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Physiology and nutritional contents of lettuce (Lactuca sativa L.) plants induced by Pseudomonas fluorescens

Fisiologia e conteúdo nutricional de plantas de alface (Lactuca sativa L.) induzidas por Pseudomonas fluorescensv

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ABSTRACT

Sustainable agriculture and the larger scales of production needed to meet the higher demand for food each year have become models for several studies; beneficial microorganisms are one model that has been used as an alternative for greater production of and reductions in the use of chemical fertilizers. Thus, the objective of this work was to study the physiological changes in and nutrient contents of lettuce after inoculation with *Pseudomonas fluorescens* in combination with chemical fertilization. *P. fluorescens* had a positive influence on gas exchange, with increases of 41% in *A*, 44% in *Gs*, 29% in *E*, and 100% in *A / Ci*; increased

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Concentration of chlorophyll *a* and *b*, of 220% more chl a and 106% *chl* b; increased chlorophyll a fluorescence, higher relative water content; and nutrient content increases of 19% for nitrogen, 24% for phosphorus, and 105% for potassium. Plants inoculated with BRM-32111 presented increases for MFT, MFC and FF, in 37%, 34% and 26% respectively. However, when combined with fertilization, antagonism occurred, and the evaluated parameters were negatively affected.

Keywords: rhizobacteria. Biostimulants. growth. Synergism

RESUMO

A agricultura sustentável e as maiores escalas de produção necessárias para atender à maior demanda por alimentos a cada ano tornaram-se modelos para vários estudos; microorganismos benéficos são um modelo que tem sido utilizado como alternativa para maior produção e redução do uso de fertilizantes químicos. Assim, o objetivo deste trabalho foi estudar as alterações fisiológicas e o conteúdo de nutrientes da alface após a inoculação com Pseudomonas fluorescens em combinação com a fertilização química. P. fluorescens teve uma influência positiva nas trocas gasosas, com aumentos de 41% em A, 44% em Gs, 29% em E e 100% em A / Ci; aumento da concentração de clorofila aeb, 220% mais chl a e 106% chl b; aumento da fluorescência da clorofila a, maior teor relativo de água; e o teor de nutrientes aumenta 19% para nitrogênio, 24% para fósforo e 105% para potássio. As plantas inoculadas com BRM-32111 apresentaram aumentos para MFT, MFC e FF, em 37%, 34% e 26%, respectivamente. No entanto, quando combinado com a fertilização, ocorreu antagonismo e os parâmetros avaliados foram afetados negativamente.

Palavras-chave: rizobactérias. Bioestimulantes. crescimento. Sinergismo

1. INTRODUCTION

Lettuce is one of the most consumed vegetables in the world; it is generally consumed raw and is rich in nutrients and water. Currently, in the context of nutritious food that is free of fertilizers and an environment that is free of soil contaminants, the increasing use of chemical fertilizers has become a great challenge in horticulture (Oliveira et al., 2017; Fincheira et al., 2016).

To reduce or eliminate the use of chemical fertilizers for sustainable agriculture and for the consumption of organic products, mainly raw lettuce, studies are needed to identify tools that facilitate reductions in chemical inputs. Therefore, biopromoters such as fungi and bacteria have been used because these microorganisms enhance disease resistance and plant growth via mechanisms that promote an increase in the synthesis of enzymes and hormones that help augment root development, thus increasing the absorption of water and nutrients (Filippi et al., 2011; Nascente & Lanna, 2016; Bueno et al., 2017). Some genera of rhizobacteria, such as Pseudomonas and Bacillus, among others, have already been described as plant growth promoters (Devi et al., 2017; Tahir et al., 2017).

The main responses of plants to the use of biopromoters can be reflected in the parameters of gas exchange, since photosynthesis is the main mechanism for obtaining energy for the metabolic processes of the plant (Su et al., 2015). A supply of plants with higher nutritional contents is important since lettuce is widely used in the preparation of salads.

Thus, it is very important to show that the use of Pseudomonas fluorescens BRM 32111, which has already been described as a growth promoter in rice that increases biomass and root development (Rêgo et al., 2014) and positively influences gas exchange, fluorescence, and nutrient levels during initial development, results in more vigorous plants with higher nutrient contents. The objective of this work was to study the changes in chlorophyll a fluorescence, chlorophyll, gas exchange, stomatal density, water use and nutrient levels in lettuce plants inoculated with a BRM 32 111 isolate.

2. MATERIAL AND METHODS

The experiment was carried out at the Plant Protection Laboratory (LPP) and nursery at the Federal Rural University of Amazonia (latitude 01º27'25" S, longitude 48º26'36" W) in Belém, Pará, Brazil.

Preparation and inoculation of isolates

The Pseudomonas fluorescens isolate (BRM-3211) and isolates from commercial fields of the rice cultivar BRS Primavera in the municipality of Paragominas-PA collected in 2008/09 were tested in a greenhouse and in vitro, as were isolates from rice, stored in the microrganisms collection of the LPP / UFRA. Suspensions of the rhizobacteria were prepared as described by Filippi et al., (2011).

Greenhouse

Five seeds per polyethylene pot (300 mL) were sown in Ferrasol soil with the following characteristics: pH (water) 4.2, 18.80 g dm^{-3} M.O., 2 mg dm^{-3} P, 4 mg dm^{-3} K, 18 mg dm^{-3} Na, 0.05% N, 0.2 mmol dm^{-3} Ca, and 0.3 mmol dm^{-3} Ca + Mg. On the seventh day after sowing (AS), all but one plant was removed from the pot and maintained under an average daytime air temperature of 27°C, relative humidity of 74% and light intensity of 1300 µmol .m-2.s-1 (HOBO data logger). The experiment included three treatments (control-water, inoculation with BRM 32 111 and inoculation with BRM 32 111 + fertilization with NPK 20-05-15) (Trani et al., 2014).

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2.1 QUANTIFICATION OF PIGMENTS

Five plants per treatment were harvested at 28 °C; a total of 15 mg of leaves was macerated in 250 µL of 98% ethanol (EtOH) on ice and in the absence of light, incubated at 80 °C for 20 minutes, and centrifuged at 4 °C at 14,000 rpm for five minutes. The supernatant was collected immediately, and the pellet was subjected to two more extractions, one in 80% and one in 50% EtOH. The resulting supernatants were harvested and homogenized. The chlorophyll content was quantified as described by Porra, Thompson & Kriedemann (1989). A 35 µL aliquot of ethanol extract from each sample was added to a reaction mixture containing 120 mL of 98% EtOH and 15 mL of mixed acetate (final volume of 170 µL per well / sample). After preparing the reaction mixture, the absorbance of the samples was estimated at wavelengths (λ) of 645 nm and 665 nm. The chlorophyll a and b concentrations could be estimated from the absorbance; the total content was determined using formulas A and B and then normalized to the fresh weight of each of the samples (Porra, Thompson & Kriedemann, 1989).

$$(A)\text{chlorophyll a} = 5.48 ** \text{Abs}665 - 2.16\text{Abs}(\mu g/\text{well})$$

(B) Chlorophyll b = 5.48 * * Abs665-2.16 Abs 645 (µg / well)

Determination of chlorophyll a fluorescence

The fluorescence was measured from the 3rd leaf using a device (IG 6400-40, LI-COR Inc.) embedded in a portable open-flow gas exchange system. The leaves were illuminated with modulated measuring beams (0.03 µmol m-2 s-1) to obtain the initial fluorescence (F0). Saturated white light pulses of 6000 µmol of photons m^{-2} s^{-1} were applied for 0.8 s to ensure maximum fluorescence emission (Fm), of which the variable photochemical efficiency ratio up to the maximum was calculated, Fv / Fm = [Fm-F0) / Fm)] (Oxborough & Baker, 1997).

2.2 DETERMINATION OF GAS EXCHANGE PARAMETERS

The gas exchange parameters were analyzed using a portable open flow gas exchange system (LI-6400XT, LI-COR Inc., Lincoln, NE). The rate of CO_2 assimilation (A) was estimated using the 3rd leaf. Stomatal conductance to water vapor (Gs), intercellular carbon (Ci), the transpiration rate (E) and the instantaneous water use efficiency (WUE) were assessed 28 days after sowing. The evaluations were performed from 8:00 a.m. to 9:30 a.m., the maximum active photosynthetic radiation period (1300 mol photons m^{-2} s^{-1} at leaf level and 400 mol CO_2 mol -1 ar). All measurements were performed at 28 °C (Bohm, 1994).

2.3 STUDY OF STOMATA

The size of the stomata was determined based on the equivalent area of the ellipsoid representing the area of the stomatal pores (APE) using the following formula: (π × length x width) / 4. The stomatal density was considered to be the number of stomata per unit area and was measured using an ocular aid millimeter, Motic Microscope (BA-400), and ANTI QUANT 2 software (Minnocci, Panicuca, vitagliano, 1995; Bartolini, Minnocci, vitagliano, 1997).

2.4 WATER HOLDING CAPACITY (WHC)

The water content was evaluated by subtracting the dry weight of the leaf samples from each treatment from their fresh weight; the difference obtained corresponds to water retention (Souza et al., 2000).

2.5 MACRONUTRIENT CONTENTS

The samples were maintained in a greenhouse with forced air circulation at temperatures ranging from 65 to 70°C until reaching a constant weight. To ensure the homogenization of the sample, milling was carried out in Willey mills with knives in a stainless steel chamber and with sieves of 0.5 or 1 mm in diameter (20-40 mesh). For the nutrient analysis of the leaf tissue, N, P, K, Mg, and Na were evaluated (Carmo et al., 2000).

2.6 HYDROPONIC SYSTEM

The experiment was a completely randomized design with two treatments (plants moistened in water: control and plants inoculated with BRM 32111) and 10 replications distributed in an area of 24m2 of hydroponic system. Controlled and inoculated seeds with BRM-32111, prepared as described by Filippi et al., (2011). They were sown in trays containing coconut fiber. After four days after germination the seedlings followed for 11 days in the maternity in cells containing coconut fiber, being replanted in hydroponic profiles of 50 mm of width for 14 days in the Pre-Growth. After pre-growth, the final growth phase was started in hydroponic profiles of 75 mm until reaching the harvest point, for 20 days. The evaluation of total fresh matter (TFM) and fresh commercial matter (FCM), lettuce productivity (LP), based on total and commercial fresh mass, respectively, and the leaf quality (LQ) of the plant estimated by the number of leaves above of 10 cm (FERREIRA et al., 2015, FERREIRA et al., 2016).

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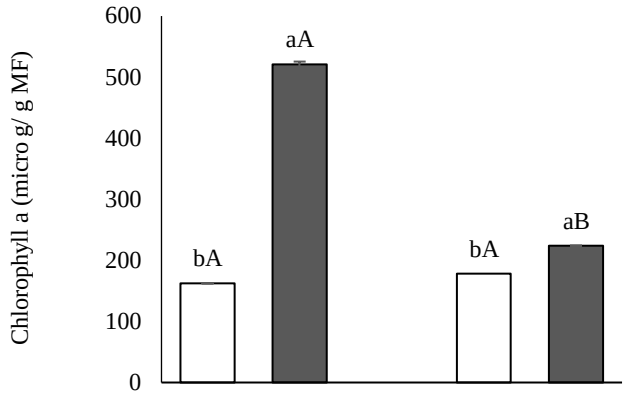
2.7 STATISTICAL ANALYSIS

The averages of the data were compared by the t test using the assistat software 7.7.

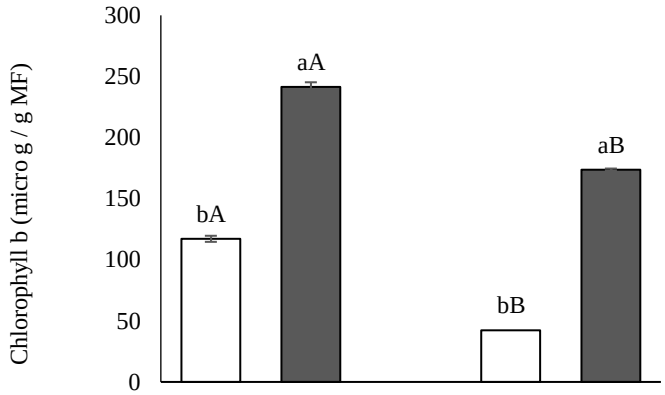
3. RESULTS

3.1 CONCENTRATION OF CHLOROPHYLL

Biochemical methods showed that plants inoculated with BRM 32111 but not fertilized had more chlorophyll than the control without fertilization and than the fertilized control. Relative to the control plants (without fertilization), the plants inoculated with BRM 32111 had 220% more chl *a*, 106% more chl *b* and 173% more total chl (Figure 1, A-C). When inoculated with BRM 32111 and fertilized, there was a reduction in chlorophyll content.



(A.)



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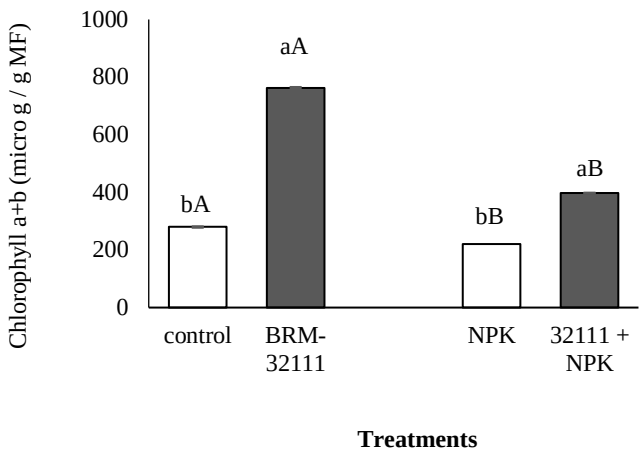
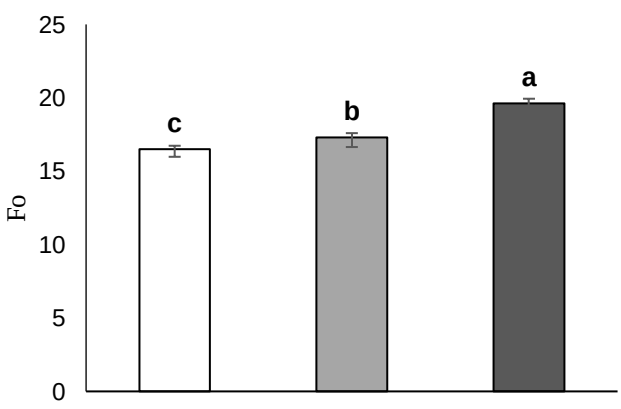


Figure 1 Levels of chlorophyll a (A), chlorophyll b (B), and chlorophyll a + b (C) in lettuce plants 28 days after germination in soil with and without fertilization, in uninoculated control plants and in plants inoculated with Pseudomonas fluorescens (BRM 32111). Bars with the same lowercase letter did not differ between treatments (Duncan, $p < 0.05$), standard error ($p < 0.05$). Bars with the same capital letter do not differ between plants with the same tested inoculation ($p < 0.05$).

3.2 DETERMINATION OF CHLOROPHYLL A FLUORESCENCE

All fluorescence variables of chlorophyll a were positively affected in lettuce plants inoculated with BRM 32111; the initial fluorescence (*F0*) increased by 19% and 14% in comparison to the absolute control and the fertilized control, respectively (Figure 2A). In terms of *Fm*, plants inoculated with BRM 32111 showed increases of 33% and 37% compared to the absolute control and fertilized control, respectively (Figure 2B). In plants inoculated with BRM 32111, the maximum photochemical efficiency (*Fv / Fm*) increased by 22% and 18% in relation to the absolute control and the fertilized (NPK) control, respectively (Figure, 2C).



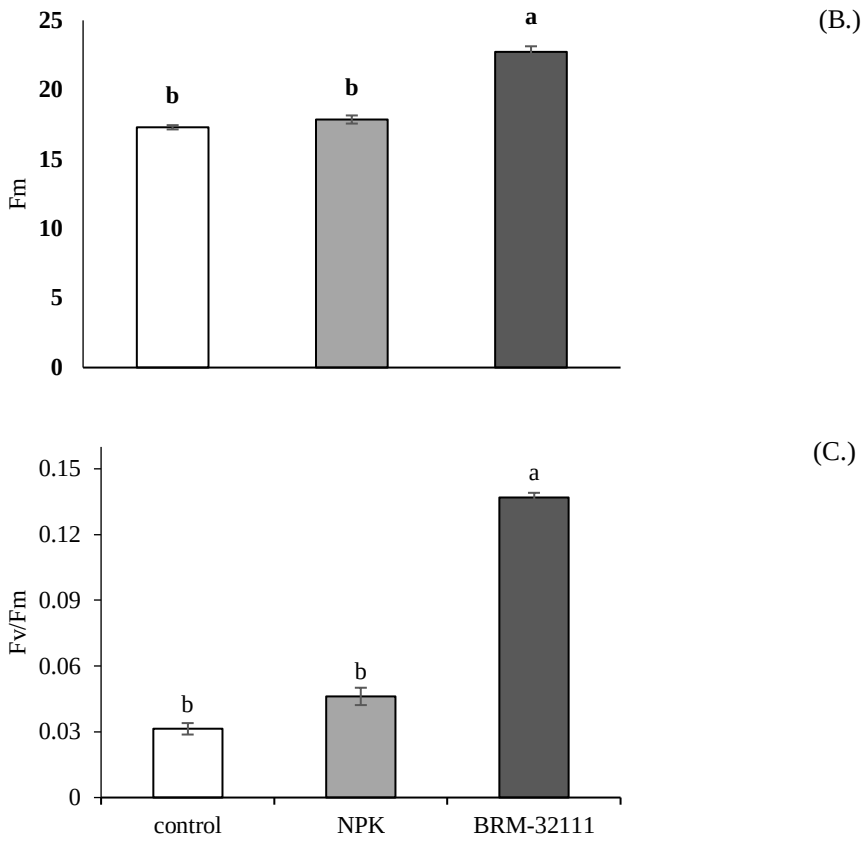


Figure 2 Initial fluorescence (F0) (A), minimum fluorescence (Fm) (B), and (Fv / Fm) (C) determined using areas of foliar tissue of uninoculated plants, uninoculated plants in soil fertilized with N-P-K and in plants inoculated with Pseudomonas fluorescens BRM-3211. Bars followed by the same lowercase letter did not differ between treatments (Duncan, $p < 0.05$), standard error ($p < 0.05$).

3.3 PARAMETERS OF GAS EXCHANGE

The plants inoculated with BRM-32111 had a 41% increase in the net CO2 assimilation rate (*A*), a 44% increase in the stomatal conductance of water vapor (*Gs*), a 29% increase in the transpiration rate (*E*), and a 100% increase in the carboxylation efficiency (*A / Ci*) (Figure, 3-D). However, when the plants were inoculated with BRM 32111 and fertilized, they showed reductions in gas exchange of 18% for Gs, 15% for E, and 13% for *A / Ci* (Figure 3A-D).

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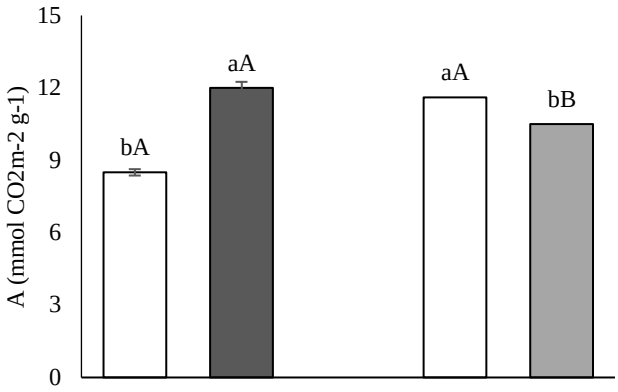
(A.)



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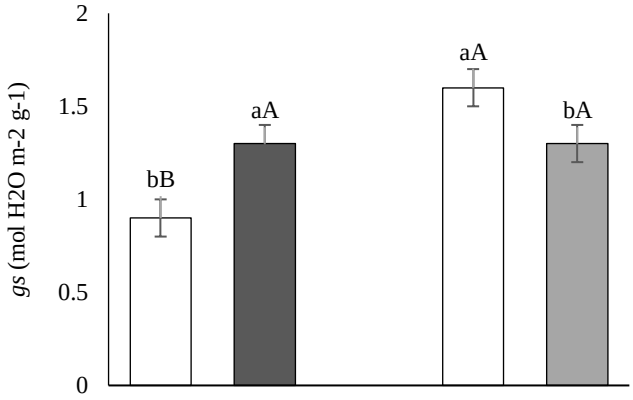
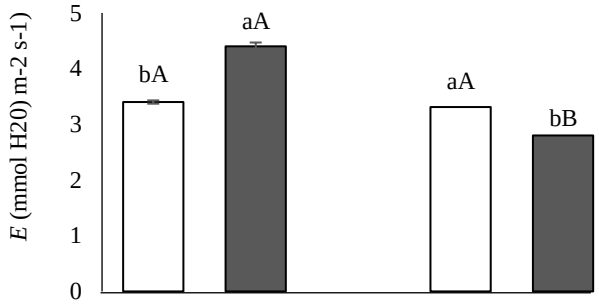


(C.)



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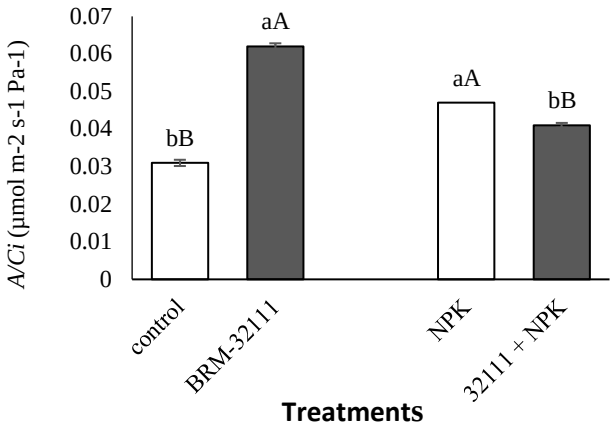
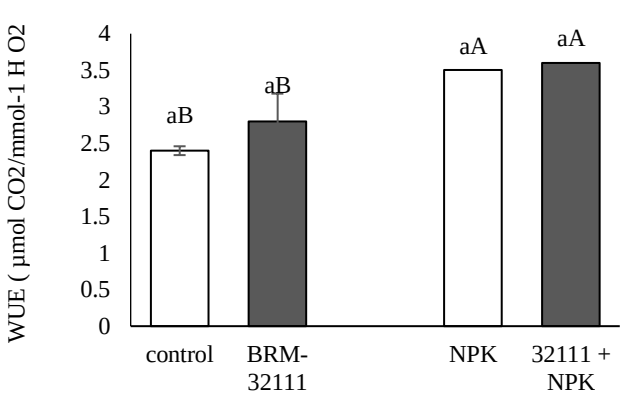


Figure 3 Liquid assimilation rate of carbon (A) mmol H2O m-2 S-1) (A), stomatal conductance to water vapor (gs) (mmol H2O m-2 S-1) (B), transpiration rate (E) (mmol H 2 O m-2 S-1) (C), and instantaneous carboxylation efficiency (D) determined from uninoculated plants, uninoculated plants on NPK-fertilized soil,inoculated with Pseudomonas fluorescens BRM-32111 and plants on NPK-fertilized soil and Pseudomonas fluorescens BRM-32111. Bars followed by the same lowercase letter did not differ between treatments with soil residue and soilless sampling (Duncan, p <0.05), standard error (p <0.05). Bars followed by the same capital letter do not differ between plants with the same tested inoculation (p <0.05)

3.4 DETERMINATION OF WATER USE EFFICIENCY (WUE) AND WATER HOLDING CAPACITY (WHC)

Efficiency in water use and water retention capacity was positively affected by BRM 32 111; WUE increased by 16% and CRA increased by 123% in plants inoculated with BRM 32111 compared to those in the absolute controls (Figure 4). However, there was a 3% reduction in WUE in plants inoculated with BRM 32111 and fertilized (NPK) (Figure 4)



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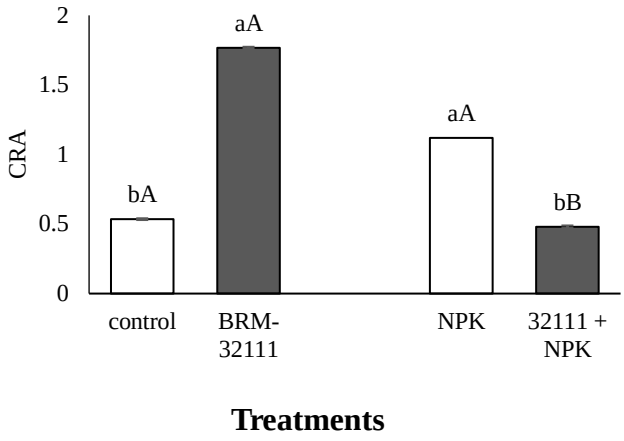
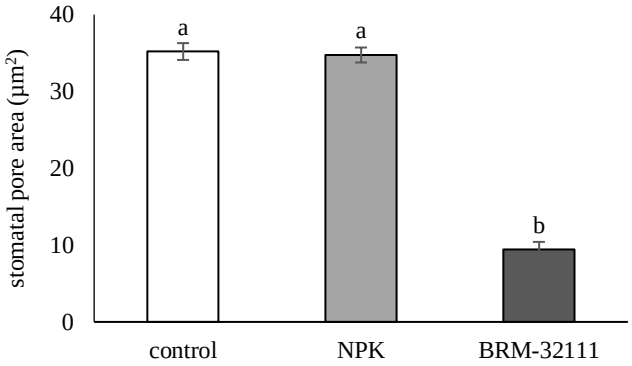


(B.)

Figure 4 Instantaneous water use efficiency (A) and water retention capacity (WRC) (B) determined from non-inoculated plants, uninoculated plants in soil fertilized with NPK and plants inoculated with Pseudomonas fluorescens BRM-32111. Bars followed by the same lowercase letter did not differ between treatments with soil residue and soilless sampling (Duncan, $p < 0.05$), standard error ($p < 0.05$). Bars followed by the same capital letter do not differ between plants with the same tested inoculation ($p < 0.05$)

3.5 STUDY OF STOMATA

Inoculation with BRM 32 111 increased the stomatal density by 190% but reduced the area of the stomatal pores by 73% in relation to the unfertilized control. Compared to fertilized plants (NPK), the stomatal density increased by 81% compared to the fertilized control (NPK) (Figure 5).



(A.)

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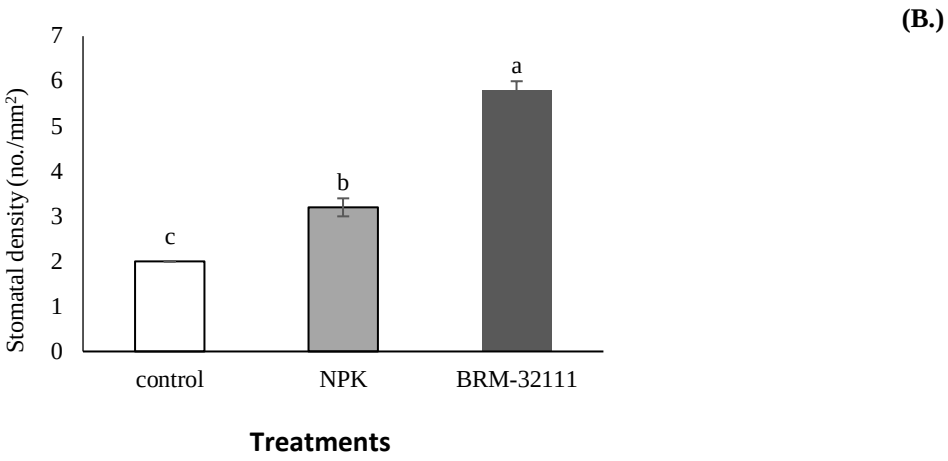

Figure 5 Stomatal density (A) and stomatal pore area (B) determined from uninoculated plants, uninoculated plants on N-P-K fertilized soil and plants inoculated with Pseudomonas fluorescens BRM-32111. Bars followed by the same lowercase letter did not differ between treatments with soil residue and soilless sampling (Duncan, $p < 0.05$), standard error ($p < 0.05$)

3.6 MACRONUTRIENT CONTENT

Inoculation with BRM 32111 had a positive effect on the macronutrient content of lettuce plants; in relation to the control, nitrogen content increased by 19%, phosphorous increased by 24%, and potassium increased by 105%. Compared to the fertilized plants (NPK), the lettuce plants inoculated with P. fluorescens showed increases of 10% in nitrogen content, 15% in phosphorus content, and 25% in calcium content (Table 1).

Table 1. Nitrogen, phosphorus, potassium, sodium, calcium, and magnesium contents of uninoculated lettuce plants, lettuce grown in soil fertilized with N-P-K and lettuce inoculated with Pseudomonas fluorescens BRM-32 111.

Treatment	N	P	K	Mg	Na
	g/Kg	g/Kg	g/Kg	g/Kg	g/Kg
Control	24 c ±0.7*	2.46 c ±0.1*	12.13 b±0.8*	2.58 a ±0.2	1.82 a ±0.3
NPK	26 b ±0.7*	2.66 b ±0.1*	27.44 a±0.6	3.29 a ±0.2	2.63 a ±0.1
BRM 32 111	29 a ±0.8*	3.06 a ±0.02	24.89 a±0.3*	3.06 a ±0.03	2.32 a ±0.1

3.7 HYDROPONIC SYSTEM

Plants inoculated with BRM-32111 presented increases for TFM, CFM and LQ, in 37%, 34% and 26% respectively. Treatment with BRM3211 also increased lettuce yield by 42% (Table, 2).

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Table 2. Production of lettuce inoculated and not inoculated with BRM-32111.

Treatments	TFM (g/plant)	CFM (g/plant)	LQ (unity)	LP (Kg/m2)
BRM-32111	343,4*	239,75 *	24,4 *	1,7
Control	251,3	178,79	19,4	1,2

4. DISCUSSION

The rhizobacterium P. fluorescens (BRM 32111) increased the chlorophyll content, physiological parameters and nutrient contents in lettuce plants. The genus Pseudomonas promotes plant growth in lettuce and rice via both direct and indirect mechanisms of action (Rêgo et al., 2014; Ugochi et al., 2016). However, in the present study, chlorophyll a and fluorescence changes were observed, as were gas exchanges that resulted in N, P and K increases, indicating the positive interaction of Pseudomonas with lettuce plants (Ahmad et al., 2015). On the other hand, when chemical fertilization was combined with BRM 32111 treatment of seeds, there was an antagonistic effect on chlorophyll, gas exchange and the water retention capacity.

In the present study, lettuce plants inoculated with BRM 32111 had an increase in stomatal density that was associated with a smaller stomatal pore area, indicating that the increased number of stomates allowed a greater influx of CO2 without excessive water loss, resulting in increased rates of CO2 assimilation, transpiration and carboxylation (Silva et al., 2015). The relative contents of chlorophyll a and chlorophyll b as well as chlorophyll a + b increased in plants treated with BRM 32111, which can be attributed to the increase in the nitrogen concentration in the leaves (Chapman & Barreto, 1997). BRM 32111 can contain the nitrogenase enzyme complex, which is common to the genus *Pseudomonas* and reduces atmospheric nitrogen to ammonia; this process increases the availability of N, which is used to synthesize amino acids, DNA, and RNA, in addition to chlorophyll (Xu et al., 2017; Bulgarelli et al., 2013).

The use of chlorophyll a fluorescence parameters to evaluate the performance of photosystem II (PSII) in plants inoculated with PGPR is poorly studied. However, the fluorescence emission of chlorophyll a is an important indicator of the integrity of photosystem II (PSII), a system that permits the production of ATP and NADPH. In our study, inoculation with BRM 32111 promoted an increase in the photochemical phase yield (Fv / Fm), which

increases the ATP and NADPH that will be used in CO2 fixation, resulting, in part, in the increase in liquid photosynthesis (Murchie & Lawson, 2017).

The increase in gas exchange and the carboxylation rate in plants inoculated with BRM 32111 can be directly associated with a larger leaf area and greater stomatal density, with stomata being the main mechanism of water loss through transpiration and CO2 fixation (Lamaud et al., 1966; Vavasseur & Raghavenda, 2005).

The nutrient content of lettuce leaves in plants inoculated with BRM 32111 and the chemically fertilized plants were found to be within the range suitable for lettuce cultivation in relation to N, P and K, according to the values described by Hartz and Johnstone (Hartz et al, 2007). This result indicates the positive effect of rhizobacteria on the nutritional status of lettuce plants, in addition to the N and P contents being higher than those in the fertilized plants. The highest N content in lettuce leaves inoculated with BRM 32111 is directly related to the results obtained in this work because N is an essential constituent of nucleotides, membrane lipids and amino acids, as well as a component of chlorophylls (Millard & Grelet, 2010; Sanchez & Bragado, 2014). In beans, inoculation with P. fluorescens and Azospirillum lipoferum increased the amount of N fixed, and increased seed production and plant protein content (Yadegari, 2010).

The increased P content in the plants inoculated with BRM 32111 may be related to the ability of the rhizobacteria to solubilize this element in the soil and to make it more available to plants (Ahemad & Kibret, 2014). through the production of organic acids with low PQQ and a cofactor (PQQ), forming the metabolic base for the solubilization of phosphate by species such as Pseudomonas (Rodriguez & Fraga, 1999; Otieno et al., 2015).

These strategies of phosphate solubilization are determinant in tropical soils, where high P fixation occurs, reaching levels above 80% insolubility after the application of chemical phosphate fertilizers in the soil (Guedes et al., 2016). As observed in the present study, lettuce plants fertilized with NPK showed lower P contents in the leaves than did plants inoculated with BRM 32111.

Chemical fertilization (NPK) combined with BRM 32111 inoculation induced losses in the physiological parameters of lettuce plants, indicating an antagonistic effect of the chemical fertilization on the rhizobacteria. This effect occurred after applying the recommended dose of nitrogen fertilizer to wheat (Saubidet et al., 2002). In the case of maize plants, nutritional status may affect the rhizobacteria by repressing the genes associated with

protein synthesis or affecting the composition of root exudates during the growth phase, for example, providing greater uptake in shoots from low fertilizer contents (Galvez et al., 2001).

All production variables were positively affected with the inoculation of rhizobacterium BRM-32111. What reveals the changes occurred with the inoculation of the bacteria made in the lettuce seeds last until the productive cycle, resulting in high yield of leaves with quality. In the case of microbial seed propagation, it has a positive effect on the plants with increased production, can guarantee less chemical use, seedling protection in the early stages of development and lower volume of water per application (Ludwig et al., 2013). Thus, the use of growth promoting bacteria can be inserted into the hydroponic lettuce cultivation system in regions of high temperature and humidity, leading to increased yield and product quality.

In this way, we demonstrate that the use of Pseudomonas fluorescens BRM 32 111 positively influences the gas exchange parameters, chlorophyll a fluorescence, chlorophyll *a* and *b* pigments, and nutrient contents in lettuce plants. In addition to having the most positive effect on plant development, BRM 32 111 can act as a biofertilizer.

5. CONCLUSION

The use of BRM 32 111 promotes better photosynthetic efficiency in lettuce plants during initial development and promotes increased nitrogen, potassium and phosphorus contents. These results show the importance of further studies that address the behavior of lettuce plants after transplanting.

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