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Influence of Bioprotector with Microbial Inoculation on Green Pepper Yield and Improvement on Soil Nutrients Availability

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***Abstract* - The aim of the study is to evaluate the effectiveness of PK rock biofertilizer mixed with organic matter inoculated with diazotrophic bacteria (NPKB) and the bioprotector with fungi chitosan by *Cunninghamella elegans* (PNPK) on yield of green pepper (*Capsicum annuum*) and improvement in soil available nutrients. The experiment was carried out during March – August, 2011 in a factorial using eight fertilization treatments with and without chitosan application on leaves, with four replicates. The fertilizer treatments were: (1) NPKF soluble fertilizers applied in recommended rate - RR; (2) NPKB (50% RR); (3) NPKB (100% RR); (4) NPKB (150% RR); (5) NPKP (50% RR); (6) NPKP (100% RR); (7) NPKP (150% RR); (8) Control treatment (earthworm compound). The NPKB and NPKP increased green pepper yield especially when applied in the highest rate, reduced soil pH and increased positively total N, available P and K, exchangeable Ca and Mg. The study focus the potential of biofertilizer with free living diazotrophic bacteria (NPKB) and the bioprotector with fungi chitosan (NPKP) as alternative to NPK fertilization.**

***Keywords* – Capsicum Annuum, Cunninghamella Elegans, Biopolymer, Biological Fertilizers, Fungi Chitosan, Nutrient Availability.**

I. Introduction

The growing world population and the demands for fertilizers and pesticides have led to sensible changes in agricultural systems and intensify the use of new techniques to maximum yields (Goy *et al.*, 2009). Fertilization with NPK is one of the most important factors that affect horticultural productivity and the nutrient availability in soil which increase yield and maximize the agricultural crop system, especially in tropical soils (Stamford *et al.*, 2008).

Biofertilizers produced from powdered P and K rocks plus elemental sulfur inoculated with *Acidithiobacillus thiooxidans*, which metabolically produces H_2SO_4, contributes decisively to increase availability of the nutrients contained in rocks. It is known that N is the nutrient required in greater amount by plants and is not supplied by powdered rocks to promote better plant growth. To increase N content in PK rock biofertilizer with low pH values, is necessary the addition of organic matter (OM) inoculated with free living diazotrophic bacteria as proposed by (Lima *et al.*, 2010).

In biological studies crustaceous chitosan are frequently used to increase the resistance to plant pathogens, and moreover chitosan have chelating properties greater than others natural polymers by the presence of the amino groups, and may release nutrients to the environment (Boonlertnirun *et al.*, 2008; Goy *et al.*, 2009). In this study fungus biomass (*Cunninghamella elegans*) was applied to NPKB biofertilizer for production of the bioprotector that promote chitin deacetylation by effect of the acidity promoted by the sulfur oxidative bacteria *Acidithiobacillus.* The use of chitosan from fungi biomass has great advantages compared to crustaceous chitosan, such as independence of seasonal factors and simultaneous extraction of chitin and chitosan (Franco *et al*., 2004).

The use of fungi chitosan application in agriculture as bioprotector does not appear in the specific literature and it was not found any published research using *C. elegans* to produce biofertilizer for addition of fungi chitosan. This study was carried out to describe the production of bioprotector and to evaluate the stimulating effects of biofertilizer and bioprotector with fungi chitosan on green pepper growth and nutrient uptake. The main objective of the research was to study the feasibility of PK rocks plus organic mater enriched in N by free living diazotrophic bacteria and addition of fungi chitosan for use as alternative to conventional fertilizers and to observe the influence on nutrients in the soil. Furthermore the product could present potential to be used as bioprotector against phytopatogenic microorganisms in further researches.

II. Materials and Methods

Production of Biofertilizers (BNPK) and Protector (PNPK)

Biofertilizers from phosphate and potash rocks were produced at the Horticultural Experimental Station of the Federal University Rural of Pernambuco (UFRPE) using two furrows (each 10.0-m long, 1.0-m wide and 0.5-m deep). For each biofertilizer, 4000 kg of natural phosphate with total P of 240 g kg^{-}1, purchased from Irecê (Bahia), Brazil, and 4000 kg of potash rock (biotite) with total K of 100 g kg^{-1}, from Santa Luzia (Paraiba), Brazil, was mixed with 400 kg of elemental sulfur that is inoculated with *Acidithiobacillus thiooxidans* bacteria, following the procedure described by Stamford *et al.* (2007).

The sulfur-oxidizing bacteria were grown in 2000 ml Erlenmeyer flasks that contained 1000 ml of specific culture medium (El Tarabily *et al.*, 2006) and had been sterilized for 30 min at 120 °C. The Erlenmeyer flasks were shaken (150 rev/min) for 5 days at 30 °C. The materials (phosphate and potash rocks mixed with elemental sulfur) were incubated for 60 days, and the humidity was maintained at a level that was near the field holding capacity. To avoid the effects of excessive humidity due to rain and to increase the efficiency of the sulfur-oxidizing bacteria, the furrows were covered daily using black plastic.

Analysis of the PK rock biofertilizer (PKB), extracted by (A) Mehlich 1 solution and (B) citric acid according to Embrapa (2009), yielded 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) = 0.5 (g kg^{-1}).

The biofertilizer (NPKB) was produced in field conditions using PK rock biofertilizer (PKB) and organic matter (OM) obtained from sugar cane cake, mixed in proportion (PKB: OM) equivalent to (1: 4), inoculated with free living bacteria (NFB 10001) selected in previous assays. The diazotrophic bacteria was cultured in LG liquid media (50 ml) in 125 ml Erlenmeyer flasks and shaken (180 rpm) for 96 h at 28 ± 5 °C temperature, according to the methodology of Lima et al. (2010). After inoculation, the material was incubated for 30 days following the same process described above for the PK rock biofertilizer, and the humidity was maintained near water holding capacity. Samples were collected, and the total N determined by the Kjeldhal method, using the Kjeltec auto analyzer (1030 Model). The results of the chemical analysis of the mixed biofertilizer (NPKB) are as follows: pH 6.90; organic carbon 120.7 g kg^{-1}; total N 19.8 g kg^{-1}; total sulfur 10.9 g kg^{-1}; total P 10.1 g kg^{-1}; total K 15.1 g kg^{-1}.

The protector (NPKP) represents the biofertilizer (NPKB) with addition of micelial biomass of the fungus *Cunninghamella elegans* (UCP 542), which contains a considerable amount (7-8%) of chitosan in the cellular wall. The fungus *C. elegans* was purified in Petri dishes on medium PDA grown 10 days at 28 °C. The monosporic culture of the *C. elegans* was obtained grown the Mucorales fungus in Potato-Dextrose (BD) medium as recommended by Franco et al. (2004), using 2000 mL Erlenmeyer flasks kept under shaking (180 rotations per minute) at 28 °C by 96 h. The micelial biomass (10 L) was diluted (1 L culture per 10 L of distilled water) and added to the substrate by manual application, and then incubated for 35 days. Weekly samples were collected for chemical analyses (pH, total N, available P and K) as described in the NPKB production.

Experimental conditions and soil analyses - field experiment

A field experiment with sweet pepper (cv. All Big) was carried out at the IPA Experimental Station located at the rainforest region of Pernambuco State, Brazil, in the District of Vitoria de Santo Antão, situated in Latitude South (8º 8' 00'), Longitude west (35º 22' 00"), and Altitude 146 m.

During the field experimental period (December 2010–March 2011) the photoperiod remained close to 12 h of darkness and 12 h of light. The temperature oscillated between 28 and 36°C and the relative humidity was 60-80 %. The soil was prepared for crop by cutting and removing all of the vegetation from the experimental area. Soil was prepared by the conventional tillage using one plowing and two disking, and followed the rows were opened to transplant the green pepper seedlings. The rows were made systematically to maintain declivity around 0.2-0.5% to avoid soil run off.

Seeds were pre-germinated in trays (128 cells per tray) and transplant to the field 38 days after seed plantation. The fertilizers were mixed with the surface soil (10 cm deep), and realized the seedling plantation. The sub plot (8.4 m^2) measured 2.8 m long and 3 m wide and plants spaced 1.0m x 0.40m. To estimate yield 10 plants were collected from each sub plot from the central rows. Plants were collected weekly and realized four harvests to evaluate the total fruit yield and the number of fruits.

Soil analyzes were carried out in samples from 10 plants of the central rows of each sub plot. Soil (composite samples) were collected at 20 cm deep in furrows with 10 cm distance to plant base, and analyzed following the Embrapa (2009) methodology: pH, total N, available P, and K, exchangeable Ca^{+2} and Mg^{+2}. Soluble $S\text{-}SO_4^{-2}$ was determined using Merck specific Kits and the Merck Spectrophotometer model TR 420.

The NPKF mixed fertilizer with ammonium sulfate, simple superphosphate, potassium sulfate was prepared based in the recommended rate (RR) following soil analyzes and the recommendation for irrigated green pepper in the state of Pernambuco, Brazil (IPA, 2008). Fertilizers NPKF were applied at seedling transplant and for N and K used more two dressing fertilization applying ammonium sulfate and potassium sulfate. The treatments with NPKB and NPKP were: 50; 100 and 150 (g $plant^{-1}$), which correspond to 50% RR, 100% RR and 150% RR, applied at seedling transplantation and in the two dressing fertilization. In the control treatment applied farmyard manure (2.4 L $plant^{-1}$) at seedling transplantation and applied the same amount in the two dressing fertilization. The soil used was classified as a Red Yellow Latosol (Embrapa, 2006) located in the rainforest region of Pernambuco state, Brazil. The soil analysis (Embrapa, 2009) showed: pH (H_2O) = 6.1; total N = 0.55 g kg^{-1}, available P = 2.7 mg dm^3, available K= 10.4 mg dm^{-3}, exchangeable cations ($mmol_c$ dm^{-3}): Ca = 16 and Mg = 4.1.

Experimental design and statistical analyzes

After the fruits harvest, soil samples were collected at 0-20 cm deep, to analyze the chemical attributes: pH, available P and K (Mehlich 1), exchangeable sodium, calcium and magnesium, in accord to Embrapa (2009) methodology.

The experiment was set up in a factorial (8x2), in randomized split plot design with 4 replicates. The treatments were: (1) NPKF soluble fertilizers applied in recommended rate - RR; (2) NPKB (50% RR); (3) NPKB (100% RR); (4) NPKB (150% RR); (5) NPKP (50% RR); (6) NPKP (100% RR); (7) NPKP (150% RR); (8) Control treatment (farmyard manure). All fertilizer treatments were applied with and without shrimp chitosan (90% purity, 95 % deacetylation degree) purchased from Sigma Industry, applied on leaves seven days after seedling transplantation, to observe the natural occurrence of root pathogenic fungi.

The statistical calculations for the assay for production of the bioprotector and for the field experiment parameters were achieved using the software Program SAS 9.2 version (SAS Institute 2011). Analyzes of variance and averages were compared by the Tukey test at probability $p<0.05$.

III. Results

Green pepper yield

Rock biofertilizers mixed with earthworm compound inoculated with diazotrophic bacteria (NPKB) and adding *C. elegans* (NPKP) showed to be more effective than the conventional fertilizer (NPKF) and increase sweet pepper yield (Figure 1). The fertilization with PNPK (150 % RR) showed higher green pepper fruit yield, that presented 21.36 t ha^{-1} followed by PNPK (100 % RR) and BNPK (150 % RR) with fruit yield 19.14 and 19.07 t ha^{-1}, respectively. The treatment with soluble fertilization (FNPK) produced 17.38 t $ha^{-1}$and the control treatment (farmyard manure - 2.4 L $plant^{-1}$) had the lower fruit yield (15.65 t ha^{-1}). However, it is interesting to observe that all the fertilizers treatments applied showed yield higher than the average for the state of Pernambuco for irrigated green pepper (15 t ha^{-1}).

(Figure 1)

Soil pH in the field experiment after green pepper harvest

The soil pH in function of the fertilization sources showed significant and high effects of the treatments (Figure 2). The protector (NPKP) and the mixed biofertilizer (NPKB) followed by the control treatment showed the high values of soil pH and application of commercial soluble fertilizers (NPKF) have not effect in soil reaction.

The effect of NPKP and NPKB increasing soil pH may be explained by the use of very high amount of organic matter applied 4 times the PK rock biofertilizer (proportion 4:1) when produced these substrates in field conditions, and the organic (earthworm compound) has pH 7.9 and rock biofertilizers pH 3.5, and in the control treatment was applied high amount of organic matter (pH 6.7).

Figure 2.

Total N, available P and soluble SO_4^{-2} in soil after harvest

The results of total N in soil, after green pepper harvests are presented in Figure 3. The total N in soil increased with application of the different fertilization treatments, compared with the control. The best results were obtained when applied PNPK (BNPK+ *C. elegans*). The mixed biofertilizer (BNPK) applied in highest rate (150% RR) incremented total N up 50%, and PNPK, applied in 150% RR, increased total N in 100%, compared with mineral fertilizer (FNPK). Probably the N released by the mineral fertilizer (ammonium sulphate) had been used by plants to promoted growth, and due to its greater solubility may be percolating from soil by the effect of the heavy precipitations that had occurred in the region during the experimental period.

Figure 3.

The results of available P in soil are presented in Figure 3. It may be observed the positive and significant effects of NPKB and NPKP in available P in soil after green pepper harvest. It was observed low available P content in soil when applied the conventional fertilizer (NPKF) and the control treatments.

The results for SO_4^{-2} are also showed in Figure 3. It may be observed that the values increased with the mineral mixed fertilizer NPKF, followed by NPKB applied in the highest rate (150% RR). The mineral fertilizer probably displayed higher soluble SO_4^{-2} values because the fertilizer mixture were used that used ammonium sulfate, simple super phosphate and potassium sulphate, that have sulphate as constituinte in the fertilizer. Besides sulphate is a weak base, normaly is not percolate in soil, especialy in presence of Ca and Mg, and also because green pepper is not very sulfate exigent. It may be observed that in the treatments BNPK e PNPK the soluble SO_4^{-2} in soil showed high values, as expected, due to the metabolic production of sulfuric acid which release SO_4^{-2} to the soil that may be used by the plant and besides other amount may be displayed to the soil and have potential to be used in the consecutive crops.

Exchangeable potassium, calcium and Magnesium in soil

The results of available K in soil are presented in Fig.4. The biofertilizer and the bioprotector promoted significant increase in available K. When applied higher rate NPKB increase available K, and with Bioprotector occurred inverse effect of these treatments.

The available K in soil increases when mixed mineral fertilizers were applied, probably due to the higher K concentration in the soluble fertilizer, followed by the treatment with NPKB applied in the higher rate (150% RR). Figure 4.

The results for exchangeable Ca are present in Figure 4. Exchangeable Ca content in soil showed significant effect of the fertilization treatments, especially when applied the NPKP in highest rate (150% RR) obtaining the highest amount in exchangeable Ca in soil , followed by NPKP in rate 100% RR. The soluble mineral fertilizer (NPKF) and the control treatment showed low exchangeable Ca. Furthermore, it may be observed that the Ca content in soil increased in a considerable amount in comparison with the values observed in the analyzed soil before the start of the experiment.

The results for exchangeable Mg were not significant for the fertilization treatments (Figure 4). Despite no significant effect of the fertilization treatments application, it can be observed that exchangeable Mg increase compared to the Mg in soil analyzed before carried out the field experiment.

The highest values of exchangeable Mg in soil may be also explained by the solubilization of Mg contained in the mineral (biotite) used to produce the K rock biofertilizer, and most probably by the effect of the sulphuric acid produced metabolically by *Acidithiobacillus* in the presence of elemental S.

IV. Discussion

Green pepper yield

Similar results on yield were obtained by Moura et al. (2007) applying P and K rock biofertilizers and organic matter (earthworm compound) compared with P and K mineral fertilizers in a Brazilian Argisol. Oliveira *et al.* (2010) applying organic matter from castor bean (10 t ha^{-1}) observed increase in melon yield and the authors reported that the effect have occurred because the organic matter increases nutrient solubilization.

The difference observed on yield when applied the bioprotector is due to effect of the fungi *Cunninghamella elegans* addition to the biofertilizer (NPKP), which produced inorganic polyphosphate and increase P and N due to the chitosan amino charges observed in the chitosan deacetylation (Franco et al., 2004). 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.

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.

Soil pH in the field experiment

Stamford *et al.* (2011) in a study applying rock biofertilizer mixed with organic matter in grape observed similar effect in soil pH. Silva *et al.* (2011), evaluate melon growth in two soils of Rio Grande do Norte State, using three sources of P (triple superphosphate, P rock biofertilizer, and mixed triple superphosphate plus phosphate rock) and observed a slight increase in soil pH when applied P rock biofertilizer in a red Yellow Latosol. Lima *et al.* (2007) verified the effect of P and K rock biofertilizers produced with P and K rocks with Elemental rock inoculated with *Acidithiobacillus* oxidative bacteria mixed with earthworm compound in two crops of lettuce and observed that in general the pH was not affected by the fertilization treatments.

Oliveira *et al*. (2010), in a study to evaluate the agronomic effectiveness of castor bean residues in soil attributes observed a linear reduction of soil pH with organic matter rates and promoted variation from 6,0 to 5,0 on values on pH. Stamford *et al*. (2004, 2006, 2009,) and Moura *et al.* (2007), showed the effect of P and K biofertilizers reducing soil pH after P and K rock biofertilizers plus elemental sulfur inoculated with *Acidithiobacillus*. The authors attribute that the acidity effect was produced by the metabolic H_2SO_4 produced by the oxidative e bacteria. However, it is important to observe that the P and K biofertilizer was not applied mixed with organic matter, unlike what was done in the present study.

Total N, available P and soluble SO_4^{-2} in soil after harvest

In the treatment NPKP with addition of fungi chitosan increased the total N in soil, in agreement with the results obtained by Stamford *et al*. (2009) evaluating the agronomic effectiveness of rock biofertilizers from PK rocks mixed with earthworm compound in cowpea compared with soluble fertilizer FNPK. The authors also described the positive effects of the mixed biofertilizer with earthworm compound enriched in N by inoculation with the free living diazotrophic bacteria (NFB 10001).

The effects of chitosan application probably occurred because in the treatments with higher amounts of elemental sulfur inoculated with *Acidithiobacillus* the acid production increase available P and K in soil as reported by Stamford *et al*. (2006, 2007, 2008) and also because chitosan increase the levels of N, P and K in the substrates as described by Kowalski *et al*. (2006), Goy et al. (2009) and Stamford *et al*. (2014).

The results show the effect of the sulfur-oxidizing bacteria *Acidithiobacillus thiooxidans* on solubilization of minerals contained in the P and K rocks as described by Stamford *et al*. (2006, 2007, 2011) in different soils cropped with sugar cane, yam bean and grapes, respectively. Also, the increase in available P effect may be explained because others bacteria native from soil besides *Acidithiobacillus*, and some soil fungi that promote P solubility produce phosphatases chitin and chitosan as *Cunninghamella elegans* (Franco *et al*., 2011) and increase solubility of P and others nutrients.

Applying organic matter from castor bean in rate 10 t ha^{-1}, Oliveira *et al.* (2010) obtained similar results in the soil available P. The authors reported that the effect have occurred because the organic matter can increase nutrient solubilization which promote the balance between K and Ca and resulted in greater phosphate availability.

Silva *et al.* (2011) in a study applying different P sources in melon growth verified the positive effect of P rock biofertilizer with highest amounts of available P in soil. In an Argisol from the semiarid region (San Francisco Valley) Stamford *et al.* (2009) also observed significant effect of PK rock biofertilizers increasing available P compared with conventional soluble fertilizers.

The effectiveness of biofertilizers from P and K rocks plus elemental S inoculated with *Acidithiobacillus thiooxidans* were reported by Lima *et al*., 2009. The authors described that the rock biofertilizers mixed with earthworm compound promote higher residual effect of the biofertilizers compared with mineral fertilizers on lettuce in a Yellow Latosol of the “Cariri” region, in two consecutive crops.

Exchangeable potassium, calcium and magnesium in soil

It is important to know that in the specific literature there are not many references about the effects of K biofertilizers produced from powdered rocks. In soils of the coastal tableland of Pernambuco State, after sugar cane crop, Stamford *et al*. (2006) described the increase in available K in soil when applied K rock biofertilizer with elemental S inoculated with *Acidithiobacillus thiooxidans*, and the results seems to be similar to the results found in the present study. Lima *et al*. (2007) observed positive and significant effect of P and K fertilization in available K in soil, after lettuce harvest in the Brazilian semiarid region of "Cariri", and the authors described best results when applied conventional fertilizer at rate 160 kg ha^{-1}, quite similar to K biofertilizer (KB) applied in the recommended rate. In an Argisol of the San Francisco valley Stamford *et al.* (2009) showed increment in the available K in soil when applied the mineral fertilizer (NPKF) and PK biofertilizer applied in the highest rate.

The greater results are probably due to the solubilization of Ca contained in the phosphate rock. The results are similarly to those found by Stamford *et al.* (2006) with sugar cane grown in a soil of the coastal tableland of the Pernambuco State, Brazil, where higher values of exchangeable Ca were obtained by application of P and K rock biofertilizer.

Similar results were obtained in a experiment with sugar cane carried out in a tableland soil of the coastal humid region of Pernambuco state (Stamford *et al.,* 2006), with melon in the San Francisco Valley, semiarid region of Pernambuco state (Stamford et al., 2009), in grapes (Stamford *et al*., 2011) and in cowpea (Stamford *et al*., 2014) which showed high amount of exchangeable Mg in soil when applied P and K rock biofertilizer. Significant effect in exchangeable Mg when applied organic matter (10 t ha^{-1}) which varied from 39 $mmol_c$ dm^{-3} to 1,62 $cmol_c$ dm^{-3} compared with the control treatment without organic matter application were reported by Oliveira *et al.* (2010).

V. Conclusion

Biofertilizers with PK rocks mixed with earthworm compound (NPKB) enriched in N by inoculation with diazotrophic bacteria (NFB 1001) and the BNPK with *C. elegans* (PNPK) are effective and promote significant increase in sweet pepper fruit yield, and also increase nutrients availability in soil. The BNPK and PNPK should to be alternative for NPK mineral fertilization.

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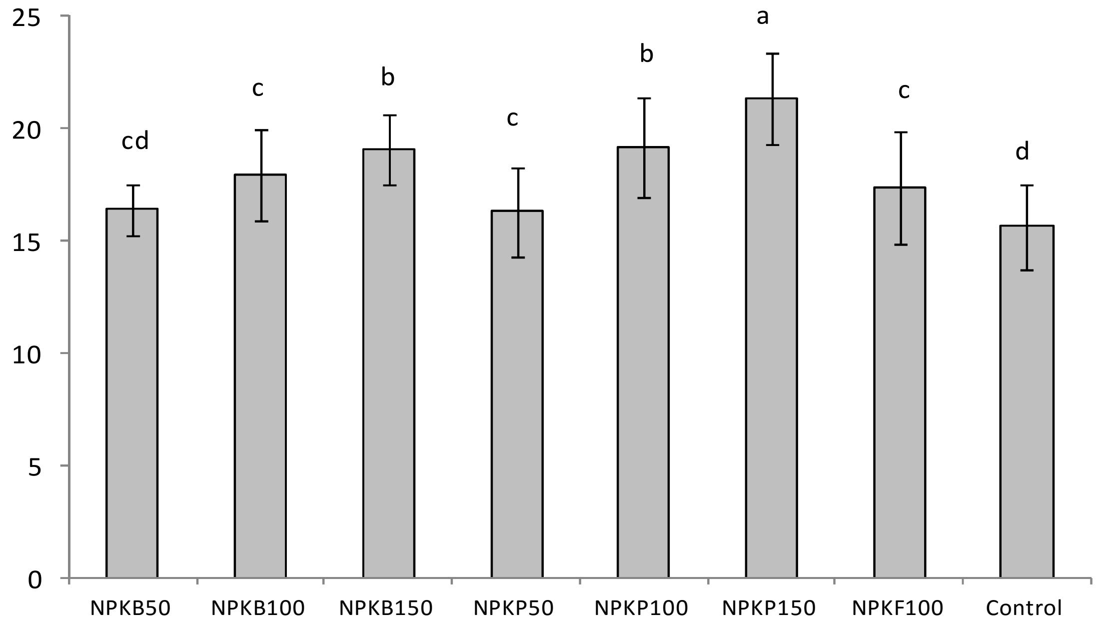

Fig.1. Green pepper yield at the first harvest, as affected by the different fertilization treatments (sources and rates). Means of four replicates

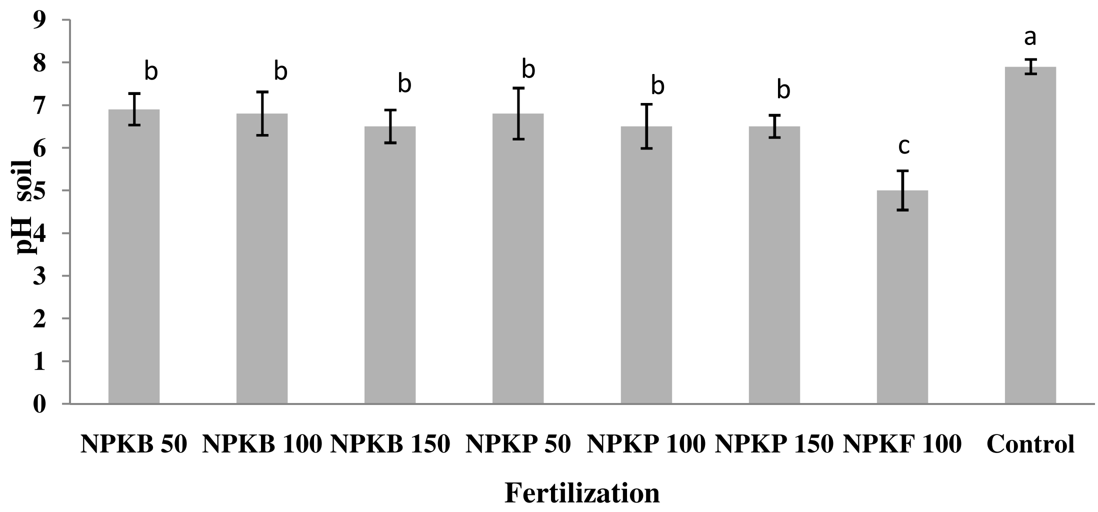

Fig.2. Soil pH after green pepper harvest, as affected by the different fertilization treatments (sources and rates). Means of four replicates.

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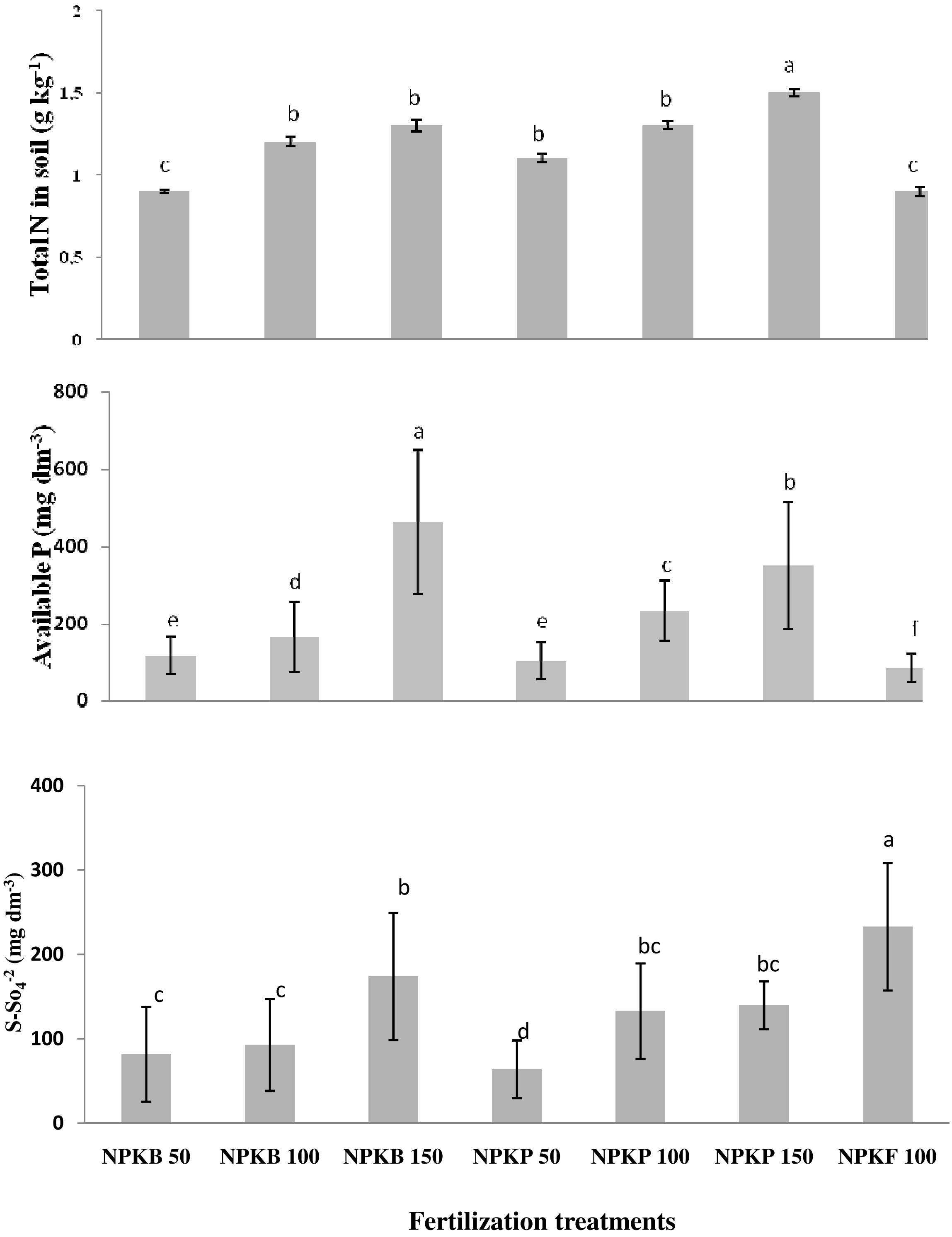

Fig.3. Total N, Available P and soluble SO_4^{-2} in soil after green pepper harvest, as affected by the different fertilization treatments (sources and rates). Means of four replicates.

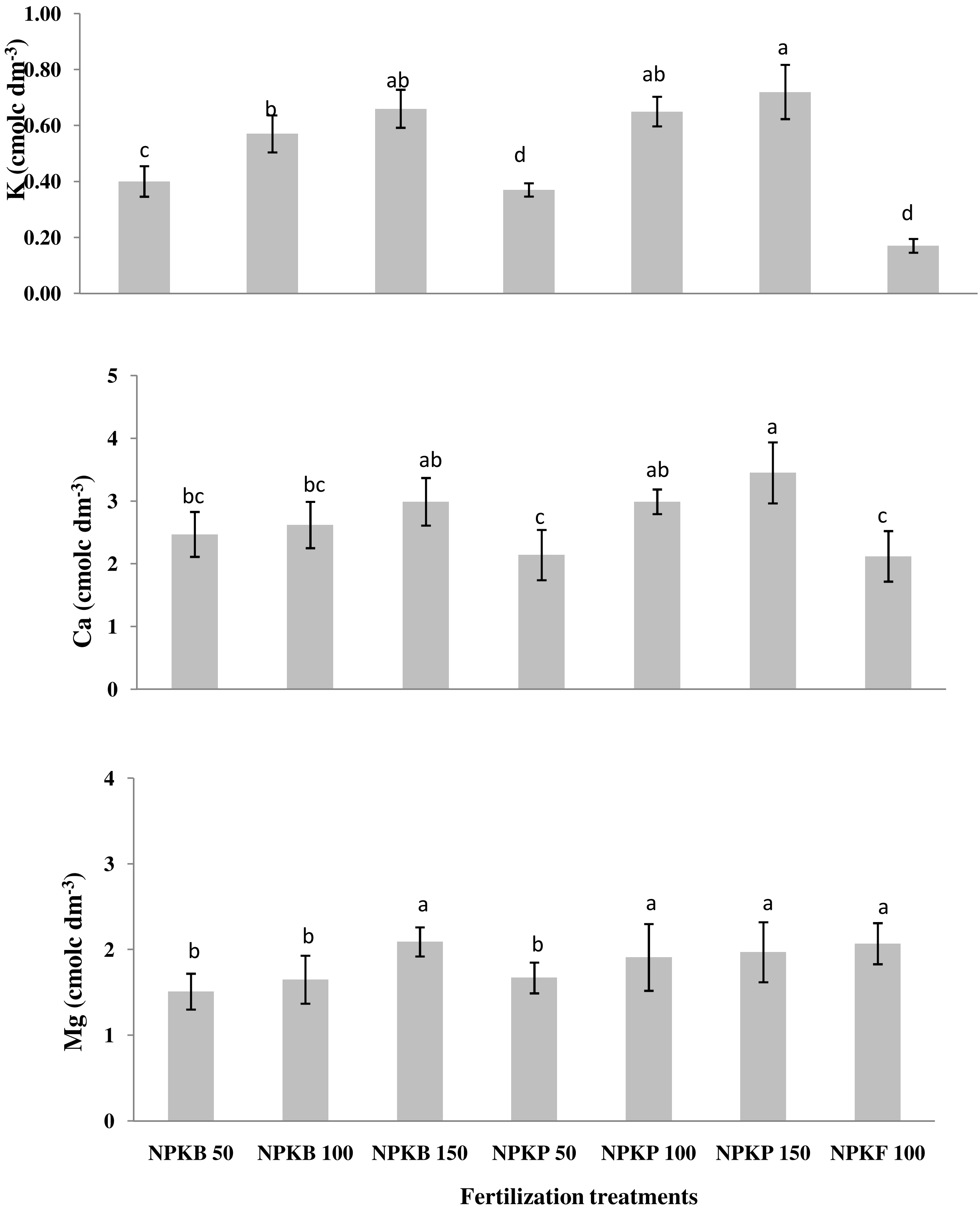

Fig.4. Available K, Ca and exchangeable Mg in soil after green pepper harvest, as affected by the different fertilization treatments (sources and rates). Means of four replicates