International Journal of Recycling of Organic Waste in Agriculture (2022)11: 229-245
Doi: 10.30486/IJROWA.2021.1909458.1134

ORIGINAL RESEARCH

Azospirillum brasilense and organomineral fertilizer co-inoculated with *Bradyrhizobium japonicum* on oxidative stress in soybean

Fernanda Carvalho Barros[1], Reginaldo de Camargo[1], Regina Maria Quintão Lana[1], Miguel Henrique Rosa Franco[1], Mayara Cristiana Stanger[1], Vanderley José Pereira[1], Ernane Miranda Lemes[1*]

Received: 14 September 2020 / Accepted: 08 December 2021 / Published online: 05 April 2022

Abstract

Purpose Nitrogen-fixing bacteria (NFB) are essential for biologically fixing nitrogen in natural and soybean crop systems. The use of organomineral fertilizers is a promising crop management tool made from the mixing of an organic source and mineral fertilizers. This study evaluated the efficacy of an organomineral fertilizer (sewage sludge-based) as a carrier for the *Azospirillum brasilense* inoculation and the influence of *A. brasilense* plus *Bradyrhizobium japonicum* on the soybean antioxidant metabolism.

Method The experiment was performed under greenhouse conditions. The soybean cultivar (AS 3680 IPRO) was evaluated in a *4×2(+1)* factorial scheme, corresponding to four doses of *A. brasilense* [0, 100, 200, 300 mL ha^{-1} (2x10^{8} viable cells mL^{-1}) per 50 kg^{-1} of soybean seeds], with or without *B. japonicum* [100 mL ha^{-1} (5x10^{9} viable cells mL^{-1}) per 50 kg^{-1} of soybean seeds], and a control treatment (without inoculants or fertilizers). Soybean antioxidant metabolism (enzymes, oxidative processes, proline amino acid) was evaluated in different plant stages.

Results The seed inoculation with *A. brasilense* via organomineral fertilizer enhanced the soybean plant protection factors. Plant protection to stresses happened mainly by reductions in the superoxide dismutase activity, lipid peroxidation, and hydrogen peroxide concentration. The co-inoculation of *A. brasilense* with *B. japonicum* enhanced the proline activity at the V3 and R5 soybean phenological stage compared to *A. brasilense* inoculated alone.

Conclusion The studied organomineral fertilizer is an efficient inoculant carrier for *A. brasilense* in soybean plants via seed treatment. The protective results observed for *A. brasilense* were improved when co-inoculated with *B. japonicum*.

Keywords *Glycine max*, Biological nitrogen fixation, Growth-promoting bacteria, Joint inoculation, Antioxidant enzymes

Introduction

The symbiosis between nitrogen-fixing bacteria (rhizobia) and the soybean plants (*Glycine max* L.) forms the most important biological nitrogen fixation (BNF) system known. The process regularly occurs in structures formed in the roots known as nodules (Hungria et al. 2013). After the microorganism establishes these nodule formations, bacteria start to fix atmospheric nitrogen into plant-useful organic compounds. Soybean is a very important crop and is also highly dependent on BNF for high crop yields.

In addition to rhizobia symbiosis, other plant growth-promoting bacteria (PGPB) can associate and perform a wide range of biological processes that benefit plants, including the production of growth hormones and BNF. *Azospirillum* is a genus of PGPB that has been extensively studied; one of the expected effects of *Azospirillum* sp. inoculation is the increase in root hair production and root growth, which increase the absorption of water and nutrients by the plant and reduce plant stresses (Bashan et al. 2014; Chibeba et al. 2015).

✉ Ernane Miranda Lemes
ernanefito@gmail.com

[1] Federal University of Uberlândia (ICIAG - UFU), Minas Gerais state, Brazil

However, the mechanism of plant association with PGPBs can be directly affected by climate, soil variables, and biological interactions such as the population of phytonematodes (Elhady et al. 2020), raising the need for improved inoculation strategies.

The BNF efficiency can be improved with the co-inoculation (joint inoculation) with another inoculant, which is aligned with the current approach of modern agriculture: improved productivity (production per area) integrated with the sustainability of the agricultural, economic, social, and environmental concerns (Bárbaro et al. 2008, 2011). Besides being environmentally sustainable, co-inoculation is an up-and-coming technology for crop production (Hungria and Nogueira 2014).

The continuous demand for agricultural advances in crop production raises the need for studies on the dynamics of rhizobial populations in soils, as well as the application of new inoculation technologies, selection of cultivars and strains with a higher symbiotic affinity, and *Azospirillum* co-inoculation with *Bradyrhizobium* (Bárbaro et al. 2011). Basal and Szabó (2020) also indicated that plants inoculated with *B. japonicum* under water stress present a higher leaf area index than do non-inoculated plants. The studies on the physiological and molecular effects of organomineral fertilizers on plants under stress conditions are relatively recent but of great importance.

Although seed treatment is a routine operation, research has shown that products used in seed treatment may, in certain situations, decrease the survival of seed-associated organisms (Dan et al. 2011). Bacterial and fungal communities represent key soil quality bioindicators, playing essential roles for cycling and maintaining nutrient availability (Kadian et al. 2020). These communities respond differently depending on the crop products applied and the source of organic matter present, indicating that biodiversity shifts in the bacterial and fungal populations are prone to happen.

Other organic sources were studies and found to be unable to improve soil microbiota. This might be associated with the availability of specific nutrient sources provided by the composting materials (Escobar et al. 2020). Bouhia et al. (2021) reported that the application of biochar negatively impacted mycorrhizal symbiosis was not significant for other soil microorganisms studied; the authors also indicated that the short duration of the experiment (less than three months) could have affected the final results.

The use of mineral fertilizer material mixed with an organic source - called organomineral fertilizer - has several benefits, including modifying the property of an efficient soil conditioner. For high crop yield, large amounts of fertilizers must be implemented in quantity and balanced proportions. Organomineral fertilizer (Brasil 2005) research is being pushed due to the use and relative abundance of raw materials (organic residues), which are usually environmental liabilities of other production systems (Benites et al. 2010). Other organic sources, such as filter cakes, were initially designated as waste and residues of agribusiness but show great potential to prepare organominerals (Oliveira et al. 2017; Mota et al. 2019).

Camargo et al. (2020) reported recent advances in the knowledge and management techniques of organomineral fertilizers. They indicated that the inclusion of other technologies in this class of fertilizers contributes to the rise of its market share, representing about 30% of today's Brazilian fertilizer market. Silva et al. (2020) observed that organomineral fertilizers formulated with sugarcane filter cake could replace mineral fertilizer and increase soybean plant growth. These authors also indicated quantitative changes in lipid peroxidation, catalase, urease, and peroxidase activity after organomineral fertilizer application.

The effects of environmental stresses on plants, including nutritional deficiencies, cause the production of reactive oxygen species (ROS), which inactivate enzymes and damage the integrity of the cellular components. Examples of ROS include superoxide radicals ($O_2^{\circ-}$), hydroxyl radicals (OH°), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) (Scandalios 2005). The plant's reaction to eliminate ROS is a complex antioxidant system, including the use of several enzymes (Hernandez et al. 2001; Bartoli et al. 2012). When plants are subjected to environmental stresses - extreme temperatures, drought, salinity, ultraviolet radiation, pests - the damage caused can raise ROS production (Apel and Hirt 2004; Foyer and Noctor 2005).

To prevent the accumulation of ROS, plants use efficient non-enzymatic antioxidant defense systems [a) vitamins C and E, b) glutathione (GSH), c) β-carotene, d) phenolic compounds, e) tocopherols, and f) prolines (PROL)]; and enzymatic defense systems [a) enzymes superoxide dismutase (SOD), b) catalases (CAT), c) peroxidases (POD), d) glutathione peroxidase (GPX), e) ascorbate peroxidase (APX), f) glutathione reductase (GR) and g) glutathione S-transferase (GST)] (Scandalios 2005). This defense system allows the elimination of these reactive species and protection against oxidative damage (Hernandez et al. 2001; Fagan et al. 2016).

There are still many challenges to be solved that could result in significant yield gains. This improvement requires research, including investigating farmer's perceptions and difficulties, to subsequently provide viable technologies, such as the co-inoculation of PGPBs, to potentialize the gains from the BNF (Libório et al. 2018). However, more information about the integration of PGPBs with organomineral fertilizers as inoculum carriers is missing. Thus, the objective of this study was to evaluate the responses of the soybean antioxidant metabolism to the application of *A. brasilense* with organomineral fertilizer, alone or with *B. japonicum*.

Material and methods

Experimental conditions

The study was performed in a covered greenhouse located in Glória Campus of the Federal University of Uberlândia, Brazil. The soil used was classified as Acric Red Latosol, according to Santos et al. (2018). A representative sample was collected for chemical analysis after soil homogenization, drying, and sifting (2 mm). The soybean cultivar AS 3680 IPRO was used.

Experimental design and treatments

The experiment was conducted in a randomized block design with five replications, in a *4×2(+1)* factorial scheme, corresponding to four doses of *A. brasilense* [0, 100, 200, and 300 mL of Masterfix Gramíneas® ($2x10^8$ viable cells mL^{-1}) per 50 kg^{-1} of soybean seeds], with or without *B. japonicum* [100 mL ha^{-1} of Masterfix Soja® ($5x10^9$ viable cells mL^{-1} - SEMIA 5019 and SEMIA 5079 strains) per 50 kg^{-1} of soybean seeds], and a control treatment (without inoculants and fertilizers), totaling nine treatments replicated five times (45 plots). The treatments are as following: T1 - 0 *A. brasilense* with *B. japonicum*; T2 - 0 *A. brasilense* without *B. japonicum*; T3 - 100 *A. brasilense* with *B. japonicum*; T4 - 100 *A. brasilense* without *B. japonicum*; T5 - 200 *A. brasilense* with *B. japonicum*; T6 - 200 *A. brasilense* without *B. japonicum*; T7 - 300 *A. brasilense* with *B. japonicum*; T8 - 300 *A. brasilense* without *B. japonicum*; T9 - without inoculants and fertilizer.

All plants, except the control (T9), were fertilized with pelletized organomineral fertilizer. The sewage sludge used in the composition of the organomineral fertilizer came from the Municipal Department of Water and Sewage of Uberlândia (DMAE), located in the municipality of Uberlândia, Brazil. The sewage sludge was sanitized according to the methodology used by Alves Filho et al. (2016) to eliminate pathogens and reduce humidity. This organic fraction was mixed with the mineral fertilizer by the Geociclo Biotechnology S/A (Uberlândia, Brazil) to produce the 3-17-10 (N-P-K) formulation with B (0.2%), Zn (0.3%), and Mn (0.3%).

The chemical characteristics of the sewage sludge are presented in Table 1. The Geociclo Company also performed microbiological (total and thermotolerant coliforms) and chemical analysis in the resulting organomineral (USEPA 1992; EMBRAPA 2009). The results for heavy metals (cadmium, chromium, nickel,

Table 1 Chemical characterization of sewage sludge used in the composition of the organomineral

Attribute			Attribute		
pH $CaCl_2$	pH	8.1	Total mineral	%	51
Density	g cm^{-3}	0.66	Boron	mg kg^{-1}	10
Total N	%	0.99	Sodium	mg kg^{-1}	201
Org. matter total	%	50	Manganese	mg kg^{-1}	209
Total carbon	%	28	Copper	mg kg^{-1}	135
C/N relation		28/1	Zinc	mg kg^{-1}	1,042
Phosphorus	%	2.80	Iron	mg kg^{-1}	27,236
Potassium	%	0.30	Cadmium	mg kg^{-1}	1.4
Calcium	%	8.25	Mercury	mg kg^{-1}	0.7
Magnesium	%	2.48	Chrome	mg kg^{-1}	158.6
Sulphur	%	1.31	Nickel	mg kg^{-1}	250

N - [N Total] = sulfuric digestion. P, K, Ca, Mg, S, Cu, Fe, Mn, Zn = nitro perchloric digestion. B = colorimetric azomethine-H.

and lead) and the levels of total and thermotolerant coliforms were within the acceptable values by the Brazilian Resolution n°. 375 (CONAMA 2006).

The *A. brasilense* inoculation was performed on the fertilizer, which was placed in plastic bags containing doses of microorganisms; 0.5 mL of water was added to facilitate the distribution of the inoculant. The mix was agitated for homogenization and then distributed to the soil in the experimental area. The same procedure was used for inoculation with *B. japonicum*, but it was performed on the soybean seeds.

The soil used was classified as Acric Red Latosol, according to Santos et al. (2018). After homogenization, drying and sifting, samples were taken for chemical analysis. The dose of organomineral fertilizer was based on soil P content, following the "*Recommendation for the use of correctives and fertilizers in Minas Gerais State*" (CFSEMG 1999) and corresponded to 120 kg ha^{-1} of P_2O_5.

Study procedure

The sowing was performed at a depth of 3 cm in pots of approximately 8 L and placed eight seeds per pot, arranged in a circular shape, about 7 cm from the center (Fig. 1). In the center of the plastic pot, the fertilizer was placed at 4 cm deep. Fifteen days after sowing, the least developed plants were thinned, leaving three plants per pot. Two of these plants were used for destructive analysis, with only one plant remaining per plastic pot. Each experimental plot consisted of six plastic pots (8 L capacity).

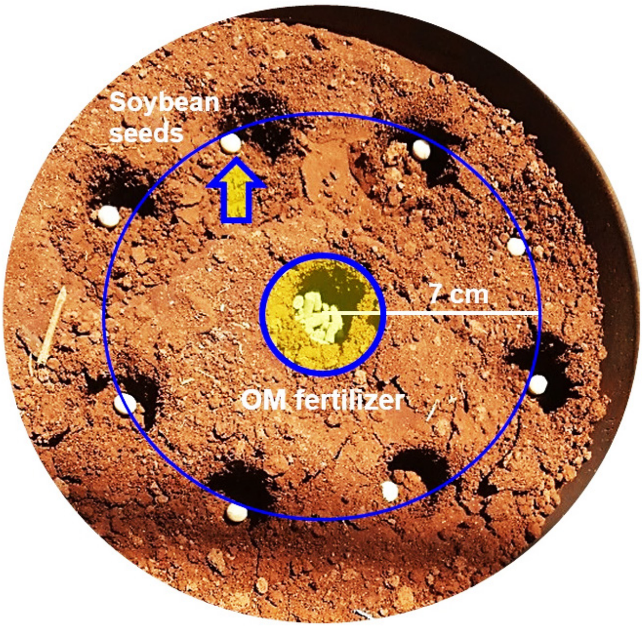

Fig. 1 Distribution of eight soybean seeds (AS 3681 IPRO) and organomineral (OM) fertilizer at sowing in an 8-liter pot filled with an Acric Red Latosol. Image author: F.C. Barros

Evaluated variables

Samplings for biochemical determinations were performed at V3 (second trifoliate leaf fully developed), V6 (fifth trifoliate leaf fully developed), R2 (open flower in one of the last two nodes of the main stem with fully developed leaf), and R5 (3 mm long bean in one of the last four nodes of the main stem with fully developed leaf) soybean phenological stages. The soybean leaves were sampled between 8 and 10 h in the morning, placed in identified plastic bags, wrapped in aluminum paper, conditioned in a thermal box with ice, and subsequently frozen with liquid nitrogen and then kept frozen in a standard freezer. These procedures were intended to paralyze the biochemical reactions.

The soybean leaf samples were transported to the Center of Research in Plant Physiology and Stress of the University Center of Patos de Minas (UNIPAM), Brazil. The peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) enzymatic activity was assessed, as well as the lipid peroxidation (LP), hydrogen peroxide (H_2O_2) concentration, and proline amino acid (PROL).

The extraction of plant material used for CAT, POD, and SOD determination started with the maceration of the leaf material in liquid nitrogen. Subsequently, 200 mg of plant material was macerated with 4 mL of 0.1 mol L^{-1} of potassium phosphate buffer at pH 6.8. The samples were transferred to microtubes and centrifuged at 10,000 rpm for 30 min at 4 °C. In the end, the samples were stored at -20 ºC (Kar and Mishra 1976).

The determination of the activity of POD was performed according to the methodology described by Teisseire and Guy (2000); SOD according to Beauchamp and Fridovich (1971), CAT according to Havir and McHale (1987), H_2O_2 content according to Alexieva et al. (2001), LP according to Heath and Packer (1968), and PROL according to Bates et al. (1973) technique.

Statistical analysis

The data obtained were initially tested for the analysis of variance (ANOVA) presumptions of normality of residues (Shapiro-Wilk test), homogeneity of variances (Levene test), and block additivity (Tukey test) at 0.01 significance, using the SPSS (V. 20.0). The data were then submitted to ANOVA performed by F test, at 5% probability, compared by Tukey and Dunnett test at 5% probability, using Assistat software (V. 7.7). For the doses of *A. brasilense* doses, regressions were performed, and the most adjusted model was chosen based on the significance of regression coefficients, at 5% probability, by F test, and the highest coefficient of determination (R^2), using Sisvar software (V. 5.6).

Results and discussion

Superoxide dismutase

At the V3 soybean phenological stage, high SOD activity was observed only when *B. japonicum* was inoculated. This result indicates a higher stress level (Table 2) and implies that *A. brasilense* could protect plants from the stressful inoculation caused by *B. japonicum*. In the V6 soybean stage, the SOD activity presented, in general, lower activities with the presence of *A. brasilense* than the control treatment (Table 2).

Table 2 Enzyme superoxide dismutase (SOD) activity in soybean plants at different growth stages, submitted in different doses of *A. brasilense,* with and without *B. japonicum* (100 mL ha^{-1}) compared to the control (without inoculant or organomineral fertilizer)

	SOD (U μg^{-1} [protein])			
	B. japonicum		*B. japonicum*	
A. brasilense (mL 50 kg^{-1} of seeds)	With	Without	With	Without
	V3 growth stage		V6 growth stage	
0	69.26 A*	B 21 21	27.92 A	19.33 B*
100	23.17 A	A 75 26	10.64 B*	18.64 A*
200	29.80 A	A 06 20	12.65 A*	13.66 A
300	22.32 A	A 26 28	17.01 A*	14.33 A*
Mean				
Control	27.29		33.82	
CV (%)	45.97		28.65	
	R2 growth stage		R5 growth stage	
0	22.61*	*12.59	28.23 A	12.56 B*
100	25.20	*17.06	31.33 A	18.87 A*
200	19.08*	*15.37	21.40 A*	17.53 A*
300	13.92*	*17.17	10.79 A*	19.04 A*
Mean	20.20 A	A 15.55		
Control	37.68		42.04	
CV (%)	28.25		45.41	

Means followed by distinct uppercase letters in the line differ by Tukey's test; means accompanied by an asterisk differ from the control by Dunnett's test, both at 0.05 probability. CV (%): coefficient of variation.

The results remained similar at the R2 soybean stage, but the plants inoculated with only *B. japonicum* showed some stress recovery. This recovery was expected since *B. japonicum* is a microorganism co-evolved with the soybean plant species (Ribeiro et al. 2013; Xu et al. 2013). In the R5 soybean stage, the control treatment presented an increased SOD activity which is related to plant senescence. High ROS activity during plant senescence is a known plant response (Strothe 1988; Bor et al. 2003; Bieker et al. 2018). The *A. brasilense* inoculation can improve plant protection since inoculated plants present lower SOD activity (Table 2).

The SOD activity is the main line of defense against ROS by dismuting the superoxide (O_2^-) to hydrogen peroxide (H_2O_2) (Scandalios 2005). The enzyme reduction occurs by superoxide forming oxygen. The reduced enzyme reacts with another superoxide ion forming H_2O_2 as the product; H_2O_2 is then dismutated by catalase, or other peroxidases, originating H_2O and O_2 (Berg et al. 2004). Similar results were verified by Müller (2016), who observed higher SOD activity in maize hybrids when *A. brasilense* was not inoculated (control treatment). In contrast, Bulegon et al. (2016) observed that the inoculation of ruzigrass (*Urochloa ruziziensis*) leaves with *A. brasilense* increased the SOD activity under severe water stress.

The *A. brasilense* doses presented significant adjustment to a second-degree polynomial model for SOD activity of soybean plants at V3 and V6 phenological stages. Equations demonstrate that the lowest SOD activities, 21.02 and 8.45 U μg^{-1} [protein], were achieved with doses of 211.9 and 193 mL of *A. brasilense* per 50 kg^{-1} of seeds, with *B. japonicum* inoculation, respectively (Fig. 2A and 2B); therefore, these doses caused lower stress levels signaled by the SOD activity.

Catalases

At R2 and R5 soybean phenological stages, *A. brasilense* doses presented significant adjustment using a linear model. The SOD activity in plants decreased linearly 0.0322 and 0.0623 U μg^{-1} [protein] for every 1 mL dose of *A. brasilense* inoculant co-inoculated with *B. japonicum* at the R2 and R5 stage, respectively (Fig. 2C and 2D). These results demonstrated a reduction of the stress level, signaled by the SOD activity, with an increasing dose of *A. brasiliense* co-inoculated with *B. japonicum*.

Catalases are oxidoreductase enzymes present in all plants, animals, and aerobic microorganisms. These enzymes are commonly found in peroxisomes and glyoxysomes organelles. CAT and SOD enzymes are considered the most efficient among antioxidant enzymes and can rapidly degrade H_2O_2 into H_2O and O_2; therefore, they are very important in the antioxidant system (Scandalios 1993; Gill and Tuteja 2010; Hasanuzzaman et al. 2020).

The CAT enzyme activity at the V3 and V6 stages presented no difference between the presence and absence of *B. japonicum*, but was lower than the control (Table 2). In these phenological stages, the control (not fertilized with organomineral) presented higher CAT activity than the other treatments, indicating the significant effect of the organomineral fertilizer on the CAT activity. Silva et al. (2020) also reported a reduction in CAT activity in soybean with 150 kg ha^{-1} of organomineral fertilizer.

According to Vasconcelos et al. (2009), organomineral fertilizers contain various compounds with antioxidant properties, such as humic acids. Thus, it is hypothesized that organomineral fertilizers can increase the activity of defensive enzymes to oxidative stress and turn plants more tolerant. According to García et al. (2012), applying humic acids to rice plants in water stress conditions increased the activity of antioxidant enzymes, which minimized the adverse effects of oxidative stress. Rocha (2018) reported that the humic acid extracted from sewage sludge could generate biostimulants that allow plant remediation against ROS.

Peroxidases

No differences were observed in the R2 and R5 soybean phenological stages compared to the control (Table 3).

The POD activity was higher in V3 and V6 soybean stage only in the treatments containing *B. japonicum* (Table 4). The plants inoculated only with *B. japonicum* presented greater POD activity than the plants from the control in the V3 phenological stage (Table 4). The same was observed for SOD, since POD decomposes H_2O_2 produced in SOD-catalyzed reactions (Bor et al. 2003).

At the R2 soybean stage, the treatments without inoculants and *A. brasiliense* (300 mL per 50 kg^{-1} of soybean seeds) or co-inoculated with *B. japonicum*, presented lower POD activity than the control treatment. These same treatments also showed lower SOD activity (Table 2). In the R5 soybean stage, no difference was observed (Table 4).

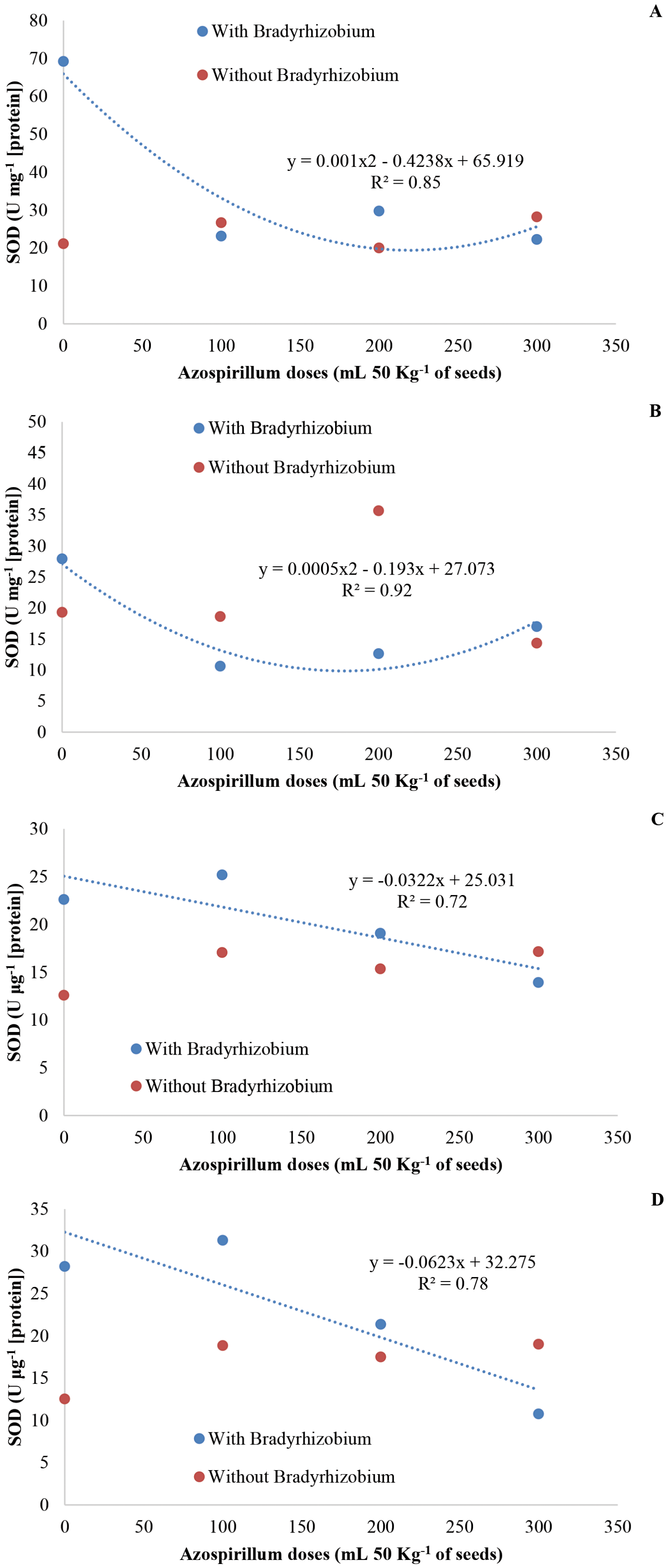

Fig. 2 Superoxide dismutase (SOD) content in soybean plants at V3 (A), V6 (B), R2 (C), and R5 (D) stages, in the presence of *A. brasilense*, with and without *B. japonicum* inoculation

Table 3 Enzyme catalase (CAT) activity in soybean plants at different growth stages, submitted to different doses of *A. brasilense*, with and without *B. japonicum* (100 mL ha^{-1}) compared to the control (without inoculant or organomineral fertilizer)

	CAT (µmol min^{-1}µg^{-1} [protein])			
	B. japonicum		*B. japonicum*	
A. brasilense (mL 50 kg^{-1} of seeds)	With	Without	With	Without
	V3 growth stage		V6 growth stage	
0	161.36*	89.01*	28.41*	3.89*
100	18.16*	39.94*	15.69*	10.61*
200	35.35*	19.03*	23.23*	42.15*
300	32.32*	48.76*	9.46*	22.50*
Mean	61.80 A	49.19 A	19.20 A	19.78 A
Control	203.00		139.35	
CV (%)	51.24		107.12	
	R2 growth stage		R5 growth stage	
0	48.58 A	A 37.23	56.43	25.17
100	72.80 A	B 22.29	75.54	30.51
200	43.84 A	A 56.37	58.12	83.72
300	51.32 A	A 30.03	56.63	67.87
Mean	-	-	61.68 A	51.82 A
Control	50.13		66.24	
CV (%)	58.36		45.41	

Means followed by distinct uppercase letters in the line differ by Tukey's test; means accompanied by an asterisk differ from the control by Dunnett's test, both at 0.05 probability. CV (%): coefficient of variation.

Table 4 Peroxidase enzyme (POD) activity in soybean plants at different growth stages, submitted to different doses of *A. brasilense*, with and without *B. japonicum* (100 mL ha^{-1}) compared to the control (without inoculant or organomineral fertilizer)

	POD (µmol min^{-1}µg^{-1} [protein])			
	B. japonicum		*B. japonicum*	
A. brasilense (mL 50 kg^{-1} of seeds)	With	Without	With	Without
	V3 growth stage		V6 growth stage	
0	3.07 A*	1.08 B	1.82 A	0.78 B
100	1.17 A	1.33 A	1.34 A	1.21 A
200	0.98 A	0.94 A	1.44 A	1.59 A
300	0.97 A	1.53 A	1.04 A	1.22 A
Mean				
Control	1.44		1.24	
CV (%)	34.26		29.15	
	R2 growth stage		R5 growth stage	
0	1.28	1.13*	1.61	1.26
100	1.32	1.28	1.74	1.63
200	1.35	1.48	1.44	2.19
300	0.94*	1.14*	1.02	1.43
Mean	1.22 A	1.26 A	1.45 A	1.63 A
Control	2.05		1.89	
CV (%)	23.86		29.90	

Means followed by distinct uppercase letters in the line differ by Tukey's test; means accompanied by an asterisk differ from the control by Dunnett's test. both at 0.05 probability. CV (%): coefficient of variation.

Regarding the POD activity, *A. brasilense* doses presented significant adjustment at the V3 and V6 soybean stages using a second-degree polynomial model and a linear model, respectively. The polynomial equation demonstrates that the lowest POD activity, 0.8511 µmol min^{-1} µg^{-1} [protein], was achieved with the dose of 207 mL of *A. brasilense* with *B. japonicum* inoculation per 50 kg^{-1} of soybean seeds (Fig. 3A). The linear equation shows that POD activity in plants decreased linearly by 0.0022 µmol min^{-1} µg^{-1} [protein] for every 1 mL dose of *A. brasilense* inoculant co-inoculated with *B. japonicum* (Fig. 3B).

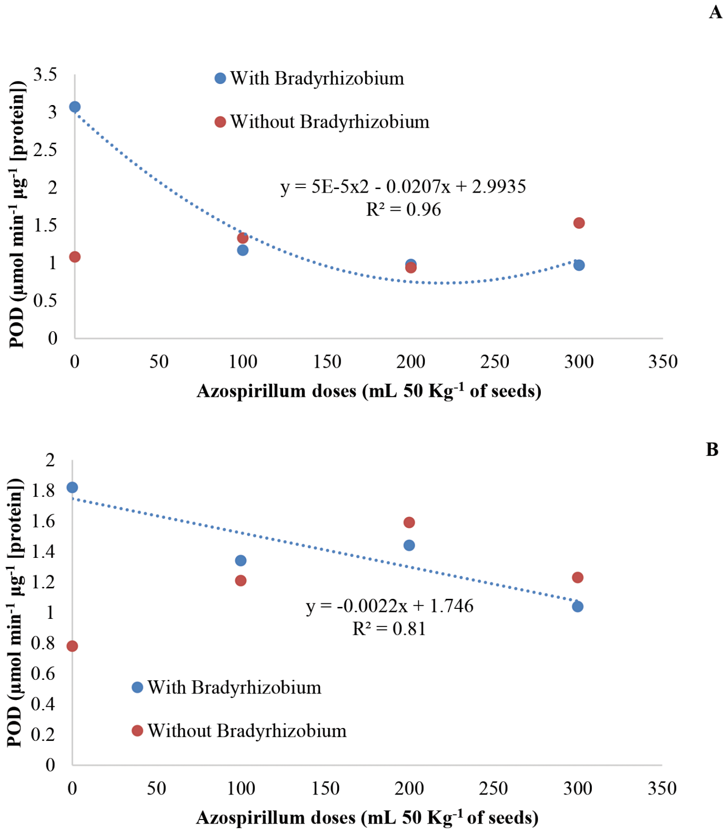

Fig. 3 Peroxidases (POD) content in soybean plants at V3 (A) and V6 (B) stages, in the presence of *A. brasilense*, with and without *B. japonicum* inoculation

The peroxidases are hydrogen acceptor-specific oxidoreductase heme proteins that are considered the most important enzymes for H_2O_2 elimination in cytosol and chloroplasts (Inzé and Van Montagu 1995; Alfenas 1998; Bela et al. 2015; Maruta and Ishikawa 2017). The POD activity is directly linked to stress and is often increased for this reason (Siegel 1993; Pandey et al. 2017).

Lipid peroxidation

The average lipid peroxidation (LP) activity at the V3 soybean stage presented no significant differences among the treatments (Table 5). In other soybean phenological stages evaluated, all treatments presented lower LP activity when compared to the control treatment (Table 5), indicating that the application of organomineral fertilizer can stimulate a protective function. The peroxidation of the lipids in the cell membrane is one of the most significant oxidative stress events, causing reductions in membrane fluidity, changing ion permeability, and disturbing other membrane-associated functions (Queiroz et al. 1998; Xu et al. 2011; Slama et al. 2017). Also, high LP activity was already reported in soybean plants when organomineral fertilizers were applied (Silva et al. 2020).

The LP presented a significant adjustment to a second-degree polynomial model for the *A. brasilense* dose at the R5 soybean stage. The polynomial equation demonstrates that the highest LP activity, 0.03871 µmol g^{-1} [fresh weight], was reached with 207 mL of *A. brasilense* with the *B. japonicum* inoculation per 50 kg^{-1} of seeds (Fig. 4).

Table 5 Lipid peroxidation (LP) of soybean plants at different growth stages, submitted to different doses of *A. brasilense*, with and without *B. japonicum* (100 mL ha^{-1}) compared to the control (without inoculant or organomineral fertilizer)

	LP (µmol [TBARS] g^{-1} [fresh weight])			
	B. japonicum		*B. japonicum*	
A. brasilense (mL 50 kg^{-1} of seeds)	With	Without	With	Without
	V3 growth stage		V6 growth stage	
0	72.20	67.79	33.59*	32.49*
100	76.34	69.48	33.52*	34.92*
200	69.28	67.72	37.27*	31.32*
300	66.40	61.84	32.38*	38.90*
Mean	71.05 A	66.71 A	34.19 A	34.41 A
Control	61.80		52.88	
CV (%)	15.12		13.21	
	R2 growth stage		R5 growth stage	
0	37.52 A*	*A 39.99	39.30 B*	43.44 A*
100	33.18 A*	*A 37.52	35.74 A*	29.97 B*
200	32.10 B*	*A 41.47	35.55 B*	42.63 A*
300	29.87 A*	*A 32.38	26.99 B*	31.45 A*
Mean				
Control	54.97		47.20	
CV (%)	13.48		8.43	

Means followed by distinct uppercase letters in the line differ by Tukey's test; means accompanied by an asterisk differ from the control by Dunnett's test. both at 0.05 probability. CV (%): coefficient of variation.

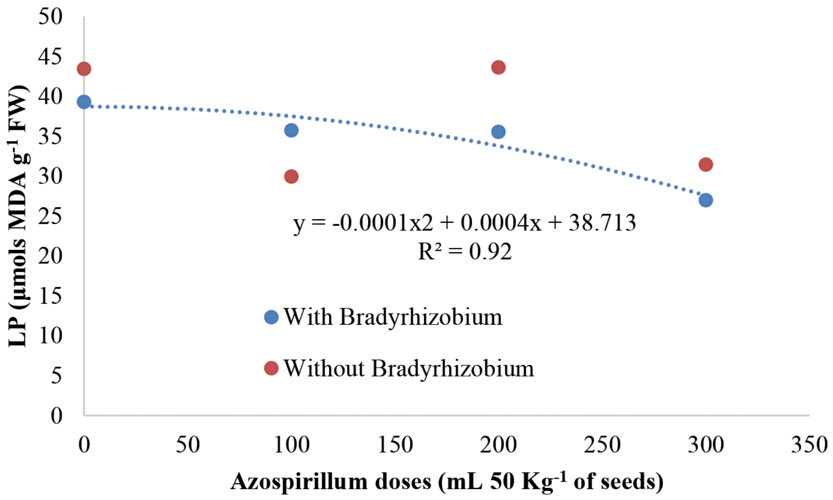

Fig. 4 Lipid peroxidation (LP) activity in soybean plants at R5 stage, in the presence of *A. brasilense*, with and without *B. japonicum* inoculation. MDA: malondialdehyde. FW: fresh weight

Hydrogen peroxide

The H_2O_2 leaf content was similar between the inoculated treatments and the control treatment at the V3 soybean stage, except for the 100 mL of *A. brasilense* per 50 kg^{-1} of seeds without *B. japonicum* (Table 6).

In the V6 soybean stage, the control treatment, *B. japonicum* solely and co-inoculated with *A. brasiliense* at 300 mL per 50 kg^{-1} of seeds, and the *A. brasilense* at 200 mL per 50 kg^{-1} of seeds, presented similar H_2O_2 concentration. The other treatments showed lower H_2O_2 concentrations (Table 6).

Table 6 Concentration of hydrogen peroxide (H_2O_2) in soybean plants at different growth stages, submitted to different doses of *A. brasilense*, with and without *B. japonicum* (100 mL ha^{-1}) compared to the control (without inoculant or organomineral fertilizer)

	H_2O_2 (µmol g^{-1} [fresh weight])			
	B. japonicum		*B. japonicum*	
A. brasilense (mL 50 kg^{-1} of seeds)	With	Without	With	Without
	V3 growth stage		V6 growth stage	
0	6.07 A	7.50 A	8.64	7.38*
100	5.76 B	8.98 A*	6.87*	7.27*
200	4.72 A	5.19 A	6.95*	8.47
300	6.10 A	5.10 A	8.39	6.86*
Mean	-	-	7.72 A	7.49 A
Control	5.96		10.55	
CV (%)	23.50		17.62	
	R2 growth stage		R5 growth stage	
0	8.33*	6.26*	8.45 A*	8.23 A*
100	7.14*	6.12*	6.76 A*	7.57 A*
200	6.93*	7.89*	7.09 A*	7.84 A*
300	8.68*	7.81*	9.70 A*	6.85 B*
Mean	7.77 A	7.02 A	-	-
Control	11.86		11.46	
CV (%)	18.26		8.43	

Means followed by distinct uppercase letters in the line differ by Tukey's test; means accompanied by an asterisk differ from the control by Dunnett's test. both at 0.05 probability. CV (%): coefficient of variation.

At the R2 and R5 soybean stages, all treatments presented lower H_2O_2 concentration than the control (without inoculant or organomineral fertilizer), which corroborates with the LP results observed at V6, R2, and R5 stages (Table 6). The application of organomineral fertilizer may have activated other antioxidant enzymes to respond to stressful conditions (Rady 2012; Benidire et al. 2021).

Azospirillum brasilense doses showed significant adjustment to a second-degree polynomial model for H_2O_2 concentration at V6 and R5 soybean stages. The polynomial equations show that the lowest activity of H_2O_2, 6.71 and 6.47 µmol g^{-1} [fresh weight] were achieved at 154.38 and 141.5 mL of *A. brasilense* per 50 kg^{-1} of seeds, with inoculation of *B. japonicum* (Fig. 5A and 5B). Concentrations above the mentioned *A. brasilense* doses raise the ROS activity.

The results presented a significant linear model for *A. brasilense* doses at R5 soybean stage when *B. japonicum* was not applied. The linear equation demonstrates that the concentration of H_2O_2 in plants linearly decreased 0.0039 µmol g^{-1} [fresh weight] for every 1 mL added of *A. brasilense*, indicating the potential of this inoculant to neutralize ROS (Fig. 5B). In addition, H_2O_2 is a small reactive ROS that can cross cell membranes, migrating through different compartments and forming hydroxyl radical (OH), the most reactive oxidant of the ROS family (Gadjev et al. 2008; Karuppanapandian et al. 2011; Khan et al. 2018; Smirnoff and Arnoud 2019).

Proline

At the V3 soybean stage, the treatments with *B. japonicum* presented higher proline levels than the control, except for the co-inoculated treatment with 300 mL of *A. brasiliense* per 50 kg^{-1} of seeds. In its highest dose, this microorganism may have protected the plant from the stress of the inoculation with *B. japonicum*. At the V6 and R2 soybean stage, there were no differences between the treatments with inoculants and the control. At the R5 soybean stage, as in the V3 soybean stage, higher levels of PROL can be verified in treatments with *B. japonicum* alone or in co-inoculation with 100 mL of *A. brasilense* per 50 kg^{-1} of seeds compared to the control (Table 7).

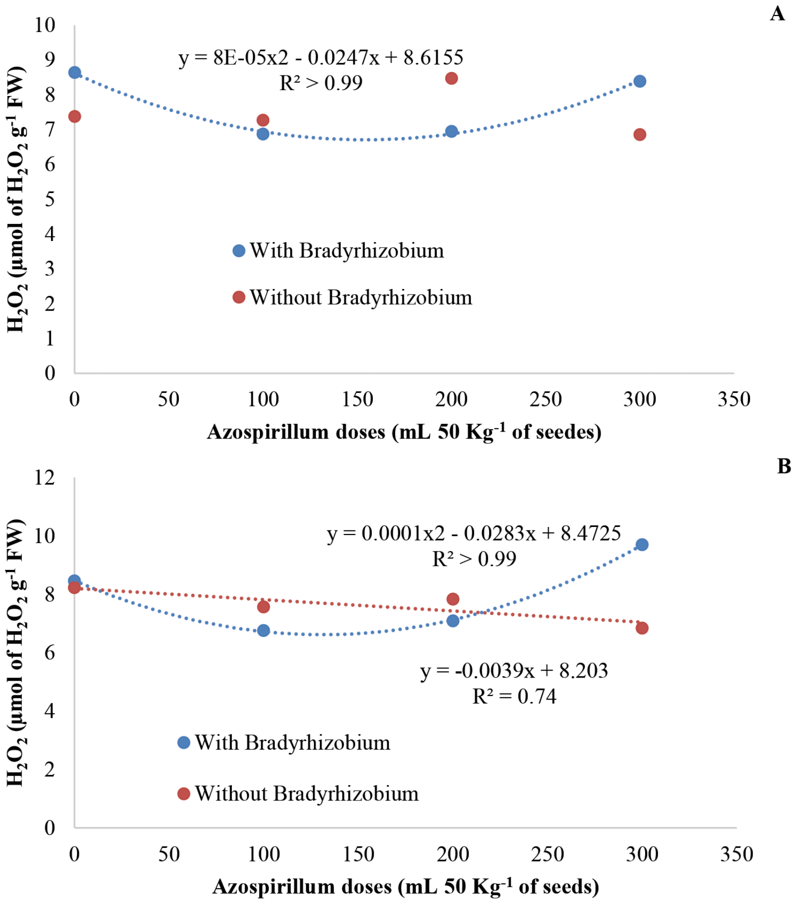

Fig. 5 Hydrogen peroxide (H_2O_2) activity in soybean plants at V6 (A) and R5 (B) stages, in the presence of *A. brasilense*, with and without *B. japonicum* inoculation. FW: fresh weight

Table 7 Proline (PROL) content in soybean plants at different growth stages, submitted to different doses of *A. brasilense*, with and without *B. japonicum* (100 mL ha^{-1}) compared to the control (without inoculant or organomineral fertilizer)

	PROL (µmol g^{-1} [fresh weight])			
	B. japonicum		*B. japonicum*	
A. brasilense (mL 50 kg^{-1} of seeds)	With	Without	With	Without
	V3 growth stage		V6 growth stage	
0	2.04 A*	1.09 B	0.56	0.70
100	1.82 A*	1.04 B	0.53	0.59
200	1.60 A*	1.18 A	0.61	0.76
300	0.96 A	1.07 A	0.68	0.67
Mean			0.60 A	0.68 A
Control	0.47		0.61	
CV (%)	42.38		23.31	
	R2 growth stage		R5 growth stage	
0	1.20 A	0.45 B	0.63 A*	0.13 B
100	0.94 A	0.47 A	0.48 A*	0.14 B
200	0.62 A	0.49 A	0.15 A	0.15 A
300	0.76 A	0.50 A	0.14 A	0.07 A
Mean	-	-	-	-
Control	0.50		0.16	
CV (%)	20.07		30.52	

Means followed by distinct uppercase letters in the line differ by Tukey's test; means accompanied by an asterisk differ from the control by Dunnett's test. both at 0.05 probability. CV (%): coefficient of variation.

Proline is a non-enzymatic antioxidant amino acid usually produced in cytosol and chloroplasts (Rejeb et al. 2014). It plays an important role in plant cell detoxification and stabilizes cell membranes, subcellular structures, and cellular functions (Ahmad et al. 2010; Kaur and Asthir 2015). Also, proline can act as a signaling molecule and may increase antioxidant enzyme activity in response to different types of stress (Hayat et al. 2012; Carvalho et al. 2013; Rejeb et al. 2014; El Moukhtari et al. 2020).

High contents of synthesized proline must be harmful to plants because this antioxidant consumes between 0.4 and 0.6% of the total N in leaves; thus, when proline is presented in high concentrations, there is a consumption of the plant N that will not be used for plant growth (Ernst et al. 2000). Proline can also be applied to improve plant protection against stresses (El Moukhtari et al. 2020), root nodulation (El Sabagh et al. 2017), and crop production (Alam et al. 2016; Wani et al. 2016).

Azospirillum brasilense doses presented significant adjustment to a linear model. Proline content in plants decreased linearly 0.0035 and 0.0018 µmol g^{-1} [fresh weight] for every 1 mL added of *A. brasilense* with *B. japonicum* inoculation at the V3 and R5 stages, respectively (Fig. 6A and 6B). These results also indicated that *A. brasilense* in high doses co-inoculated with *B. japonicum* could improve plant protection to stresses.

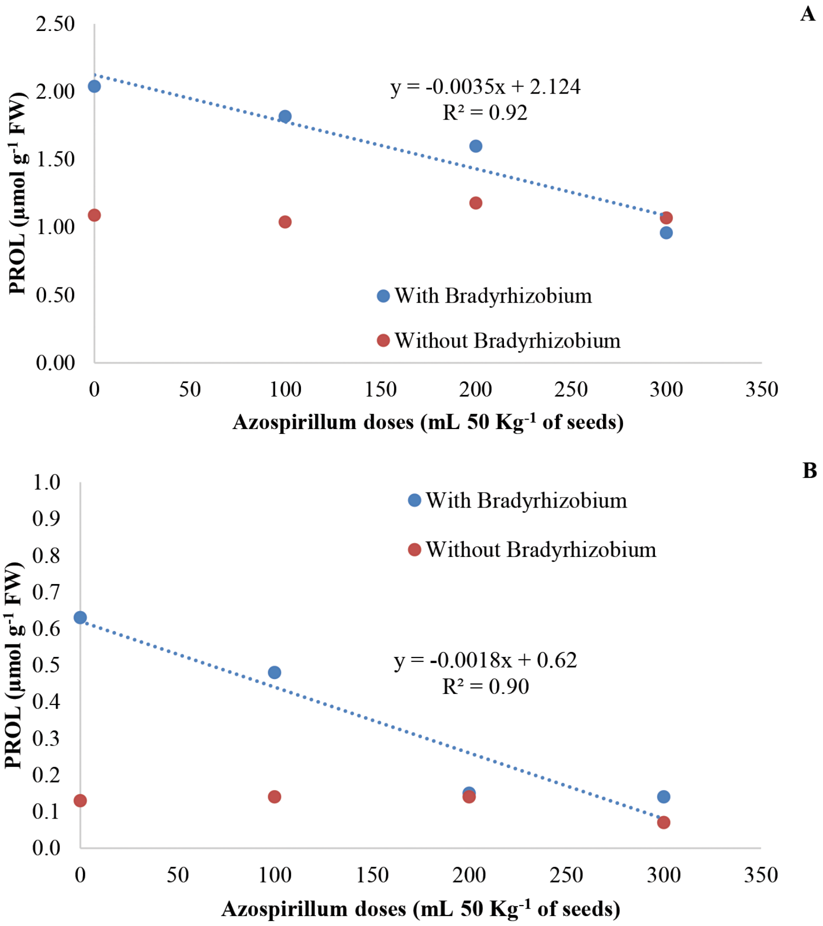

Fig. 6 Prolines (PROL) activity in soybean plants at V3 (A) and R5 (B) stages, in the presence of *A. brasilense*, with and without *B. japonicum* inoculation. FW: fresh weight

Perspectives and upcoming approaches

The meaning of the results observed in the present study corroborates with the observation of multiple plant benefits when microbiological plant inoculation is implemented to crop production (Bashan et al. 2014; Smercina et al. 2019; Escobar et al. 2020; Lopes et al. 2021). Positive responses of dual inoculation (*Azospirillum* and *Bradyrhizobium*) to soybean plants have already been reported (Chibeba et al. 2015). The results observed here demonstrated that seed inoculation affects the enzymatic responses of soybean plants in the vegetative and reproductive stages. The results also indicated that organominerals could be used as a substrate for inoculum adhesion. It is a viable technique that adds another option for the crop inoculation routine.

Future inoculant and organomineral researchers should consider the results already available in the literature and the findings reported here to evaluate the co-inoculation applied on the pellets of organomineral fertilizers. The study of organominerals based on other organic sources must be considered. Also, the continuous application of plant inoculants via organomineral over time (biological residual) should be assessed. Using beneficial microorganisms to increase plant performance is a safer technology and has less impact from the ecological perspective. It should be a rational step to improve the production efficiency (productivity) and the sustainability of the cropping process in modern agriculture.

Conclusion

The use of *Azospirillum brasilense* in soybean seed inoculation changes the activity of superoxide dismutase, lipid peroxidation, and hydrogen peroxide compared to the control (without inoculants or fertilizers). The co-inoculation of *A. brasilense* with *Bradyrhizobium japonicum* enhanced the proline activity compared to the control and the *A. brasilense* alone at the V3 e R5 soybean phenological stage. These responses improve the plant's protection against stresses in critical soybean plant stages. Organomineral fertilizer based on sewage sludge is an efficient carrier for the *A. brasilense* inoculation of soybean plants via seed treatment.

Acknowledgment Federal University of Uberlândia (UFU). Coordination of Superior Level Staff Improvement (CAPES).

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest associated with this study.

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