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Microbial biofertilizer increases nutrient uptake on grape (*Vitis labrusca* L) grown in an alkaline soil reclaimed by sulfur and *Acidithiobacillus*

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Abstract

Saline soils require application of gypsum or organic matter for improvement. The reclamation of alkaline soil may be more effective when elemental sulfur is applied and soil inoculated with *Acidithiobacillus*. This study has focused to evaluate the effects of biofertilizer with interactive microorganisms on nutrient status of grape (*Vitis labrusca* cv. Isabel) grown in a reclaimed alkaline soils in semiarid region by application of S and inoculation with *Acidithiobacillus* in two consecutive years. The plants were harvested 110-120 days after pruning and samples were used to determine the nutrient status in the two consecutive cycles. Organic Biofertilizers inoculated with *Beijerinckia indica* and *Cunninghamella elegans* were applied at different rates (B1.0, B1.5 and B2.0), at two depths (0-20 and 20-40 cm). This is the first time that the interactive effects of biofertilizer favoring nutrient uptake in grape (*Vitis labrusca* cv. Isabel) in reclaimed alkaline soil by utilising sulfur inoculated with *Acidithiobacillus* is reported. The results showed that biofertilizer applied in higher rate at 0-20 cm depth increases the nutrients status in the leaves. The organic biofertilizer produced from rocks mixed with organic matter inoculated with *Beijerinckia indica* and *Cunninghamella elegans* may be alternative to soluble conventional fertilizers.

Keywords: *Cunninghamella elegans;* fungic chitosan; nutrient status; organic fertilizer.
Abbreviations: B_biofertilizer; C_control; S_soluble conventional fertilizer.

Introduction

The world population is growing. The demands for fertilizers and pesticides have pushed sensible changes in agricultural systems to improve the use of new techniques for maximum yield (Goy et al., 2009). Fertilization is one of the most important factors that affect grape yield and fruit quality and furthermore, the nutrient availability in the soil for maximization of the agricultural crop system, especially in tropical soils (Stamford et al., 2009).

Several studies have been conducted in tropical regions, showing that soil salinity may reduce plant yield and nutrient uptake. These studies have indicated that the soil reclamation is very important to control this specific problem and to improve productivity and the quality of crops (Stamford et al., 2008).

The sulfur-oxidizing bacteria produce sulfuric acid that may reduce soil alkalinity. The most important *Acidithiobacillus* species occur naturally in the soils but are relatively less abundant in agricultural soils. However, for satisfactory and effective reduction of soil pH the bacteria should be introduced to the soil (Qadir et al., 2008).

Elemental sulfur inoculated with *Acidithiobacillus* produces metabolic H_2SO_4, which contributes decisively to release nutrients contained in rocks (Stamford et al., 2004; Stamford et al., 2007; Stamford et al., 2008). However, it is known that powdered rocks cannot supply N in sufficient quantity to support adequate plant growth. Thus, it is necessary to use organic matter to increase N content in the rock biofertilizer and to neutralize the low pH (3.0-3.5). Therefore, inoculation with selected free living diazotrophic bacteria can further N into the soil addition according to Lima et al. (2010). The adequate supply of this nutrient favors the productivity.

Crustaceous chitosan is frequently used in biological studies to increase the resistance to plant pathogens. Moreover, chitosan demonstrates chelating properties greater than others natural polymers by the presence of the amino groups, by which the salinity in the environment may be alleviated (Boonlertnirun et al*.,* 2008).

The main objective of this research was to study the potential of sulfur inoculated with *Acidithiobacillus* in reclamation of soil salinity, and subsequently to observe the feasibility of biofertilizer enriched in N by free living diazotrophic bacteria and fungi chitosan (*C. elegans*). The treatments were applied in two depths (0-20 cm and 20-40 cm) as alternative for soluble conventional fertilizer and to improve the nutrient status of grape (*Vitis labrusca* – cv. Isabel).

Results and discussion

Total N in plants (blades and petioles)

The results of total N uptake in grape leaves (blade and petiole) are shown in Fig. 1**.** Total N uptake in the leaf blades only showed significant differences with the control treatment (earthworm compost at the rate of 2 L plant^{-1}). No difference was observed at two depths (0-20 and 20-40 cm).

The total N uptake increased in the leaves of grape (cv. Isabel) with application of the biofertilizer and showed significant difference ($p \leq 0.05$). The lower concentration of total N was observed in the control treatment and with soluble fertilizer application (in recommended rate). The higher concentration of total N uptake was obtained when the biofertilizer in higher rate (150 % RR) applied.

The current literature is so poor on the effects of biofertilizers from powdered rocks mixed with organic matters on nutrient status of plants. The soluble fertilizer with ammonium sulphate is more soluble and may be more available to plants and probably influence the absorption in grape leaves. Fontes (2006) reported that the total N uptake in the grape leaves occur in critical level in low values up to 20 g kg^{-1}.

The biofertilizer inoculated with the diazotrophic bacteria increased the total N in the soil, in accordance with the results of Lima et al. (2010) who evaluated the agronomic effectiveness of rock biofertilizers mixed with organic matter (earthworm compost). Stamford et al. (2009) also compared the biofertilizer with conventional soluble fertilizer in a soil of the semiarid region.

In the present paper, authors also described the positive effects of the biofertilizer produced with earthworm compost, enriched in N by inoculation with the free living diazotrophic bacteria (NFB 1001). Rock biofertilizer mixed with organic matter, inoculated with free-living diazotrophic bacteria constitutes the mixed biofertilizer and might justify the increase in the total N uptake, because the substrate was enriched in N by the process of biological nitrogen fixation (Lima et al*.*, 2010).

Total P in plants (blades and petioles)

The total P uptake in blades and petioles of grape (cv. Isabel) are shown in Fig. 2. The total P in the blades showed significant difference with the applied biofertilizers, compared with the rates of the soluble conventional fertilizer and with the control treatment (earthworm compost – 2 L plant^{-1}.

The soluble conventional fertilizer and the control (earthworm compost) showed similar response and are not different at two applied depths. The total P did not present any significant effect on petioles due to fertilization treatments, even compared with the control (earthworm compost).

The increase in total N uptake also contributes to increase the P availability in the soil due to the production of inorganic polyphosphate promoted by the addition of *C. elegans* in accordance with Franco et al. (2004). Similar results were observed by Oliveira et al*.* (2016) using different fertilization treatments (biofertilizer, bioprotector, soluble fertilizers). The results reflect the effect of the *Acidithiobacillus* bacteria, used to produce the phosphate and potassic rock biofertilizers, on availability of minerals contained in the P and K rocks as described by Stamford et al. (2006, 2007 and 2011) in different soils grown with sugar cane, yam bean and grapes, respectively. The increase in total P uptake also may be explained due to the occurrence of others bacteria native from soil besides *Acidithiobacillus*, and some soil fungi as *C. elegans* that promotes P solubility producing inorganic phosphates as described by Franco et al. (2004).

The total P uptake in fruits of grapes showed no significant variation and the highest values were obtained in the blade leaves. The higher values were observed with biofertilizer applied in highest rate (B2.0), and the low values with application of the soluble conventional fertilizer in recommended rate (F1.0) and in the control treatment with earthworm compost applied in rate (2 L plant^{-1}). It was observed that the biofertilizer was very effective and it shows great potential for replacement of soluble conventional fertilizer on grape fertilization.

Total K in plants (blades and petioles)

The results of total K uptake in the blade and petioles of grape leaves (cv. Isabel) are presented in Fig. 3. The total K in the blades showed significant effect of the fertilization only when compared with the soluble conventional fertilizer and with the control treatment (earthworm compost – 2 L plant^{-1}). The best results were obtained when the fertilizers were applied in the depth 0-20cm. In general, no significant difference in blades was observed by application of the fertilization treatments at two depths.

The total K uptake in leaves of grape (cv. Isabel) showed variable effects, and the best results were found in the petioles. The organic biofertilizer applied in highest rate presented similar results when compared with the soluble conventional fertilizer and the control treatment. In the petioles, the total K showed the best results, when the biofertilizer was applied at the highest rate (B2.0) and with the soluble conventional fertilizer treatment (F2.0). No significant effect was observed upon depth of fertilizer application, except for the control treatment that showed higher absorption when applied at depth of 0-20cm.

Stamford et al. (2009) observed significant effect of P and K rock biofertilizers on total K uptake in melon in an acidic soil of the semiarid region (San Francisco Valley), compared with conventional soluble conventional fertilizers. In a study with different sources of fertilizers, Silva et al. (2011) verified positive effect of rock biofertilizer on melon, promoting highest total K uptake.

In an Oxisol of the coastal tableland of Pernambuco State, Stamford et al. (2006) described the increase in total K uptake on sugarcane, when applied K rock biofertilizer plus elemental S inoculated with *Acidithiobacillus*. These results are in accordance with the data found in the present study. In an Argisoil of the San Francisco valley, Moura et al. (2007) reported significant increment in the total K uptake in melon, compared with soluble conventional fertilizer and biofertilizer from phosphate and potassic rocks inoculated with *Acidithiobacillus*.

Santana et al. (2014) described the availability of P and K to the soil and plants using organic biofertilizer from phosphate and potassic rocks with elemental sulfur inoculated with the

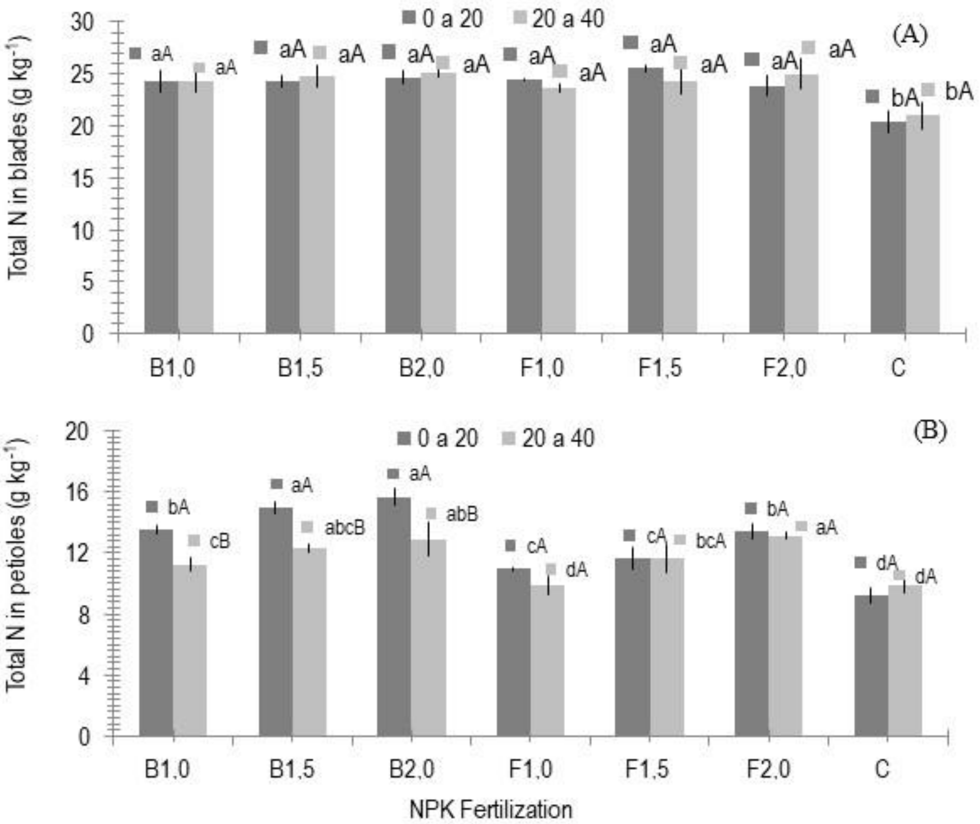

Fig 1. Total N in blades (A) and in petioles (B) of grape leaves (*Vitis labrusca* cv. Isabel) under fertilization treatments (B) - Biofertilizer and (F)- soluble conventional fertilizer, in three rates (100%, 150% and 200% recommended rate – RR), and the control with earthworm compost - 2L plant^{-1} (C) applied in two depths (0-20 and 20-40 cm). Small letters compare the different fertilizer treatments in the same depth, and capital letters compare the two depths in the same fertilizer using the Tukey test ($p<0.05$).

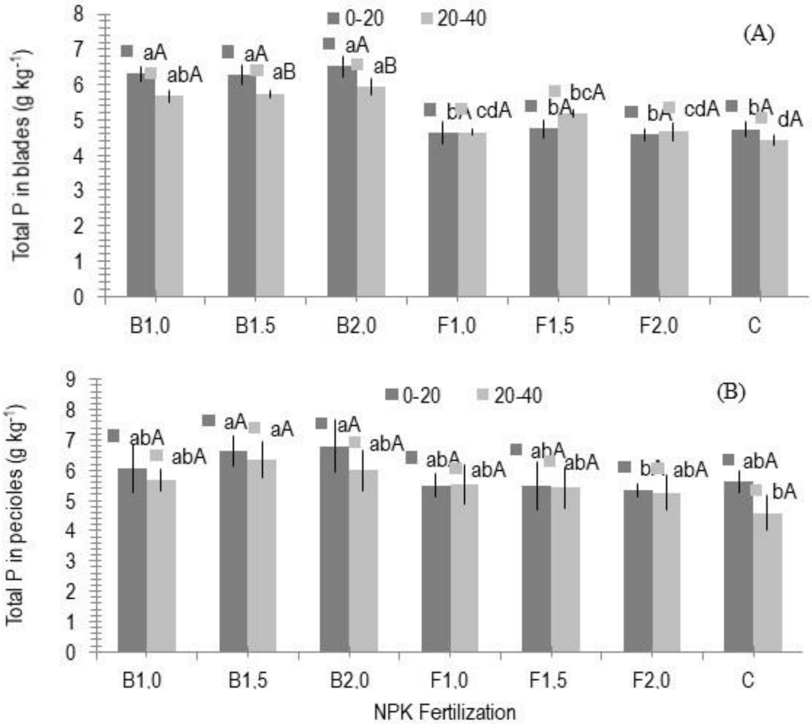

Fig 2. Total P in blades (A) and in petioles (B) of grape (*Vitis labrusca* cv. Isabel) leaves of under fertilization treatments (B) - Biofertilizer and (F)- soluble conventional fertilizer, in three rates (100%, 150% and 200% recommended rate – RR), and the control with earthworm compost - 2L plant^{-1} (C) applied in two depths (0-20 and 20-40 cm). Small letters compare the different fertilizer treatments in the same depth, and capital letters compare the two depths in the same fertilizer using the Tukey test ($p<0.05$).

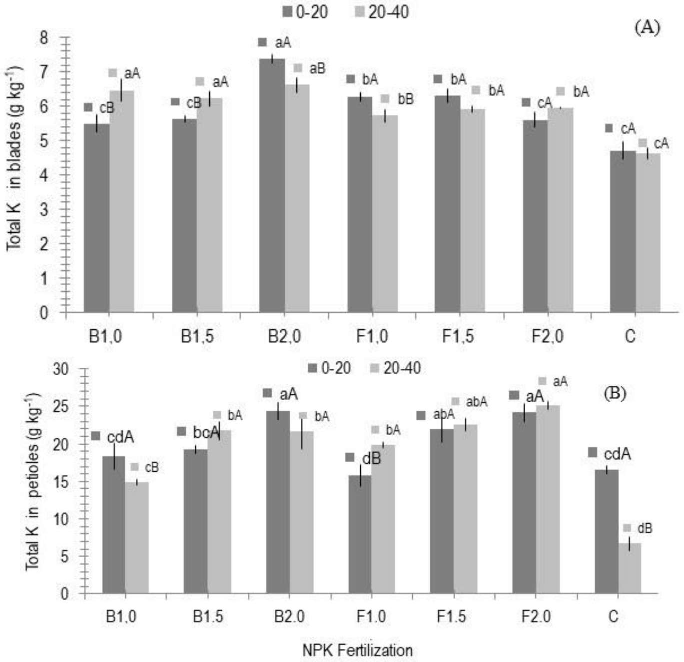

Fig 3. Total K in blades (A) and in petioles (B) on the grape (*Vitis labrusca* cv. Isabel) leaves under fertilization treatments (B) - Biofertilizer and (F)- soluble conventional fertilizer, in three rates (100%, 150% and 200% of recommended rate – RR), and the control with earthworm compost - 2L plant^{-1} (C) applied in two depths (0-20 and 20-40 cm). Small letters compare the different fertilizer treatments in the same depth, and capital letters compare the two depths in the same fertilizer using the Tukey test (p<0.05).

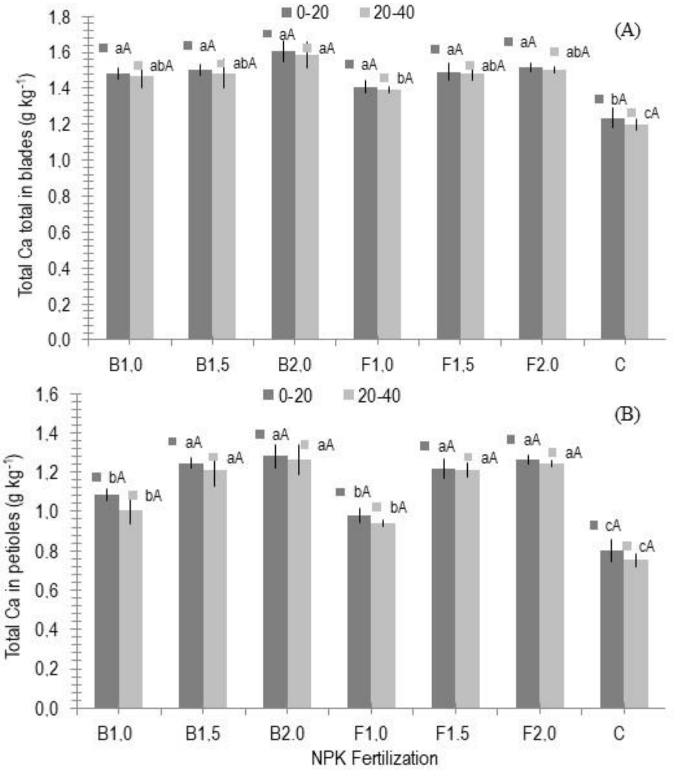

Fig 4. Total Ca in blades (A) and in petioles (B) of grape (*Vitis labrusca* cv. Isabel) leaves under fertilization treatments B) - Biofertilizer and (F)- soluble conventional fertilizer, in three rates (100%, 150% and 200% of recommended rate – RR), and the control with earthworm compost - 2L plant^{-1} (C) applied in two depths (0-20 and 20-40 cm). Small letters compare the different fertilizer treatments in the same depth, and capital letters compare the two depths in the same fertilizer using the Tukey test (p<0.05).

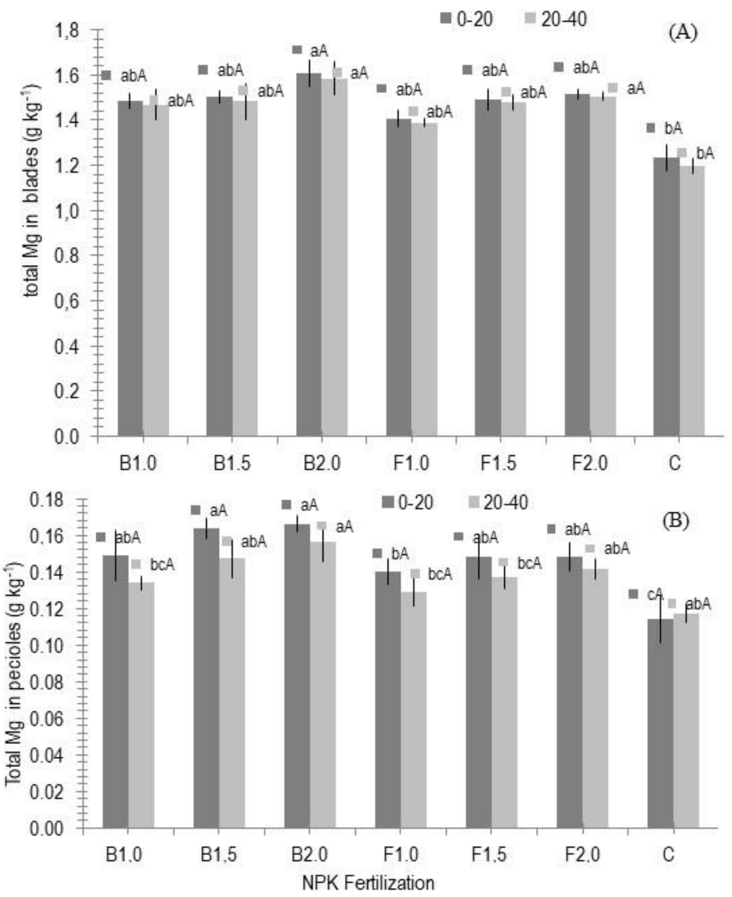

Fig 5. Total Mg in blades (A) and in petioles (B) of grape (*Vitis labrusca* cv. Isabel) leaves under fertilization treatments B) - Biofertilizer and (F)- soluble conventional fertilizer, in three rates (100%, 150% and 200% of recommended rate – RR), and the control with earthworm compost - 2L plant^{-1} (C) applied in two depths (0-20 and 20-40 cm). Small letters compare the different fertilizer treatments in the same depth, and capital letters compare the two depths in the same fertilizer using the Tukey test ($p<0.05$).

sulfur oxidative bacteria *Acidithiobacillus* mixed with earthworm compost. The favorable effect obtained when the biofertilizer applied. It may be due to increase in availability of P and K contained in the phosphate and potassic rocks and the effect of the sulfuric acid produced by the oxidative bacteria *Acidithiobacillus*.

The chitosan can be used as carbon source to soil microorganisms and accelerate the process of organic matter mineralization that increases the nutrients availability for plants (Boonlertnirun et al., 2008). In this way, the plant system can increase the nutrient absorption from the soil. This fact may explain the best results, when the biofertilizer is applied to grape fertilization.

The organic biofertilizer by effect of the fungi chitosan contained in the cell walls, increases the plant response to nutrient absorption, especially when applied in the highest rate. It guarantees the best plant growth and greater effectiveness, comparing with application of soluble conventional fertilizer as found by Santana et al. (2014). On cowpea grown in a tableland soil of the Brazilian rain forest region, Berger et al. (2013, 2016) applied the organic biofertilizer with *C elegans* and showed positive effect on plant nutrition and also increased the content of nutrients in soil and on cowpea plants. Similar results were found by Oliveira et al. (2016), in which a greenhouse study was conducted and authors evidenced positive effects with increase in nutrient absorption and on growth of melon, when they applied the biofertilizer produced with phosphate and potassic rocks mixed with sulfur inoculated with *Acidithiobacillus*.

Total Ca in plants (blades and petioles)

The results of total Ca content in function of fertilization treatments at two depths (0-20 and 20-40 cm) in blades (A) and in petioles (B) on leaves of grape (cv. Isabel) are shown in Fig. 4. The Total Ca in blades were not affected by the fertilizer treatments, and only promoted significant difference when the biofertilizer applied in the highest rate (at depth of 0-20 cm), when compared to the control treatment (earthworm compost – 2 L ha^{-1}). The biofertilizer present the best result (1.60 g kg^{-1}), and the control showed the lower values (1.23 g kg^{-1} and 1.19 g kg^{-1}), applied at depths of 0-20 cm and 20-40cm, respectively.

The total Ca in the petiole of the grape leaves showed significant effect of the fertilizer treatments, when compared with the control. The total Ca in the petiole was increased significantly using biofertilizer and soluble conventional fertilizer application comparing the low rates (B1.0 and F1.0) with the others rates applied. The best results were obtained with application of biofertilizer at rates B1.5 and B2.0 (1.28 g kg^{-1} and 1.26 g kg^{-1}), respectively. We also observed that no significant effect was occurred at different depths using the same fertilization treatment.

The favorable effect by application of the biofertilizer produced from phosphate rock may be due to the increase in the availability of Ca contained in the phosphate rock and the effect of the sulfuric acid produced by the oxidative bacteria *Acidithiobacillus* that releases available elements from the rocks (Lima et al., 2010; Oliveira et al., 2016).

Total Mg in plants (blades and petioles)

The data for total Mg uptake in function of the fertilizer treatments and for the two depths of application (0-20 and 20-40 cm) in blade (A) and in the petiole (B) on grape leaves are present in Fig. 5.

The results of total Mg uptake in blade and in petiole of grape leaves showed significant response ($P<0.05$) of the fertilization treatments and the fertilizer depth, when compared with the control treatment. The total Mg in blade only showed low effect compared with the control treatment. However, no difference was observed among depth. The best results were obtained with biofertilizer applied in the highest rate (150 % RR). The total Mg in petiole was significant, when the biofertilizer applied in the highest rate compared with the soluble conventional fertilizer and the control treatment.

Similar to the effects on Ca availability, the data for total Mg uptake by application of biofertilizer with sulfur inoulated with *Acidithiobacillus* were probably due to the solubilization of the Mg contained in the biotite potassic rock (biotite) (Lima et al., 2010).

Stamford et al. (2006) studied the effectiveness of rock biofertilizer in sugarcane and observed positive effect on Mg uptake. Similar results were found in an Argisoil of the San Francisco Valley in the semiarid region of Pernambuco state. Stamford et al. (2009) and Oliveira et al. (2016) also found positive effects in Mg availability in melon. In these crops the authors observed a significant increase in the total Mg uptake when applied K rock biofertilizer using biotite rock. Oliveira et al. (2010) also found significant increase in total Mg uptake on melon when applied organic matter (10 t ha^{-1}) compared with the control treatment (with no application of organic matter).

Materials and Methods

Biofertilizer production in the field

The phosphate and potassic rock biofertilizers were produced at the Federal Rural University of Pernambuco according to Stamford et al. (2007). The analysis of the PK rock biofertilizers (Embrapa, 2009) showed the following: (P-biofertilizer) pH= 3.5, available P= 60 (g kg^{-1}); (K-biofertilizer) pH= 3.3, available K= 10 (g kg^{-1}).

The organic biofertilizer enriched in N was produced using earthworm compost inoculated with the selected free living diazotrophic bacteria *Beijerinckia indica* (NFB 1001), mixed with PK rock biofertilizer according to Lima et al. (2010). The organic biofertilizer produced the bioprotector by addition of *C. elegans* fungus that contains chitosan in the cellular walls which increases P availability. The analyses of the biofertilizer at 30 days of incubation showed: pH (H_2O) = 6.9; total N= 18 (g kg^{-1}); available P= 10 (g kg^{-1}) and available K= 8.9 (g kg^{-1}).

Site and soil

The field experiment was carried out in a grape area located in the District of Santa Maria da Boa Vista, semiarid region of Pernambuco, Brazil (Latitude South 8º 58’ 00’, Longitude west 40º 22’ 19”, and Altitude 300 m), and the climate classified as BSwh, according to the Köppen classification.

The soil was classified as an Alkaline Sodic Soil with a medium texture by USDA (2014). The soil samples were collected at two depths (0-20 cm and 20-40 cm), air dried, sieved (5 mm sieve) and well-mixed. The chemical analysis showed the following at 0-20 cm: pH (H_2O) =8.5; electrical conductivity (E.C.) =12.7 dS m^{-1}; exchangeable cations (cmol$_c$ dm^{-3}) Na=25.5; K=8.7; Ca=27.9 and Mg=19.6. 20-40 cm: pH (H_2O) = 9.0; electrical conductivity (E.C.) =15.6 dS m^{-1}; exchangeable cations (cmol$_c$ dm^{-3}) Na= 27.8; K^+= 7.8; Ca= 28.6 and Mg = 15.8.

Experimental conditions

The soil of the experimental area was cleaned by removing debris, and then the transplantation of grape seedlings was proceeded. The alkaline soil was previously reclaimed by application of sulfur inoculated with *Acidithiobacillus* (4 t ha^{-1}), and applied a leach blade (40 cm depth) using water from the San Francisco river. The soil analysis was carried out one month after reclamation, shown the follwoing values: pH = 6.0, E.C. = 0.2 dS m^{-1}; exchangeable cations (cmol$_c$ dm^{-3}) Na=2.4, K=3.0, Ca=8.0 and Mg=2.7.

The grape (*Vitis labrusca* cv. Isabel) was obtained from the Botticelli Company due to the high commercial value for juice production. Grape seedlings were grown in black plastic recipients, irrigated daily and transplanted to the field at 70 days after seed plantation.

The grape cultural practices followed the Embrapa methodology used by the Botticelli Company. The grapes were pruned to 2 shoots, each with eight to ten buds. Two months before harvest, the shoots were wrapped onto fruiting wires. The grapes were harvested 110-120 days after pruning and at this moment the juice analysis revealed values between 15-18 Brix°.

*Experimental design, treatments and statistical analyse*s

The experiment was set up in a factorial arrangement (2x3+1)x2, in randomized split plot design with four replicates. The fertilization treatments with two fertilizers (soluble conventional fertilizer and biofertilizer) were applied in three rates (1.0, 1.5 and - 2.0) that correspond to 100%, 150% and 200% of the recommended rate (RR). The fertilization treatments, including the control with earthworm compost - 2L plant^{-1} (C) were applied at two depths (0-20 and 20-40 cm). The fertilization treatments were chosen based on recommendations for grapes in the San Francisco Valley (IPA, 2008).

The soluble conventional fertilizer was produced with ammonium sulfate (20% N), simple superphosphate (20% P_2O_5) and potassium sulfate (50% K_2O) and applied according to the soil analyzes and the recommendation for irrigated grape (cv. Isabel) for the state of Pernambuco, Brazil (IPA, 2008). The treatments were applied at seedling transplantation and in two dressing fertilization. The control treatment (earthworm compost) was used at the rate of 2 L plant^{-1} at seedling transplantation and the same amount in the two dressing fertilization.

The analyses of leaves (blades and petioles) nutrients (N, P, K, Ca and Mg) was carried out in samples (average of 10 plants) collected in two rows of each sub plot, for all the fertilization treatments applied at two depths (0-20 and 20-40 cm).

The experimental data were processed by the SAS Statistical Program (SAS Institute, 2011). The analysis of variance for significant effects was conducted. The comparison of the means were also carried out using the Tukey test ($p<0.05$). The mathematical model assumptions for errors normality were also considered.

Conclusion

This study for the first time reports that application of biofertilizer enriched with N by inoculation of diazotrophic bacteria (*B. indica*) and fungus (*C. elegans*) can improve nutrients uptake on grape (*Vitis labrusca* cv. Isabel) grown in a saline soil reclaimed by application of sulfur inoculated with *Acidithiobacillus*. We demonstrate that the organic biofertilizer produced from PK rocks mixed with organic matter (earthworm compost) enriched in nutrients by inoculation with interactive microorganisms (*B. indica and C. elegans*) may be alternative to soluble conventional fertilizers depending on the rate applied.

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