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Full Length Article

Interference of Herbicides in Association of Diazotrophic Bacterium *Nitrospirillum amazonense* and Sugarcane Pre-Sprouted Seedlings

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Abstract

Microbial inoculant containing cells of *Nitrospirillum amazonense* is a recent technology that has been used in association with pre-sprouted seedlings to sustainably increase the productivity of sugarcane. This study aimed to assess the sensitivity of the rhizobacterium *N. amazonense* to the herbicides imazapic and indaziflam and the effect of this inoculation and herbicide treatments on sugarcane pre-sprouted seedlings. The *In vitro* sensitivity of the *N. amazonense* to the herbicides was assessed using the minimum inhibitory concentration technique (first assay). In this research, we evaluated imazapic (200 g a.i. ha^{-1}) and indaziflam (100 g a.i. ha^{-1}) at five doses: recommended dose (1×D), twice the recommended dose (2×D), one and a half of the recommended dose (1.5×D), half the recommended dose (0.5×CD), a quarter of the recommended dose (0.25×CD) and control treatment. The sensitivity of *N. amazonense* to imazapic and indaziflam applied at commercial doses on autoclaved soil was assessed in the second assay. The bacterial population count was performed using the most probable number technique (McCrady Table). The third assay assessed five herbicide treatments (clomazone (720 g a.i. ha^{-1}), imazapic (200 g a.i. ha^{-1}), tebuthiuron (800 g a.i. ha^{-1}), indaziflam (75 g a.i. ha^{-1}), sulfentrazone (800 g a.i. ha^{-1}) and control without herbicide) applied in pre-planting of pre-sprouted seedlings of the variety RB 966928 in the presence and absence of the inoculant *N. amazonense*. The results showed that the presence of indaziflam did not interfere with the *In vitro* growth of the bacterium *N. amazonense*, regardless of the dose. Imazapic caused significant inhibition of bacterial *In vitro* growth from the recommended dose (200 g a.i. ha^{-1}). The *N. amazonense* count in the soil of treatments that received indaziflam and imazapic application did not differ compared to the soil without herbicide. The pre-sprouted seedlings of the variety RB966928 showed high sensitivity to the herbicide imazapic, regardless of *N. amazonense* inoculation. Clomazone, tebuthiuron, and sulfentrazone did not interfere with the growth-promoting effect of *N. amazonense*. The results showed that the recommended dose of the herbicides tested does not impair the growth promoting effect of *N. amazonense*, and the inoculation of the pre-sprouted seedlings does not alter their sensitivity to herbicides, although the selectivity of the seedlings is differential among herbicides. Therefore, it may be concluded that the combined use of these technologies is a viable alternative to increase sugarcane productivity in a more sustainable way.

Keywords: Pre-sprouted seedlings; Plant growth-promoting bacteria; Intoxication; Herbicides

Introduction

The growing demand in the sugar-energy sector has led to the search for new sugarcane production technologies aimed at increasing raw material productivity and quality. In this sense, the multiplication system through pre-sprouted seedlings (PSS) and the use of inoculants based on plant growth-promoting bacteria are among the technological innovations employed in the sector (Pereira *et al.* 2013; Garcia 2016).

The PSS technology is a multiplication system used to implement previously treated seedlings in the plantation, providing high phytosanitary quality to the sugarcane field, high clonal standard, homogeneity, and vigor, and reduction in the volume of plant material used in the planting process (Ventura 2017).

On the other hand, plant growth-promoting bacteria (PGPB) consists of a group of microorganisms with the ability to associate with plants and stimulate their growth (Oliveira *et al.* 2003). The PGPB mechanisms differ between species and can benefit plant growth directly through phytohormone production, phosphate solubilization, and biological nitrogen fixation, or indirectly through siderophore production and induction of resistance

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systemic to pathogens (Costa *et al.* 2014). PGPB can promote an increase in the bud sprouting rate of associated plants and the rapid establishment of lateral and adventitious roots through phytohormone production, resulting in the exploration of a higher soil volume and, consequently, better water and nutrient absorption (Lopes 2013).

Sugarcane can associate with a large number of species of plant growth-promoting bacteria. Thus, the inoculation of these microorganisms in the crop has become a viable alternative to the sugar-energy sector to increase sustainably of the raw material productivity and quality, reducing costs and environmental impacts (Ferreira *et al.* 2018). Studies applied in the area have led to the development of a liquid microbiological inoculant from the bacterium *Nitrospirillum amazonense* specific for sugarcane cultivation. The product can generate sugarcane productivity gains of up to 18% (Embrapa 2018).

The presence of agrochemicals can compromise the efficiency of PGPB performance, as the contact with these molecules can cause specific damage to bacterial cells, such as inhibition of protein synthesis, DNA alterations, and oxidative destruction of membranes, leading to bactericidal and bacteriostatic effects or even harming the biological nitrogen fixation effectiveness of these microorganisms (Procópio *et al.* 2013; Lino 2018).

Studies with several herbicides commonly used in sugarcane have shown their toxic effect on PGPB development, such as the products imazapyr, ametryne, and oxyfluorfen (Procópio *et al.* 2014), paraquat, amicarbazone, clomazone, diuron, metribuzin, 2,4-D (Procópio *et al.* 2013), and isoxaflutole (Silva *et al.* 2014).

In contrast, Pies *et al.* (2017) observed beneficial effects of the application of diuron, imazapic, and clomazone on the development of the diazotrophic bacterium *Burkholderia tropica*. In this case, these herbicides acted to stimulate the microorganism development, which can be explained by the ability of some bacteria to degrade herbicide molecules, using their chemical compound as a source of energy and carbon.

Das and Debnath (2006) also reported stimulant effects of herbicides on diazotrophic microorganisms, as the presence of the herbicide oxyfluorfen led to an increase in microbial activity, resulting in higher atmospheric nitrogen fixation and phosphate solubilization by these microorganisms.

Pre-sprouted seedlings are more sensitive to soil residues because they are transplanted with a root system already formed, which may cause intoxication by herbicides applied in pre-and post-planting (Silva *et al.* 2018). PGPB inoculation stimulates the rapid growth of lateral and adventitious roots (Chaves *et al.* 2015), which may cause increased sensitivity of the seedlings to phytotoxicity by herbicides present in the soil.

Thus, studies on the compatibility of herbicides to PGPB associated with the sugarcane and the herbicide interference in the sensitivity of sugarcane pre-sprouted seedlings inoculated with these microorganisms are required. This study aimed to assess the compatibility of the herbicides imazapic and indaziflam with the bacterium *N. amazonense* and the sensitivity of inoculated pre-sprouted seedlings for the application of the herbicides clomazone, imazapic, tebuthiuron, indaziflam, and sulfentrazone.

Materials and Methods

In vitro assays of herbicide compatibility with the bacterium *N. amazonense*

Minimum inhibitory concentration assessment: The experiment was carried out at the Laboratory of Agricultural and Molecular Microbiology (LAMAM) of the Center for Agricultural Sciences at UFSCar, Araras, SP, Brazil.

The tests were carried out using the strain of *N. amazonense* (BR 11145) obtained from the Diazotrophic Bacteria Collection at Embrapa Agrobiology. In inoculant preparation, the cells of the bacterium *N. amazonense* were activated and multiplied in 200 mL of nutrient broth (NB), whose formulation, in g/L of distilled water, consisted of: 1.0 (meat extract), 2.0 (yeast extract), 5.0 (peptone), and 5.0 (sodium chloride). The culture was incubated in a shaker at 30°C and 150 rpm until the medium became cloudy, reaching an optical density (OD_{600nm}) of approximately 0.8.

The experimental design was completely randomized in a 2 × 5 factorial scheme, consisting of two herbicides and five doses, with three replications. Two herbicides registered for sugarcane, imazapic (recommended dose – 200 g a.i. ha^{-1}) and indaziflam (recommended dose –100 g a.i. ha^{-1}) were tested at five doses (recommended dose (1 × D), twice the recommended dose (2 × D), one and a half of the recommended dose (1.5 × D), half the recommended dose (0.5 × D), a quarter of the recommended dose (0.25 × D) and control treatment, each dose being considered a treatment.

In the preparation of herbicide solutions, the herbicides were submitted to serial dilutions to obtain the concentrations that represented the previously established doses. Subsequently, they were filtered on a membrane with 0.2-micrometer pores for sterilization.

The methodology adopted to assess the minimum inhibitory concentration was based on that described by Procópio *et al.* (2011). Therefore, the herbicide solutions were mixed in a 125 mL Erlenmeyer flask with 50 mL of NB. The control treatments received the same volumes of sterile distilled water. Finally, 0.1 mL of the microbial inoculant was added to the medium.

The treatments were incubated in a shaker at 30 ± 2°C and 150 rpm for 48 h. The *N. amazonense* cells were quantified by absorbance in a spectrophotometer (600 nm) through the correlation in a standard curve from a pre-culture of a pure sample in NB, according to the methodology based on Silva *et al.* (2008).

Evaluation of *N. amazonense* resistance to soil herbicide

application: The soil used in this experiment was collected from a native forest, located at the Center for Agricultural Sciences (CCA–UFSCar), Araras, SP, Brazil, at a depth of 0.10 m, without previous pesticide application. The soil chemical analysis was carried out by the Laboratory of Soil Chemistry and Fertility of the CCA/UFSCar (Table 1).

Soil samples (1000 g) were crushed, sieved through a 2-mm mesh, homogenized, and subjected to the tyndallization process, which consists of soil sterilization to eliminate microorganisms (Basseto *et al*. 2008). Therefore, the soil was placed under steam pressure from an autoclave for 20 minutes for three consecutive days, according to the methodology described by Hungria and Araújo (1994).

The experimental design in this assay was completely randomized with two herbicides in the presence and absence of the bacterium *N. amazonense*, with four replications. Control treatments, one without herbicide and inoculant and another with only inoculant, were also assessed. The doses for the herbicides imazapic and indaziflam were 200 and 100 g a.i. ha^{-1}, respectively. Before being applied to the sterile soil, the herbicides were serially diluted to obtain the established doses and previously filtered on 0.22 μm membranes to sterilize the solution.

The inoculant with *N. amazonense* cells was prepared as described in the firsty assay. The soil microbial inoculation was carried out with the inoculant application at a dose equivalent to 1.5 L ha^{-1}. The same volumes of sterile distilled water were applied for the control treatments. The soil samples were incubated at room temperature for 48 h.

The methodology used for quantifying *N. amazonense* cells was based on that proposed by Videira *et al.* (2007). After the incubation period, 10 g of soil were collected from each treatment, being diluted in 90 mL of saline solution and then serially diluted by adding 1 mL of the original dilution into test tubes with 9 mL of saline solution. This process was repeated until the 10^{-6} dilution. A sample (in triplicate) of 0.1 mL from each dilution was inoculated into flasks with 5 mL of semi-solid LGI culture medium (Table 2), which is a semi-selective medium for *N. amazonense* isolation. Subsequently, the inoculated flasks were incubated at 30°C for 7 days.

The bacterial population count was performed using the most probable number (MPN) technique, using the McCrady Table for three replicates of each dilution. Bacterial growth was detected by visualizing the formation of a characteristic veil-shaped surface film on the semi-solid medium.

Sensitivity of sugarcane pre-sprouted seedlings inoculated with *N. amazonense* to herbicide application

The experiment was carried out in a greenhouse and the experimental units consisted of polyethylene pots with a 6.0-L volumetric capacity filled with soil samples classified as Latossolo Vermelho distrófico (Oxisol), whose physico-chemical analysis was carried out by the Laboratory of Soil Chemistry and Fertility of the CCA/UFSCar (Table 1).

Table 1: Chemical analysis of soil samples used in the experiment

Latossolo Vermelho Escuro (Oxisol)									
P	OM	pH	K	Ca	Mg	H+Al	SB	CEC	V
mg/dm^3	g/dm^3	$CaCl_2$	$mmol_c/dm^3$			%			%
19	32	5.4	2.7	60	10	31	72.7	103.7	70

*pH measured in $CaCl_2$ 0.01 *M* solution

Table 2: Composition of the LGI medium

Reagent	Quantity/L
Granulated sugar	5 g
Agar	1.4 g/L
0.5% bromothymol blue in 0.2 N KOH	5 mL
1% w/v calcium chloride dihydrate	2 mL
1% w/v ferric chloride hexahydrate	1 mL
Yeast extract	0.02 g/L
10% w/v dibasic potassium phosphate	2 mL
10% w/v monobasic potassium phosphate	6 mL
0.1% w/v sodium molybdate dihydrate	2 mL
Potassium nitrate	1 g/L
10% w/v magnesium sulfate heptahydrate	2 mL

Source: Adapted from Döbereiner *et al.* (1999)

Sugarcane planting was carried out using pre-sprouted seedlings (PSS) with the technology AgMusa. The seedlings of the variety RB966928 were planted 60 days after bud sprouting. This variety has characteristics including high tillering, medium useful period of industrialization, and early to medium maturation. The variety stands out as the most planted in the state of São Paulo (Ridesa 2020). The seedlings were irrigated by a sprinkler system, according to the evapotranspiration demand.

The experimental design for pre-planting application was completely randomized with five replications in a 6 × 2 factorial scheme. The first factor consisted of the application of the herbicides clomazone (720 g a.i. ha^{-1}), imazapic (200 g a.i. ha^{-1}), tebuthiuron (800 g a.i. ha^{-1}), indaziflam (75 g a.i. ha^{-1}), and sulfentrazone (800 g a.i. ha^{-1}), in addition to the non-herbicide application (control treatment). The herbicide treatments were applied with a CO_2-pressurized knapsack sprayer set at a constant pressure of 245.16 kPa and a boom equipped with four flat fan spray tips (110.03). The spray solution volume was 200 L ha^{-1}. The second factor was (i) presence, and (ii) absence of the microbial inoculant.

The methodology used for *N. amazonense* inoculation was based on Reis and Urquiaga (2009), being carried out by immersing the seedling root system in a solution with a concentration of 1×10^{-8} CFU mL^{-1}. Seedling transplanting was carried out immediately after the product inoculation.

The assessments were carried out at 7, 14, 28, and 56 days after herbicide application (DAA). Visual assessments of herbicide toxicity were carried out in a range between 0 (absence of symptoms) and 100 (plant death), according to the methodology proposed by Velini *et al.* (1995).

The sugarcane plants were assessed at 56 DAA and their height (cm) was determined considering the distance from the base to the first leaf insertion, while the leaf area

Table 3: Log_{10} of the most probable number of CFU of *N. amazonense* per gram of soil

Treatment	g a.i. ha^{-1} + L ha^{-1}	log MPN CFU g^{-1} of soil
Imazapic + *N. amazonense*	200 + 1.5	5.82 a
Indaziflam + *N. amazonense*	100 + 1.5	5.37 a
N. amazonense	0 + 1.5	5.28 a
Control	-	0.00 b
LSD	0.63	
CV%	7.25	

*Means followed by the same letter do not differ statistically from each other by Tukey's test at the 5% probability

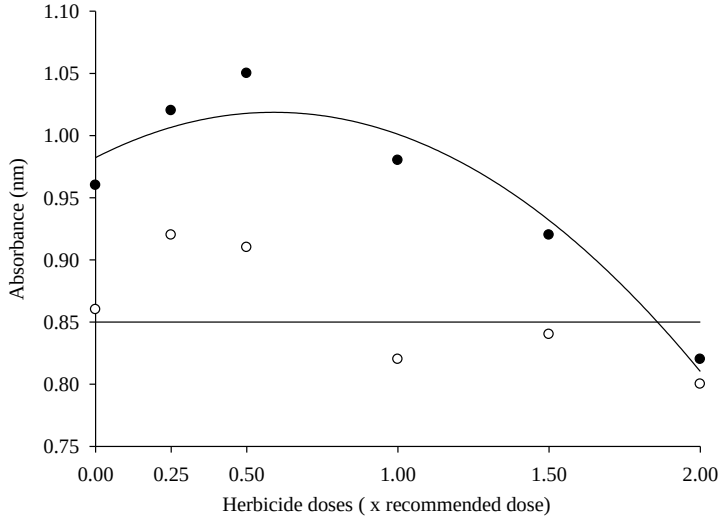

Fig. 1: Absorbance of *N. amazonense* in a medium with different doses of the herbicides imazapic and indaziflam

(cm^2) was obtained using an LI-COR LI-3000C portable leaf area meter. Then, the plants were cut close to the ground and the shoot dry biomass (g) was determined in an oven at 65°C for 48 h. The pots were disassembled, and the roots were washed and dried in an air-circulation oven at 65°C for 48 h to determine the length (cm) and root dry biomass (g).

Statistical analysis

The minimum inhibitory concentration assessment data were subjected to analysis of variance and regression curves were constructed using the SIGMA-Plot when significant. The results obtained from the McCrady Table were subjected to logarithmic transformation and later submitted to analysis of variance. In the second and thirdy assay the means were compared by Tukey's test at the 5% probability when the analysis of variance was significant.

Results

In vitro compatibility between the herbicides imazapic and indaziflam and the diazotrophic bacterium *N. amazonense*

The results of absorbance regarding the growth of *N. amazonense* cells in the medium with different imazapic doses (Fig. 1) showed a negative interference of the herbicide on the microbial growth from the commercial dose. The *N. amazonense* growth in medium with the herbicide indaziflam did not differ from the control treatment (Fig. 1) for all tested doses (0.25 × D, 0.5 × D, 1 × D, 1.5 × D and 2 × D), showing herbicide selectivity.

Sensitivity of *N. amazonense* to soil-applied herbicides

The sensitivity of the bacterium *N. amazonense* to the herbicides imazapic and indaziflam applied to the soil is shown in Table 3. Fig. 2 shows the bacterial growth through the formation of a typical film and change in the culture medium color. The growth of the bacterium *N. amazonense* occurred in the presence of both herbicides. No significant differences were observed in the MPN of CFU g^{-1} of soil between the treatments that received the herbicides imazapic and indaziflam relative to the treatment that received only inoculant (Table 3).

Sensitivity of sugarcane pre-sprouted seedlings inoculated with *N. amazonense* to herbicide application

A significant interaction was observed between herbicides and the inoculant applied to sugarcane pre-sprouted seedlings (Table 4). The assessment carried out at 7 DAA, considering non-inoculated seedlings, showed that the herbicides did not differ from each other regarding

Table 4: Percentage of phytotoxicity of herbicides applied in the pre-planting of sugarcane pre-sprouted seedlings of the variety RB966928 with and without *N. amazonense* inoculation assessed at 7, 14, 28, and 56 DAA

Treatment		7 DAA		14 DAA		28 DAA		56 DAA	
	g a.i. ha^{-1}	I	NI	I	NI	I	NI	I	NI
indaziflam	75	0.00 aB	41.00 aA	20.00 bcB	45.00 aA	50.00 abA	50.00 aA	70.00 bA	56.00 bA
imazapic	200	10.00 aA	5.00 bA	62.00 aA	40.00 aB	64.00 aA	53.00 aA	97.00 aA	88.00 aA
clomazone	720	11.00 aA	5.00 bA	54.00 aA	33.00 abB	45.00 abA	50.00 aA	32.00 cA	26.00 cA
tebuthiuron	800	0.00 aA	0.00 bA	1.00 cA	11.00 bcA	0.00 cA	0.00 bA	0.00 dA	0.00 dA
sulfentrazone	800	5.00 aA	3.00 bA	38.00 abA	46.00 aA	39.00 bB	55.00 aA	16.00 cdB	36.00 cA
Control	–	0.00 aA	0.00 bA	0.00 cA	0.00 cA	0.00 cA	0.00 bA	0.00 dA	0.00 dA
LSD		15.69	5.87	20.45	8.49	17.64	6.22	13.10	6.18
CV (%)		167.48	165.17	49.86	54.62	37.08	34.50	26.57	33.07

*I = inoculated.; NI = non-inoculated. Means followed by the same lowercase letters in the column and uppercase letters in the row do not differ statistically from each other by Tukey's test at the 5% probability

Table 5: Height, leaf area, and shoot biomass of sugarcane pre-sprouted seedlings of the variety RB966928 with and without inoculation of *N. amazonense* assessed at 56 DAA

Treatment	g a.i. ha^{-1}	Height (cm)		Leaf area (cm^2)		Biomass (g)	
		I	NI	I	NI	I	NI
indaziflam	75	13.00 bA	14.00 bcA	40.61 cA	90.33 bcA	4.42 bcA	2.97 abA
imazapic	200	7.60 bA	11.40 cA	8.92 cA	35.46 cA	1.64 cA	1.48 bA
clomazone	720	25.20 aA	21.20 abA	412.08 abA	257.70 aB	7.38 aA	4.28 abB
tebuthiuron	800	27.00 aA	21.80 aA	408.30 abA	257.01 aB	7.18 abA	4.18 abB
sulfentrazone	800	25.40 aA	18.20 abcB	342.90 bA	183.89 abB	6.93 abA	4.06 abB
control	–	31.00 aA	22.40 aB	469.75aA	276.42 aB	8.40 aA	5.26 aB
LSD		5.37	2.30	82.84	34.54	2.33	0.68
CV (%)		19.24	21.71	25.41	27.94	34.24	26.38

*I = inoculated.; NI = non-inoculated. Means followed by the same lowercase letters in the column and uppercase letters in the row do not differ statistically from each other by Tukey's test at the 5% probability, within each biometric parameter

phytotoxicity, with low values. However, the herbicide indaziflam promoted 41% phytotoxicity with the previous seedling inoculation, with 0% in the treatment without inoculation. The other herbicides did not differ from the control, regardless of the presence or absence of the inoculant (Table 4).

The assessment carried out at 14 DAA showed that only tebuthiuron did not cause phytotoxicity in the seedlings with or without inoculation. A distinct response was found between herbicides regarding the effect of inoculation. In this sense, the inoculated seedlings continued with higher phytointoxication caused by indaziflam compared to the non-inoculated seedlings. However, the opposite occurred for imazapic and clomazone, with injuries corresponding to 62 and 54% in inoculated seedlings and 40 and 33% in non-inoculated seedlings, respectively.

The herbicide sulfentrazone showed no influence of microbial inoculation in PSS on the phytotoxicity assessed at 14 DAA. However, the assessments carried out at 28 and 56 DAA showed a higher recovery of plants inoculated with rhizobacteria, with final phytotoxicity of 16% in inoculated plants and 36% in non-inoculated plants. This result shows that seedling inoculation using rhizobacteria may assist in reducing the phytotoxicity of some herbicides.

The herbicides indaziflam, imazapic, clomazone, and tebuthiuron showed no statistical differences between inoculated and non-inoculated plants at the final assessments (28 and 56 DAA). Imazapic showed the highest phytotoxicity. Phytotoxicity values of 97 and 88% were observed at 56 DAA for seedlings with and without inoculation, respectively.

The herbicide indaziflam showed lower phytotoxicity than that promoted by imazapic but still considered high (70 and 56% for inoculated or non-inoculated seedlings, respectively).

The herbicides clomazone (720 g a.i. ha^{-1}) and sulfentrazone (800 g a.i. ha^{-1}) showing values ranging from 16 to 36% of phytotoxicity. Tebuthiuron (800 g a.i. ha^{-1}) was the most selective herbicide in the study, not differing from the control without application.

Seedling height showed an interaction between herbicides and inoculation of the rhizobacterium *N. amazonense* (Table 5). The herbicide sulfentrazone and the control without application showed significant differences regarding the inoculation factor, with a favorable inoculation effect.

As observed for height, the herbicides imazapic and indaziflam also stood out negatively for leaf area and shoot dry biomass, with lower means compared to the control without application (Table 5). These herbicides showed no significant differences between the seedlings that were or were not previously inoculated for the assessed parameters.
The inoculation of pre-sprouted seedlings promoted increases above 40% in leaf area and shoot dry biomass compared to non-inoculated seedlings for clomazone, tebuthiuron, sulfentrazone, and control.

The analysis of seedling growth variables allows us to state that the application of the herbicides clomazone (720 g a.i. ha^{-1}), tebuthiuron (800 g a.i. ha^{-1}), and sulfentrazone (800 g a.i. ha^{-1}) does not interfere with the growth-

Table 6: Root length and root dry biomass of sugarcane pre-sprouted seedlings of the variety RB966928 transplanted with and without inoculation of *N. amazonense* assessed at 56 DAA

Treatment		Root length (cm)		Root biomass (g)	
	g a.i. ha^{-1}	I	NI	I	NI
indaziflam	75	13.00 cA	20.90 bA	10.94 bA	9.43 bA
imazapic	200	7.00 cA	7.00 bA	4.66 cA	5.23 bA
clomazone	720	65.50 abA	64.40 aA	18.13 aA	9.05 bB
tebuthiuron	800	56.80 bA	68.80 aA	12.76 abA	9.70 bA
sulfentrazone	800	54.80 bA	59.80 aA	11.52 bA	8.12 bA
control	–	73.20 aA	65.20 aA	17.17 aA	15.15 aA
LSD		10.74	4.98	4.12	1.49
CV (%)		16.48	20.14	26.69	25.52

*I = inoculated.; NI = non-inoculated. Means followed by the same lowercase letters in the column and uppercase letters in the row do not differ statistically from each other by Tukey's test at the 5% probability

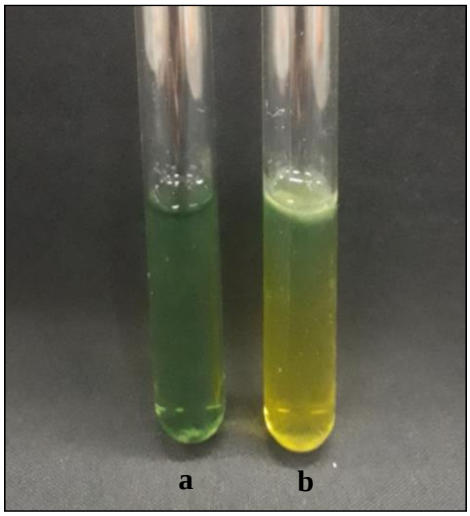

Fig. 2: Surface film formation and color change (Tube b) confirm the growth of *N. amazonense* on the semi-solid LGI culture medium. Araras 2021

promoting effect of the bacterium, considering that the seedlings showed a favorable effect of inoculation by *N. amazonense* even in the presence of these herbicides. The inoculation of seedlings also did not increase their sensitivity to herbicide treatments applied in the pre-planting of pre-sprouted seedlings. Thus, the use of inoculation is suitable in this planting system.

Length and root dry biomass showed no inoculation effect, except for the treatment with clomazone (720 g a.i. ha^{-1}), which showed higher root dry biomass in inoculated seedlings (Table 6).

Discussion

The results regarding *in vitro* compatibility between the herbicides imazapic and indaziflam and the diazotrophic bacterium *N. amazonense* showed that bacterial growth was inhibited by the herbicide imazapic from the recommended dose. Whereas herbicide indaziflam did not interfere with the *In vitro* growth of *N. amazonense* at the doses evaluated. Schwerz *et al.* (2017a) also observed no effect of imazapic on the *Azospirillum amazonense* growth when it was grown in a medium with imazapic at the commercial dose under *In vitro* conditions. The selectivity of imazapic at commercial doses has been observed over other species of diazotrophic bacteria. Procópio *et al.* (2014) also found no toxic effect of this herbicide on *Herbaspirillum seropedicae* growth. Also, other studies have found no changes in the growth and biological nitrogen fixation activity of bacterial cells of *Gluconacetobacter diazotrophicus* when grown in a medium with the herbicide imazapic (Procópio *et al.* 2011, 2013).

Imazapic is an acetolactate synthase (ALS)-inhibiting herbicide. The ALS enzyme participates in the biosynthesis of the amino acids valine, leucine, and isoleucine in microorganisms and plants (Christofoletti 2001). The highest interference of herbicides on the soil microbiota occurs when they act on the biosynthesis of amino acids or metabolic pathways common to microorganisms and plants (Santos *et al*. 2006).

Imazapic showed selectivity to *N. amazonense* up to the recommended dose but considering literature reports confirming its selectivity over other species of diazotrophic bacteria, we cannot infer that the selectivity occurs due to a resistance of these microorganisms to the mechanism of

action of the herbicide. Procópio *et al.* (2014) observed selectivity of imazapic on the bacterium *H. seropedicae*, but a significant inhibition in the growth of the microorganisms was found when imazapyr, an herbicide belonging to the same mechanism of action and chemical group, was assessed.

The selectivity of an herbicide is not only associated with the active ingredient, mechanism of action, or chemical group but also with factors related to the physico-chemical characteristics of the commercial product. Compounds present in agro-chemical formulations, such as solvents, surfactants, and wetting agents, may be directly associated with the toxic herbicide effect on microorganisms (Santos *et al*. 2004).

The increased herbicide dose potentiated its negative effect on strain growth. Childs (2007) observed in *In vitro* tests that herbicides are potentially toxic to microorganisms at high concentrations, with frequent inhibitory effects on the quantity and activity of these organisms. However, the contact between the herbicide and microorganisms in *In vitro* tests is theoretically higher than under field conditions.

On the contrary, Schwerz *et al.* (2017a) observed no negative interferences in the growth of the bacterium *A. amazonense* with an increase in the dose of the herbicide imazapic since no change was observed in the medium turbidity compared to the control treatment when the product was applied at the highest concentration. Similarly, Pies *et al.* (2017) verified that the growth of the diazotrophic bacterium *Burkholderia tropica* in a medium with different imazapic doses, instead of reducing, had an increase in optical density values with increasing herbicide doses.

Tironi *et al.* (2009) emphasized that microorganisms are subjected to maximum exposure to toxic herbicide molecules in *In vitro* tests, which does not occur under field conditions, where external factors act on the chemical, reducing its toxicity. Thus, herbicides identified as selective to microorganisms in laboratory tests are likely to present selectivity under field conditions.

Similar to our results regarding sensitivity of N. amazonense to soil-applied herbicides, Koçak *et al.* (2021) found no negative or positive effects of the herbicide indaziflam applied to the soil on the microbial population. Torres *et al.* (2018) found that the application of the herbicide indaziflam did not cause damage to soil microorganisms, increasing the microbial population. Several studies in the literature have reported the absence of negative effects of the herbicide imazapic on species of diazotrophic bacteria associated with sugarcane (Procópio *et al*. 2011, 2013, 2014; Pies *et al*. 2017; Schwerz *et al*. 2017b).

Pesticide application can positively affect the soil microbiota when the molecules are likely to be metabolized by microorganisms, or negatively interfere with it when they intoxicate the microbial population (Ferreira 2016). Several strategies can be used by microorganisms to metabolize herbicides: (a) catabolism: the herbicide molecule is absorbed and broken down, generating energy; and (b) cometabolism: the herbicide is transformed by metabolic reactions, but it is not used as an energy source (Childs 2007).

In the catabolism process, microorganisms use energy from herbicide molecules for cell formation and multiplication. However, Monquero *et al.* (2012) reported that initial increases in the soil microbial population from the metabolization of herbicide molecules are usually followed by a decrease.

The results obtained in this experiment demonstrated that the application of the commercial dose of the herbicides imazapic (200 g a.i. ha^{-1}) and indaziflam (100 g a.i. ha^{-1}) do not harm the bacterium *N. amazonense* present in the inoculant. The use of herbicide molecules and formulations not harmful to diazotrophic bacteria associated with sugarcane allows an increase in agricultural productivity without compromising the system's sustainability.

Results about sensitivity of sugarcane pre-sprouted seedlings inoculated with N. amazonense to herbicide application indicated distinct effects of PSS inoculation observed in the first assessments regarding the impact of herbicides may be related to the complex dynamics involved in the association between bacterium and plant and the impact of the presence of herbicides on these organisms.

Crop-associated diazotrophic bacteria can promote biological nitrogen fixation, siderophore synthesis, phosphate and potassium solubilization, and the production of growth-promoting phytohormones. These microorganisms facilitate nutrient absorption and, consequently, provide higher vigor to the plant physiological system (Simões *et al.* 2018). The availability and balance in the absorption of essential nutrients allow an increase in the plant's capacity to carry out its metabolic functions without undergoing damage when subjected to biotic and abiotic stresses, such as those caused by herbicide applications (Andrade 2020).

On the other hand, the increase in absorption efficiency of the root system can favor the absorption of herbicides present in the soil solution, increasing the risk of phytotoxicity due to increased exposure to these molecules (Perez 2017).

The explanation for the higher tolerance to PROTOX-inhibiting herbicides is related to the ability of plants to metabolize peroxidative stress, potentially through antioxidant systems (Carbonari *et al.* 2012). Thus, the higher recovery of inoculated seedlings with the application of sulfentrazone may be associated with a higher physiological plant efficiency, resulting from the beneficial action of the diazotrophic bacterium *N. amazonense*.

The growth-promoting effect may be related not only to an increase in root length but also to morphological changes in the root system. The higher development of lateral roots and root hairs, although thinner and shorter, allows for higher efficiency in water and nutrient absorption (Matoso *et al.* 2016). The biological response regarding the

inoculation of diazotrophic bacteria can be variable, as it is related to several factors, such as plant genotype and environmental characteristics, and there may be changes in the initial development and allocation of biomass between different varieties and within the same variety, considering the genotype-environment interaction (Silva *et al.* 2009; Santos *et al.* 2012).

Imazapic stood out negatively regarding the effects of herbicides on root length and dry biomass, with a reduction of up to 10-times in root length relative to the control. Imazapic acts by inhibiting the acetolactate synthetase (ALS) enzyme, preventing the synthesis of essential amino acids (leucine, isoleucine, and valine) and the production of new cells. The stoppage in shoot growth and reduction in the number and length of roots are characteristic symptoms of herbicides that have this mechanism of action (Marchi *et al*. 2008), which explains their suppressive effect on the seedling roots.

Conclusion

The herbicide indaziflam did not interfere with the *in vitro* growth of *N. amazonense* at the assessed doses. Bacterial growth was inhibited by the herbicide imazapic from the recommended dose. The herbicides imazapic (200 g ai ha^{-1}) and indaziflam (100 g ai ha^{-1}) applied to the soil were not harmful to *N. amazonense* growth. Sugarcane pre-sprouted seedlings of the variety RB966928 were highly susceptible to the herbicide imazapic, regardless of the *N. amazonense* inoculation. Clomazone, sulfentrazone, and tebuthiuron did not interfere with the growth-promoting effect of *N. amazonense* in pre-sprouted seedlings of the variety RB966928. Inoculation with *N. amazonense* did not change the sensitivity of pre-sprouted seedlings to herbicides.

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Author Contributions

Luana Carolina Gomes Jonck: Formal analysis, Investigation, Methodology, writing original draft; Marcia Maria Rosa Magri: Conceptualization, Data curation, supervision; Patricia Andrea Monquero: Resources; Writing review and editing, Funding Acquisition.

Conflicts of Interest

Authors declare no conflicts of interests among institutions.

Ethics Approval

Not applicable to this article.

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