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ORIGINAL PAPER

Impact of pesticides in properties of *Bradyrhizobium* spp. and in the symbiotic performance with soybean

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Abstract

Soybean [*Glycine max* (L.) Merr.] has great economic and nutritional importance mainly due to its high protein content. All plant's N needs can be met by the symbiosis with elite *Bradyrhizobium* strains applied as inoculants to the seeds at sowing time; however, the increasing use of pesticides in seed treatments can impair the contribution of the biological nitrogen fixation. In this study, we report decreases in cell survival of two strains, *B. japonicum* SEMIA 5079 and *B. elkanii* SEMIA 587 in seeds inoculated and treated with StandakTop™, composed of the fungicides pyraclostrobin and thiophanate-methyl and the insecticide fipronil, the pesticides most used in soybean seed treatment in several countries. Cell death was enhanced with the time of exposure to the pesticides, and *B. elkanii* was less tolerant, with almost no detectable viable cells after 15 days. Change in colony morphology with smaller colonies was observed in the presence of the pesticides, being more drastic with the time of exposure, and attributed to an adaptive response towards survival in the presence of the abiotic stress. However, morphological changes were reversible after elimination of the stressing agent and symbiotic performance under controlled greenhouse conditions was similar between strains that had been or not exposed to the pesticides. In addition, no changes in DNA profiles (BOX-PCR) of both strains were observed after the contact with the pesticides. In two field experiments, impacting effects of the pesticides were observed mainly on the total N accumulated in grains of plants relying on both N_2-fixation and N-fertilizer. Our data indicate that StandakTop® affects parameters never reported before, including colony morphology of *Bradyrhizobium* spp. and N metabolism and/or N remobilization to soybean grains.

Keywords Biological nitrogen fixation · Inoculant · Pesticides · *Glycine max* · *Bradyrhizobium*

Introduction

Nitrogen (N) is the nutrient required in largest amount by the soybean [*Glycine max* (L.) Merr.] crop, with about 80 kg of N required per 1,000 kg of grains produced (Hungria and Mendes 2015; Hungria and Nogueira 2019). The plant's N requirement can be supplied by N-fertilizers, a source with implications in environmental impact and economic costs. However, soybean can establish a symbiotic partnership with strains of *Bradyrhizobium* spp., taking advantage of the biological nitrogen fixation process, with reported contributions of up to 450 kg of N ha^{-1} (Hungria et al. 2006; Ormeño-Orrillo et al. 2013).

Brazil became the leading world's soybean producer in 2020, and along with Argentina, Uruguay, Paraguay and Bolivia is now responsible for 57% of the global production (USDA 2020). In these South American countries, it has been broadly shown that inoculation with elite *Bradyrhizobium* strains can fulfill soybean N needs, achieving high yields with sustainability (Hungria et al. 2006, 2020; Hungria and Mendes 2015; Hungria and Nogueira 2019). In 2019 the inoculant market in Brazil commercialized about 70 million doses, more than 90% for the soybean crop (Santos et al. 2019), and about 50 million doses are used in the other South American producing countries. The economic

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impact in Brazil is highlighted by estimates that the replacement of N-fertilizers by the biological nitrogen fixation (BNF) saves about U$ 15 billion per year (Hungria and Mendes 2015).

The great majority of the inoculants are applied to the seeds at sowing time; however, more than 90% of soybean seeds are treated with pesticides in Brazil, and 70% of the pesticides carry two or more active ingredients (a.i) (Hungria and Nogueira 2019). Pesticides are recommended to ensure the best establishment of the plants, protecting the seeds against soil pathogens, especially critical when drought periods after sowing occur, not allowing prompt seed germination (Embrapa Soja 2013). However, the toxic effects of pesticides may impair rhizobial survival, nodulation and nitrogen fixation efficiency (Campo et al. 2009; Kunal and Sharma 2011; Costa et al. 2013). The problem is greater with the increased adoption of anticipated inoculation, also broadly called as pre-inoculation, in which *Bradyrhizobium* cells remain in contact with the soybean seeds treated with pesticides for days or weeks, that may go to up to 60 days (Araujo et al. 2017; Machineski et al. 2018; Hungria et al. 2020), with little information about the impact on rhizobial cells and symbiotic performance.

Here we report the results of studies performed under laboratory, greenhouse and field conditions to investigate the effects of StandakTop™, containing the fungicides pyraclostrobin and thiophanate-methyl and the insecticide fipronil, the most common combination of pesticides used for the treatment of soybean seeds in Brazil and in several other countries, on properties of *Bradyrhizobium* cells and on the symbiotic performance with soybean in greenhouse and field experiments.

Material and methods

Strains and growth conditions

The bradyrhizobial strains used in the experiments were *Bradyrhizobium elkanii* strain SEMIA 587 (=CNPSo 14), *Bradyrhizobium japonicum* strain SEMIA 5079 (=CPAC 15, =CNPSo 07), and *Bradyrhizobium diazoefficiens* strain SEMIA 5080 (=CPAC 7, =CNPSo 06); the three strains are used in commercial inoculants in Brazil (MAPA 2011). For the laboratory and greenhouse conditions, *B. elkanii* SEMIA 587 and *B. japonicum* SEMIA 5079 were chosen because they show different intrinsic properties in relation to tolerance of several molecules, such as antibiotics (Kuykendall et al. 1988; Boddey et al. 1997). Contrarily, *B. japonicum* and *B. diazoefficiens* show similarity in properties such as lower tolerance of antibiotics (Boddey and Hungria 1997; Delamuta et al. 2013). For the field experiments the combination of *B. japonicum* strain SEMIA 5079 + *B. diazoefficiens* strain SEMIA 5080, that compose the great majority of the soybean inoculants used in Brazil (Santos et al. 2020) was used. For all experiments the strains were grown on modified-YMA or modified-YM medium (Hungria et al. 2016), at 28 °C, in the dark, for 7 days, at 120 rpm.

The *Azospirillum brasilense* strains used in the field experiments were Ab-V5 (=CNPSo 2083) and Ab-V6 (=CNPSo 2084). The two strains are used in commercial inoculants in Brazil since 2009 (MAPA 2011; Santos et al., 2019). The strains were grown on DYGS medium (Santos et al. 2020), at 30 °C, in the dark, for 5 days, at 120 rpm.

All strains are deposited at the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja" (WFCC Collection # 1213, WDCM Collection # 1054), in Londrina, State of Paraná, Brazil. For long-term preservation the strains are cryopreserved in modified-YM (yeast-mannitol) medium (Hungria et al. 2016) with 30% (v/v) of glycerol at -80 ºC and -150 ºC, and lyophilized.

Cell recovery from inoculated soybean seeds

The methodology used to estimate the bradyrhizobia cells recovered from inoculated soybean seeds was based on Penna et al. (2011), Araujo et al. (2017), and Santos et al. (2020); the methodology is also included in the Brazilian legislation for inoculants (MAPA 2010). Four groups of 500 g of soybean seeds (cultivar M5947 IPRO) were split into two groups, one control with seeds not treated with pesticides, and the second one receiving 1 mL of StandakTop™ (BASF) (composition: piraclostrobin, 25 g L^{-1}; thiophanate-methyl, 225 g L^{-1}; fipronil, 250 g L^{-1}; including fungicides and insecticide), as recommended by the manufacturer; the seeds were mixed to homogenize the distribution of the pesticides and dried at room temperature for 30 min. Following, 1 mL of an inoculum [3×10^9 CFU (colony forming units) mL^{-1}] of *B. elkanii* strain SEMIA 587, prepared as described in the previous item of strains and growth conditions, was applied to each of the seed groups, with and without pesticides. The same procedure was repeated with *B. japonicum* strain SEMIA 5079. This procedure was realized to have samples representing the times of exposure of 2 h, and 7, 15 and 30 days after inoculation. Seeds were stored in kraft paper bags at 25 ± 5 °C and humidity greater than 45%.

At each sampling time, three biological samples, each with 100 seeds were transferred to sterile Erlenmeyer flasks containing 100 mL of sterile saline solution (0.85%) with Tween 80 (0.4 mL L^{-1}), and the samples were submitted to horizontal agitation at 150 rpm for 20 min, resulting in the dilution 10^0. From this suspension, decimal serial dilutions of 10^{-1} to 10^{-7} were prepared and spread in Petri dishes containing modified-YMA medium with 10% of Congo red (25 mg L^{-1}) as indicator of contaminants, that appear as dark red color colonies, and 200 µL cycloheximide (56 µg L^{-1}

in ethanol) and vancomycin (1 µg mL^{-1}) to decrease natural seed contamination. Inoculated plates were incubated at 28 °C ± 2 °C, in the dark, for 7 days. After this period, the CFU of each plate were evaluated, considering the plates with number of colonies ranging from 30 to 300 CFU.

At each sampling time evaluated, using a caliper rule, the diameter of the colonies of each treatment was estimated on 25% of all the colonies grown in each plate, by dividing each plate considered for the CFU counting in quarters and analyzing one quarter. Colony color and mucoidy were also evaluated.

In the last evaluation of cell recovery from inoculated soybean seeds treated with the pesticide, eight colonies were randomly chosen from the plate used for CFU counting. Each colony was transferred to modified-YMA medium by streaking. Plates were incubated at 28 °C ± 2 °C, in the dark, for 7 days. Following, after confirming the purity, each bacterium from each plate was cryopreserved at − 80 ºC in 1.5 mL of modified-YM medium with 30% of glycerol.

Twenty-µL of three cryopreserved colonies were grown in 10 mL of modified-YM medium at 28 °C for seven days at 120 rpm, diluted in saline solution at the 10^6 factor and 100 µL of the suspension was scattered in modified-YMA medium containing Congo red. Plates were incubated at 28 °C, in the dark, for seven days. The diameter of the colonies was estimated based on 25% of the colonies from each plate to verify if colony diameter remained the same as when they were recovered from seeds. Colony color and mucoidy were also evaluated.

A genomic fingerprint using the BOX-A1R primer was generated. The method was chosen for been broadly used for rhizobial strain identification (e.g. Menna et al. 2009) and adopted by the Brazilian legislation to confirm the identity of the strains in commercial inoculants (MAPA 2010). The DNA extraction, primers used and amplification conditions were as described by Chibeba et al. (2017). Bacteria used in this analysis were the cryopreserved colonies, selecting two colonies from the treatments with and without the pesticide with 2 h and 7 days of storage. For the 15-day period of storage, four cryopreserved colonies were selected per treatment. Therefore, the evaluation included a control not recovered from seeds, and 17 samples from each strain. For BOX-PCR identification all strains were first grown in modified-YM medium, followed by DNA extraction and amplification conditions were as described by Chibeba et al. (2017).

Symbiotic performance under controlled greenhouse conditions

The eight cryopreserved colonies from each treatment recovered from the seed treatments with pesticides and with anticipated inoculation of 15 days were transferred to modified-YM medium and grown at 28 ± 2 °C, in the dark, for 7 days, at 120 rpm. Following, an aliquot of each pre-inoculum was transferred to modified-YM medium and grown under the same conditions. After the incubation period, the concentration of each culture was adjusted by optical density (OD at 600 nm) to 0.300–0.350, corresponding to approximately 4×10^8 CFU mL^{-1}. The procedure was repeated with cryopreserved cells that had not been in contact with seeds, using the same procedure, starting from growth in modified-YM medium.

Soybean seeds of cultivar M5947 IPRO were surface-disinfected in 70% ethanol for 1 min, followed by 10% hypochlorite for 4 min, and then washed six times in sterile water. Seeds were dried at room temperature under sterile conditions. For each treatment, five modified Leonard jars (Yates et al. 2016) containing ground coal and sand 1:3 (v:v) and N-free nutrient solution of Broughton and Dilworth (Yates et al. 2016) were prepared and sterilized. Five seeds were sown per jar and then 1 mL of each bacterium inoculum was applied per seed. A non-inoculated control was included. Five days after emergence (DAE), shoots were thinned to two per vessel.

Soil–plant analysis development (SPAD) was applied to estimate SPAD index at 33 DAE (days after emergence), and harvest was carried out at 35 DAE. Roots and shoots were separated at the cotyledonary node. Nodulated roots were washed, and shoots and nodulated roots were dried at 50 °C until constant weight. Nodules were detached and count. Shoot dry weight was determined and shoots were ground (18 mesh); following, N content in shoots was determined based on the Kjeldahl digestion of sulfuric digests by the green salicylate method (Feigl and Anger 1972).. Nodule dry weight was also determined.

A flowchart of the steps used for cell counting, analysis of colony morphology, and in vivo experiments is shown in supplementary Fig. 1.

Symbiotic performance under field conditions

Sites description, area preparation, sowing and trials conduction

Two field experiments were carried out, in Lutécia, SP, Brazil (22º12′23.1″S, 50º25′59.5″W, 460 m altitude), and in Paranavaí, PR, Brazil (22º57′29.9″S, 52º27′88.3″W, 405 m altitude; both sites had no previous history of cropping soybean and expected to have close to zero population of compatible soybean bradyrhizobia. In both sites, the climate is classified as *Cfa* (mesothermic, subtropical humid), according to Köppen-Geiger classification, and in both sites soils were predominantly sandy. The sowing in Paranavaí and Lutécia was performed in 23 and 30 of October of 2018, respectively. Before sowing, soil samples (0–20 cm) were collected for determination of

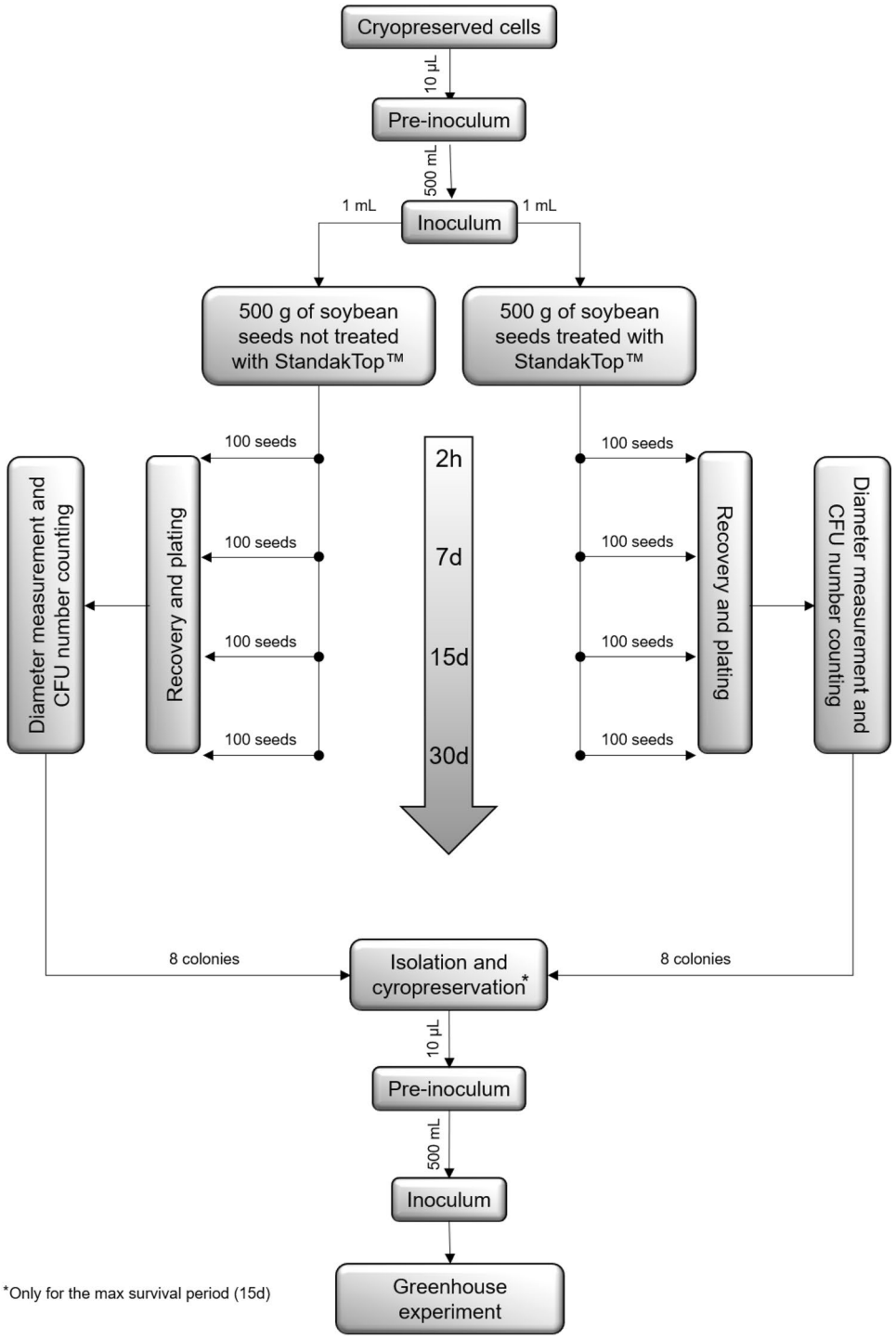

Fig. 1 Flowchart of the steps used for cell counting, analysis of colony morphology, cell recovery from seeds, and greenhouse experiment

soil chemical (Silva 2009) and granulometric (Donagema et al. 2011) properties (Table 1). The soybean-nodulating rhizobial population was estimated using the most probable number (MPN) method (O´Hara et al. 2016), using soybean cultivar BRS 1010IPRO as trapping host; as both sites were a first-year cropping, estimates confirmed no compatible soybean bradyrhizobial population (Table 1). Monthly data of rainfall, potential evapotranspiration, and maximum and minimum air temperatures during the cropping season in Lutécia and Paranavaí were obtained from the Integrated Center for Agrometeorological Information (https://www.ciiagro.sp.gov.br/ciiagroonline/) and the agrometeorological station of the Agronomic Institute of Paraná (www.iapar.br), respectively, and are shown in Fig. 2. The cropping season was unusually drier, since potential evapotranspiration was higher than rainfall

Table 1 Soil chemical and granulometric properties, and soybean bradyrhizobia population before sowing, at the 00–20 cm layer, in the experiments performed in Paranavaí-PR and Lutécia-SP

Site	Depth cm	pH ($CaCl_2$)	P mg dm^{-3}	Al^{3+} $cmol_c$ dm^{-3}	H + Al	Ca^{2+}	Mg^{2+}	K^+	SB	CEC	S %	C g dm^{-3}	Clay %	Silt	Sand	Rhizobia cells g^{-1}
Paranavaí	00–20	5.1	2.6	0.0	1.88	0.73	0.41	0.14	1.28	3.16	41	5.4	6.5	2.85	90.65	Zero
Lutécia	00–20	5.1	2.9	0.0	1.99	0.91	0.72	0.13	1.75	3.74	47	6.3	10.30	3.10	86.60	Zero

SB sum of bases; *CEC* cation exchange capacity; *S* saturation of bases

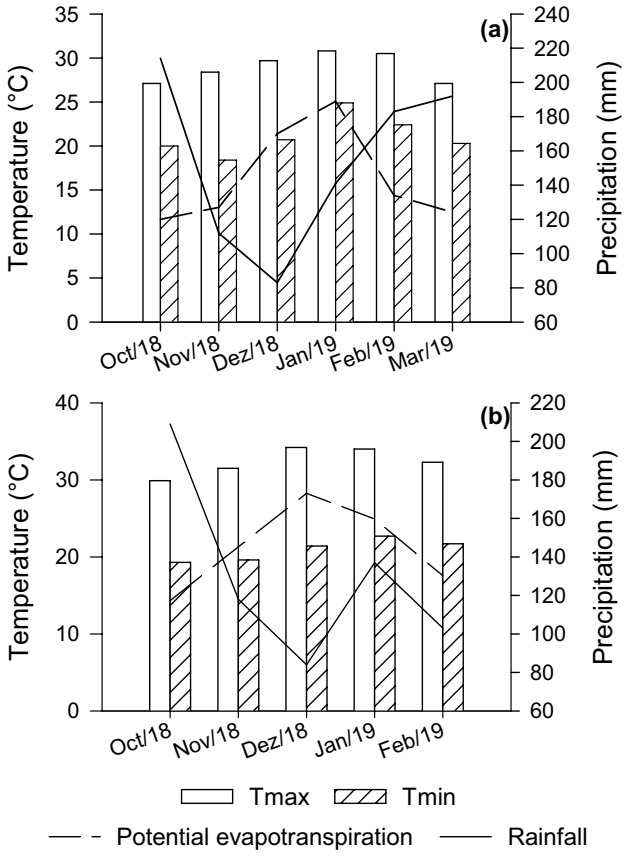

Fig. 2 Climate data in the experimental sites during the trials period in **a** Lutécia-SP, and **b** Paranavaí-PR

between November to January in both sites, extending until February in Paranavaí (Fig. 2).

Fifty days before sowing, the pH of the soil was evaluated and lime was applied to reach approximately 5.5. Thirty days before sowing. glyphosate was applied (1.5 L ha^{-1}). Immediately before sowing. 300 kg of the N-P-K formulation 00–20-20 (60 kg ha^{-1} of P_2O_5 and 60 kg ha^{-1} of K_2O) were applied in-furrow in all treatments using a no-till sowing machine. In the NI + N treatment, 100 kg ha^{-1} of N as urea (215 kg of urea, 46,6% of N) were applied by surface spreading and slight incorporation.

Treatments were defined in a factorial design of 2 × 4 with six replicates. The first factor was represented by the absence or presence of StandakTop™ (2 mL of pesticide kg^{-1} of seed). The second factor consisted of the following treatments: (1) Non-inoculated control (NI); (2) Non-inoculate control with N-fertilizer (NI + N); (3) Inoculated with *B. japonicum* strain SEMIA 5079 + *B. diazoefficiens* strain SEMIA 5080, applied to deliver 1.2×10^6 cells $seed^{-1}$ (I); (4) Co-inoculated with *Bradyrhizobium* spp. strains SEMIA 587 and SEMIA 5079 applied to deliver 1.2×10^6 cells $seed^{-1}$ and *Azospirillum brasilense* strains Ab-V5 and Ab-V6, applied to deliver 1.2×10^5 cells $seed^{-1}$. The NI + N treatment received 200 kg N ha^{-1} as urea (46.6% N), half at the sowing by surface spreading and slight incorporation, and half when soybean reached R1 stage of soybean growth (Fehr and Caviness 1977), spread on the surface as topdressing. Each replicate plot measured 6 m × 4 m, consisting of 8 rows of 6 m spaced 0.5 m apart, and between plots 1 or 2 m, to prevent contamination by superficial run-off containing bacteria or fertilizer, caused by heavy rains that often occur in the summer season. Plant density was of about 300,000 plants ha^{-1}.

When the soybean reached the V5 stage of soybean growth (Fehr and Caviness 1977), 20 g ha^{-1} Mo (as $Na_2MoO_4.6H_2O$) and 2 g ha^{-1} Co (as $CoCl_2.6H_2O$) were applied as foliar spray. At the R1 stage of soybean growth (Fehr and Caviness 1977), 100 kg of N (215 kg of urea, 46,6% of N) were spread on the surface as topdressing in the NI + N treatment. All cultural and phytosanitary procedures followed the recommendations for the soybean crop in Brazil (Embrapa Soja, 2013).

Plant sampling, harvesting and analyses

At the V5 stage of growth (Fehr and Caviness 1977) six plants were randomly harvested from the second and seventh rows. At the laboratory, shoots were separated from roots at the cotyledonary node and dried at 50 °C until a constant weight was obtained (about 72 h). Nodules were removed from roots, counted, dried again and then weighed. Shoot dry weight was evaluated, as well as shoot N content based on the Kjeldahl digestion of sulfuric digests by the green salicylate method (Feigl and Anger 1972). Total N in shoots was obtained by multiplying the shoot N concentration by the shoot dry weight.

At physiological maturity, plants were harvested in the central area of each plot (6.75 m^2). The grains were cleaned, weighed and, after determination of moisture in a grain moisture analyzer (Gehaka, model AGRI G800), the mass was corrected to 13% of moisture. The N concentration in grains was also determined as for shoots. Total N in grains (kg ha^{-1}) was obtained by multiplying the N concentration in grains by grain yield.

Statistical analyses

All data obtained were tested for the normality of variables and variance homogeneity, followed by an analysis of variance (ANOVA) at $p < 0.05$. In case of significance of the ANOVA, on laboratory and greenhouse trials, means were compared by the Tukey test and, on field trials, means were compared by the SNK test, both at $p < 0.05$. In cases where the variables did not show normality and/or variance homogeneity, data were transformed to $\sqrt{(x+1)}$. These tests were performed using the ExpDes package of the R software (version 3.5.3, 2019).

For the data of rhizobia survival on seeds, regressions were performed with the number of CFU mL^{-1} transformed to log $(x+1)$ using the SMA (Standardized Major Axis) method (Warton et al. 2006). The significance ($p < 0.05$) of each regression was tested and, subsequently, the likelihood ratio test was performed to test the difference between the slopes of the lines in the absence and presence of the pesticide ($p < 0.05$) (Warton and Weber 2002) using the smatr package (Standardised Major Axis Tests and Routines) (Warton et al. 2012) of the R software (version 3.5.3, 2019).

The means of all variables in all treatments, except for the non-inoculated, but with N-fertilizer, were correlated with each other by Pearson's correlation and a heatmap was elaborated in R software (version 3.5.3, 2019) from this correlation.

Results

Cell number, colony morphology and DNA fingerprinting of strains recovered from inoculated soybean seeds

Although viable cells populations of both *B. elkanii* SEMIA 587 and *B. japonicum* SEMIA 5079 recovered from the seeds decreased with time even in the absence of the pesticides, the contact with StandakTop™ drastically decreased the survival of the inoculated bacteria, such that very low populations were detected in seeds carrying pesticides after 15 days of inoculation (Fig. 3). The results were confirmed by the likelihood ratio test, confirming an increased mortality rate in the presence of the pesticides (Fig. 4).

The longer the storage period, the smaller the sizes of the colonies recovered were, and the colony size reduction was significantly intensified by the presence of the pesticides (Fig. 5). *B. japonicum* (Fig. 5b) was less sensitive than *B. elkanii* (Fig. 5a) to morphological changes in the evaluation performed 7 days after inoculation. In relation to color and

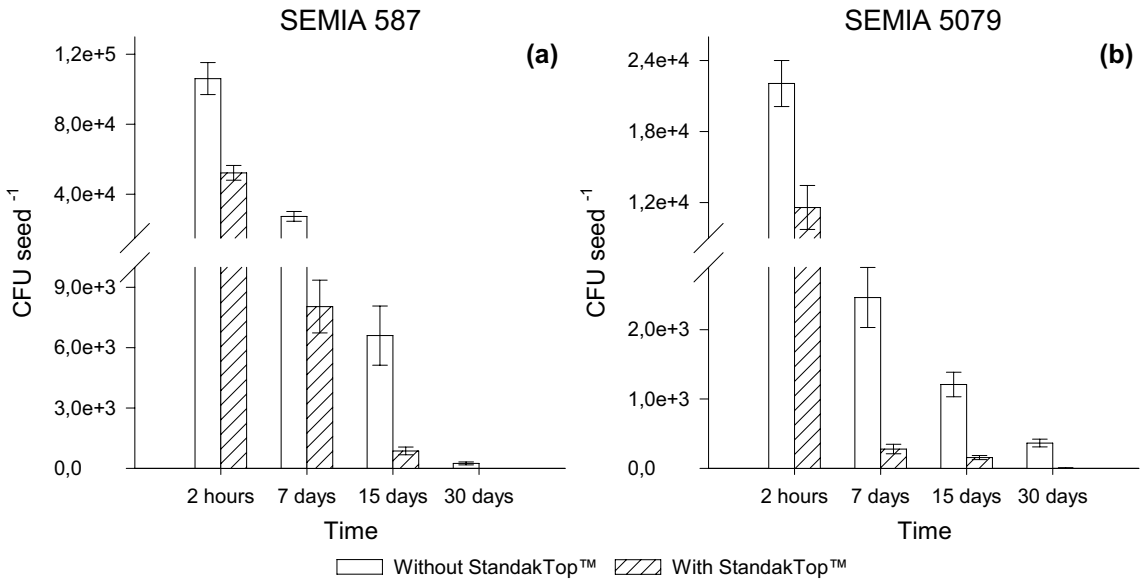

Fig. 3 Recovery of viable cells of **a** *Bradyrhizobium elkanii* strain SEMIA 587 and **b** *Bradyrhizobium japonicum* strain SEMIA 5079 from inoculated soybean seeds, in the presence or abscence of the pesticides and with different times of storage. Data represent the means of three replicates, and vertical bars denote standard deviation

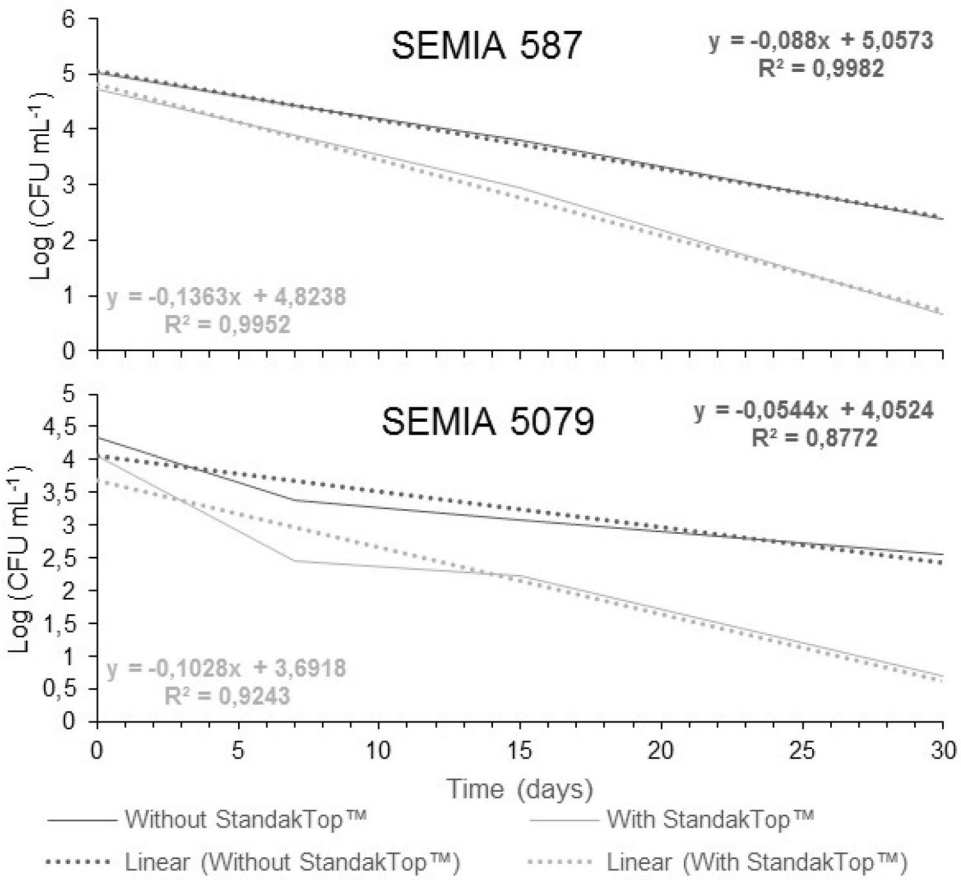

Fig. 4 Linear estimation with the log of the cell number of **a** *Bradyrhizobium elkanii* SEMIA 587 and **b** *Bradyrhizobium japonicum* SEMIA 5079 recovered from soybean seeds. Mathematical formulas in dark gray represent the linear functions in the treatments without pesticides and in light gray the linear functions in the treatments with pesticides. By the likelihood ratio test there were significant differences between slopes of the treatments with and without pesticides on both species ($p < 0.05$)

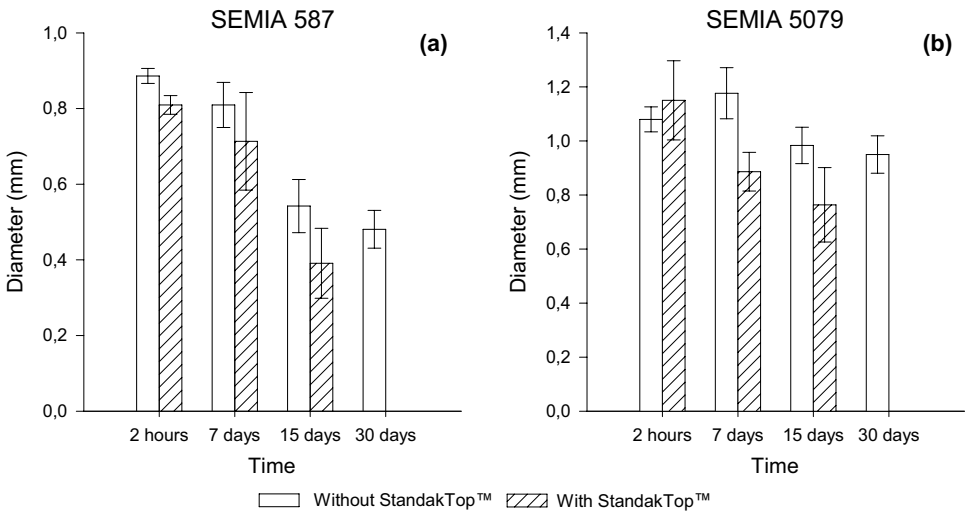

Fig. 5 Diameter of the colonies of **a** *Bradyrhizobium elkanii* SEMIA 587 and **b** *Bradyrhizobium japonicum*, SEMIA 5079 recovered from soybean seeds treated or not with pesticides and with different times of storage. Data represent the means of three replicates per treatment, and vertical bars denote standard deviation

mucoidy of the colonies, we observed no differences. Figure 6 illustrates two plates of cells recovered from inoculated seeds after 7 days of treatment in the absence and in the presence of pesticides.

Despite the reduction in colony sizes, when the smaller colonies were grown in modified-YMA, followed by cryopreservation, and then grown again in the same culture medium, they recovered their original colony sizes, indicating that the changes in colony sizes were not permanent (Fig. 7).

The BOX-PCR analysis indicated no changes in the DNA profiles of both SEMIA 587 and SEMIA 5079, neither by the exposure to the pesticide, nor by the storage time, nor by the interaction of pesticide x storage time (Fig. 8).

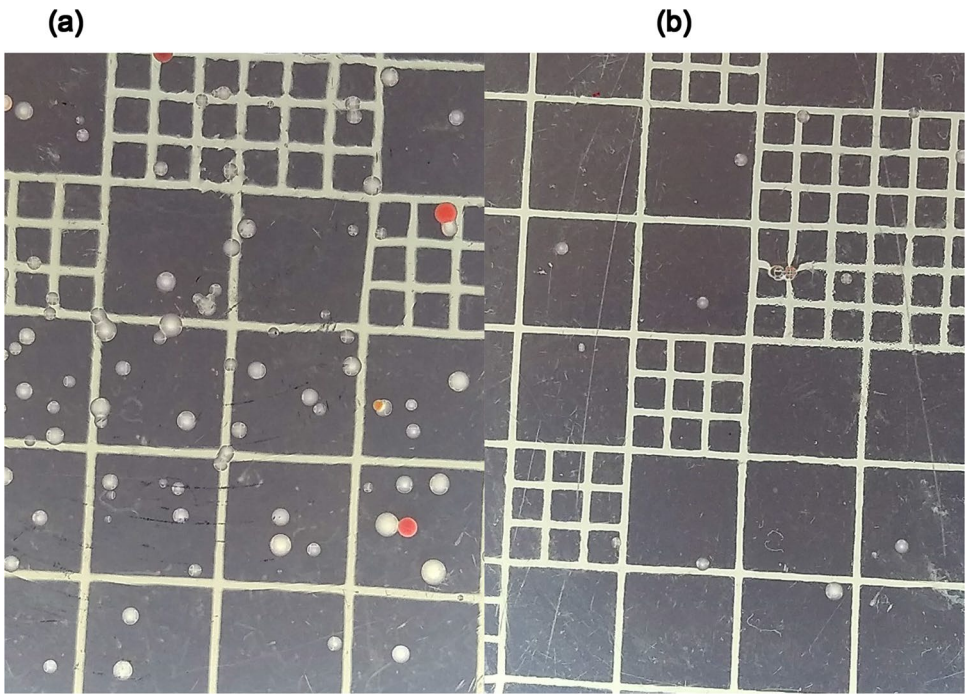

Fig. 6 Colonies of *Bradyrhizobium japonicum* strain SEMIA 5079 recovered from seeds of soybean after seven days of inoculation and **a** non-treated or **b** treated with pesticides. Colonies with red color refer to contaminants usually found in soybean seeds, easily recognized in culture medium containing Congo red as indicator

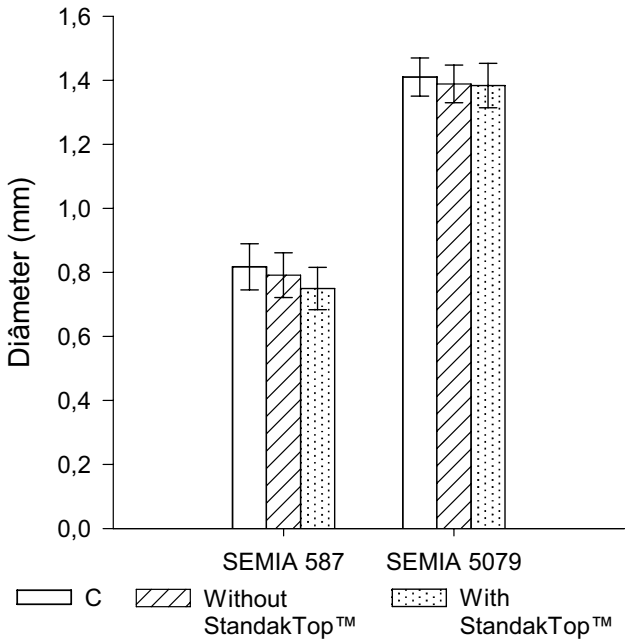

Fig. 7 Diameter of the colonies of *Bradyrhizobium elkanii* SEMIA 587 and *Bradyrhizobium japonicum* SEMIA 5079 of the control treatment (C) with bacteria from the culture collection that had never been exposed to pesticides or used as inoculant, and bacteria recovered from soybean seeds treated or not with the pesticides and stored by 15 days, followed by growth in culture medium without pesticides. Data represent the means of three replicates, and vertical bars denote standard deviation. There were no significant differences between the treatments in each rhizobial species by the Tukey test ($p < 0.05$)

Symbiotic performance

Experiment under controlled greenhouse conditions

For both strains, no differences between the treatments were verified considering treatment with the pesticides and time of treatment in any of the evaluated parameters: nodule number (NN) and dry weight (NDW), root dry weight (RDW), shoot dry weight (SDW), SPAD index and shoot N concentration (SNC) (Fig. 9). Therefore, under controlled greenhouse conditions, the symbiotic performance was not affected by the pesticides, the storage time, or the interaction of pesticides x storage time. Differences were observed only in the comparison of the strains, with better symbiotic performance of *B. japonicum* SEMIA 5079.

Experiments under field conditions

It is important to highlight again that both areas had no previous history of cropping soybean, showing no detectable population of compatible soybean bradyrhizobia (Table 1), and under these conditions the survival of inoculated cells is even more critical for nodulation. In Paranavaí, at the V5 plant growth stage, the pesticides increased nodule dry weight (NDW) of plants inoculated with *Bradyrhizobium* spp. strains SEMIA 5079 + SEMIA 5080, but not of plants co-inoculated with the same strains of *Bradyrhizobium* and with *A. brasilense* strains Ab-V5 + Ab-V6 (Table 2). In addition, the highest NDW was observed in the co-inoculated treatment, both in the absence, and in the presence of the

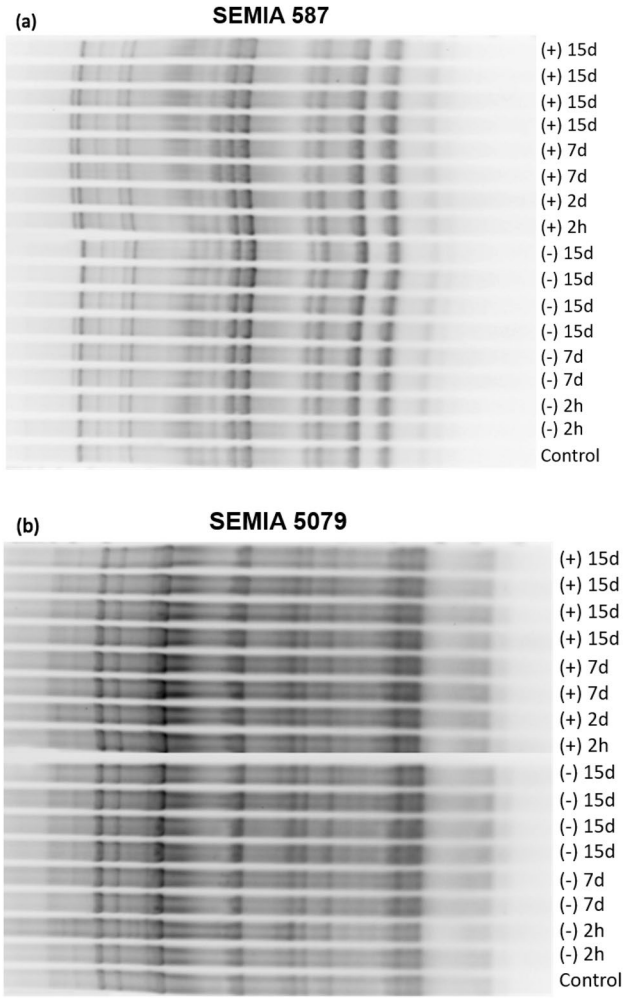

Fig. 8 DNA profiles obtained by BOX-PCR of bacteria recovered from soybean seeds treated or not with pesticides, after different periods of storage (2 h, 7 and 15 days). **a** *Bradtrhizobium elkanii* strain SEMIA 587, **b** *Bradyrhizobium japonicum* strain SEMIA 5079. With: bacteria from seeds treated with StandakTop™; Without: bacteria from seeds not treated with StandakTop™; Control: bacteria that were not recovered from seeds

pesticides, significantly higher than with the single inoculation with *Bradyrhizobium*. At this same evaluation time, no effects of the pesticides were observed in shoot dry weight (SDW), shoot N concentration (SNC) and total N accumulated in shoots (TNS), except for a negative effect on SNC observed in the non-inoculated control treatment with N-fertilizer (NI + N). In general, the NI + N treatment had superior performance in all these parameters, in comparison to the other treatments.

In Paranavaí, at the physiologic maturity stage, in the treatments without StandakTop™, grain yield (GY) was higher in the NI + N treatment, followed by co-inoculation with *Bradyrhizobium* + *Azospirillum* (I + Azo), single inoculation with *Bradyrhizobium* (I), and non-inoculated control without N-fertilizer (NI). When treated with pesticides, GY of NI + N, I and I + Azo treatments were similar and higher than the NI treatment. Both in the absence and in the presence of the pesticides, total N accumulated in grains (TNG) was higher in the NI + N treatment, followed by the I + Azo and the I treatment. StandakTop™ decreased GY in both NI + N and I + Azo treatments, and TNG in all treatments (Table 2).

In Lutécia, at the V5 stage, nodulation was higher in the inoculated and co-inoculated treatments, and in both cases was not affected by the pesticides (Table 2). On the contrary, SDW was reduced by the treatment with StandakTop™ in all treatments (Table 2). The pesticides increased SNC but not TNS in the treatment single inoculated with *Bradyrhizobium*.

At the physiological maturity in Lutécia, treatments NI + N, inoculated and co-inoculated produced more grains than the NI treatment, both in the absence and in the presence of the pesticides (Table 2). In relation to TNG, the highest accumulation was always achieved with the co-inoculation of *Bradyrhizobium* and *Azospirillum*. Although GY was not affected by the presence of StandakTop™, TNG was reduced by the pesticides in all treatments. (Table 2).

A heatmap was built and showed that in Paranavaí (Fig. 10a) the treatments with and without the pesticides in the group single inoculated *Bradyrhizobium* (I) were clustered in clades of low similarity. In the absence of the pesticides, both non-inoculated controls were clustered, and in general, negative correlations were observed with the variables. In the presence of the pesticides, the treatment of inoculation (I) was clustered together with the co-inoculation (I + Azo), with positive correlations with the variables. It was also verified that the nodulation parameters showed greater relationship with the SDW and TNS parameters than with the grain yield (GY) and TNG.

In Lutécia, the heatmap (Fig. 10b) exhibited a different correlation pattern than in Paranavaí (Fig. 10a). The treatment inoculated with *Bradyrhizobium* and treated with the pesticides showed lower similarity with the NI treatments (Fig. 10b). Still contrasting with the results obtained in the experiment of Paranavaí, the nodulation showed higher relationship with GY and TNG than with SDW and TNS.

Discussion

Soybean has been considered an outstanding crop in relation to the benefits that can achieve with biological nitrogen fixation, and the right combination of plant genotypes and bradyrhizobia strains can fully supply N demands even of high-yielding cultivars (e.g. Hungria and Mendes 2015; Hungria and Nogueira 2019). Care should then be taken to maintain the biological contribution. Our data confirm

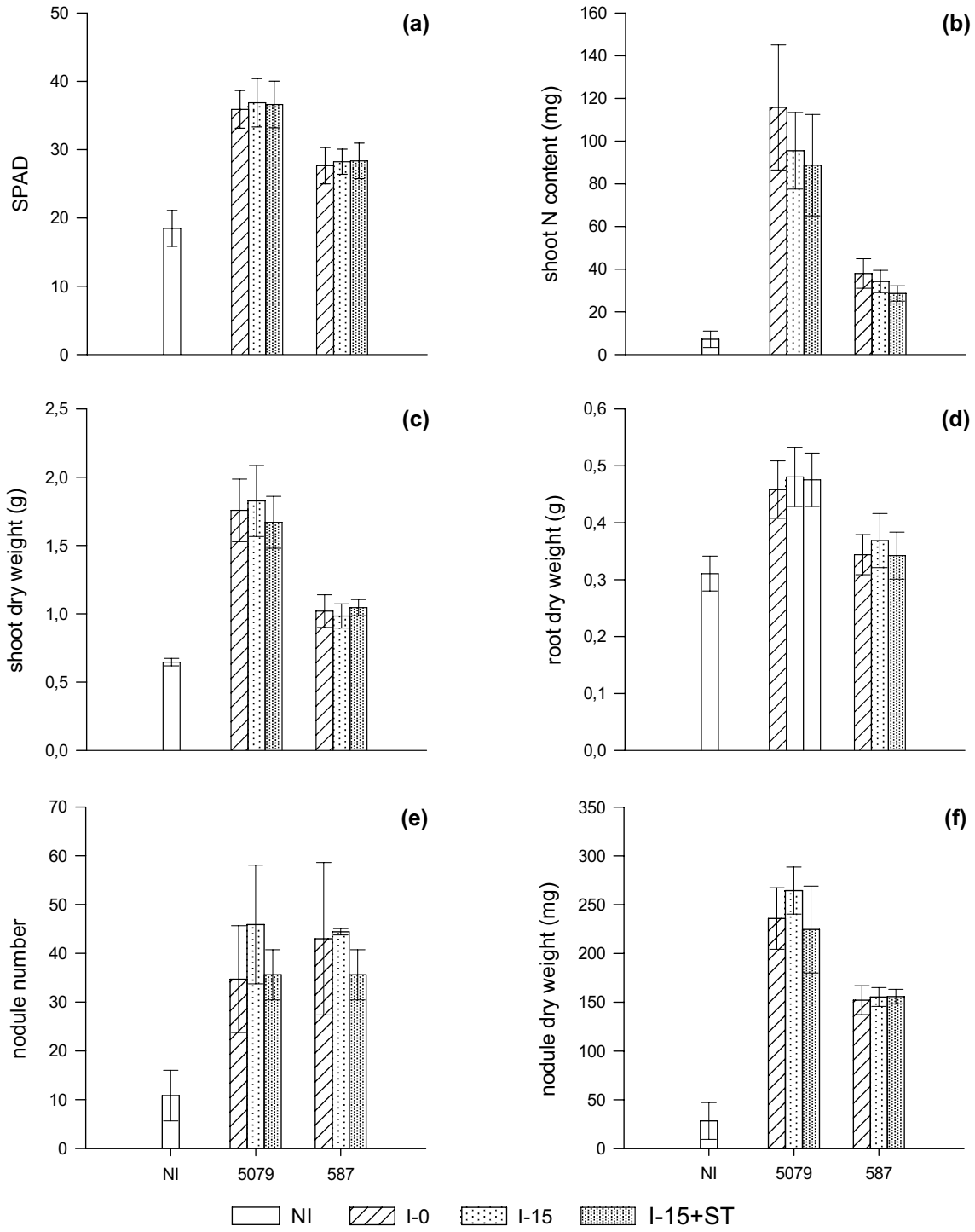

Fig. 9 Evaluation of symbiotic parameters on soybean under controlled greenhouse conditions: **a** SPAD (Soil–plant analysis development) index, **b** shoot N content, **c** shoot dry weight, **d** root dry weight, **e** nodule number, **f** nodule dry weight. In the abscissas axis: (NI) non-inoculated; (5079) inoculated with *Bradyrhizobium japonicum* SEMIA 5079; (587) inoculated with *Bradyrhizobium elkanii* SEMIA 587. Legends: (NI) non-inoculated, (I-0) inoculated at the sowing day; (I-15) inoculated with bacteria recovered 15 days after inoculation; (I-15 + ST) inoculated with bacteria recovered 15 days from seeds treated with StandakTop™. Vertical bars represent standard deviation

Table 2 Symbiotic performance of soybean with seed treatments consisting of treatment or not with the pesticide StandakTop™ and the inoculation treatments of control with no-inoculation without (NI) or with 200 kg of N-fertilizer ha^{-1} split 50% at sowing and 50% at early flowering (NI + N), inoculated with Bradyrhizobium japonicum strain SEMIA 5079 + Bradyrhizobium diazoefficiens strain SEMIA 5080 (I) and co-inoculated with the same strains of *Bradyrhizobium* and *Azospirillum brasilense* strains Ab-V5 + Ab-V6 (I + Azo). Experiments performed in Paranavaí-PR and Lutécia-SP, Brazil

		Paranavaí							Lutécia						
		NN [a]	NDW	SDW	SNC	TNS	GY	TNG	NN	NDW	SDW	SNC	TNS	GY	TNG
		V5 stage					Maturity		V5 stage					Maturity	
		(n° pl^{-1})	(mg pl^{-1})	(g pl^{-1})	(mg N g^{-1})	(mg N pl^{-1})	(kg ha^{-1})	(kg N ha^{-1})	(n° pl^{-1})	(mg pl^{-1})	(g pl^{-1})	(mg N g^{-1})	(mg N pl^{-1})	(kg ha^{-1})	(kg N ha^{-1})
Without StandakTop™	NI	0.0 Ac[b]	0.0 Ac	2.7 Ac	17.5 Ac	47.4 Ac	1021 Ad	53 Ad	0.2 Ab	2.1 Ab	6.0 Aa	19.6 Aa	117.5 Aa	2319 Ab	133 Ac
	NI + N	0.0 Ac	0.0 Ac	5.4 Aa	29.8 Aa	162.0 Aa	2659 Aa	157 Aa	0.8 Ab	0.3 Ab	7.5 Aa	22.2 Aa	165.3 Aa	3128 Aa	176 Aab
	I	2.6 Bb	17.4 Bb	2.5 Ac	21.2 Abc	52.4 Ac	1532 Ac	88 Ac	10.9 Aa	81.9 Aa	5.1 Aa	19.2 Ba	98.6 Aa	2728 Aa	153 Ab
	I + Azo	9.3 Ba	97.8 Aa	3.5 Ab	24.8 Ab	87.6 Ab	2052 Ab	127 Ab	14.9 Aa	100.2 Aa	5.0 Aa	22.4 Aa	111.9 Aa	3026 Aa	176 Aa
With StandakTop™	NI	0.0 Ac	1.0 Ac	2.0 Ac	21.4 Aa	40.5 Ad	815 Ab	24 Bd	0.1 Ab	1.5 Ab	5.2 Ba	21.8 Aa	112.8 Aa	2156 Ab	80 Bc
	NI + N	0.3 Ac	1.1 Ac	6.0 Aa	23.1 Ba	138.2 Aa	1607 Ba	101 Ba	0.1 Ab	0.8 Ab	5.3 Ba	20.7 Aa	108.8 Aa	2769 Aa	126 Bab
	I	6.3 Ab	64.2 Ab	3.3 Ab	22.6 Aa	74.6 Ac	1442 Aa	59 Bc	11.8 Aa	69.2 Aa	4.3 Ba	23.5 Aa	101.3 Aa	2845 Aa	120 Bb
	I + Azo	12.0 Aa	118.3 Aa	3.5 Ab	25.1 Aa	83.1 Ab	1550 Ba	70 Bb	17.5 Aa	84.3 Aa	5.4 Ba	20.1 Aa	108.4 Aa	2863 Aa	134 Ba
C.V. (%)		28.22 [c]	26.79*	20.25	17.24	11.5*	19.63	22.04	37.51*	38.87*	12.06*	14.41	15.7*	10.18	14.08

[a]*NN* nodule number; *NDW* nodule dry weight; *SDW* shoot dry weight; *SNC* shoot N concentration; *TNS* total N accumulated in shoots; *GY* grain yield; *TNG* total N accumulated in grains

[b]Data represent the means of six replicate, capital letters represent the comparison within the inoculant treatment by the SNK test ($p < 0.05$) and lower case letters represent the comparison within of a same pesticide treatment by the SNK test ($p < 0.05$)

[c]Values transformed into $\sqrt{(x+1)}$ for the statistical analysis

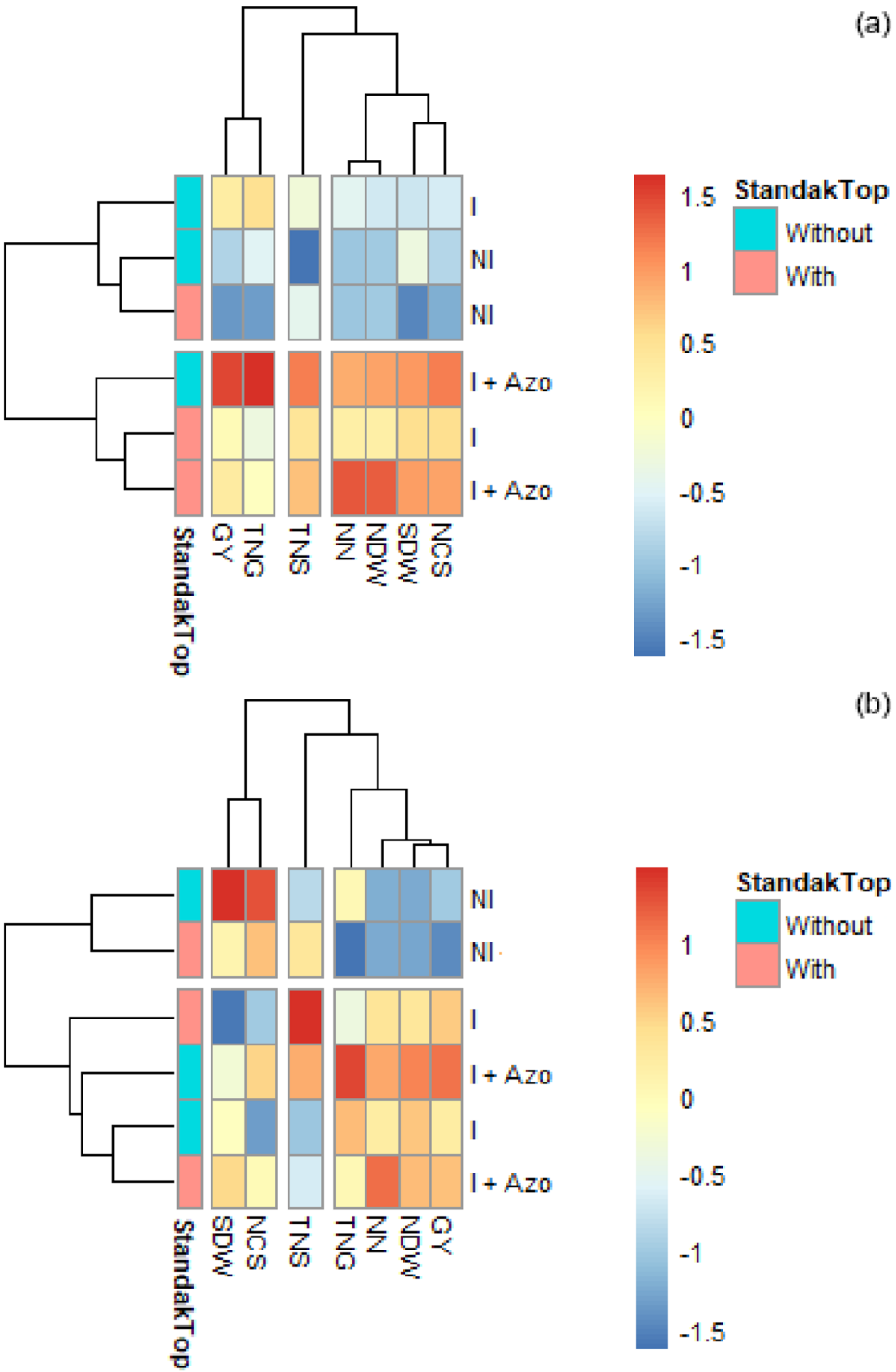

Fig. 10 Heatmaps correlating the variables and treatments obtained in the field experiments performed in **a** Paranavaí and **b** Lutécia. Lines cluster treatments and columns cluster variables. NI: non-inoculated; I: inoculated with *Bradyrhizobium*; I + Azo: co-inoculated with *Bradyrhizobium* and *Azospirillum*. *NN* nodule number; *NDW* nodule dry weight; *SDW* shoot dry weight; *NCS* N concentration in shoots; *TNS* total N accumulated in shoots; *GY* grain yield; *TNG* total N accumulated in grains

previous reports showing the negative impact of pesticides on rhizobial survival (e.g. Campo and Hungria 2000; Campo et al. 2009; Ferreira et al. 2011; Kunal and Sharma 2011; Costa et al. 2013), drastically decreasing the number of viable cells recovered from inoculated seeds with time, such that no surviving cells were detected 30 days after inoculation, neither of *B. japonicum* SEMIA 5079, nor of *B. elkanii* SEMIA 587. Therefore, the intrinsic properties of strains of the *B. elkanii* group, of higher tolerance of antibiotics and other molecules (Kuykendall et al. 1988; Delamuta et al. 2013) did not confer higher tolerance to StandakTop™, composed of the fungicides pyraclostrobin and thiophanate-methyl and the insecticide fipronil.

In both *Bradyrhizobium* strains, the contact with the pesticides resulted in morphological change, reducing colony sizes, but not color or mucoidy. However, the reduction in colony size was not permanent, being recovered when the stress was eliminated. Cell wall thickening was observed in *Bradyrhizobium lupini* WU425 after transfer from broth cultures into peat, and suggested as an adaptive response for long-term survival under nutrient-limited conditions (Feng et al. 2002). This increased capacity for desiccation tolerance was confirmed in *B. diazoefficiens* CB 1809 cultured in peat in comparison to liquid broth, and suggested as multifactorial involving the accumulation of trehalose, increased expression of proteins involved in protection of the cell envelope, repair of DNA damage, oxidative stress responses, and maintenance of stability and integrity of proteins (Casteriano et al. 2013). We found no studies with rhizobia reporting changes and recovery of colony size after exposure to pesticides. However, in *Burkholderia pseudomallei*, Chantratita et al. (2007) reported that stresses such as nutrient starvation, high temperature and iron limitation resulted in morphological change, also attributed to a process of adaptation, and were also reversible after elimination of the stressing conditions. In another report, chemical stress caused by excess of N resulted in morphological changes in *Ralstonia solanacearum* (Wang et al. 2020). It is worth mentioning that we detected no genetic changes in the strains in the BOX-PCR DNA profile. Therefore, we may hypothesize that the changes in colony size observed in our study should represent an adaptive response towards survival in the presence of the pesticides, also reversible after elimination of the stressing conditions.

Under controlled greenhouse conditions, no effects of the pesticides in the symbiosis were observed, as the inoculant composed by strains that had been in contact with pesticides showed the same performance as the inoculant with non-exposed strains. However, one must consider that growth under optimized conditions is very different from the field. Usually, soybean optimum nodulation does not exceed 20 to 60 nodules per plant, as observed in the greenhouse experiment and, theoretically, only 20 to 60 *Bradyrhizobium* cells would allow this number of nodules, as each rhizobial cell can result in one nodule. On the contrary, under field conditions, this very low number of surviving cells would not result in proper nodulation, e.g. under field conditions in Brazil it has been shown that at least 1.2 million cells seed^{-1} are required to achieve successful nodulation and grain yield (Hungria et al. 2017).

Methyl thiophanate is a non-active component of StandakTop™, but it can be transformed into carbendazim, a molecule with fungicidal activity (Fleeker et al. 1974). In Brazil, under field conditions, Zilli et al. (2009) showed that the application of carbendazim + thiram reduced nodulation in soybean inoculated with *B. japonicum* SEMIA 5079 + *B. diazoefficiens* SEMIA 5080 and with *B. elkanii* SEMIA 587 + SEMIA 5019 strains, while Campo and Hungria (2000) observed that the combinations of carbendazim and captam, or thiram or tolyfluanid also decreased nodulation of plants inoculated with SEMIA 5079 and SEMIA 5080 strains. However, there are also studies showing no deleterious effects of pesticides in the symbiosis. Bueno et al. (2003) observed no effects on nodule number of soybean inoculated with *B. elkanii* SEMIA 5019 + *B. japonicum* SEMIA 5079 with the applications of carbendazim + thiram or thiophanate-methyl + tolylfluanid, while Gomes et al. (2017) detected no effects on nodulation by *B. japonicum* SEMIA 5079 and *B. diazoefficiens* SEMIA 5080 with the treatment of carbendazim and thiram with the insecticide fipronil;however, both studies were performed under controlled greenhouse conditions, with the limitations of requirement of low number of cells discussed above. In our study, under field conditions, NDW was not affected by the treatment with StandakTop™. We may attribute the lack of effect to the application of a high concentration of cells per seed and immediately sowing, highly recommended for a successful nodulation under these conditions (Hungria and Nogueira 2019). The surviving cells would be capable of promoting nodulation according to the plant´s capacity under these conditions, resulting in similar nodulation in the absence and in the presence of the pesticides. Interestingly, despite similar nodulation, some negative effects in plant growth and N accumulation started to be observed at this same harvest, such as on SDW in Lutécia.

Co-inoculation of soybean with the same strains of *Bradyrhizobium* spp. and *A. brasilense* used in our field experiments can result in considerable improvements in soybean nodulation and grain yield (Hungria et al. 2013, 2015; Chibeba et al. 2015), and can also improve symbiotic performance by mitigating drought stress (Cerezini et al. 2016). Our hypothesis was that the co-inoculation could help to mitigate the negative impact of the pesticides under field conditions, although the survival and colonization capacity of *A. brasilense* can also be affected by pesticides (Santos et al. 2020). Improvement in nodulation due to the co-inoculation in comparison to the single nodulation with *Bradyrhizobium* was observed, with statistical difference in Paranavaí, where also resulted in higher accumulation of N shoots.

The effectiveness of the biological nitrogen fixation process even in edaphoclimatic limiting conditions as those of the two field experiments, and in first-year cropping areas was highlighted, as grain yield of inoculated and co-inoculated treatments was statistically similar to those of the non-inoculated treatment receiving 200 kg of N ha^{-1} in three out of the four experiments, confirming previous reports by our group (Hungria et al. 2016, 2017, 2020, 2020; Hungria and Mendes 2015; Hungria and Nogueira 2019). In the

only experiment with superior performance of the NI + N, in Paranavaí without pesticides, indicating limiting conditions to the nitrogen fixation, co-inoculation favored GY in relation to the single inoculation, confirming previous reports (Hungria et al. 2013, 2015), indicating help in the mitigation of the limitation conditions. StandakTop™ impacted negatively GY in only two treatments, NI + N and co-inoculation in Paranavaí.

For the N accumulated in grains, co-inoculation always resulted in higher TNG than single inoculation, and in Lutécia did not differ from the NI + N treatment. Interestingly, StandakTop™ reduced TNG in all treatments and both sites. During grain filling, N is remobilized from leaves to grains (Sinclair and Wit 1976; Mastrodomenico and Purcell 2012), resulting in reduction of chlorophyll content and yellowing of leaves. In soybean, piraclostrobin + boscalid applied to the seeds may cause green stem disorder (Hill et al. 2013), indicating that the molecules may cause effects on N remobilization from leaves to grains. Under drought conditions, piraclostrobin also causes senescence delay in wheat (*Triticum aestivum* L), through changes in ACC (aminocyclopropane-1-carboxylic acid) synthase activity and ethylene synthesis, and increase of green leaf number and photosynthetic rate in sugarcane (*Saccharum* spp.) (Köehle et al. 2002; Kanungo and Joshi 2014; Lopes et al. 2018). This could explain the relevant decreases in TNG observed in our experiment, in plants relying both on biological nitrogen fixation and on N-fertilizer. Other studies reported that applications of combinations of carbendazim with thiram, captan or tolylfluanid reduced GY and TNG of soybean inoculated with *Bradyrhizobium* spp. (Campo and Hungria 2000; Zilli et al. 2009).

Nitrogen is key for protein synthesis and in soybean protein plays a key economic role, being equivalent, in quality, to animal protein, and higher than in other plants used as protein sources (UNCTAD 2016). The low N accumulation in the grains may cause impacts in the productive chain, for example, in the livestock, processed soybean is used as protein source to animal feed; therefore, strategies to increase soybean protein content must be searched for and biological nitrogen fixation plays a key role (e.g. Hungria et al. 2020). In our study, as the effect of the pesticides on N content of the grains persisted in all treatments, the effect may be attributed to biological nitrogen fixation, or to the assimilation of mineral N, or also to mechanisms of N mobilization from the shoot to the grains. Further investigation to clarify this observation is needed, as it can highly affect protein production.

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Author contributions Conceived and designed the experiments: TFR, MAN, MH. Performed the experiments: TFR, FRB, AWSS, EF. Analyzed the data: TFR, FRB, AWSS, EF, MAN, MH. Contributed reagents/materials/analysis tools: MH Wrote the paper: TRF, MAN, MH. All authors read and approved the final manuscript.

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Data availability All datasets generated or analyzed during this study are included in the manuscript, and complementary dataset will be available upon request to the corresponding author.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval The authors declare no ethical conflicts.

Informed consent Authors declare that they have consented to participate in the manuscript and publish it.

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