



## ***Herbaspirillum seropedicae* as a degrading bacterium of monofluoroacetate: effects of its inoculation in goats by ingesting *Amorimia septentrionalis* and the concentrations of this compound in plants sprayed with the bacterium<sup>1</sup>**

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**ABSTRACT.**- Pessoa D.A.N., Lopes J.R.G., Souza E.M., Campos E.M., Medeiros R.M.T., Cook D., Lee S.T. & Riet-Correa F. 2019. *Herbaspirillum seropedicae* as a degrading bacterium of monofluoroacetate: effects of its inoculation in goats by ingesting *Amorimia septentrionalis* and the concentrations of this compound in plants sprayed with the bacterium. *Pesquisa Veterinária Brasileira* 39(10):802-806. Unidade Acadêmica de Medicina Veterinária, Centro de Saúde e Tecnologia Rural, Universidade Federal de Campina Grande, Avenida Universitária s/n, Bairro Santa Cecília, Patos, PB 58700-970, Brazil. E-mail: [danipessoavet14@gmail.com](mailto:danipessoavet14@gmail.com)

*Herbaspirillum seropedicae* is a nitrogen-fixing bacterium capable of using toxic compounds as a source of carbon. Bacteria with this capacity can be used to make animals resistant to plant poisoning containing monofluoroacetate (MFA), such as *Amorimia septentrionalis*. The aim of this study was to evaluate if *H. seropedicae* is efficient in the degradation of MFA present in *A. septentrionalis* and if the inoculation of this bacterium in goats confers protection to *A. septentrionalis* intoxication. Two experiments were performed: in the first experiment 12 goats were divided into 2 groups. Goats in Group 1 were orally administered a solution containing the *H. seropedicae* bacterium for 10 days. From day 10 onwards, they received a daily dose of 5g/kg of *A. septentrionalis* with the bacteria until clinical signs of intoxication were observed. Group 2 goats received only the plant at the same dose, also until the observation of clinical signs of intoxication. The amount of MFA found in *A. septentrionalis* used in the experiment with goats was 1.6±0.058µg/mg. The total plant dose ingested by all goats in Group 1 was 80.83±12.81g/kg (129.33±20.50mg/kg MFA), which were significantly greater (p<0.05) than those of Group 2 goats (39.16±19.08g/kg plant and 62.66±30.53mg/kg MFA). Group 1 goats took an average of 16.16±2.56 days to develop clinical signs of intoxication, significantly longer (p=0.0012) than Group 2 goats (7.83±3.81 days). Two Group 2 goats died on the same day that they developed clinical signs of intoxication. At necropsy of these two animals, no significant changes were observed. In the second experiment, samples of *A. septentrionalis* were sprayed with a solution containing *H. seropedicae*. Before and eight days after spraying, the samples were pressed and dried for quantitation of MFA. The amount of MFA present in samples of *A. septentrionalis* 8 days after spraying with *H. seropedicae* was significantly lower (p=0.017) than that found prior to spraying. It can be concluded

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that administration of *H. seropedicae* in goats is capable of causing greater resistance to *A. septentrionalis* intoxication, and spraying the plant with this bacterium significantly reduces the amount of MFA in the plant.

INDEX TERMS: *Herbaspirillum seropedicae*, degrading bacterium, monofluoroacetate, goats, *Amorimia septentrionalis*, plants sprayed, poisoning, toxic plants.

**RESUMO.- [Herbaspirillum seropedicae como bactéria degradadora de monofluoroacetato de sódio: efeitos de sua inoculação em caprinos ingerindo Amorimia septentrionalis e nas concentrações deste composto em plantas pulverizadas com a bactéria.]**

*Herbaspirillum seropedicae* é uma bactéria fixadora de nitrogênio, capaz de utilizar compostos tóxicos como fonte de carbono. Bactérias com essa capacidade podem ser utilizadas para tornar os animais resistentes à intoxicação por plantas que contêm monofluoroacetato (MFA), como *Amorimia septentrionalis*. O objetivo do presente estudo é avaliar se *H. seropedicae* é eficiente na degradação do MFA presente em *A. septentrionalis* e se a inoculação dessa bactéria, em caprinos, confere proteção à intoxicação por *A. septentrionalis*. Foram realizados dois experimentos: no primeiro experimento foram utilizados 12 caprinos, divididos em dois grupos. Os caprinos do Grupo 1 receberam diariamente, oralmente, uma solução contendo a bactéria *H. seropedicae* durante 10 dias. A partir do décimo dia passaram a receber, diariamente, além da solução com a bactéria 5g/kg de *A. septentrionalis* até a observação de sinal clínico de intoxicação. Os caprinos do Grupo 2 receberam apenas a planta na mesma dose, também até que a observação de sinais clínicos de intoxicação. A quantidade de MFA encontrada em *A. septentrionalis* utilizada no experimento com caprinos foi de  $1,6 \pm 0,058 \mu\text{g}/\text{mg}$  de planta em média. A dose total de planta ingerida por todos os caprinos do Grupo 1 foi de  $80,83 \pm 12,81 \text{g}/\text{kg}$  ( $129,33 \pm 20,50 \text{mg}/\text{kg}$  de MFA), valores significativamente maiores ( $p < 0,05$ ) do que os dos caprinos do Grupo 2 ( $39,16 \pm 19,08 \text{g}/\text{kg}$  de planta e  $62,66 \pm 30,53 \text{mg}/\text{kg}$  de MFA). Os caprinos do Grupo 1 demoraram em média  $16,16 \pm 2,56$  dias para desenvolver sinais clínicos da intoxicação, período significativamente maior ( $p = 0,0012$ ) que os caprinos do Grupo 2 ( $7,83 \pm 3,81$  dias). Dois caprinos do Grupo 2 morreram no mesmo dia que desenvolveram sinais clínicos da intoxicação. Na necropsia desses dois animais não foram observadas alterações significativas. No segundo experimento, amostras de *A. septentrionalis* foram pulverizadas com uma solução contendo a bactéria *H. seropedicae*. Antes e oito dias após a pulverização, as amostras foram prensadas e secas para posterior quantificação do MFA. A quantidade de MFA presente nas amostras de *A. septentrionalis* oito dias após a pulverização com *H. seropedicae* foi significativamente menor ( $p = 0,017$ ) do que a encontrada antes da pulverização. Pode-se concluir que a administração de *H. seropedicae* em caprinos é capaz de causar uma maior resistência à intoxicação por *A. septentrionalis*, e a pulverização da planta com esta bactéria reduz significativamente a quantidade de MFA na planta.

TERMOS DE INDEXAÇÃO: *Herbaspirillum seropedicae*, bactéria degradadora, monofluoroacetato de sódio, caprinos, *Amorimia septentrionalis*, plantas tóxicas.

## INTRODUCTION

Ruminant poisoning by toxic plants causes significant losses in the Brazilian herd. It is estimated that 52,675 to 63,292 goats die each year in Brazil from plant poisoning. In cattle this number may exceed 1 million head of cattle, and half of these deaths

are due to poisoning by plants containing monofluoroacetate (MFA) (Pessoa et al. 2013).

In Brazil, 22 species of plants that cause sudden death associated with exercise in ruminants are known, among them *Amorimia septentrionalis*, previously identified as *Amorimia (Mascagnia) rigida*, and popularly known as tingui. *A. septentrionalis* found in northeastern Brazil, mainly in the States of Paraíba, Pernambuco and Ceará (Duarte et al. 2013, Nascimento et al. 2018) and is responsible for outbreaks of poisoning in goats and sheep in Paraíba (Vasconcelos et al. 2008) and in cattle in both Paraíba and Pernambuco (Albuquerque et al. 2014).

Control and prevention of this type of poisoning by eliminating the plant or by avoiding its consumption by animals is not always effective (Barbosa et al. 2003). New alternatives are suggested to make animals resistant to poisoning, including: repeated administration, at alternate periods, of non-toxic doses of the plant (Duarte et al. 2014); the transfer of ruminal fluid from ruminants inoculated with MFA-degrading bacteria that became resistant to intoxication to susceptible ruminants (Silva et al. 2015); the administration of MFA-degrading bacteria isolated from the rumen of goats, soil and plants containing MFA (Pessoa et al. 2015, Silva et al. 2016, Pessoa et al. 2018); and the use of lithium chloride as a conditioned aversion technique (Brito et al. 2016). Among these alternatives is the use of bacteria capable of degrading the MFA present in the plant. When animals are administered these bacteria daily and continuously, the animals develop different degrees of resistance to poisoning by plants containing MFA (Silva et al. 2016, Pessoa et al. 2018).

*Herbaspirillum seropedicae* is a gram-negative, rod-shaped, aerobic, positive-motility bacterium belonging to the  $\beta$  subdivision of proteobacteria that uses organic and inorganic acids as a carbon source (Schmid et al. 2006). It is also considered diazotrophic and endophytic facultative, i.e. it is able to fix nitrogen under microaerophilia conditions and colonize the leaves, stems, and roots of grasses of great economic interest such as corn, wheat, sorghum, rice and sugarcane (Roncato Maccari et al. 2003, Baldani & Baldani 2004, Rodrigues et al. 2006). It has flexible metabolism, capable of degrading various organic and inorganic compounds, most often through the production of enzymes, such as dehalogenases, which are able to catalyze the breakdown of the carbon-halogen bond in halogenated compounds, such as monofluoroacetate, rendering it non-toxic (Fetzner & Lingens 1994, Pedrosa et al. 2011). Due to this versatility in the degradation capacity of toxic compounds, the objective of the present study was to evaluate the use of *H. seropedicae* as an MFA-degrading bacterium, either by oral administration in goats exposed to *Amorimia septentrionalis* poisoning or by field spraying of *A. septentrionalis* specimens with the bacterium. For this, two experiments were performed, one with the administration of bacteria in goats, and the other by spraying the plant with the bacteria.

## MATERIALS AND METHODS

**Experiment 1.** *Amorimia septentrionalis* used in the study was collected in the municipality of Teixeira (7°12.24' S 37°15.11' W; elevation 749m), Paraíba. A plant *exsiccata* was deposited at the Herbarium of the "Universidade Federal de Campina Grande" (UFCG), at the Patos Campus, Paraíba, Brazil (registration No. 6701). To determine the amount of monofluoroacetate present in the plant, a five plant samples, each with 10 leaves, were collected, pressed and dried, and then sent to the Poisonous Plant Laboratory, Agricultural Research Service, United States Department of Agriculture, Logan, USA for analysis by high performance liquid chromatography mass spectrometry (Lee et al. 2012).

The degrading bacterium of MFA *Herbaspirillum seropedicae*, used in this study, is part of the SmR1 strain. It was obtained in partnership with the Department of Biochemistry of the UFCG.

To obtain the solution used, *H. seropedicae* was grown in NFBHP lactate agar medium supplemented with  $\text{NH}_4\text{Cl}$  for 48 hours at 30°C (Machado et al. 1991) in a bacteriological greenhouse incubator. After it was diluted in 60mL NaCl at 0°C. 9% sterile until reaching Mac Farland scale grade 1 (Gregg et al. 1998). After testing by the plate colony counting method, it was determined that at this degree of turbidity each 1mL of the solution contained approximately  $8.6 \times 10^7$  bacterial colony forming units.

Twelve crossbred goats were used, ranging in age from 1 to 4 years, and weighing 25 to 45kg, coming from free areas of plants containing MFA. These were divided into two groups, each with six animals. Group 1 goats were daily administered an oral dose of a 60mL of the solution containing *H. seropedicae* for 10 days (Silva et al. 2016). From the tenth day and until clinical signs of intoxication were observed, 5g/kg of *A. septentrionalis* was associated with the solution with the bacteria. Group 2 goats received only *A. septentrionalis* at the same dose, also until clinical signs of intoxication were observed.

The plant was offered to goats on a voluntary basis and those that did not eat received it by administering small amounts directly into their mouths. After administration, all animals were stimulated to move for 10 minutes. Daily, before and after administration of the plant, rectal temperature, heart rate, respiratory rate and ruminal movements of all goats were measured and the presence of clinical signs of intoxication were observed. Animals that died were necropsied and organ fragments of the abdominal and thoracic cavities and central nervous system were collected, fixed in 10% formaldehyde, processed according to routine methods, stained with hematoxylin-eosin (HE), and subsequently evaluated histopathologically.

For statistical analysis, the variables were: day of observation of clinical signs of intoxication, amount of plant ingested by each goat, and amount of MFA ingested per kg of body weight. These values were submitted to the Shapiro-Wilk normality test and then expressed as means and standard deviations. The comparison between the values observed in the groups was made by Student's t-test for independent samples, both for the day of observation of clinical signs of intoxication and for the amount of plant ingested and MFA ingested (Zar 1999). The program used to perform the analyses was BioEstat 5.3, and the significance level adopted was 5%.

**Experiment 2.** For the spray test, *H. seropedicae* was diluted in 60ml of 0.9% sterile NaCl until it reached Mac Farland grade 1 climbing. Thereafter it was mixed in one liter of NFBHP lactate broth supplemented with  $\text{NH}_4\text{Cl}$  and was then incubated in a bacteriological oven for 48 hours at 30°C (Machado et al. 1991). Eight liters of the medium solution with the bacteria were used for the study. After

the incubation period, they were placed in sterile hand sprayers for subsequent spraying of the plants.

The area chosen for spraying was Sítio Lopes, a property in the city of Teixeira (7°12.24' S 37°15.11' W; elevation 749m), Paraíba, Brazil, which had a significant amount of *A. septentrionalis*. Twenty-five plant specimens were sprayed over an area of 100 m<sup>2</sup>, where approximately every plant received 320 ml of solution. Spraying was carried out during the dry season, early in the morning, once. After eight days the plants were collected, pressed and dried. Five samples, each with 10 leaves, were sent to the Poisonous Plant Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Logan, USA, for the quantitation of MFA (Lee et al. 2012).

For statistical analysis, the MFA concentrations before and after spraying were subjected to the Shapiro-Wilk normality test and then expressed as means and standard deviations. The comparison between means before and after spraying was done by Student's t-test for related samples (Zar 1999). The program used to perform the analyses was BioEstat 5.3, and the significance level adopted was 5%.

## RESULTS

### Experiment 1

All goats showed clinical signs of intoxication, which consisted of tachypnea, tachycardia, arrhythmia, bleating, tremors, motor incoordination, reluctance to movement, anorexia, apathy, fall, distended neck, and engorged jugular vein with positive venous pulse (Fig.1). The period for the development of clinical signs of poisoning in Group 1 goats that received the bacterium was  $16.16 \pm 2.56$  days, significantly longer ( $p=0.0012$ ) than Group 2 goats, which received was  $7.83 \pm 3.81$  days (Table 1).

Considering that the amount of MFA found in *Amorimia septentrionalis* was  $1.6 \pm 0.58 \mu\text{g}/\text{mg}$ , the 5g/kg dose of plant supplied to the animals corresponds on average to 8 mg MFA per kg of body weight. The total plant dose ingested by Group 1 goats was  $80.83 \pm 12.81 \text{g}/\text{kg}$ , therefore goats ingested  $129.33 \pm 20.50 \text{mg}/\text{kg}$  MFA in total, while Group 2 goats ingested  $39.16 \pm 19.08 \text{g}/\text{kg}$  plant and  $62.66 \pm 30.53 \text{mg}/\text{kg}$  MFA (Table 1).



Fig.1. Control goat with severe depression and sternal decubitus after ingestion of *Amorimia septentrionalis*.

**Table 1. Period of observation of clinical signs in *Amorimia septentrionalis* poisoning, amount of plant ingested and amount of MFA ingested until the onset of clinical signs in Group 1 goats, which ingested *Herbaspirillum seropedicae* and *A. septentrionalis*, and Group 2 goats, which ingested only the plant**

Group/Goats	Day of observation of clinical signs of intoxication*	Dose of ingested plant (g/kg)*	MFA intake dose (mg/kg)*
Group 1			
1	18	90	144
2	20	100	160
3	14	70	112
4	14	70	112
5	17	85	136
6	14	70	112
Average ± standard deviation	16.16 ± 2.56 <sup>a</sup>	80.83 ± 12.81 <sup>a</sup>	129.33 ± 20.50 <sup>a</sup>
Group 2			
7	5	25	40
8	6	30	48
9**	11	55	88
10**	3	15	24
11	13	65	104
12	9	45	72
Average ± standard deviation	7.83 ± 3.81 <sup>b</sup>	39.16 ± 19.08 <sup>b</sup>	62.66 ± 30.53 <sup>b</sup>

\* Student's t-test, \*\* animals 9 and 10 died on the same day showing clinical signs of intoxication; <sup>a,b</sup> different letters in the same column indicate statistical difference (p<0.05) between the groups.

**Table 2. Amount of MFA measured before and eight days after spraying *Amorimia septentrionalis* specimens with *Herbaspirillum seropedicae* bacteria**

Sample	Amount of MFA (µg/mg)	
	Before spraying	Eight days after spraying
A 1	0.61	0.23
A 2	0.85	0.27
A 3	1.2	0.32
A 4	1.4	0.19
A 5	2.0	0.22
Average ± standard deviation*	1.21 ± 0.53 <sup>a</sup>	0.24 ± 0.05 <sup>b</sup>

\* Student's t-test for related samples; <sup>a,b</sup> different letters in different columns represent statistically significant difference (p<0.05).

Both the amount of plant and MFA ingested by Group 1 goats were significantly greater (p<0.05) than Group 2 goats.

Goats in both Groups (1 and 2) recovered on average three days after the end of plant ingestion. However, in two Group 2 goats, the clinical signs of poisoning evolved to death on the same day they were observed, and no significant changes were observed in the necropsy of these animals.

## Experiment 2

The amount of MFA present in *A. septentrionalis* samples eight days after *Herbaspirillum seropedicae* spraying (0.24±0.05µg/mg) was significantly lower (p=0.017) than before spraying (1.21±0.53µg/mg) (Table 2).

## DISCUSSION

The results of the present study show that goats that ingested *Herbaspirillum seropedicae* took longer to develop clinical signs of *Amorimia septentrionalis* poisoning, containing 0.16% MFA, than goats that did not receive the bacteria.

In addition, in the goats that received the bacteria, no deaths were recorded, while in the group that did not receive the bacteria two goats died. This suggests that the bacteria likely degraded the MFA contained in the plant. These results are similar to those obtained with other MFA-degrading bacteria (Pessoa et al. 2015, 2018, Silva et al. 2016).

Silva et al. (2016) presented more satisfactory results than those of this study since resistance remained during the 30 days of administration of *Ralstonia* sp. and *Burkholderia* sp. without goats showing any clinical signs of intoxication. In the study by Silva et al. (2016) the authors do not mention the MFA concentration of the plant used. However, the amount of MFA detected in *A. septentrionalis* samples used in experiment 1 (0.16%) was higher than that found in previous studies: 0.002% (Lee et al. 2012), 0.0021% (Albuquerque et al. 2014) and 0.00074% (Lopes et al. 2019). Marked variations in MFA content in *A. septentrionalis* are also reported by Lee et al. (2012). Lee et al. (2012) reported variations in MFA content in *A. septentrionalis*. It is likely that *H. seropedicae* does not survive or multiply in the rumen to effectively hydrolyze significant amounts of MFA as found in previously reported studies.

The bacteria *Ralstonia* sp., *Burkholderia* sp., *Paenibacillus* sp. and *Cupriavidus* sp. induce resistance to intoxication by *A. septentrionalis*; however, this resistance ends after a period of 55 days after the end of bacterial ingestion (Pessoa et al. 2018). In the case of *H. seropedicae*, which is an endophytic bacterium, it was also tested for colonization of the plant by degrading MFA. The results show a significant reduction in MFA concentrations in plants that were treated with *H. seropedicae*, suggesting that *H. seropedicae* may be able to colonize *A. septentrionalis*. Further experiments should be performed to prove these data and to verify the persistence of *H. seropedicae* in *A. septentrionalis* and probably in other plants containing MFA, mainly in *Palicourea* species, which contain higher concentrations of MFA and are more widespread in Brazil.

## CONCLUSION

It was concluded that the inoculation of *Herbaspirillum seropedicae* in goats was able to cause greater resistance to *Amorimia septentrionalis* poisoning. Spraying samples of *A. septentrionalis* with *H. seropedicae* was able to significantly decrease the amount of MFA present in the plant.

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**Conflict of interest statement.**- The authors declare no conflict of interest.

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