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Yield of grape (*Vitis labrusca* cv. Isabel*)* and soil nutrients availability affected by biofertilizer with diazotrophic bacteria and fungi chitosan

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Abstract

Fertilization by biological products is often reported as one of the most important factors for a modern and sustainable agricultural system. The scope of this study is to evaluate the effects on grape (*Vitis labrusca* cv. Isabel) and nutrients availability by application of biofertilizer (NPKB) enriched in N by inoculation with free living diazotrophic bacteria and *Cunninghamella elegans* a fungus that contains chitosan in the cell wall. The experiment was carried out in the Botticelli fine wine industry at San Francisco Valley grown for February 2010 to May 2012), comparing the biofertilizer (NPKB) and the mineral fertilizer (NPKF) applied in 50, 100 and 150 % recommended rate (RR), at two depths (0-20 and 20-40 cm). A control treatment with grape waste compound (2.4 kg plant^{-1}) was applied. Grape yield and soil attributes (pH, total N, soluble SO_4^{-2}; available P and K; exchangeable Ca^{2+} and Mg^{2+}) were evaluated. The NPKB and NPKF increased up to 10 t ha^{-1}grape yield compared to the control. The soil pH was reduced when NPKF applied in rate 100% RR. The NPKB influence positively on total N, available P, available K, soluble S-SO_4^{2-}, exchangeable Ca^{2+} and Mg^{2+} in soil especially when applied at 0-20 cm depth. The study focuses the potential of the biofertilizer inoculated with diazotrophic bacteria and *C. elegans* which may be an alternative for NPK fertilization favoring long-term soil fertility. The NPKB may be recommended for grape in rate 150% RR applied at 0-20 cm deep.

Keywords: *Vitis labrusca,* free living diazotrophic, fungi chitosan, organic fertilizer, soil fertility.
Abbreviations: NPKB_biofertilizer with NPK nutrients; NPKF_soluble mineral fertilizers; RR_Recommended Rate.

Introduction

The growing world population and increased demands for fertilizers and pesticides have led to sensible changes in agricultural systems and the use of new techniques to maximize yields (Goy et al., 2009). Fertilization with NPK affects nutrient availability in the soil; this increases yield and maximizes the productivity of the agricultural crop system (Stamford et al., 2008). In order to reduce the production costs and the use of finite natural nutrient resources, many researches are looking for alternatives to substitute synthetic mineral fertilizers with organic and rock biofertilizers (Van Straaten, 2007). Recent studies described the potential of rock biofertilizers from phosphorus and potassium bearing rocks as alternatives to synthetic fertilizers on economic crops and applied in different soils (Moura et al., 2007, Lima et al., 2007, Stamford et al., 2007, 2008). However, the phosphorus and potassium bearing rocks and minerals contain no nitrogen and thus do not supply N to increase plant growth and yield. In order to produce a more complete biofertilizer it is important to improve the N concentration of the organic matter, for example by adding effective free living diazotrophic bacteria to enrich the N content of the organic material (Lima et al., 2010). In biological studies, crustaceous chitosan is frequently used to increase resistance to plant pathogens. Moreover, chitosan has chelating properties greater than those of other natural polymers due to the presence of amino groups, and it may release nutrients into the environment (Boonlertnirun et al. 2008; Goy et al., 2009). In this study, fungus biomass (*Cunninghamella elegans*) was applied to an NPKB biofertilizer for the production of a bioprotector that promoted chitin deacetylation through the acidity promoted by the sulfur oxidative bacteria *Acidithiobacillus.* The use of fungi chitosan has advantages over crustaceous chitosan, such as an independence from seasonal factors and the simultaneous extraction of chitin and chitosan contained in the cell wall of some fungi from the Mucorales Order (Franco et al. 2004). Fungi chitosan application in agriculture as a bioprotector does not appear in the literature. No published research on the use of *C. elegans* to produce biofertilizer with the addition of fungi chitosan was found. This study describes the production of a bioprotector and evaluates the stimulating effects of this biofertilizer and bioprotector containing fungi chitosan applied in two deeps (0-20 and 20-40cm) on yield of grape (*Vitis labrusca* cv. Isabel) and increasing availability of soil nutrients after plant harvest. The main objective of this study was to determine the feasibility of using PK rocks mixed with organic matter enriched in N by inoculation with free-living diazotrophic bacteria and addition of chitosan (*C. elegans*) as an alternative to conventional fertilizers. Furthermore, this product could potentially be used as a bioprotector against phytopathogenic microorganisms in further studies.

Results

Grape yield in the subsequent harvests

In the specific literature there are not references about the effects of biofertilizers produced from powdered rocks mixed with organic matters enriched in N by inoculation with diazotrophic bacteria and addition of fungi chitosan in plant yield. The results of grape yield (*Vitis labrusca* - cv. Isabel) submitted to the fertilization treatments (NPKB, NPKF and grape waste compound) are presented in Fig. 1. The biofertilizer enriched in N by inoculation with diazotrophic bacteria and fungi chitosan from *C. elegans* (NPKB) showed significant response on grape yield, and the results are similar to the NPKF. The fertilization with NPKF (150 % RR) showed higher grape yield, followed by NPKB (150 % RR). The control treatment (grape waste compound) showed the lower grape yield. However, it is interesting to observe that all the fertilizers treatments produce grape yield superior to the average (15 t ha^{-1}) for irrigated grape (cv. Isabel) in the state of Pernambuco in accordance with IPA (2008). The greater yieds were obtained in the second harvest (May 2012), with greater difference for the first harvest (December 2011), especially when applied BNPK in 50% RR and the control treatment.

Soil pH after grape harvest

In the same way described for plant yield, there are not references that describe the influence of biofertilizers from rocks mixed with organic matters inoculated with diazotrophic bacteria and fungi chitosan in soil pH, especially when applied in different rates and depths (Fig. 2). The soil pH after the second grape harvest had shown not significant differences in function of the fertilizers treatments. The control treatment (grape waste compound) presented higher values of soil pH and are not significantly different comparing to NPKB. The application of NPKF promoted slight reduction in soil reaction and the changes between the two depths were not significant. In soil pH the effect of the two depth application was not significant ($p \leq 0.05$), but in a general greater values were observed at 0.20cm depth.

Total N and SO_4^{2-} *in soil after grape harvest*

The total N in soil after the second grape harvest are presented in Fig. 3A. The total N in soil increased with application of the different fertilization treatments, compared with the control. The best results were obtained when applied NPKB in highest rate (150% RR) which incremented total N up 100%, compared with NPKF (150% RR), and up 200% compared with the control treatment. In a general the NPKF and the control treatment was not affected by the application in the two depths, although, showed a great significance for the NPKB and best results were observed when applied in 0-20cm deep. The results for soluble SO_4^{2-} (Fig. 3B) showed that these values increased directly in interaction with the NPKF and NPKB, especially when applied in the highest rate (150% RR). The mineral fertilizer probably displays higher soluble SO_4^{2-} because the fertilizer mixture used ammonium sulphate, simple super phosphate and potassium sulphate, and all of them have sulphate as nutrient. Sulphate is a weak base, and normally is not percolate in soil, especially in presence of Ca^{+2} and Mg^{+2}, and grape may be not very exigent in sulphate nutrient. Also it may be observed that in the treatments with NPKB the soluble SO_4^{2-} in soil showed very high values (1200 mg dm^3) due to the metabolic production of sulphuric acid which release SO_4^{2-} to the soil that can be used by the plant and besides may be displayed to the soil and have potential to be used in the consecutive crops. The two different depths showed significant response for soluble SO_4^{2-} especially when applied the NPKB.

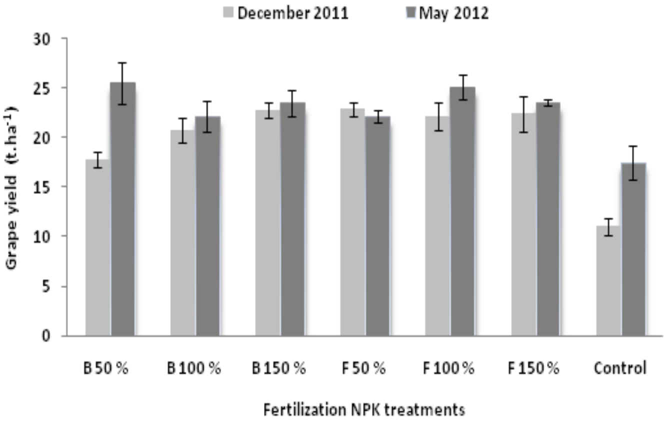

Fig 1. Yield of grape (*Vitis labrusca* - cv. Isabel) grown in the Brazilian San Francisco Valley, as affected by fertilization treatments with NPKB biofertilizers (B), NPKF mineral fertilizer (F) applied at 50%, 150% and 150% recommended rate (RR), and control with grape waste compound (2.4 L $plant^{-1}$) at two consecutive harvests (December 2011 and May 2012). Means with the different letters indicate significant differences at Tukey test ($p \leq 0.05$).

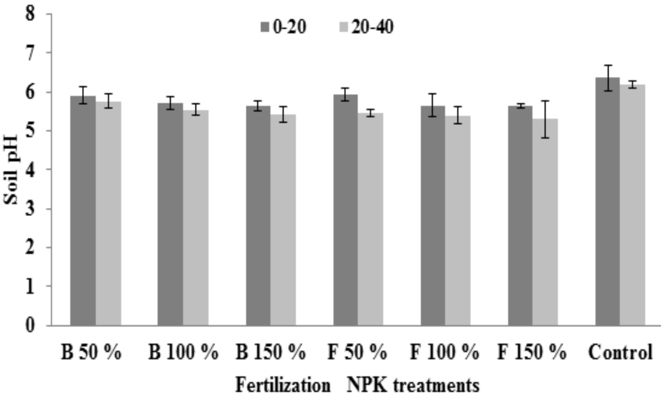

Fig 2. Soil pH after the second harvest (May 2012) as affected by fertilization treatments with NPKB biofertilizers (B), NPKF mineral fertilizer (F) applied at 50%, 150% and 150% recommended rate, and control with grape waste compound (2.4 L $plant^{-1}$), in depth (0-20 cm and 20 – 40 cm). Means with the different letters indicate significant differences at Tukey test ($p \leq 0.05$).

Available P and K in soil after grape harvest

The results of available P in soil are shown in Fig. 4A. It may be observed the positive and significant effects of NPKF and NPKB treatments in available P in soil after the grape harvest. The best results are observed when applied NPKB in rate 150% that was up to 50%, compared with NPKF. It is shown low available P content in soil when applied the control treatment (grape compound) in the level used by the local farmers, and represents only 10% of available P in soil (120 mg kg^{-1}) compared with BNPK in rate 150% (1200 mg kg^{-1}). It was observed effect of the two depths, especially when applied NPKB in 150% RR and with NPKF in the three rates. The results of available K in soil (Fig. 4B) showed that the fertilizers promoted significant increase in available K. The available K in soil increases when NPKF 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). For available K in soil it was observed effect of the two depths when applied NPKF in rate 100% and 150% RR.

Exchangeable Ca^{2+} and Mg^{2+} in soil after grape harvest

The results for exchangeable Ca^{2+} are presented in Fig. 5A. Exchangeable Ca^{2+} content in soil showed significant influence of the fertilization treatments, especially when applied NPKB in highest rate (150% RR) that presented the greater amount in exchangeable Ca^{2+} in soil (4.5 cmol$_c$ dm^{-3}), followed by NPKB applied in rate 100% RR. The NPKF in recommended rate showed similar results compared to NPKB in rate 50% RR. The control treatment showed low exchangeable Ca^{2+}. Furthermore, it may be observed that the Ca content in soil increased in a considerable amount in comparison with the soil analysed before the seedling transplantation (1.6 cmol$_c$ dm^{-3}), and the BNPK showed high conditions to maintain long-term fertility. The results for exchangeable Mg^{2+} (Fig. 5B) showed no substantial significance of the fertilizers, comparing NPKB and NPKF in rate 150% RR (3 cmol$_c$ dm^{-3}) that were significantly different to the control treatment (1.5 cmol$_c$ dm^{-3}). However, despite to this effect it may be observed an increase in the exchangeable Mg^{2+} when compared to the exchangeable Mg^{2+} in soil analyses before the conduction of the field experiment (0.4 cmol$_c$ dm^{-3}). The increase for exchangeable Mg^{2+} in soil may be also explained by the solubilization of Mg contained in the mineral (biotite) used to produce the K rock biofertilizer, probably by the effect of the sulphuric acid produced by *Acidithiobacillus* in the presence of elemental sulfur. For Ca^{2+} and Mg^{2+} the different depth showed effect and higher values were found when applied NPKB in the rates, and NPKF in 150% RR, with best results when applied the fertilizers in 0-20cm depth.

Discussion

The effective results obtained by application of biofertilizer produced from phosphate and potash rocks mixed with organic matter inoculated with free living diazotrophic bacteria and *C. elegans* (NPKB) are in accord with Stamford et al. (2011) that evaluate the effectiveness of PK rock biofertilizers mixed with organic matter (earthworm compound) in grape yield cropped in the Botticelli fine wine industry at the San Francisco Valley. Conclusive results by application of P and K rock biofertilizer mixed with organic matter (earthworm compound) increasing plant yield were reported by Lima et al. (2007) on lettuce grown in a Latossol from the Ceará state, and by Moura et al. (2007) and Stamford et al. (2006), respectively with cowpea and sugarcane in a Brazilian Spodosol from Pernambuco state. However, these results are not significant as the obtained in the present paper, especially due to the influence of N and P nutrients in yield. The NPKB was enriched in the NPKB by the inoculation with free living diazotrophic bacteria, that increase the N content influenced by the process of nitrogen fixation, and the increment in available P by the production

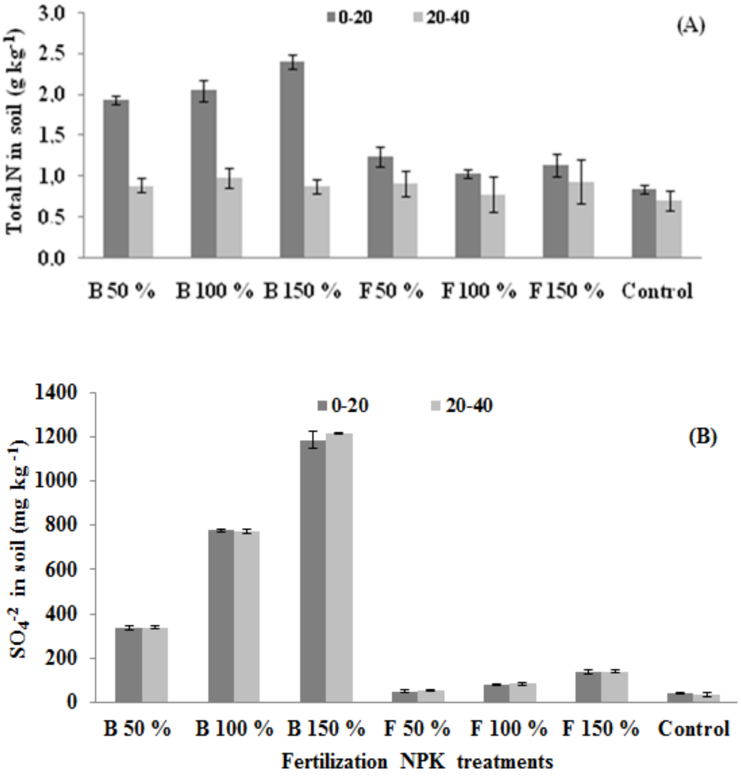

Fig 3. Total N and SO_4^{-2} in soil after the second harvest (May 2012) as affected by fertilization treatments with NPKB biofertilizers (B), NPKF mineral fertilizer (F), applied at 50%, 150% and 150% recommended rate, and control with grape waste residue (2.4 L plant^{-1}), in depth (0-20 cm and 20 – 40 cm). Means with the different letters indicate significant differences at Tukey test ($p \leq 0.05$).

of inorganic polyphosphate from the *C. elegans* (Franco et al., 2011). Low pH values were observed in response to the mineral fertilizers and 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 effect of the NPKB increasing soil pH may be explained by the use of organic matter (earthworm compound). In the production process the organic matter is applied 4 times the PK rock biofertilizer (equivalent proportion 4:1) when NPKB was produced in field conditions. It could be observed that the organic matter (earthworm compound) has pH 7.9 and rock biofertilizers pH 3.5, and in the control treatment was applied organic matter in greater amount (2.4 kg plant^{-1}). In the NPKB inoculation with diazotrophic bacteria and addition of fungi chitosan increased the total N in soil, in agreement with the results obtained by Stamford et al. (2009) that evaluated the agronomic effectiveness of rock biofertilizers from phosphate and potash rocks mixed with earthworm compound affecting the cowpea growth. In the present paper the authors also described the positive effects of the mixed PK rock biofertilizer with earthworm compound enriched in N by inoculation with the free living diazotrophic bacteria (NFB 10001)) and by the increment of inorganic polyphosphate as reported by Franco et al. (2011), and the results are in accord with Lima et al. (2010). Probably the N released by the NPKF (ammonium sulphate) had been used by plants to promoted growth, and due to the greater solubility may be percolating from soil by the effect of the heavy precipitations

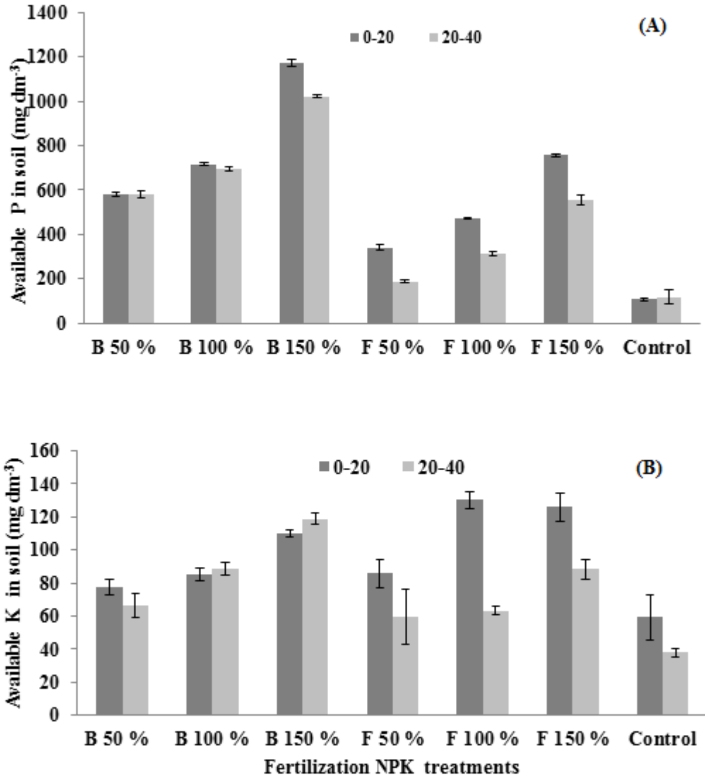

Fig 4. Available P and K in soil after the second harvest (May 2012) as affected by fertilization treatments with NPKB biofertilizers (B), NPKF mineral fertilizer (F) applied at 50%, 150% and 150% recommended rate, and control with grape waste residue (2.4 L plant^{-1}), in depth (0-20 cm and 20 – 40 cm). Means with the different letters indicate significant differences at Tukey test ($p \leq 0.05$).

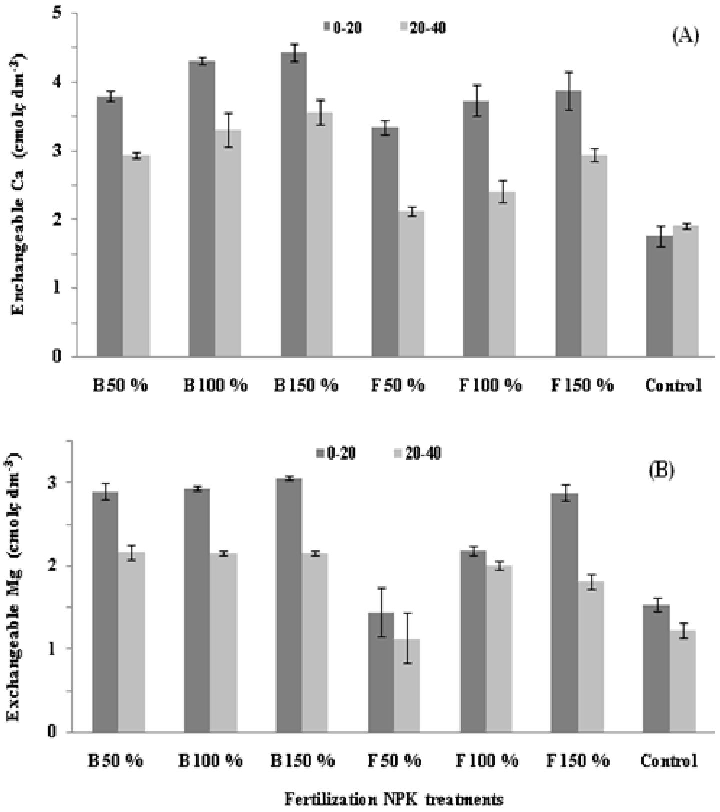

Fig 5. Exchangeable Ca^{2+} and Mg^{2+} in soil after the second harvest (May 2012) as affected by fertilization treatments with NPKB biofertilizers (B), NPKF mineral fertilizer (F) applied at 50%, 150% and 150% recommended rate, and control with grape waste residue (2.4 L plant^{-1}), in depth (0-20 cm and 20 – 40 cm). Means with the different letters indicate significant differences at Tukey test ($p \leq 0.05$).

that occurred in the region during the experimental period. The effects of the fungi chitosan addition 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 the production of inorganic polyphosphate (Franco et al. 2011). Also, it may be observed that chitosan increases the levels of N, P and K in the substrates as observed by Kowalski et al. (2006) and Goy et al. (2009). The results represent the effect of the sulfur oxidizing bacteria *Acidithiobacillus* on solubilization of minerals contained in the P and K rocks as reported by Stamford et al. (2006, 2007, 2011) applied in different soils on growth of sugar cane, yam bean and grapes, respectively. Also, the influence in the increment of available P may be explained because others bacteria native from soil besides *Acidithiobacillus*, and some soil fungi that produce phosphatases increase P solubility. Furthermore, the addition of chitin and chitosan by Mucorales fungi as *C. elegans* produce inorganic polyphosphate (Franco et al. 2011) and increase solubility of P and others nutrients. Oliveira et al. (2010) applying organic matter from castor bean in rate 10 t ha^{-1} obtained the same effects in reference to the increase of soil available P. The authors reported that the increment in soil available P have occurred because the organic matter applied in higher rates 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 on melon growth verified the positive effect of P rock biofertilizer and resulted in higher 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 in available P compared with mineral fertilizers. Lima et al. (2007) observed the effectiveness of biofertilizers from P and K rocks plus elemental S inoculated with *Acidithiobacillus* oxidizing bacteria. 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 Latossol of the “Cariri” region in two consecutive crops. In soils of the Brazilian coastal tableland of Pernambuco State, after sugar cane crop, Stamford et al. (2006) reported the increase in available K in soil when applied K rock biofertilizer plus elemental S inoculated with *Acidithiobacillus*, 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”. However, the authors described best results when applied mineral 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 and PK biofertilizer applied in the highest rate (two times the recommended rate). The higher results obtained for exchangeable Ca^{2+} in soil with NPKB application are probably due to the solubilization of total Ca^{2+} contained in the natural phosphate used in the production process. 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^{2+} were displayed in the soil by application of PK rock biofertilizer. Similar results were obtained in experiments realized in the San Francisco Valley to test the effects of rock biofertilizer on melon (Stamford et al., 2009) and on grape (Stamford et al., 2011) with a significant

increase in the exchangeable Mg^{2+} in soil when applied PK rock biofertilizer. Oliveira et al. (2010) also reported significant increase in exchangeable Mg^{2+} in soil when applied organic matter (10 t ha^{-1}).

Materials and Methods

Experimental site and plant material

The field experiment was carried out at the Botticelli Company (Latitude South 8º 58' 00', Longitude west 40º 22' 19", and Altitude 300 m), located in the San Francisco Valley in Pernambuco state in the semiarid region of Northeastern Brazil. The climate is classified as BSwh, according to the Köppen classification. The grape species (*Vitis labrusca*), variety Isabel, was indicated by the Botticelli Company interested in the commercial value of the cultivar for juice production and due to their great yield in the San Francisco Valley with two harvests in the same year. Grape seedlings were grown in black plastic recipients, irrigated daily and transplanted to the field at 70 days after seed plantation.

Soil and experimental conditions

The soil used was classified as a Red Yellow Latossol (Embrapa, 2006) and 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^{2+}= 16 and Mg^{2+}=4.1.

During the course of the field experiment (February 2010 – May 2012), the photoperiod remained close to 12 h of darkness and 12 h of light. The temperature oscillated between 28°C and 36°C, and the relative humidity was 60-80%. The soil was prepared for the crop by cutting and removing all of the vegetation from the experimental area. Soil was prepared by conventional tillage with one plowing and two disking. Furrows (40x40x40 cm) were prepared two weeks prior the grape seedling plantation. The soil water was maintained near field capacity using computer-controlled micro-irrigation system. The cultural practices, excluding pesticides, were applied weekly after pruning, according to the methods of Embrapa (Brazilian Agricultural Research Company) and as required followed the fruit Integrated Program (FIP) fresh fruit export guidelines. The grapes were pruned to 2 shoots, each with eight to ten gens, two months before the both harvests, and the shoots were wrapped onto fruiting wires. The grapes were harvested 110-120 days after pruning and the juice analysis revealed values between 15-18 Brix°. Throughout the field experimental period (February 2010 – May 2012) 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 air humidity was 60-80 %.

Experimental treatments and soil analysis

The mineral fertilizer (NPKF) was prepared mixing the simple N-P-K fertilizers: ammonium sulfate (20% N), simple superphosphate (20% P_2O_5) and potassium sulfate (50% K_2O), applied in accord with the NPKF treatments, following soil analyzes and the recommended rate (RR) for irrigated grape (cv. Isabel) for the state of Pernambuco, Brazil (IPA, 2008). The three rates (50%, 100% and 150% RR) for the mixed mineral fertilizers (NPKF) applied 50; 100 and 150 (g $plant^{-1}$); and for the NPKB used 500; 1000 and 1500 (g $plant^{-1}$). The treatments were applied at seedling transplantation and in the two dressing fertilization. For the control treatment (grape waste compound) were used 2.4 L $plant^{-1}$ at seedling transplantation and the same amount applied in the two dressing fertilization. The analyses of grape waste compound available at the Botticelli Company and in the regional market showed: pH 8.0; total N 0.8 (g kg^{-1}); available P 50 (mg kg^{-1}) and available K 10 (mg kg^{-1}). Soil analyzes were carried out in randomized composite samples collected from 10 plants of two rows of each sub plot. For soil analysis were collected composite samples at the two depths (0-20 and 20-40 cm) in furrows opened 20-30cm away to plant base. In soil samples were analyzed: pH, total N, available P, and K, soluble S-SO_4^{2-}, exchangeable Ca^{2+} and Mg^{2+} (Embrapa, 2009).

Experimental design and statistical analysis

The field experiment was set up in a factorial arrangement (2x3) +1x2, in randomized split plot design with four replicates. The fertilization treatments were: two sources (Biofertilizer–NPKB and soluble mineral fertilizers - NPKF) applied in 3 rates (50, 100 and 150 % Recommended Rate - RR). A control treatment was applied with grape waste compound (2.4 L $plant^{-1}$), following the practice of local producers. All the fertilization treatments were applied in two depths (0-20 and 20-40cm) and constitute the sub plots.

The experimental data were evaluated and analysed by ANOVA, with significant effects comparing the means by the Tukey's test ($p \leq 0.05$), using the SAS Statistical Program (SAS Institute, 2011).

Conclusions

The present study demonstrate that biofertilizer (NPKB) produced from PK rock inoculated with *Acidithiobacillus* bacteria mixed with organic matter (earthworm compound) enriched in N by inoculation with diazotrophic bacteria and *C. elegans* may be used as source of nutrients to increment plant yield and increase soil nutrients (total N, soluble SO_4^{2-}, available P, available K, exchangeable Ca^{2+} and Mg^{2+}). The NPKB produced with phosphate and potash rocks mixed with organic matter (earthworm compound) inoculated with diazotrophic bacteria and addition of fungi chitosan may be alternative for replacement of soluble mineral fertilizers. The NPKB supply nutrients to the soil, which may be used to improve plant yield and showed great power to maintain successive yields, especially when applied in 0-20 cm depth.

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