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Potentially harmful effects of seed treatment and pre-inoculation on soybean biological nitrogen fixation and yield

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ABSTRACT

Biological nitrogen fixation (BNF) is a crucial process for the successful development of soybean plants. Nevertheless, many management decisions can affect the symbiosis between the bacteria and the plant, impacting soybean growth and yield. The use of pesticides in soybean seed treatment and the application of *Bradyrhizobium* sp. inoculant to the seeds a few days or even weeks before sowing are two management practices that can have a detrimental effect on the BNF process and always raise concerns. To shed light on the potential impact of these two management practices, the objective of this study was to evaluate under laboratory, greenhouse, and field conditions the effects of pre-inoculating soybean seeds with *Bradyrhizobium* sp. for up to 30 days prior to sowing with and without common pesticides used for seed treatment. One laboratory, one greenhouse, and six field experiments were conducted from 2016 to 2019. Pre-inoculation time (3 h and 30 days before sowing) and pesticides seed treatment (control - without pesticide application; pyraclostrobin + thiophanate-methyl + fipronil; thiabendazole + fludioxonil + metalaxyl-M; and carbendazim + thiram) were the two fixed effects controlled by the experimental design. Results from laboratory and greenhouse trials showed that pre-inoculation and pesticide seed treatment can negatively affect the recovery of colony-forming units of *Bradyrhizobium elkanii* inoculated to the seed, ureides concentration in plant shoots, BNF efficiency, and plant growth. Pooled analysis of the six field experiments demonstrated that although none of the BNF variables assessed were affected by pre-inoculation or pesticide seed treatment compared to the control, thiabendazole + fludioxonil + metalaxyl-M caused significant yield loss, whereas the weight of thousand grains for inoculation 3 h before sowing was significantly higher than inoculation 30 days before sowing.

1. Introduction

Soybean (*Glycine max* (L.) Merrill) is considered the primary source of plant protein for human and animal feed. The high protein content of the seed implies a demand of ~ 80 kg of nitrogen per Mg of grain yield, of which 75% are exported from the field (Bender et al., 2015; Salvagiotti et al., 2008). A considerable part of this demand is met by biological nitrogen fixation (BNF) with *Bradyrhizobium* sp., which eliminates the need for N fertilizer. The lack of response to N fertilizers, which rely on fossil fuel for manufacturing, provides economic and environmental benefits, making BNF a strategically sustainable option for protein production.

The BNF process occurs in root nodules, where atmospheric N_2 is converted into NH_3 and later into NH_4^+ (Mulder et al., 2002; Baral et al., 2012, 2014). In exchange, the host plant provides dicarboxylic acids (e.g., malate) (Udvardi and Day, 1997) as a source of carbon and energy to

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the fixing bacteria. In soybeans, the final products of BNF are primarily transported to the shoots as ureides, such as allantoin and allantoic acid (Baral et al., 2016). The amount of ureides in the plant tissue increases along the plant cycle, peaking between R_3 and R_5 and decreasing at R_7 (Osborne and Riedell, 2011; Zapata et al., 1987), and their relative abundance in the plant xylem is considered an indicator of the BNF activity (Herridge, 1982; Duran and Todd, 2012).

The annual reinoculation of soybeans with *Bradyrhizobium* is commonly made with commercial inoculants (reinoculation). This is a far more common management practice in South America than in the United States (US). In Brazil and Argentina, approximately 80% of soybean fields are inoculated yearly (Perticari, 2015; Santos et al., 2019), while only 15% in the US (Graham et al., 2004). According to Leggett et al. (2017), reinoculation showed a yield increase of 14% and 9.5% in areas of low yield potential in the US and Argentina, respectively. In high yield potential areas, the differences were 0.6% and 3.5% in US and Argentina, respectively (Leggett et al., 2017). In Brazil, consistent results point to a yield increase of 8% with annual inoculation (Hungria and Mendes, 2015) and 16% in co-inoculation with *Azospirillum* sp. (Hungria et al., 2013).

Inoculants are commonly applied via seed treatment or in-furrow. Via seed treatment, the bacteria are exposed to fungicides, insecticides, nematicides, micronutrients, and biostimulants, which may negatively impact rhizobia survival (Campo et al., 2009; Rodrigues et al., 2020; Santos et al., 2021). Furthermore, inoculants are often applied many days or a few weeks before the sowing date due to logistics and practicality (Hungria et al., 2020). This practice substantially extends the time of exposure and likely increases the negative effect on the bacteria survival. Therefore, the objective of this study was to evaluate under laboratory, greenhouse, and multiple field conditions the impact of pre-inoculating soybean seeds with *Bradyrhizobium* sp. for up 30 days prior to sowing, with and without common pesticides used for seed treatment.

2. Material and methods

2.1. Experiments

One laboratory, one greenhouse, and six field experiments were conducted over three cropping seasons. The laboratory trial was set up to check the effect of pre-inoculation and pesticide seed treatment on the recovery of colony-forming units (CFU) of *Bradyrhizobium elkanii* per seed (Campo and Hungria, 2007; MAPA, 2010). A hundred seeds were transferred to a sterile Erlenmeyer flask containing 90 mL of sterile saline solution (0.85%) with Tween 80, and the samples were submitted to horizontal agitation at 150 rpm for 20 min. This step was repeated twice, and the final volume of the suspension was 200 mL. From this volume, 10 mL were transferred to another sterile Erlenmeyer flask containing 90 mL of sterile saline solution in order to obtain the dilution 10^{-1}. From this suspension, decimal serial dilutions of 10^{-2} to 10^{-7} were prepared and spread in Petri dishes containing semi-selective Ikuta medium. Inoculated plates were incubated at 28 °C ± 2 °C, in the dark, for 7 days. After that, the CFU of each plate was evaluated, considering the number of colonies ranging from 30 to 300 CFU.

In the greenhouse experiment, 10 seeds of cultivar TMG7062 IPRO RR2 were planted per pot in a mixture of sand and vermiculite (2:1) in 9 dm^3 pots, containing 0.5% of organic matter and pH in $CaCl_2$ of 5.4. Fertilization was performed with 0.385 g of K, 0.786 g of P, and 3.458 g of S per pot. No nitrogen fertilizer was used. K fertilization was divided into two applications, the first at sowing and the other at 21 days after planting. Plants were trimmed seven days after planting, leaving 2 plants per plot. Plants were watered daily with a regular hose. Temperature and light time were not controlled.

In the 2016/2017 crop season, two field experiments were conducted, one in Ponta Grossa-PR and the other in Piracicaba-SP with cultivar TMG7062 IPRO RR2. The former was sown on 11/05/2016 with 50 kg ha^{-1} of P and 50 kg ha^{-1} of K in a Latossolo Vermelho distrófico soil (Santos et al., 2018) containing 2.9% of organic matter and pH in $CaCl_2$ of 4.6, previously cropped with soybeans in the last 10 seasons. The latter was sown on 11/20/2016 with 50 kg ha^{-1} of P and 25 kg ha^{-1} of K in a Nitossolo Vermelho eutroférrico soil (Santos et al., 2018) containing 1.8% of organic matter and pH in $CaCl_2$ of 5.5, without soybean in the last 4 crop seasons.

In 2017/2018, two other field experiments were conducted with cultivar TMG7062 IPRO RR2, both in Piracicaba-SP. The experiments were sown on 12/02/2017 with 50 kg ha-1 of P and 25 kg ha^{-1} of K in a Nitossolo Vermelho eutroférrico soil (Santos et al., 2018) soil containing 1.4% of organic matter and pH in $CaCl_2$ of 5.8, without soybean in the last 3 crop seasons.

Finally, in 2018/2019, two more field experiments with cultivar TMG7062 IPRO RR2 were conducted in Piracicaba-SP. One of them was sown on 11/20/2018 with 80 kg ha^{-1} of P and 50 kg ha^{-1} of K in an Argissolo Vermelho-Amarelo distrófico soil (Santos et al., 2018) containing 0.5% of organic matter and pH in $CaCl_2$ of 5.0. The other was sown on the same day with 40 kg ha^{-1} of P and 30 kg ha^{-1} of K in a Nitossolo Vermelho eutroférrico soil (Santos et al., 2018) containing 1.8% organic matter pH in $CaCl_2$ of 6.1. Both areas had been cropped with soybeans in the last season.

All field trial soils had 10^5 soybean-nodulating rhizobia cells g^{-1}, according to the MPN method (Campo and Hungria, 2007). The cultivar TMG7062 IPRO RR2 was used in all field experiments without applying N-fertilizer. The experiment in Ponta Grossa (2016/2017) and Piracicaba (2018/2019) were rainfed, while all others were irrigated via a central pivot.

2.2. Treatments and experimental design

Pre-inoculation and pesticide seed treatment were the two fixed effects controlled by the experimental design. The first was related to the storage time of pre-inoculated seeds, which were 3 h and 30 days. The second factor was the type of seed treatment: i) pre-inoculated control without pesticide application; ii) Standak Top® (pyraclostrobin 0.050 g kg^{-1} of seeds + thiophanate-methyl 0.450 g kg^{-1} of seeds + fipronil 0.500 g kg^{-1} of seeds); iii) Maxim Advanced® (thiabendazole 0.188 g kg^{-1} of seeds + fludioxonil 0.031 g kg^{-1} of seeds + metalaxyl-M 0.025 g kg^{-1} of seeds) and iv) Derosal Plus® (carbendazim 0.300 g kg^{-1} seed + thiram 0.700 g kg^{-1} of seeds). All experiments were conducted in a 2 × 4 factorial arrangement, except the one in Ponta Grossa (2016/2017), in which there was no control without pesticide treatment. All experiments used a randomized complete block design with five replications.

Bradyrhizobium elkanii formulated as a peat inoculant (5×10^9 colony-forming units [CFU] g^{-1}; 4 g kg^{-1} of seeds) was used. In addition, colorant polymer (Poliplus® Forquímica – 3 mL kg^{-1} of seeds), osmoprotectant polymer (S30® BASF – 3 mL kg^{-1} of seeds), and powder-drier (Alldry® Forquímica – 4 g kg^{-1} of seeds) were added in all treatments in the following order: first, the pesticides seed treatments were mixed with the colorant and osmoprotectant polymers, applying the resulting slurry to the seeds. Subsequently, with the seeds still wet, the inoculant was added. Finally, the powder-drier was added after mixing the treated seeds with the inoculant.

2.3. Evaluations

2.3.1. Plant biometry

Two whole plants in the greenhouse experiment and five in the field trials were randomly collected per experimental plot at the V_4 phenological stage (Fehr et al., 1971) and V_4 + R_3 phenological stages, respectively. The root nodules were counted for only the greenhouse experiment and weighed after oven drying for 72 h at 60 °C. The shoots of both greenhouse and field trials had their leaves separated from petioles and stems and were also oven dried for 72 h at 60 °C to determine the shoot dry matter.

2.3.2. Ureides and biological nitrogen fixation efficiency

The same plants sampled for the plant biometry were used to evaluate ureides and biological nitrogen fixation efficiency. After drying, petioles and stems were ground in a Wiley mill (Herridge, Peoples, 1990). For the determination of ureides and nitrate, 0.1 g of the processed sample was placed in 15 mL Falcon vials, added to 10 mL of distilled water, and placed in a water bath for 1 h at 45 °C (Teixeira et al., 2018). The suspension was centrifuged at 15,344 × *g*, and the supernatant was transferred to new 15 mL Falcon vials. The determination of ureide was performed according to Young and Conway (1942), adapted by Teixeira et al. (2018). Nitrate determination was performed only for the field experiments using the salicylic acid method proposed by Cataldo et al. (1975), adapted by Teixeira et al. (2018). Ureides and nitrate concentrations were used to calculate the efficiency of BNF (EF_{BNF}) in the field experiments, as proposed by McClure et al. (1980), Herridge (1982), and Herridge and Peoples (1990), using Eq. 1, where EF_{BNF} is given as a percentage, and ureides and nitrate are given in mM g^{-1} of dry matter of stem and petioles. Constant 4 refers to the ratio of nitrogen atoms in an allantoin (ureide) molecule compared with a nitrate molecule, which is 4:1.

$$EF_{BNF} = \frac{4 \times [ureides]}{(4 \times [ureides]) + [nitrate])} \quad (1)$$

2.3.3. Yield components

In the field trials, grain yield was determined at maturity, harvesting 5.4 m^2 (4 m in length from the three central rows) of each plot. The grains were cleaned and weighed, and the yield was estimated based on 13% moisture content. Weight of thousand grains was recorded by weighing grains from five subsamples taken randomly from each plot.

2.4. Data analysis

Statistical analysis was performed using R Studio software (R Core Team, 2021). Shapiro-Wilk and Bartlett tests were performed to determine variance normality and homogeneity. When residues were neither normally distributed nor homogenous ($p \leq 0.05$), data were transformed using a rank methodology (Shah and Madden, 2004). All experiments were pooled and analyzed with pre-inoculation time and pesticide seed treatment considered fixed effects, and replication nested within experiments considered a random effect, using a hierarchical mixed-model (*lme4* package - *glmer* function). When the fixed effect factors showed significant differences or interaction between them ($p \leq 0.1$), the analysis was unfolded and compared using the least squares mean test with the *emmeans* package (*emmeans* function).

3. Results

3.1. Laboratory and greenhouse experiments

Both pesticide seed treatment and pre-inoculation significantly affected the recovery of colony-forming units (CFU) of *B. elkanii* (Fig. 1). There was a significant decrease in CFU from 3 h to 30 days even for the control treatment without pesticides. Overall, pesticide seed treatment increased the deleterious effect of pre-inoculation, and treatment containing pesticide seed treatment and inoculation 30 days prior to sowing had the lowest values of CFU (Fig. 1).

Pesticides used in seed treatment and pre-inoculation significantly affected the number of nodules and nodule dry matter in the greenhouse environment (Table 1). Inoculation 3h before sowing resulted in a greater number of nodules than 30 days, whereas seed treatment with carbendazim + thiram resulted in the lowest value regardless of the period the inoculum was exposed to the chemicals before planting.

The storage of inoculated seeds for 30 days decreased the concentration of ureides in the shoots by 60%. Considering the effect of pesticides in the seed treatment, the treatment thiabendazole + fludioxonil + metalaxyl-M resulted in the highest value. Regarding the shoot biomass, the carbendazim + thiram treatment decreased the shoot biomass compared with pyraclostrobin + thiophanate-methyl + fipronil and thiabendazole + fludioxonil + metalaxyl-M but was not different from the control without pesticides. The storage of inoculated seeds for 30 days negatively impacted the shoot dry matter, reducing it by 18% (Table 1).

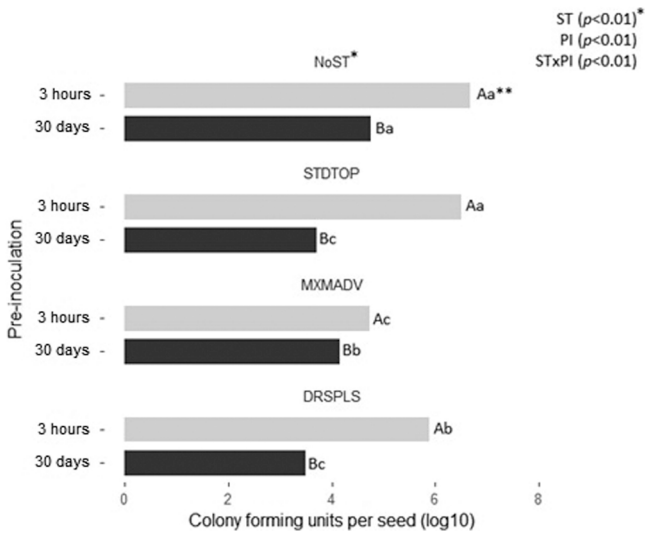

Fig. 1. Effect of pre-inoculation and pesticide seed treatment on the recovery of colony-forming units of *Bradyrhizobium elkanii* per soybean seed. *p*-values are indicated in the upper right corner of the plot. * NoST (control without pesticide); STDTOP (Standak Top®, pyraclostrobin + thiophanate-methyl + fipronil); MXMAVD (Maxim Advanced®, thiabendazole + fludioxonil + metalaxyl-M); DRSPLS (Derosal Plus®, carbendazim + thiram); ST (seed treatment); PI (pre-inoculation). * *Lowercase letters indicate statistical differences between pesticide seed treatments within the same level of pre-inoculation, whereas uppercase letters indicate statistical differences in pre-inoculation within the same level of pesticide seed treatment. Means followed by the same letter denote no statistical differences among treatments ($p \leq 0.1$).

3.2. Field experiments

The interaction between the pesticide seed treatment and pre-inoculation was not significant ($p > 0.1$) for any of the variables assessed. Pre-inoculation and/or seed treatment were responsible for more than 50% of the variance only for ureides at the beginning of pod formation (R_3 growth stage) and yield. Seed treatment significantly ($p < 0.1$) influenced ureides concentration at the V_4 stage, although multiple comparison procedure found no differences between treatments. Ureides concentration at the beginning of pod formation (R_3) was also significantly ($p < 0.1$) influenced by pre-inoculation and seed treatment. Seed inoculation 30 days prior to sowing had significantly greater ureides concentration than inoculation on planting day and seeds treated with thiabendazole + fludioxonil + metalaxyl-M had significantly higher levels of ureides than those treated with carbendazim + thiram at R_3 growth stage (Table 2). The average ureides concentration across treatments and inoculum exposure to pesticides at V_4 and R_3 phases was 1.31 and 3.14 mM g^{-1}, respectively (Table 2).

The average BNF efficiency across site-years was 34.4% for the V_4 phase and 67.3% for the R_3 phase. None of the factors significantly ($p > 0.1$) influenced BNF efficiency at the V_4 stage (Table 2). On the other hand, seed treatment significantly ($p < 0.01$) affected this variable at the beginning pod stage (R_3). In this case, seeds treated with pyraclostrobin + thiophanate-methyl + fipronil had significantly greater BNF efficiency than those treated with carbendazim + thiram. (Table 2).

Yield was only significantly ($p < 0.05$) affected by seed treatment. Seeds treated with thiabendazole + fludioxonil + metalaxyl-M resulted

Table 1
Effect of seed treatments with different pesticides and pre-inoculation on the number of nodules, nodule dry matter, ureides concentration, and shoot dry matter of soybean plants in the V_4 phenological stage in the greenhouse trial.

Seed Treatment (ST)*	Pre-inoculation (PI)		
	Number of nodules (n° plant^{-1})		
	3 h	30 days	Mean
NoST	26	16	21a*
STDTOP	25	13	19a
MXMADV	26	14	20a
DRSPLS	17	8	12b
Mean	23a	13b	
p-values	(ST)= 0.013	(PI)< 0.001	ST × PI= 0.934
	Nodule dry matter (mg plant^{-1})		
	3 h	30 days	Mean
NoST	75.6	72.3	74.0a
STDTOP	86.2	59.0	72.6a
MXMADV	75.8	60.2	68.0ab
DRSPLS	65.1	37.8	51.5b
Mean	75.7a	57.3b	
p-values	(ST)= 0.011	(PI)< 0.001	ST × PI= 0.272
	Ureides concentration (mM g^{-1})		
	3 h	30 days	Mean
NoST	1.14	0.57	0.85b
STDTOP	1.26	0.47	0.87b
MXMADV	1.86	0.74	1.30a
DRSPLS	0.82	0.27	0.54b
Mean	1.27a	0.51b	
p-values	(ST)< 0.001	(PI)< 0.001	ST × PI= 0.2299
	Shoot dry matter (g plant^{-1})		
	3 h	30 days	Mean
NoST	0.789	0.713	0.751ab
STDTOP	0.917	0.702	0.809a
MXMADV	0.888	0.677	0.783a
DRSPLS	0.689	0.598	0.643b
Mean	0.821a	0.673b	
p-values	(ST)= 0.005	(PI)< 0.001	ST × PI= 0.2677

* NoST (control without pesticide); STDTOP (Standak Top®, pyraclostrobin + thiophanate-methyl + fipronil); MXMAVD (Maxim Advanced®, thiabendazole + fludioxonil + metalaxyl-M); DRSPLS (Derosal Plus®, carbendazim + thiram)
* Means followed by the same letter denote no statistical differences among treatments ($p \leq 0.1$)

in significantly lower yield than those without any seed treatment regardless of pre-inoculation for 3 h or 30 days (Table 2). The average yield of the experiments was 3610 kg ha^{-1}, ranging from 2947 to 4503 kg ha^{-1} (data not presented). The weight of thousand grains was significantly affected by pre-inoculation ($p \leq 0.1$), whereas seed treatment did not affect this trait. Treatments that received inoculation on the sowing day had a greater weight of thousand grains than those that were inoculated 30 days before sowing.

4. Discussion

Storage of inoculated seeds for 30 days may be detrimental to BNF even without seed treatment, as shown in the laboratory and greenhouse experiments (Fig. 1 and Table 1). There was a significant reduction in all variables evaluated in the greenhouse experiment where seeds inoculated and stored for 30 days before sowing were negatively affected compared with plants from seeds inoculated and sown on the same day (Table 1); a likely consequence of the decrease in CFU of the *B. elkanii* inoculated cells as shown in Fig. 1. Additionally, the negative effect on shoot biomass was permanent across the crop cycle, and at the phenological stage R_3, it was still possible to observe significant differences between the two storage periods (data not shown). Although pre-inoculation did not significantly affect the concentration of ureides, BNF efficiency, or yield in the pooled analysis of the field trials, it was found that inoculation on sowing day resulted in a greater weight of thousand grains than inoculation 30 days prior to sowing, regardless of the seed treatment (Table 2). It is also important to highlight that despite the pooled analysis of yield from the six field trials revealing no

Table 2
Effect of seed treatments with different pesticides and pre-inoculation with *Bradyrhizobium elkanii* (3 h or 30 days) before sowing on ureides concentration, BNF efficiency, and grain yield. Results represent the estimated marginal means of each treatment across the six field experiments from 2016 to 2019.

Seed Treatment (ST)●	Pre-inoculation (PI)		
	Ureides – V_4 (mM g^{-1})		
	3 h	30 days	Mean
NoST	1.35	1.15	1.25 ^{ns}
STDTOP	1.43	1.29	1.36
MXMADV	1.23	1.27	1.25
DRSPLS	1.35	1.36	1.36
Mean	1.34 ^{ns}	1.27	
p-values	(ST)= 0.098	(PI)= 0.199	ST × PI= 0.617
	Ureides – R_3 (mM g^{-1})		
	3 h	30 days	Mean
NoST	3.10	2.97	3.04ab*
STDTOP	3.22	3.34	3.28ab
MXMADV	3.08	3.56	3.32a
DRSPLS	2.81	2.99	2,90b
Mean	3.05b	3.22a	
p-values	(ST)= 0.061	(PI)= 0.084	ST × PI= 0.438
	BNF efficiency – V_4 (%)		
	3 h	30 days	Mean
NoST	35.6	35.3	35.5 ^{ns}
STDTOP	35,1	34.1	34.6
MXMADV	33.9	33.0	33.5
DRSPLS	33.7	34.0	33.9
Mean	34.6 ^{ns}	34.1	
p-values	(ST)= 0.375	(PI)= 0.801	ST × PI= 0.979
	BNF efficiency – R_3 (%)		
	3 h	30 days	Mean
NoST	68.4	66.3	67.4ab
STDTOP	70.9	70.8	70.9a
MXMADV	68.0	65.6	66.8ab
DRSPLS	64.6	63.5	64.1b
Mean	68.0 ^{ns}	66.6	
p-values	(ST)= 0.004	(PI)= 0.492	ST × PI= 0.938
	Yield (kg ha^{-1})		
	3 h	30 days	Mean
NoST	3781	3692	3737a
STDTOP	3702	3599	3651ab
MXMADV	3567	3425	3496b
DRSPLS	3538	3574	3556ab
Mean	3647 ^{ns}	3573	
p-values	(ST)= 0.046	(PI)= 0.172	ST × PI= 0.728
	Weight of thousand grains (g)		
	3 h	30 days	Mean
NoST	212	213	213 ^{ns}
STDTOP	219	212	216
MXMADV	215	210	213
DRSPLS	216	214	215
Mean	216a	212b	214
p-values	(ST)= 0.581	(PI)= 0.090	ST × PI= 0.294

●NoST (control without pesticide); STDTOP (Standak Top®, pyraclostrobin + thiophanate-methyl + fipronil); MXMAVD (Maxim Advaced®, thiabendazole + fludioxonil + metalaxyl-M); DRSPLS (Derosal Plus®, carbendazim + thiram)
^{ns}Not statistically significant
*Means followed by the same letter denote no statistical differences among treatments ($\alpha = 0.1$)

differences caused by pre-inoculation, statistically significant yield loss caused by pre-inoculation was observed for the seed treatment with pyraclostrobin + thiophanate-methyl + fipronil in the experiment in Piracicaba in crop season 2016 /2017 and thiabendazole + fludioxonil + metalaxyl-M in one of the experiments in Piracicaba in the crop season 2018/2019 (data not shown).

The pesticide seed treatment with carbendazim + thiram decreased the number of CFU of *B. elkanii* per seed even in inoculation on the sowing day (Fig. 1) and impacted nodule number and dry matter, although no significant effect was found on the concentration of ureides when compared with the control (Table 1). This pesticide seed treatment also did not differ from the control without pesticides in any of the variables assessed in the field experiments (Table 2). The adverse effect

of carbendazim and thiram, alone or in combination, on BNF, has been reported in previous studies (Bikrol et al., 2005; Campo et al., 2009; Martyniuk et al., 2016). In addition, other active ingredients from the same chemical groups (benzimidazoles and dithiocarbamates) have been reported to be harmful to Bradyrhizobial strains, affecting the number and dry weight of nodules (Campo et al., 2009; Anupama et al., 2005). Fungicides of the dithiocarbamates group have multisite action and affect the biochemical processes of various organisms (Oliver and Hewitt, 2014).

Seeds treated with thiabendazole + fludioxonil + metalaxyl-M resulted in higher ureides concentration in both greenhouse and field trials (Tables 1 and 2), although it decreased the number of CFU of *B. elkanii* per seed (Fig. 1), and no clear explanation to this observation might be drawn at this time. Nevertheless, the combination of thiabendazole + fludioxonil + metalaxyl-M was the only treatment that resulted in a lower yield than the control without pesticides (Table 2) in the field experiments.

The combination of pyraclostrobin + thiophanate-methyl + fipronil only impacted the recovery of CFU of *B. elkanii* on seeds inoculated 30 days before sowing (Fig. 1). It did not differ from the control in any of the variables assessed in both greenhouse and field trials. Other authors have also reported similar results in recent years. Araujo et al. (2017) found no differences in yield comparing the use of inoculant associated with pyraclostrobin + thiophanate-methyl + fipronil on pre-inoculated seeds for up to 30 days in four experiments carried out in soils with *Bradyrhizobium* population varying from 0 to 10^4 MPN g^{-1} of soil. In addition, the authors found no reduction in the number and mass of soybean nodules in the vegetative phase. Although no differences in yield were found in the field trials, the authors demonstrated a reduction from 6.70×10^7 to 2.31×10^3 CFU $seed^{-1}$ with the storage for 30 days before sowing, which would be below the recommended level to ensure good symbiotic performance under Brazilian conditions (Hungria et al., 2017). Rodrigues et al. (2020) found that, although the presence of pyraclostrobin + thiophanate-methyl + fipronil had no impact on yield in two field experiments on sandy soils devoid of rhizobia, the seed treatment resulted in a significantly higher rate of CFU decrease over time of both *B. elkanii* (SEMIA 587) and *B. japonicum* (SEMIA 5079). Moreover, the authors showed that the presence of pyraclostrobin + thiophanate-methyl + fipronil associated with *Bradyrhizobium* sp. inoculation significantly decreased the total nitrogen accumulated in grains compared with the treatment without pesticides in the two field trials.

The relationship between soybean yield and biological nitrogen fixation is not always clear. For example, inoculation influenced soybean nodulation but not yield in a study by Sanginga et al. (2000). Conversely, in Brazil, inoculation increased yield but did not affect the nodulation parameters (Hungria et al., 1998). Even when there are linear responses in the number and mass of nodules with an increase in inoculant rates, there may not be a corresponding effect on yield (Hungria et al., 2017). This may happen because, whereas BNF assessments happen in specific growth stages of soybean development, the yield evaluation is influenced by many factors along the whole crop cycle. Therefore, when a nodulation assessment is done during the vegetative stages, the plant can be exposed to water or temperature stress later, eventually impacting yield (Franchini et al., 2016).

Despite the fact of being an excellent management strategy for several diseases and pests (Dorrance and McClure, 2001; Urrea et al., 2013), the cost-effectiveness of using soybean seed treatment is a topic of increasing discussion, especially because of the large variations in yield results (Bradley, 2008). Rossman et al. (2018), testing combinations of fungicides, fungicides + insecticides, and insecticides + fungicides + nematicides over three crop seasons in seven different environments, observed that the chemicals increased soybean plant stand in V_C/V_1 growth stages when compared with the control without any seed treatment. However, only the combination of fungicide and insecticide showed an increase in yield. The authors further demonstrated that although the yield correlates with plant stand ($r = 0.16$, $p < 0.0001$), the increase in plant stand resulted in increased yield in only one location and one crop season when the plant population of control treatment fell below 247,000 plants ha^{-1}. That study showed a statistical gain in yield comparing seed treatment with and without fungicides in only two out of 21 production environments. Similarly, our results have shown that none of the pesticides seed treatments had greater yield than the control without pesticides, which can be explained by the fact that all field trials did not have incidence of soil-borne pathogens such as *Fusarium* sp., *Phytophthora* sp., or *Rhizoctonia* sp. When seed and soil-borne diseases constitute a significant problem, seed treatment can result in greater yield, and even for rhizobia inoculated seeds, as shown by Golden et al. (2016) that in some cases, soybean inoculated with nitrogen-fixing bacteria may have greater yield with the use of pesticide-treated seeds when compared with the inoculated control without pesticide.

5. Conclusion

This study has demonstrated that pre-inoculation of soybean seeds for up to 30 days may negatively affect the recovery of colony-forming units, biological nitrogen fixation, plant growth, and weight of thousand grains. Additionally, pesticide seed treatments did not increase soybean grain yield. The only pesticides seed treatment that was similar to the non-treated control in both greenhouse and field experiments was the combination of pyraclostrobin + thiophanate-methyl + fipronil, whereas the other two were negative for one or more variables. Therefore, the results presented here show that pre-inoculation should be discouraged and, when necessary, farmers should give preference to less-impacting pesticides on seed treatment to avoid potentially harmful effects on biological nitrogen fixation. Moreover, further research should seek more effective strategies for cell protection in pre-inoculation with pesticides.

CRediT authorship contribution st6atement

Felipe Fadel Sartori: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Project administration, Funding acquisition. **Thaise Dieminger Engroff:** Investigation, Project administration, Funding acquisition. **Thais H. Godoi Sanches:** Investigation. **Julia Soave:** Investigation. **Mila V. Pessoto:** Investigation. **Guilherme Felisberto:** Investigation. **Valter E.Hilgemberg Jr.:** Investigation. **André Fróes Borja Reis**: Formal analysis, Writing – review & editing. **Mariangela Hungria:** Writing – review & editing. **Marco A. Nogueira:** Writing – review & editing. **David de Souza Jaccoud-Filho:** Writing – review & editing, Supervision. **Fernando Dini Andreote:** Writing – review & editing, Supervision. **Durval Dourado-Neto:** Conceptualization, Writing - original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Felipe Fadel Sartori reports financial support was provided by National Council for Scientific and Technological Development. Thaise Engroff Dieminger reports financial support was provided by National Council for Scientific and Technological Development. Durval Dourado-Neto reports equipment, drugs, or supplies was provided by Agrisus Foundation Sustainable Agriculture. Durval Dourado-Neto reports equipment, drugs, or supplies was provided by State of Sao Paulo Research Foundation.

Data Availability

Data will be made available on request.

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