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Biological Control of the Root-knot Nematode, *Meloidogyne incognita* on Tobacco Using Native Biological Agents

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The nematicidal potential of three native biological agents containing the fungus *Trichoderma harzianum* strain YZL229, the bacteria *Pseudomonas fluorescens* strain P-72-10 and *Bacillus subtilis* strain Itb162 which collected from *Meloidogyne* spp. infested tobacco fields and infected roots in Chongqing, against the root-knot nematode, *Meloidogyne incognita*, infecting tobacco, was assessed in vitro and under greenhouse. All treatments displayed nematicidal potentials through ovicidal and larvicidal assays in vitro and resulted in significant improvement in tobacco growth in greenhouse. The significant suppression of gall index (GI) and eggmass index (EMI), the reduction of nematode population in soil and eggs per eggmass were obviously observed following the application of these biocontrol agents, especially *Trichoderma harzianum* strain YZL229. Generally, the results of this study indicated that the tested native biological agents could prove to play a significant role in integrated root-knot nematode management on tobaccos.

Key words: Biological control, Native biological agents, *Meloidogyne incognita*, *Trichoderma harzianum*, *Pseudomonas fluorescens*, *Bacillus subtilis*.

Tobacco (*Nicotiana tabacum*) is one of the most important economic crops all over the world which brings income worth of billion dollars to the tobacco farmers annually (Mujeebur *et al*. 2011). It is commonly being cultivated in most agricultural countries like USA, Sweden, Turkey, New Zealand, India and China (Raveendra *et al.* 2011). Root-knot nematodes, *Meloidogyne* spp., which tobacco is highly susceptible to, are important pathogens of tobaccos to a big part of tobacco-growth areas, including China. *Meloidogyne incognita* is ubiquitous in tobacco lands which are widely distributed in the tobacco-growth areas of China (Yu *et al*. 2008). It is a sedentary and endoparasitic nematode that possessing high reproduction capacity and whose life cycle can be completed in a short time on tobacco (Arens *et al*. 1980).

Generally, *M. incognita* infestation often occurred on the tobacco plants and tobacco show symptoms after transplantation (Motha *et al*. 2010). *M. incognita* sets up feeding locations on tobacco root where it deforms the root cells and establishes giant cells, result in a nodule or gall, when the roots of tobacco were attacked by *M. incognita*. Tobacco plants have shown obviously stunted and poor growth in oval patterns in the field when early symptoms of nematode damage on tobacco was observed. The leaves of nematodes infected tobacco plants, whose color was pale-green, were

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gradually turned to yellow, and the condition known as 'rim firing' was observed at the necrosis of leaf tips and leaf margins at severe stages. These complicated symptoms of nematodes damage on tobacco could be misdiagnosed as caused by lack of nutrient uptake and water supply until the galls and knots which induced by nematodes were examined in the tobacco roots. In addition, root-knot nematode could make tobacco plants more susceptible to other wilt diseases.

Due to the big damage, which was caused by *M. incognita* on tobacco,have affected the yield and quality of tobacco both in nursery garden as well as field, there are different strategies that can be combined to manage root-knot disease on tobacco, such as crop rotation, soil steaming, soil solarization, the growth of resistant varieties, the field application of nematicides and biological control (Noling and Becker 1994). Chemical fumigants can control nematode directly and effectively in agricultural industry up to now, but in consideration of environment concerns and increased regulations on use of chemical fumigants, more management strategies such as biological control should be implemented to control *Meloidogyne* spp. (Burkett-Cadena *et al*. 2008). The biological control agents whose bio-control effects have been assessed can limit nematode abundance including nematode-trapping fungi, egg-parasitic fungi, bacteria, and polyphagous predatory nematodes (Gray, 1988; Kerry, 1988; Kerry and Hidalgo-Diaz, 2004; Kiewnick and Sikora, 2005).

Trichoderma harzianum which colonizes and grows on plant roots, causes a physical barrier for nematodes to penetrate, and also enhances the plant growth and nutrient absorption (Sahebani and Hadavi, 2008). *Pseudomonas fluorescens*, which might produce compounds such as phenazine-type antibiotics or hydrogen cyanide against *M. incognita*, was widely used to control nematodes (Siddiqui and Shaukat, 2003; Siddiqui *et al*. 2005). *Bacillus subtilis*, which has a unique ability to produce endospores when environmental conditions are stressful, was also widely used to control nematode. It was reported that nematode infection and root-knot formation on cowpea plant in the soil inoculated with *B. subtilis* are reduced (Shahnaz *et al*. 2008).

This study was conducted to assess the effect of using several biological agents individually for the control of *M. incognita* on tobacco aiming at obtaining nematode free healthy plants through this eco-friendly pest control methods.

MATERIALS AND METHODS

Biological agent strains

The fungus *T. harzianum* strain YZL229 was originally isolated from the egg-mass of *M. incognita* which parasite on tobacco plants, the bacterium *P. fluorescens* strain P-72-10 was originally isolated from the rhizosphere of healthy tobaccos, and the bacterium *B. subtilis* strain Itb162 was originally isolated from the tissue of healthy tobaccos. All tobacco plants were collected from Chongqing and all strains were was stored at -20°C. A single colony of *P. fluorescens* and *B. subtilis*, pure spores of *T. harzianum* were cultured in 100 ml of nutrient broth respectively. *P. fluorescens* and *B. subtilis* were incubated at 28°C on a rotary shaker at 180 rpm for 48 h, while *T. harzianum* was incubated for 72h at 28°C on a rotary shaker at 180 rpm. They were all harvested by centrifugation at 10000 rpm for 10 min and the supernatants were sterilized through 0.22μm filters. Then the supernatants were diluted with sterilized water to 50% and 20% respectively to study their effect on *M. incognita*. The precipitates were resuspended with sterilized water and the final density of *P. fluorescens* and *B. subtilis* were adjusted to approximate 1×10^{10} CFU ml^{-1}. And the density of *T. harzianum* was adjusted to approximate 1×10^{6} CFU ml^{-1}. Those preparations were designated to study their bio-control effects on *M. incognita* in greenhouse experiments.

Nematodes

The root-knot nematode, *M. incognita* used in this experiment was initially isolated from tobacco in Chongqing and maintained in the greenhouse on tomato (*Solanum lycopersicum*). Eggs were extracted from heavily galled tomato roots with 0.5% NaOCl (Hussey and Barker, 1973), and fresh J2 that were collected after hatching for 24h were adjusted to approximate 600 juveniles $5ml^{-1}$. And fresh J2 must be used for inoculation immediately.

In vitro egg hatch (Ovicidal) test

80 fresh eggs of *M. incognita* were

carefully transferred into each well of 24-well microliter plate and each well was added 500µL supernatants of each biological agent strain of 100%, 50% and 20% concentrations. The plates were incubated at 27°C for 120 h and the hatched eggs in each well were counted microscopically. Treatment with distilled water was used as negative control and each treatment was repeated three times for the accuracy of the results.

In vitro mortality (Larvicidal) test of J2

100 freshly hatched J2 in distilled water suspension were added into each dish of petri dish (2cm diameter) containing 1000µL supernatants of each biological agent strain of 20%, 50% or 100% concentrations. The petri dishes were incubated at 27°C for 48 h and the J2's populations in each dish were counted after 1, 6, 12, 24 and 48 h exposure period microscopically. Treatment with distilled water was used as negative control and each treatment was repeated three times for the accuracy of the results.

Tobacco plants

Tobacco cultivar Hong Hua Da Jin Yuan (*Nicotiana tabacum*), which is susceptible to *M. incognita* was used in this experiments. The sterile tobacco seeds were sown in the planting trays containing autoclaved soil and the planting trays were maintained in with 24±2°C and 16 h diurnal light. After 15 days, the tobacco plantlets were transplanted into 10 cm plastic pots containing approximate 300 g of autoclaved sand-soil, mixed 2:1 (v:v), and were transferred to a greenhouse set at 28± 3°C and 16 h diurnal light, fertilized with Hoaglands fertilizer 5 ml each plantlet weekly.

Greenhouse bio-control experiments

15 days old tobacco plants were inoculated with *T. harzianum* strain YZL229 or *P. fluorescens* strain P-72-10, *B. subtilis* strain Itb162 or water (control), and each treatment was repeated 10 times. 5ml suspension of YZL229, P-72-10, Itb162 and water should be drench around the tobacco plant respectively and repeated again 1 week later. 2 weeks after the first suspension inoculated, each tobacco plants was treated with 5ml suspension contains approximate 600 freshly hatched *M. incognita* J2 which were dispensed into five 2-cm-deep holes around the tobacco plant base.

60 days after nematode inoculation, roots of tobacco plant were slightly rinsed under a slow stream of water and gall index (GI) and egg-mass index (EMI) were determined base on 0-5 scales (Taylor and Sasser, 1978): 0 = no galls/egg-masses; 1 = one-two; 2 = three-ten; 3 = 11-30; 4 = 31-100; and 5 ≧100 galls or egg-masses per root system. Plant growth was determined by measuring root length, shoot height, leaf surface areaÿ fresh and dry weight of root and shoot. Root and shoot dry weights were determined after drying in a hot air oven at 75°C. The total number of *M. incognita* of each pot was also determined. 300g soil sample from each of the replicative pots of all the treatments was collected individually and *M. incognita* from a sample of 300 g soil were extracted by means of modified Cobb's sieving and decanting technique followed by Baermann funnel method (Southey, 1986). Ten egg-masses which randomly isolated from the ten galled roots of each treatment were stained by 0.015 % (w/v) Phloxine B (Dababat and Sikora, 2007) and released into individual with the 2ml centrifuge tube. The number of *M. incognita* and number of eggs per egg-mass was counted and calculated microscopically.

Data analysis

Data was statistically analyzed by statistical software PASW Statistics version 18.0. Analysis of variance (ANOVA) techniques were used for the statistical analysis of the data. To compare treatment means for the significance of difference between any two variables, least significant difference (LSD) was calculated at 5% probability level (P = 0.05).

RESULTS

Ovicidal and lavicidal effects of different supernatants of bio-agents on *M. incognita*

The ovicidal potentials of *T. harzianum* strain YZL229, *fluorescens* strain P-72-10 and *B. subtilis* strain Itb162 as estimated by in vitro egg hatch test for *M. incognita* were shown in Fig. 1. It was observed that all the supernatants of the bio-agent were able to reduce the hatching numbers of *M. incognita* eggs and the hatching numbers of *M. incognita* eggs decreased with the increasing concentration of the supernatants of bio-agents. It was shown in Fig. 1 that the hatching numbers of *M. incognita* eggs reduced significantly in all treatments compared with the control after 120h. The highest ovicidal potential was shown in *T. harzianum* treatment, in which only less than 33

eggs were hatched at 100% concentration, while more than 70 eggs were allowed to hatch in negative control that treated with distilled water.

Table 1 has summarized the larvicidal potentials of *T. harzianum* strain YZL229, *fluorescens* strain P-72-10 and *B. subtilis* strain Itb162 on the J2's at different concentrations. It was observed that all the supernatants of the bio-agents at different concentrations were able to increase the mortality of J2 and the mortality of J2 increased with the increasing concentration of the supernatants of bio-agents and increasing processing time. *T. harzianum* was shown to have the highest mortality effect when compared with other supernatants of bio-agents. It reduced the number of J2 by 87.3% after treating for 48h at 100% concentration.

Effect of different bio-agents on GI and EMI

GI and EMI of tobacco which were treated with *T. harzianum* strain YZL229, *fluorescens* strain P-72-10 and *B. subtilis* strain Itb162 were shown in Fig.2. It was observed that the GI and EMI of tobacco in treatement with *T. harzianum* reduced significantly compared with the control after 60 days after inoculation. No significant difference of GI and EMI was found between nematodes treated with *B. subtilis* and *fluorescens*. They both cause reduction of GI and EMI by 2.67 and 3.33, respectively. In addition, *T. harzianum* showed the highest reduction of GI and EMI by 2.33 and 2.67 when compared with the control (3.33 and 4).

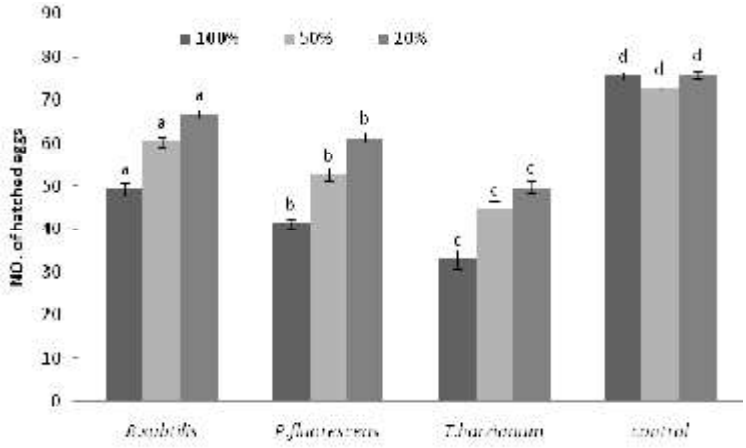
Effect of different bio-agents on plant growth parameters

The plant growth parameters (including shoot length, root length and leaf surface area) of tobacco treated with *T. harzianum* strain YZL229, P. *fluorescens* strain P-72-10 and *B. subtilis* strain Itb162 were shown in Fig.3. The shoot length of tobacco increased significantly in all treatments when compared with untreated inoculated control and all treatments have promoted the growth of shoot length when compared with untreated inoculated control. Only the tobacco treated with *B. subtilis* has shown the significant reduction in the shoot length when compared with untreated control. No significant difference of shoot length was found between tobacco treated with *B. subtilis* and *P. fluorescens*. And no significant difference of root length and leaf surface areas was found among tobacco treated with *B. subtilis*, *P. fluorescens* and *T. harzianum*. All treatments significantly increased root length and leaf surface area compared with untreated inoculated control. But when comparing with untreated control, all treatments significantly reduced the root length and leaf surface area. The results significantly correlate to the bio-control agents, effective as growth promoting when compared with untreated inoculated control.

Table 1. Larvicidal effect of different supernatants of bio-agents on *M. incognita* (in vitro)

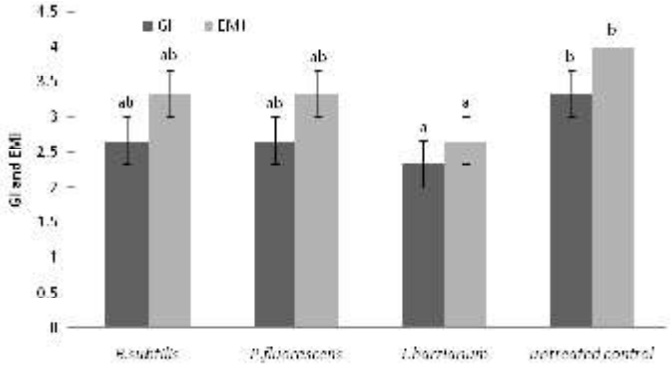
Treatments	100 %				
Time after inoculation	1h	6h	12h	24h	48h
B. subtilis	59.3±1.45	68.3±1.76	78.3±1.20	82.3±1.20	87.3±0.88
P. fluoresens	62±1.73	72.7±1.45	81.7±1.45	87.3±1.20	91±0.57
T. harzianum	65.3±1.20	79±1.73	84.7±1.45	91.3±1.88	94.7±0.33
control	0	0	1.15±0.67	3.0±0.58	4.0±1.00
		50%			
B. subtilis	49.7±2.03	60.7±1.45	69.3±1.45	72.3±0.88	77.0±0.58
P. fluoresens	56.0±1.53	64.3±1.76	71.7±1.45	74.0±1.15	84.7±0.88
T. harzianum	58.0±1.73	69.0±0.58	74.0±1.73	77.0±1.15	87.0±0.88
control	0	0	1.15±0.67	3.0±0.58	4.0±1.00
		20%			
B. subtilis	36.3±1.76	45.7±0.88	52.0±1.73	57.7±0.88	62.3±0.57
P. fluoresens	39.3±2.02	49.7±0.88	57.0±1.73	64.3±0.33	70.3±0.88
T. harzianum	42.3±1.45	52.7±0.88	61.3±1.86	68.7±0.88	72.7±0.88
control	0	0	1.15±0.67	3.0±0.58	4.0±1.00

Data in the table indicated dead count of J2-juveniles (mean value) with ± standard error (SE)



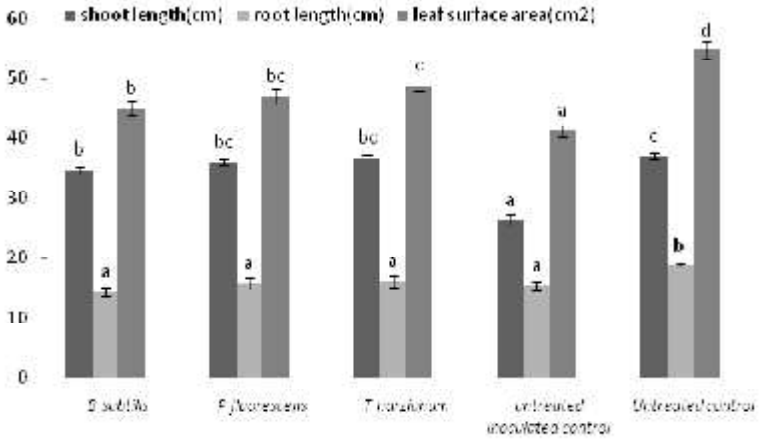
Columns in the same color and followed by the same letter(s) are not significantly different at P ≤ 0.05.

Fig. 1. Ovicidal effect of different supernatants of bio-agents on *M. incognita* (in vitro)



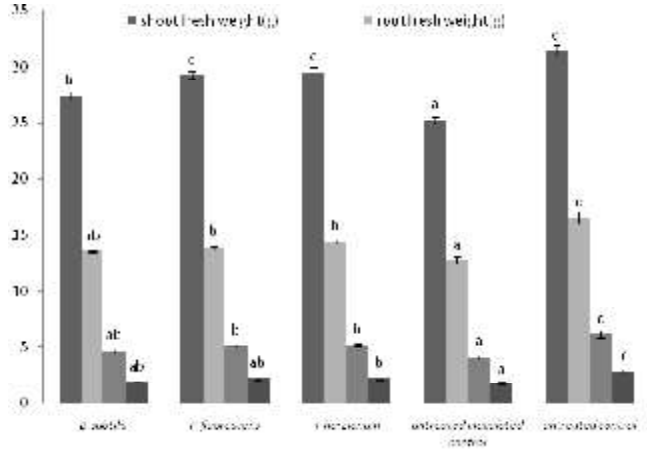
Columns in the same color and followed by the same letter(s) are not significantly different at P ≤ 0.05

Fig. 2. Effect of different bio-agents on GI and EMI in tobacco



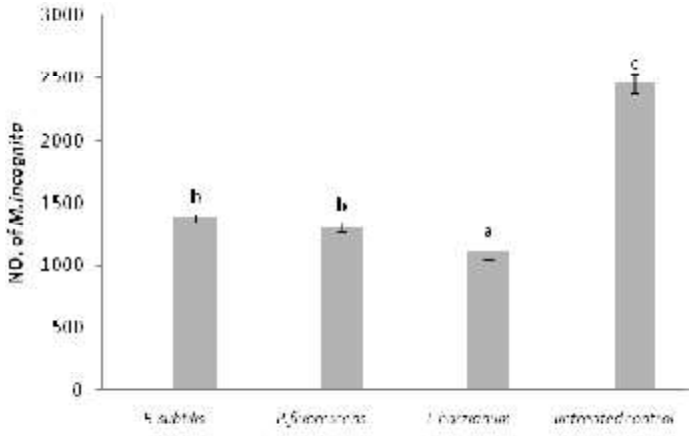
Columns in the same color and followed by the same letter(s) are not significantly different at P ≤ 0.05

Fig. 3. Effect of different bio-agents on plant growth parameters in tobacco



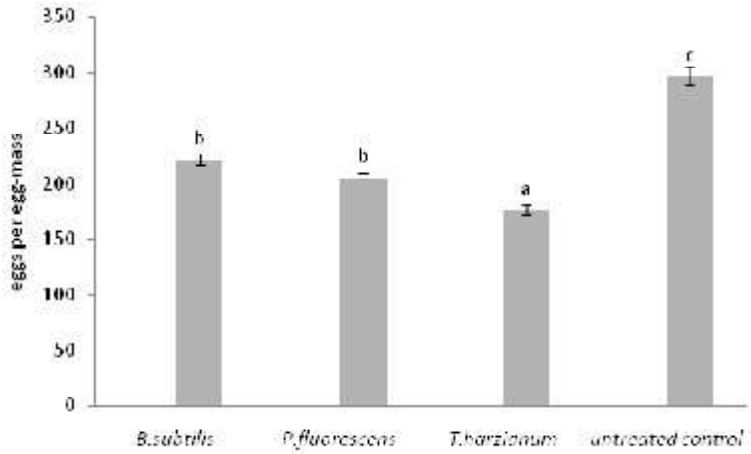
Columns in the same color and followed by the same letter(s) are not significantly different at P ≤ 0.05

Fig. 4. Effect of different bio-agents on root and shoot fresh and dry weight in tobacco



Columns in the same color and followed by the same letter(s) are not significantly different at P ≤ 0.05.

Fig. 5. Effect of different bio-agents on number of *M. incognita* per pot (300g soil) in tobacco



Columns in the same color and followed by the same letter(s) are not significantly different at P ≤ 0.05

Fig. 6. Effect of different bio-agents on number of eggs per egg-mass in tobacco

Effect of different bio-agents on root and shoot fresh and dry weight

The results for this study depicted by root and shoot fresh and dry weight in tobacco revealed significant mean differences against control in Fig.4. The application of the various treatments increased plant growth parameters of tobacco when compared with untreated inoculated control. All treatments have shown a significant increase of shoot fresh weight in tobacco compared with untreated inoculated control. But when comparing with untreated control, a significant reduction of root and shoot fresh and dry weight in tobacco was shown in all treatments. A significant increase of root fresh weight and shoot dry weight was shown in the tobacco treated with *P. fluorescense* and *T. harzianmu* compared with untreated inoculated control, but a significant reduction was shown when compared with untreated control. Only treatment with *T. harzianum* significantly increased root dry weight compared with untreated control.

Effect of different bio-agents on number of *M. incognita* per pot and number of eggs per egg-mass

The application of the various treatments significantly suppressed nematode soil population and eggs per egg-mass when compared with untreated control (Fig.5. and Fig.6.). The number of *M. incognita* and the number of eggs per egg-mass were significantly reduced in tobacco treated with *T. harzianum*, which possessed the highest suppression potentials of nematode soil population when compared with other treatment. No significant difference of suppression when reducing the number of *M. incognita* and the number of eggs per egg-mass were found between the tobaccos treated with *B. subtilis* and *P. fluorescens*.

DISCUSSION

Research on the use of bio-agent to manage plant parasitic nematodes was receiving increasingly greater attention (Hallman *et al*. 2009). As shown in this study, three tested organisms, *B. subtilis* strain Itb162, *P. fluorecense* strain P-72-10 and the *T. harzianum* strain YZL229 were investigated for their effectiveness in controlling tobacco root-knot disease in vitro and under greenhouse. In vitro assays, all bio-agents expressed different degrees of ovicidal and larvicidal potentials towards *M. incognita*. Inhibiting egg hatching is helpful in reducing populations of *M. incognita* in soil and roots (Meyer *et al.* 2004), and larvicidal effect is helpful in reducing the penetration of J2 directly. It is obviously suggested that *T. harzianum* strain YZL229 possessed the best ovicidal and larvicidal potentials invitro towards *M. incognita* when compared with *B. subtilis* strain Itb162 and *P. fluorecense* strain P-72-10 base on the result. In Greenhouse bio-control experiments, all bio-agents not only expressed different degrees of effect on reducing the numbers of galls on tobacco roots and J2 of the nematode in the soil towards *M. incognita*, but also enhanced the growth on tobacco, resulting in the increased weight and length of tobacco's shoots and roots.

The fungus *T. harzianum* strain YZL229 gave encouraging results on the control of *M. incognita* as its highest bio-control potential which shown in this study in both vitro assays and greenhouse experiments. Many achievements can support our result in this study, for example, *T. harzianum* could effectively reduce the incidences of *M. javanica* infecting sunflower when they were used as a soil amendment (Hammad and Zaid 2007). A lot of researches have proved that using the isolates of *Trichoderma* spp. for the management of root-knot nematodes in tobacco was a viable strategy (Spiegel and Chet 1998; Dababat and Sikora 2007; Sahebani and Hadavi 2008; Affokpon *et al.* 2011). Several studies have shown that root colonization by *T. harzianum* not only lead to direct parasitism of eggs and junveniles through the increase in chitinase and protease activities (Sharon *et al.* 2001), but also a slight increasing in tomato growth due to inoculating the seedlings with *T. harzianum* in the presence of *M. incognita* (Dababat and Sikora 2007).

The bacteria *B. subtilis* strain Itb162 and *P. fluorecense* strain P-72-10 also expressed their good bio-control potentials towards *M. incognita* both in vitro assays and greenhouse experiments based on the result of this study. The well-known bio-control mechanisms that mediated by plant growth promoting bacteria such as *B. subtilis* and *P. fluorecense*, were competition for nutrients, accumulation of toxins, enzymes and secondary metabolites, enhancing plant growth and induction

of systemic acquired resistance (Sikora *et al*. 2007). Besides that, there were many achievements can support our result in this study. It is reported that a kind of noncellular extract of *B. subtilis* possessed a high degree of larvicidal effect towards root-knot and cyst nematodes (Gokte and Swarup 1988).It is also reported that the growth of a wide range of plants could be promoted by *B. subtilis* ((De Freitas *et al.* 1997; Kokalis-Burelle et al. 2002)

Similarly, *P. fluorecense* has been reported to significantly enhance banana growth and reduce root-knot nematode populations on bananas in India (Jonathan *et al.* 2006). The good bio-control potential of *P. fluorecense* towards *M. incognita* could be attributed to antagonism effect as a result of the interaction between secondary metabolites synthesized by *P. fluoresens* and plant-parasite nematode (Siddiqui *et al*. 2005), and a broad-spectrum antibiotic 2, 4-diacetylphloroglucinol, which was synthesized by *P. fluoresens* has already been demonstrated to be a major determinant in nematode bio-control (Cronin *et al*. 1997; Siddiqui *et al*. 2003).

CONCLUSIONS

In summary, treatment with different bio-agents not only induced suppressiveness against *M. incognita* on tobacco both in vitro and greenhouse experiments but also promoted tobacco growth. The results presented in our study show that the bio-agents play a significant role in protecting plants against diseases and enhancing their growth. Additionally, the combinative use of different bio-agents against plant-parasitic nematodes may provide greater protection, but further studies are required. And the mechanisms through which these bio-agents exist and respond to their surroundings must be further understood.

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