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Biological control of the bacterial wilt *Ralstonia solanacearum* by bioprotector with fungi chitosan from *Cunninghamella elegans* on tomatoes

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Fertilization is one of the most important means to improve plant production and nutrient uptake. Tomatoes plants are very exigent on fertilizers and sensitive to diseases. For satisfactory yield and fruit quality soil fertility and diseases need to be controlled. The aim of this paper was to evaluate the effectiveness of a bioprotector that contains fungi chitosan as an alternative biofertilizer, which releases nutrients and induces resistance against tomatoes wilt by *Ralstonia solanacearum* bacteria. The treatments were: 1- soluble NPK fertilizers (NPKF) in recommended rate (RR), 2- bioprotector (NPKP) with fungi chitosan in half of recommended rate (50% RR), 3– NPKP 100% RR, and 4– NPKP 150% RR. Treatments without bacterial inoculation (PO) and with bacterial inoculation (P1) were added to evaluate the plant characteristics (plant height, shoot diameter, number of leaves in ramification, fresh and dry matter of shoots). The symptom classes used to observe the induction of resistance were: (--) plants with no disease symptoms; (-) plant with slight symptoms; (+) plants with drastic symptoms and (++) plants died. Plants receiving the soluble fertilizers (NPKF) showed drastic disease symptoms one week after *R. solanacearum* inoculation, and all the plants died two weeks after inoculation. Plants with NPKP that contains *Cunninghamella elegans* in rates 50, 100 and 150% RR induced resistance for bacterial disease and promote better plant characteristics. The results showed that the bioprotector displays normal characteristics. The protector may be used as alternative for conventional fertilizers, especially inducing resistance for bacterial control.

Key words: *Lycopersicon lycopersicum*, *Ralstonia solanacearum*, biopolymers, organic bioprotector, phytopatogenic bacteria, resistance induction.

INTRODUCTION

Soluble fertilizers are of great importance for plant growth and yield but their use by low-income farmers is prohibitive due to the high price. Furthermore, the soluble nutrients may lixiviate to the deeper soil layers and can promote environmental problems (van Straaten, 2007). In a modern and sustainable agriculture, the application of soluble fertilizers and soil amendments are applied for increment of food production, meet economic criteria to

increase soil fertility and to minimize environmental damage (Stamford et al., 2008).

In general, Brazilian soils contain low available P and K content and these nutrients need to be supplied by the farmer. So, to increase the use of renewable natural sources of phosphate, it is necessary to study the effectiveness of different fertilizers (Araújo et al., 2008). An alternative for effective and economic fertilization is the use of biofertilizers made from phosphate and potash rocks with elemental sulfur inoculated with *Acidithiobacillus.* This combination achieves greater nutrient availability since the bacteria produce sulfuric acid, and thus increase both phosphorus and potassium availabilities, with results comparable to traditional fertilizers in several experiments with different economic crops (Stamford et al., 2006; van Straaten, 2007; Stamford et al., 2008).

Nitrogen is one of the most important nutrients due to its role in proteins and nucleic acids, and PK rock biofertilizers have no available N content to promote normal plant growth, although, in mixture with earthworm compound, inoculated with free living diazotrophic bacteria, has been shown to be effective as a N source (Lima et al., 2010).

Chitosan from crustaceous has been frequently used in assays to increase resistance against plant pathogens (Berger et al., 2013), while at the same time, it has greater chelating properties as compared to other natural biopolymers, and can release nutrients to the environment (Boonlertnirun et al., 2008; Goy et al., 2009). On the other hand, chitosan from fungi biomass, as compared to that from crustaceous sources, is independent of seasonal factors, and allows simultaneous extraction of chitin and chitosan (Franco et al., 2004). Up to now, there are no reports on its use as a bioprotector (Franco et al., 2004).

This paper aims to evaluate the effectiveness of the bioprotector (NPKP) with addition of fungi chitosan from *Cunninghamella elegans*, on characteristics of tomatoes grown in a Brazilian tableland Argisol. The NPKP bioprotector compared with the mineral soluble fertilizer (NPKF) showed possibility for use as alternative for replacement of conventional NPK soluble fertilizer and especially for use as fungicide.

MATERIALS AND METHODS

Production of the bioprotector

The PK rock biofertilizers were produced at the Federal Agricultural University of Pernambuco (UFRPE) in accordance with El Tarabily et al. (2006) and Stamford et al. (2007). Analysis of the P and K biofertilizer by the Embrapa (2009) methodology showed: (P-biofertilizer)- pH = 3.8, available P (A) = 60 (g kg^{-1}) and (K biofertilizer) - pH = 3.3, available K = 10 (g kg^{-1}).

The production of the biofertilizer (NPKB) was processed by mixing PK rock biofertilizers with organic biofertilizer (earthworm compost) enriched in N by inoculation with the selected free-living bacteria *Beijerinckia indica* (NFB 10001), in accordance with Lima et al. (2010). The analysis of the earthworm compound presented: pH 7.95; organic carbon (100.7 g kg^{-1}); total N (8.6 g kg^{-1}); total S (2.98 g kg^{-1}); total P (1.12 g kg^{-1}). The rock biofertilizer (PKB) and the organic biofertilizer (OB) were mixed in proportion 1:4 (PKB: OB), inoculated with free-living bacteria (NFB 10001) and maintained in incubation for 30 days.

The bioprotector (NPKP) represents the biofertilizer (NPKB) by addition of *C. elegans* (UCP 542), fungi that contains chitosan in their cellular wall (Franco et al., 2004). The fungus *C. elegans* was purified in Petri dishes in potato dextrose agar (PDA) grown for 10 days at 28°C. The monosporic culture of the *C. elegans* was obtained growing the Mucorales fungus in Potato Dextrose (BD), in 2000 mL Erlenmeyers flasks (containing 1000 mL) kept under shaking (180 rotations per minute) for 96 h at 28°C. The culture diluted in distilled water (20 L^{-1}) was applied by manual irrigation. For production of the bioprotector (NPKP), the NPKB from PK rocks was mixed with earthworm compound and incubated for 30 days. The chemical analyses of the bioprotector (NPKP) at the final period of incubation showed: pH = 6.4, total N = 20 g kg^{-1}; available P = 21 g kg^{-1} and available K = 19 g kg^{-1}.

Site, soil and experimental conditions

A greenhouse experiment was realized (November to December 2015) using samples of a “Yellow Argisol medium texture” (Embrapa, 2013) with low available P and K, and predominantly cultivated with horticultural crops, tropical fruits and cowpea legume. The chemical analyzes of soil, collected at 0-20 cm deep, showed: pH (H_2O) = 6.2; organic matter (g kg^{-1}) = 12.31; P (Mehlich 1) = 2 mg dm^{-3}; exchangeable cations ($cmol_c$ dm^{-3}) K = 0.22; Ca = 1.05; Mg = 0.6; Al = 0.4. The physical analyzes showed: particle density (g cm^{-3}) = 2.61; bulk density (g dm^{-3}) = 1.40; sand (g kg^{-1}) = 700; lime (g kg^{-1}) = 100 and clay (g kg^{-1}) = 200.

One month before transplanting to the pots at the greenhouse experiment, the seedlings of tomato (UC 82) purchase from the Isla Pak Industry, were grown in polypropylene trays (450 cells) with the commercial substrate “Vivatto Slim”. The seedlings were manually planted in November 02, 2015, and after 13 days of growth, they were transplanted to pots with soil (4 kg). Irrigation were processed daily, based in the pot weight, applying distilled water to maintain the moisture near field holding capacity. The NPK fertilizers treatments were applied at the planting date, before seedling transplantation. The current cultural practices were realized in accord with the usual recommendations for commercial tomatoes cultivated in the Brazilian rainforest region.

The greenhouse experiment was conducted in randomized block design, with four replicates. The fertilization treatments were: (1)-treatment with conventional NPK fertilizer (NPKF) in recommended rate (RR), (2)- bioprotector (NPKP) with fungi chitosan in half of recommended rate (50% RR), (3)- NPKP 100% RR, and (4)- NPKP 150% RR. All fertilization rates followed the current recommendation for irrigated tomatoes in Pernambuco (IPA, 2008). Treatments without bacterial inoculation (PO) were used to

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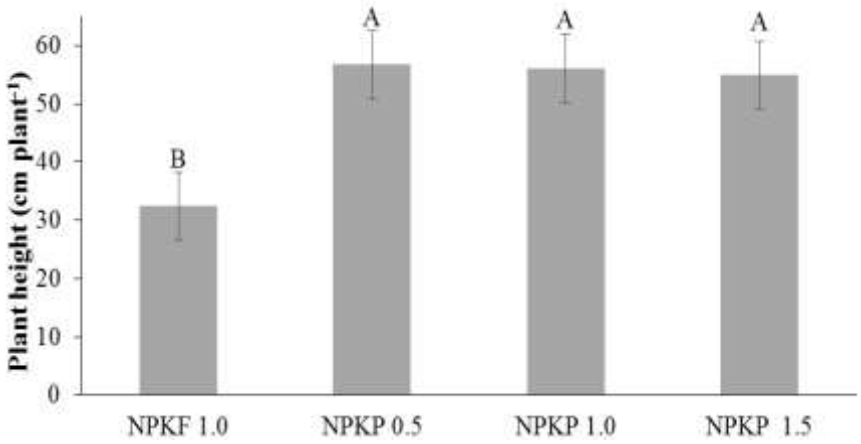

Figure 1. Plant height (45 days after transplantation) affected by the fertilization treatments NPKF 1.0 (100% recommended rate- RR), NPKP 0.5 (50% RR), NPKP 1.0 (100% RR), NPKP 1.5 (150% RR). Means with the same letter are not different by the Tukey test ($p < 0.05$).

evaluate the plant characteristics and the plants with bacterial inoculation (P1) were used to evaluate tomato resistance to *Ralstonia solanacearum*. The analysis of soil fertility evaluates the influence of the fertilization treatments and the resistance for the plant disease tomatoes wilt promoted by *Ralstonia solanacearum* bacteria in plants after inoculation of the pathogen was observed.

The pathogenic bacteria *R. solanacearum* was isolated from an area with tomatoes plants characterized with bacterial wilt symptoms, confirmed and identified by biochemistry analyzes. Isolation was processed in NYDA (dextrose 10 g, meal extract 3 g, yeast extract 5 g. peptone 3 g and agar-agar 18 g), by the continuous risks method, and Petri dishes were incubated for 48 h at 28°C. Healthy plants were inoculated by making a semicircular cut in the root system with a sterilized scalpel, and was added, per pot 20 mL of bacterial suspension (UFC $5x10^8$ mL^{-1}) in accordance with Garcia et al. (2013).

Determinations and statistical analyzes

After 45 days of growth, when flowering was beginning, the plants with PO treatment (not inoculated with pathogenic bacteria) were harvested to determine the plant characteristics (height, diameter, number of ramification and fresh and dry shoot weight). Soil samples were collected immediately after the plant harvest, for analysis of fertility (soil pH, available P and K and exchangeable Ca^{+2}, Mg^{+2} and Al^{+3}).

One week after the pathogen inoculation, the disease symptoms classified into: (--) plants with no disease symptoms; (-) plants with slight disease symptoms (+) plants with drastic disease symptoms and (++) plants died were observed.

The statistical calculations for plant characteristics and soil analysis used SAS 9.2 (SAS Institute 2011) through analysis of variance and means comparison using Tukey's test at probability $p \leq 0.05$.

RESULTS AND DISCUSSION

Tomatoes plants characteristics

Plants receiving bioprotector (NPKP) were significantly ($P<0.05$) higher, with larger diameter and had more leaves in ramifications than plants receiving the soluble conventional fertilizer. There were no significant differences between the bioprotector treatment applied in the three rates (50, 100 and 150% RR).

The plant characteristics (height, shoot diameter and average leaves in ramifications) are present in Figures 1, 2 and 3. The results showed positive and significant increase with application of the different bioprotector treatments, compared with the soluble fertilizer treatments. In a general, the best results were found with NPKP applied in the higher rates, and the soluble fertilizer (NPKF) showed the lowest results.

In reference to fresh and dry shoot weight (Figures 4 and 5), the best results were obtained when the NPKP was applied in higher rates (100 and 150% RR), when compared with NPKP in the lower rate (50% RR) and NPKF in recommended rate, which achieved the lowest results. The obtained results showed the effectiveness of the NPKP that promote good nutritional response in tomato plants in the greenhouse experiment.

In two consecutive harvest of lettuce, applying the biofertilizer (NPKB), Lima et al. (2007) reported the positive and significant effect when compared with soluble fertilizer (NPKF). Similar results on melon grown in soil of the Brazilian semiarid region were described by Oliveira et al. (2014), Costa et al. (2011) and Moura et al. (2007).

In reference to the bioprotector effects, in general, the best results were shown, when compared with the soluble fertilizer treatment, which can be due to the metabolic action of the oxidative bacteria *Acidithiobacillus* that acidify the soil and the acidity release nutrients contained in the rocks used to produce the bioprotector. Stamford et al. (2006, 2008) in greenhouse experiments reported positive and significant effects of the PK rock

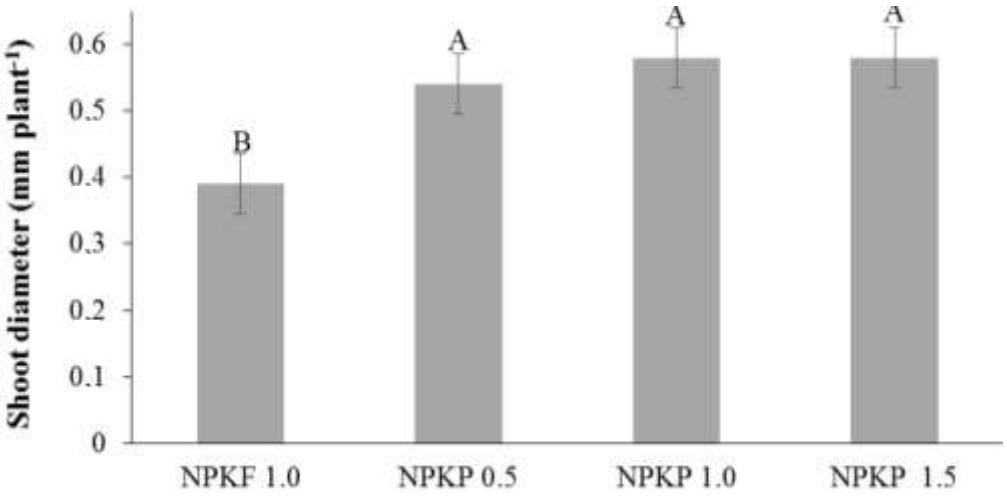

Figure 2. Shoot diameter with 45 days after transplantation as affected by the fertilization treatments NPKF 1.0 (100% recommended rate- RR), NPKP 0.5 (50% RR), NPKP 1.0 (100% RR), NPKP 1.5 (150% RR). Means with the same letter are not different by the Tukey test ($p < 0.05$).

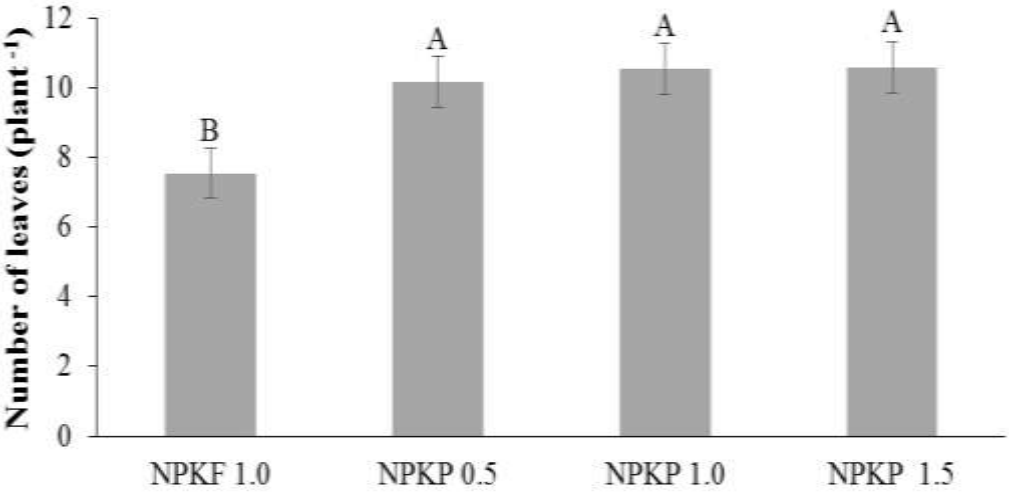

Figure 3. Number of leaves in ramifications with 45 days after transplantation as affected by the fertilization treatments NPKF 1.0 (100% recommended rate - RR), NPKP 0.5 (50% RR), NPKP 1.0 (100% RR), NPKP 1.5 (150% RR). Means with the same letter are not different by the Tukey test ($p < 0.05$).

biofertilizers inoculated with *Acidithiobacillus* in some characteristics of sugarcane and observed best effectiveness when comparing the PK soluble fertilizer. When applying the rock biofertilizer in higher amount, the authors observed reduction in plant characteristics, probably promoted by the soil acidification due to the low pH of the rock biofertilizers (pH 3.0 to 3.5).

The best results for the plant characteristics display the same behavior obtained when the different rates of the bioprotector treatments were applied. The obtained results are in accordance with Echart and Cavalli-Molina (2001) and Degenhardt et al. (1998) that observed damage in the plant growth and inhibition on roots development as affected by the acidification that reduce soil pH, especially in the presence of exchangeable aluminum, which may promote nutritional deficiency and reduction in shoot and root growth.

Soil analyses

The soil analyzes determined in soil collected after tomatoes harvest at 45 days of growth are present in Table 1. The treatment with NPKF applied in recommended

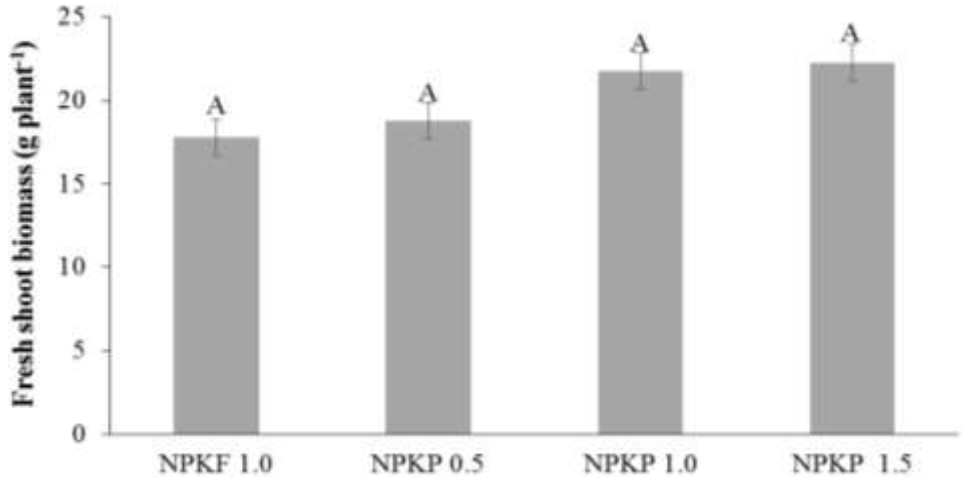

Figure 4. Fresh shoot biomass of tomato plants with 45 days after transplantation as affected by the fertilization treatments NPKF 1.0 (100% recommended rate - RR), NPKP 0.5 (50% RR), NPKP 1.0 (100% RR), NPKP 1.5 (150% RR). Means with the same letter are not different by the Tukey test ($p < 0.05$).

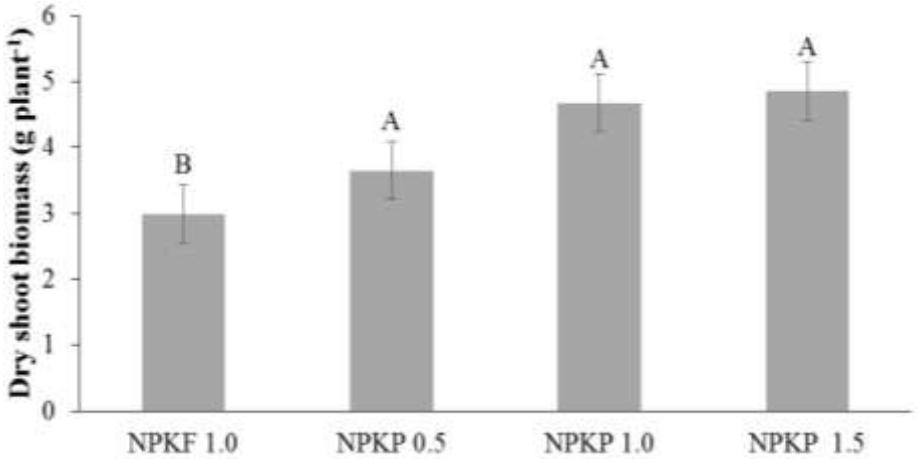

Figure 5. Dry shoot biomass of tomato plants with 45 days after transplantation as affected by the fertilization treatments NPKF 1.0 (100% recommended rate - RR), NPKP 0.5 (50% RR), NPKP 1.0 (100% RR), NPKP 1.5 (150% RR). Means with the same letter are not different by the Tukey test ($p < 0.05$).

Table 1. Soil analyzes after harvesting tomatoes plants submitted to fertilization treatments with NPKF 100% recommended rate (RR), NPKP 50% RR, 100% RR, and 150% RR, inoculated with *R. solanacearum*.

Fertilization treatments	pH	Exchangeable			Available P
		Al	Ca	Mg	
	H_2O	---------- $cmol_c\ dm^{-3}$ ----------			$mg\ dm^{-3}$
FNPK 100% RR	4.6b	1.00a	1.45a	1.0b	30b
PNPK 50% RR	5.3a	0.02b	1.08a	3.2a	40a
PNPK 100% RR	5.4a	0.02b	1.11a	4.9a	46a
PNPK 150% RR	5.5a	0.01b	1.18a	5.1a	50a

Data with the same letter have no statistical difference by the Tukey's test ($p \leq 0.05$)

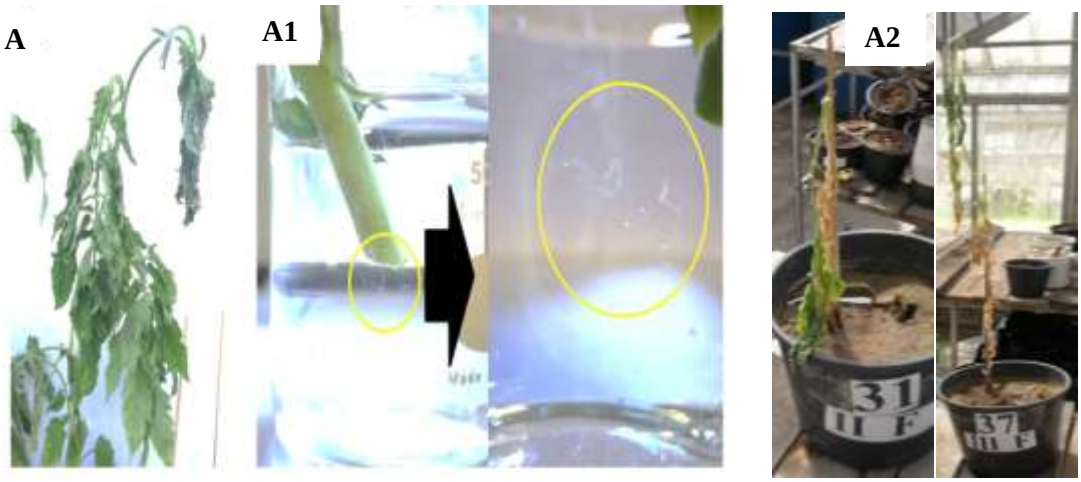

Figure 6. A- Tomato plants with wilt symptoms of the disease promoted by the *R. solanacearum* bacteria with treatment NPKF 100% (recommended rate) at one week after bacteria inoculation. A1- plant exudate with evident reaction of the wilt disease by the test in the symptomatic tissue showing the bacteria grown in the base of stem. A2- Tomato plants died at two weeks after wilt bacteria inoculation.

Table 2. Disease symptoms showed by tomatoes plants one and two weeks after inoculation with *Ralstonia solanacearum* as affected by the different fertilization treatments (Bioprotector at 50%, 100% and 150% Recommended Rate - RR and soluble Fertilizers 100% RR).

Response in growth	One week (mg plant^{-1})	Two weeks (mg plant^{-1})
NPKP 50	(--)	(-)
NPKP 100	(--)	(--)
NPKP 150	(--)	(--)
NPKF* 100	(+)	(++)

*Plants with no symptoms of disease (--); Plants with slight symptoms of disease (-); Plants with drastic symptoms of disease (+); plants that died (++).

rate revealed lower pH, higher Al, and also did not differ among the different rates of NPKP applied. Although, the experimental design confirmed these results because in the production of bioprotector the low pH is neutrized by the addition of organic matter as earthworm compound that present very high pH (pH 7.9).

The results of nutrients in soil especially Mg and P are in accordance with the literature of biofertilizer produced with phosphate and potash rocks. The addition of sulfur inoculated with *Acidithiobacillus* may be taken to confirm the production of sufficient sulfuric acid by the bacteria to increase P and Mg solubility as previously proposed by Stamford et al. (2008, 2009). A secondary possibility for causal mechanism is a direct effect of the chitosan as proposed by Kowalski et al. (2006) and Goy et al. (2009). The values of P in the soil are higher than in the NPKF treatment, because chitosan increase the levels of N and P in the substrates as proposed by Kowalski et al. (2006) and Goy et al. (2009).

Resistance to wilt disease (*R. solanacearum*)

The tomatoes wilt symptoms were observed in Figure 6. One week after inoculation, all plants that received the treatments with soluble mineral fertilizers (NPKF) showed drastic symptoms of the wilt disease (Figure 6 1A) and the effects were confirmed by the plant exudate test in the symptomatic tissue (Figure 6 A1). Statistical analysis was not necessary because the wilt disease occurred in plants with application of soluble fertilizer (NPKF) and all the tomatoes plants died two weeks after the pathogenic inoculation (Figure 6 A2). The plants that received the fertilization treatments with NPKP with the different rates do not present wilt disease and maintained satisfactory growth (Table 2).

Hayward (1994) reported that several factors influence the success of infection promoted by *R. solanacearum* bacteria, and the most important is cultural practice, especially fertilization. Berger et al. (2013) observed the

effects of fungi chitosan in the activity of some enzymes and on growth of cowpea plants, in a table land soil from the Brazilian Northeast, rainforest region with low content in P and K nutrients, applying fungi chitosan and biofertilizer (NPKP), although, in the study, symptoms of disease (*Fusarium oxysporum*) were not observed in cowpea plants supplied with soluble fertilizer (NPKF).

Plants not supplied by sufficient nutrients such as N, P and K, influence growth and may contribute to increase in the susceptibility of diseases as reported by Ghormade et al. (2010) and Guazzelli et al. (2007). However, the plants of the experiment were normally supplied in nutrients at the 54 days of growth and these arguments, and the plants with NPKF only showed symptoms of disease after the pathogen inoculation.

Chitosan release from the biomass of *C. elegans* fungi, during the process of PNPK production probably acts in the protection against *R. solanacearum* inducing plant resistance, and therefore may act in the mineralization process, releasing nutrients for plants absorption as reported by Boonlertnirun et al. (2008).

The reduction of bacterial wilt disease by the action of compounds that induce resistance such as acibenzolar-S-methyl (Araujo et al., 2008), were tested for *Pseudomonas* (Peixoto, 1997); cultural processes such as biofumigation and solarization (Baptista et al., 2006) are not proven to control bacterial wilt, and these practices are expensive and produce problems in the soil and in the environment; although, there is no study on tomato wilt control in the literature by application of biofertilizers and bioprotector.

Conclusions

The application of bioprotector with fungi chitosan controls tomatoes bacterial wilt with no addition of fungicides, while its fertilizer effects allowed normal plant growth than conventional soluble fertilizer. The bioprotector may be used in replacement of NPK fertilizers.

Conflict of Interests

The authors have not declared any conflict of interests.

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