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Plant and soil characteristics affected by biofertilizers from rocks and organic matter inoculated with diazotrophic bacteria and fungi that produce chitosan

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Abstract

The aim of this study was to evaluate the effectiveness of a mixed biofertilizer with phosphate and potash rocks (PK biofertilizer) combined with an earthworm compound inoculated with free living diazotrophic bacteria and *Cunninghamella elegans*, fungi that produces chitosan, on cowpea nodulation, biomass yield and nutrient uptake. The effects of some chemical attributes from an acidic soil of the Brazilian Northeast were also studied. The treatments were as follows: a) biofertilizer enriched in N by free living diazotrophic bacteria(NPKB), applying crustaceous chitosan (ChCru) at a rate 2 mg mL^{-1}; b) NPKB and ChCru at a rate 4 mg mL^{-1}; c) NPKB and ChCru at a rate 6 mg mL^{-1}; d) NPKB and fungi chitosan (ChFu, 2 mg mL^{-1}); e) NPKB+*C. elegans* (NPKP); f) NPKB without chitosan; g) mineral fertilizers (NPKF); and h) control without NPK fertilizer and chitosan. Biofertilizer treatments increased cowpea nodules biomass, shoot biomass, and total N, P, and K in the shoots. The largest increase was obtained with ChCru, and the highest rate was obtained with NPKP. Furthermore, biofertilizers reduced soil pH and increased the total N and available P and K. These results reveal the potential of rock biofertilizer mixed with earthworm compound inoculated with free living diazotrophic bacteria and *C. elegans* (fungi chitosan) for plant production and nutrient uptake. The biofertilizer may be an alternative for NPK fertilization that slows the release of nutrients, favoring longterm soil fertility.

Keywords: Biopolymer, free living diazotrophic bacteria, *Cunninghamella elegans*, nitrogen fixation, *Vigna unguiculata*.

1. Introduction

The growing world population and demand for fertilizers have led to changes in agricultural cropping systems and intensified the use of new techniques to produce maximum yields from field crops. The use of legume species is of great importance because they may provide nitrogen to the system through N_2 fixation and supply nitrogen without the application of mineral fertilizers (Kuhn, 2007).

Soil acidity is one of the most important factors that requires consideration, especially because of the effects of increased exchangeable aluminum on the nutrient uptake reduces the soil's pH, which negatively affects nutrient availability (Stamford *et al*., 2008). Several studies conducted on acidic soil from rainforest regions have shown that plant yield is reduced as a function of acidity. These studies indicated that it is controlling this specific problem will improve the productivity and quality of crops (Stamford *et al*., 2007, 2008).

Biofertilizers produced with P and K rocks that are sulfur inoculated with oxidizing bacteria that produces sulfuric acid (El Tarabily *et al*., 2006) contributes to reduced soil pH. Therefore, it is necessary to use organic matter with high pH to control the acidic effects of rock biofertilizers (Lima *et al*., 2010). Studies carried out in tableland soil from the Brazilian Northeast reported that sulfur inoculated with *Acidithiobacillus* is a promising material for soil fertilization because it releases P and K from rock minerals, which reduces the nutrient deficiency of acidic soils used to grow different crops (Stamford *et al.*, 2008, Lima *et al*., 2007, Moura *et al.*, 2007).

The incorporation of organic matter increases the microbiological activity and enhances the physical and chemical conditions of soil. However, in many cases, these materials contain low concentrations of N. Biological nitrogen fixation (BNF) is one of the most fundamental processes used to supply N to the soil, especially in combination with organic matter (Lima *et al*., 2010).

To date, there have been no reports describing the effects of biofertilizers inoculated with diazotrophic bacteria and fungi that produce chitosan. However, recent studies suggest the potential use of rock biofertilizers from phosphorus- and potassium-bearing rocks and minerals as alternatives to synthetic fertilizers. Moura *et al*. (2007) and Stamford *et al*. (2007, 2008) evaluated the effects of these alternative materials on different economic crops and soils. However, the phosphorus- and potassium- bearing rocks contain no N, and thus, they do not supply N for improved plant development and yield. To produce a more complete biofertilizer, it is important to improve the N concentration of the organic matter. One method for achieving this is by adding free living diazotrophic bacteria to enrich the N content of the organic material (Lima *et al.*, 2010).

Crustaceous chitosan is frequently used in biological studies with different objectives, including to increase the resistance to plant pathogens. In our laboratory, we obtained good results using chitosan from Mucorales fungi, such as *Cunninghamella elegans, Mucor rouxii,* and *Mucor circineloides* (Franco *et al*., 2004). Fungi biomass has not been used for chitosan addition after the production of biofertilizers from rocks plus elemental sulfur inoculated with the sulfur oxidizing bacteria *Acidithiobacillus*. The final pH of the biofertilizers is very acidic (3.0 – 3.5) due to the metabolic production of sulfuric acid, which may involve the formation of reacting chitosan under homogeneous conditions, as proposed by Bautista-Baños *et al*., (2006).

The use of chitosan from fungi biomass has great advantages, such as independence from seasonal factors, wide scale production, simultaneous extraction of chitin and chitosan, and the fact that the process of chitosan extraction is simple and cheap, resulting in reduced time and cost. Moreover, this strategy avoids protein contamination, particularly from proteins that could cause allergic reactions in individuals with shell fish allergies (Franco *et al.*, 2004).

Chitosan has better chelating properties than other natural polymers due to their amino groups, as nitrogen is a donor of electron pairs, although hydroxyl groups participate in combining these reactive groups with metal ions. Based on these chemical properties, chitosan may stimulate the plant growth and yield as well as induce the immunologic system to promote resistance to plant pathogens (Boonlertnirun *et al.*, 2008). Furthermore, the addition of *C. elegans*, which are fungi that produce chitosan and polyphosphate, may increase the availability of phosphate in the soil (Franco *et al.*, 2011).

This study aimed to compare the effects of mineral NPK fertilizers with those of biofertilizers produced from rocks with *Acidithiobacillus* and earthworm compound inoculated with free living diazotrophic bacteria and *C. elegans* on cowpea nodulation, biomass yield, and nutrient uptake. The application of chitosan from fungi biomass and from crustaceous by chemical processes was also studied.

2. Material and Methods

2.1. Production of PK rock biofertilizers - BPK

P and K rock biofertilizers were produced at the Federal Agricultural University of Pernambuco (UFRPE) Horticultural Experimental Station using two furrows (each 10.0 m long, 1.0 m wide and 0.5m deep). For each biofertilizer, 4,000 kg of natural phosphate with 11 g kg^{-1} total P (purchased from Irecê (Bahia), Brazil, were applied with4,000 kg of potash rock (biotite) containing 10 g kg^{-1} total K (purchased from Santa Luzia (Paraiba), Brazil, following the procedure described by Stamford *et al*. (2007).

The sulfur oxidizing bacteria were grown in 2,000 mL Erlenmeyer flasks containing 1,000 mL of specific culture medium (El Tarabily *et al.* 2006), and the cultures were sterilized for 30 min at 120 °C. The Erlenmeyer flasks were shaken (150 rpm/min) for 5 days at 30 °C. The materials (phosphate and potash rocks mixed with elemental sulfur) were incubated for 60 days, and humidity was maintained at a level near the field holding capacity. To avoid excessive humidity due to rain and to increase the efficiency of the oxidative bacteria, the furrows were covered with black plastic.

The natural rock P and K biofertilizer was analyzed using the (A) Mehlich 1 and (B) an extraction using citric acid (2 g kg^{-1}) methods, according to Embrapa, (2009), yielding the following results: (P biofertilizer)- pH = 3.8, available P (A) = 60 (g kg^{-1}) and (B) = 48 (g kg^{-1}); (K biofertilizer-BK)- pH = 3.3, available K (A) = 10 (g kg^{-1}) and (B) = 5 (g kg^{-1}).

2.2. Production of biofertilizer with free living diazotrophic bacteria and C. elegans

The organic biofertilizer was produced by applying the selected free living bacteria to the earthworm compound. The isolate (NFB 1001) was cultured in LG liquid media (50 mL) in 125 mL Erlenmeyer flasks, shaken (180 rpm) for 96 h at ±28 °C and applied at 100 mL per tray, according to Lima *et al*. (2010). After inoculation, the trays were incubated for 30 days at 28 ±5 °C. The humidity was maintained near the water holding capacity using distilled water, according to Stamford *et al*. (2008). Samples were collected, and the total N content was determined by the Kjeldhal method, using the Kjeltec auto analyzer (1030 Model). Chemical analysis of the earthworm compound revealed the following: pH 7.15, organic carbon 100.7 g kg^{-1}, total N 8.6 g kg^{-1}, total sulfur 2.98 g kg^{-1} and total P 11.2 g kg^{-1}.

The earthworm compound (3 dm^3) and the PK biofertilizers (1 dm^3) were mixed to produce the biofertilizer (NPKB). After the production of the biofertilizer (NPKB), we added a micelial biomass of the Mucorales fungi, *Cunninghamella elegans* (UCP 542), which produce sufficient chitin (140 mg g^{-1}) and chitosan (80 mg g^{-1}) in the cellular wall, as reported by Franco *et al*. (2004). The fungi were grown in

Petri dishes with BDA medium for 10 days at 28 °C, producing a final concentration of 106 spores/ mL^{-1}, to which 5 mL of spore suspension were applied; the mixture was then shaken for 96 hours (150 rpm) at room temperature. The fungi biomass was sieved in a nylon membrane and washed (three times) with cold distilled water. After 10 days of incubation, the organic matter plus PK biofertilizer (NPKB) was analyzed (Embrapa, 2009) and exhibited the following characteristics: pH (H_2O) = 6.9; total N (21 g kg^{-1}); available P (20 g kg^{-1}) and available K (19 g kg^{-1}).

2.3. Soil site and analyzes

Were used an acidic soil classified as sand texture Spodosol (Embrapa, 2006). The soil (0-30cm layer) was collected from a small farm area in the County of Itapirema in the rain forest region of the Pernambuco state (Northeast Brazil; $7^0$59' 0'', of south latitude, $38^0$19' 16''of west longitude). The soil was air dried, sieved (5 cm sieve), mixed, analyzed (Embrapa, 2009), and stored at 10 kg pot^{-1}. Soil chemical analysis produced the following results: pH (H_2O) =5.2; exchangeable cations: K^+=7 $mmol_c$ dm^{-3}; Ca^{2+}=7.9 $mmol_c$ dm^{-3}; and Mg^{2+}9 $mmol_c$ dm^{-3}. Soil analyses were processed using the Absorption Spectrophotometer and atomic emission Perkin Elmer 3110, and total N was determined by the Kjeldhal method, using the N auto analyzer Kjeltec 1030.

2.4. Treatments and experimental design

The greenhouse experiment was set up in a factorial arrangement (8x2), and it was conducted in a completely randomized design with 4 replicates. The fertilization treatments were as follows: (a) mixed biofertilizer (NPKB) + chitosan from crustaceous (ChCru; purchased from Heppe Gmb, German Industry) was applied at a rate of 2 $mgmL^{-1}$; (b) NPKB + (ChCru) at a rate of 4 $mgmL^{-1}$; (c) NPKB + (ChCru) at a rate 6 $mgmL^{-1}$; (d) NPKB + fungi chitosan (ChFu), produced in laboratory and obtained from *C. elegans*, at a rate of 2 mg mL^{-1}; (e) Biofertilizer (NPKB) –plus *C. elegans*; (f) NPKB without chitosan; (g) NPKF (mineral fertilizer – NPK), mixing ammonium sulfate, simple superphosphate and potassium sulfate, applied at the recommended rate for cowpea; (h) Control (without NPK fertilization and without chitosan).

Fertilizer treatments were estimated following the recommendation for irrigated cowpea grown in the Pernambuco state (IPA, 2008) using soluble N fertilizer (ammonium sulfate, 500 kg ha^{-1}), soluble P fertilizer (simple super phosphate, 300 kg ha^{-1}), and soluble K fertilizer (potassium sulfate, 80 kg ha^{-1}). The amount of biofertilizer NPKB was calculated based on the N content (20 g kg^{-1}), which corresponded to the application of 5,000 kg ha^{-1} of organic matter (earthworm compound). The seeds were inoculated with the rhizobia strain (NFB 700) recommended for cowpea by the Nucleus of Nitrogen Fixation of the University Federal Rural of Pernambuco (UFRPE).

2.5. Experimental conditions and statistical analysis

Cowpea (*Vigna unguiculata* cv. IPA 206) was utilized because it is resistant to *Fusarium oxysporum* f. sp. *Tracheiphilum*. Seeds were surface-sterilized in ethyl alcohol (70%) for 1 min, immersed in $HgCl_2$ (1:500) for 0.5 min, and washed 6 times with sterile water. The seeds were sown (4 seeds pot^{-1}) at a depth of 5 mm, and when emergence was completed (5 DAE), the seedlings were thinned to one per pot and inoculated by applying 2 mL pot^{-1} of the liquid inoculant liquid containing more than 108 UFC mL^{-1}. The Bradyrhizobium (NFB 700), was used because it has displays nitrogenase activity in the presence of mineral nitrogen (Stamford *et al.*, 1995) and has shown effective nodulation in acidic soil (Stamford *et al.*, 2008).

Seeds were sowed in the dry season (2008, July), which is characterized by low humidity and high temperature. A phytosanitary control was not necessary because insects and disease were not observed. Water was applied daily to the pots, maintaining the moisture near field holding capacity. During the experimental period, the photoperiod remained close to 12 h of dark and 12 h of light. The temperature oscillated between 28 and 36 °C,

and the relative humidity was 60-80%, resembling the natural growth conditions of the culture. Nitrogen was applied in dressed fertilization on the planting date and 15 days after seed planting.

The dry biomass of nodules and shoots was determined at plant harvest, prior to the emission of flowers (45 days after planting). Nodules and shoots were harvested separately, and their dry biomass was measured. Total N content was analyzed by the semi-micro Kjeldhal method, using the automatic N analyzer (Kjeltec - Model 1030). Total P and K were analyzed by nitroperchloric digestion.

Total P content was analyzed by the spectrophotometer method, and total K was measured by atomic emission (Perkin Elmer 3110).

The statistical analysis was assessed using analysis of variance, which included the main effects of fertilization treatments, using SAS software 3.2 (SAS Institute, 2011). Differences among the mean treatment values were determined by Tukey's test ($p \leq 0.05$). All parameters were normally distributed, including the nodule biomass, because the zero values were not determined in the uninoculated treatment group.

Table 1. The pH, total N, and available P and K of biofertilizers from PK rocks mixed with earthworm compound inoculated with free living diazotrophic bacteria (NPKB) and with addition of *C. elegans* (NPKP) incubation times.

Time of incubation (days)	pH (1.0:2.5)	Total N	Available P	Available K
NPKB biofertilizer		($g\ kg^{-1}$)	--------($g\ dm^{-3}$)--------	
10	6.4a	10b	13c	34b
20	6.1b	12b	20b	35b
30	6.0b	17a	26ab	41a
NPKP (NPKB+*C. elegans*)				
10	6.3a	14b	20b	40a
20	6.0b	13b	25ab	45a
30	6.0b	19a	29a	45a
CV- Coefficient variation, %	8.9	9.8	12.9	18.3

Means followed by different letters, in the rows, are significantly different at p=0.05 by Tukey's test.

3. Results and Discussion

3.1. Biofertilizer with diazotrophic bacteria and C. elegans

The chemical analyses (pH, total N, and available P and K) are shown in Table 1. The pH results significantly differed between both products in regard to period of incubation, especially from 10 to 20 days. The reduction in pH values was evident in the biofertilizer (NPKB) with inoculation of the free living bacteria (NFB 1001) and with the addition of *C. elegans*. Similar pH results were obtained by Lima *et al*. (2010) in a study evaluating the effect of diazotrophic bacteria on the chemical parameters of biofertilizer produced from earthworm compound inoculated with different strains of free living diazotrophic bacteria. Comparing the results from the mixed biofertilizer (NPKB) and the biofertilizer with the addition of the fungi *C. elegans,* there was no change in pH reduction, likely due to the short incubation time (10 days). The fact that the pH stabilized at 6.0 is advantageous because the highest yield of tropical crops are obtained in soil from pH 6.0 to 6.5, as described by Stamford *et al.* (2008).

The effects on total N and available P and K in the biofertilizers were the opposite of those observed for the pH values. There was a substantial increase in the content of these nutrients in the substrates. In recent assays, an increase of up to 100% of total N was observed when the free living bacteria (NFB 1001) was used to inoculate the earthworm compound (Lima *et al.*, 2010). However, in our study, it was not evident that the time of incubation was sufficient to produce the greatest enzyme activity, as determined by total N enrichment. The results suggest that more than 20 days of incubation is necessary to further increase nutrient levels with the application of *C. elegans*. The level of total N increased with the length of incubation, and the values were stabilized when the fungi and *C. elegans* were applied. The best results for complete production of NPKB biofertilizer was approximately 30 days of incubation when the earthworm compound was inoculated with the free living diazotrophic bacteria, and the production of the protector when *C. elegans* was added need more than 20 days to produce the highest yield of total N.

There was a significant difference in the level of available P over the period of incubation. The highest available P was obtained with 30 days of incubation, resulting in an increase of up to 100% relative to the initial time. The increase in available K was also significant, and the highest values were obtained with 30 days of incubation in the NPKB biofertilizer. However, the increase in available K was only approximately 20% with NPKB with *C. elegans* compared to the NPKB biofertilizer.

3.2. Nodules and shoot biomass

The dry biomass of cowpea nodules and shoots grown in a Brazilian Spodosol and harvested 45 days after emergence are presented in Table 2. The effect of NPK fertilizers on cowpea nodulation and biomass yield was dependent on the fertilization treatments. The highest nodule biomass was obtained with chitosan from fungi (ChFu-2 mg mL^{-1}) and the NPKB with *C. elegans*. Ali *et al.* (1997) and Costales *et al.* (2005, 2007) described similar results in studies on the effects of applying chitin and chitosan to soil for soybean nodulation.

A detrimental effect of mineral fertilizer was observed on nodule biomass, confirming a report from Stamford *et al*., (2008) in cowpea grown in a Brazilian Spodosol. They reported that high levels of mineral nitrogen promoted total plant reduction and no nodules formation, but in this case, nodule production was not totally damaged but also the dry nodule biomass was reduced by more than 90%. It is interesting to observe that when organic matter was applied at the same rate as nitrogen, the cowpea biomass yield increased by more than 250% compared with the control treatment lacking N application.

The fertilization treatments significantly increased the cowpea shoot biomass compared with the control treatment, resulting in a 2.5 fold increase in nodule

biomass production. No significant difference was observed between the fertilizer treatments; however, the best dry shoot yield was obtained using biofertilizer (NPKB) and crustaceous chitosan applied at the highest rate (ChCru 6 mg mL^{-1}), followed by the chitosan from fungi (ChFu-2 mg mL^{-1}) and NPKB with *C. elegans* and NPKB without chitosan. The control treatment showed the lowest value of shoot biomass.

Maia *et al*. (2009) and Mazaro (2008) also observed that the application of chitosan by foliar pulverization in the shoots at different rates showed no significant effects on the shoots, roots or fruit yield of grapes. The authors agree that chitosan do not positive effect plant yield because the induction of resistance may promotenterference and deviation of the metabolic routes and the syntheses of defensive components. Negative effects were observed in cowpea, and Kuhn (2007) described that the metabolic energetic cost promotes the redirection of photo assimilates that are used for plant defense against diseases, which reduce plant yield.

The large amount of N in chitosan (6.9 to 8.7%) may increase vegetative and reproductive plant growth, consistent with reports by Otha *et al.* (2004) and Rabea *et al.* (2003). They observed that when chitosan was applied to soil as a mixed fertilizer, the resulting high rates of nitrogen, phosphorus, and potassium increase plant growth compared to control treatments.

Results described by Otha *et al.* (2004) in ornamental plants differed from those observed in the present study, which compared the effect of chitosan application to soil with mineral fertilizers using the same rate of nitrogen. However, we observed that chitosan increased the dry and fresh shoot and root biomass and reduced the time of flowering compared with others fertilizer treatments.

Table 2. The effect of biofertilizers enriched in N by free living diazotrophic bacteria (NPKB), and foliar application of chitosan from crustaceous (ChCru) and fungi chitosan (ChFu), NPKB inoculated with *C. elegans* (NPKP), mineral fertilizers (NPKF) and the control treatment on nodule biomass (NB) and shoot biomass (SB) of cowpea.

Treatments	Nodules Biomass (NB)	Shoot Biomass (SB)
	----------g plant^{-1}----------	
NPKB + ChCru(2mg mL^{-1})	0.86 ± 0.03 b	29.68 ± 0.58 a
NPKB + ChCru(4mg mL^{-1})	0.96 ± 0.06 b	29.01 ± 0.87 ab
NPKB + ChCru(6mg mL^{-1})	1.01 ± 0.04 ab	31.19 ± 1.38 a
NPKB + ChFu(2mg mL^{-1})	1.26 ± 0.08 a	29.81 ± 0.50 a
NPKB + *C. elegans* (NPKP)	1.10 ± 0.09 ab	29.15 ± 0.49 a
NPKB without chitosan	1.07 ± 0.05 ab	29.98 ± 0.96 ab
NPKF (mineral fertilizer)	0.13 ± 0.03 d	25.69 ± 0.55 b
No NPK applied and without chitosan	0.48 ± 0.03 c	12.60 ± 1.01 c
Coefficient of Variation, C.V.(%)	6.27	6.36

Means followed by different letters, in rows, are significantly different at p=0.05 by Tukey's test.

Table 3. The effect of biofertilizers enriched in N by free living diazotrophic bacteria (NPKB), and foliar application of chitosan from crustaceous (ChCru) and fungi chitosan (ChFu), NPKB inoculated with *C. elegans* (NPKP), mineral fertilizers (NPKF) and the control treatment on total N, P and K accumulation in cowpea shoot biomass.

Fertilization treatments[1]	Total N	Total P	Total K
	----------mg plant^{-1}----------		
NPKB + ChCru(2mg mL^{-1})	218 ± 6.5 a	10.6 ±0.1 ab	99.0 ± 8.4 b
NPKB + ChCru(4mg mL^{-1})	243 ±26.1 a	9.4 ±0.9 b	110.7 ±13.4 b
NPKB + ChCru(6mg mL^{-1})	248 ±21.6 a	11.2 ±0.6 ab	111.3 ±12.6 b
NPKB + ChFu(2mg mL^{-1})	210 ±20.5 a	8.4 ±1.0 b	112.6 ± 6.1 b
NPKB + *C. elegans* (NPKP)	228 ±36.2 a	13.0 ±1.1 a	126.8 ± 3.3 a
NPKB without chitosan	200 ±14.1 ab	10.6 ±1.0 ab	103.2 ± 4.8 b
NPKF (mineral fertilizer)	251±24.0 a	13.2 ±0.4 a	134.0 ±12.0 a
No NPK applied and without chitosan	174 ± 4.0 b	8.2 ± 0.5 b	90.0 ± 6.2 c
Coefficient of Variation, C.V.(%)	12.28	18.03	13.18

Means followed by different letters, in rows, are significantly different at p=0.05 by Tukey's test.

3.3. Nutrient uptake

Total N, P, and K accumulation in the shoot biomass of cowpea (mg plant^{-1}), 45 days after emergence, are present in table 3. Total N and P accumulation in the shoot dry matter were significantly different between the fertilizer treatments and the control. In general, there were no differences between the fertilization treatments; however, the mineral fertilizer, biofertilizer +ChCru (4 mg mL^{-1}) and NPKB plus *C. elegans* were only 35% superior to the control treatment.

A significant difference was in total K accumulation in the shoot dry biomass of cowpea was observed between the treatments. The mineral fertilizer (FNPK) produced the highest amount of total K in shoot biomass, and it was 20% superior to the other fertilization treatments. There were no significant differences between the treatments with NPKB.

Boonlertnirun *et al*. (2008) reported that the period of chitosan availability in soil may be longer when it is applied to the biopolymer in the shoot, and prolonged contact of the plant root and soil favored the interaction between the positive charges of chitosan and the negative charges of the nutrients contained in soil, which may influence nutrient absorption by plants and contribute to increase plant yield.

3.4. Soil attributes

The soil pH and total N data are shown in the Table 4, and the available P and K in the soil is shown in Table 5. The soil pH only varied slightly between the different fertilization treatments and the control, and the changes were not significant. Low pH values (approximately pH 5.0) were observed in response to the mineral fertilizers. This effect was likely due to the addition of ammonium sulfate (N mineral fertilizer),

which can increase acidity, as described by Chien *et al*. (2008) and Stamford *et al*. (2008). The NPKB biofertilizer is produced with earthworm compound with very high pH (7.9), which helps to control the acidity of the PK rock biofertilizer, as observed by Moura *et al*. (2007) Lima *et al*. (2007).

According to our study, the applied substrates significantly affected the total N content in soil. The highest amount of total N in soil was found when NPKB with *C. elegans* was added to substrate that produces chitosan, and this biopolymer increased the nutrient content, especially nitrogen, due to the high amount of N in the acetylated chain of chitosan from *C. elegans* (Franco *et al*. 2004, 2011). The available P and K in soil after the treatments is shown in Table 5. In response to different fertilizer treatments, the available P was significant different compared to the control without the addition of fertilizers, and the highest amount of available P in soil was observed with the application of soluble fertilizers. The NPKB treatments showed no difference. Significant differences were observed in available K between the fertilizers treatments and the control. However, the available K measured in the soil was highest after treatment with FNPK fertilizers, NPKB (ChCru 6 mg mL^{-1}) and (ChFu 2 mg mL^{-1}), with the addition of *C. elegans*. The effects of chitosan were likely observed in the treatments with higher amounts of substrates that were sulfur inoculated with *Acidithiobacillus,* as the acid production increases the available P and K in soil (Stamford *et al.*, 2006, 2007, 2008). Furthermore, chitosan increased the levels of N, P and K in the substrate (Kowalski *et al.*, 2006, Goy *et al*., 2009). The application of sulfur with *Acidithiobacillus* must be thoroughly evaluated due to the acidification effect of sulfuric acid, which in some cases and particularly in acidic soils, may result in the displacement of exchangeable Al^{+3} that is harmful to plant growth (Stamford *et al.*, 2006). The results of this study suggest that further investigation is necessary to evaluate the effect of amendments in different soils due to their effect on soil pH. Reducing the pH of sodic soils to values approximately 6.0-6.5 may improve plant growth, especially in tropical crops.

Table 4. The effect of biofertilizers enriched in N by free living diazotrophic bacteria (NPKB), and foliar application of chitosan from crustaceous (ChCru) and fungi chitosan (ChFu), NPKB inoculated with *C. elegans* (NPKP), mineral fertilizers (NPKF) and the control treatment on soil pH and total N in a Brazilian Spodosol after cowpea growth (45 days).

Fertilization treatments	pH H_2O (1.0 : 2.5)	Total N (mg g^{-1})
NPKB + ChCru(2mg mL^{-1})	5.73 ± 0.15 a	0.35 ± 0.09 c
NPKB + ChCru(4mg mL^{-1})	5.73 ± 0.21 a	0.55 ± 0.90 ab
NPKB + ChCru(6mg mL^{-1})	5.72 ± 0.24 a	0.65 ± 0.18 a
NPKB + ChFu(2mg mL^{-1})	5.72 ± 0.28 a	0.55 ± 0.09 ab
NPKB + *C. elegans* (NPKP)	5.62 ± 0.08 a	0.65 ± 0.13 a
NPKB without chitosan	5.62 ± 0.08 a	0.35 ± 0.09 c
NPKF (mineral fertilizer)	4.82 ± 0.08 b	0.40 ± 0.10 b
No NPK applied and without chitosan	5.60 ± 0.10 a	0.30 ± 0.10 c
Coefficient of Variation (%)	2.57	13.81

Means followed by different letters, in rows, are significantly different at p=0.05 by Tukey's test.

Table 5. The effect of biofertilizers (NPKB) enriched in N by free living diazotrophic bacteria, with foliar application of chitosan from crustaceous (ChCru) and fungi chitosan (ChFu), NPKB with *C. elegans* (NPKP), mineral fertilizers (NPKF) and the control without NPK and chitosan on the available P and K in Brazilian Spodosol after cowpea growth (45 days).

Fertilization treatments	Available P	Available K
	--- mg dm^{-3} ---	--- $mmol_c$ dm^{-3}---
NPKB + ChCru(2mg mL^{-1})	17.54 ± 1.18 a	55 ± 1.5 ab
NPKB + ChCru(4mg mL^{-1})	17.74 ± 1.08 a	47 ± 0.6 ab
NPKB + ChCru(6mg mL^{-1})	17.68 ± 0.83 a	61 ± 1.2 ab
NPKB + ChFu(2mg mL^{-1})	17.57 ± 0.08 a	65 ± 0.5 ab
NPKB + *C. elegans* (NPKP)	17.87 ± 0.57 a	75 ± 0.5 a
NPKB without chitosan	17.81 ± 0.80 a	50 ± 0.5 b
NPKF (mineral fertilizer)	19.70 ± 1.39 a	75 ± 1.8 a
No NPK applied and without chitosan	1.20 ± 0.20 b	12 ± 0.1 c
Coefficient of Variation (%)	15.0	16.11

Means followed by different letters, in rows, are significantly different at p=0.05 by Tukey's test.

4. Conclusions

The results of this study indicate that the incubation period affected the nutrient content in both substrates. In general, the best time for NPKB biofertilizer production is 30 days, and the best time for the biofertilizer with *C. elegans* was 20 days until the final production of this substrate.

Biofertilizers with PK rocks and earthworm compound inoculated with diazotrophic bacteria and *C. elegans* is effective for nodulation and nutrient uptake and might increase the availability of nutrients in soil.

A combined effect of *Acidithiobacillus,* free living diazotrophic bacteria, and *C. elegans* was observed on cowpea nodulation and nutrient uptake. Thus, the biofertilizer seems to be an alternative for the substitution of soluble fertilizers.

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