandan et al., 2019).

Species of microalgae from genus *Spirulina* and *Scenedesmus* are promising alternatives to obtain

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biofertilizers and biostimulants. *S. platensis* has an amount of essential nutrients, such as calcium, iron, phosphorus, copper, magnesium, manganese, potassium, boron, molybdenum, selenium and zinc within which as which plant uptakes (Silva et al., 2017; Guedes et al., 2018). Microalgae from genus *Scenedesmus* are also requested by presenting as main constituents the chlorophylls a and b, xanthophylls (lutein and prasinoxanthin) and carotenoids α, β and γ. (Soares et al., 2018).

In fruit production systems, during the implantation phase, the use of microalgae can be applied in order to obtain seedlings with homogeneity and quality, since their application induces a series of positive responses, acting as a growth promoter, seed germination, chlorophyll synthesis and plant defense mechanisms (Ertani et al., 2018).

Therefore, the aim of this study was to evaluate the morphophysiology of *Punica granatum* L. seedlings testing the application of *Spirulina platensis* and *Scenedesmus* sp. nanoparticles in controlled environment.

Material And Methods

The experiment was carried out in controlled environment (greenhouse) at the Federal University of Campina Grande - UFCG, located in the municipality of Pombal, Paraíba, Brazil, with geographic coordinates 6°47'20"latitude and 37°48'01"longitude, and altitude of 194 m.

The design was a completely randomized design (CRD) in factorial scheme (2 × 4), presenting two types of microalgae (*Spirulina platensis* and *Scenedesmus* sp.) and four doses of microalgae extracts (0, 5, 10, and 15%), with five replications, totaling 40 experimental plots. In order to obtain the solutions according to proposed doses, microalgae were weighed with the following proportions: 0% - 0 g, 5% - 0.125 g, 10% - 0.250 g and 15% - 0.375 g, and later diluted in 250m of distilled water, under constant stirring until complete dissolution. It was used 50mL per plant of each volume in a single application. Microalgae solutions were sprayed reaching all the seedling leaves, at 120 days after planting (DAP) considering each treatment.

Seedlings from semi-hardwood cuttings presenting 15 cm in length and 4 to 5 mm in diameter were taken from the median portion of vigorous and healthy branches in pomegranate matrices of 'Molar' variety. Cuttings were washed with water and disinfested at 2% of sodium hypochlorite solution for 5 minutes. Two bevel incisions, at 1 cm of length, were made on the base of cuttings and they were planted by burying 2/3 of the base in polyethylene bags (20 x 25 cm) containing substrate composed of soil, Basaplant® substrate and tanned bovine manure at ratio of 1: 1: 1. The substrate was previously sterilized and autoclaved at 127 °C and 1.5 atmospheric pressure with the following chemical constitution.

Cuttings were cultivated in greenhouse covered with sombrite providing 50% of luminosity. The avarage temperature and relative humidity were 36.5 °C and 42%, respectively. Daily irrigations occurred in the early morning and late afternoon when the volume through the drainage lisimetry was established. Rooted cuttings were transplanted to 8dm^3 capacity pots which were filled with substrate composed of soil, Basaplant® substrate and tanned bovine manure at ratio of 3: 1: 1 (**Table 1**). There was a pruning to keep two young lateral buds at 15 days after transplanting of cuttings.

The number of branches in plants was evaluated at 135 DAP, counting new buds after application of treatments. Shoot length was measured from the neck to the apex of plant. The number of leaves (NF) was counted through green leaves from new buds.

The physiological parameters evaluated were gas exchanges, estimating stomatal conductance (*gs*) (mol H_2O m^{-2} s^{-1}), CO_2 assimilation rate (*A*) (µmol CO_2 m^{-2} s^{-1}), transpiration (*E*) (mol H_2O m^{-2} s^{-1}) and internal CO_2 concentration (*Ci*) (µmol CO_2 mol air^{-1}), and through these data, water use efficiency (*WUE*) (*A*/*E*) [(µmol m^{-1}) and instantaneous carboxylation efficiency (*ICE*) (*A*/*Ci*) [(µmol m^{-2} s^{-1}) (µmol mol^{-1})$^{-1}$] were also obtained. These variables were measured by using a portable infrared gas analyzer (IRGA), model LCpro + portable photosynthesis system (ADC BioScientific Limited, UK), operating with photosynthetic photon flux of 1,200 µmol m^{-2} s^{-1} under environmental conditions of air temperature and CO_2 internal concentration, in the morning, from 7 to 9 o'clock.

A portable fluorometer equipment was used, the Plant Efficiency Analyzer -PEA II® (Hansatech Instruments Co., UK) for chlorophyll '*a*' fluorescence measurements.

Table 1. Chemical analysis of substrate used to produce pomegranate seedlings.

Chemical Characteristics										
pH	P	K^+	Na^+	Ca^{+2}	Mg^{+2}	Al^{3}	H+Al	SB	CEC	OM
H_2O	mg dm^3	---------- cmol$_c$ dm^3 ----------								g kg^{-1}
6.1	337.21	416.22	0.36	10.95	1.91	0.00	6.86	14.29	21.17	41.36

pH= Potential of hydrogen; S-SO_4^{2}= Sulfate; K^+= Potassium; Na^+= Sodium; Ca^{2+}= Calcium; Mg^{+2}= Magnesium; Al^{3}= Aluminum; H+Al= Aluminum hydroxide; SB= Sum of bases; CEC= total cation exchange capacity; OM= Organic matter.

Leaves for analysis were pre-adapted to the dark with appropriate clips, and after 30 minutes, readings were taken. The variables evaluated were the initial fluorescence (F_0), variable fluorescence (F_v), maximum fluorescence (F_m) and photochemical efficiency of Photosystem II (F_v/F_m). When chlorophyll fluorescence parameters of dark-adapted state were definied, the same leaves were submitted to the actinic light associated to the distant red light, which enabled to achieve FS (fluorescence when electron transport processes and biochemical reactions from carbon reduction are balanced), MF (maximum fluorescence in state adapted to light) and F_O' (minimum fluorescence in state adapted to light). From these parameters, the effective photochemical efficiency (Y) and electron transport rate (ETR) were estimated.

Data were subjected to normality (Shapiro-Wilk) and homogeneity of variances (Bartlett) tests. Subsequently, an analysis of variance ($p \leq 0.05$) was performed, In cases of significance, Tukey's test ($p \leq 0.05$) was used for types of microalgae extracts linear or quadratic polynomial regression analysis according to the highest coefficient of determination (R2) obtained for doses of microalgae solutions, with software SISVAR version 5.6 (Ferreira, 2014).

Results and Discussion

According to results from analysis of variance, the number of branches, shoot length and number of leaves in function of different doses of microalgae extracts showed significant difference ($p \leq 0.05$). Unfolding the factors, it was possible to verify isolated significant effect on microalgae extracts for the number of branches and shoot length, while the interaction between the extracts and doses promoted significant effect on the number of leaves (**Table 2**).

Regarding the number of branches, significant effect for doses of microalgae extract was found, regardless of types, and data were adjusted to the quadratic model presenting maximum increment at the highest dose (15%), which pomegranate plants induced, on average, 15 new branches per plant, increasing 4.05% if compared to the control treatment (**Figure 1**A).

For shoot length, there was positive effect for the application at dose of 6%, obtaining the maximum increase at 27.4 cm, with rise of 29.8% if compared to the dose of 15%, which minimum values (21.1 cm) were

Table 2. Summary of analysis of variance for the number of branches (NB), shoot length (PL), number of leaves (NL) in pomegranate seedlings in function of different doses of Spirulina platensis and Scenedesmus sp. extracts

Source of variation	DF	Mean Square		
		NB	SL	NL
Microalgae	1	0.62^{ns}	21.75^{ns}	2.50^{ns}
Doses	3	30.15*	104.9*	13.66*
M x D	3	9.42^{ns}	16.62^{ns}	18.56*
Residue	32	10.23	35.98	4.71
CV(%)		25.05	24.25	25.5
Average		12.77	24.73	8.50

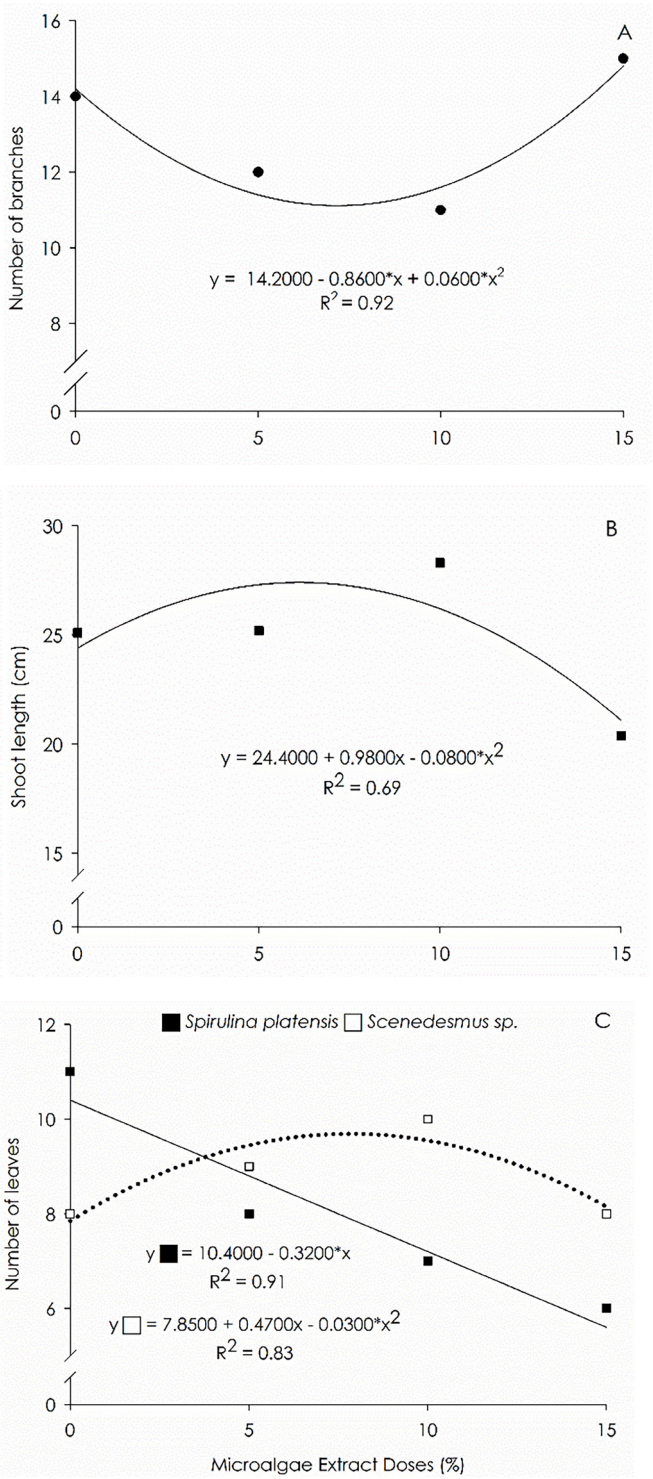

Figure 1. Number of branches (A), shoot length (B) and number of leaves (C) in pomegranate seedlings submitted to foliar application of *Spirulina platensis* and *Scenedesmus* sp. extracts

achieved (Figure 1B).

Significant effect was checked for interaction between the types of microalgae and the doses in relation to the number of leaves, which the application of *Spirulina platensis* adjusted to decreasing linear effect, losing 46.15% of leaves compared to the highest dose with the control treatment. However, when the application of *Scenedesmus* sp. extracts was adopted, data were better adjusted to the quadratic model, showing the highest foliar emission on plants submitted to 8% of dose with 9.69 leaves per plant, reaching more than 23.4% compared to the values of plants that did not receive the extract (Figure 1C).

Regarding the analysis of variance for physiological parameters, there was only significant effect between extracts and doses for stationary fluorescence (FS) and effective photochemical efficiency (Y). Both types of microalgae extracts promoted effect on stomatal conductance (*gs*), internal CO_2 concentration (*Ci*) and electron transport rate, while isolated effect on doses influenced the minimum light-adapted fluorescence (FO') and electron transport rate (ETR) in pomegranate seedlings (**Table 3**).

The stationary fluorescence was also influenced by the interaction between factors, with values for application of *Spirulina platensis* adapted to the increasing linear model, showing maximum increase at dose of 15% with 188.06 photons, which corresponded to above 39.5% compared to values in control treatment. The application of *Scenedesmus* sp. best fit to the the quadratic model, with maximum increment in plants submitted at dose of 6% with 191.93 photons (**Figure 2**A).

For photochemical efficiency of PSII, there was effect on the interaction of factors, which the application of *S. platensis* and *Scenedesmus* sp. had values that fit the quadratic model with 0.622 and 0.639 at doses of 3.0 and 10%, respectively (Figure 2B).

Between the types of microalgae extracts, regardless of doses, the application of *Spirulina platensis* promoted the highest stomatal conductance (gs) in pomegranate seedlings (0.164 mol H_2O m^{-2} s^{-1}), overpassing *Scenedesmus* sp. in 26.2% with 0.130 mol H_2O $m^{-2}s^{-1}$ (**Figure 3**A). The internal CO_2 concentration had also similar behavior, which plants submitted to the application of *Spirulina platensis* had the highest CO_2 concentration with 282.75 µmol CO_2 m^{-2} s^{-1} (Figure 3B). Pomegranate plants submitted to *Scenedesmus* sp. had average of 256.65 µmol CO_2 mol air^{-1} for internal CO_2

Table 3. Summary of analysis of variance for stomatal conductance (*gs*), CO_2 assimilation rate (*A*) transpiration (*E*), internal CO_2 concentration (*Ci*), water use efficiency (*WUE*), instantaneous carboxylation efficiency (*iCE*), initial fluorescence (F0), variable fluorescence (F_v), maximum fluorescence (F_m), photochemical efficiency of Photosystem II (F_v/F_m), stationary fluorescence (SF), maximum fluorescence in state adapted to light (MFS), minimum fluorescence in state adapted to light (F_O'), effective photochemical efficiency (Y) and electron transport rate (ETR) in pomegranate seedlings in response to different doses of *Spirulina platensis* and *Scenedesmus* sp

Source of variation	DF	Mean Square					
		gs	*A*	*E*	*Ci*	*WUE*	*ICE*
Microalgae	1	0.01122*	0.00001^{ns}	0.059^{ns}	6812.10**	1.51^{ns}	0.000087^{ns}
Doses	3	0.00348^{ns}	3.0129^{ns}	0.297^{ns}	1041.33^{ns}	1.17^{ns}	0.000075^{ns}
M x D	3	0.00211^{ns}	12.614^{ns}	0.208^{ns}	485.96^{ns}	4.17^{ns}	0.000219^{ns}
Residue	32	0.002008	4.6976	0.403	405.82	1.76	0.000085
CV(%)		30.5	26.9	22.6	7.47	44.4	30.3
Avarage		0.14	8.04	2.80	269.7	2.98	0.03
Source of variation	DF	Mean Square					
		F0		Fv	Fm	Fv/Fm	
Microalgae	1	1380.62^{ns}		64400.62^{ns}	84640.0^{ns}	0.00017^{ns}	
Doses	3	2299.69^{ns}		41138.62^{ns}	48751.0^{ns}	0.00029^{ns}	
M x D	3	188.49^{ns}		28128.67^{ns}	29946.9^{ns}	0.00017^{ns}	
Residue	32	1108.83		44321.47	54194.2	0.00019	
CV(%)		4.56		9.06	7.62	1.86	
Avarage		730.27		2323.8	3054.1	0.75	
Source of variation	DF	Mean Square					
		SF	MFS	F_O'	Y	ETR	
Microalgae	1	2656.9^{ns}	483.02^{ns}	315062.50^{ns}	0.0090^{ns}	4763.74*	
Doses	3	3027.8^{ns}	7014.8**	133389.61^{ns}	0.0177^{ns}	3269.92*	
M x D	3	5185.3*	605.15^{ns}	247107.67^{ns}	0.0206**	1443.17^{ns}	
Residue	32	1198.6	1989.62	122925.14	0.0070	500.46	
CV(%)		20.4	10.6	159.1	14.2	55.8	
Avarage		169.9	419.02	220.25	0.59	33.96	

* Significant at 5%; ** significant at 1%; ^{ns} not significant

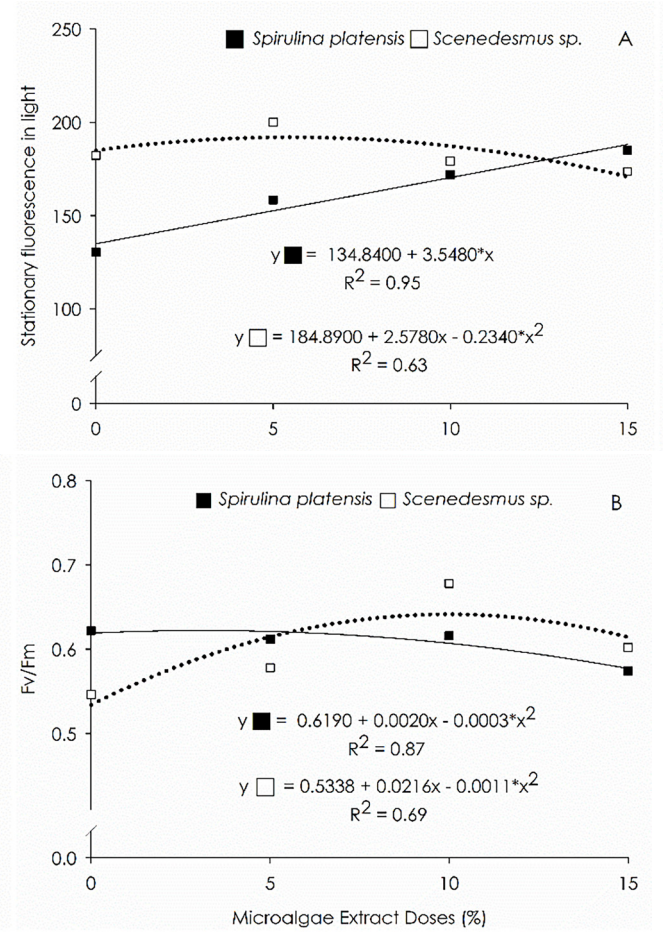

Figure 2. Stationary fluorescence – FS (A) and photochemical efficiency of PSII – Fv/Fm (B) in pomegranate seedlings submitted to foliar application of Spirulina platensis and Scenedesmus sp. Extracts.

concentration (Ci), corresponding to a 10.2% reduction when compared to *Spirulina platensis* (Figure 3B).

Electron transport rate (ETR) had isolated effect for treatments, which the application of Spirulina platensis promoted the highest increase with 44.87 µmol of electrons m $^{-2}$ s^{-1}, representing more than 94.6% when compared to Scenedesmus sp., with 23.05 µmol of electrons m^{-2} s^{-1} (Figure 3C).

Maximum fluorescence after adaptation to saturating light was influenced by doses with values adjusting to the quadratic model, with maximum increment in plants submitted to 11% of dose and 440.05 photons, followed by decreases as doses increased. (**Figure 4**A).

The effect of doses for ETR had values that fit the quadratic model, ranging from 23.16 µmol of electrons m^{-2} s^{-1} (control) to 45.44 µmol of electrons m^{-2} s^{-1} (plants at dose of 8%), decreasing as the doses increased (Figure 4B).

The application of the extracts of *Spirulina platensis* and *Scenesdesmus* sp., stimulated an increase

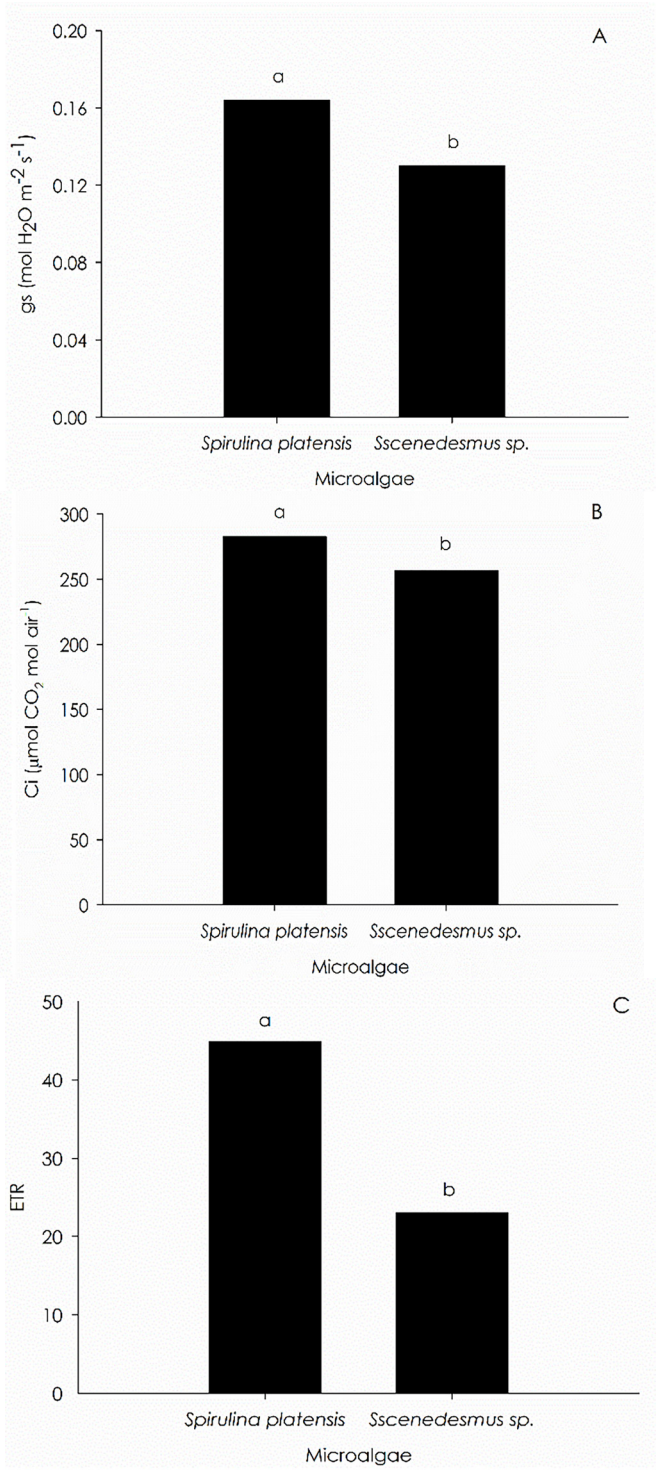

Figure 3. Stomatal conductance - gs (A), internal CO_2 concentration – Ci (B) and electron transport rate – ETR (C) in pomegranate seedlings submitted to the application of *Spirulina platensis and Scenedesmus* sp.

in the production of new branches in the pomegranate seedlings, due to the presence in their constitution of growth promoting substances, such as plant hormones, such as brassinosteroids (BRs), whose function induce new shoots and when associated with other hormones. For example, auxins and gibberellins, they also control the growth and development of seedlings (Han et al.,

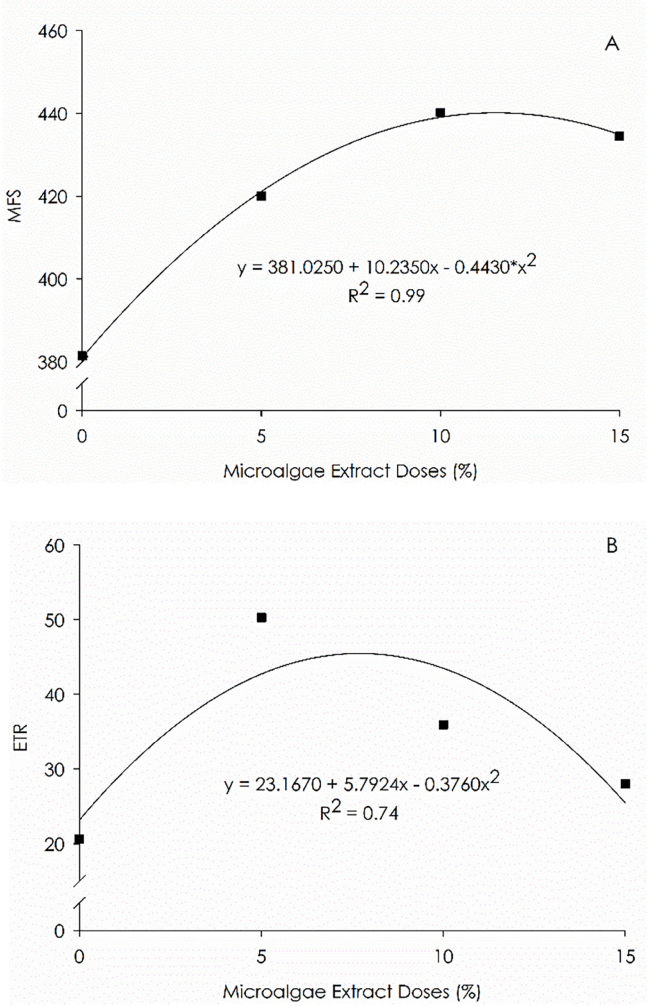

Figure 4. Maximum fluorescence after adaptation to saturating light - MFS (A) and Electron transport rate - ETR(B) submitted to different doses of microalgae extract.

2018). According to Hartmann et al. (2011), the initial development and growth of branches on cuttings are due to endogenous substances, produced by own plants. Then, the application of microalgae biomass extracts is absorbed in plant tissues, promoting hormonal stimulation through gene expression and signaling (Yakhin et al., 2017).

As well as in the production of branches the content of substances contained in the extracts of microalgae increased the growth of the aerial part of the seedlings and number of leaves, highlighting the presence of phytohormones such as auxins, cytokinins, gibberellins, abscisic acid, ethylene and other compounds such as betaines, brassinosterols, jasmonates, polyamines that promote biological effects, stimulating plant growth and development (Saa et al., 2015; Michalak et al., 2016).

The effect promoted by the application of extracts is associated with its chemical constitution and applied concentration, since it contains a diversity of components, such as macro and micronutrients and growth regulators that, depending on the applied concentration, can promote direct and effects on plant growth (Castellanos-Barriga et al., 2017).

The effect of extracts of microalgae associated with plant growth have been found in several studies, as Guedes et al. (2018), studying papaya seedling production with *Spirulina platensis* doses, verified rises in values for plant length when applications and doses of these microalgae increased. Silva et al. (2016), working with *Ascophyllum nodosum* extract found that 2 mL L^{-1} of dose improved the growth of *Annona glabra*.

The greater efficiency for stationary fluorescence in light with the application of *Scenesdesmus* sp., can occur due to several factors, in which the extract is directly influenced by the high levels of lipids that the microalgae has in its constitution, favoring a greater uptake of light by the plant emitted constantly by the photons, with a greater assimilation of CO_2 by the photosynthesis process in the plant. *Scenesdesmus* sp., Is a species of microalgae rich in lipids, which acts on cell organization and structure, performing vital functions such as cell control and signaling and energy source (Bermudez-Sierra, 2018).

The photochemical efficiency of PSII may be used as indicator of biotic and abiotic stress condions on plants. Thus, doses up to 3.0 and 10% for both extracts promote better light absorption by photosynthetic pigments, whereas values above these rates cause stress to plant. Taiz et al. (2017) described that the photosynthetic process is related to fluorescence and light absorption by chloroplast pigments, which transform light energy into heat and ATP along the reaction centers of photosystems I and II. However, each plant is able to transport electrons and store absorbed energy, and the excess is released in the form of fluorescence, energy dissipation through light and heat (Farooq et al., 2018).

It may be attributed to the amount of amino acids in microalgal biomass, which work as modulators of stomatal opening, and thus control stomatal conductance and photosynthetic rate (Cristiano et al., 2018). Higher stomatal conductance at doses above 10% may induce salt stress, resulting in reduction of *gs* to save water and imporove water use efficiency. Similarly, Li et al. (2014) found that exogenous application of *Chlorella vulgaris* on *Vicia faba* plants induced stomatal closure through the production of reactive oxygen species (ROS) by NADPH oxidase.

The application of *S. platensis* extract promoted

improvements in the physiology of pomegranate seedlings, increasing stomatal conductance and CO_2 assimilation, since the influx of CO_2 in the leaf mesophile is directly associated with stomatal opening, allowing greater assimilation (Taiz et al., 2017). While the extract of *Scenesdesmus* sp., Reduced the assimilation of CO_2, due to stomatal closure, it is directly related to the decrease in the internal concentration of CO_2 (Harb et al., 2010; Elliott-Kingston et al., 2016).

The effect of using microalgae extracts on plant physiology is still conflicting. Xu and Leskovar (2015), working with 0.1 mL of *Ascophyllum nodosum* extract applied on spinach plants under water stress, found positive effect for stomatal conductance and photosynthesis. On the other hand, Spann and Little (2011), working with the same seaweed extract under 0.0 to 8.0 mL L^{-1} proportion of doses, did not verify any significant effect on the physiology of orange plants.

The application of extracts stimulates maximum fluorescence rates, once higher than 15,5% in relation to the control treatment. Silva (2017), working with 'Tahiti' lime under different irrigation blades, did not find any significant effect for this variable. However, it presented higher values, ranging from 550 to 800 photons compared to those obtained in this study.

The electron transport rate showed a great variation between the doses of applied microalgae extracts, possibly due to environmental conditions, since this variable is susceptible to environmental variations (PIMENTEL et al., 2011).Thus, these oscillations in ETR m ay be associated with the short evaluation period and environmental conditions during evaluation, because photosynthetic capacity of plants may be changed by biotic or abiotic stresses which plants are able to pass through, such as temperature, radiation, water deficiency, salinity, presence of insects or fungi, etc (Bown et al., 2002; Kollist et al., 2019).

The increase for ETR in seedlings sprayed with *Spirulina platensis* until the dose of 10% is possibly due to the rise in oxidation of the primary quinone acceptor in the chloroplast electron transport chain related to the high electrical conductivity of extracts. The increase in electrical conductivity is caused by rises in the concentration of ions, mainly Cl^- and K^+ contained in microalgae constitution (Manrich, 2017).

Conclusion

Spirulina platensis and *Scenedesmus* sp. extracts stimulated growth in pomegranate seedlings, providing the development of shoot length and number of shoots and leaves.

Spirulina platensis extract promoted the highest efficiency for stomatal conductance, internal CO_2 concentration and electron transport rate.

Scenedesmus sp. extract improved stationary fluorescence and photochemical efficiency of PSII in pomegranate seedlings.

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