Acta Scientiarum

http://www.periodicos.uem.br/ojs/
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v44i1.58683

BIOTECHNOLOGY

Inoculation of corn seeds with *Azospirillum brasilense* in different temperatures

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ABSTRACT. Growth-promoting bacteria *Azospirillum brasilense*, used as an inoculant in corn culture, can be severely affected by the exposure temperature, with a lethal effect above 35°C, when cultivated alone under laboratory conditions. Such effects may limit the associative interaction between plant-bacteria, with reduced inoculation efficiency, resulting in a lower growth rate of the plant and an increase in oxidative stress. Thus, the objective of the research was to evaluate the efficiency of the inoculation process with *A. brasilense* in seeds and in the initial growth of seedlings of two corn cultivars submitted to different temperatures. Were utilized corn hybrids seed Syn 488 and Syn 505. The experimental design was completely randomized in a 2 x 4 factorial scheme (with and without inoculation of *A. brasilense* x 4 sowing temperatures: 20, 25, 30, and 35°C), with four replications. The inoculation efficiency in corn seedlings submitted to different temperatures was evaluated through the following tests: germination, first count, seedling length and dry weight. In addition, responses at the biochemical level of the interaction (temperatures x inoculation) for the content of photosynthetic pigments and hydrogen peroxide (H_2O_2), antioxidant enzymes and lipid peroxidation were evaluated. The inoculation with *A. brasilense* changed the morphological and biochemical responses of corn seedlings, and the inoculation process was efficient for all temperatures tested. The best results were under temperatures 25, 30, and 35°C. Plant and bacteria when exposed to temperature conditions (30°C inoculated) showed a significant increase in plant biomass and activity of antioxidant enzymes.

Keywords: Antioxidant enzymes; germination; oxygen reactive species; growth-promoting bacteria.

Received on April 15, 2021.
Accepted on October 5, 2021.

Introduction

Environmental factors such as CO_2 concentration, humidity, temperature, or drought can influence plants and soil microorganisms, in addition to modulating the plant-bacteria interaction mechanisms via changes in the quantity and type of root exudates, rhizospheric colonization, microbial growth, and the degree of association achieved (Classem et al., 2015; Nottingham, Baath, Reischke, Salinas, & Meir, 2018). Among these factors, the soil temperature when sowing crops plays an essential role in the initial development of seedlings in the field and can affect the absorption of water and nutrients and the speed with which the physiological and biochemical processes occur (Carvalho & Nakagawa, 2000). Projections by the Intergovernmental Panel on Climate Change indicate that in the next 100 years there could be an increase in the global average temperature between 1.8 and 4.0°C, which could significantly affect human activities and terrestrial ecosystems.

Thermal stress can promote membrane disruption, changes in redox status, and oxidative damage in plant cells, significantly reducing biomass production and grain productivity (Noblet, Leymarie, & Bailly, 2017; Tiwari & Yadav, 2019; Nakagawa et al., 2020), with better plant responses for a maximum percentage of germination and seedling growth exposed from 25 to 30.8°C (Sanchez, Rasmussen, & Porter, 2014; Santos et al., 2018). Furthermore, *Azospirillum brasilense* growth-promoting bacteria, used as an inoculant in corn crops, can be severely affected by temperature, with lethal effects at more than 35°C and extremely slow growth below 28°C, under laboratory conditions (Romero-Perdomo, Camelo-Rusinque, Criollo-Campus, & Bonilla-Buitrago, 2015; Molina et al., 2018), combined with changes in the insertion of nitrogenous bases, capable of affecting the dynamics of microbial populations and their functionality (Bérard, Sassi, Kaisermann, & Renault, 2015). In this sense, early sowing of corn, especially in southern Brazil, may lead to delays in seed germination and changes in inoculation efficiency with plant growth-promoting bacteria, which may have their activity restricted depending on the environmental conditions.

Although many studies have demonstrated the beneficial effects of inoculation in grasses via changes in the architecture of the root system, biological nitrogen fixation (BNF), phytohormones production, and modulation of the plant's antioxidant system (Fukami, Cerezini, & Hungria, 2018; Barnawal, Singh, & Singh, 2019; Bulegon, Guimarães, Battistus, Inagaki, & Da Costa, 2019; De lima et al., 2020), 30 to 40% of the experiments report the inefficiency of the process, questioning the use of this technology (Cassán & Diaz-Zorita, 2016). Therefore, research aimed at the influence of temperature during the sowing period of the culture under inoculation should be expanded to understand the relationship between plant and microorganism since the data in the literature emphasize the optimal growth conditions of the laboratory-isolated bacteria without considering the plant-microorganism interaction. Thus, this research's objective was to evaluate the influence of temperature on the process of inoculation of corn seeds with *Azospirillum brasilense* and the effects on germination and early development of the seedlings.

Material and methods

Experiment location and plant material

The experiment was conducted in the Plant Physiology Laboratory at the *Universidade Federal de Santa Maria* (UFSM), Brazil. Seeds from two corn cultivars, Syn 488 and Syn 505 (Syngenta®), were used. The seeds had undergone a chemical treatment with insecticides and fungicide before inoculation. The experimental design was completely randomized in a 2 x 4 factorial scheme (with and without inoculation of *Azospirillum brasilense* x four sowing temperatures: 20, 25, 30, and 35°C), and four replicates of 50 seeds, for each corn cultivars.

Inoculation with *Azospirillum brasilense*

The seeds were inoculated with *Azospirillum brasilense* one hour before sowing in plastic bags with five liters' capacity. The commercial inoculum Azototal®, contained strains Abv5 and Abv6, with a minimum guarantee of 2×10^8 colony forming units (CFU) mL^{-1}, in the dose of 1.66 mL for every 1,000 seeds, according to the product recommendations. Distilled water was added to maintain the same moisture level as the seeds for treatments without inoculation.

Germination and development of corn seedlings

After the inoculation process, the seeds of both corn cultivars were sown on germitest paper towels moistened 2.5 times the mass of the substrate with distilled water. The rolls were packed in hermetically sealed plastic bags with five liters' capacity to maintain humidity. BOD type (Biological Oxygen Demand) growth chamber was used to submit the seeds and bacteria to different temperature conditions in the germination process, maintaining constant light and temperature adjusted according to the treatment, with counts for each corn cultivar performed according to the rules for seed analysis (Brasil, 2009).

After seven days inside the germination chamber, the germitest paper rolls were removed, and the following evaluations were conducted:

First germination count and final germination: performed at four and seven days after sowing, by directly counting the number of normal seedlings emerging on the paper (Brasil, 2009). After the final count, the results were converted and expressed as a percentage of normal seedlings.

Root and shoot length, and dry seedling weight: 20 seeds were distributed on the upper third of the pre-moistened paper in a longitudinal direction, with four replicates per treatment. Subsequently, paper rolls similar to the germination test were made and placed to germinate in BOD for seven days. After this period, ten normal seedlings were randomly harvested from each replicate. The length of the roots and shoot of these seedlings was measured using a graduated ruler, separating them into two portions (root and shoot), stored in an oven at 60°C until obtaining a constant weight to obtain the dry mass. The results were expressed in cm $seedling^{-1}$ and dry weight $seedling^{-1}$ (Sbrussi & Zucareli, 2012).

Biochemical variables

Eight replicates of 50 seeds of each treatment were placed on germitest paper towels to evaluate biochemical changes in corn seedlings, following the same methodology used for final germination. Forty normal seedlings were immediately collected from each replicate, separated into roots and shoot, and frozen in liquid nitrogen for later storage in an ultra-freezer at -80°C. The frozen samples were macerated in liquid nitrogen for the determination of the following variables:

Pigment concentration: Total chlorophylls (*a* and *b*) and carotenoids were evaluated using the methodology described by Hiscox and Israelstam (1979) and the Lichtenthaler equation (1987). 50 mg of fresh weight of each sample's plant tissue (shoot) were incubated in 5 mL of dimethylsulfoxide (DMSO) for 45 minutes at 65°C for the complete removal of the pigments. Subsequently, 4 mL of the supernatant liquid was pipetted into glass tubes (2 mL for each replicate), and the absorbance values were determined on a spectrophotometer at the wavelengths of 663, 645, and 470 nm, for chlorophyll *a*, *b*, and carotenoids, respectively. The total chlorophyll content was obtained by adding the values of chlorophyll *a* and *b*.

Guaiacol peroxidase (POD): 0.5 g of fresh tissue was homogenized in 3 mL of 0.05 M sodium phosphate buffer (pH 7.8), containing 1 mM EDTA and 2% (M/V) polyvinylpyrrolidone (PVP). The homogenized sample was centrifuged at 13,000 g for 20 minutes at 4°C, and the supernatant was stored in an eppendorf to determine the enzymatic activity. POD activity was determined using a digital UV/VIS spectrophotometer (Fentoscan) at 470 nm, according to Zeraik, Desouza, Fatibello-Filho, and Leite (2008).

Superoxide dismutase (SOD): 0.5 g of plant tissue was homogenized following the same protocol described for POD. The SOD enzymatic activity was evaluated using a Mix with potassium phosphate buffer (pH 7.8), 13 mM methionine, 0.1 μM EDTA, 75 μM NBT, and 2 μM riboflavin. 300 μL of sample and 2.5 mL of the mix were incubated under a fluorescent lamp (15 watts) for five minutes (Giannopolitis & Ries, 1977). Later, the absorbance readings were performed on a spectrophotometer (Bel Photonics, 1105, Brazil) at 560 nm. The results were expressed in U mg^{-1} protein.

Hydrogen peroxide (H_2O_2) content: 0.3 g of fresh plant mass was homogenized in 3 mL of 0.1% trichloroacetic acid (TCA), according to the methodology described by Loreto and Velikova (2001). The homogenized liquid was centrifuged at 12,000 rpm for 15 minutes at 4°C. 0.5 mL of the supernatant was added in 0.5 mL of potassium phosphate buffer (pH 7.0) with 1 mL of potassium iodide (KI) to determine the H_2O_2 content in the plant tissue, reading the absorbance in a spectrophotometer (Bel Photonics, 1105, Brazil) at 390 nm. The values were expressed in μmol g^{-1} of fresh weight.

Lipid peroxidation (TBARS): The level of lipid peroxidation was assessed from the accumulation of malondialdehyde (MDA), as a product of the peroxidation of unsaturated fatty acids, determined by the reaction to thiobarbituric acid (TBA) (El-Monshaty, Pike, Novackya, & Seghal, 1993). 0.5 g of fresh plant tissue mass was homogenized in 3 mL of citrate phosphate buffer (pH 6.5) and centrifuged at 20,000 g for 15 minutes. The supernatant fraction was added to glass tubes containing TCA + TBA, shaken, and incubated at 95 °C for 40 minutes. After this, the samples were placed on ice for 15 minutes and centrifuged at 10,000 g for 15 minutes. Absorbance was measured on a spectrophotometer (Bel Photonics, 1105, Brazil) at 532 and 600 nm, with results expressed in nmol MDA g^{-1} fresh weight.

Statistical analysis

The data were submitted to the normality test by Shapiro Wilk and homogeneity test by Bartlett, with analysis of variance (ANOVA) by the F test ($p < 0.05$) for each corn cultivar. The treatment means were compared by the Scott-Knott test ($p < 0.05$). The SISVAR software was used for data analysis (Ferreira, 2014).

Results

Germination and initial growth of genotype Syn 488 seedlings

The inoculation x sowing temperature interaction significantly affected all germination and vigor parameters, except for shoot length (SL), root dry weight (RDW) and shoot dry weight (SDW), which demonstrated isolated effects for each factor (Table 1). There was no significant effect of inoculation for final seed germination (G) in most temperatures, except 35°C, where a 9.36% reduction was observed in seeds that were not inoculated. Such behavior differed for the first germination count, showing responses to seed inoculation from 25°C.

Negative impacts of inoculation were verified for root length (RL) and total length (TL) at a temperature of 20°C when evaluating parameters related to the initial seedling development, an average of 20% lower than uninoculated seedlings, with no differences for other temperatures tested. Additionally, the variables SL, RDW, and SDW demonstrated isolated effects for temperature and inoculation, for which the seedling-bacteria interaction reduced SL (9.31%), RDW (6.6%), and SDW (5.8%) for genotype Syn 488 (Table 1). The temperature of 30°C provided the highest averages for most growth variables, followed by 25, 20, and 35°C, except for SDW, which presented behavior similar that at 30°C.

Table 1. Variables of first germination count, final germination, root length, shoot length, total length, root dry weight, and shoot dry weight, cultivar Syn 488.

Inoculation	Sowing Temperature				
	20ºC	25ºC	30ºC	35ºC	
		First Count (%)			Mean
Without *Azo*	94.00 ± 1.0 Aa*	82.00 ± 1.6 Cb	88.00 ± 1.9 Bb	60.00 ± 5.5 Db	81.00
With *Azo*	97.00 ± 2.5 Aa	93.00 ± 5.0 Aa	94.00 ± 0.0 Aa	73.00 ± 6.0 Ba	89.00
Mean	95.00	87.00	91.00	66.00	
		Germination (%)			Mean
Without *Azo*	100.00 ± 1.0 Aa	96.00 ± 1.6 Aa	97.00 ± 2.6 Aa	78.00 ± 2.5 Bb	93.00
With*Azo*	98.00 ± 1.9 Aa	99.00 ± 1.0 Aa	98.00 ± 1.6 Aa	86.00 ± 3.0 Ba	95.00
Mean	99.00	97.00	98.00	82.00	
		Root length (cm)			Mean
Without *Azo*	14.02 ± 0.1 Ba	15.53 ± 0.3 Aa	15.75 ± 2.2 Aa	4.08 ± 0.9 Ca	12.34
With *Azo*	11.03 ± 1.0 Bb	16.48 ± 0.4 Aa	15.07 ± 1.4 Aa	3.79 ± 0.4 Ca	11.59
Mean	12.52	16.00	15.41	3.93	
		Shoot length (cm)			Mean
Without *Azo*	7.12 ± 0.6	9.80 ± 0.5	10.24 ± 0.7	6.34 ± 0.3	8.38 a
With *Azo*	5.98 ± 0.9	8.75 ± 0.1	9.37 ± 0.3	6.31 ± 0.4	7.60 b
Mean	6.55 B	9.28 A	9.80 A	6.33 B	
		Total length (cm)			Mean
Without *Azo*	21.14 ± 0.6 Ba	25.33 ± 0.5 Aa	25.99 ± 2.4 Aa	10.42 ± 0.9 Ca	20.72
With *Azo*	17.00 ± 1.1 Bb	25.23 ± 0.4 Aa	24.43 ± 1.6 Aa	10.09 ± 0.8 Ca	19.19
Mean	19.07	25.28	25.21	10.26	
		Root dry weight (g seedling^{-1})			Mean
Without *Azo*	25.63 ± 1.5	25.41 ± 3.7	36.93 ± 3.2	20.83 ± 0.2	27.20 a
With *Azo*	21.85 ± 1.0	26.20 ± 1.7	35.75 ± 2.0	17.80 ± 0.7	25.40 b
Mean	23.74 B	25.80 B	36.34 A	19.32 C	
		Shoot dry weight (g seedling^{-1})			Mean
Without *Azo*	21.50 ± 1.3	25.59 ± 2.5	28.30 ± 1.6	28.17 ± 2.0	25.89 a
With *Azo*	17.17 ± 1.7	24.62 ± 0.6	28.48 ± 0.6	26.67 ± 2.2	24.37 b
Mean	19.61 C	25.10 B	28.38 A	27.42 A	

*Uppercase letters in the line compare the temperatures in each inoculation system (with or without *Azo*), and lowercase letters in the column compare inoculation systems in each temperature by the Scott-Knott test ($p < 0.05$). Values represent mean ± standard deviation.

Biochemical data of genotype Syn 488

Interaction effects (temperature x inoculation) were identified for the content of total chlorophyll, carotenoid, hydrogen peroxide (H_2O_2) and activity of the enzymes guaiacol peroxidase (POD) in the roots and superoxide dismutase (SOD) in the roots and shoot (Figures 1 and 2). Isolated effects of temperature on the POD of the shoot were also identified. The inoculation reduced the levels of total chlorophylls and carotenoids by an average of 30% for most sowing temperatures, except at 35ºC (Figure 1). Changes were evidenced regarding H_2O_2 (ROS) content for both structures (roots and shoot). According to the results (Figure 1C), in uninoculated plants, the temperature of 20ºC promoted the highest concentrations of H_2O_2 in the roots (77% higher) regarding the inoculated plants. There were no significant variations in the results for the temperature of 25°C, while for 30 and 35°C, the inoculated plants showed an increase of around 54% for H_2O_2 in the roots tissue (Figure 1C). On the other hand, POD activity increased at 25 and 30°C in non-inoculated and to 30°C in inoculated ones (Figure 1E).

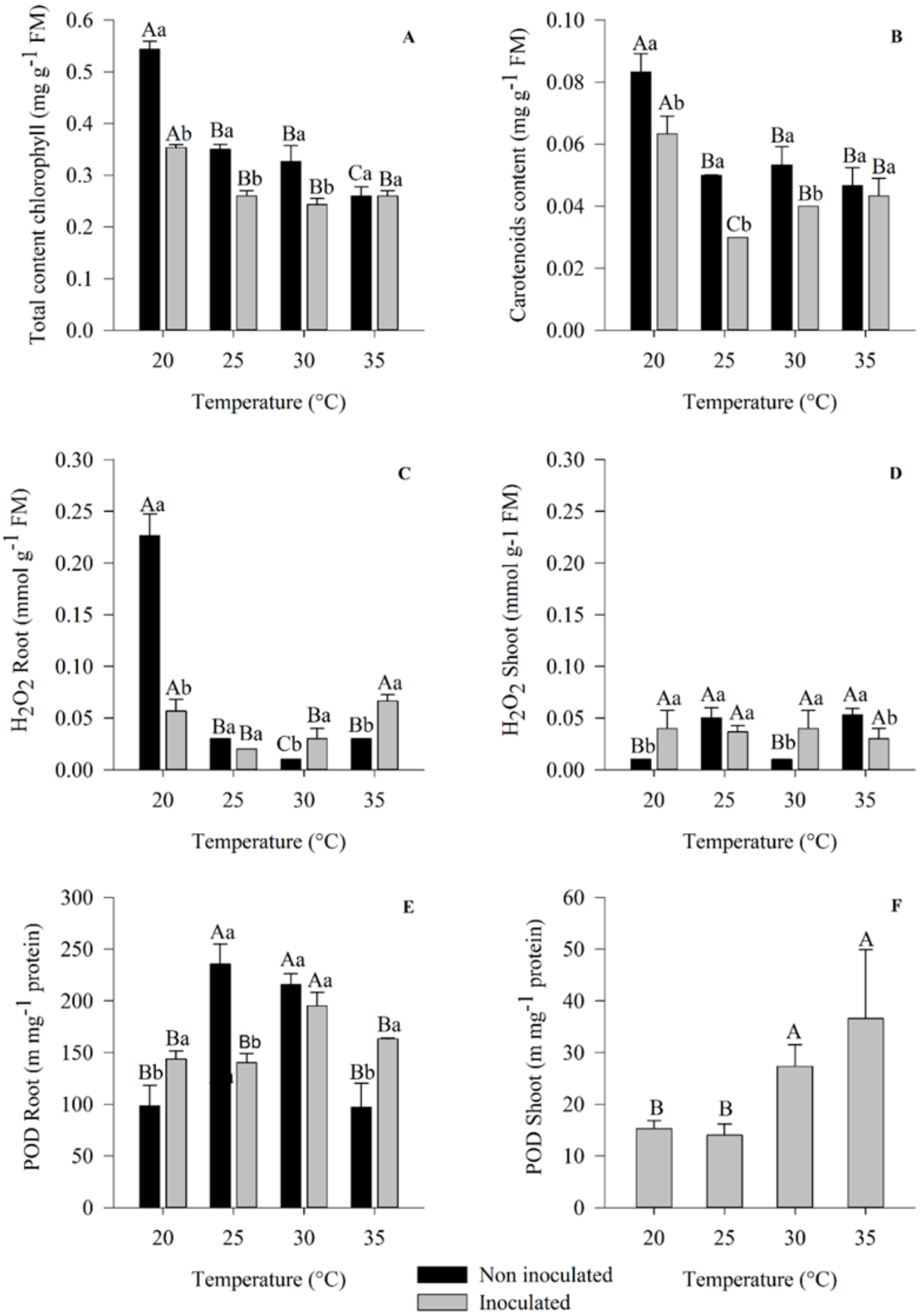

Figure 1. Total chlorophyll content, carotenoids, H_2O_2 content, and guaiacol peroxidase (POD) activity obtained from corn seedlings submitted to different temperatures and inoculation systems. Uppercase letters compare the effects of temperatures on each inoculation system, and lowercase letters determine the presence or absence of the bacteria by the Scott-Knott test ($p < 0.05$). Bars represent mean ± standard deviation.

Such observations differ for the content of H_2O_2 in the shoot, with a greater production in seedlings inoculated at 20 and 30°C (Figure 1D). However, the POD in the shoot showed an isolated effect for temperature, without direct correlation with the previous data. Nonetheless, as the temperature increased, an increase in enzyme activity was verified. The SOD activity presented a behavior similar to the results already presented for H_2O_2 and POD in the roots of inoculated seedlings, showing a higher activity at 20 and 35°C (Figure 2A) response to the production of ROS under these conditions. The SOD in the shoot showed no effects for inoculation regardless of temperature, except at 25°C. However, a lower content of carotenoids was found in the tissues at this temperature, which may be linked to less stress on the plant, without the need to improve the enzymatic system.

The level of oxidative damage determined by lipid peroxidation (Tbars) (Figure 3) indicated that the interaction of Syn 488 corn seedlings with the bacterium *Azosprillum brasilense* promoted an average increase of 42% in lipid peroxidation at the roots for the different temperatures tested, except 25°C, at which lipid peroxidation remained low and with no difference from the control condition. Such a negative impact of inoculation corroborates the growth data (Table 1) and pigments (Figure 1A and B) with reduced SL, chlorophyll, and carotenoid content, indicating the excessive production of ROS in the process of root infection/colonization, reducing plant performance. On the other hand, isolated effects were observed for lipid peroxidation in the shoot, obtaining higher malondialdehyde values (one of the byproducts of lipid peroxidation) in samples exposed to 35°C (Figure 3C), in addition to inoculation reducing the level of damage by 12% in inoculated seed samples.

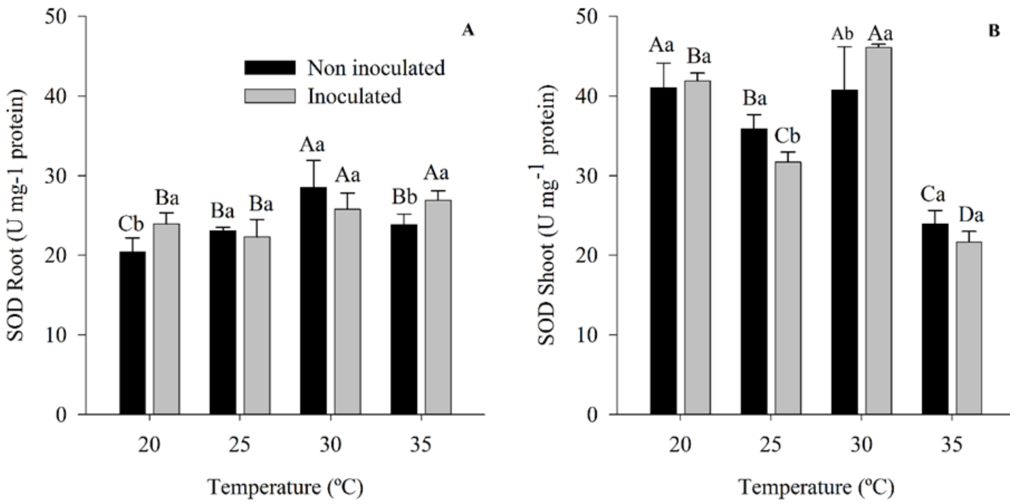

Figure 2. Superoxide dismutase (SOD) enzyme activity determined from corn seedlings submitted to different temperatures and inoculation systems. Uppercase letters compare the effects of temperatures in each inoculation system, and lowercase letters determine the presence or absence of the bacteria by the Scott-Knott test ($p < 0.05$). Bars represent mean ± standard deviation.

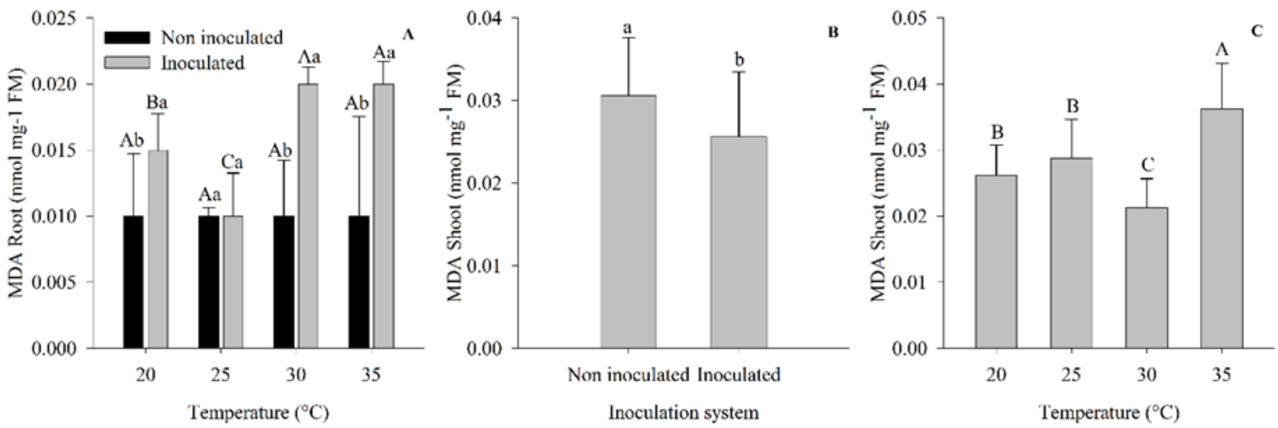

Figure 3. Lipid peroxidation (Tbars) determined from corn seedlings submitted to different temperatures and inoculation systems. Uppercase letters compare the effects of temperatures in each inoculation system, and lowercase letters determine the presence or absence of the bacteria by the Scott-Knott test ($p < 0.05$). Bars represent mean ± standard deviation.

Germination and initial growth of genotype Syn 505 seedlings

The inoculation x sowing temperature interaction significantly affected all germination and vigor parameters, except for the first count (FC), root length (RL), shoot length (SL), and shoot dry weight (SDW), which presented isolated effects for each factor (Table 2). The temperatures did not influence the emergence of normal seedlings in FC. However, seeds inoculated with the bacteria increased the total number of seedlings that emerged at four days by 6%. Additionally, final germination was affected by the treatments seven days after the installation of the experiment. The results showed that, regardless of the temperature, inoculated seeds could germinate and reach high levels (above 98%) compared to seeds not inoculated, which germinated approximately 5% less when at 25 and 35°C, confirming the influence of the association with microorganisms even in a short period.

Table 2. Variables of first germination count, final germination, root length, shoot length, total length, root dry weight, and shoot dry weight, cultivar Syn 505.

Inoculation	Sowing Temperatures 20°C	25°C	30°C	35°C	
	First Count (%)				Mean
Without *Azo*	90.00 ± 3.7	83.00 ± 2.5	86.00 ± 4.3	82.00 ± 8.5	85.00 b
With *Azo*	90.00 ± 2.3	85.00 ± 6.2	92.00 ± 1.9	93.00 ± 2.6	90.00 a
Mean	90.00	84.00	89.00	87.00	
	Germination (%)				Mean
Without *Azo*	99.00 ± 1.9 Aa*	94.00 ± 1.6 Bb	97.00 ± 1.0 Aa	91.00 ± 1.2 Cb	95.00
With *Azo*	98.00 ± 1.9 Aa	98.00 ± 1.6 Aa	99.00 ± 1.9 Aa	96.00 ± 1.6 Aa	98.00
Mean	98.00	96.00	98.00	94.00	
	Root length (cm)				Mean
Without *Azo*	16.14 ± 0.7	17.11 ± 0.7	19.93 ± 1.1	5.75 ± 0.4	14.73 b
With *Azo*	17.53 ± 0.9	18.48 ± 0.7	19.59 ± 1.1	6.87 ± 0.2	15.62 a
Mean	16.83 C	17.80 B	19.76 A	6.31 D	
	Shoot length (cm)				Mean
Without *Azo*	10.02 ± 0.8	9.61 ± 0.5	11.76 ± 0.7	5.07 ± 0.5	9.11 b
With *Azo*	10.66 ± 0.8	10.18 ± 0.6	11.96 ± 0.2	6.60 ± 0.3	9.85 a
Mean	10.34 B	9.89 B	11.86 A	5.84 C	
	Total length (cm)				Mean
Without *Azo*	26.15 ± 1.1 Bb	26.72 ± 1.2 Bb	31.69 ± 0.8 Aa	10.81 ± 0.8 Cb	23.84
With *Azo*	28.18 ± 0.8 Ba	28.67 ± 1.3 Ba	31.54 ± 1.0 Aa	13.48 ± 0.3 Ca	25.47
Mean	27.17	27.69	31.61	12.14	
	Root dry weight (g seedling^{-1})				Mean
Without *Azo*	32.90 ± 1.4 Ca	36.20 ± 1.0 Ba	44.50 ± 1.3 Aa	20.78 ± 1.1 Db	33.59
With *Azo*	34.07 ± 1.5 Ca	37.47 ± 1.1 Ba	42.25 ± 2.4 Aa	26.25 ± 2.7 Da	35.01
Mean	33.49	36.83	43.38	23.51	
	Shoot dry weight (g seedling^{-1})				Mean
Without *Azo*	25.90 ± 1.7	25.33 ± 1.0	30.35 ± 2.9	30.80 ± 0.6	28.09
With *Azo*	25.95 ± 1.7	25.38 ± 1.0	29.70 ± 0.8	32.35 ± 2.9	28.34
Mean	25.93 B	25.35 B	30.03 A	31.58 A	

*Uppercase letters in the line compare the temperatures in each inoculation system (with or without *Azo*), and lowercase letters in the column compare inoculation systems in each temperature by the Scott-Knott test ($p < 0.05$). Values represent mean ± standard deviation.

The length of roots and shoot was affected in isolation by inoculation x temperatures. In other words, seed inoculation was not affected by the exposure to environmental conditions. In this sense, there was a linear increase for the parameters analyzed according to the rise in temperature, with 30°C promoting the most significant elongation of plant structures. Furthermore, both RL and SL increased by an average of 7% in the presence of *Azospirillum brasilense*. However, seed inoculation was superior at all temperatures regarding the total length of seedlings, with no statistical difference at 30°C.

Although RDW and SDW showed different effects when comparing the treatments, the best temperature for maximum dry matter accumulation was around 30°C, regardless of inoculation. However, even under high temperatures (35°C), the bacteria formed a 20% larger root system than the uninoculated plants.

Biochemical data of genotype Syn 505

Effects of the temperature x inoculation interaction were verified for most of the biochemical variables evaluated, except for total chlorophylls and lipid peroxidation (Tbars) in the shoot, which demonstrated isolated effects for each factor (Figures 4, 5, and 6). Regardless of the condition to which the seedlings were exposed, *Azospirillum brasilense* contributed to the content of total chlorophylls and carotenoids (Figure 4), with a 26% increase over the content of chlorophylls and 18, 41, and 32% for the carotenoid at the temperatures of 20, 30, and 35°C, respectively. Although seed inoculation at 25°C showed a 30% decrease for content carotenoids (Figure 4C), such results can be explained by the lower stress condition on the plant, with a lower concentration of H_2O_2 in the tissues and an increase in the POD activity by the bacteria (Figures 4D, 4E, 5A, and 5B), without the need to increase other molecules of the antioxidant system.

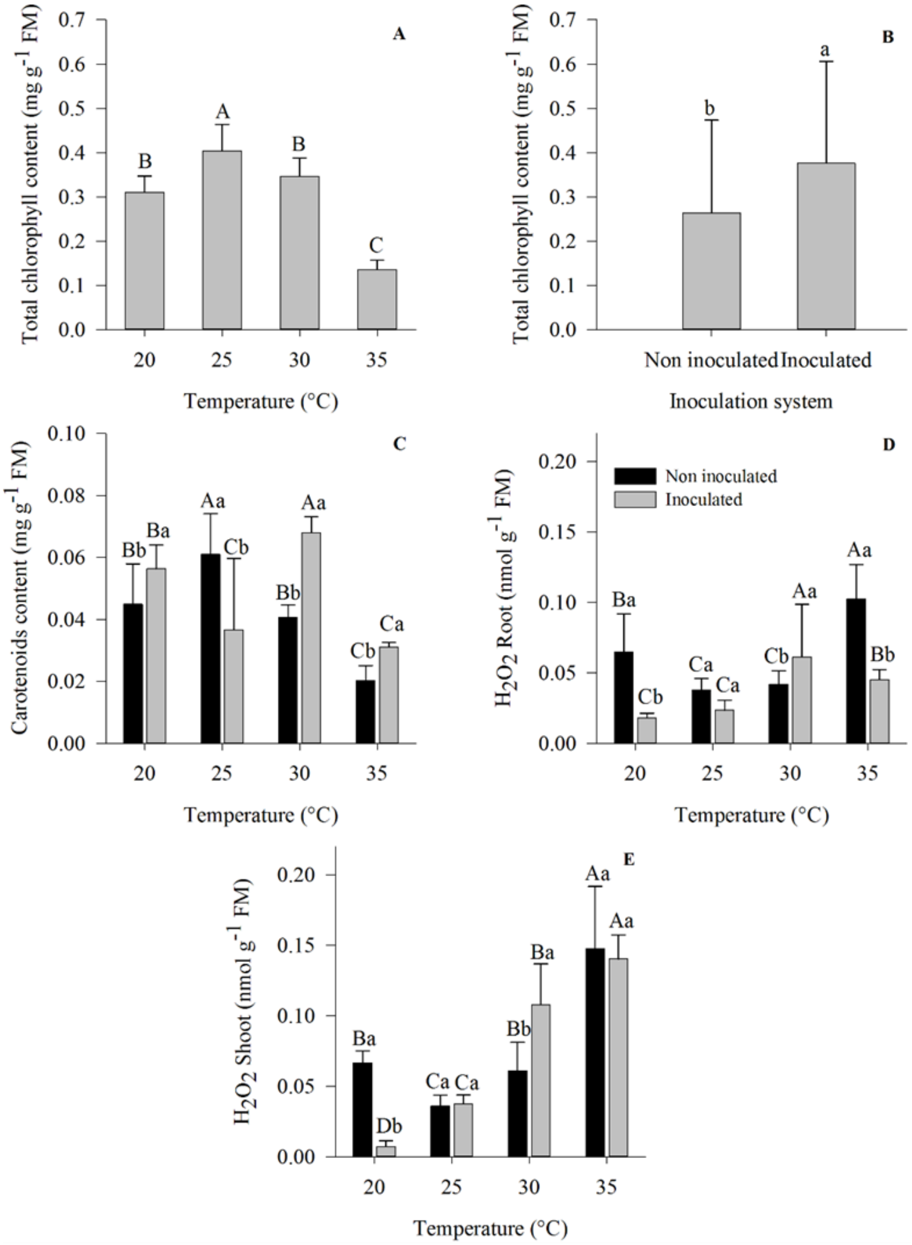

Figure 4. Total chlorophyll content, carotenoids, H_2O_2 content determined from corn seedlings submitted to different temperatures and inoculation systems. Uppercase letters compare the effects of temperatures in each inoculation system, and lowercase letters determine the presence or absence of the bacteria by the Scott-Knott test ($p < 0.05$). Bars represent mean ± standard deviation.

There was a 37% increase in the concentration of H_2O_2 in both plant structures of inoculated seedlings when submitted to 30°C (Figure 4D and E), which could indicate oxidative stress. Additionally, POD enzymatic activity in inoculated seedlings increased about 50% both in the roots and in the shoot at 25°C (Figure 5), even with no difference in the concentration of H_2O_2 at this temperature.

POD and SOD activity reduced when the seeds were inoculated and exposed to higher temperatures, 30 and 35°C (Figure 5). Although such behavior indicates possible damage to plant metabolism, the carotenoid content was statistically higher than in uninoculated plants for the respective temperatures (Figure 4C), indicating its possible efficiency in combating molecules harmful to the system.

The level of lipid peroxidation determined by Tbars (Figure 6) makes it evident that the plant-bacteria interaction promoted a significant reduction of oxidative damage in inoculated plants, regardless of the temperature to which they were exposed, except at 20 and 25°C, in which there was no statistical difference for the absence of the bacteria (Figure 6A). However, seed inoculation generally reduced ROS oxidative damage by 22%, considering that ROS are generated in large quantities when plants are exposed to higher temperatures (Figure 6B). Furthermore, it was observed that both in inoculated and non-inoculated there was an increase in lipid peroxidation, increasing as the temperature increased (Figure 6C).

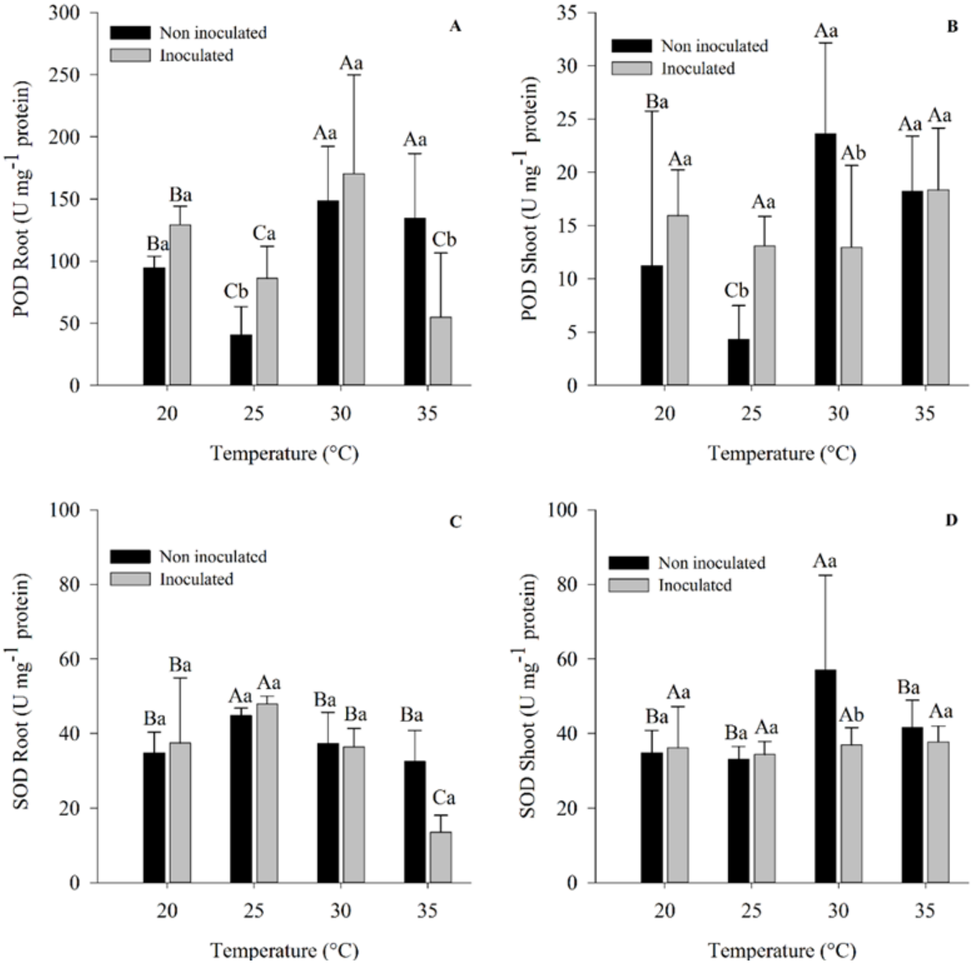

Figure 5. Guaiacol peroxidase (POD) and superoxide dismutase (SOD) enzyme activities determined from corn seedlings submitted to different temperatures and inoculation systems. Uppercase letters compare the effects of temperatures in each inoculation system, and lowercase letters determine the presence or absence of the bacteria by the Scott-Knott test ($p < 0.05$). Bars represent mean ± standard deviation.

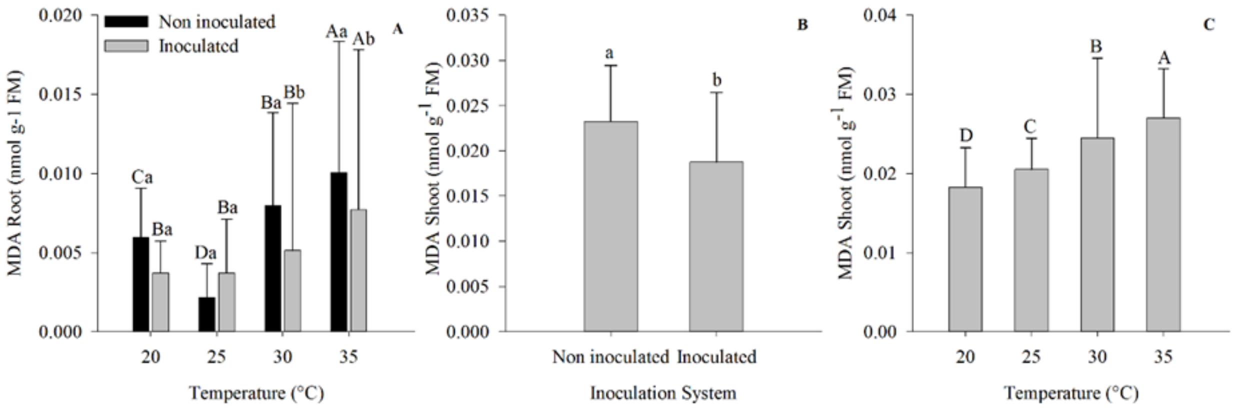

Figure 6. Lipid peroxidation (Tbars) determined from corn seedlings submitted to different temperatures and inoculation systems. Uppercase letters compare the effects of temperatures in each inoculation system, and lowercase letters determine the presence or absence of the bacteria by the Scott-Knott test ($p < 0.05$). Bars represent mean ± standard deviation.

Discussion

Many studies have characterized the potential of using bacteria of the *Azospirillum* genus to promote plant growth, which is directly related to the production of phytohormones, such as auxins and gibberellins, and biological nitrogen fixation (BNF) (Cassán et al., 2009; Fukami et al., 2018). In this study, the first germination count was statistically higher for inoculated seeds regardless of the temperature tested in both genotypes, except for 20°C in the genotype Syn 488 (Tables 1 and 2). Although the final seed germination did not differ from the uninoculated plants for most temperatures, there was a reduction for the variable in uninoculated seeds when exposed to 35°C, indicating that the bacteria remained active even under high temperatures. This differs from studies conducted by Romero-Perdomo et al. (2015), who found a lethal effect of high temperatures (34 and 36°C) in *Azospirillum brasilense* cultivated in a specific culture medium. Such data make it clear that the plant-microorganism interaction allows greater tolerance to stress factors for both conditions.

Moreover, side effects, such as changes of the plant's defense system, are reported in the literature, presenting an increase in antioxidant enzymes and pigments (Alen'kina, Romanov, & Nikitina, 2018), with a response significantly linked to the modulation of the expression of genes related to plant growth and development (Masuda et al., 2018; Thomas et al., 2019). In this sense, *Azospirillum brasilense* can promote changes in seed germination even in a short period, with direct effects on the number of normal seedlings emerged and their germination speed due to the more significant synthesis of gibberellin, which possibly triggers greater enzyme activity linked to carbohydrate degradation and translocation to the embryo (Dartora, Marini, Guimarães, Pauletti, & Sander, 2013; De Morais, De Brito, Brandão, & Rezende, 2016). Genotypes responded differently in terms of inoculation to seed vigor, photosynthetic pigments, and antioxidant enzymes, showing a direct correlation between the change in growth and the biochemical variables of the plant, mainly for the antioxidant defense system. Seed inoculation significantly increased lipid peroxidation in Syn 488 seedlings, especially in roots (Figure 3), with higher production of H_2O_2 in roots of plants inoculated at 30 and 35°C (Figure 1C). Although the plant-bacterial interaction promotes an increase in ROS production under higher temperatures, the plant's defense system was also stimulated, increasing an average of 33% in POD activity (Figure 1E). ROS can be naturally produced in plants, regardless of the environmental condition they are exposed to, and can be considered signaling molecules in low concentrations (Foyer, 2018). However, excess radiation, temperature, or plant/soil microorganism interactions (symbiotic or pathogenic) can change these molecules' formation (Nanda, Andrio, Marino, Pauly, & Dunand, 2010; Awasthi, Bhandari, & Nayyar, 2015). Thus, the results obtained in this experiment prove that the plant-microorganism interaction increased ROS in some situations but, contrary to what was expected, it resulted in oxidative damage, reducing the content of chlorophylls and carotenoids for Syn 488 (Figure 1A and B). Consequently, lower values were observed for SL, RDW, and SDW (Table 1). These data can be explained by the intrinsic relationship between photosynthetic pigments and the capture of light energy for later conversion into photoassimilates by the plant. Therefore, any reduction in pigment content may constitute a type of oxidative stress, with consequences on the production and accumulation of biomass (Taibi et al., 2016).

The initial interaction is highly dependent on the communication established between the bacteria and the host, mediated by lectins present in the bacteria's cell wall, with carbohydrate-binding domains capable of adhering to the root surface and initiating the infection process, triggering a series of events at the level of cell signaling, with changes in the content of ROS (Alen'kina et al., 2014; Alen'kina & Nikitina, 2020). These authors also reinforce the changes in the enzymatic system of wheat plants exposed to different concentrations of lectins produced by *Azospirillum*, with an increase in SOD activity in the interaction's initial moments. Such data corroborate the results found by Méndez-Gómez, Castro-Mercado, Alexandre, and García-Pineda (2015), who showed more significant production of superoxide anion in wheat seedlings exposed to *Azospirillum brasilense* extracellular enzymes in the first hours of infection. Additionally, Méndez-Gómez, Castro-Mercado, Alexandre, and García-Pineda (2016) found a reduction in root length, lower ROS content, and increased SOD and peroxidase activity in wheat roots inoculated with the bacteria. These authors also reinforce that, although the interaction with the bacteria promoted a significant reduction of the root system, the number of cells in the meristematic region and proliferation of root hairs was higher than at the control condition.

On the other hand, the inoculation of Syn 505 seeds showed a reduction in the content of MDA in the samples, both in the roots and in the shoot (Figure 6A and B), in response to a lower concentration of H_2O_2, mainly at 20 e 35°C in roots (Figure 4D). Furthermore, photosynthetic pigments and POD enzyme activity also increased in the presence of the bacteria, at specific temperatures, which resulted in a suppressive effect of ROS damaging the plant's metabolism (Figures 4 and 5). Thus, RL, SL, and TL increased significantly with inoculation, proving the direct relationship between the enzyme system and plant development. In this sense, many studies demonstrate that the increase in biomass and change in plant architecture is a result of the ability of *Azospirillum brasilense* to produce phytohormones (auxins), influencing cell division, proliferation, and differentiation (Spaepen, Bossuyt, Engelen, Marchal, & Vanderleyden, 2014; Duca, Patten, Rose, & Glick, 2014; Çakmakçi, Mosber, Milton, Alaturk, & Ali, 2020). However, recent studies prove that auxins can act as signaling molecules in the cell, stimulating the activity of NADH-oxidases, which will increase the production of superoxide anion and H_2O_2 in the cell wall, loosening cellulose and hemicellulose fibers and increasing cell size (Schmidt, Kunkowska, & Schippers, 2016; Mangano et al., 2017; Majda & Robert, 2018). Although the auxin content was not evaluated in the present study, the H_2O_2 content in inoculated seedlings was higher when the best results were obtained (30°C inoculated) (Figure 4D and E), which resulted in more significant seedling development (Table 2).

Such adaptations of the host species to a higher concentration of ROS in the initial period of infection and cell colonization, associated with the modulation of the antioxidant system and architecture of the root system, can determine the success of the interaction, which can be harmful from the moment the antioxidant system is not able to maintain ROS homeostasis in the plant. In this sense, Alquéres et al. (2013) reported the fundamental role of antioxidant enzymes in the efficiency of inoculation in rice plants, which considerably increased ROS production after coming into contact with the bacteria. Therefore, such statements could explain the negative performance of cultivar Syn 488 since the carotenoid content was reduced and POD and SOD activity remained very close to the control condition. Such data reinforce the idea that the genetic characteristics of the hybrids can influence inoculation in response to greater or lesser root exudation and the type of metabolite released (Pereira et al., 2020), combined with the genetic role of the hybrids in terms adhesion root to bacterial cells (Rojas et al., 2013; De león, Castellanos, Ling, Rojas-Hernández, & Roder, 2015), as well as the presence and transcriptional regulation of genes linked to molecular signaling and plant growth (Vargas et al., 2012; Masuda et al., 2018). Moreover, metabolic routes linked to plant defense must also change so that the infection/inoculation process is efficient without damaging the host plant (Thomas et al., 2019).

Considering the effects of temperature on the inoculation of compatible corn seedlings (Cultivar Syn 505), the inoculation was not negatively affected by temperature for germination and growth parameters (Table 2). However, the highest rate of plant growth occurred when the seedlings were exposed to 30ºC. In this sense, Riley (1981) and Rilkey (1981) found that corn seeds showed higher activity of enzymes linked to degradation and reserve mobilization under temperatures close to 28°C, combined with the synthesis of new proteins, which reduced as a result of inhibiting mRNA synthesis when exposed to temperatures above 30°C. Liu et al. (2019) found a negative impact of thermal stress (38°C) on germination and the initial establishment of rice seedlings, in response to the accumulation of abscisic acid (ABA) and H_2O_2 in seeds, in the early stages of development. Additionally, modeling experiments and historical harvest data reinforce that the increase in temperature can significantly impact corn development, reducing the duration of the phenological stages and low pollen viability with losses of up to 80% in grain productivity (Hatfield, 2016; Lizaso et al., 2018).

Researches have already widely characterized the influence of temperature on the activity of diazotrophic bacteria, given the negative impact on the bacterial population, production of auxin, and biological nitrogen fixation when exposed to temperatures above 30°C (Romero-Perdomo et al., 2015; Molina et al., 2018). These authors reported that bacteria of the *Azospirillum* genus suffered a significant decrease in the expression of genes linked to phytohormone synthesis when under thermal stress, which directly impacts inoculation responses in plants, concluding that the optimal condition for the bacteria is approximately 30°C, which is similar to the results obtained in this study. However, this experiment's results make it clear that, from the moment the inoculation process becomes efficient, both organisms can increase their capacity to tolerate stress factors (such as a temperature of 35°C), which would be impossible in isolated conditions. This is consistent with Trolldenier (1982), who evaluated the activity of diazotrophic bacteria in rice plants submitted to 35°C, obtaining positive responses of the association, even under thermal stress.

Conclusion

Inoculation with *Azospirillum brasilense* changes the initial developmentand biochemical responses of corn plants. Moreover, the inoculation response was beneficial at all temperatures tested, but with better results under 25, 30, and 35°C. However, when the seedling and bacteria were exposed to a temperature of 30°C and inoculated, an increase in plant biomass and activity of antioxidant enzymes was higher.

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