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Original article

Bradyrhizobium sp. inoculation ameliorates oxidative protection in cowpea subjected to long-term composted tannery sludge amendment

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ABSTRACT

Oxidative stress can strongly affect biological nitrogen fixation (BNF) in nodules; therefore, the antioxidant system in nodules works to reduce the damage caused by the deleterious effects of oxidizing compounds and maintain adequate BNF. In this context, the objective of this work was to evaluate variables related to BNF and oxidative metabolism in the nodules of cowpea inoculated with *Bradyrhizobium* sp. and grown in soils subjected to different levels of composted tannery sludge amendment. A randomized block design was used with a 4 × 2 factorial scheme, involving four doses of composted tannery sludge (0, 5, 10, and 20 t ha^{-1}) for uninoculated cowpea and cowpea inoculated with *Bradyrhizobium* sp. The cowpea inoculated with *Bradyrhizobium* sp. showed a better growth response than the cowpea inoculated with native rhizobia. Increases in the number of nodules, nodule dry mass, nitrogen fixation efficiency and nitrogen content were recorded in the cowpea inoculated with *Bradyrhizobium* sp. grown in soils with composted tannery sludge. The cowpea nodules colonized by *Bradyrhizobium* sp. showed lower hydrogen peroxide levels, while leghemoglobin was maintained at the highest levels. The catalase and phenol peroxidase enzymes were positively modulated in the nodules of the cowpea inoculated with *Bradyrhizobium* sp. We conclude that the presence of composted tannery sludge affects the establishment and development of the symbiosis between rhizobia and cowpea, mainly between native soil rhizobia and cowpea. When cowpea was inoculated with *Bradyrhizobium* sp., it was concluded that these plants were able to maintain better plant growth and nitrogen capture and lower oxidative stress in their nodules. Thus, inoculation with *Bradyrhizobium* sp. could be a useful tool for minimizing the deleterious effects of exposing plants to composted tannery sludge and for ensuring plant growth and productivity.

1. Introduction

The interactions between plants and microorganisms that occur in the rhizosphere have important implications for agriculture worldwide [1,2]. Plants and microbes perform important ecological functions such as biological nitrogen fixation (BNF) when they are intrinsically connected. BNF takes place in bacteroids, which are differentiated forms of rhizobia present in the nodules of leguminous plant roots, such as peanut, cowpea, lentil, common bean,

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lima bean, and soybean roots [3]. In this process, atmospheric nitrogen is converted to ammonia, which is an assimilable form of nitrogen for plants [4]. Members of the rhizobia group, particularly the *Rhizobium* and *Bradyrhizobium* genera, are nitrogen-fixing bacteria that interact with specific forms of host plants and thus infect a limited range of hosts [1].

BNF depends on the host plant genotype, host photosynthesis and strain of rhizobia and is responsive to pedoclimatic factors [2]. Cowpea (*Vigna unguiculata* [L.] Walp) displays nodules in the presence of various species of rhizobia bacteria [5]. Several studies have shown that some strains of rhizobia can promote increased productivity through effective symbiosis and therefore promote high agronomic efficiency [6,4]. Cowpea has been used in studies aimed at optimizing BNF by selecting strains [7] or by producing new vehicles for inoculation [8]. However, the establishment and development of cowpea-rhizobia symbiosis under adverse conditions, such as high heavy metal concentrations, have been poorly studied [9,10].

Chromium (Cr) is a heavy metal that is present at high concentrations in tannery sludge, a sub-product of the tannery industry that is produced during the tanning process [9]. In addition to the presence of high concentrations of Cr, organic matter originating from animals, inorganic salts, and carbonates are also present in sludge [10]. Landfilling is the most commonly used method for disposing of tannery sludge, but other methods, such as the production of organic compost that may be used in agricultural soils, have been suggested [10,11]. When properly executed, composting tannery sludge reduces Cr levels [12]. Composted tannery sludge can be used in agriculture; however, the long-term application of composted tannery sludge results in increased salinity, soil alkalinity, and Cr accumulation [9].

Although previous studies have indicated that composted tannery sludge has positive effects on plant growth and productivity [10,11,13], the efficiency of cowpea-rhizobia symbiosis in such environments remains poorly understood. Cr accumulation has toxic effects on plant-soil ecosystems [14,15] and can induce oxidative stress [16]. Oxidative stress occurs when the amount of reactive oxygen species (ROS) formed inside the cell is greater than the amount of ROS that the cell can remove [17,18]. In this context, the activity of the antioxidant defense system, which involves both antioxidative compounds and enzymes, represents an important mechanism for combating or controlling the possible oxidative damage induced by the presence of heavy metals [19,20]. In bacteroids, oxidative stress increases oxidative reactions and damage to cellular constituents, which accelerates nodule senescence, reduces BNF, alters photosynthesis, and increases lipid peroxidation and protein oxidation, which decreases plant productivity [3,20].

The establishment and development of symbiosis involves a complex molecular dialogue between the host and symbiont and is mediated by chemical signals [21]. Hydrogen peroxide (H_2O_2) is a ROS that acts as a signaling molecule in many physiological processes and probably mediates the establishment of legume-rhizobia symbiosis [19,20]. It is possible that H_2O_2 acts as a defense mechanism against the oxidative stress caused by excess Cr in the presence of effective plant-microbe symbiosis. In this situation, the antioxidative enzymes in nodules are stimulated, and high rates of BNF are maintained. We hypothesize that effective cowpea-rhizobia symbiosis minimizes the deleterious effects of excess Cr compared with cowpea nodulated with native rhizobia. Thus, this study aimed to evaluate the variables related to BNF and antioxidative metabolism in the nodules of cowpea inoculated with *Bradyrhizobium* sp. and grown in soils subjected to six years of successive applications of composted tannery sludge.

2. Materials and methods

2.1. Long-term tannery sludge application: general aspects

A long-term field experiment was initiated in 2009 and has been used to study the changes in a plant-soil ecosystem after successive applications of composted tannery sludge to the soil at doses of 2.5, 5, 10, and 20 t ha^{-1} (dry basis). This research was conducted in the experimental area of the Center of Agricultural Science located at the Federal University of Piaui (Teresina, Piaui, Brazil). Tannery sludge was continuously composted using the aerated-pile method after being mixed with sugarcane straw and cattle manure at a ratio of 1:3:1 (v/v/v), and chemical analyses were performed on the composted tannery sludge. This sludge was spread over the soil surface (0–20 cm) in plots of 20 m^2 in a completely randomized design, with rows spaced 1.0 m apart. After spreading, the composted tannery sludge was incorporated using a harrow. Each composted tannery sludge application rate was used for four plots. For soil sampling, a 12 m^2 area in each plot was considered as the usable area. The soil was classified as a Fluvisol, with 10% clay, 28% silt and 62% sand in the 0–20 cm layer.

2.2. Preparation of the inoculant

The BR 3267 strain of *Bradyrhizobium* sp. used in the greenhouse experiments was obtained from the National Center for Research in Agrobiology (Seropédica, Rio de Janeiro, Brazil). The BR 3267 strain was purified in yeast mannitol agar (YMA) medium with 0.25% (w/v) Congo red as an indicator. After purification, the BR 3267 strain was multiplied in tubes with YMA medium without the indicator. To prepare the inoculant, the BR 3267 strain was placed in Erlenmeyer flasks with yeast mannitol (YM) liquid medium and incubated for 96 h in a rotator shaker set at 220 rpm (28 °C).

2.3. Greenhouse experiment

For the greenhouse experiment, soil was collected from the different plots maintained in the previously described long-term field experiment. To fill the pots, soil collected from the 0–20-cm soil layer was sieved (2.0-mm-mesh sieve) and homogenized after air-drying. Pots containing 3.8 kg of the unsterilized soil were amended with $CaCO_3$, P_2O_5 and K_2O according to the recommendations obtained from the soil chemical analysis (Table 1). Soil from an adjacent area without the application of composted tannery sludge was used as a control. In each pot, cowpea (*Vigna unguiculata* [L.] Walp. cv. BRS Guariba) seeds were sown after being disinfected. For disinfection, the cowpea seeds were immersed in 70% (v/v) ethanol for 30 s and 2% (v/v) sodium hypochlorite for 60 s. Subsequently, the seeds were rinsed seven times with sterile distilled water.

Cowpea seeds were sown in pots containing soil amended with composted tannery sludge (5, 10, or 20 t ha^{-1}) and simultaneously inoculated with 1.0 mL of YM medium containing the BR 3267 strain at 10^8 CFU mL^{-1}. Additionally, uninoculated cowpea seeds were sown in pots containing soils amended with composted tannery sludge (5, 10, or 20 t ha^{-1}). For the control (zero level), uninoculated cowpea seeds or cowpea seeds inoculated with *Bradyrhizobium* sp. were grown in the soil in the absence of composted tannery sludge and collected in an adjacent area, as noted above. On the seventh day after planting, two plants were left in each pot. All plants were irrigated with Hoagland and Arnon's nutritive solution modified according to Silveira et al. [22] and without nitrogen.

The adopted experimental design consisted of a randomized block design with four replications. The treatments were arranged in a 4 × 2 factorial design involving four levels of composted

Table 1
Chemical analysis of the soil used in the experiment. The soil was collected after the application of 5, 10 and 20 t ha^{-1} of composted tannery sludge for six consecutive years and utilized to fill pots. An adjacent area that did not receive composted tannery sludge was used to fill the control pots (level zero). TOC is total organic carbon.

Soil samples	pH	TOC	P	K	Na	Al	Ca	Mg	Cr
	(H_2O)	(g kg^{-1})	(mg dm^{-3})		($cmol_c$ dm^{-3})				(mg kg^{-1})
Adjacent area	6.2	5.60	3.3	42.3	1.0	0.07	0.77	0.10	3.8
5	7.0	7.39	7.9	51.0	1.1	0.07	1.51	0.16	55.9
10	7.3	7.74	11.2	49.5	1.2	0.06	1.32	0.19	75.2
20	8.1	10.50	18.6	58.5	1.2	0.06	1.61	0.48	253.5

tannery sludge (0, 5, 10, and 20 t ha^{-1}) in uninoculated cowpea and cowpea inoculated with *Bradyrhizobium* sp. The experimental unit was composed of a pot with two plants.

2.4. Measurements

Throughout the experimental period, the heights of the cowpea plants were measured and used to calculate absolute growth rate. At harvest (65 days after planting), the length of the major root of each collected plant was measured using a measuring tape, and the collected plant material was partitioned into shoots, roots, and nodules. The numbers of nodules were manually counted. The dry masses of the shoots, roots, and nodules were determined after drying in a forced aeration oven at 65 °C until a constant weight was achieved. The nitrogen contents in the shoots and nodules were measured using the methods described by Bremner [23]. Based on the aforementioned data, the nitrogen fixation efficiency [24], shoot dry matter nitrogen content, accumulated nitrogen content, and specific nodulation were calculated.

Fresh cowpea nodules were used to quantify leghemoglobin using Drabkin's reagent according to the methodology described by Smagghe et al. [25], and the resulting data were expressed in mg g^{-1} fresh weight (FW). After extraction with 5% (w/v) trichloroacetic acid, fresh nodules were used to measure their H_2O_2 content and lipid peroxidation. The H_2O_2 content was determined according to the method described by Brennan and Frenkel [26], and the resulting data were expressed as μmol of H_2O_2 g^{-1} FW. Lipid peroxidation was quantified by measuring of the malondialdehyde-thiobarbituric acid (MDA-TBA) complex, as described by Heath and Packer [27], and the resulting data were expressed as ηmol MDA-TBA g^{-1} FW. The catalase (CAT) and phenol peroxidase (POX) activities were measured in fresh nodules as described by Havir and McHale [28] and Amako et al. [29], respectively.

The enzymatic extract employed in the assays was the supernatant collected after centrifugation of the macerated fresh nodules at 14,000 g for 25 min at 4 °C in the presence of 100 mM K-phosphate buffer (pH 7.0) containing 1.0 mM EDTA. CAT activity (EC 1.11.1.6) was assayed in aliquots of the enzymatic extract after mixing with 50 mM K-phosphate buffer (pH 7.0) containing 20 mM H_2O_2 at 30 °C, and the decrease in absorbance at 240 nm was monitored. CAT activity was calculated based on an extinction coefficient of 36 mM^{-1} cm^{-1} and expressed as μmol H_2O_2 g^{-1} FW min^{-1}. To determine the POX activity (EC 1.11.1.7), aliquots of the enzymatic extract were mixed with 50 mM K-phosphate buffer (pH 7.0) containing 20 mM pyrogallol. The reaction initiated by the addition of 20 mM H_2O_2 was stopped after 300 s by adding 0.5% (v/v) H_2SO_4. The absorbance of the samples was obtained at 420 nm, and POX activity was calculated using an extinction coefficient of 2.47 mM^{-1} cm^{-1} and expressed in μmol pyrogallol g^{-1} MF min^{-1}.

2.5. Statistical analysis

The results were subjected to analysis of variance (ANOVA) preceded by the F test at the 5% probability level. Initially, the data were analyzed using the Shapiro-Wilk test to evaluate normality. To compare the means of each level of composted tannery sludge, Student's *t*-test ($p < 0.05$) was utilized. Dunnett's test ($p < 0.05$) was used to identify the effects of *Bradyrhizobium* sp. (BR 3267) inoculation relative to uninoculated cowpea for each level of composted tannery sludge. Pearson's correlation coefficient and simple regression analysis were carried out to seek a correlation between the obtained variables and the composted tannery sludge dose, Cr concentration, and soil pH (*p*-values less than 0.05 were considered statistically significant).

3. Results

The uninoculated cowpea and cowpea inoculated with *Bradyrhizobium* sp. (BR 3267) displayed different growth responses when cultured in soils with different doses of composted tannery sludge. The growth variables of the uninoculated cowpea and the cowpea inoculated with *Bradyrhizobium* sp., including the absolute growth rate, root length, shoot and root dry weight, number of nodules, and nodule dry weight, were analyzed in response to the sludge dose, Cr level and soil pH using simple regression and Pearson's correlation analyses. ANOVA showed significant interactions between the composted tannery sludge dose and inoculation with *Bradyrhizobium* sp. that affected the absolute growth rate ($F = 77.7$, $p < 0.0001$). Regression and correlation analysis showed that the absolute growth rate of the uninoculated cowpea is not significantly and linearly correlated with increasing sludge dose, Cr levels or soil pH (Table 2 and Fig. 1A).

The cowpea inoculated with *Bradyrhizobium* sp. and grown in soils without composted tannery sludge exhibited an absolute growth rate increase of 120% when compared with uninoculated plants grown under the same conditions. Composted tannery sludge application resulted in an increase in the absolute growth rate (~30%) in the uninoculated plants, but this increment was reduced by approximately 10% when the doses of composted tannery sludge were increased (Fig. 2A). In the cowpea inoculated with *Bradyrhizobium* sp., a significant linear relationship and negative correlations between the absolute growth rate and sludge dose, Cr level and soil pH were observed, indicating decreasing growth with increasing sludge dose, Cr level and soil pH (Fig. 1B). Although the cowpea inoculated with *Bradyrhizobium* sp. exhibited lower absolute growth rates when cultivated with increasing doses of composted tannery sludge, the absolute growth rate remained higher than that of the uninoculated plants.

Significant interactions between sludge dose and inoculation with *Bradyrhizobium* sp. for root length were observed when using ANOVA ($F = 29.4$, $p < 0.0001$). The root length of the uninoculated cowpea did not display significant linear relationships or correlations with sludge dose, Cr level or soil pH (Table 2; Fig. 1A). As illustrated in Fig. 2B, the lengths of the uninoculated cowpea roots increased by 20% when cultivated in the presence of 5 or 10 t ha^{-1} of composted tannery sludge; however, when 20 t ha^{-1} of sludge was applied to the soil, the lengths of the uninoculated cowpea roots were not significantly different from the lengths of the

Table 2
Regression analysis (R^2-value) between the variables measured in this study and the doses of composted tannery sludge, Cr levels in soil or soil pH.

Variables	Uninoculated			Inoculated		
	Sludge	Cr levels	Soil pH	Sludge	Cr levels	Soil pH
Absolute growth rate	0.210	0.129	0.381	0.954**	0.873**	0.918**
Root length	0.108	0.051	0.281	0.888**	0.906**	0.811**
Root dry weight	0.520*	0.384*	0.702**	0.432*	0.397*	0.603*
Shoot dry weight	0.744**	0.711**	0.901**	0.788**	0.933**	0.669**
Number of nodules	0.285	0.159	0.722*	0.920**	0.763**	0.962**
Nodule dry weight	0.323	0.219	0.506*	0.320	0.211	0.505
Accumulated nitrogen	0.630**	0.502*	0.810**	0.954**	0.943**	0.843**
Specific nodulation	0.476*	0.604**	0.394*	0.867**	0.798**	0.965**
Nitrogen (N) content	0.742**	0.585**	0.880**	0.952**	0.824**	0.986**
N-fixation efficiency	0.972**	0.857**	0.988**	0.966**	0.891**	0.876**
Leghemoglobin	0.826**	0.649**	0.754**	0.883**	0.764**	0.974**
Lipid peroxidation	0.141	0.129	0.287	0.002	0.002	0.017
Hydrogen peroxide	0.063	0.030	0.185	0.841**	0.682**	0.754**
Catalase	0.777**	0.645**	0.900**	0.014	0.001	0.044
Phenol peroxidase	0.965**	0.854**	0.965**	0.991**	0.927**	0.958**

Double asterisk (**) - significant at 1% ($p < 0.01$); Single asterisk (*) - significant at 5% ($0.01 =< p < 0.05$).

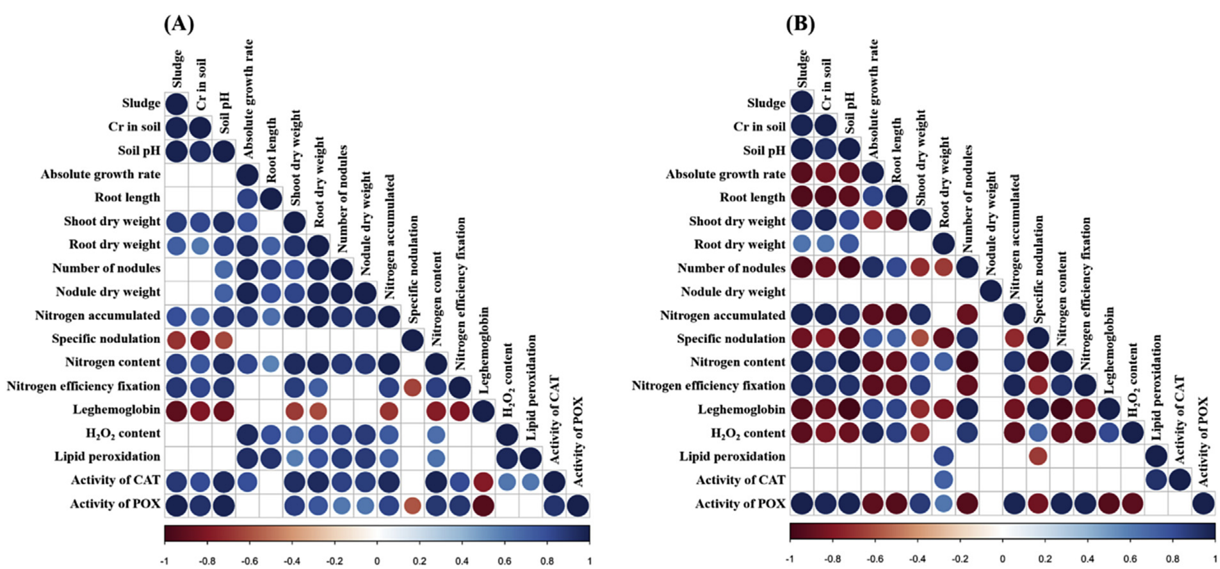

Fig. 1. Cross-correlograms between the variables obtained in uninoculated cowpea (A) or cowpea inoculated with *Bradyrhizobium* sp. (B) and the composted tannery sludge doses, Cr levels, and soil pH values. Circles are used to indicate the strengths of the associations between these elements (factors and/or variables). The blue circles show positive correlations, and the red circles show negative effects, with the intensity of the color indicating the strength of the association. The blank cells in the correlogram indicate that the correlation was not significant ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

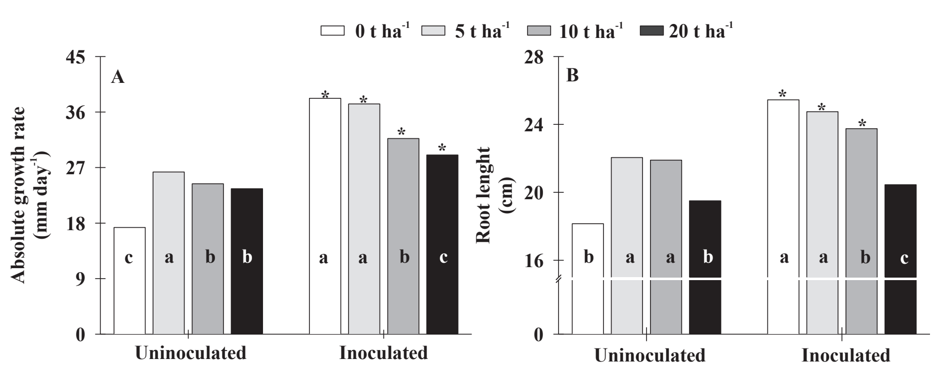

Fig. 2. Absolute growth rate (A) and root length (B) of uninoculated cowpea or cowpea inoculated with *Bradyrhizobium* sp. (BR 3267) and grown in soils amended with zero, 5, 10, and 20 t ha^{-1} of composted tannery sludge. Means followed by different lowercase letters show a significant difference between each level of composted tannery sludge (Student's *t*-test, $p < 0.05$). An asterisk (*) indicates a significant difference between the uninoculated cowpea and the cowpea inoculated with *Bradyrhizobium* sp. for each level of composted tannery sludge application (Dunnett's test, $p < 0.05$).

uninoculated cowpea roots and the roots of the cowpea cultivated in soils without composted tannery sludge. When compared with the uninoculated cowpea plants, the cowpea plants inoculated with *Bradyrhizobium* sp. and cultivated in soils without composted tannery sludge exhibited a 35% increase in root length.

For the cowpea inoculated with *Bradyrhizobium* sp. and cultivated in soils with composted tannery sludge, a gradual reduction in root length was observed in response to increasing levels of composted tannery sludge (Fig. 1B). This reduction was supported by the regression and correlation analysis, which indicated a significant and negative linear relationship (Table 2 and Fig. 1B) between the root length of cowpea inoculated with *Bradyrhizobium* sp. and the tested factors (sludge dose, Cr level and soil pH). When 20 t ha^{-1} of composted tannery sludge was applied, which was the highest level of sludge application, the cowpea plants inoculated with *Bradyrhizobium* sp. showed a decrease in root length of 16% relative to the cowpea plants inoculated with *Bradyrhizobium* sp. and cultivated in soil without composted tannery sludge. As shown in Fig. 2B, the lengths of the cowpea roots in the uninoculated and *Bradyrhizobium* sp. inoculated treatments with 20 t ha^{-1} composted tannery sludge were statistically similar.

All of the applied treatments were compared with the uninoculated cowpea, and the root and shoot dry weight data are presented as percentages (Fig. 3). Notably, significant interactions between sludge dose and *Bradyrhizobium* sp. inoculation were observed for root and shoot dry weight (ANOVA, $p < 0.0001$). After regression and correlation analyses, it was observed that root and shoot dry weight were positively influenced by sludge dose (Table 2; Fig. 1). An increase in the root dry weight of the cowpea plants exposed to different doses of sludge was observed, mainly when the cowpea plants were inoculated with *Bradyrhizobium* sp. (Fig. 3). The increase of root dry weight in the uninoculated cowpea was approximately 170% relative to the control (without sludge), while the cowpea inoculated with *Bradyrhizobium* sp. displayed increases of more than 240% relative to the control. The shoot dry weight percentage was greatly increased in the uninoculated cowpea and the cowpea inoculated with *Bradyrhizobium* sp. in the absence of sludge, particularly under 20 t ha^{-1}. At this level, the cowpea inoculated with *Bradyrhizobium* sp. displayed a 120% increase in shoot dry weight compared with the uninoculated plants cultivated without sludge.

The variables related to nodule development presented higher values for the cowpea inoculated with *Bradyrhizobium* sp., even

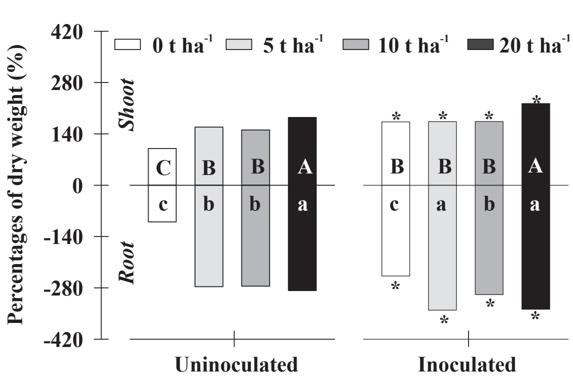

Fig. 3. Percentages (%) of shoot and root dry weight in uninoculated cowpea or cowpea inoculated with *Bradyrhizobium* sp. (BR 3267) and grown in soils amended with zero, 5, 10, and 20 t ha^{-1} of composted tannery sludge. Means followed by a different lowercase letter (roots) or capital letter (shoot) show a significant difference between each level of composted tannery sludge (Student's *t*-test, $p < 0.05$). An asterisk (*) indicates a significant difference between the uninoculated cowpea and the cowpea inoculated with *Bradyrhizobium* sp. under each level of composted tannery sludge applied (Dunnett's test, $p < 0.05$).

when sludge was present in the soil (Fig. 4). ANOVA showed significant interactions between sludge level and inoculation with *Bradyrhizobium* sp. for the number of nodules, nodule dry weight, accumulated nitrogen content, and specific nodulation ($p < 0.0001$). The cowpea plants inoculated with *Bradyrhizobium* sp. exhibited a greater number of nodules than the cowpea plants nodulated by native soil rhizobia (Fig. 4A). On average, the increase in the number of nodules in the cowpea inoculated with *Bradyrhizobium* sp. and cultivated in soils with sludge was greater than 60% relative to the uninoculated plants cultivated under the same conditions (Fig. 4A). It is likely that increasing the sludge level was responsible for the observed decreased number of nodules in the cowpea inoculated with *Bradyrhizobium* sp. In fact, the number of nodules in these plants was negatively correlated with increasing sludge dose (Fig. 1B). When 20 t ha^{-1} of the sludge was applied to the plants inoculated with *Bradyrhizobium* sp., the number of nodules was reduced by 33% when sludge was added.

Uninoculated plants and plants nodulated by native rhizobia exhibited low nodule dry weights relative to cowpea inoculated with *Bradyrhizobium* sp., even when the plants were cultivated in soils with sludge (Fig. 4B). The cowpea inoculated with *Bradyrhizobium* sp. did not exhibit different nodule dry weights in response to the different sludge doses. Positive correlations were observed between the accumulated nitrogen and composted tannery sludge doses for the uninoculated cowpea and the cowpea inoculated with *Bradyrhizobium* sp., while negative correlations were observed between the specific nodulation of the uninoculated cowpea or the cowpea inoculated with *Bradyrhizobium* sp. and the sludge level (Fig. 1). Furthermore, the accumulated nitrogen measured in the cowpea inoculated with *Bradyrhizobium* sp. and exposed to different levels of sludge was much higher than that recorded in the uninoculated plants, particularly when 20 t ha^{-1} of this sludge was applied (Fig. 4C). Likewise, specific nodulation was higher in the plants inoculated with *Bradyrhizobium* sp. (Fig. 4D); however, this variable was reduced as the level of sludge in the soil was increased. When 20 t ha^{-1} of the sludge was applied, specific nodulation was reduced by 40% relative to the control plants inoculated with *Bradyrhizobium* sp.

Variables related to nitrogen absorption, including the nitrogen content based on shoot dry weight and the nitrogen fixation efficiency, were measured (Fig. 5), and significant interactions between sludge levels and the inoculation with *Bradyrhizobium* sp. were observed for these variables (ANOVA, $p < 0.0001$). The nitrogen contents in the dried shoots of the uninoculated cowpea or the cowpea inoculated with *Bradyrhizobium* sp. increased in a dose-dependent manner in response to the applied level of sludge (Fig. 5A), which was confirmed by regression and correlation analysis (Fig. 1). Moreover, the nitrogen contents in the cowpea inoculated with *Bradyrhizobium* sp. were much greater than those in the uninoculated cowpea. Likewise, the nitrogen fixation efficiency increased in a dose-dependent manner in the cowpea inoculated with *Bradyrhizobium* sp. and subjected to different levels of sludge addition (Fig. 5B). Correlation analysis showed a strong positive relationship between the nitrogen fixation efficiency of the cowpea inoculated with *Bradyrhizobium* sp. and the sludge dose ($p < 0.0001$). The cowpea inoculated with *Bradyrhizobium* sp. exhibited nitrogen fixation increases of 99, 77, 104, and 123% relative to the uninoculated plants when zero, 5, 10, and 20 t ha^{-1} of sludge was added, respectively.

The uninoculated cowpea and the cowpea inoculated with *Bradyrhizobium* sp. displayed different responses with regard to the examined indicators of oxidative stress/protection in the presence of different levels of sludge. According to ANOVA, the interaction between sludge level and inoculation with *Bradyrhizobium* sp. was statistically significant at 1% for leghemoglobin, H_2O_2 content, and

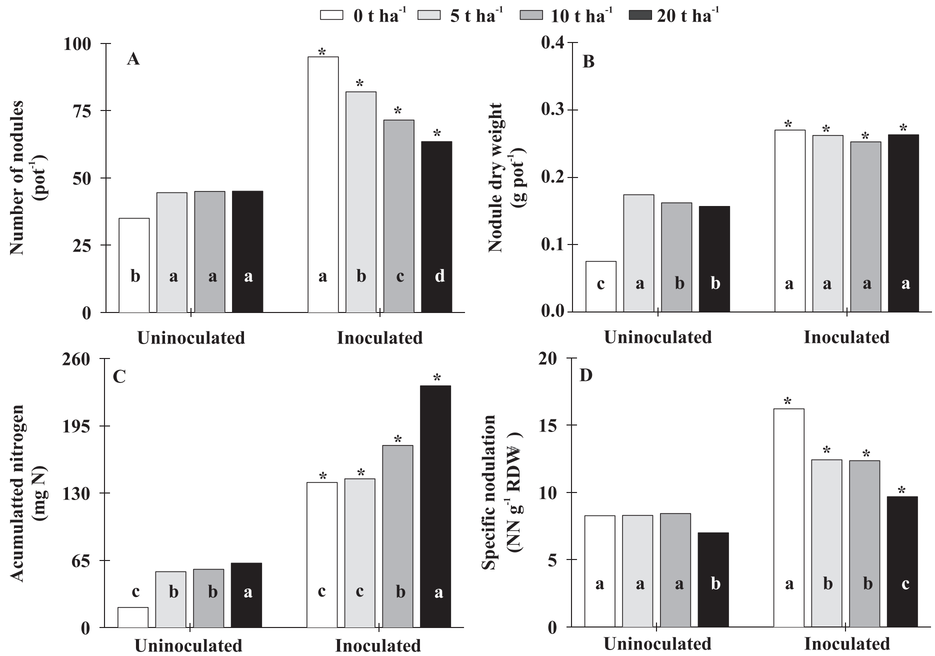

Fig. 4. Variable related to nodule development: number of nodules (A); nodule dry weight (B); accumulated nitrogen (C); and specific nodulation (D) of uninoculated cowpea or cowpea inoculated with *Bradyrhizobium* sp. (BR 3267) and grown in soils amended with zero, 5, 10, and 20 t ha^{-1} of composted tannery sludge. Means followed by a different lowercase letter show a significant difference between each level of composted tannery sludge (Student's *t*-test, $p < 0.05$). An asterisk (*) indicates a significant difference between the uninoculated cowpea and the cowpea inoculated with *Bradyrhizobium* sp. under each level of composted tannery sludge applied (Dunnett's test, $p < 0.05$).

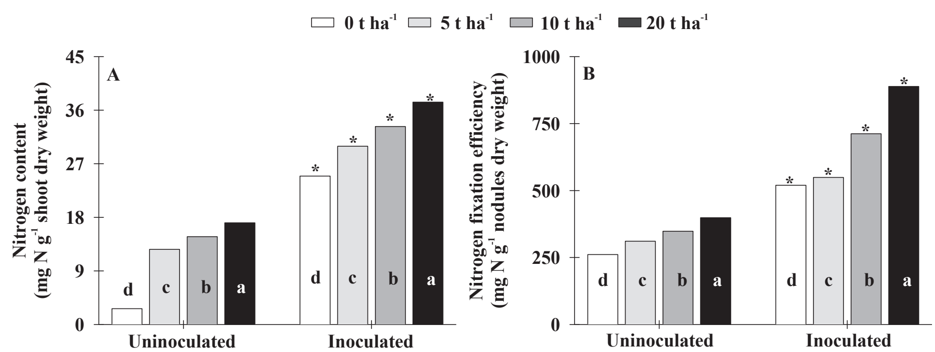

Fig. 5. Variable related to nitrogen absorption: nitrogen content (A) and nitrogen fixation efficiency (B) of uninoculated cowpea or cowpea inoculated with *Bradyrhizobium* sp. (BR 3267) and grown in soils amended with zero, 5, 10, and 20 t ha^{-1} of composted tannery sludge. Means followed by a different lowercase letter show a significant difference between each level of composted tannery sludge (Student's *t*-test, $p < 0.05$). An asterisk (*) indicates a significant difference between the uninoculated cowpea and the cowpea inoculated with *Bradyrhizobium* sp. under each level of composted tannery sludge applied (Dunnett's test, $p < 0.05$).

the antioxidative enzymes CAT and POX, and the interaction between lipid peroxidation and these factors was significant at 5%. In the uninoculated plants, the leghemoglobin in the nodules was reduced when these plants were exposed to 10 and 20 t ha^{-1} of sludge, a reduction of approximately 30% compared with the control (Fig. 6). In fact, a negative correlation between the leghemoglobin and sludge levels was observed for the uninoculated plants (Fig. 1). The cowpea inoculated with *Bradyrhizobium* sp. displayed leghemoglobin reductions of 16, 19, and 28% when grown in soils with 5, 10, and 20 t ha^{-1} of sludge relative to the cowpea inoculated with *Bradyrhizobium* sp. and grown in soils without sludge (Fig. 6). Despite the reduction in the leghemoglobin content in response to increased levels of sludge, the leghemoglobin concentration recorded in the presence of 10 and 20 t ha^{-1} of sludge was 60% higher than that observed in the uninoculated plants.

Lipid peroxidation was quantified in the nodules of the uninoculated cowpea and the cowpea inoculated with *Bradyrhizobium* sp. and exposed to composted tannery sludge (Fig. 7A). When the uninoculated cowpea and the cowpea inoculated with *Bradyrhizobium* sp. were compared, it was possible to observe that the lipid peroxidation in the uninoculated plants was superior to that in the cowpea plants inoculated with *Bradyrhizobium* sp., even when the plants were grown in soils amended with sludge. The greatest lipid peroxidation was observed in the uninoculated cowpea plants or the cowpea plants inoculated with *Bradyrhizobium* sp. with 5 t ha^{-1} of sludge, with an increase of approximately 30% relative to the respective controls. The lipid peroxidation in the nodules of the uninoculated cowpea amended with 20 t ha^{-1} of the sludge was 20% greater than the lipid peroxidation of the uninoculated cowpea grown without sludge. Regression and correlation analysis shows that lipid peroxidation in the nodules of uninoculated cowpea or cowpea inoculated with *Bradyrhizobium* sp. is not significantly and linearly related or correlated with increasing sludge dose, Cr level or soil pH (Table 2; Fig. 1).

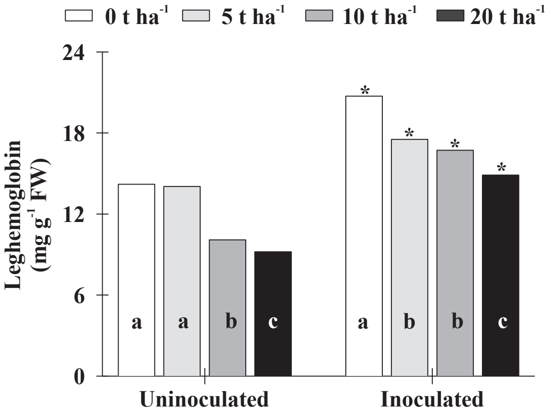

Fig. 6. The contents of leghemoglobin in the nodules of uninoculated cowpea or cowpea inoculated with *Bradyrhizobium* sp. (BR 3267) and grown in soils amended with zero, 5, 10, and 20 t ha^{-1} of composted tannery sludge. Means followed by a different lowercase letter show a significant difference between each level of composted tannery sludge (Student's *t*-test, $p < 0.05$). An asterisk (*) indicates a significant difference between the uninoculated cowpea and the cowpea inoculated with *Bradyrhizobium* sp. under each level of composted tannery sludge applied (Dunnett's test, $p < 0.05$).

Bradyrhizobium sp. grown in soils with 10 or 20 t ha^{-1} of sludge were reduced by approximately 20% relative to the cowpea inoculated with *Bradyrhizobium* sp. and cultivated in soils without composted tannery sludge (Fig. 7B). Moreover, 27 and 32% reductions in the H_2O_2 content were observed in the nodules of the cowpea inoculated with *Bradyrhizobium* sp. compared with the uninoculated plants under the same conditions.

The antioxidative enzymes CAT and POX were measured in this study and exhibited different responses to the applied treatments (Fig. 7). The CAT activity in the nodules of the uninoculated cowpea displayed a significant linear relationship and were positively correlated with increasing sludge dose, Cr level and soil pH (Table 2; Fig. 1) because the CAT activity in the nodules of the uninoculated cowpea treated with sludge was superior to the CAT activity in the uninoculated plants grown in soils without sludge (Fig. 7C). Furthermore, the cowpea plants inoculated with *Bradyrhizobium* sp. showed much higher CAT activities in their nodules than in the nodules of the uninoculated plants. In the nodules of the cowpea plants inoculated with *Bradyrhizobium* sp., no correlation was observed between CAT activity and increasing sludge dose (Table 2; Fig. 1B). When composted tannery sludge was applied at 20 t ha^{-1}, the CAT activity in the nodules of the uninoculated

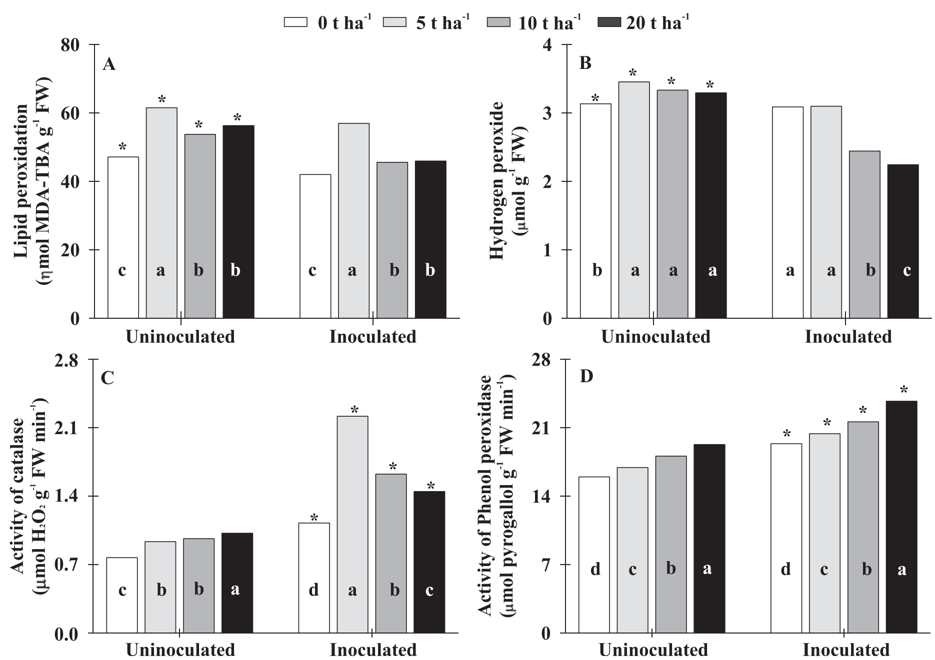

Fig. 7. Variables related to oxidative stress: lipid peroxidation (A), hydrogen peroxide content (B), and activities of catalase (C) and phenol peroxidase (D) in the nodules of uninoculated cowpea or cowpea inoculated with *Bradyrhizobium* sp. (BR 3267) and grown in soils amended with zero, 5, 10, and 20 t ha^{-1} of composted tannery sludge. Means followed by a different lowercase letter show a significant difference between each level of composted tannery sludge (Student's *t*-test, $p < 0.05$). An asterisk (*) indicates a significant difference between the uninoculated cowpea and the cowpea inoculated with *Bradyrhizobium* sp. under each level of composted tannery sludge applied (Dunnett's test, $p < 0.05$).

Uninoculated cowpea plants exhibited greater nodule H_2O_2 contents than cowpea plants inoculated with *Bradyrhizobium* sp. for all doses of sludge (Fig. 7B). The H_2O_2 contents in the nodules of the uninoculated plants were not significantly and linearly related or correlated with increasing sludge dose (Table 2; Fig. 1) but slightly increased relative to the uninoculated plants grown in soils without sludge amendment (Fig. 7B). For the cowpea plants inoculated with *Bradyrhizobium* sp., it was observed that the H_2O_2 content was negatively influenced by the sludge dose (Fig. 1B). The H_2O_2 contents in the nodules of the cowpea inoculated with cowpea or the cowpea inoculated with *Bradyrhizobium* sp. increased by approximately 30% relative to the respective controls (zero level). Cowpea inoculated with *Bradyrhizobium* sp. and grown in soils with 5 t ha^{-1} of composted tannery sludge showed the highest CAT activity in the nodules relative to the other treatments (Fig. 7C).

The POX activity measured in the nodules of the uninoculated cowpea or cowpea inoculated with *Bradyrhizobium* sp. increased in a dose-dependent manner in response to the applied levels of composted tannery sludge (Fig. 7D). In fact, simple regression analysis showed a significant linear relationship between the POX activity in the uninoculated cowpea or the cowpea inoculated with *Bradyrhizobium* sp. and sludge dose (Table 2). Additionally, a positive correlation was observed between the POX activity in the uninoculated cowpea and the cowpea inoculated with *Bradyrhizobium* sp. and the increase in sludge dose (Fig. 1). The POX activity measured in the nodules of the uninoculated cowpea was lower (~25%) than that in the nodules of the cowpea inoculated with *Bradyrhizobium* sp. under all composted tannery sludge doses. When the uninoculated cowpea or the cowpea inoculated with *Bradyrhizobium* sp. subjected to 20 t ha^{-1} of composted tannery sludge were compared with their respective controls, a POX activity increase of approximately 25% was observed in the nodules of the plants exposed to sludge (Fig. 7D). An increase in POX activity was also detected in the nodules of the uninoculated cowpea or the cowpea inoculated with *Bradyrhizobium* sp. and grown in soils treated with 5 or 10 t ha^{-1} of composted tannery sludge relative to the uninoculated plants.

4. Discussion

Cr is a toxic metal present in composted tannery sludge that can disturb vital plant metabolism processes such as photosynthesis, resulting in detrimental effects on plant growth [30]. In this study, uninoculated cowpea exhibited a dose-dependent decrease in absolute growth rate when cultivated in soils with composted tannery sludge, i.e., this variable was reduced as the sludge level increased. The absolute growth rate reflects the daily plant growth rate and is therefore related to carbon sequestration by photosynthetic processes [31]. Although they displayed greater absolute growth rates than the uninoculated plants, the cowpea plants inoculated with *Bradyrhizobium* sp. had lower absolute growth rates with increasing sludge dose. In *Pithecellobium dulce*, *Pongamia glabra* and *Cassia auriculata*, which are three species of the Fabaceae family, the shoot length was reduced when 50 mg kg^{-1} of Cr was added to the soil [30].

Changes in the photosynthetic rate, coupled with decreases in protein synthesis and photosynthetic pigment contents, may be responsible for reducing plant growth and have been reported as a response to Cr toxicity [32]. As previously reported, the composted tannery sludge used in this study displayed higher Cr contents (Table 1). High Cr levels may alter the effectiveness of diazotrophic bacteria in symbiosis with legumes due to the reduction of nitrogenase enzyme activity [33]. However, under low or moderate Cr levels, successful establishment of plant-microbe symbiosis has been reported [10]. Considering that photosynthesis is closely related to the BNF performed by diazotrophs, mainly by species of the *Azorhizobium*, *Bradyrhizobium* and *Rhizobium* genera, in symbiosis with legumes [3], it is possible that high concentrations of Cr result in unbalanced carbon fixation and consequently delay plant growth.

Cowpea plants inoculated with *Bradyrhizobium* sp. and grown in soils containing composted tannery sludge were more efficient in retaining Cr in their root cells, thereby avoiding its translocation to photosynthetic tissues. Our study group previously reported that cowpea exhibited greater Cr contents in the plant leaves when cultivated in soils with sludge under field conditions [10]; however, this increase represented approximately 10% of the Cr quantified in the soil, suggesting that this element was accumulated on the roots of these plants. According to Singh et al. [15], plant roots can accumulate 100 times more Cr than plant shoots, and Cr is likely is stored as insoluble chemical complexes deposited in the vacuoles of root cells. Furthermore, large amounts of Cr in the rhizosphere result in physiological and metabolic alterations of the plant roots [34]. Indeed, we observed changes in the growth of cowpea roots when the plants were exposed to composted tannery sludge. Moreover, cowpea inoculated with *Bradyrhizobium* sp. had shorter roots in response to the composted tannery sludge treatments; however, the root length of these plants was significantly greater than that observed in the uninoculated plants (Fig. 2B).

Some authors have reported that the cultivation of plants in soils with high Cr concentrations may cause damage to the root system and thereby reduce water and nutrient absorption [11,13,34,35]. The presence of Cr inside root cells can cause symptoms of Cr toxicity, including the inhibition of cell division, delayed cell elongation or other cellular damage [15,33]. In accordance with Kirschbaum [31], low primary root growth reflects a reduction in the final size of differentiated root cells and the number of cells entering cell division. With fewer roots, a plant explores a smaller volume of soil, which can compromise the formation of shoot organs [13,35]. An alternative to the inhibition of primary root growth is the stimulation of lateral root production from the translocation of photosynthetic carbon from the plant shoots [34].

The high availability of organic matter and nutrients identified in the composted tannery sludge may be primarily responsible for the observed increases in plant biomass [10,11,13]. Indeed, in addition to the increases in root dry mass, the application of composted tannery sludge was effective for increasing the shoot dry mass of uninoculated cowpea or cowpea inoculated with *Bradyrhizobium* sp. (Fig. 3). Similar findings have been recorded for *Capsicum* [11], *Tagetes minuta* [36], and *Ocimum basilicum* [37] cultivated in soils amended with tannery sludge, which suggests that these plants are tolerant of the Cr present in the sludge. Together, the growth data presented here indicate that cowpea may be tolerant of the high concentrations of Cr in composted tannery sludge, which allows it to maintain growth.

Considering that the metabolic energy that sustains plant growth comes from the maintenance of the photosynthetic process [31], which is inhibited in the presence of Cr [32], it is likely that the retention of Cr in roots represents a tolerance strategy employed by cowpea. In pea plants cultivated in the presence of different concentrations of Cr (20–2000 mg L^{-1}), it was observed that plant roots accumulate more Cr than plant shoots under different treatments [32]. Likewise, the increase in root dry weight, which is potentially linked to the increase in lateral root growth, could be an important strategy for storing Cr. According to Singh et al. [15], the Cr absorbed by plants generally accumulates in the plant roots, which form a barrier to decrease the translocation of Cr to the plant shoots. Previous studies performed using tannery sludge at levels greater than 10 mg kg^{-1} have shown positive effects on plant growth [11,36,37]; however, the efficiency of cowpea-rhizobia symbiosis in the presence of Cr in the rhizosphere remains poorly understood [10].

Rhizobia are bacteria that can establish symbiotic interactions through the colonization and formation of root nodules with legumes [2]. Here, the number of nodules in cowpea inoculated with *Bradyrhizobium* sp. decreased in a dose-dependent manner in response to increasing levels of sludge, although the nodule dry weight was not altered in response to the employed treatments. These results show that a small number of nodules increase their mass to maintain a higher nitrogen fixation rate and provide a continuous flux of nitrogen to the plant. In fact, the dry weight of each nodule in cowpea inoculated with *Bradyrhizobium* sp. increased in response to the investigated sludge levels (data not shown). The shoot/root carbohydrate reserves were likely mobilized to a small number of nodules, resulting in the maintenance of a constant total nodule dry weight in all treatments involving cowpea inoculated with *Bradyrhizobium* sp. grown in soils amended with sludge. Nodules are active sinks; therefore, carbohydrate reserves are useful for maintaining the full functions of these nodules [2,21]. According to Furlan [18], carbon remobilization is a basic strategy in stress mitigation.

Effective plant-microbe interactions induce plant growth, mainly by maintaining proper carbon-nitrogen flow between the plant and its symbiont [38]. Thus, the selection of elite strains has shown that symbiotic nitrogen fixation can successfully replace the use of mineral nitrogen in cowpea cultivation, even in stressful environments. Soils exhibiting excess heavy metal concentrations, water restrictions, or high salt concentrations are considered stressful environments for both plants and microorganisms [17]. Under these conditions, plant-microbe interactions can be maximized if the microorganisms possess resistance mechanisms [38]. In environments with high metal concentrations, the secretion of exopolysaccharides, which are deposited around microbial cells, has been reported as a mechanism of microbial resistance [14,38]. According to these authors, these exopolysaccharides can sequester metal pollutants, thereby preventing their entry into microbial cells, and are responsible for protecting the microbes against this type of stress.

Through plant-microbe interactions, microorganisms offer nitrogen to the plant in the form of amino acids, and the plants respond by providing carbohydrates to bacteria as a source of energy to complete their life cycle in a satisfactory manner [3,7]. In the present study, variables related to the capture of nitrogen and its flow from rhizobia to plants (i.e., the nitrogen-fixing efficiency and nitrogen content in the shoots) significantly increased in response to the application of composted tannery sludge. BNF is a mechanism for supplying nitrogen to plant species in symbiosis with bacteria [3]. Although organic matter, which is a potential source of nitrogen after mineralization, is present at high concentrations in composted tannery sludge [10], the better performances of cowpea inoculated with *Bradyrhizobium* sp. reinforce the notion that the interactions between these partners are the most effective for providing nitrogen and maintaining vegetative growth, particularly in the presence of the investigated sludge.

The metabolism of microorganisms in symbiosis with plants can also be altered when high Cr concentrations are present in the rhizosphere [19]. The root nodules of *Cicer arietinum* [35] and *Pithecellobium dulce*, *Pongamia glabra*, and *Cassia auriculata* [30] show reduced leghemoglobin in response to increased Cr levels. Here, root nodules produced by *Bradyrhizobium* sp. on cowpea exhibited more leghemoglobin than the root nodules formed by native rhizobia in uninoculated plants; however, reductions in leghemoglobin content were observed in the nodules of these plants in response to increasing sludge amendment. Interestingly, the reduction in the leghemoglobin contents in the nodules of uninoculated cowpea was not correlated with the increase in the H_2O_2 concentrations in these nodules (Fig. 1A). However, a positive relationship between leghemoglobin and H_2O_2 concentration was observed in the cowpea inoculated with *Bradyrhizobium* sp. (Pearson's correlation coefficient $[r] = 0.819$, $p < 0.0001$).

Leghemoglobin is a protein that binds molecular oxygen, preventing its diffusion inside nodules and allowing for nitrogenase activity in microaerobic environments [39]; thus, leghemoglobin is essential for symbiotic nitrogen fixation in legume root nodules induced by rhizobia [17,18]. The reduction in leghemoglobin content observed in this study potentially resulted from the different metabolic strategies employed by the cowpea symbionts. Thus, it is likely that the strong reduction in leghemoglobin content observed in the root nodules of uninoculated cowpea must be involved with the autoxidation of leghemoglobin, whereas the reduction observed in the root nodules of cowpea inoculated with *Bradyrhizobium* sp. should be involved with the H_2O_2 homeostasis in the nodules. Indeed, leghemoglobin can undergo autoxidation in the presence of toxic metals such as Cr and can result in the formation of superoxide radicals ($O_2^{\bullet-}$), which can spontaneously form H_2O_2 [20]. In the root nodules of cowpea plants inoculated with *Bradyrhizobium* sp., the reduction in leghemoglobin content may be due to their action in the decomposition of H_2O_2 to water, similarly to plant peroxidases [17,32,39].

Increasing ROS contents, especially H_2O_2, have been reported in response to the exposure of cells to Cr [30,32,35] and inhibit BNF in the nodules of several legumes [17]. In fact, the uninoculated cowpea plants treated with composted tannery sludge displayed increasing H_2O_2 concentrations in their root nodules relative to the uninoculated cowpea plants grown in soils without sludge amendment (Fig. 5B). On the other hand, cowpea inoculated with *Bradyrhizobium* sp. and grown in soils with high sludge amendment levels exhibited decreased H_2O_2 contents relative to the control. H_2O_2 plays a dual role in cells, acting as a signal transduction molecule and as a ROS that oxidizes cellular macromolecules [17]. Overall, oxidative stress stimulates the formation of ROS, resulting in damage to the plant membranes via lipid peroxidation [20]. In this study, no correlation was observed between lipid peroxidation in uninoculated cowpea and increasing sludge dose, Cr level or soil pH (Fig. 1A), but a positively relationship was observed between lipid peroxidation and H_2O_2 content in the uninoculated cowpea ($r = 0.895$, $p < 0.0001$). It is possible to conclude that the lipid peroxidation exhibited by the uninoculated plants was a response to increased H_2O_2 concentrations. Indeed, increases in H_2O_2 are frequently associated with protein and lipid damage and with the activation of the ROS-scavenging system [4,40].

To form an environment that is compatible with nitrogenase activity, rhizobacteria possess a complex array of antioxidant molecules and antioxidative enzymes that maintain the ROS concentrations at a low and steady-state levels [3,19]. This ROS-scavenging system includes different antioxidative enzymes, such as CAT, superoxide dismutase (SOD), ascorbate peroxidase (APX) and POX, which play particularly important roles in ROS scavenging [17,20]. CAT and POX are metalloenzymes that use different mechanisms to degrade the H_2O_2 produced in nodules and can be activated in different situations because they are an important primary line of protection against oxidative stress [3,20]. Overall, the CAT activity was strongly enhanced in the nodules of cowpea plants inoculated with *Bradyrhizobium* sp. and treated with composted tannery sludge. Similarly, nodules of alfalfa (*Medicago sativa* L.) were observed to display increased CAT activity in response to the supplementation of soils with sewage sludge, which is similar to tannery sludge (i.e., rich in nitrogen and containing high heavy metal concentrations) [41].

CAT activation is considered an important mechanism that is involved in metal detoxification [16] and might control the H_2O_2 content with the assistance of POX in nodules in stressful environments. POX is an enzyme that decomposes H_2O_2 through the oxidation of co-substrates, such as phenolic compounds and/or antioxidants, and has much higher activities than CAT [4,20]. In nodules of a tolerant genotype of common bean, the activation of POX activity has been shown to occur in environments with salt stress [42]. Here, increases in POX activity were observed in the nodules of cowpea inoculated with *Bradyrhizobium* sp. in response to the application of composted tannery sludge. In cowpea inoculated with *Bradyrhizobium* sp., increases in the POX activity were correlated with decreases in H_2O_2 content and root length and with increases in root dry weight. Taken together, these results indicate that POX can be involved in H_2O_2 homeostasis and the formation of a physical barrier against heavy metal inputs by inducing lignin biosynthesis, as reported by Bhaduri and Fulekar [40]. Overall, H_2O_2 at low levels in the nodules of cowpea inoculated with *Bradyrhizobium* sp. is maintained by the synchronic actions of leghemoglobin, CAT and POX.

The present study demonstrated a significant positive effect of inoculation with *Bradyrhizobium* sp. (BR 3267) on the growth, nitrogen content and antioxidative responses of cowpea relative to uninoculated plants when grown in soils amended with composted tannery sludge, particularly at a concentration of 10 mg kg^{-1}. Taken together, our data reinforce the notion that favorable plant-microbe interactions improve plant performance. Furthermore, possible biochemical changes that may occur in the presence of sludge or, more specifically, the changes that result from the stressful environment caused by the presence of Cr in the sludge, can be minimized through appropriate interactions between plants and microorganisms. In nodules formed by *Bradyrhizobium* sp. on cowpea, it is possible that H_2O_2 induces defenses against the oxidative stress that results from the presence of excess Cr in the surrounding area as a strategy to maintain BNF at high rates. This hypothesis is supported by significant correlations between the increases in nitrogen-fixing efficiency and POX activity as the H_2O_2 content in the nodules of the cowpea plants inoculated with *Bradyrhizobium* sp. decreased. Although the high concentrations of Cr (253.5 mg kg^{-1}) present in tannery sludge could result in metabolic changes, inoculation with *Bradyrhizobium* sp. was observed to be a useful tool for minimizing such detrimental effects and for ensuring plant growth and productivity.

5. Conclusion

We conclude that the presence of composted tannery sludge affects the establishment and development of the symbiosis of rhizobia with cowpea, mainly when this symbiosis occurs with native soil rhizobia. When cowpea was inoculated with *Bradyrhizobium* sp., the plants were able to maintain better plant growth and nitrogen cavitation and flux and had lower oxidative stress in their nodules.

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