Article

Cocksfood forage yield inoculated with PGPB bacteria

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Abstract

The PGPB bacteria have beneficial effects on crop yields. The aim of the present study was to determine the effect of five plant growth promoting bacteria on yield, plant height, SPAD units and content of protein in cocksfood defoliated every five weeks in spring and summer, under greenhouse conditions. A completely random design was used, with factorial arrangement 5x2x2, being the experimental unit a pot with ten stems, with four repetitions. The bacteria evaluated were: *Ewingella americana* (digestate), *Ewingella americana* (Soil), *Pseudomonas clororaphis*, *Bacillus toyonensis* and *Microbacterium oxidans*; compared with each other, and with the positive (triple 17) and negative (soil without fertilization) controls. The highest values of dry matter yield in spring were recorded by *E. americana* (3.5 g DM pot), while in summer it was *B. Toyonensis*. The height values did not register differences ($p > 0.05$) in both epochs, the SPAD units only in the summer and *E. americana* registered the lowest values (1.8). The protein content evidenced that the controls were inferior to all the treatments that were inoculated. The evaluated bacteria recorded effects on all evaluated variables and were superior to inorganic fertilization and unfertilized soil.

Keywords: *Bacillus toyonensis*, *Ewingella americana*, *Microbacterium oxidans* and *Pseudomonas clororaphis*.

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Introduction

The rhizospheric microorganisms colonize the rhizosphere and improve the development of plants, promoting an efficient use of minerals, through a wide variety of mechanisms such as mineralization of organic matter, biological control against soil pathogens, biological fixation of N, P, K and Zn, among other micronutrients and synthesize siderophores, which encourages the development of roots since, in this component of the plant.

Complex interactions occur between photosynthetic root exudates and other physiological processes of the plant, soil, and microbiome, which is inhabited by a wide range of microorganisms including fungi, bacteria, actinomycetes, algae, and nematodes, where dominance is determined by the product of the root exudates (carbohydrates, amino acids, fatty acids, nucleotides, organic acids, phenolics, growth regulators, putrescence, sterols, sugars and vitamins, among other compounds), which affects the population and functional dynamics of soil microorganisms, which differs from the rhizospheric zone to the rest of the population present in the soil or substrate (De-Bashan *et al*., 2007; Rashid *et al*., 2012; Esquivel-Cote *et al*., 2013; Hungria *et al*., 2016; Mora *et al*., 2017; Singh *et al*., 2017; Posada *et al*., 2018; Ramakrishna *et al*., 2019).

Cocksfood (*Dactylis glomerata* L.) is a species cultivated and exploited for its high nutritional value, rapid regrowth, DM yield, stem production and for being a species associated with legumes that allow more efficient use of radiation and soil resource (Castro *et al*., 2012; Castro *et al*., 2013; Villareal *et al*., 2014; Flores-Santiago *et al*., 2018).

The information published on the use of biofertilizers or plant growth promoting bacteria (PGPB) in this species is null; however, these have been reported in other pastures of the genus Penissetum (Criollo *et al*., 2012), Brachiaria (Lopes *et al*., 2018), Zea (Tejada *et al*., 2016), Sorgum and Tritricum (Naiman *et al*. , 2009; Rangel *et al*., 2014). Reporting differences ($p< 0.05$) due to the inoculation or effect of the bacteria compared to the control, which generally refer to the unfertilized soil.

Montalvo-Aguilar *et al*. (2018), evaluated the effect of fertilization with digestate at different concentrations and reported that the increase in yield was gradual ($p< 0.01$) in relation to the concentration, which also presents an interaction with the frequency of pasture harvest. In this experiment, the effect of digestate (sterile and non-sterile) as a means of inoculating bacteria was evaluated; as well as the effect of bacteria in sterile and non-sterile soil, that is why the objective was to evaluate the effect of bacteria that promote plant growth on the yield of cocksfood in greenhouse conditions.

Materials and methods

The experiment was carried out in a tunnel-type plastic greenhouse with side windows of the Center for Research in Applied Biotechnology of the National Polytechnic Institute (IPN), located in Tepetitla de Lardizabal, Tlaxcala (19° 16’ 50.3” north latitude, 98° 21’ 58.1” west longitude, 2 221 masl). The outdoor temperature is shown in Figure 1.

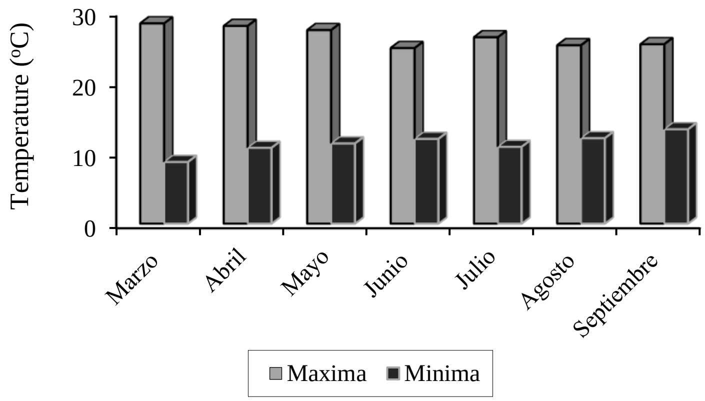

Figure 1. Maximum average temperature and minimum average environmental temperature in Tepetitla de Lardizabal, Tlaxcala. https://www.accuweather.com/es/mx/tepetitla/240244/weather-forecast/240244.

Cocksfood seeds (*Dactylis glomerata* L.) were donated by the forage laboratory of the Postgraduate in Livestock of the College of Postgraduates. The digestate was obtained from the Experimental Farm of the Department of Zootechnics of the Autonomous University Chapingo (UACH). The compost made from garden waste was donated by the Zacatenco composting unit of the National Polytechnic Institute. The soil used as substrate was obtained from the experimental plot of the CIBA IPN Unit Tlaxcala, which was identified as Fluvisol with a sandy texture.

Sowing of cocksfood was performed by placing 15 seeds in the substrate containing 1.5 kg of soil in plastic pots (experimental unit). Once the seedlings emerged, a manual thinning was performed to leave only 10 stems per pot and an establishment period of 45 days was left after sowing, later a uniform cut was made at 5 cm in height, to reduce the effect of covariate and plant growth-promoting bacteria (PGPB) were inoculated directly into the rhizosphere of the grass by means of plastic syringes.

Selection and inoculation of PGPB batteries

Serial dilutions were made of the soil, compost and digestate samples. 1 mL was grown in Petri dishes at three dilutions 10^{-2}, 10^{-4} and 10^{-6}, with an incubation period of 24 h at 30 °C. Pure cultures were obtained to describe the particular characteristics and with the selected morphotypes they were inoculated into the selective and specific media: Paenibacillus, Variovorax, Lysobacter, Azospirillum, Streptomyces, Streptomyces, Pikovskaya, Ashby, NFIP and NBRIP (Bashan and Holguin, 1997; Noumavo *et al*., 2013; Beghalem *et al*., 2017).

Bacteria that grew in these media were re-inoculated into Pikovskaya, Ashby, NFb and NBRIP specific media to evaluate their potential as potassium solubilizers, nitrogen fixers and phosphorus solubilizers, respectively. The strains selected for this experiment were identified as *Ewingella americana*, *Pseudomonas clororaphis*, *Bacillus toyonensis* and *Microbacterium oxidans*, which were previously identified by means of 16S rRNA sequencing.

The inoculation of the bacteria was carried out directly in the soil, in the rhizosphere of the tillers at the beginning of the season, at a concentration of 1 x 10^8 CFU mL^{-1} and the inoculation medium was the liquid digestate. A completely czar design with a 5 x 2 x 2 factorial arrangement was used, where the factors were: bacteria (five strains of bacterial), inoculation medium (sterile and non-sterile digestate) and substrate (sterile soil and non-sterile soil). In addition to this, experimental units with uninoculated soil (negative control) and experimental units with chemical fertilization (triple 17) were established as positive control. Protein values were plotted in SigmaPlot V.10.0 and statistical analyzes were performed using the GLM procedure in SAS® Statistical Software Version 9.0 for Windows®. The treatment means were compared using Tukey at a significance level of 5%. The media and substrates were sterilized in a Prado brand autoclave, model AH-80170.

Variables evaluated

The DM yield was determined every five weeks (Velasco *et al*., 2001) at a harvest height of five centimeters, placing all the cut forage in previously identified paper bags. The harvested plant material was washed and weighed fresh, and then dried in a forced air stove at 70 °C, for 48 h to a constant weight, and the dry matter content was determined.

Before each defoliation, the height of the forage was recorded with a fifty centimeter ruler and an accuracy of 0.1 cm, in randomly chosen plants, with the ruler placed completely vertical from the base of the plant to the youngest top leaf (Castillo *et al*., 2009; Castro *et al*., 2011). Likewise, the chlorophyll content (SPAD units) was measured before each cut, taking 3 samples per bunch, placing the sensor of the Apogee instruments MC-100 instrument on the exposed upper leaves with the ligule well differentiated.

The protein content in leaves was obtained from a sample of each treatment in the middle of each evaluated period, which was ground in a porcelain mortar and sieves with 0.5 and 0.17 mm opening were used, the material obtained was introduced into 1.5 ml Appendort tubes, which were analyzed by the Thermo Scientific team (Flash 2000 Series, Organic Elemental Analyzer).

Results and discussion

Cocksfood forage yield recorded that both in spring and summer there were differences ($p< 0.05$) due to the effects of different bacteria, in spring *P*. *clororaphis* was the one with the lowest record (2.74 g DM pot^{-1}) and it was not different ($p< 0.05$) from the rest of the bacteria except for *E*. *132mericana* (soil), which was the one that registered ($p< 0.05$) the highest value (3.5 g DM pot), surpassing *P*. *chlororaphis* 27% (lowest record), in 20% to *M*. *oxidans* and *B*. *toyonensis*, respectively and in 5% to the same species, which was obtained from the digestate. While in the summer the behavior of the yield changed and the bacteria (*E*. *132mericana* (soil)) that obtained the highest yield in spring, now registered ($p< 0.05$) the lowest yield (2.98 g DM pot^{-1}), but it was not different ($p> 0.05$) to its same species that was obtained from the digestate, nor to *P*. *clororaphis* and *M*. *oxidans*, where the latter were similar ($p> 0.05$) to *B*. *toyonensis*, which was the one that registered ($p< 0.05$) the highest value (3.74 g DM pot^{-1}), exceeding *M*. *oxidans* and *P*. *clororaphis* by 14 and 18%, respectively, and *E*. *132mericana* of digestate and compost, respectively, and 21 and 25%.

The inoculation medium (digestate) did not register differences ($p > 0.05$) between being sterilized and non-sterilized, which infers that the result in performance is due to bacteria and not to the medium, while the same result was obtained with the ground; that is, the effect is the same whether the soil is sterile or not, at the time of inoculation. Regarding the effect of the main factor, it was only registered that the strain ($p > 0.05$) had an effect on the yield both in spring and summer, while the soil and digestate did not ($p > 0.05$) and in the interactions only in the summer it was recorded that the strain∗soil was the one that had an effect on yield (Table 1).

The SPAD units that are closely related to the chlorophyll content and as a consequence of the nitrogen in the plant, did not register differences in spring (p> 0.05) between bacteria; however, in the summer, with the exception of *E. americana* (soil), which registered the lowest value (1.8) ($p < 0.05$), in the rest of the bacteria there were no differences ($p < 0.05$), registering that, *B. toyonensis* , *E. americana* (digestate) and *P. chlororaphis* were the best ($p < 0.05$) despite the fact that there was no difference between them and exceeding the one with the lowest value by an average of 70%. In the factorial analysis it was recorded that neither the medium nor the substrate had an effect ($p > 0.05$). However, there was only an effect of the strain in the summer season, while the rest of the factors and the interactions were not significant (Table 1).

Table 1. Yield of dry matter (g DM pot), SPAD units and height of (cm) of cocksfood, inoculated with PGPB bacteria in sterilized and non-sterilized media and substrates.

Factor		Yield		SPAD		Height	
		Spring	Summer	Spring	Summer	Spring	Summer
Bacteria	*Microbacterium oxidans*	2.92AB	3.26AB	2.07	2.3AB	22.3	19.7
	Bacillus toyonensis	2.92AB	3.74A	2.31	3.1 A	23.2	22.9
	Pseudomonas clororaphis	2.74B	3.16AB	2.27	2.9A	22.7	20.9
	Ewingella americana Soil	3.5A	2.98B	2.07	1.8B	22.2	19.7
	Ewingella americana Digestate	3.31AB	3.09 B	2.71	3 A	24.5	18.4
Digestate	Sterile	2.91	3.29	2.34	2.49	23.5	20.1
	Not sterile	3.25	3.24	2.23	2.81	23.7	20.4
Soil	Sterile	3.18	3.28	2.05	2.59	23.5	19.5
	Not sterile	2.98	3.24	2.52	2.71	23.7	21
Sign.	Strain	*	*	ns	*	ns	ns
	Soil	ns	ns	ns	ns	ns	ns
	Digestate	ns	ns	ns	ns	ns	ns
	Strain∗Soil	NS	*	ns	ns	ns	*
	Strain∗Digestate	ns	ns	ns	ns	ns	ns
	Digestate∗Strain	ns	ns	ns	ns	ns	ns

Different capital letters in columns are statistically different Tukey ($p < 0.05$); Sing.= significance; *= 0.05; ns= not significant.

Regarding the height of the forage, both in spring and summer, there were no differences (p> 0.05). However, in the summer time in the factor analysis the interaction strain∗soil, recorded an effect on height. However, the mean test did not show differences, despite the fact that the difference in height between the highest value (*B. toyonensis*) and the lowest (*M. oxidans* and *E. americana*) was 16% (Table 1).

Protein content recorded that with the exception of *P. clororaphis* in the rest of the treatments, higher protein content was recorded in the spring than in the summer (Figure 2). Likewise, in the spring it was evident that with the exception of the negative control (soil only) and *P. chlororaphis*, in the rest of the treatments a higher nitrogen content was found in the leaves, with *E. americana* (digestate) having the highest value (23%), surpassing 90% to the soil without fertilizer and 20% to chemical fertilizer. In the summer, the behavior of the protein in leaves registered that *P. chlororaphis* was the highest (15%), exceeding the control treatments on average by 61% (Figure 2).

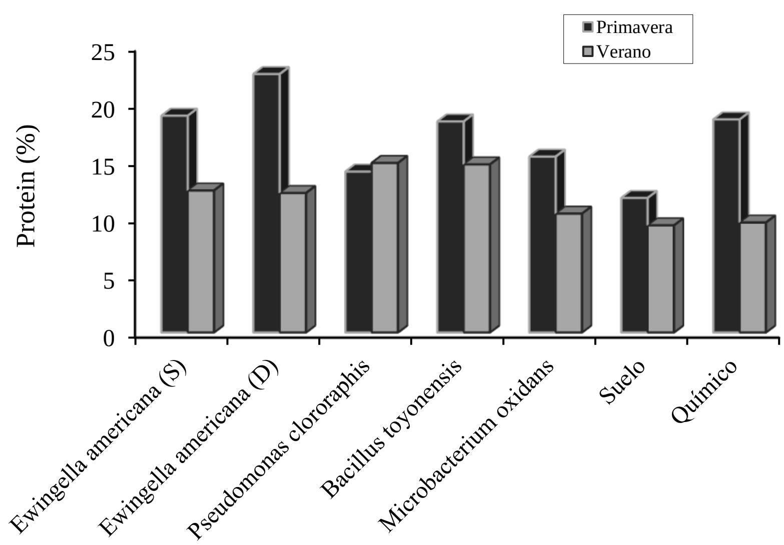

Figure 2. Protein content in cocksfood leaf, fertilized with PGPB bacteria, negative control (soil) and chemical fertilizer in the spring and summer of 2018.

Different authors, such as Hernández-Garay *et al*. (1997); Moliterno (2002), Ganderats *et al*. (2003), Mendoza-Pedroza *et al*. (2018), Gaytan *et al*. (2019), mention that forage yield is determined by the interaction of different environmental factors in a given climate or place, but the environmental variable that most influences the performance of a forage species is the environmental temperature (McKenzie *et al*., 1999).

However, in this experiment it is shown that this variable affects not only the plant but also the soil and, consequently, the microbiota of the rhizosphere, the difference between the spring and summer season was 1.7 °C in the maximum temperature recorded (Figure 1) and this difference was sufficient to show that in spring *Ewingella americana* registered the greatest effect on yield, while in summer it recorded *Bacillus toyonensis* (Figure 3).

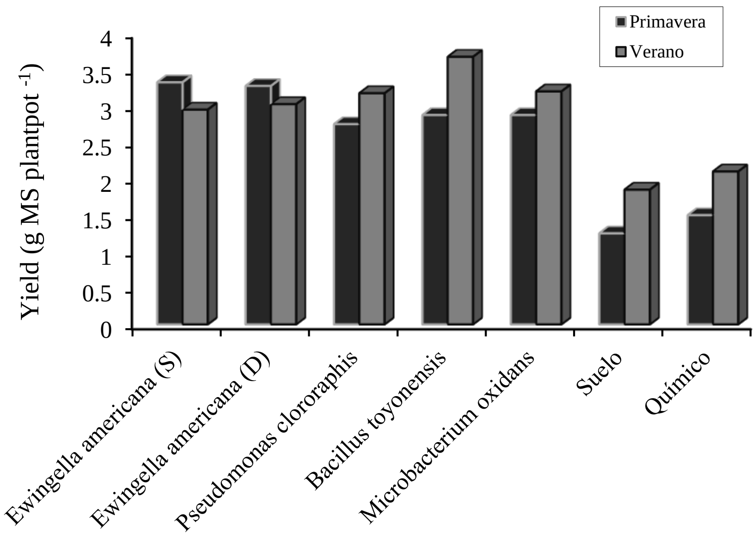

Figure 3. Cocksfood dry matter yield, fertilized with PGPB bacteria, negative control (soil) and chemical fertilizer in the spring and summer of 2018.

Meanwhile *Psedumona. clororaphis*, *Bacillus toyonensis* and the controls had more effect on the yield than in spring, which allows us to infer that, at different times of the year, the populations of rhizosphere bacteria that have symbiosis with grasses differ depending on the ambient temperature. . However, other publications have only evidenced and suggested that inoculating PGPB bacteria has effects on yield in grasses (Lopes *et al*., 2016), for grain production (Rangel *et al*., 2014), in forages of tropical climate (Humgria *et al*., 2016), in legumes (Pérez-Montaño *et al*., 2014) and in temperate pastures (Criollo *et al*., 2012), but in no article did they mention the effect of temperature or a conclusion regarding this variable.

SPAD units is a colorimetric of the leaves, which are correlated with the nitrogen content in the plant, and this element is at the same time the protein content. In this experiment it was recorded that in spring *E. americana* (soil), was the one that showed the highest value of SPAD units and at the same time of protein, while, in the summer, the lowest value of protein was obtained by *M. oxidants* (Figure 2) that also recorded the lowest values of SPAD units (Table 1). Rodríguez *et al*. (1998) and Gonzáles-Torres *et al*. (2009), mention that the determination coefficients between SPAD units and the chlorophyll content in the plant is 0.79 on average. However, these authors made the nitrogen determination in the entire plant and correlated it with the values obtained in the chlorophyll meter. Whereas, in this experiment, only the record of the portable meter was taken in the leaves, which are the ones that contain the highest protein content in a grass.

Regarding the digestate and the soil, the results obtained show that the condition of the application (sterile or not) does not influence the dry matter yield of cocksfood, despite the fact that the non-sterile medium was 12% higher than the sterilized one (digestate) and in the soil only a difference of 3% was registered. In the case of digestate, the difference in the percentage could be due to the microbial load that has beneficial effects on the soil, while the nutrient supply remains the same.

This statement coincides with what Tilvikiene *et al*. (2018), who reported the benefits of microorganisms and the contribution of organic matter when fertilizing cocksfood with digestate for five consecutive years. Montalvo-Aguilar *et al*. (2018), reported that the higher the concentration of the digestate, the forage yield has an upward trend in the yield of *Lolium perenne*; likewise, Walsh *et al*. (2012) reported that fertilization with digestate in Ballico perennial, exceeds (p< 0.05) in the yield to grasslands associated with white clover (*Trifolium repens*). Tempere and Viiralt (2014) reported that in temperate pastures fertilizing with digestate increases the yield by an average of 2.4 t DM ha^{-1}, compared to unfertilized meadows and is 85% higher than chemical fertilization.

Regarding forage height, it was recorded that, with the exception of the negative control (p< 0.05), in the rest of the treatments in spring, higher records were obtained (Figure 4), and the treatments with bacteria were higher (p< 0.05) to the control in this variable. Some authors have shown that the forages with the highest heights are not necessarily the most productive, since as the height of the forage species increases and yield increases, in the lower strata, the dead or senescent material also accumulates, suggesting that as plant height increases, forage yield is a result of green or live biomass and decomposing tissue (Hernández-Garay *et al*., 1997; Velasco *et al*., 2003; Montes *et al*., 2016; Mendoza-Pedroza *et al*., 2018; Gaytan *et al*., 2019).

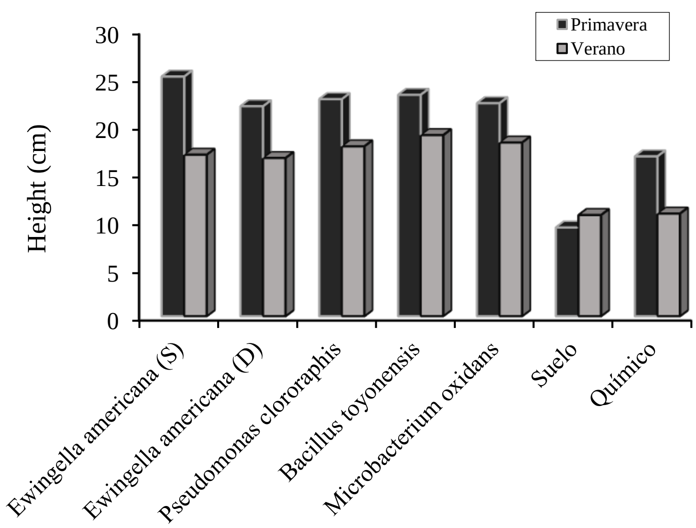

Figure 4. Forage height of cocksfood, fertilized with PGPB bacteria, negative control (soil) and chemical fertilizer in the spring and summer of 2018.

Conclusions

Plant growth promoting bacteria have a direct effect on the forage yield of cocksfood, and the level of influence is determined by the environmental temperature, so more should be evaluated in this regard, and thus a management of the inoculation with PGPB bacteria at different times of the year. In this experiment, all bacteria treatments were superior to controls, regardless of whether the media (digestate) and substrates (soil) were sterile or not.

Acknowledgments

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