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Original article

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

Nayara de Jesus Moraes [a], Vicente Paulo da Costa Neto [a], Ademir Sérgio Ferreira de Araújo [b], Marcia do Vale Barreto Figueiredo [c], Aurenivia Bonifacio [d], Artenisa Cerqueira Rodrigues [e,*]

[a] Federal University of Piaui, Department of Agricultural Engineering and Soil Science, Teresina, PI, Brazil
[b] Federal University of Piaui, Department of Agricultural Engineering and Soil Science, Laboratory of Soil Quality, Teresina, PI, Brazil
[c] Agronomical Institute of Pernambuco, IPA/SEAGRI, Laboratory of Soil Biology, Recife, PE, Brazil
[d] Federal University of Piaui, Department of Biology, Laboratory of Plant Physiology, Teresina, PI, Brazil
[e] Federal University of Piaui, Department of Agricultural Engineering and Soil Science, Laboratory of Soil Microbiology, Teresina, PI, Brazil

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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,

* Corresponding author. Laboratory of Soil Microbiology, Department of Agricultural Engineering and Soil Science, Federal University of Piaui, CEP 64049-550, Teresina, PI, Brazil.

E-mail addresses: nayaramoraes@yahoo.com.br (N.J. Moraes), costanetovp@gmail.com (V.P. Costa Neto), asfaruaj@yahoo.com.br (A.S.F. Araújo), mbarreto@elogica.com.br (M.V.B. Figueiredo), bonifacio.a@live.com (A. Bonifacio), artenisacerqueira@hotmail.com (A.C. Rodrigues).

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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