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Effect of diazotrophic bacteria and thiamine on growth and regrowth of millet

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Abstract
New technologies are constantly being launched, however studies that point to the effects of their joint application are lacking. The objective of this study was to evaluate the effect of the isolated and joint use of *Azospirillum brasilense* inoculation and the application of thiamine, on seed or leaf, on the development of millet in two cycles. A randomized complete block design was used in five replicates. The treatments were composed of the isolated or combined inoculation with *Azospirillum brasilense* and the application of thiamine, on seed (10 ml kg^{-1}) or leaf spray (10 ppm). The relative levels of chlorophyll a, b and total, plant height, number of shoots, fresh and dry mass production and dry matter index of plants were evaluated. In the first cycle, treatments were only effective for the relative chlorophyll contents, and in general, the treatment with isolated inoculation of *A. brasilense* stood out, differing significantly from the treatment with thiamin applied on seed. In the second cycle, there was significant effect of the treatments on the variables of plant height and number of shoots, in which a maximum height of 165 cm and production of 140 shoots m^{-2} with application of thiamin on seed and with the use of *A. brasilense* and thiamine applied on the leaf, respectively. Thus, it was concluded that inoculation with *A. brasilense* increases the relative chlorophyll content in millet during the first cycle. The joint use of inoculation with *A. brasilense* and the application of thiamine can be used as a strategy to increase the number of shoots.
Keywords: *Pennisetum glaucum* L.; biostimulant; vitamin B1; biological nitrogen fixation.

Efeito de bactérias diazotróficas e tiamina sobre crescimento e rebrota de milheto

Resumo
Novas tecnologias estão constantemente sendo lançadas, no entanto faltam estudos que apontem para os efeitos da aplicação conjunta destas. O presente estudo teve como objetivo avaliar o efeito da utilização isolada e conjunta da inoculação com *Azospirillum brasilense* e aplicação de tiamina, via semente ou foliar, sobre o desenvolvimento do milheto, em dois ciclos. Foi utilizado o delineamento de blocos ao acaso, em cinco repetições. Os tratamentos foram compostos pela utilização isolada ou conjunta da inoculação com *Azospirillum brasilense* e aplicação de tiamina, via semente (10 ml kg^{-1}) ou spray foliar (10 ppm). Foram avaliados os teores relativos de clorofila a, b e total, altura de planta, número de brotações, produção de massa fresca e seca e o índice de matéria seca das plantas. No primeiro ciclo houve resposta aos tratamentos apenas para os teores relativos de clorofila, sendo que, de modo geral, o tratamento com inoculação isolada de *A. brasilense* sobressaiu-se, diferindo significativamente do tratamento com aplicação de tiamina via semente. No segundo ciclo houve efeito dos tratamentos sobre as variáveis de altura de planta e número de brotações, nos quais se observou altura máxima de 165 cm e produção de 140 brotações por m^2 com aplicação de tiamina via semente e com a utilização conjunta do *A. brasilense* e da tiamina aplicada via spray foliar, respectivamente. Assim, concluiu-se que a inoculação com *A. brasilense* aumenta o teor relativo de clorofila no milheto durante o primeiro ciclo. O uso conjunto de inoculação com *A. brasilense* e a aplicação de tiamina podem ser utilizados como estratégia para aumento do número brotações.
Palavras-chave: *Pennisetum glaucum* L.; bioestimulante; vitamina B1; fixação biológica de nitrogênio.

Introduction

About one-third of the world's arable land is occupied for animal feed, an effect of increasing demand for products of this origin (FAO, 2017a). China emerges as the largest beef producer, followed by Brazil, which holds about 13.0% of the 300 million tons produced worldwide in 2015 (FAO, 2017b). In that same year, the country had a herd of 172 million animals (IBGE, 2017), which is mostly pastured, reducing production costs and increasing market competitiveness (DIAS-FILHO, 2016). Thus, the quality of pasture has great national importance and technologies must be sought in order to provide greater sustainability of the productive system.

Millet (*Pennisetum glaucum* L.) is a grass used as a forage species with high nutritional value (GÖRGEN *et al*., 2016) and characteristics of erect growth, high size, uniform development and good tillering, even when submitted to stress conditions due to lack of water, which favors its use in tropical regions (KISSMANN, 1997). In Brazil, the species started to arouse the interest of producers, becoming a crop of commercial value, such as forage, mainly after the introduction of hybrids with high productive potential (DAN *et al*., 2010). However, their cultivation still demands information to improve the production system.

Among the existing technologies, it is the ability of some bacteria to perform atmospheric nitrogen fixation, thanks to the functional nitrogenase enzyme, which converts the nitrogen present in the atmosphere (N_2) into forms that can be used to build plant tissues (HUNGRIA, 2011; BATTISTUS *et al*., 2014). The process is much explored in the cultivation of several plant species belonging to the Fabaceae family, such as soybeans and common bean, using bacteria of the genus *Rhizobium* (PEREIRA *et al*., 2013, ROHRIG *et al*., 2016). In grasses, studies involving biological nitrogen fixation are more recent and involve bacteria of the genus *Azospirillum*, which can promote vegetative development, improving production in quality and quantity (HUNGRIA, 2011; LONGHINI *et al*., 2016).

The application of bacteria of the genus *Azospirillum* in species used as forage has presented promising results. In a study with inoculation of different strains of *Azospirillum* in *Brachiaria* grass, Guimarães *et al*. (2011) observed that some of the bacteria positively influenced the development and the relative chlorophyll index. Also Vogel *et al*. (2014) carried out a survey on the inoculation of forage species with *A. brasilense*, verified the potential use of the bacteria as a promoter of vegetative development and forage quality and can be used as an alternative to partially replace the nitrogen fertilization.

In addition to the inoculation with diazotrophic bacteria, new techniques are sought that help in the efficiency of the productive systems. In this sense, the introduction of vitamins has been demonstrating the potential to increase the performance of species of economic interest, especially vitamins belonging to the B complex (HENDAWY; EL-DIN, 2010; MOHSEN *et al*., 2013; SOLTANI *et al*., 2014).

Thiamine, or vitamin B1, has a complex action on plant metabolism, improving the absorption of water and nutrients, increasing the nutritional and energy reserves and contributing to the activation of defense mechanisms (AHN *et al*., 2005; BOUBAKRI *et al*., 2012, KAYA *et al*., 2014). These effects culminate in gains in the vegetative and reproductive development of different plant species (OERTLI, 1987; HENDAWY; EL-DIN, 2010; SOLTANI *et al*., 2014). In this sense, increases in the vegetative organs of the mustard crop were observed (VENDRUSCOLO *et al*., 2017), as well as yield increases for the sweet corn crop (VENDRUSCOLO *et al*., 2018).

The benefits related to the inoculation with diazotrophic bacteria and the use of thiamine can be of great value to the increase of pasture production for animal feed. However, studies should be conducted in order to define methodologies for the use of these products in an effective way, as no work on this subject is found.

The objective of this study was to evaluate the effect of the isolated and joint use of the inoculation with *Azospirillum brasilense* and the application of thiamine, on seed or leaf, on the development of millet in two cycles.

Material and Methods

The study was conducted in a protected arc-style environment, covered with clear plastic and containing anti aphid screen on the sides, located in the city of Goiânia, Goiás, Brazil. The municipality is located in the central region of the State, 16 ° 40 'S, 49 ° 15' W and altitude of 750 m.

It presents as average climatic indicators: annual precipitation of 1575 mm and average monthly temperature of 22.9ºC, with predominance of Aw climate, characterized by tropical climate with rainy season from October to April and a period with precipitations below 100 mm monthly between May to September (CARDOSO *et al*., 2015).

The installation of the experiment was performed on November 30, 2016. Ten millet seeds were arranged in 18-liter pots filled with soil classified as a latossolo vermelho (SANTOS *et al*., 2013), present in the experimental area. After 12 days of sowing the thinning of the plants was carried out, keeping only two per pot. The soil used was submitted to laboratory analysis, presenting the following characteristics: Ca^{2+}= 2,0 $cmol_c$ dm^{-3}, Mg^{2+}= 0,81 $cmol_c$ dm^{-3}, K^{+}= 131,0 mg dm^{-3}, P (Mehlich I)= 3,5 mg dm^{-3}, Organic matter = 7,0 g dm^{-3}, Al^{3+}= 0,0 $cmol_c$ dm^{-3}, H+Al= 2,5 $cmol_c$ dm^{-3} and pH ($CaCl_2$)= 4,6, CTC=5,6 $cmol_c$ dm^{-3}, V%= 55,7% (DONAGEMMA *et al*., 2011). The soil granulometric analysis showed 48 g kg^{-1} of clay in the layer 0-0,2m (SILVA, 2009).

The experimental design was a randomized block design for six treatments, in five replications. For the composition of the treatments the following combinations were used: T1 - Control, without application; T2 - Inoculation with *Azospirillum brasilense* (10 ml kg^{-1}) (Masterfix Gramíneas, Stoller, Campinas, São Paulo, Brazil); T3 - Thiamine application on seed (10 mg kg^{-1}); T4 - Inoculation with *A. brasilense* (10 ml kg^{-1}) and application of thiamine on seed (10 mg kg^{-1}); T5 – Foliar Application of thiamine (10 ppm); T6 - Inoculation with *A. brasilense* (10 ml kg^{-1}) and foliar application of thiamine (10 ppm). The doses were based in previous studies (OERTLI, 1987).

The inoculation with *A. brasilense* and the application of thiamine via seeds were carried out with a graduated pipette directly on the seeds, followed by stirring in polythene bags for one minute for homogeneous distribution of the products. Leaf application of thiamine was carried out 13 days after planting, using a hand sprayer with a flow rate of 10ml s^{-1}, for 10 s.

The data were collected after two productive cycles, when the plants were in pre-flowering phase. The first cut was performed at 58 days after sowing, while the second cut was performed at 80 days after sowing, 22 days after the first cut. The plant height was measured with a graduated tape, the relative chlorophyll a, b and total content were obtained by the chlorophyll meter reading (Falker®) and the number of shoots was counted before cutting the plants. The plants were then cut to obtain the fresh and dry mass, by drying in forced air ventilation oven at 65 °C until obtaining a constant mass. The dry mass index was obtained by the ratio between the dry mass and the fresh mass of the plants. All the parameter were evaluated for both cut, first and second.

The data were submitted to analysis of variance and the means were compared by Tukey test to the probability of 5% on Sisvar statistical software (FERREIRA, 2014).

Results and Discussion

It was observed that the treatments applied differentially affected the behavior of millet plants during the first and second cycle. In the first cycle, only the relative levels of chlorophyll a, b and total were affected, while in the second cycle the characteristics of plant height and number of shoots were affected (Table 1).

Table 1. Relative chlorophyll content and developmental characteristics of millet plants submitted to inoculation with *A. brasilense* and treatment with thiamine.

Treatments	1st Harvest							
	CA	CB	CT	HP	SN	FM	DM	DMI
	(SPAD)	(SPAD)	(SPAD)	(cm)	(m^2)	(kg m^2)	(kg m^2)	(%)
Control	35.73ab*	12.45ab	48.18ab	94.86a	45.00a	6.39a	0.85a	13.20a
A. brasilense	40.42a	17.77a	58.18a	95.15a	41.00a	6.06a	0.77a	12.60a
Thiamine (seed)	33.81b	11.65b	45.46b	113.95a	45.00a	5.33a	0.74a	13.80a
A. brasilense + Thiamine (seed)	39.14a	16.73ab	55.86ab	131.02a	45.00a	6.04a	0.85a	14.00a
Thiamine (spray)	36.18ab	13.70ab	49.88ab	119.94a	42.00a	6.29a	0.79a	13.00a
A. brasilense + Thiamine (spray)	36.75ab	14.66ab	51.41ab	114.77a	41.00a	5.64a	0.77a	13.60a
MSD	5.22	6.02	11.08	98.25	13.40	2.83	0.09	2.40
CV%	7.21	21.22	11.00	45.00	15.83	24.26	25.53	9.16

Treatments	2nd Harvest							
	CA	CB	CT	HP	SN	FM	DM	DMI
	(SPAD)	(SPAD)	(SPAD)	(cm)	(m^2)	(kg m^2)	(kg m^2)	(%)
Control	39.29a	15.01a	54.30a	142.80abc	92.70b	7.44a	1.07a	14.29a
A. brasilense	40.10a	15.26a	55.36a	131.03bc	91.30b	6.89a	0.95a	13.78a
Thiamine (seed)	41.73a	16.85a	58.58a	165.00a	90.00b	6.52a	1.04a	16.07a
A. brasilense + Thiamine (seed)	40.23a	15.72a	55.95a	139.83abc	103.30ab	7.55a	1.13a	14.98a
Thiamine (spray)	41.33a	15.33a	56.55a	157.00ab	90.00b	8.08a	1.11a	13.78a
A. brasilense + Thiamine (spray)	41.30a	15.33a	56.63a	128.25c	140.00a	7.45a	1.02a	13.73a
MSD	3.15	3.97	6.90	27.06	37.00	2.93	0.40	2.03
CV%	3.90	12.82	6.17	9.45	18.38	20.09	19.20	7.07

*Means followed for the same letter do not present statistical difference when submitted to the Tukey test with 5% of probability. Relative index of chlorophyll a (CA), b (CB) and Total (CT); Height of plants (HP); Shoot number (SN); Fresh matter (FM); Dry mass (DM); Dry mass index (DMI); Minimum significant difference (MSD) Coefficient of variance (CV).

For the relative contents of chlorophyll obtained at the time of first cut of millet plants, it was observed that the treatment with isolated inoculation of *A. brasilense* stood out, but there was a significant difference only on the treatment with thiamine alone application via seed treatment. However, there was no significant residual effect of the inoculation on the second cut.

Having a direct relation with the internal contents of pigments and leaf nitrogen in grass species (ROCHA *et al*., 2010; SILVA *et al*, 2014), the increase of chlorophyll relative levels is related to the fixation capacity of atmospheric nitrogen by bacteria of the genus *Azospirillum* (BATTISTUS *et al*., 2014), that can culminates in a bigger protein content.

At the time of the second cut of the millet plants, a slight superiority of the treatment composed by the application of thiamine on seed was observed for the height of the plants, with a significant difference only for the isolated inoculation treatments of *A. brasilense* and combined foliar application of thiamine (Table 1).

It was also observed that the lower height for the combined treatment with inoculation with *A. brasilense* and foliar application of thiamine showed an inverse relation to the number of shoots and this treatment was superior in relation to this characteristic, with an increase of 51.0% in the number of shoots per m^2 in relation to the control treatment. However, there was no significant difference in relation to the treatment composed by inoculation with *A. brasilense* and thiamin on seed. This result can be a consequence of a negative interaction between the bacteria and the vitamins, as it was observed for sweet maize (VENDRUSCOLO *et al*., 2018), since the variation on thiamine content can be a signal against stress effect (GOYER, 2010).

However, the positive results, increases on vegetative and reproductive characteristics, are probably due to the promotion capacity of the production of endogenous hormones in plant tissues, observed for both bacteria and vitamin (OERTLI, 1987; EL-LATTIEF, 2016). In addition, the joint use of these products may have favored the root development by the action of the bacteria (COSTA *et al*., 2015) and the accumulation of energy reserves in the root tissues during the first cycle due to the improvement of photosynthetic capacity (KAYA *et al*. 2014), favoring the emission of larger shoots (FOLONI *et al*., 2008).

In studies conducted to evaluate the regrowth of grass species, it is observed that the use of nitrogen fertilization positively affects the emission of new shoots (MORAIS *et al*., 2006; FOLONI *et al*., 2008) and the fixation of the atmospheric nitrogen, carried out by *A. brasilense*, can exert such influence, since the bacterial fixing capacity is enough to increase the development of several types of grasses (HUNGRIA, 2011). In this sense, it is observed that the increase in nitrogen availability tends to reduce the relationship between auxins and cytokines (WILLIAMSON *et al*., 2012). The increased participation of cytokines in this relationship culminates in a greater development of shoots, since auxin suppression results in a decrease in bud dormancy.

Based on the results obtained and on the effects observed with the thiamine application alone or with the inoculation with *A. brasilense*, it is possible to infer that there is potential for the application of these techniques in obtaining higher pasture performance. However, the realization of new studies is essential to determine the dosages and methods of application of the treatments.

Conclusions

The inoculation with *A. brasilense* increases the relative chlorophyll content of millet during the first cycle.

The joint use of inoculation with *A. brasilense* and application of thiamine, via seed treatment or via foliar sprays can be used as strategy for improvement of the shoot number of the plants after regrowth.

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