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Scientific Article

Biocontrol of *Pratylenchus zeae* and sugarcane growth promotion by rhizobacteria

Biocontrole de *Pratylenchus zeae* e promoção de crescimento de cana-de-açúcar por rizobactérias

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ABSTRACT - The root lesion nematode (*Pratylenchus zeae*) is one of the main phytosanitary problems of sugarcane, and the biological control is an important tool in the integrated nematode management. Thus, the objective of this study was to evaluate the potential of rhizobacteria in the biocontrol of *P. zeae* and their plant growth promotion (PGPR) in sugarcane. Seedlings of sugarcane 'RB008347' were microbiolized with individual suspensions of nine bacterial isolates in a greenhouse. After 15 days, the plants were transplanted to pots containing sterilized soil, and then were inoculated or not with 1000 specimens of *P. zeae*/plant under greenhouse conditions. Non-microbiolized seedlings inoculated or not with the nematode were used as controls. Ninety days after inoculation, each plant was evaluated for its development, number of nematodes/root system, nematode/g of roots and nematode reproduction factor. In an *in vitro* bioassay, the nematicidal activity of these bacteria on specimens of *P. zeae* was evaluated. Additionally, the bacterial isolates were characterized biochemically regarding production of compounds related to nematode biocontrol and PGPR. The majority of isolates promoted significant increases in the number of tillers, shoot fresh and root fresh mass, and suppressed *P. zeae* reproduction (48-74%) compared to the control. In the *in vitro* bioassays, the isolates XT23, XT26, XT51, XT56, XT37 and P17 showed nematicidal activity and produced at least one compound related to the biological control of phytonematodes and PGPR. In this way, the microbiolization of sugarcane seedlings with rhizobacteria demonstrates its effectiveness as a bionematicide and growth promoter for sugarcane crops.

Keywords: Biological control, PGPR, Root-lesion nematode, *Saccharum* spp.

RESUMO – O nematoide das lesões radiculares (*Pratylenchus zeae*) é um dos principais problemas fitossanitários da cana-de-açúcar, e, o controle biológico é uma ferramenta importante no manejo integrado de fitonematoides. Assim, foi objetivo deste estudo, avaliar o potencial de rizobactérias no biocontrole de *P. zeae* e na promoção de crescimento de plantas (PCPR) de cana-de-açúcar. Mudas de cana -de-açúcar 'RB008347' foram microbiolizadas com suspensões individuais de nove isolados bacterianos em casa de vegetação. Decorridos 15 dias, as plantas foram transplantadas para vaso contendo solo esterilizado e, a seguir, inoculadas ou não com 1000 espécimes de *Pratylenchus zeae*/planta. Como testemunhas, utilizaram-se mudas não microbiolizadas inoculadas ou não com o nematoide. Decorridos 90 dias, cada planta foi avaliada quanto ao seu desenvolvimento, número de nematoides/sistema radicular, número de nematoides/g raízes e fator de reprodução do nematoide. Em bioteste conduzido *in vitro*, avaliou-se a atividade nematicida dessas bactérias sobre *P. zeae*, bem como procedeu-se a caracterização bioquímica dos isolados quanto à produção de compostos relacionados ao biocontrole e PCPR. A maioria dos isolados bacterianos promoveu aumento significativo do número de perfilhos, da massa fresca da massa fresca da parte aérea e das raízes, bem como suprimiu a reprodução do nematoide (48-74%) comparativamente à testemunha. Nos testes *in vitro*, os isolados XT23, XT26, XXT51, XT56, XT37 e P17 apresentaram atividade nematicida e produziram pelo menos um composto relacionado ao controle biológico de fitonematoides e/ou à PCPR. Dessa forma, a microbiolização de mudas com rizobactérias demonstra sua efetividade como bionematicida e promotor de crescimento para cultura da cana-de-açúcar.

Palavras-chave: Controle Biológico, PGPR. Nematoide-das-lesões, *Saccharum* spp.

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INTRODUCTION

Sugarcane (*Saccharum* spp. L Hybrid) was introduced in Brazil during the sixteenth century and soon became an economically important crop, fostering the expansion of agribusiness and the family farming agriculture in the country. Nevertheless, the crop is subject to various limiting factors, such as lack or excess of rainfall, extreme temperatures, low irradiation, and phytosanitary problems, which hamper maintenance of high yields and productivity (SILVA et al., 2016).

Among the phytosanitary problems associated with low productivity, diseases caused by nematodes stand out for causing damage to sugarcane crops, reducing yields by 20-30%. These phytoparasites generally have high destructive potential for parasitizing the root system, drastically decreasing the uptake and translocation of nutrients and impairing the physiology and nutrition of the crop. In Brazil, the species *Pratylenchus zeae* (Godfrey) Filipjev & Schuurmans Stekhoven, *Meloidogyne javanica* (Treub) Chitwood and *M. incognita* (Kofoid & White) Chitwood are considered the most common nematodes affecting sugarcane crops (DINARDO-MIRANDA, 2022; SILVA et al., 2016). High population

levels of these nematodes can drastically reduce sugarcane productivity in the different regions of the country. *Pratylenchus zeae* is the species most often found affecting Brazilian sugarcane crops (DINARDO-MIRANDA, 2022). This pathogen causes multiple problems, including reddish-purple or brown lesions and necrotic areas in the parasitized roots, resulting in the weakening of tussocks and yellowing of their leaves, stunted root and shoot development and reduction of the number and weight of stalks (BARBOSA et al., 2013).

Among the practices to manage these pests, the use of chemical nematicides is most common in sugarcane fields in the country. However, the effect of these products in controlling nematodes is ephemeral, and new infestations by phytonematodes occur about 90 days after application (MOURA, 2020). In this context, biological control methods, consisting of application of beneficial microorganisms such as plant growth-promoting rhizobacteria (PGPR), are gaining importance. These bacteria colonize the rhizosphere of plants and can provide bioprotection against phytopathogens such as phytonematodes, which prevent the hatching, development and/or reproduction of parasites (SUBEDI et al., 2020).

Considering the lack of sugarcane genotypes resistant to root-lesion nematodes available in the market, the low efficiency of chemical nematicides and their negative impact on the environment, the objectives of this study were to evaluate the potential of eight rhizobacteria for the biocontrol of *P. zeae* and the promotion of sugarcane growth under *in vitro* and *in vivo* conditions.

MATERIALS AND METHODS

Eight bacterial isolates from the microorganism collection of Embrapa Clima Temperado, selected *in vitro* by Bisognin (2017) regarding their ability to colonize the root system of sugarcane plants, were evaluated for plant-growth promotion and biocontrol of root-lesion nematodes, under laboratory and greenhouse conditions. The bacteria were multiplied and preserved in test tubes containing Kado-Heskett 523 medium at 4 °C and stored in 20% (V/V) glycerol at -20 °C in a freezer (MARIANO; SILVEIRA, 2005).

We used the bacterial isolates XT23, XT33 and XT39 [*Micrococcus luteus* (Schroeter 1872) Cohn 1872], XT37 [*Serratia liquefaciens* (Grimes and Hennerty 1961 (sic) Bascomb et al. 1971], XT38 [(*Exiguobacterium acetylium* (Levine and Soppeland 1926) Farrow et al. 1994)], XT56 (*Bacillus megaterium* Bary), XT26 (*Pseudomonas alcaligenes* Monias, 1928), XT67 (*Microbacterium* sp. Takeuchi and Hatano, 1998), and XT51 (*Pseudomonas* sp. Walter Migula, 1894, 1900), previously identified at the genus and species level by molecular analysis using the oligonucleotide primers 1492R (5-TACGGYTACCTTGTTACGACT-3) and 27F (5-GAGAGTTTGATCCTGGCTCAG-3) for the gene 16S rRNA (LANE, 1991).

For the nematode inoculum, we used a pure population of *P. zeae* (Godfrey) Filipjev & Schuurmans Stekhoven from sugarcane plants, previously characterized morphologically and morphometrically (CASTILLO; VOVLAS, 2007) and maintained in pots containing sorghum plants ('BRS 506' genotype).

Greenhouse bioassay for biocontrol and growth promotion of sugarcane plants

Sugarcane seedlings of the RB008347 genotype, maintained in tubes with sterilized soil, were first microbiolized with eight bacterial isolates (XT56, XT67, XT51, XT33, XT23, XT39, XT26, XT37), separately, seven days after sprouting. For each treatment, each seedling was microbiolized by depositing 15 mL of bacterial suspension in the soil (A_{540} = 0.5), previously prepared in a saline solution (0.85% NaCl) and adjusted using a spectrophotometer.

One week after microbiolization, each seedling was individually transplanted into a pot with 5 L capacity containing sterilized soil. Then, the seedlings were inoculated with 1000 specimens of *P. zeae*/plant (initial population -Pi) with six repetitions per treatment in a completely randomized design. As controls, we used seedlings that received 15 mL of a 0.85% saline solution (without bacterial suspension), inoculated or not with the nematodes. The seedlings were watered daily.

Ninety days after inoculation, the plants were evaluated for collar diameter (CD) (mm) with a digital caliper and their height (cm) was measured with a ruler, followed by counting of the number of tillers (NT). Subsequently, we separated the aerial parts (shoots) from the roots of each plant for measurement of the root fresh mass (RFM) and shoot fresh mass (SFM) (g) with a digital precision scale. Finally, the roots of each plant were processed as described by Coolen and D´Herde (1972) to determine the final population (Pf) of nematodes and to calculate the nematode reproduction factor (RF=Pf/Pi), according to Oostenbrink (1966) and determine the number of nematodes per gram of roots (NGR).

In vitro nematicidal activity of the bacterial isolates

We evaluated the *in vitro* nematicidal potential of these bacteria on *P. zeae* specimens in a bioassay performed with ELISA microplates as described by Wille, Gomes and Mota (2019). Each well in the microplate was considered a repetition, in which 50 µL of distilled water containing 25 *P. zeae* specimens was added, followed by 50 µL of the bacterial suspension of each isolate, previously grown for 48 hours and standardized with a spectrophotometer (A_{540}= 0.5) using four repetitions per treatment in a bioassay in completely randomized design. As control, we used wells containing only 50 µL of saline solution with 25 nematode specimens. Next, the plates of the different treatments were covered with plastic film and incubated in a BOD chamber at 28 °C in the dark.

After incubation for 24 hours, 10 µL of NaOH (1N) was added to each well of the plates. Then, nematode mortality was determined (number of dead nematodes = NDN); specimens with completely distended body for 40 seconds after addition of NaOH were considered dead (CHEN; DICKSON, 2000). The NDN values were then used to determine the mortality percentage (nematicidal effect) in each repetition.

Biochemical characterization of the bacterial isolates regarding production of compounds related to phytonematode biocontrol and plant growth promotion

We assessed the ability of the isolates to produce (+) or not (-) compounds related to biocontrol, such as protease and

lipase, besides nitrogen compounds such as ammonia and hydrocyanic acid (HCN). The proteolytic capacity of the isolates was determined in gelatin 12% and milk agar + casein as substrate (MARIANO; SILVEIRA, 2005). The lipolytic capacity was evaluated by the presence of a halo of precipitates surrounding the bacteria colonies in Tween 80 medium at 1% (FAHY; PRESLEY, 1983).

The production of ammonia was considered positive when the isolates formed a yellow-orange precipitate in a test tube containing peptone broth (MARIANO; SILVEIRA, 2005). HCN compound was determined according to the protocol of Bakker and Schippers (1987), where the change in color of a filter paper strip to reddish orange in TSA medium supplemented with glycine and iron chloride hexahydrate indicated the ability to produce this acid.

The production by the isolates of compounds related to plant growth promotion was assessed by their ability to dissolve phosphate (phosphatase) at pH 4.5 and 7.0 (NAUTIYAL, 1999) and potassium at pH 7.0 (SUGUMARAN; JANARTHANAM, 2007). The presence of a halo around the colonies in the culture medium specific for each method was considered positive for this evaluation. Additionally, we tested the ability of the bacteria to produce auxins by colorimetry (GORDON; WEBER, 1951), and bacterial isolates that had reddish color at the end of the process were considered positive.

Next, the values of the different variables related to the *in vitro* bioassay (mortality percentage, transformed to arcsine $\sqrt{x}/100$), and *in vivo* test (SFM, RFM, CD, NT, height and RF and NGR) were subjected to ANOVA, and the means of each treatment were compared to each other using the Scott-Knott grouping test at 5% significance level in SASM-Agri statistical software.

RESULTS AND DISCUSSION

Biocontrol of the root-lesion nematode and promotion of growth of sugarcane plants with rhizobacteria

In the *in vivo* experiment, all the isolates tested suppressed reproduction of *P. zeae* in the microbiolized sugarcane seedlings ($P<0.05$) in comparison with the non-microbiolized control considering the lower values of RF nematode ($7.0>RF>3.4$) and NGR (Table 1), and the greatest nematode suppression was obtained with isolate XT23 (≈75%).

With regard to the influence of the bacterial treatments on the developmental parameters of the sugarcane plants (Table 1), all the treatments increased the root fresh mass by at least two times in comparison with the respective controls inoculated with the nematode without bacteria. The majority of the bacterial treatments promoted significant increases in the number of tillers/plant, of 2.3 times, except the treatment with the isolate XT56. The isolates XT23 and XT33 (*M. luteus*) promoted a significant increase in the shoot fresh mass (10%) in comparison with the respective controls. Only XT56 (*B. megaterium*) promoted a significant increase in plant height in comparison with the plants that were inoculated and not microbiolized. For the variable collar diameter, there were no significant effects of the microbiolization of plants with the bacteria ($P>0.05$).

Table 1. Biocontrol of *Pratylenchus zeae* and promotion of growth of sugarcane plants evaluated by the, reproduction factor (RF), nematode/g roots (NGR) and potential control (PC) of the nematode, root fresh mass (RFM), shoot fresh mass (SFM), number of tillers (NT), height, and collar diameter (CD) of plants subjected to microbiolization with different bacterial isolates under greenhouse conditions.

Bacterial isolates	RF	NGR	PC (%)	RFM (g)	SFM (g)	NT	Height (cm)	CD (mm)
Test (-N)	-	-	-	89.4a	161.4b	2.3b	171.6a	12.5^{ns}
Test (+N)	13.4a	363.2a	-	36.9b	152.4b	1.5b	151.6b	13.2
XT67 (*Microbacterium* sp.)	7.0b	84.56b	48.3	82.8a	156.0b	5.0a	135.5b	11.6
XT33 (*M. luteus*)	5.3c	70.71b	60.7	75.1a	176.4b	4.6a	148.5b	13.5
XT51 (*Pseudomonas* sp.)	4.9c	58.70b	63.7	83.3a	151.5b	4.1a	149.4b	11.4
XT39 (*M. luteus*)	5.4c	68.39b	59.6	79.1a	163.7b	5.0a	143.3b	12.2
XT26 (*P. alcaligens*)	4.6c	60.52b	65.5	76.0a	146.7b	4.2a	150.6b	11.9
XT37 (*S. liquefaciens*)	4.4c	54.40b	67.3	80.9a	149.4b	4.6a	147.8b	10.8
XT56 *(B. megaterium*)	3.9c	47.40b	70.8	82.7a	162.2b	1.5b	176.3a	13.3
XT23 (*M. luteus*)	3.4d	37.29b	74.8	91.3a	178.9a	3.5a	147.6b	11.4
CV (%)	20.3	27.1		20.7	20.6	23.1	10.2	18.3

*Means followed by distinct letters in the rows differ from each other by the Scott-Knott test at 5%. NS - not significant; control -N= plants not inoculated with *P. zeae* and not microbiolized; control +N= plants not inoculated;; CV - coefficient of variation.

In vitro nematicidal activity and biochemical characterization of the bacterial isolates related to nematode biocontrol and plant growth promotion

The isolates XT23 (*M. luteus*), XT37 (*S. liquefaciens*), XT56, XT26 (*P. alcaligenes*), P17 (*Microbacterium* sp.) and XT51 (*Pseudomonas* sp.) had nematicidal activity on specimens of *P. zeae*, resulting in mortality rate of 45% in comparison with the control where the nematodes were subjected to treatment with only salt water (Table 2).

According to the methods used for biochemical characterization of the isolates (Table 2), the majority of them

produced at least one compound associated with biocontrol and/or plant growth promotion (Table 1). With regard to the biocontrol capacity, according to the evaluation of the proteolytic activity, the isolates XT56, XT23, XT37, XT39 and XT51 produced proteases in sugar-milk medium, with the first two also being positive for proteolytic activity using the gelatin method. Furthermore, except for XT51, the other five bacterial isolates produced ammonia. In relation to the production of the enzyme lipase, only the XT37 isolate was positive for this lytic enzyme and none of the isolates produced HCN.

Table 2. Nematicidal activity and biochemical characterization of eight bacterial isolates (BI) regarding capacity for biocontrol of *P. zeae* and promotion of growth of sugarcane plants.

Bacterial isolates	Nematode mortality (%)	PG	PLA	L	A	HCN	P^1	P^2	K	AIA	NCP
XT51	49.4a*	+	-	-	+	-	-	-	-	-	2
XT23	44.6a	-	+	-	+	-	-	-	-	+	3
XT67	44.3a	-	-	-	-	-	+	+	-	-	1
XT37	42.9a	+	-	+	-	-	-	+	-	-	3
XT56	41.2a	+	+	-	+	-	-	-	-	-	3
XT26	38.4a	-	-	-	-	-	-	-	-	-	0
XT33	31.8b	-	-	-	-	-	-	-	-	-	0
XT39	23.8b	-	+	-	+	-	-	-	-	+	3
Control (SW)	32.6b										
CV (%)	9.03										

*Means followed by the same letter in the column do not differ from each other by the Scott-Knott test at 5%; ** data transformed to arcsine $\sqrt{x|100}$. Production of proteases in gelatin (PG) and in milk-sugar (PMS); production of lipases in Tween 80 (L); production of ammonia (A); production of hydrocyanic acid (HCN); production of phosphatase at pH 5.0 (P^1) and pH 7.0 (P^2), solubilization of potassium (K), production of auxin (AIA), production of the compound (+) or no production (-); Number of compounds produced (NCP) and salt water control (SW).

With regard to the production of compounds associated with promoting plant growth, isolates XT67 and XT37 were able to dissolve phosphorus in NBRIP medium at pH 7.0, but at acidic pH (5.0), only the first isolate was effective. No bacterial isolate was able to solubilize potassium, and only isolates XT23 and XT39 were positive for production of auxin.

Among the bacterial isolates evaluated here, at least four of them (XT23, XT37, XT38 and XT39) suppressed the reproduction of the root-knot nematode (*M. graminicola*) in a study with irrigated rice (BRUM, 2017). In that study, the author verified that *M. luteus* (XT23) caused a reduction of over 50% in the number of galls and from 30 to 68% in the reproduction of the pathogen in three rice cultivars whose control levels were similar to those observed here. Additionally, this isolate promoted growth of the microbiolized plants, demonstrating its potential for use as a biocontrol of that pest under rainfed and irrigated (flooded) conditions in a greenhouse.

The *in vitro P. zeae* mortality with the isolates XT23, XT37, XT56, XT26, P17 and XT51 corroborates their nematicidal activity, mainly in light of the production of proteases, facilitating penetration of the bacteria in the cuticle of the nematodes, as found in a study with *Bacillus* sp. (LIAN et al., 2007) and another with *Pseudomonas* sp. (SIDDIQUI; HAAS; HEEB, 2005). The production of protease can be considered a virulence factor in the pathogenesis of phytonematodes. Likewise, the majority of the isolates analyzed produced ammonia, which is a compound with strong nematicidal activity (WILLE; GOMES; MOTA, 2019). Moreover, XT37 also showed lipolytic activity, in which this enzyme is associated with degradation of eggshell and body cuticle of nematodes, negatively affecting hatching and development of these pathogens, as previously observed in various bacterial genera on *Meloidogyne incognita* (WILLE; GOMES; MOTA, 2019) and *P. brachyurus* (HARNI; SUPRAMANA; SUPRIADI, 2012). Although none of the bacterial isolates produced HCN, and this compound contributes to the biological control of phytonematodes (RAZA; YOUSAF; RAYER, 2016), the production of other lytic enzymes previously mentioned in association with other mechanisms of action such as resistance induction may have positively influenced the levels of nematode control, as already observed in *in vivo* tests in other studies.

The suppression of phytonematodes on sugarcane by bacteria has been observed in other studies of root-knot nematodes. Pacheco et al. (2016) evaluated the effectiveness of two isolates from *M. luteus* (XT39 and XT23) for the biocontrol of *M. javanica* in sugarcane seedlings and observed the effectiveness of the bacteria in suppressing this phytoparasite under greenhouse conditions. In turn, Cardozo and Araújo (2011), when evaluating the efficiency of *Bacillus subtilis* in the same pathosystem, verified that the application of this bacterium in sugarcane under field conditions, verified suppression of *M. javanica* at rate of more than 50% besides a significant increase in the shoot dry mass of the plants.

In another study, Ferreira et al. (2017) investigated the treatment of soil with *B. subtilis*, *B. firmus* and *B. amyloliquefaciens*, and observed a more than two-fold increase in the number of tillers of sugarcane plants, but did

not observe any effect of the treatments on biocontrol of the nematode. In two other studies, Dinardo-Miranda et al. (2022) observed that the application of biological products based on *B. subtilis* and *B. licheniformis* on sugarcane for two years, resulted in suppression of *P. zeae* and *M. javanica* for 30 days to four months, and these treatments resulted in a significant increase in productivity at 5% probability level in the second year. Similarly, Schoen-Neto et al. (2019), when investigating the potential of *Bacillus* spp. and *Trichoderma harzianum* for *P. zeae* control in sugarcane, observed that the treatments negatively affected the nematode, penetration in the roots and suppressed this pest nematode, although the control percentages were lower than those observed in our study, and there was no plant growth promotion.

Although the isolates XT33 and XT39 did not have *in vitro* nematicidal effect, they promoted the *in vivo* suppression of 60% of *P. zeae* on the microbiolized plants compared to the untreated control plants. In this case, the bacterial isolates might have induced resistance of the plants. According to Brum (2017), the treatment of rice seedlings with another bacterial isolate of *M. luteus* (XT23) not only resulted in an increase of root fresh and shoot fresh mass of plants, but it also restricted the damage caused by *M. graminicola* in the roots. Additionally, in these microbiolized plants, the activity of polyphenoloxidase and peroxidase resistance enzymes was reduced by over 50% in comparison with the plants inoculated with the nematodes without the bacterium, and there was also a delay in the development of the nematodes to reach their adult stage. Therefore, this bacterial isolate has the ability to synthesize lytic enzymes, substances with nematicidal action (LIAN et al., 2007), toxic metabolites (OKA; CHET; SPIEGEL, 1993), induce resistance in plants (KLOEPPER; RYU; ZHANG, 2004) and/or compete for nutrients or space. This fact can not only restrict the entry of the pathogen into the roots but also inhibit its development and reproductive capacity, to the benefit of the plants.

In recent years, over 50 biological products per year have been submitted for registration by the Brazilian Ministry of Agriculture for biological control of phytonematodes (AGROFIT, 2024). These inputs constitute additional tools for management of these pathogens, besides enabling reduction in the contamination of the soil by the use of chemical nematicides, especially in the case of sugarcane (DINARDO-MIRANDA et al., 2022). The majority of the biological nematicides are registered for use against root-knot nematode (*Meloidogyne* spp.) and the root lesion nematode *P. brachyurus* with few being targeted against *P. zeae*, justifying the analysis and selection of other agents that cause stronger suppression and have broad effect against these two phytonematodes. Therefore, further studies are necessary involving the biocontrol agents analyzed in this study, as another option for suppression of phytonematodes that attack sugarcane crops, to promote greater yield and lower risks to human health or the environment.

CONCLUSION

The microbiolization of sugarcane seedlings with rhizobacteria results in the suppression of the lesion nematode *P. zeae* and also promotes development of plants by increasing their leaf and root mass and number of shoots.

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