Flumioxazin, imazethapyr and their mixture in the rhizosphere of soybean: effect on early nodulation and biological N fixation[1]

Mariane Pertile[2], Sandra Mara Barbosa Rocha[2], Indrid Stephanie da Costa Silva[2], Jadson Emanuel Lopes Antunes[2], Francisco de Alcântara Neto[3], Ademir Sergio Ferreira de Araújo[2]*

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ABSTRACT

The application of flumioxazin and imazethapyr can affect the interactions between rhizobia and soybean in the rhizosphere. However, it remains unclear the effect of both herbicides, even when applied in mixture, on early nodulation and biological N fixation (BNF) in soybean, mainly related to some important biochemical traits in nodules. This study assessed the effect of flumioxazin, imazethapyr and their mixture on early nodulation and BNF in rhizosphere of soybean. The plant emergence, early nodulation, growth, and N accumulation were evaluated in soybean grown in sandy and clayey soils. The leghemoglobin content and glutamine synthetase (GS) activity were also assessed in fresh nodules. Individually, flumioxazin and imazethapyr did not affect the plant emergence, nodulation and N accumulation in soybean, while the mixture decreased significantly these parameters. Also, the values of leghemoglobin content and GS activity did not decrease with the application of the herbicides individually. The application of herbicides imazethapyr and flumioxazin separately negatively influenced the growth of soybean, while they do not influence the nodulation and N accumulation. However, the use of these herbicides in mixture can negatively influence the early nodulation and N fixation by native rhizobia.

Keywords: soybean rhizosphere; rhizobia; nodulins; environmental issues.

INTRODUCTION

Herbicides are important inputs applied in modern agriculture to control weeds and their use has significantly increased, mainly after the introduction of herbicide-resistant crops. In soybean, the herbicides flumioxazin and imazethapyr have been used to control weeds and the main reason is that both herbicides provide higher spectrum of action against weeds, being even applied in mixture (Alister *et al.*, 2008; Thiour-Mauprivez *et al.*, 2019). Although being recommended to soybean, the application of flumioxazin and imazethapyr can bring negative effect on soybean rhizosphere and its associated-microbes, mainly rhizobia. Usually, herbicides decrease nodulation as a direct effect on population of rhizobia and biochemical processes related to symbiosis (Zobiole *et al.*, 2010).

Indeed, some studies have reported negative effect of flumioxazin and imazethapyr on bacterial community (Pertile *et al.*, 2021), nodulation and biological N fixation (BNF) in the rhizosphere (Bohm *et al.*, 2009; Gonçalves *et al.*, 2018). For instance, Bohm *et al.* (2009) observed imazethapyr reducing soybean growth but with no significant effect on nodulation and N fixation. However, Gonçalves *et al.* (2018) reported imazethapyr decreasing the root system and nodulation in soybean. In the case of flumioxazin, Gonzalez *et al.* (1999) reported a decreased nodulation with its application on soybean field.

The application of herbicides can also influence the process of reduction and assimilation of N from the atmosphere and decrease the efficiency of BNF. Particularly, glutamine synthetase (GS) and leghemoglobin are essential to BNF, acting on the conversion of

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[2] Universidade Federal do Piauí, Centro de Ciências Agrárias, Departamento de Engenharia Agrícola e Solos, Teresina, Piauí, Brazil. mariane_pertile@hotmail.com; sandramarabr@yahoo.com.br; ingrid.agro@hotmail.com; jadsonantunes2016@gmail.com; ademir@ufpi.edu.br
[3] Universidade Federal do Piauí, Centro de Ciências Agrárias, Departamento de Fitotecnia, Teresina, Piauí, Brazil. fneto@ufpi.edu.br
*Corresponding author: ademir@ufpi.edu.br

ammonium into glutamin and maintain suitable O_2 to nitrogenase, respectively (Gonnet and Diaz, 2000; Appleby, 1984). Therefore, some process that reduces GS and leghemoglobin in nodules leads to a decrease on BNF in soybean.

Although some studies have reported the effects of flumioxazin and imazethapyr on growth and nodulation of soybean, the effect of both herbicides, applied in mixture, on early nodulation and BNF in soybean remains poorly understood. Thus, this study assessed the effect of flumioxazin and imazethapyr, and their mixture on early nodulation and BNF of soybean.

MATERIALS AND METHODS

Soil samples were collected in two soybean fields located at Sambaiba, Maranhao, Brazil (7°31'59''S and 46°2'6''W, 243 m). The soils from field 1 and 2 are sandy (63% sand, 27% silt, 10% clay) and clayey (35% sand, 27% silt, 38% clay), respectively. Both soils had no history of application of flumioxazin, imazethapyr, and their mixture. In each field, five soil subsamples were collected randomly, at 0-20 cm depth, in an area with 1,000 m^2, and pooled together to form a composite sample. All samples were transported in bags to the greenhouse. The chemical characteristics of soil were analyzed in laboratory by methodology described by Tedesco *et al.* (1995) and are shown in Table 1.

The experiment was conducted in a greenhouse belonging to Centro de Ciencias Agrarias from Universidade Federal do Piauí, Brazil. Pots (3.2 L) were filled with both soils collected as reported above. The treatments consisted of a control without herbicides (control), flumioxazin (Flu), imazethapyr (Ima), and their mixture (Flu + Ima) under a completely randomized design with four replications. For this study, each soil was evaluated separately. Before sowing, the herbicides were applied at the recommended field rates, being 0.9 mg of flumioxazin (0.6 mg of Flumyzin 500® with a purity of 500 g a.i. kg^{-1}), 2.4 mg of imazethapyr (7.5 µL of Imazethapyr Plus Nortox® with a purity of 106 g a.i. L^{-1}), and a mixture of 2.4 mg imazethapyr and 1.2 mg flumioxazin (3.75 µL of Zethamaxx® with a purity of 212 g a.i. L^{-1} imazethapyr + 100 g a.i. L^{-1} flumioxazin) that were diluted in 30 mL of water and sprayed into each pot, according to the treatment. As a control, 30 mL water was sprayed into each pot. After 24h from the application, soil microbial biomass C (MBC) and the number of bacteria, by the most probable number (MPN), were assessed in soil samples collected inside the pots. MBC was estimated by method of chloroform fumigation-extraction (Vance *et al.*, 1987) using an extraction efficiency coefficient of 0.38 to convert the difference in C between fumigated and unfumigated soil in MBC. MPN was assessed by the plate counts method (Araujo *et al.*, 2003). Briefly, 10 g (dry weight) of soil samples were used to provide a dilution series of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}. Thus, 0.1 mL of dilution were added to each of 5 nutrient agar spread-plates. The plates were incubated at 28 °C for 72 h.

Ten days later, soybean (*Glycine max* L., hybrid TMH 1188 RR) was sown. The seeds were surface-disinfested (5% sodium hypochlorite for 3 min) and were rinsed with sterile distilled water. In this study, the seeds were not inoculated by an external inoculum. Before sowing, all pots received the application of P_2O_5 (2.5 g per pot) and K_2O (2.0 g per pot). The fertilization with N was not used in this study. At sowing, ten seeds were placed per pot and ten days after sowing, the emergence of plants was evaluated by counting the plants that presented both cotyledons above soil surface. Ten days after plant emergence, plants were thinned, leaving one plant per pot. Pots were irrigated daily to maintain soil moisture.

At 45 days after emergence, plants were collected, and shoots were separated from the roots. Shoots and roots were dried at 65 °C for 72 h and then weighed, so obtaining both shoot and root dry weight (SDW and RDW, respectively). The accumulation of N in shoot was estimated according to Keeney and Nelson (1982) by the shoot dry weight. Nodule number, dry weight (at 65 ºC, 72 h) and distribution on roots were recorded. The nodule distribution (number and dry weight) was evaluated as crown region (all nodules distributed until 5 cm of the taproot immediately below the cotyledons) and secondary root (all nodules distributed laterally after the crown region) (Peoples *et al.*, 1989).

The determination of leghemoglobin and glutamine synthetase (GS) activity were assessed in nodules. GS activity (EC 6.3.1.2) was determined by the hydroxamate biosynthesis method (Farnden and Robertson, 1980), while leghemoglobin was determined by the cyanmethe-

Table 1: Chemical properties of the soils used in this study

Soil	pH	Ca^{2+}	Mg^{2+}	K^+	P	TOC
	H_2O		$cmol_c\ kg^{-1}$		$mg\ kg^{-1}$	$g\ kg^{-1}$
Sandy	5.8	0.92	0.37	0.2	2.83	10.72
Clayey	6.1	1.18	0.48	0.3	5.04	23.81

TOC – total organic C.

moglobin method (Wilson and Reisenauer, 1963) using human hemoglobin as standard.

The normality and homogeneity of variance of data were assessed by Shapiro-Wilk and Bartlett tests, respectively. All data were statistically analyzed using an analysis of variance (ANOVA) and the means were compared by the Tukey test. All data were analyzed using the R software.

RESULTS AND DISCUSSION

The responses of all evaluated parameters were different according to the herbicides. MBC decreased significantly after the application of each herbicide, while the MPN of bacteria was reduced by the application of the mixture of herbicides (Table 2).

The herbicides negatively influenced the emergence and growth of soybean. The emergence of soybean was

Table 2: Soil microbial biomass and number of bacteria detected in soil after application of flumioxazin and imazethapyr

	Soil microbial biomass(mg kg^{-1})		Number of bacteria(CFU g^{-1})	
	Sandy	Clayey	Sandy	Clayey
C	214 a	238 a	7.9×10^7 a	8.5×10^7 a
Flu	108 b	117 b	5.5×10^7 ab	6.8×10^7 ab
Ima	103 c	98 b	4.6×10^7 ab	5.1×10^7 ab
Flu + Ima	65 d	71 c	2.9×10^7 b	3.5×10^7 b

C – control; Flu – flumioxazin; Ima – imazethapyr; Flu + Ima – flumioxazin + imazethapyr; CFU – Colony forming unit
Means with similar lower case letters, in each column, do not differ significantly by the Tukey test ($p < 0.05$).

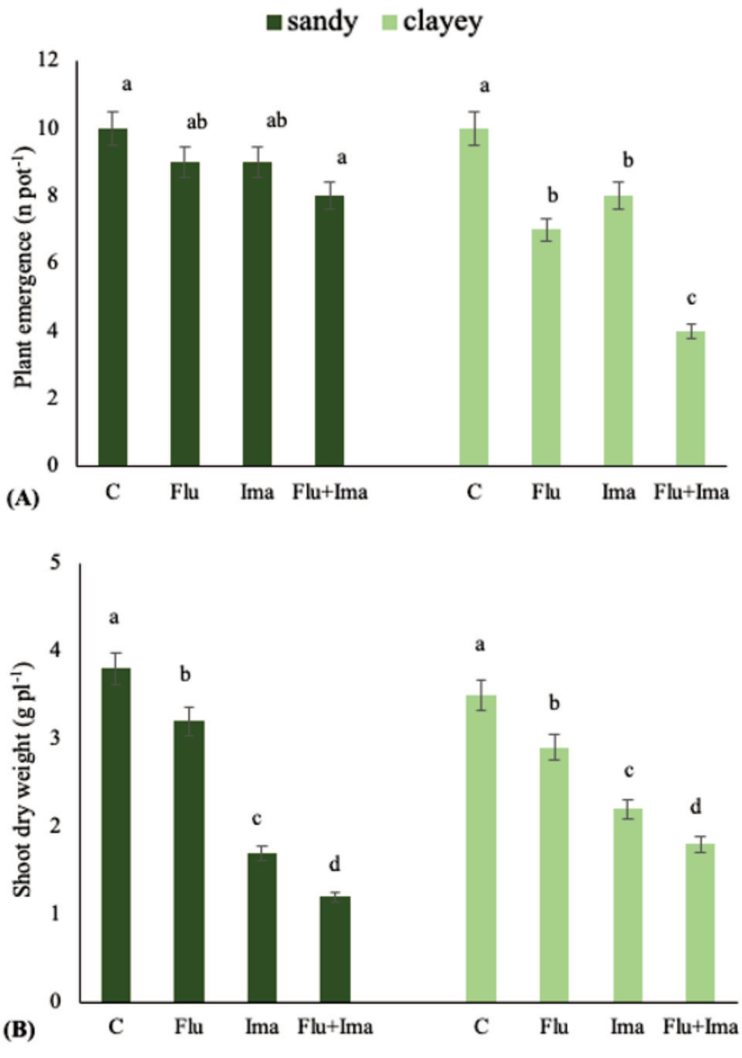

Figure 1: Plant emergence (A) and shoot dry weight (B) of soybean after application of flumioxazin, imazethapyr and their mixture. C – control; Flu – flumioxazin; Ima – imazethapyr; Flu + Ima – flumioxazin + imazethapyr. Means and standard error. Means with similar lower case letters, in each soil type, do not differ significantly by the Tukey test ($p < 0.05$).

affected by the application of the herbicides, mainly when the mixture was applied in the soil (Figure 1A). SDW (Figure 1B) and RDW (Figure 2A) decreased with the application of herbicides, mainly imazethapyr and the mixture (imazethapyr + flumioxazin). Although the herbicides decreased the growth of soybean, its accumulation of N in shoots was not affected by imazethapyr or flumioxazin, when applied separately. However, the application of the mixture decreased the accumulation of N in soybean (Figure 2B).

The nodulation in soybean was reduced after the application of the herbicides, mainly when the mixture was applied (Table 3). However, the negative effect of herbicides was more pronounced on nodulation found on the crown than in the secondary roots. At the crown region, the nodulation decreased significantly by the application of each herbicide or their mixture (flumioxazin + imazethapyr). At the secondary roots, the nodulation decreased with the application of all herbicides, but did not vary between them. In general, the lowest nodulation at the secondary roots was observed with the application of the mixture (flumioxazin + imazethapyr). Although the application of the herbicides negatively influenced the nodulation in soybean, the leghemoglobin content and GS activity did not show significant differences, except when the mixture was applied in the soil (Figure 3).

In this study, the effect of two herbicides recommended to soybean was assessed on biological N fixation in this legume. In general, the results have shown flumioxazin and imazethapyr presenting negative effects on all evaluated parameters, mainly when the mixture was applied. Firstly, the application of the herbicides significantly decreased the number of bacteria and the values of soil microbial biomass. It corroborates with previous studies that shown imazethapyr negatively influencing the accumulation of microbial C into microbes (Thiour-Mauprivez *et al.*, 2019), while

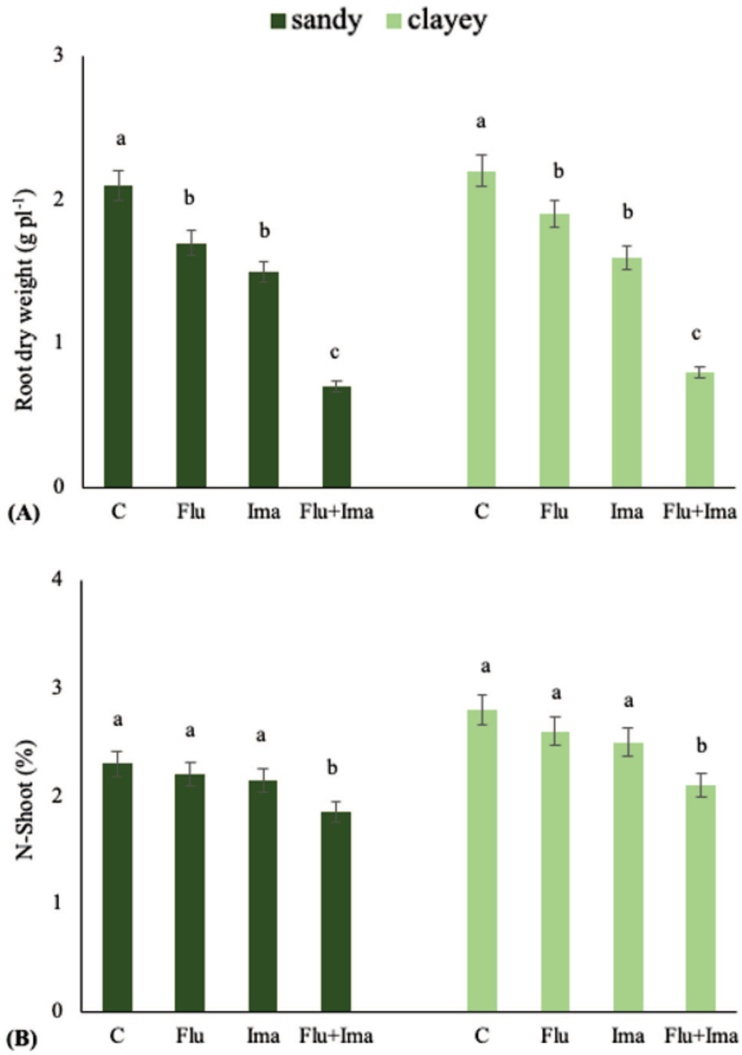

Figure 2: Root dry weight (A) and N-shoot (B) of soybean after application of flumioxazin, imazethapyr and their mixture. C – control; Flu – flumioxazin; Ima – imazethapyr; Flu + Ima – flumioxazin + imazethapyr. Means and standard error. Means with similar lower case letters, in each soil type, do not differ significantly by the Tukey test ($p < 0.05$).

flumioxazin presents an anti-microbial effect (Kushwaka and Kaushik, 2016). Thus, these results observed for MBC and number of bacteria can be explained by the negative effect of these herbicides on soil microbes.

The nodulation was also significantly affected by the herbicides, and some studies reported that the symbiosis legume-rhizobia can be affected by herbicides through of some mechanisms: a) reduction of the availability of root hairs to infection (Kremer and Means, 2009); b) inhibition of biochemical signaling by plants to initiate nodulation (Ahemad and Khan, 2011); and c) reduction of cell division, also inhibiting nodule development (Datta *et al.*, 2009). Particularly, some previous studies found that imazethapyr, even when applied at recommended rates, decreased the nodulation in soybean (Parsa *et al.*, 2013; Gonçalves *et al.*, 2018). The results disagree

Table 3: Distribution of nodule number (NN) and dry weight (NDW) at the crown and secondary roots of soybean rhizosphere after application of flumioxazin, imazethapyr and their mixture

	NN at the crown (nodule pl^{-1})		NN at SR* (nodule pl^{-1})		NDW at the crown (mg pl^{-1})		NDW at SR (mg pl^{-1})	
	Sandy	Clayey	Sandy	Clayey	Sandy	Clayey	Sandy	Clayey
C	29 a	30 a	40 a	33 a	121 a	135 a	132 a	140 a
Flu	23 a	12 b	32 a	36 a	60 b	51 b	109 b	102 b
Ima	18 a	10 b	31 a	31 a	53 b	42 b	96 b	63 c
Flu + Ima	7 b	4 c	28 a	8 b	26 c	23 c	93 b	21 d

C – control; Flu – flumioxazin; Ima – imazethapyr; Flu + Ima – flumioxazin + imazethapyr; *SR (secondary roots).
Means with similar lower case letters, in each column, do not differ significantly by the Tukey test ($p < 0.05$).

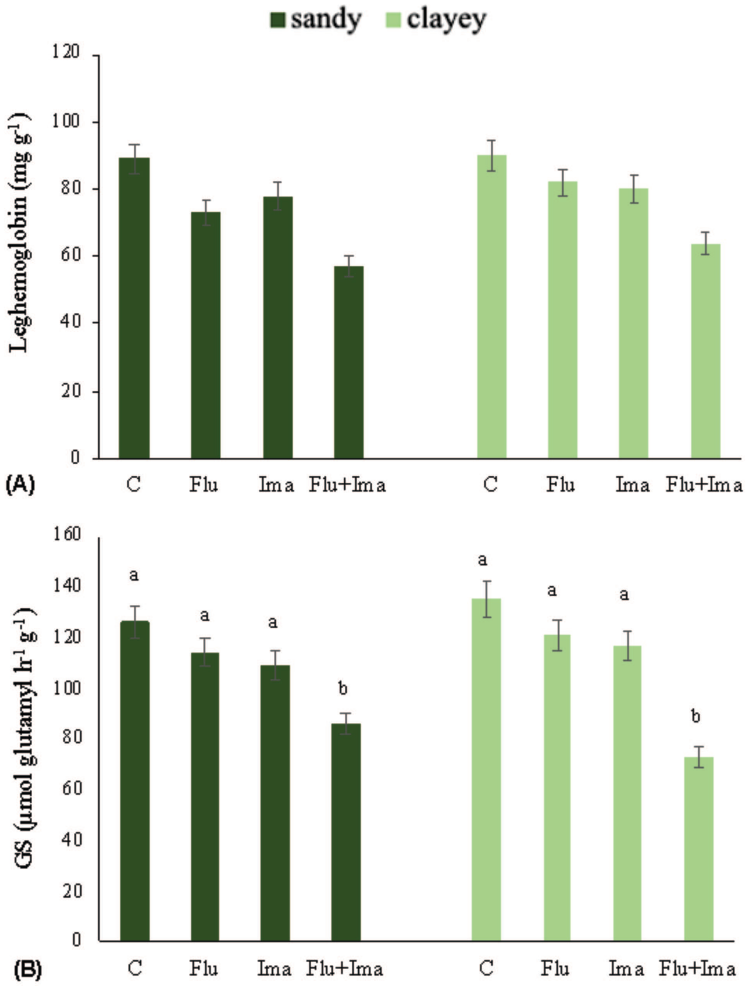

Figure 3: Leghemoglobin content (A) and glutamine synthetase activity (B) in nodules of soybean after application of flumioxazin, imazethapyr and their mixture. C – control; Flu – flumioxazin; Ima – imazethapyr; Flu + Ima – flumioxazin + imazethapyr. Means and standard error. Means with similar lower case letters, in each soil type, do not differ significantly by the Tukey test ($p < 0.05$).

with Gonzalez *et al.*, (1999) who applied flumioxazin, at recommended rate, and did not observe negative effect on nodulation in inoculated soybean. Thus, these different results found in this study those observed by Gonzalez *et al.*, (1999) can be explained by the absence of inoculation. In this study, the soybean was not inoculated, and the nodulation occurred by the rhizobia from the soil. By the way, the lowest nodulation found in soybean by rhizobia from the soil also can be explained by the effect of herbicides on soil microbes. (Pertile *et al.*, 2021).

Interestingly, this study showed that the nodulation in the crown region was more affected than that found at the secondary roots, so suggesting a negative effect on early nodulation. Thus, the application of herbicides promoted a negative effect on the initial infection of rhizobia to the roots, while afterwards there would be a better condition to infection at the secondary roots. It could be explained by the degradation of these herbicides which decrease their toxicity to soil rhizobia. The degradation time of imazethapyr and flumioxazin in soils are about 15 days (PPDB, 2019). Anyway, the significant effect of the herbicides on the early nodulation in soybean contribute to decrease the early N fixation and plant growth.

The emergence of soybean was significantly decreased in clayey soil and one possible explanation would be the higher capacity of clayey soil in maintain the water and, thus, leave the herbicides in longer contact with the seeds. On the other hand, in sandy soils, the herbicides could be rapidly percolated into the soil and the damage on plant emergence would be lower. As reported by Razavipour and Farrokh (2014), sandy soils have higher water percolation rate than clayey soils. The herbicides negatively influenced the growth of soybean, and the results agree with previous studies that also found a decrease in soybean growth, at least initially, as affected by flumioxazin (Priess *et al.*, 2020) and imazethapyr (Papiernik *et al.*, 2005). The results also showed a decrease in the accumulation of N in soybean, and it can be a reflect of lower nodulation in plant submitted to herbicides. Anyway, the N accumulated by soybean probably came from the soil organic matter (SOM) as also observed by Bohm *et al.*, (2009) who assessed the effect of imazethapyr on nodulation and N accumulation in soybean and found low nodulation and high N accumulation in plants due to the greater organic matter decomposition. Although soybean can accumulate N from SOM, so maintaining its yield, this process will contribute for decreasing SOM and N reserves, affecting the soil sustainability.

When considering the effects of the herbicides on biochemical parameters, the results suggest no detrimental effects of each separated herbicides on biological N fixation in soybean. However, when the mixture is applied the effect is negative on the leghemoglobin content and GS activity. This decreasing in leghemoglobin found in nodules submitted to the mixture of herbicides indicates a low synthesis of globin and contributes to lower BNF rates (Araujo *et al.*, 2007). In addition, the lower GS activity found in nodules affected by the mixture suggests a deleterious effect on the N metabolism in nodules and its diverse metabolic and developmental regulation (Harrison *et al.*, 2003). Some previous studies have reported that the application of glyphosate and imazethapyr promoted negative effects on leghemoglobin content in soybean (Reddy and Zablotowicz, 2003; Bohm *et al.*, 2009).

CONCLUSIONS

The application of herbicides imazethapyr and flumioxazin separately negatively influenced the growth of soybean, while they do not influence the nodulation and N accumulation. When applied in mixture, the herbicides enhance the negative effect on the growth and even harm N fixation in soybean. Therefore, the mixture of herbicides seems to be more harmful to soil microbes and, consequently, their ability to fix N in soybean.

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