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Co-inoculation of *Anabaena cylindrica* and *Azospirillum brasilense* during initial growth and chloroplast pigments of corn[1]

Co-inoculação de *Anabaena cylindrica* e *Azospirillum brasilense* durante o crescimento inicial e pigmentos cloroplásticos do milho

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HIGHLIGHTS:
Anabaena cylindrica *associated with* Azospirillum brasilense *favored the development and growth of corn.*
Plant growth-promoting bacteria promote morphoanatomical alterations in the roots.
Anabaena cylindrica *and* Azospirillum brasilense *provide efficient water and nutrients absorption.*

ABSTRACT: Although the isolated effects of the cyanobacteria *Anabaena cylindrica* and the bacteria *Azospirillum brasilense* are well-known, the co-inoculation of both can promote other benefits to corn plants' biological nitrogen fixation and growth. The aim was to evaluate the initial growth and chloroplast pigment levels of corn hybrids co-inoculated with *A. brasilense* and *A. cylindrica*. The experiment was conducted in greenhouse conditions using the completely randomized design in a factorial scheme 2 × 4 with two hybrids, Balu 184 and Balu 280 Pro, and four inoculation treatments: no inoculation, inoculation of *A. cylindrica*, inoculation of *A. brasilense*, and the co-inoculation of *A. cylindrica* + *A. brasilense*. Co-inoculation resulted in higher root growth and dry mass of the aerial part in both hybrids; however, the hybrid Balu 280 Pro presented higher responsivity. The co-inoculation of *A. brasilense* and *A. cylindrica* increased initial corn growth without altering the photosynthetic pigment levels.

Key words: *Zea mays* L., *Anabaena cylindrica*, diazotrophs bacteria, microalgae, plant-growth-promoting bacteria

RESUMO: Embora comprovados os efeitos isolados das cianobactérias *Anabaena cylindrica* bem como da bactéria *Azospirillum brasilense*, na fixação biológica de nitrogênio e na promoção de crescimento de plantas de milho, a co-inoculação de ambas pode ampliar outros benefícios às plantas. O objetivo foi avaliar o crescimento inicial e teores de pigmentos cloroplastídicos de híbridos de milho co-inoculados com *A. brasilense* e *A. cylindrica*. O experimento foi conduzido em condições de casa de vegetação sob o delineamento inteiramente casualizado, em esquema fatorial 2 × 4, com dois híbridos: Balu 184 e Balu 280 Pro e quatro tratamentos de inoculação: ausência de inoculação, inoculação de *A. cylindrica*, inoculação de *A. brasilense* e a co-inoculação de *A. cylindrica* + *A. brasilense*. A co-inoculação resultou no maior crescimento da raiz e massa seca da parte aérea nos dois híbridos, porém com maior responsividade para o híbrido Balu 280 Pro. A co-inoculação combinada de *A. brasilense* e *A. cylindrica* incrementou o crescimento inicial do milho, sem alterações nos teores dos pigmentos fotossintéticos.

Palavras-chave: *Zea mays* L., *Anabaena cylindrica*, bactérias diazotróficas, microalgas, bactérias promotoras de crescimento vegetal

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Introduction

The co-inoculation of plant-growth-promoting bacteria (PGPB) is an advanced agricultural technology of proven efficiency that promotes higher root growth (Yegorenkova et al., 2016). The co-inoculation in corn crops of plant-growth-promoting bacteria may favor root and aerial parts growth due to efficient nutrient absorption. It also reduces the need for synthetic fertilizers, environmental and production costs and promotes more profitable and sustainable systems (Andrade et al., 2014a; Prasanna et al., 2015; Muro-Pastor et al., 2017).

Azospirillum brasilense is a widely known PGPB, commercialized and used in corn crops due to its biological nitrogen fixation (BNF) action, hormone synthesis, phosphate solubility, and increased tolerance to biotic and abiotic stresses (Strzelczyk et al., 1994; Gupta et al., 2013; Hashtroudi et al., 2013). The free-living cyanobacteria *A. cylindrica* occurs in many different environments (Shariatmadari et al., 2013) and fixate nitrogen through their heterocysts (Muro-Pastor et al., 2017). They also produce hormones and present nutrient-rich mucilage and soil conditioner bio-compounds (Prasanna et al., 2015). Nevertheless, studies with inoculated and co-inoculated cyanobacteria in agriculture are still scarce.

Evidence suggests an associative potential of *A. cylindrica* and *A. brasilense* through BNF enhancing growth and, consequently, corn development and grain yield (Prasanna et al., 2015).

Exploring the co-inoculation potential of cyanobacteria such as *A. cylindrica* combined with *A. brasilense* can reduce the use of nitrogen fertilizers, the production costs, and favor environment conservation (Hungria et al., 2015; Marini et al., 2015; Fukami et al., 2016; Milléo & Cristófoli, 2016; Gavilanes et al., 2020). The interaction with other production factors, such as the corn cultivar, must be considered. Thus, the aim of the present study was to evaluate the initial growth and chloroplast pigment levels of corn hybrids co-inoculated with *A. brasilense* and *A. cylindrica*.

Material and Methods

The experiment was conducted in a greenhouse conditions in the Agronomy Department of the Universidade Estadual de Londrina, in the municipality of Londrina, PR, located at the geographical coordinates: 23° 20' 23.45" S and 51° 12' 32.28" W, between October 2017 and January 2018.

The experiment was conducted adopting a completely randomized design in a factorial scheme 2 × 4 with four replicates using the corn hybrid cultivars Balu 184 22C (H1) and Balu 280 Pro 20C (H2), and four inoculation treatments: control (no inoculation) (T1), isolated inoculation of *A. cylindrica* (T2) and *A. brasilense* (T3), and co-inoculation of *A. cylindrica* + *A. brasilense* (T4).

The cyanobacteria were obtained from the Laboratory Collection of the Universidade Federal do Rio Grande - FURG (Rio Grande do Sul, Brazil) and reproduced in the Microbiology Laboratory of the Instituto Agronômico do Paraná, the state agricultural research center. The inoculant was prepared following Gavilanes et al. (2020) for an approximate concentration of 10^8 UFC mL^{-1}. The *A. brasilense* used was the commercial inoculant NITROBACTER AZP© containing the strains AbV5 and AbV6 with a concentration of 2.0 x 10^8 UFC mL^{-1}, following the manufacturer's instructions.

The seeds were inoculated before sowing. In the inoculation and co-inoculation, the dose of cyanobacteria applied was 5.0 mL kg^{-1} of seeds (Gavilanes et al., 2020). For the *A. brasilense*, the dose applied was 4 mL kg^{-1} of seeds, following the manufacturer's instructions.

The inoculation was conducted by adding to the seeds the bacteria *A. brasilense* and *A. cylindrica*, providing an even distribution of the inoculant in the seed. After the inoculation, the seeds were shade dried under room conditions with the lowest temperature of 16.2 °C, highest of 27.6 °C, relative air humidity of 75.7%, insolation of 2.2 hours of sunlight then seeded. Sowing was conducted with four seeds per pot and eight days after sowing (DAS), after the plants emerged, thinning was conducted to keep one plant per pot.

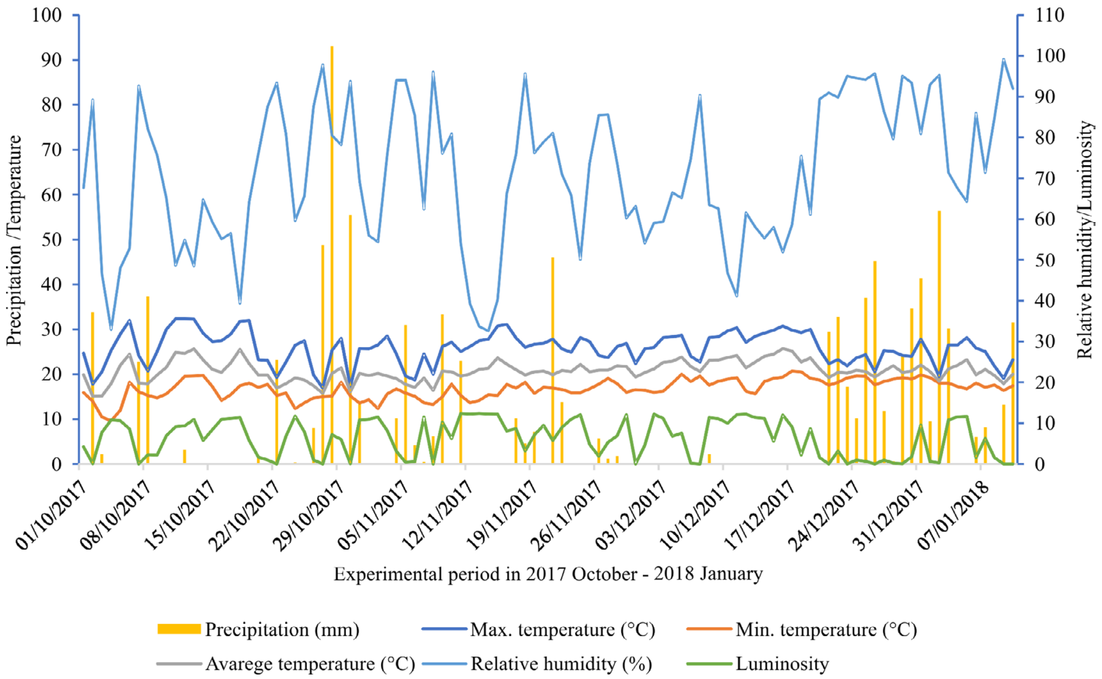

Figure 1. Meteorological data of the experimental period 2017-2018

The soil, classified as Latossolo Vermelho distróférrico (Oxisol) (EMBRAPA, 2018), after sieving was used as a substrate and conditioned in pots with a volume of 18.0 L. The soil chemical characteristics were determined before applying the treatments: pH ($CaCl_2$) 4.5; 3.48 $cmol_c\ dm^{-3}$ of Ca^{2+}; 0.8 $cmol_c\ dm^{-3}$ of Mg^{2+}; 4.96 $cmol_c\ dm^{-3}$ of H+Al; 2.22 mg dm^{-3} of P; 0.38 $cmol_c\ dm^{-3}$ of K; 1.73 g dm^{-3} of Total Nitrogen; 20.37 g dm^{-3} of OM; and 4.81 $cmol_c\ dm^{-3}$ of effective CEC. According to the soil chemical analysis, fertilization was conducted based on the recommendation for corn crops (IAPAR, 2003).

Through soil analysis, dolomitic limestone was applied at a dose equivalent to 4.5 t ha^{-1}, corresponding to the application in each pot with the substrate in a dose equivalent to 40.0 g in 18 dm^3 (31 x 30 x 25 cm). The mixture was incorporated manually in a container with a higher capacity, and after homogenization, the substrate returned to the pot. For fertilization of maintenance, a granular fertilizer was applied with the formulation NPK (4-30-10) in the dose of 400.0 kg ha^{-1}, equivalent to 4.0 g per pot. Topdressing nitrogen fertilization was performed manually on the soil surface of the pot and the plant's surroundings in the vegetative stage V4, applying ammonium sulfate (21-00-00-24S) at the dose of 50.0 kg N ha^{-1}.

The evaluation of growth characteristics was conducted in the vegetative stage V8, with the completely expanded eighth leaf. Each plant's leaf area (LA), expressed in cm^2 of leaf, was measured based on the total length (L) and width in the leaves' middle third (W). The data were obtained following Eq. 1, proposed by Francis (1969):

$$LA = 0.75\ C\ L \qquad (1)$$

where:

L - leaf length; and,

W - leaf width.

The root volume (RV) was determined by measuring the water column displacement in a graduated cylinder. After cleaning, the roots were individually put in a graded cylinder with 100 mL of water, and the difference in volume displacement provided the root volume in cubic centimeters (cm^3). Root length (RL) was determined by a graded scale measuring from the root apex to its insertion in the stem in centimeters. The root dry phytomass (RDP) and aerial part dry phytomass (APDP) were determined after drying in an oven at a constant temperature of 65.0 °C. The plant height (PH) was measured from the soil surface until the insertion of the expanded eighth leaf (V8) using a graded scale.

The level of chlorophyll 'a' (Chl 'a'), chlorophyll 'b' (Chl 'b'), and carotenoids (Car) were measured using the methodology of Whitham et al. (1971). Leaf tissues were extracted, and the mass was determined (0.2 g). After that, they were macerated in liquid nitrogen and then transferred to a test tube with 10.0 mL of acetone 80%. The tubes were wrapped in aluminum foil, with the matte side turned to the exterior and conditioned in a refrigerator at a temperature of 8.0 ± 2 °C for a week. The next procedure was the centrifugation of extracts. The supernatants were submitted to absorbance (A) readings with a spectrophotometer in the wavelengths 663, 645, and 434 nm for the levels of chlorophyll a, b, and carotenoids, respectively. The measurements of pigment levels were performed through the Eqs. 2, 3, and 4 (Barbosa et al., 2017):

$$\text{Chl a} = \left(11.24 \cdot A663 - 2.04 \cdot A645\right) \qquad (2)$$

$$\text{Chl b} = \left(20.13 \cdot A645 - 4.19 \cdot A663\right) \qquad (3)$$

$$\text{Car} = \frac{\left(1000 \cdot A434 - 1.90\text{Chl a} - 63.14\text{Chl b}\right)}{214} \qquad (4)$$

The data were submitted to normality (Shapiro Wilk) and homogeneity (Bartlett) tests, analysis of variance, and means comparison by Tukey's test at $p \leq 0.05$. The value of RL was transformed into the potency 2 and the logarithm (x) in the base 10 for RDP and APDP, following the assumptions of normality and homogeneity to perform the ANOVA, presenting a significant F-value with $p \leq 0.01$ for the respective tests.

Results and Discussions

Interactions between the hybrids and the inoculations were verified for the variables LA, RV, RL, RDP, and APDP. In the variables PH, levels of Chl a, Chl b, and Car, there was a significant effect for the isolated inoculation (Table 1).

The leaf area of the hybrid H2 at T2 (69.12 cm^2) was higher than H1 (59.64 cm^2) (Table 2). Moreover, there was a significant difference in the treatments' mean and higher value for the isolated treatment T2 with the hybrid H2 in comparison to the control treatment T1 and the combined inoculation T4. The difference between the hybrids is probably because of genetic interaction between cultivars and the inoculation of the PGPB with topdressing nitrogen fertilization increased nutrient absorption, resulting in a higher leaf area. Similar results were found by Gavilanes et al. (2019), in a greenhouse, with superior responses for LA in the interaction between cyanobacteria inoculation and nitrogen fertilization in corn crops. Zeffa et al. (2019) suggest that the positive response for the efficiency of the inoculation of *A. brasilense* (Ab-V5) for phytometric parameters varies according to the genotype.

When the co-inoculation was used, the difference between the hybrids was not observed, supporting the results obtained

Table 1. Results of the analyses of variance for initial growth and chloroplast pigment levels of the corn hybrids Balu 184 (H1) and Balu 280 Pro (H2), regarding the co-inoculation of *Azospirillum brasilense* and *Anabaena cylindrica*

Variables	Hybrid (H)	Inoculation (I)	H x I	CV (%)
LA	0.1439^{ns}	0.2639^{ns}	0.0156^{*}	27.55
RV	0.0032^{**}	$< 0.0001^{**}$	0.0003^{**}	5.14
RL (1)	0.1896^{ns}	$< 0.0001^{**}$	$< 0.0004^{**}$	3.87
RDP (2)	< 0.0001**	< 0.0001**	< 0.0001**	2.98
APDP (2)	< 0.0001**	< 0.0001**	< 0.0005**	2.96
PH	0.4018^{ns}	0.0109*	0.2838^{ns}	5.02
Chl a	0.0814^{ns}	0.0474*	0.3984^{ns}	40.50
Chl b	0.0802^{ns}	0.0488*	0.4072^{ns}	40.75
Car	0.1532^{ns}	0.0389*	0.2478^{ns}	39.49

ns - Not significant; * - Significant at $p \leq 0.05$ e ** - Significant at $p \leq 0.01$; (1) Transformation into the Potency 2; (2) Transformation into Log (x)

Table 2. Mean values and values of the interaction between hybrids vs. inoculation (H x I) for initial growth and the chloroplast pigment levels evaluated in the vegetative phase of the corn hybrids Balu 184 (H1) and Balu 280 Pro (H2), regarding the inoculation of *A. brasilense* and *A. cylindrica*, applied in the seed

Hybrid	T1	T2	T3	T4	Means
		LA (cm^2) CV (%) = 27.55			
H1	58.24 aA	49.79 aB	63.62 aA	66.92 aA	59.64
H2	56.80 bA	93.33 aA	76.46 abA	49.87 bA	69.12
		RV (cm^3) CV (%) = 5.14			
H1	104.75 bA	105.50 bA	114.50 bA	160.75 aB	121.38
H2	96.25 cA	113.25 bA	119.75 bA	186.00 aA	128.81
		RL (cm) CV (%) = 3.87			
H1	74.00 bA	65.50 cA	78.50 bB	94.50 aA	78.12
H2	67.00 cB	64.50 cA	87.25 bA	98.50 aA	79.31
		RDP (g) (1) CV (%) = 2.98			
H1	3.43 aB	6.37 bB	10.55 cB	27.30 dA	11.91
H2	8.42 cA	7.84 cA	15.55 bA	25.69 aA	14.37
		APDP (g) (1) CV (%) = 2.68			
H1	3.47 dB	6.44 cB	10.67 bB	27.61 aA	12.05
H2	8.51 cA	7.92 cA	15.72 bA	25.98 aA	14.53
		PH (cm) CV (%) = 5.02			
H1	68.25	63.25	65.25	69.50	66.56 A
H2	63.25	64.25	65.00	69.75	65.56 A
		Chl a (mg g^{-1}) CV (%) = 40.5			
H1	12.28	9.83	11.87	11.28	11.32 A
H2	12.90	3.40	9.81	8.72	8.71 A
		Chl b (mg g^{-1}) CV (%) = 40.75			
H1	1.29	1.04	1.26	1.19	1.20 A
H2	1.36	0.36	1.03	0.92	0.92 A
		Car (mg g^{-1}) CV (%) = 39.49			
H1	34.23	27.40	26.31	31.45	29.85 A
H2	35.97	9.48	27.34	24.32	24.27 A

T1 - Control; T2 - Inoculation *A. brasilense*; T3 - Inoculation *A. cylindrica*; T4 - A. brasilense + A. cylindrica; (1) Transformation Log (x); Means followed by the same letter, lower case in the row and upper case in the column, do not differ among them according to the Tukey's test at $p \leq 0.05$

by Gavilanes et al. (2020) in field research that did not observe interactions between the inoculation of hybrids with the leaf area index. The authors suggest a lack of specific responses for corn genotypes, concluding that it is a facilitator factor for the application recommendations of *A. cylindrica* associated with *A. brasilense*.

Prasanna et al. (2015) attribute a positive effect for corn growth produced by a biofilm rich in nutrients and phytohormones, synthesized by cyanobacteria and rhizobacteria, with different responses between hybrids for the agronomic characteristics. This fact highlights the necessity to investigate better the combination between hybrid and microorganisms in the inoculant.

In the variable RV, an interaction effect (Table 2) occurred in T4, with differences between the hybrids, and the mean of the hybrid H2 was higher than the hybrid H1. Moreover, T4 was higher than the other treatments with both hybrids. For the variable RL, an interaction between the hybrids H2 and H1 occurred in T3, and a higher mean for H2 (Table 2). The combined inoculation in T4, independently of the hybrids, produced a higher mean than the other treatments. The higher root growth in volume and length between the hybrids and the treatment can be attributed to the PGPB and the synergistic effect of the combined inoculation of *A. cylindrica* and *A. brasilense*, responsible for the synthesis of plant growth regulator substances. Similar studies indicate that *A. cylindrica* stimulated root growth through phytohormones (auxins and cytokinins) and other substances in different corn hybrids with different responses for the inoculation of microorganisms concerning plants' development and growth (Prasanna et al., 2015; Marini et al., 2015; Zeffa et al., 2019).

Calzavara et al. (2018), while studying *Bacillus* sp. and *A. brasilense*, attributed to the growth promoter bacteria a strong influence on root anatomy, widening the area of the metaxylem vessel elements (MVE), favoring water and nutrients absorption. According to Li et al. (2018), the application of auxins in the initial stage of corn plants stimulated the concentration in the roots, and a higher density of lateral roots was observed. However, the continual supply of exogenous auxin is fundamental to provide the morphological and anatomical changes during the plant growth stages. It is different from the supply through PGPB microorganisms, such as the co-inoculation of *A. cylindrica* (Andrade et al., 2014b; Prasanna et al., 2015) and *A. brasilense* (Cassán & Diaz-Zorita, 2016).

The interaction between the hybrids and the inoculation for the variables RDP and APDP, and the combined inoculation T4 did not significantly differ between the hybrids H1 and H2. Nevertheless, T2 and T3 of isolated inoculations resulted in an H2 higher than H1 (Table 2). In this sense, the increase of RDP and APDP of the hybrid H2 compared with H1 can be attributed to the hybrid H2's higher mass than H1, and its affinity with PGPB inoculation. Thus, RDP and APDP of H2 and H1 in the combined inoculation T4 were close, and, in the treatments' mean, T4 in both hybrids was higher than the other treatments (Table 2). Possibly, the higher biological activity in the corn rhizosphere with the co-inoculation of *A. cylindrica* and *A. brasilense*, associated with the transformations of the plants' vascular system and the nitrogen supply, promoted plant growth and the increase of corn phytomass. Calzavara et al. (2018) suggest that the inoculation of *A. brasilense* and *Bacillus* sp. with topdressing nitrogen fertilization in corn did not reduce the roots' biomass compared with the control plants. Still, the transformations in the roots influenced the increase of vegetal biomass.

Moreover, Marini et al. (2015), in a study about the interaction between corn genotype and the bacteria Azospirillum, concluded that the inoculation of *A. brasilense* increased corn APDP and LA, also noting the necessity of further studies about more responsive genotypes. Calzavara et al. (2018) related root structural alterations and increased the aerial part vegetal mass, nutrient absorption, and root exudation.

The variable PH demonstrated a significant difference between the inoculation treatments, with a lower mean for T2 than the co-inoculation T4 (Table 2). Possibly, the higher energy consumption of the plant when generating exudates by the root, which are a food source for the inoculated bacteria, influenced the lower PH of the isolated treatment T2. In the combined treatment T4, a positive synergistic effect might have occurred stimulated by PGPB bio-compounds, although it did not present a significant difference from the control treatment. In this context, the lower PH of the isolated inoculation T2 may be related to the plant's higher energy waste to liberate root exudates as a food source for diazotrophs (Lima et al., 2019).

The photosynthetic pigments (chlorophyll and b) and photoprotective (carotenoid) may sign the physiological stage of corn. The mean values obtained in the treatments indicate a significant difference only between the treatments T1 and T2, with a lower mean for T2. However, there was no significant difference for the treatments T3 and T4 compared with the control treatment (Table 2). Probably, the co-inoculation results did not present alterations because the evaluated pigment levels are generally variables of indirect evaluation regarding plant growth.

Nevertheless, Gavilanes et al. (2019) reported, in a greenhouse, an increase in chlorophyll b level with the combined inoculation of *A. cylindrica* and *A. brasilense*. In another field research, Gavilanes et al. (2020) observed higher chlorophyll levels in corn with co-inoculation than the control treatment.

In this study, the co-inoculation of *A. cylindrica* with *A. brasilense* in the seeds promoted a positive effect in the development and root growth of corn (RV, RL, and MSR) and the plant (MSA) compared with the absence of inoculation T1 and the isolated inoculation of plant-growth-promoting bacteria T2 and T3. The combined bacteria's synergy probably stimulated this fact in the production, the action of nutrient-rich PGPB bio-compounds, the hybrids more responsive to morphoanatomical alterations in the root, and the widening of the water and nutrient's uptake and absorption area.

In this study, the association of *A. cylindrica* and *A. brasilense* positively influenced root growth, revealing the great potential for higher development and grain yield in corn crops. However, more pieces of field research are necessary to verify the results obtained and the interaction between PGPB and the hybrids and other cultivation factors such as the crop environment and management.

Conclusions

1. Co-inoculation provided higher root growth and dry phytomass in the aerial part in both hybrids.
2. The co-inoculation of *A. brasilense* and *A. cylindrica* increases the initial growth of corn.

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