

The Application of Manure (Poultry Wastes) and Bio-Formulations of *Trichoderma harzianum* and *T. viride* and Their Interaction to Control Root-knot Disease on Radish and Chard

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Abstract: The study was designed to examine the effect of manure (poultry wastes) and bio-formulations of *Trichoderma harzianum* and *T. viride* separately or with some, to control root-knot disease on radish (*Raphanus sativus* L.) and chard (*Beta vulgaris var.cicla* (L.)). The study included the isolation of pathogenic nematode of both plant roots, morphological and molecular identification, examination of the pathogenicity *in vitro* and *in vivo* and green house experiments involved application of manure and fungal bio-formulations treatments. The morphological and molecular identification confirmed the identity of root-knot nematode, as *Meloidogyne javanica*, which was pathogenic to radish and chard. The recorded infection severity was 89 and 95% respectively. The green house experiment results revealed that MThTv treatment was significantly reduced infection severity to 0% for radish and chard in contaminated soil (CS) compared with control positive treatment (55.17 and 40%) respectively. MThTv treatment also showed a highest plant height for Radish in non-nematode-contaminated soil (NCS) and CS treatment (17.85 and 16.50 cm) respectively compared with control positive treatment (5.00 cm), while the highest plant height of Chard was 24.5 cm in MThTv-NCS. The wet weight index in Radish showed a superiority of MThTv and MTh in NC on other treatments (201.75 and 189.5 g.plant⁻¹) respectively followed by MThTv-NCS treatment (184.5 g.plant⁻¹) compared with 19.25 gm.plant⁻¹ in control treatment. In Chard the results showed similar pattern represented by superiority of MThTv-NC treatment (255.25 gm.plant⁻¹) followed by MThTv-NCS (190.75 gm.plant⁻¹) compared with 37.50 gm/plant for positive control.

Keywords: Nematode, *Meloidogyne javanica*, Biological control, radish, Chard, *Trichoderma*.

Introduction

Meloidogyne spp. are important plant parasitic nematodes affecting wide host range that is close to 2500 hosts. Root-knot disease is one of the most prevalent diseases in the world that causes major losses in field crops, vegetable crops, ornamental plants and weeds,

which is include Cucumbers, lettuce, papaya, onions, peaches, peppers, potatoes, soybeans, spinach, zucchini, sugarcane, sugar beets, tobacco, wheat, and hyssop, as well as radish and chard plants (Bird & Kaloshian, 2003; Davis *et al.*, 2004).

The radish (*Raphanus sativus* L.) is an edible root vegetable plant belongs to the Brassicaceae family that was originated in Asia and domesticated by Greeks and Egyptians in the ancient eras. Each 100g of roots contains 94% water, 20 calories, 1g of protein, 4g of carbohydrate, 37mg of calcium, 31mg phosphorus, 30 universal unit of vitamin A and 24 mg of vitamin C in addition to mustard oil (Matloob *et al.*, 1989).

The chard (*Beta vulgaris var.cicla*) is a leafy green vegetable of the Chenopodiaceae family. The Canary Islands and the Mediterranean Sea are origin lands of this plant (Matloob *et al.*, 1989).

The radish and chart plant mostly infected with several root diseases including root-knot disease, which is recognized by forming of root-knots on the plant root system that affecting the functional efficiency of the root, which reflected as a malfunction in absorption of the water and nutrients. As a result, wilt symptoms will appear in hot weather and regain their status in the early morning (Abad *et al.*, 2003).

For long decades, nematocides like Mocap, Fouradan, Vydate, and Nemacor were used to control root-knot nematodes, which is companied with serious adverse effects to the environment in general and to the human and animal health in particular. Alternatively, biological control considered as a best environmentally friendly solution to replace chemical control. Among several biocontrol agents, the fungi, *Trichoderma viride* and *T. harzianum* were successfully employed in the control of root-knot nematodes (Mukerji & Garg, 1987; Agrios, 2005).

Regarding the importance of this disease and the lack of studies about it Iraq, in addition to the large losses caused by, this study aimed to investigate the prevalence of

this disease and the possibility of control it biologically.

Materials & Methods

Samples collection and preparation

The radish and chard infected root samples were collected from several location of Basrah province included Al-Hotta and Al-Hartha. The samples prepared according to McClure *et al.* (1972) that involved clean up the collected roots with tap water to remove soil debris. The roots were cut in to small pieces (about 2 cm) then washed with tap water through 60 mesh sieve then transferred to the blender contained 100 ml of tap water and 500 ml of 0.1% sodium hypochlorite (6% of commercial formulation), the content mixed for 40 s at high speed. The mixture passed through several sieves (100, 400 and 500 mesh) respectively, the samples were collected with 40 ml of tap water in 50 ml test tubes then centrifuged at 1000 rpm for 40s. The supernatant (contains eggs) poured in 400 mesh sieve and washed 3 times with 100 ml of distilled water and the eggs were collected with distilled water in 205 ml conical flask.

Morphological identification

The collected eggs from the past step were incubated for 3-5 days on 28°C (McClure *et al.*, 1972). The specimen were identified morphologically by second author according to Hirschmann (1985), Luc *et al.* (1990) and Perry *et al.* (2010).

Molecular identification

The collected eggs suspension (10 ml) was centrifuged at 1000 rpm for 15 min. The supernatant was discarded and the pellet was transferred to a mortar and grinded with liquid nitrogen to a fine powder. About 20 mg of

powder was transferred to 1.5 ml Eppendorf tube for Genomic DNA extraction.

The DNA was isolated using Genomic DNA Purification kit A1120 (Promega, USA) according to the manufacturer instructions.

The PCR reaction was performed according to Aydinli & Mannen (2016) using Sequence-characterized amplified region (SCAR) primers for Fjva: GGTGCGCGATTGAACTGAGC and Rjva: CAGGCCCTTCAGTGG-AACTATAC. The reaction conditions mixture contained 25 µl of Taq DNA Master Mix RED (Amplicon, Denmark), 2.5 µl of each primer, 100 ng of DNA template and the mixture completed to 50 µl with DD-Water. The amplification was performed using MyGenie™ 96/384 thermal Block (BioNEER, Inc, Korea), the reaction conditions involved initiation on 94°C for 3min followed by 35 cycles included denaturation on 94°C for 30s, annealing on 60°C for 30s and extension on 72°C for 1min followed by 7min of final extension on 72°C.

The quantity and quality of DNA were confirmed by Nano drop device (Thermo-Scientific™, NanoDrop 2000, USA) at wavelength 260/280. The samples were sent to MacroGen Co. (Korea) to confirm PCR product.

Examining the pathogenicity of *M. javanica*

Radish and chard seeds were used to examine the pathogenicity of *M.javanica*. The experiment involved preparing a mixture of sand soil: compost (3:1), the mixture was decontaminated with formaldehyde (20ml/L of water for each 12.5 kg of mixture), the mixture packed in plastic pots (500 gm), the treatments (*M.javanica* contaminated soil “NC” and non-contaminated soil “NCS”) were considered in triplicates. The seeds were planted in the pots (5 seeds/pot) and irrigated

carefully. After 1 week, 1000 second stage juvenile were used to inoculate each pot and the infection percentage was calculated after 3 months of inoculation (Singh & Siddiqui, 2012).

Fungal isolates

T. harzianum and *T. viride* isolates were got as a gift from second author. The fungi were activated on 9 cm Petri plates containing PDA medium at 27°C for 4 days then stored on PDA slants for future use (Alwaely, 2014). The both isolates were used in all next experiment of this study.

Formulation of biological control agents *T. harzianum* and *T. viride*

The both fungal isolates were re-activated on PDA for 4 days at 27°C. Using cork poorer, a 0.5cm mycelium disks were used to inoculate 500ml conical flasks containing 200gm of 1h autoclaved mixture of Wheat bran: Sand: water (2:1:1) W/W/V then incubated at 30°C for 15 days with shaking in interval of 2 days (Papavizas *et al.*, 1982).

Field experiment

The field experiment was carried out at the research station of the College of Agriculture, University of Basrah, Garmat Ali campus on 22/2/2018. The dimensions of the experiment plot was 16 × 4m², the soil was plowed and divided in to three 16×0.5m sub-plots with a distance of 50cm among sub-plots. The sub-plots were divided in to separate experimental units (1 × 0.5m). The organic fertilizer (fermented poultry wastes) was added by mixing with soil followed by addition of biocontrol formulation (10g.m⁻¹) (Al-Waily, 2004) to the particular treatments in triplicates (table 1). After three days the seeds were planted in pits with a distance of 40cm among them then drip irrigated. A couple of weeks later, they inoculated with *M. javanica* (1000

eggs and/or second stage juvenile/plant: 2cm from plant and 3cm in depth) (Singh & Siddiqui, 2012). The considered parameters of

this experiment were infection percentage, plant high and wet weight of the plant.

Table (1): The experimental design of field experiment.

Treatment	Details
ThTvM	<i>T. harzianum</i> + <i>T. viride</i> + Manor
TvM	<i>T. viride</i> + Manor
ThM	<i>T. harzianum</i> + Manor
M	Manor
ThTv	<i>T. harzianum</i> + <i>T. viride</i>
Tv	<i>T. viride</i>
Th	<i>T. harzianum</i>
Mj	Positive control (The soil inoculated with <i>M. javanica</i>)

All treatments were applied in non *M. javanica* inoculated experimental units.

Estimation of root-knot infection severity on radish

The infection severity of *M.javanica* on radish was estimated using the root-not disease

index (designed in this study) that involved a five degrees scale (No infection= 0; 1-10 nots = 1; 11-20 nots = 2; 21-30 notes = 3; 31-40 notes = 4; more than 41 = 5).

The infection severity calculated according to Mckinney (1923) formula that reported in Al-Waily (2004):

$$\text{Infection severity \%} = \frac{\sum(\text{No. of non infected plants} \times 0 + \dots + \text{No. of dead plants} \times 5)}{\text{No. of plants} \times \text{highest degree}} \times 100$$

Estimation of root-knot infection severity on chard

The Bridge & Page (1980) disease index of root-nots was used to estimate the infection severity. The index involved six degrees scale (No notes = 0; little No. of notes hard to see = 1; some of big notes but not in main roots = 2; 50% of root were infected, notes on main roots and shrinkage of root system= 3; notes observed on most main roots = 4; All roots are infected = 5). Mickenny (1923) formula was used to calculate the infection percentage.

Results & Discussion

The morphological and molecular identification

The morphological identification results (Fig. 1) revealed that all isolated nematodes were returned to the species *M. javanica* according to the diagnostic traits (length and diameter were about: 395 × 130 μm for J2; 1400 × 30 μm for male; 700 × 380 μm for female) in Hirschmann (1985), Luc *et al.* (1990) and Perry *et al.* (2010).

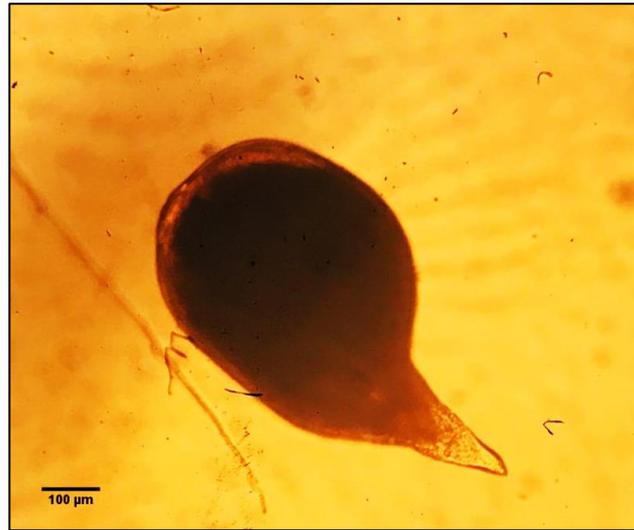


Fig. (1): *M. javanica* female, the microscope magnification is 40X.

The morphological identification of all isolates was confirmed molecularly by the electrophoresis results of a high specific PCR amplification of unique 670 bp segment (Fig. 2). The mentioned molecular method considered as a fast and accurate method usually used to identify *M. javanica* as the

SCAR primers were high specific and mostly produces a PCR product only for *M. javanica* despite of the growth stage and sample purity (the samples may contain environmental DNA contamination from host plant and/or other associated species that can affect the identification accuracy) that leads to apply easy and fast identification process (Zijlstra *et al.*, 2000; Aydinli & Mennan, 2016).

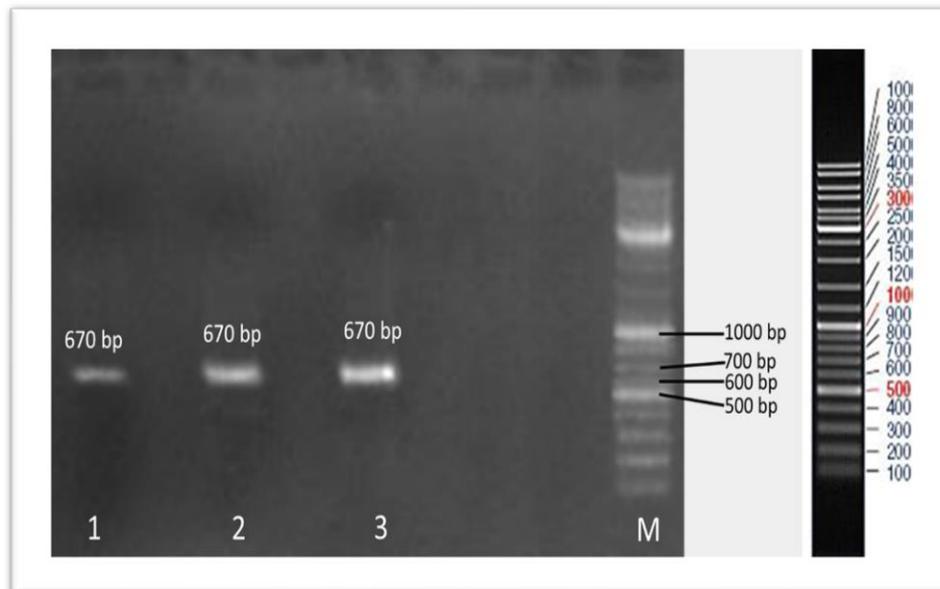


Fig. (2): Electrophoresis of three *M. javanica* PCR product using Fjav and Rjav SCAR primers. Lan 1 and 2 represent *M. javanica* that isolated from radish roots; Lan 3 represent *M. javanica* isolated from chard roots and M is the standard Ladder.

The pathogenicity of *M.javanica*

M. javanica pathogenicity tests (Fig. 3) were confirmed their ability to infect 89% of examined radish and 95% of chard plants

effectively with no significant differences between them ($P=0.157$), while no infection signs were observed on non-inoculated (control-) radish and chard plants.

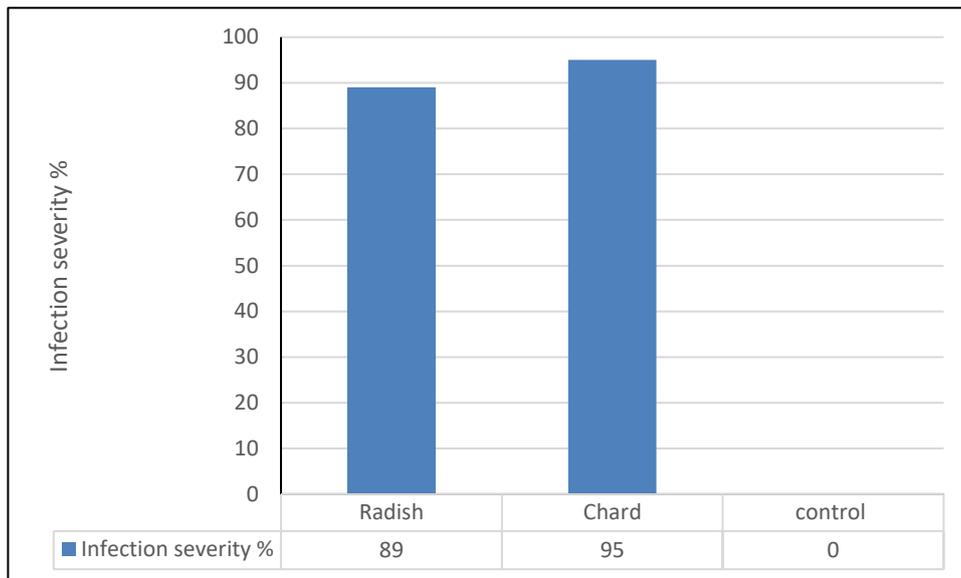


Fig. (3): The pathogenicity of *M. javanica* isolates on radish and chard plants.

Infection severity

The infection severity was varied relative to the host plant and the applied treatment (Fig. 4). On radish, no infection was observed when the plant treated with MThTv treatment which significantly surpass all other treatments followed by TvTh treatment (4.30%), while M treatment showed the lowest suppression effect (31.67%) that was significantly excel the control treatment (55.17%). On Chard, the same infection severity pattern was observed that represented by superiority of MThTv treatment (0%) followed by MTv and MTh (2.67, 5.33%) respectively and the lowest inhibition effect was recorded in M treatment. Anyhow, all the

applied treatments were exceeded the control treatment (40.00%) significantly.

The results above revealed that any treatment involved *Trichoderma* spp. Showed effective reduction in infection severity. *Trichoderma* spp. is well known effective biocontrol agent having several antagonistic mechanisms against nematodes includes the ability to surpass hatching of nematode eggs and preventing their development to the second stage juvenile that reduces the first inoculum density in soil, which in turn leads to reduce the infection development chances (Cook, 2000). Additionally, the pathogenicity, competition, and host systemic resistance induction can considered as helpful factors to prevent and/or reduce the infection severity (Baker, 1989; Okoth *et al.*, 2011).

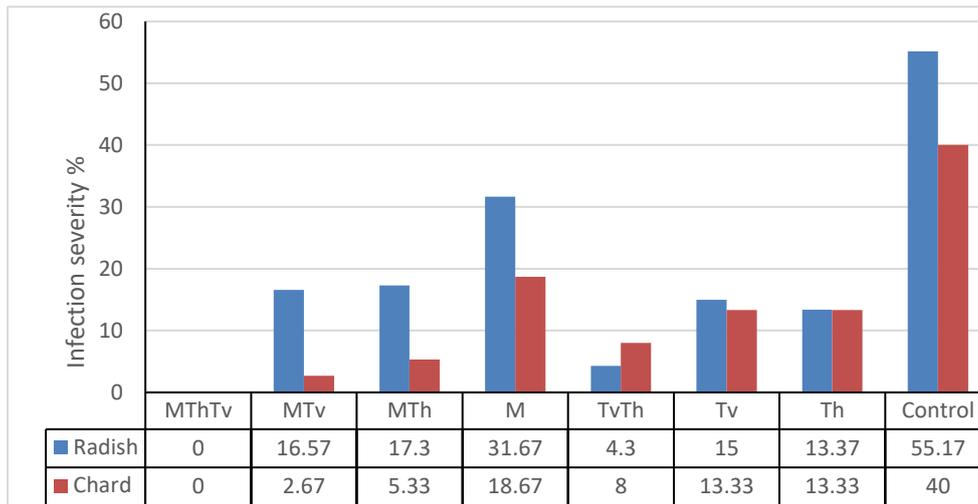


Fig. (4): The assessment of infection severity under different experimental treatments. Each mean represents 4 replicates.

Field experiment

Plant height

The radish plant height was significantly affected by the applied treatments (Fig. 5). The results showed significant differences among factors and/or factorial treatments represented by superiority of MThTv treatment on all other treatments significantly in both CS and NCS treatments (16.50, 17.25 cm) respectively while the lowest height means were observed in M treatments (9.00,

9.25 cm) respectively, which were significantly exceeded the control treatment (5.00 cm) in CS treatment, while significant decrease was recorded in NCS treatment comparing with control (11.50 cm). A significant increase in plant height was recorded in all biocontrol agent included treatments in both CS and NCS treatments comparing with control treatments separately.

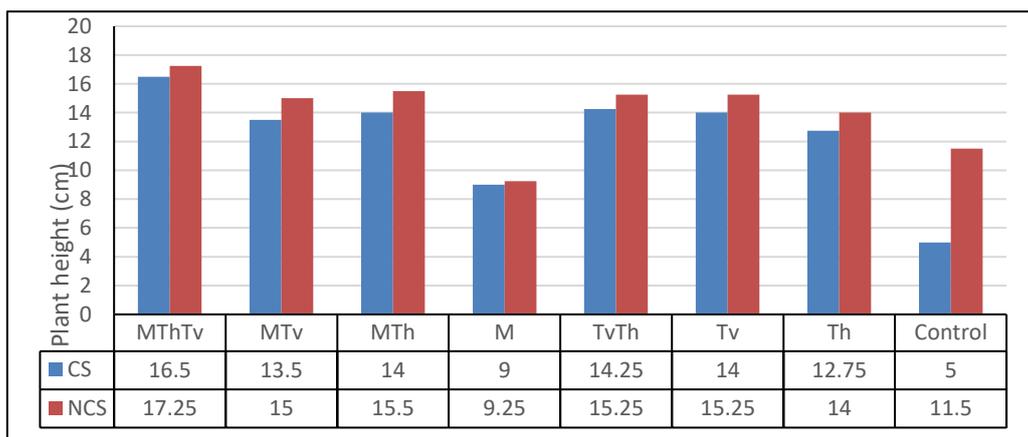


Fig. (5): The effect of experimental treatments on radish height in both *M. javanica* contaminated and non-contaminated soils. Each mean represents 4 replicates. LSD 0.05 of interaction = 0.27, LSD 0.05 of Soil = 0.38.

The chard height was also influenced significantly by field experiment treatments (Fig. 6). The results demonstrated that MThTv excel all other treatments in both Cs and NCS (17.25, 24.25 cm) respectively with a significant difference between them. The

lowest height means were also recorded in M treatments (13.75, 16.50 cm) in contaminated and nematode free soils respectively with significant differences between them. The positive controls (14.88, 19.19 cm) respectively showed significantly lower means than all other treatments.

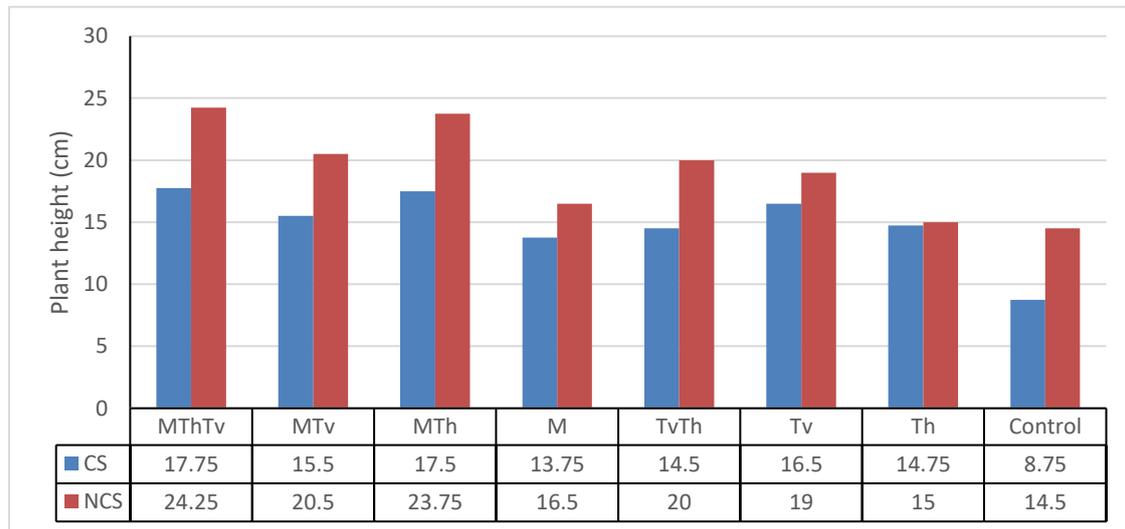


Fig. (6): The effect of experimental treatments on chard height in both *M.javanica* contaminated and non-contaminated soils. Each mean represents 4 replicates. LSD 0.05 of interaction = 0.41, LSD 0.05 of Soil = 0.59.

The plant weight

The means of vegetative wet weight showed a similar pattern to the height results (Fig. 7). All the treatments were significantly exceeded the control treatments in both Cs and NCS treatments. The highest wet weight was recorded in MThTv treatments (184.5 and 201.75 g) respectively, where the lowest wet weight observed in M treatments (39.25 and 83 g) respectively comparing with control treatments (19.25 and 36.25) respectively. Totally, the nematode infection caused a significant reduction in plant wet weight (98.5 g) comparing with control (128.56 g).

The chard wet weight means (Fig. 8) arranged ascendingly from M treatments (63.25, 73.75 gm) for CS and NCS treatments respectively to the highest wet weight in MThTv treatments (190.75 and 255.25 gm) respectively with significant differences among CS and NCS treatments separately. A significant differences were also observed between CS and NCS either totally (14.88 and 19.19 gm) respectively and separately. All the treatments were exceeded the control treatments significantly.

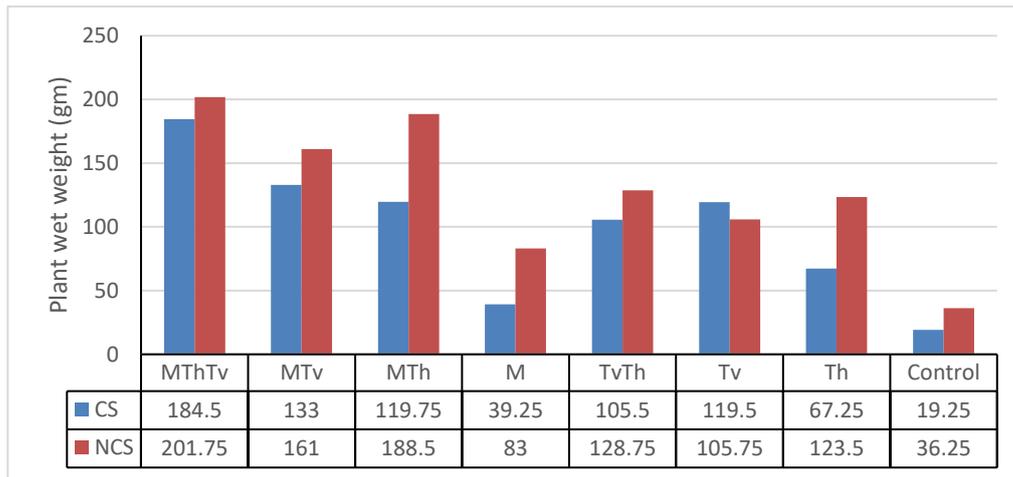


Fig. (7): The effect of experimental treatments on radish wet weight in both *M. javanica* contaminated and non-contaminated soils. Each mean represents 4 replicates. LSD 0.05 of interaction = 15.31, LSD 0.05 of Soil = 21.66.

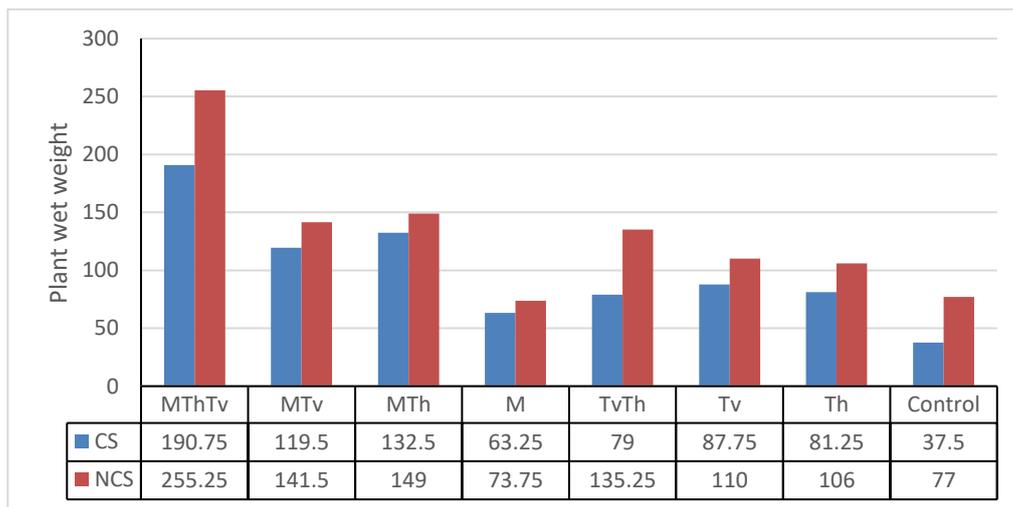


Fig. (8): The effect of experimental treatments on chard wet weight in both *M. javanica* contaminated and non-contaminated soils. Each mean represents 4 replicates. LSD 0.05 of interaction = 25.04, LSD 0.05 of Soil = 8.85.

The results revealed a notable increase in plant height and wet weight in all biocontrol agent included treatments of both CS and NCS treatments comparing with control treatments separately. Actually, there are several proposed mechanisms may interprets these results like (i) the ability of *Trichoderma* spp. to increases supplement of major and minor nutrients to the host plant, (ii) the secretion of growth hormones (like

Indole Acetic Acid) that reflecting positively on plant growth and production, and (iii) the fungus can reduces the average eggs hatching, the total nematodes population and promoting the biochemical defences of the plants, which indeed protects the plant and elevates its self-resistance level (Baker, 1989; Okoth *et al.*, 2011). The differences among CS and NCS treatments results may be due to the nature of the nematode infection that represented by

sever feeding on invaded roots and formation of root knots, which affect the ability of root system to supply plant with water and nutrient needs (Sikora & Kiewnick, 2006).

Conclusion

The radish and chard plants are severely infected by root-knot nematodes *M. javanica* causing highly economic losses to both of them. The use of the biological control by *T. harzianum* and *T. viride* in accompanying with the organic fertilizers will redacting infection severity and significantly increasing the plant vegetative growth.

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