

Full Length Research Paper

Performance of different genotypes of maize subjected to inoculation with *Azospirillum brasilense*

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The lack and/or inconsistent behavior of results found in literature have limited *Azospirillum* use in maize crops. In this regard, this study aimed at evaluating the performance and chlorophyll a fluorescence of maize hybrids inoculated with *Azospirillum brasilense* under different nitrogen levels and protected crop conditions with nutrient solution. The experiment was conducted in a greenhouse at Instituto Federal Goiano, Rio Verde Campus. Adapted jars from "Leonard" were used in the experiment. For each bottle, 1.5 kg of substrate with washed sand and vermiculite in a proportion of 1:1 and nutrient solution of Sarruge was used. The experiment was conducted in a completely randomized design in a 4 × 2 × 2 factorial arrangement with four replications, totaling 160 treatments: four maize hybrids: simple hybrid– H1 (NS 90); modified simple hybrid– H2 (AS 1581); double hybrid– H3 (DKB 310); triple hybrid– H4 (ATL 310); with absence and presence of N (NP and NA) and absence and presence of inoculation (IP and IA). Physiological evaluations of chlorophyll a fluorescence using a modulated portable fluorometer, MINI-PAM model were performed at the end of the experiment. Initial fluorescence (F_0), maximum fluorescence (F_m), potential quantum yield (F_v/F_m) variable fluorescence/maximum fluorescence), effective quantum yield ($\Delta F/F_m'$), photochemical dissipation (q_p), non-photochemical dissipation (q_n and NPQ) and electron transport rate (ETR) were determined. On average, there was a difference in ETR of about 34% between treatments. For H4, it was observed that F_v/F_m was lower with inoculant when compared with plants without inoculation, a difference of approximately 11%, on average, was observed in value of F_v/F_m ratio. Due to better efficiency in absorption and utilization of nitrogen promoted by bacteria (*A. brasilense*), inoculated maize hybrids showed a good photosynthetic performance.

Key words: biological nitrogen fixation, nitrogen economy, maize hybrids.

INTRODUCTION

Maize (*Zea mays* L.) is one of the most important consumed cereals in the world due to its productive potential and nutritional value and can be used both in food and feed (Fancelli, 2011). In Brazil, large maize areas are cultivated, about 16 million ha (crop and off-season or second crop), concentrated mainly in the states of South, Southeast and Midwest, with average

national productivity of about 4.4 tha^{-1} (Conab, 2013). In off-season maize crop, the recommendation to lower amounts of fertilizers is routine. This is justified by the lower yield potential due to higher climate risks, such as low water and heat availability and less solar radiation (Shioga et al., 2004).

Nitrogen is the most important and limiting mineral in

maize yields, requiring its application in large quantities to meet crop demand. Nitrogen is one of nutrients that have the most significant effect on increased grain yield in maize. It has great importance as constituent of protein molecules, enzymes, co-enzymes, nucleic acids and cytochromes as well as its important role as a member of chlorophyll molecule (Gross et al., 2006). According to research of Fancelli (2011), nitrogen deficiency may affect grain yield from 14 to 80%, and also reduce its protein content. The management of nitrogen fertilizations is one of the most complexes due to factors related to nitrogen fertilizer cost due to problems in the efficiency of some sources (Menezes, 2004). This nutrient is characterized by having one of the highest loss indexes, which may occur by leaching, surface runoff, erosion, volatilization and ammonium denitrification. The higher or lower loss index can be controlled by implementation, management and nutrient source to be used (Queiroz et al., 2011). Its availability in soil depends on organic matter content, climatic factors which are difficult to predict, C/N ratio and microbial activities. Nitrate (NO_3^-) and ammonium (NH_4^+) are the more readily absorbed forms by plants (Camargo and Sá, 2004). Each ton of produced grain demands on average 16.4 kg of N, which 64% is exported to grain (Setiyono et al., 2010).

Thus, due to growing demand for sustainability in agricultural production systems, some authors have presented biological nitrogen fixation (BNF) as an alternative to nitrogen fertilizer saving, which can supplement or even replace this fertilizer use (Bergamaschi, 2006).

Biological fixation may be responsible for approximately 65% of total N set on Earth, therefore it is the second most important biological process after photosynthesis (Cantarella, 2007). Among the N fixer microorganisms found in association with grass roots of species of *Azospirillum* genus are one of the most studied groups (Hartmann and Baldam, 2006). Although, *Azospirillum* genus is consisted of seven species, the most extensively studied for the use on inoculation is *Azospirillum brasilense* (Huergo et al., 2008). Despite the potential use of this technique, the application of diazotrophic *A. brasilense* through solution in seeds, with or without synthetic nitrogen rates, does not interfere with plant development and maize crop yield. The adoption of this practice does not replace the use of nitrogen fertilizers and neither allows rate reduction (Repke et al., 2013).

Plants inoculated with *Azospirillum* showed a higher rate of photosynthesis and of stomatal conductance, resulting in higher grain yield when compared with non-

inoculated plants, as well as the nutrient content in the grains was higher in inoculated plants (Saikia et al., 2007). The lack of results and inconsistent behavior of results found in literature and that of Bartchechen et al. (2010) may vary according to the cultivar, climatic conditions, research methodology and has limited the use of *Azospirillum* in maize crop.

In this regard, this study aimed at evaluating the performance and chlorophyll a fluorescence of maize hybrids inoculated with *A. brasilense* under different nitrogen levels and protected crop conditions with nutrient solution.

MATERIALS AND METHODS

The experiment was conducted in a controlled environment with temperature and relative humidity of 27°C and 70%, respectively, at Instituto Federal Goiano, Rio Verde Campus, located in the southwestern of the state of Goiás, situated at 17° 47' 53" North latitude and 51° 55' 53" South latitude with 743 m of altitude.

Adapted Leonard jars were used (Vincent, 1970) for experiment conduction, however in order to maintain sterile conditions, "pet" bottles were cut with scissors, 14 to 15 cm height from base. Subsequently, an aseptic process immersed in sodium hypochlorite (5%) for 1 h was performed. After this period, material was rinsed in running water with distilled water and autoclaved.

To each bottle, 1.5 kg of substrate with washed sand and vermiculite in a proportion of 1:1 was used. It were placed in polyethylene plastic bags and washed for sterilization in autoclave for 1 h at 121°C, for two consecutive days. Autoclaved mixture was placed on top of bottle, and nutritive solution of Sarruge (1975) was inserted at the bottom, according to Table 1. And afterwards, pots were covered with plastic bags paper of "kraft" type in order to prevent algae development.

Maize seeds were surface disinfected for 2 min with ethyl alcohol at 70% and sodium hypochlorite at 2% for 3 min (Hungria et al., 1994), then washed for 10 times in distilled water and autoclaved. Two seeds per pot were planted and after seven days of germination, manual thinning of excess plants was performed, leaving only one plant per pot.

The experiment was conducted in a completely randomized design in a 4 × 2 × 2 factorial arrangement with four replications, totaling 160 treatments: four maize hybrids (simple hybrid– H1; modified simple hybrid– H2; double hybrid– H3; triple hybrid– H4); with presence and absence of N (NP and NA); and presence and absence of inoculation (IP and IA).

Maize hybrids were chosen according to materials for planting in the region, which are widespread among crop producers in the Southwestern of the State of Goiás, (H1–NS 90; H2–AS 1581; H3–DKB 310 and H4–ATL 310), N supply was in accordance with nutrient solution (Table 1) and inoculation with Azototal® *Azospirillum brasilense* based commercial product (AbV5 and AbV6 strains) and was performed at sowing time with 100 mL of liquid inoculate for each 25 kg of seed with 2.0×10^8 CFU ml^{-1} guaranteed, applied and directly homogenized in seeds, according to the Manufacturer's specifications.

Nutrient solution was prepared in a quantity sufficient to fill the

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Table 1. Chemical composition of nutrient stock solution in molar (M), and treatments, in ml l⁻¹, used in this study (Sarruge, 1975).

Stock solution ¹	Concentration (M)	Treatments	
		Complete	-N
KH ₂ PO ₄	1	1	1
KNO ₃	1	5	-
Ca(NO ₃) ₂ .4H ₂ O	1	5	-
MgSO ₄ .7H ₂ O	1	2	2
KCl	1	-	5
CaCl ₂ .2H ₂ O	1	-	5
NH ₄ H ₂ PO ₄	1	-	-
NH ₄ NO ₃	1	-	-
(NH ₄) ₂ SO ₄	1	-	-
Mg(NO ₃) ₂ .6H ₂ O	1	-	-
Micronutrients ¹ – Fe	-	1	1
Fe – EDTA ²	-	1	1

¹Stock solution of micronutrients (g l⁻¹): H₃BO₃ – 2.86; MnCl₂.4H₂O – 1.81; ZnCl₂ – 0.10; CuCl₂.2H₂O – 0.04; H₂MoO₄.H₂O – 0.02. ²To dissolve 26.1 g of disodium EDTA on 89.6 ml of NaOH 1.0 M, to mix with 24.9 g of FeSO₄.7H₂O and complete to 1,000 ml.

bottom of jars. A total of 200 mL of nutrient solution per pot was used. Different solutions were performed according to treatments (with N "complete solution" and without N "solution – N"). Solution was remade every time that a replacement was necessary.

Physiological assessments concerned with chlorophyll a fluorescence were performed at 35 days after emergence, when all hybrids reached a total of four expanded leaves, recommended time to perform nitrogen fertilizer application. The chlorophyll a fluorescence measures were obtained using modulated portable fluorometer, MINI-PAM model (Walz, Effeltrich, Germany), equipped with a special clamp to model sheet support 2030-B (Bilger et al., 1995; Rascher et al., 2000). Initial fluorescence (F₀), maximum fluorescence (F_m), potential quantum yield (Fv/Fm = variable fluorescence/maximum fluorescence), effective quantum yield (ΔF/Fm'), photochemical dissipation (q_p), non-photochemical dissipation (q_n and NPQ) and electron transport rate (ETR) were used. The maximum quantum efficiency of photosystem II (Van Kooten and Snel, 1990) was calculated after 30 min of dark adaptation using equation $Fv/Fm = (Fm - F_0)/Fm$; where F₀ is the minimum fluorescence yield, excited by a low intensity modulated red light (0.03 mmol m⁻² s⁻¹) and F_m is the maximum fluorescence obtained by applying 0.8 s pulse of saturating actinic light (>6,000 μmol m⁻² s⁻¹). The effective quantum efficiency of photosystem II (Genty et al., 1989) will be determined by overlapping a saturation pulse in leaves prior adapted to ambient light, calculated as $\Delta F/Fm' = (Fm' - F)/Fm'$; where F is the maximum fluorescence yield during saturation pulse. The ΔF/Fm' is used to estimate the apparent rate of electron transport (ETR). According to Bilger et al. (1995) by using the equation $ETR = \Delta F/Fm' \times PFD \times 0.5 \times 0.84$, which PFD is the photon flux density (μmol m⁻² s⁻¹) incident on sheet; 0.5 is the corresponding value to fraction of excitation energy distributed to FSII (Laisk and Loreto, 1996); and 0.84 is the corresponding value to fraction of incident light that is absorbed by leaves (Ehlinger, 1981). The non-photochemical coefficient of Stern-Volmer was calculated as $NPQ = (Fm, Fm')/Fm'$ (Bilger and Bjorkman, 1990). The chlorophyll a fluorescence measurements occurred in the period between 7 and 10 a.m.

Data were tabulated and submitted to analysis of variance by F test (p≤0.05), and when significant, they were submitted to Tukey test using the statistical software SISVAR © (Ferreira, 2011).

RESULTS AND DISCUSSION

For the maximum quantum yield of photosystem II (Fv/Fm), a significant effect (p ≤ 0.05) for the interaction between inoculant and maize hybrids was observed, however there was no significant effect of nitrogen. Živčák et al. (2008) observed that Fv/Fm is insensitive to many factors that negatively affect plant photosynthesis.

Regardless of treatment, with and without inoculation, H1, H2 and H3 maize hybrids showed no difference in values of Fv/Fm ratio. For H4, it was observed that Fv/Fm was lower with inoculant when compared with plants without inoculation, a difference of approximately 11%, on average, was observed in value of Fv/Fm ratio (Figure 1). Canelas et al. (2013) observed effect on promoting greater photosynthetic capacity of the plant of corn when inoculated with *Herbaspirillum seropedicae*.

With inoculant presence, Fv/Fm mean obtained in H1, H2, H3 and H4 maize hybrids were 0.78, 0.79, 0.78 and 0.71, respectively. It was noted, therefore, that there were differences between H2 and H4 in Fv/Fm ratio. The highest value was recorded in H2 and the lowest value in H4. In plants with inoculation absence, however, there were no differences in Fv/Fm ratio among maize hybrids. The Fv/Fm mean obtained from these plants was 0.78 for H1, 0.77 for H2, 0.75 for H3 and 0.79 for H4 (Figure 2).

The Fv/Fm ratio is a sensitive indicator of plant photosynthetic performance, because it shows the functional state of photosynthetic apparatus (Björkman and Demming, 1987; Johnson et al., 1993). For most species, the values of Fv/Fm ratio are between 0.75 and 0.85 when plants are not under stress conditions; however, values lower than 0.75 would be indicative of plant exposure to stress, indicating occurrence of photoinhibition in photosynthetic apparatus (Ronquim et al., 2009).

In this study, from all evaluated maize hybrids, only H4 hybrid when inoculated with the bacteria, *A. brasilense* presented Fv/Fm values below 0.75. The other hybrids (H1, H2 and H3) maintained their Fv/Fm values within normal, independent of inoculation, indicating that there was no reduction in photochemical efficiency of FSII. However, although H4 has presented decreases in Fv/Fm, there are some studies in literature describing as normal values until 0.71, especially in crops. Thus, it can be said that there was no damage in photosystem II of maize hybrids. Araújo et al. (2014) observed that the inoculation with the Z-94 strain of *H. seropedicae* promoted increase in root volume, root length, shoot dry mass, chlorophyll content, shoot N content and N use efficiency.

The interaction between nitrogen and inoculant

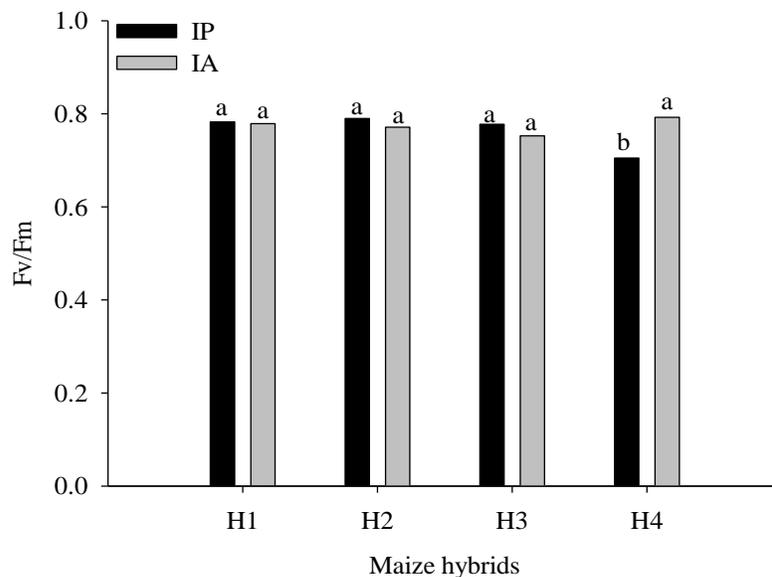


Figure 1. Maximum quantum yield of photosystem II (Fv/Fm) for different maize hybrids with and without inoculation. With inoculation presence (IP); with inoculation absence (IA); Simple hybrid (H1); simple modified hybrid (H2); triple hybrid (H3) and silage hybrid (H4).

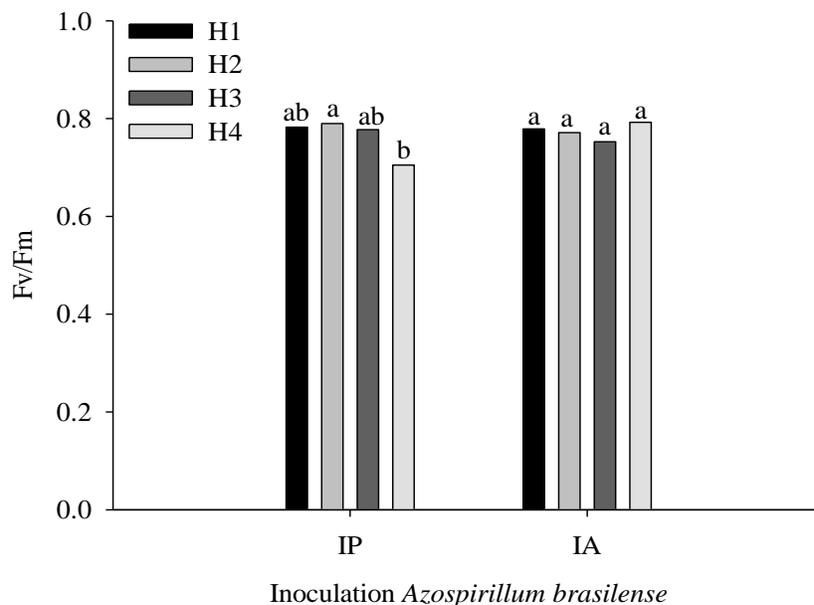


Figure 2. The maximum quantum yield of photosystem II (Fv/Fm) with and without inoculation for different maize hybrids. With inoculation presence (IP); with inoculation absence (IA); Simple hybrid (H1); Simple modified hybrid (H2); Triple hybrid (H3) and Silage hybrid (H4).

was significant for effective quantum efficiency variable of photosystem II ($\Delta F/Fm'$), according to F test, at 5%. In plants with inoculants, differences between nitrogen levels in the value of $\Delta F/Fm'$ were observed. Similar responses were found in plants without inoculation

(Figure 3). Apostol et al. (2008) observed that the effective PSII quantum yield computed as $\Delta F/Fm'$ is fluorescence parameter that gives better results in discriminating between treatments with doses intermediate to nitrogen.

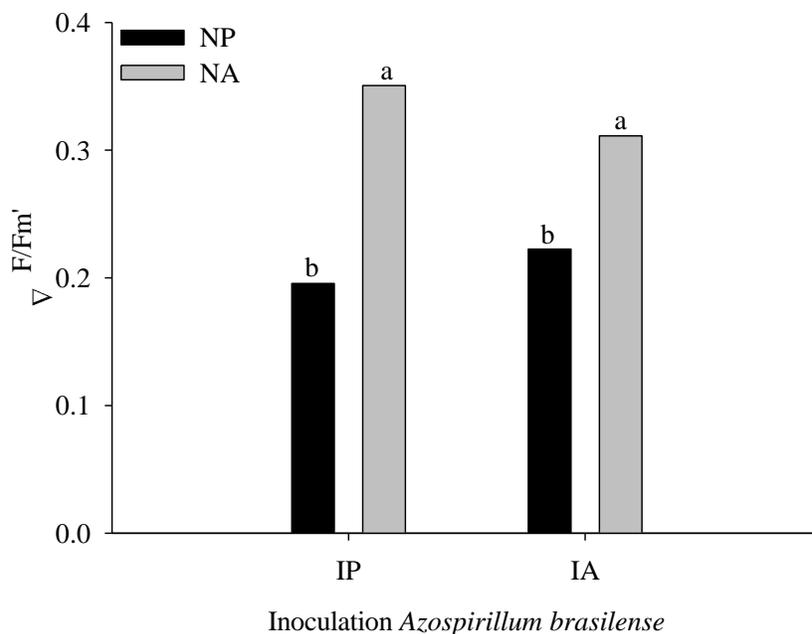


Figure 3. The maximum quantum yield of photosystem II ($\Delta F/F_m'$) for interaction between nitrogen and inoculant. With inoculation presence (IP); with inoculation absence (IA); with nitrogen presence (NP) and with nitrogen absence (NA).

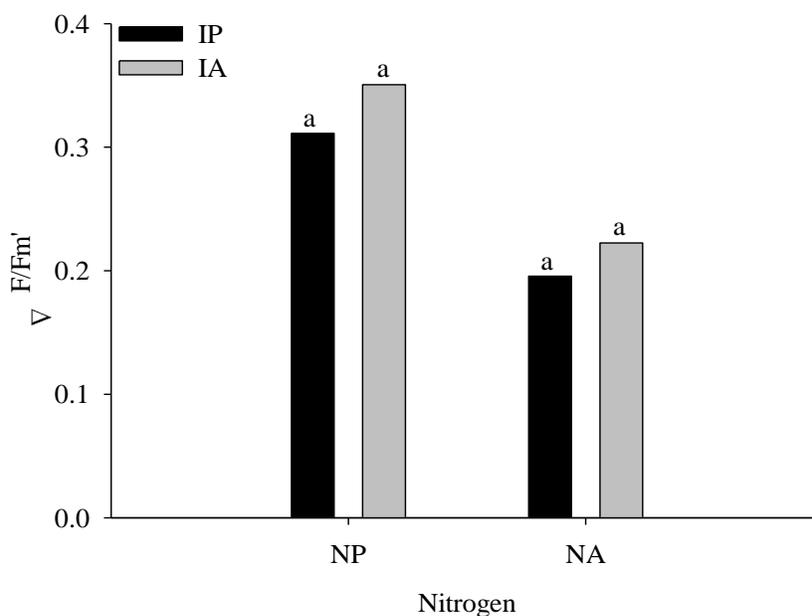


Figure 4. The maximum quantum yield of photosystem II ($\Delta F/F_m'$) for interaction between nitrogen and inoculant. With inoculation presence (IP); with inoculation absence (IA); with nitrogen presence (NP) and with nitrogen absence (NA).

With inoculant, the highest and lowest value of $\Delta F/F_m'$ was 0.35 and 0.20 in plants with nitrogen and without nitrogen, respectively. In plants without inoculant, the highest value was 0.31, with nitrogen, and the lowest value of 0.22 without nitrogen for $\Delta F/F_m'$. An increase of

56% with inoculant, and 71% without inoculation in $\Delta F/F_m'$ with nitrogen application is shown in Figure 3. In Figure 4, it is observed that, regardless of with or without nitrogen, the inoculation did not affect the $\Delta F/F_m'$ in maize hybrids. The parameters derived from rapid and

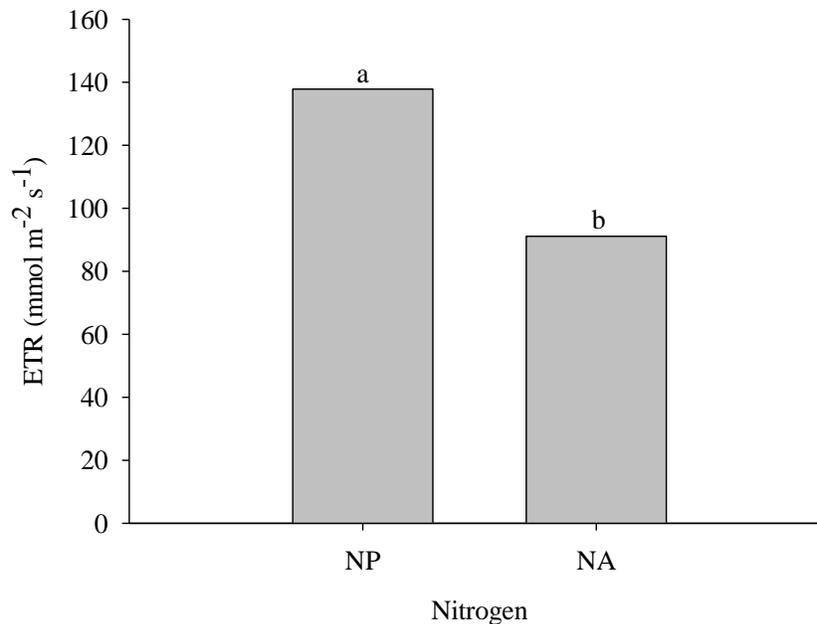


Figure 5. Electron transport rate (ETR). With nitrogen presence (NP) and with nitrogen absence (NA).

non-invasive measurements of the ChlF kinetics can serve for assessment of leaf photosynthetic performance influenced by different nitrogen nutrition, useful for crop research and practical applications (Živčák et al., 2014).

The $\Delta F/F_m'$ is the most sensitive indicator of plant physiological state and usually, decrease in values of this characteristic denotes stress condition. With nitrogen deficit, and independent of inoculation with *A. brasilense*, plants showed a smaller $\Delta F/F_m'$, thus, indicating a better physiological response of maize hybrids supplied with nitrogen. Nitrogen application provides to plants a better use of energy absorbed to photosynthetic processes. Kappes et al. (2013) observed that the application of N in coverage provided greater foliar chlorophyll content in maize plants.

Similar responses to $\Delta F/F_m'$ were obtained in ETR, which increased with nitrogen application, confirming a higher CO₂ assimilation rate as compared to plants with nitrogen deficit. It is noted, therefore, that in maize plants, absence of nitrogen caused substantial reduction in transport of electrons to carbon fixation. In contrast, H4 maize hybrid showed increase in ETR in plants with no inoculation with *A. brasilense*. Galindo et al. (2016) verified that nitrogen rates of up to 200 kg ha⁻¹ improve grain yield for corn that has been inoculated with *A. brasilense*, regardless of the N source utilized.

Electron transport rate (ETR) was significantly affected by nitrogen ($p \leq 0.01$) and interaction between inoculant and maize hybrids ($p \leq 0.05$). For maize hybrids without and with nitrogen, average ETR values of 91 and 138 mmol m⁻² s⁻¹ were obtained, respectively. On average,

there was a difference in ETR of about 34% between treatments. Therefore, with nitrogen, maize plants showed increase of 47 mmol m⁻² s⁻¹, which means increase of approximately 66% in ETR value (Figure 5).

Tang (2000), Yang et al. (2002), Duan et al. (2007) and Ji-rui et al. (2013) observed that the nitrogen fertilizer application had a great impact on the ETR and the EQY, increased the chlorophyll content of crop leaves, electron transport capacity of PS I and PS II, and extended the high-value duration of the photosynthetic rate in leaves.

Electron transport rate showed no significant difference between with and without inoculation for H1, H2 and H3, but when compared with inoculant effect in H4, there was an increase in ETR of about 67% in maize plants without inoculation (Figure 6).

In plants with inoculants, there were no differences among maize hybrids for ETR values, which for H1, H2, H3 and H4 were 125.5, 125.1, 107.2 and 93.8 mmol m⁻² s⁻¹, respectively. However, without inoculation, it was found that H4 showed the highest mean ETR value (140 mol m⁻² s⁻¹) and H3, the lowest value (94 mmol m⁻² s⁻¹) (Figure 7). Carvalho et al. (2011) observed in six maize genotypes that the stress was recorded to limit photosynthetic machinery.

The interaction between nitrogen and maize hybrids for non-photochemical quenching (NPQ) was significant ($p \leq 0.01$). By comparing nitrogen within each maize hybrid, it was found that only H3 showed no difference between NPQ values. However, in H1, H2 and H4 hybrids, differences of 33, 54 and 36%, respectively were observed in NPQ values, between plants with and without

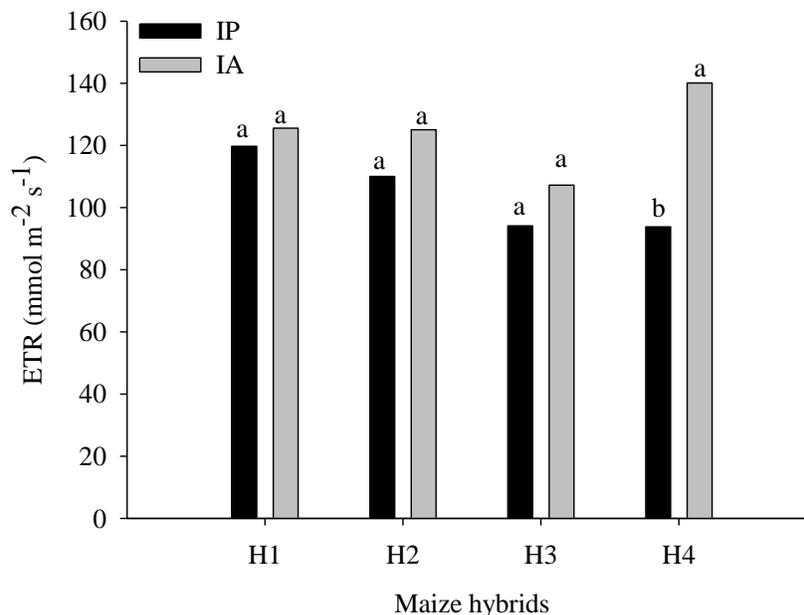


Figure 6. Electron transport rate (ETR) for interaction between inoculant and maize hybrids. With inoculation presence (IP); with inoculation absence (IA); Simple hybrid (H1); Simple modified hybrid (H2); Triple hybrid (H3) and Silage hybrid (H4).

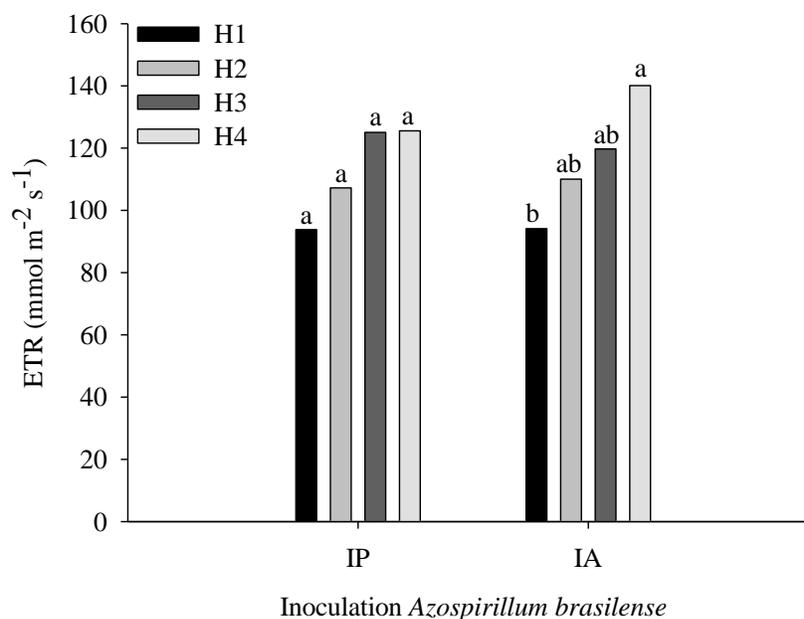


Figure 7. Electron transport rate (ETR) for maize hybrids within level without inoculation. Simple hybrid (H1); Simple modified hybrid (H2); Triple hybrid (H3) and Silage hybrid (H4).

nitrogen. It must be noted that there was high NPQ values obtained in maize hybrids with nitrogen absence (Figure 8). Souza et al. (2013) verified increase in the NPQ of maize genotypes to conserve their photosystems

from adverse effects of stress.

In plants with nitrogen, there were no differences in NPQ values among H4, H1, H2 and H3 maize hybrids with values of 0.41, 0.51, 0.65 and 0.84, respectively.

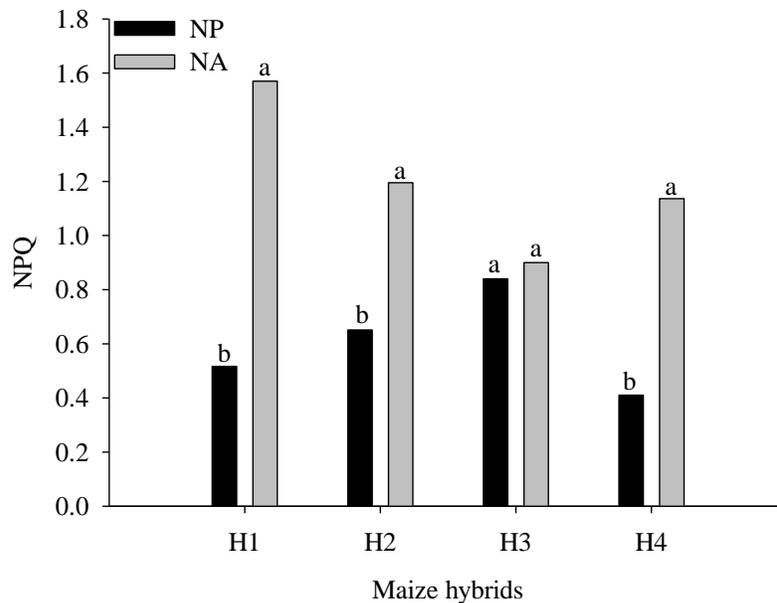


Figure 8. Non-photochemical quenching (NPQ). With nitrogen presence (NP) and with nitrogen absence (NA); Simple hybrid (H1); Simple modified hybrid (H2); Triple hybrid (H3) and Silage hybrid (H4).

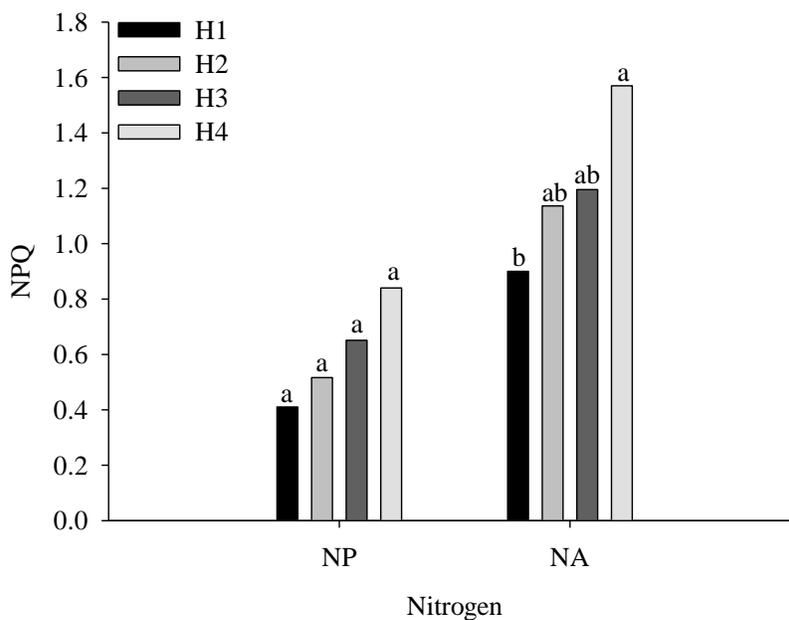


Figure 9. Non-photochemical quenching (NPQ) for hybrid within level with no nitrogen. Simple hybrid (H1); Simple modified hybrid (H2); Triple hybrid (H3) and Silage hybrid (H4).

However, H1 and H3 maize hybrids with no nitrogen whose obtained values were 1.57 and 0.90, respectively, showing a difference of approximately 57% on average, equivalent to a NPQ value of 0.67. The H2 and H4 maize hybrids do not differ from the others, in plants with no nitrogen, although it also showed higher NPQ values

(Figure 9). Kumagai et al. (2009) observed that the NPQ in the flag leaves of the six cultivars increased particularly in the nitrogen-deficient plants.

NPQ acts on excess energy dissipation absorbed by reaction centers through heat. Generally, normal NPQ values are between 0.3 and 0.7. This means that, in

plants with nitrogen, NPQ values remained as normal. In maize hybrids without nitrogen, NPQ results obtained in this study are indicative of the presence of some plant protection mechanism. Normally, when values of $\Delta F/F_m'$ and ETR are low, there is increase in NPQ in order to dissipate energy that is not being used in photochemical processes. Pandolfo et al. (2015) did not observe a positive effect from inoculation when they studied N rates in topdressing and *A. brasilense* inoculation in corn crops.

Good photosynthetic performance of maize hybrids inoculated with *A. brasilense* may be due to better efficiency in absorption and nitrogen utilization promoted by associated bacteria, probably to some plant damage control mechanism aiming at maintaining photosynthetic process by chlorophyll degradation reduction. When reviewing studies on physiological responses of plants induced by *Azospirillum*, Barassi et al. (2008) reported improvement in photosynthetic parameters, including chlorophyll content. Thus, *A. brasilense* inoculation showed great use potential in different maize hybrids, therefore contribute to nitrogen fertilizer saving, as well as provide a greater use of available resources and thus, more sustainable agriculture.

Conclusions

The silage hybrid, when inoculated with *A. brasilense*, showed values of F_v/F_m below 0.75. The simple, simple modified and triple hybrids maintained its F_v/F_m values within normal and it can be stated that there was no damage on photosystem II of maize hybrids. The nitrogen application provided to plants better use energy absorbed to photosynthetic processes ($\Delta F/F_m'$).

Also, for maize hybrids without and with nitrogen, average ETR values of 91 and 138 mol m⁻² s⁻¹, respectively, were obtained. Due to better efficiency in absorption and nitrogen utilization provided by bacteria (*A. brasilense*), inoculated maize hybrids showed good photosynthetic performance.

Conflict of Interests

The authors have not declared any conflict of interests.

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