

# Biological Control Agents in the Management of Different Initial Population Densities of *Meloidogyne javanica* in Tomato

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The efficacy of single and combined application of *Trichoderma harzianum* and *Pseudomonas fluorescens* (CHA0) in the controlling of *Meloidogyne javanica* on tomato plants was evaluated under green house conditions. Seeds of the susceptible tomato cv. Early-Urbana were sown in clean plastic pots containing 1.5 kg steam sterilized soil. Four weeks after planting, the soil of each pot was infested with a suspension of 20 ml/kg soil of *T. harzianum* ( $10^6$  spores/ml) and a suspension of 15 ml/kg soil of *P. fluorescens* (CHA0) ( $10^8$  CFU/ml). Soil of other pots were infested with the two tested bio-agents together as a combined application. Seven days later, plants in all pots, except the controls, were inoculated with *M. javanica* at initial population densities of 1, 2 or 4 eggs/cm<sup>3</sup> soil. Sixty days after nematode inoculation, the parameters of plant growth and nematode reproduction were determined. Results showed that the nematode reproduction factor (Rf) on the plants infected with 1, 2 and 4 eggs/cm<sup>3</sup> decreased by 58, 63 and 31% after the single application of *T. harzianum*, 11, 33 and 12% after the single application of *P. fluorescens* (CHA0) and 43, 55 and 49% after the combined application of the bio-agents, respectively. Combined application of the two bio-agents was found to be the most effective in controlling the higher initial population density of the nematode (4 eggs/cm<sup>3</sup>).

**Keywords:** Biological control, interaction, *Solanum lycopersicum*, root-knot nematode.

The root-knot nematodes, *Meloidogyne* spp., are one of the major detrimental agents in tomato, *Solanum lycopersicum* L., cultivation. However, the plant root surfaces could be colonized by many antagonists which can reduce the direct attack of the root by the pathogens through the production of antimicrobial components that cause induced systemic resistance (ISR) in the plants against those pathogens (Klopper and Beauchamp, 1992; Van Loon et al., 1998).

Sharon et al. (2001) reported that *T. harzianum* enhanced the accumulation of phenolic compounds and chitinase and peroxidase enzymes in tomato plants infected with *M. javanica*. Induced resistance, anti-nematode metabolites production and direct parasitism are other antagonistic mechanisms of *T. harzianum* against root-knot nematodes (Sharon et al., 2001). *Trichoderma* species are considered among the important bio-agents of plant-parasitic nematodes due to their high reproductive rates, competition mechanisms,

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direct parasitism, antibiosis and the production of extracellular enzymes (Chacon et al., 2007; Yang et al., 2007; Mallikharjuna et al., 2016).

Plant growth promoting rhizobacteria (PGPR) are free living and useful bacteria in soil that can also increase plant growth through various mechanisms (Saharan and Nehra, 2011). *Pseudomonas fluorescens* increases the growth of plants and has an antagonistic effect on root-knot nematodes (Almaghrabi et al., 2013). The action mechanisms of *P. fluorescens* against root-knot nematodes include; the production of metabolites that suppressing egg hatching, decreasing root exudates that attract nematodes to the plants and enhancing the induced resistance in the plants infected with nematodes (Sikora and Hoffmann-Hergarten, 1993).

*T. harzianum* and *T. viride* can suppress the reproduction and gall formation by *M. javanica* on tomato plants and increase tomato growth (Al-hazmi and Tariq Javeed, 2016). Similarly, nematode population indices of *M. incognita* on tomato plants treated with *T. harzianum* and *T. viride* were found to be decreased while plant growth was found to be increased (Mukhtar, 2018). It was also found that a combination of *T. viride* and *P. fluorescens* (CHA0) had a good bio-control effect against *M. javanica* infecting tomato plants as compared to the single treatment of each of them (Saeedizadeh, 2016). Combined and single treatments of salicylic acid, *P. fluorescens* (CHA0) and *T. viride* increased the plant growth indices of tomato plants infected with *M. incognita* race 2 (Esfahani et al., 2016). The placement of *M. javanica* in a filtered culture of *P. fluorescens* reduced egg hatching and increased significantly the mortality of larvae (Siddiqui and Shaukat, 2003).

It has been shown that the combination of two or more antagonists can be effective in controlling soil-borne pathogens (Schippers, 1992). Therefore, the aim of this study was to determine the effect of *Pseudomonas fluorescens* (CHA0) and *Trichoderma harzianum* on tomato plants infected with different initial population densities of *M. javanica*.

## Materials and Methods

### *Nematode preparation*

Roots infected with *M. javanica* were collected from the single egg-mass cultures established on tomato cv. "Early-Urbana Y" in the green houses of Boyer-Ahmad County, Iran. Nematode species was identified based on the morphological features of the perennial pattern as described by Taylor and Netscher (1974). The nematode eggs were extracted from the galled roots using NaOCl (Sodium hypochlorite) 0.5% solution (Hussey and Barker, 1973). The number of eggs were calculated by a manual counter.

### *Trichoderma harzianum preparation*

The isolate of *T. harzianum* was obtained from the Department of Plant Protection, Faculty of Agriculture, Shiraz University, and maintained on Potato Dextrose Agar (PDA) medium (Booth, 1977). This fungus was originally isolated from soil samples collected from different agricultural fields and green house of Shiraz, Iran, using dilution plate

method onto *Trichoderma* selective media (TSM) according to Elad and Chet (1983). The required concentration was  $10^6$  spores/ ml sterile distilled water, which was prepared using Hemocytometer and the addition of sterile distilled water.

#### *Pseudomonas fluorescens* (CHA0) preparation

The isolate of *P. fluorescens* (CHA0) was obtained from the Department of Plant Protection, Faculty of Agriculture, Tehran University (Fuqua and Greenberg, 1998). The bacterial suspension was grown on Nutrient Agar (NA) culture to obtain a pure and fresh bacterial culture, and was kept at 28 °C for 48 h. The bacteria were then harvested and mixed with sterile distilled water and finally, the concentration was adjusted to  $10^8$  CFU/ ml (Thompson, 1996).

#### Green house studies

One experiment with two trials was carried-out under greenhouse conditions ( $27 \pm 4$  °C with a 16: 8 h light to dark photoperiod) on 2017 and 2018. Plastic pots, 15 cm diam., were filled with 1.5 kg soil mixture (1 cow manure: 1 steam sterilized sandy loam soil: 2 sand), and seeded with tomato cv. “Early-Urbana”. Four weeks after planting, the tomato seedlings were thinned to one seedlings/pot. A suspension of 20 ml/kg soil of *T. harzianum* ( $10^6$  spores/ ml) and a suspension of 15 ml/kg soil of *P. fluorescens* (CHA0) ( $10^8$  CFU/ ml) were added into three holes created in soil around the stem of the seedlings. Seven days later, *M. javanica* inocula (1, 2 or 4 eggs/cm<sup>3</sup> soil = 1,500, 3,000 and 6,000 eggs/pot) were pipetted into three holes around the stem of the tomato seedlings (Siddiqui and Shaukat, 2003). Pots were arranged in a completely randomized design in the green house, irrigated and fertilized as needed till the end of the experiment. Sixty days after nematode inoculation, plant growth parameters including; plant height, fresh and dry weights of the shoot and fresh weight of the root and nematode reproduction parameters including the number of eggs as described by Hussey and Barker (1973), number of galls and egg masses on the root system as described by Taylor and Sasser (1978) and the nematode reproductive factor (Rf) were determined (Equation 1) (Taylor and Sasser, 1978).

Equation 1

$$RF = \frac{\text{Final number of nematode in soil and root system}}{\text{Initial population of nematode}}$$

#### Statistical analysis

The data of both trials were similar and they combined before statistical analysis. The experimental design was completely randomized design with five replications. Data were subjected to a factorial analysis of variance (ANOVA) using SAS statistical software ver. 9.4 (SAS Institute, Cary, NC). Whenever the *F*-test showed significant differences at  $P < 0.01$ , treatment means were compared using Fisher’s Protected Least Significant Differences (LSD) test at 0.01.

## Results

The highest shoot length was observed in nematode non-inoculated plants treated with *P. fluorescens* (CHA0), and it had no significant difference with infected plants with 1500 eggs/pot treated with *T. harzianum* (Fig. 1a). The highest shoot fresh weight was observed in nematode non-inoculated plants, treated with *P. fluorescens* (CHA0), and it had no significant difference with non-inoculated plants treated with *T. harzianum*, infected plants with 1500 eggs/pot treated with *P. fluorescens* (CHA0) and infected plants with 3000 eggs/pot treated with *T. harzianum* (Fig. 1b). The highest shoot dry weight was observed in nematode non-inoculated plants, treated simultaneously with both bio-agents, and it had no significant difference with infected plants with 1500 or 3000 eggs/pot, treated simultaneously with both bio-agents, infected plants with 3000 eggs/pot treated with *T. harzianum* and non-inoculated plants treated with *P. fluorescens* (CHA0) (Fig. 1c). Root fresh weight of infected plants with 1500 and 3000 eggs/pot treated simultaneously with both bio-agents, significantly increased compared with control plants at the same population levels (Fig. 1d).

In plants treated with *T. harzianum* and infected with 1500, 3000 and 6000 eggs/pot, the number of eggs and the reproduction factor decreased by 58, 63 and 31%, respectively, the number of galls/ root system decreased by 52, 44 and 56%, respectively, and the number of egg masses/ root system decreased by 76, 41 and 49%, respectively, compared to the control plants. In plants treated with *P. fluorescens* CHA0 and infected with 1500,

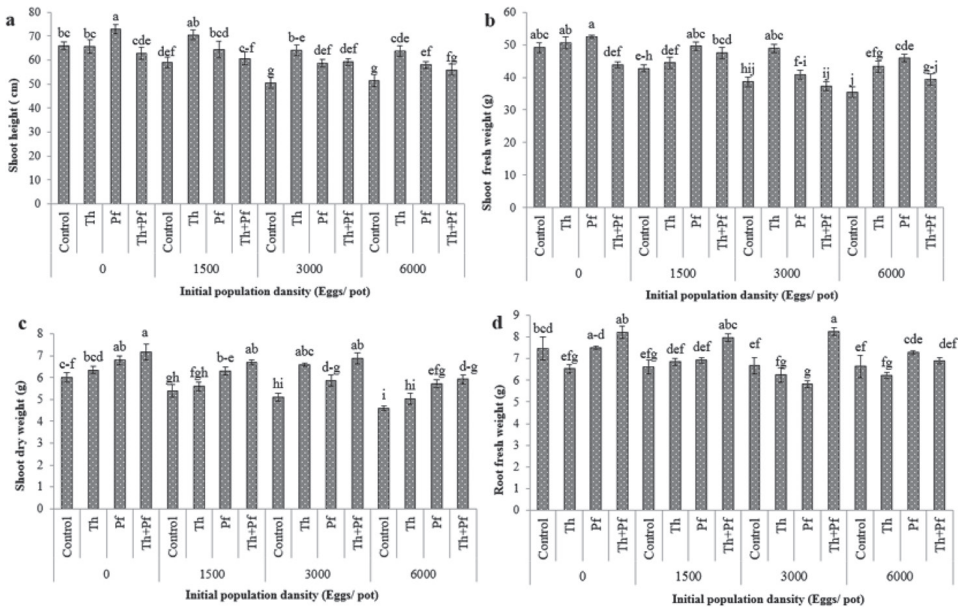


Fig. 1. Mean shoot length (a), shoot fresh weight (b), shoot dry weight (c) and root fresh weight (d) of tomato plants infected with *Meloidogyne javanica* and soil drenched by *Trichoderma harzianum* and *Pseudomonas fluorescens* (CHA0), 60 days after nematode inoculation under green house conditions.

Data are presented as the mean ± standard deviation of two independent trials with five replicates

3000 and 6000 eggs/ pot, the number of eggs and the reproduction factor decreased by 11, 33 and 12%, respectively, the number of galls/ root system decreased by 51, 32 and 56%, respectively, and the number of egg masses/ root system decreased by 54, 30 and 52%, respectively, compared to the control plants. In plants treated simultaneously with both bio-agents and infected with 1500, 3000 and 6000 eggs/ pot, the number of eggs and the reproduction factor decreased by 43, 55 and 49%, respectively, the number of galls/ root system decreased by 47, 41 and 58%, respectively, and the number of egg masses/ root system decreased by 48, 47 and 63%, respectively, compared to the control plants (Fig. 2).

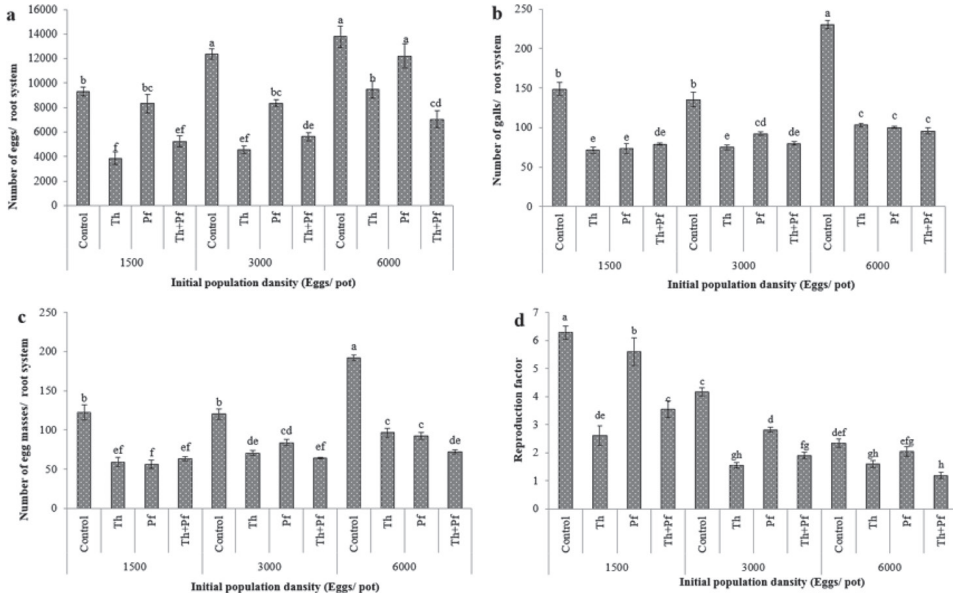


Fig. 2. Mean number of *Meloidogyne javanica* (a) eggs, (b) galls and (c) egg masses per root system and (d) reproduction factor on tomato plants soil drenched by *Trichoderma harzianum* and *Pseudomonas fluorescens* (CHA0), 60 days after nematode inoculation under green house conditions. Data are presented as the mean  $\pm$  standard deviation of two independent trials with five replicates

The highest number of eggs/ root system was observed in the plants infected with 3000 and 6000 eggs/pot without treatment of bio-agents, which had no significant difference with plants infected with 6000 eggs/pot treated with *P. fluorescens* (CHA0) (Fig. 2a). Single or joint treatments of *T. harzianum* and *P. fluorescens* (CHA0) significantly reduced the number of galls (Fig. 2b) and egg masses/ root system (Fig. 2c) compared to the control treatment at each of the nematode population levels. Single or joint treatments of *T. harzianum* and *P. fluorescens* (CHA0) significantly decreased the reproduction factor of nematode at initial population density of 1500 and 3000 eggs/pot compared with the control plants at the same population levels (Fig. 2d).

## Discussion

Previous studies have shown that the *Trichoderma*-based biocontrol mechanisms mainly rely on production of antibiotic substances (Gajera et al., 2013), competition for nutrients (Miethke 2013; Li et al., 2015), as well as induced plant resistance (Shoresh et al., 2010). Anti-nematode activity is also influenced by extracellular enzymes produced by the isolates of this fungus, including chitinase (Harman et al., 2004). Plant treatments with antagonistic agents such as *Trichoderma* increased the activity of enzymes such as chitinase, peroxidase, etc. (Sharon et al., 2001). In general, the induced systemic resistance in the plant increases the accumulation of phytoalexins, phenolic compounds, pathogenesis related proteins (PR proteins) such as peroxidase, the level of mRNA coding phenylalanine ammonia-lyase (PAL) and other protective enzymes such as polyphenol oxidase, and also lignification of the cell wall (Van Loon and Bakker, 2005). In the present study, the combined application of bio-agents, as well as single application of *T. harzianum* in reducing the nematode indices was better than the use of single application of *P. fluorescens* (CHA0). In a study, the combined application of *T. harzianum* and *P. fluorescens* (CHA0) on tomato infected with *M. javanica*, decreased significantly the nematode population indices, compared to the control (Siddiqui and Shaikat, 2003).

Previous studies have shown that induced systemic resistance in the plant increases plant activity and the production of defense enzymes and because of its negative effects on plant fitness, decreases plant vegetative indices (Molinari and Baser, 2010), and such negative effects is probably the reason for the shoot fresh weight reduction in non-inoculated plants treated simultaneously with both bio-agents, compared to the control plants. Salicylic acid-signaling pathway and ethylene biosynthesis were induced in tomato treated with *T. harzianum* when infected by *M. incognita* and limited the infection by activation of systemic acquired resistance (SAR) and ethylene production (Leonetti et al., 2017). The main suppression mechanisms used by *P. fluorescens* (CHA0) against *M. javanica* are plant defense mechanisms leading to systemic resistance and the destruction of eggs (Tavakol Norabadia et al., 2014).

Regarding the presence of chitin in the middle layers of the nematode egg shell, *T. harzianum* seems to be effective in inhibiting hatching of nematode eggs by producing chitinase enzyme (Brants et al., 2000). Al-Fattah et al. (2007) showed that *T. harzianum* caused J2s mortality of *M. javanica* by 30%, by producing extracellular metabolites. In a study by Brants et al. (2000) the effect of secretion of endochitinase enzyme from *T. harzianum* disrupted chitin formation in the egg shell and hence reduced egg hatching of *M. hapla*. *T. harzianum* suppress plant pathogens by different mechanisms such as competition, mycoparasitism and the production of enzymes and toxic compounds. It is proved that the mycelium of *Trichoderma* entered directly on the eggs and J2s of *M. javanica* and also damaged the nematode egg shell by producing hydrolyzing enzymes such as chitinase, protease, and lipase and finally decreased hatching and increased the death of larvae (Bird and McClure, 1976).

*P. fluorescens* (CHA0) is one of the most effective, active and dominant rhizobacteria, with direct antagonistic effects on the pathogens by antibiotic production, competition with the pathogen for essential nutrients such as iron, and also indirectly enhance the growth of the plant. This bacterium effectively occupies the root surface and rhizosphere and induces systemic resistance in plants (Siddiqui and Mahmood, 1998). It has been

found that increasing plant vegetative indices in the presence of *P. fluorescens* (CHA0) is due to mechanisms such as increasing available phosphorus, nitrogen fixation, production of plant hormones including auxins, cytokinins, gibberellins and, consequently, increasing plant tolerance to stresses such as salinity, drought and pesticides in plants through which the plant growth is increased (Glick, 2012). The reduction in the number of nematodes by bacteria can be due to numerous defense mechanisms in the plant (Siddiqui and Shaukat, 2003). *P. fluorescens* (CHA0) suppresses nematode by mechanisms such as producing toxic compounds and reducing root exudates. It also inhibits nematode reproduction by increasing the defense mechanisms of the plant and inducing systemic resistance in the plants (Siddiqui et al., 2001). Pre- or post-treatment application of salicylic acid and *P. fluorescens* (CHA0) on *M. javanica* infected tomato plants increased the activity of superoxide dismutase, peroxidase and catalase in plants and reduced the nematode population indices as compared to the control (Sarafraz Nikoo et al., 2014). *P. fluorescens* (CHA0) induced systemic resistance (ISR) in the host plant. This induced resistance causes cell wall fortification and also physiological changes and biochemical responses in the host against the pathogens (Van Loon et al., 1998).

## Conclusion

In the present study, for the first time we examined the efficacy of single and combined application of two bio-agents in the controlling of different initial population density of *M. javanica* on tomato plants. According to the results of this study, single application of *T. harzianum* and *P. fluorescens* (CHA0) as well as joint application of bio-agents reduced the reproduction factor of *M. javanica* compared to the control. However, single application of *T. harzianum* has been more successful in controlling low nematode initial population density (1500 and 3000 eggs/pot) compared to the single application of *P. fluorescens* (CHA0). Single application of bio-agents cannot control well the high initial population density (6000 eggs/pot), while combined application of bio-agents has been more successful in controlling this nematode density. Therefore, it can be suggested that application of *T. harzianum* and *P. fluorescens* (CHA0) has been more successful in controlling high nematode population density.

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