

EFFECTS OF HUMIC ACID, *TRICHODERMA HARZIANUM*, AND *PAECILOMYCES LILACINUS* ON *MELOIDOGYNE JAVANICA*

Ahmad S. Al-Hazmi^a, Fahad A. Al-Yahya^a, Omeed A. AbdelRafaa^b and Hamzeh A. Lafi^{a*}

^aPlant Protection Department, College of Food and Agricultural Sciences, King Saud University. P. O. Box 2460, Riyadh 11451, Saudi Arabia.

^bAlMahalliah Trading & Agriculture Co., Ltd., Riyadh, Saudi Arabia.

*Corresponding author, e-mail: hlafi@ksu.edu.sa

ABSTRACT

Humic acid, *Trichoderma harzianum*, and *Paecilomyces lilacinus* were evaluated for their potential to control root-knot nematode *Meloidogyne javanica* *in vitro* and *in vivo* tests. Exposure of *M. javanica* to increasing concentrations of humic acid significantly reduced egg hatching and increased second stage of juveniles (J2) mortality. Egg hatch was inhibited up to 16.8%–59.8% following exposure to 0.25%-1% humic acid. While test J2 mortality showed that mortality increased as the concentrations of the humic acid and exposure period were increased. The effects of humic acid, *T. harzianum*, and *P. lilacinus*, singly or combined, on egg hatch and J2 mortality were also evaluated in another *in vitro* tests. The highest inhibition (85%) of egg hatch was achieved by *P. lilacinus* and humic acid combined, followed by *T. harzianum* and humic acid, and then by *T. harzianum* alone. The effect of *P. lilacinus* with humic acid on egg hatch was significantly higher than that of *P. lilacinus* alone or of humic acid alone. On the other hand, *P. lilacinus* with humic acid resulted in the highest (78%) juvenile mortality at 10 days of exposure. In a greenhouse test, all of the three agents (the two fungi and the humic acid) were found to be effective in reducing root galling and nematode reproduction, with different levels of efficacy.

Keywords: Antagonistic fungus, control, egg-parasitic fungus, humic acid, root-knot nematode.

1. INTRODUCTION

Meloidogyne spp. is considered as a large agriculture problem, the worldwide distribution with wide host range caused much potential damage for crops (Sasser et al., 1983; Sikora and Fernandez, 2005; Karssen and Moen, 2006). Frequently, nematode management focused on chemical nematicides, but always need to repeat the application process for success results (Seenivasan, 2017). Therefore, scientists are searching for new strategies to replace or decrease using nematicides. Soil organisms and organic acids are considering clean alternatives to control nematodes (Sharon et al., 2001; Browning et al., 2006).

Several control attempts are using soil organisms (Dindal, 1990; Stirling, 1991; Coleman and Crossley, 1996). *Trichoderma* spp. are the greatest soil organisms for managing plant-parasitic nematodes (Atkins et al., 2005; Kiewniak and Sikora, 2006; Sharon et al., 2011; Javeed et al., 2016; Al-Hazmi et al., 2017; Abdelrafaa et al., 2018). Producing of nematicidal compounds is the key for root-knot nematode management (Sharon et al., 2011). Species of *Trichoderma* showed a diverse level of achievement in management against *Meloidogyne* spp. (Meyer et al., 2001; Sharon et al., 2001, 2007; Spiegel et al., 2007; Suarez et al., 2004). The egg-parasitic fungus *Paecilomyces lilacinus* is another soil-inhabiting fungus that attacks root-knot nematodes and colonizes plant roots (Siddiqui et al., 2000).

Decomposition organic products are used for enhancing crops (Khattab et al., 2012; Khan et al., 2013). Many organic acids, such as humic acid, are reported for nematode management directly and indirectly by the fungal stimulator (Jothi et al., 2009; Jothi and Poornima, 2017).

The present work established for study the direct effect of humic acid on egg hatching and juvenile (J2) mortality of *Meloidogyne javanica*, as well as the indirect effect of humic acid on *Trichoderma harzianum* and *P. lilacinus* for *M. javanica* management.

2. MATERIALS AND METHODS

A pure culture of *M. javanica* eggs was established on susceptible tomato (cv. Sultana) (Hussey and Barker, 1973). Some of the extracted eggs were left on a bench at room temperature ($24\pm 2^{\circ}\text{C}$) to hatch to second-stage juveniles (J2). The nematode eggs and newly-hatched juveniles were used in the *in vitro* and the *in vivo* tests.

Commercial water-soluble humic acid (Shenyang Humate Technology, Corporation Limited, China) was used; it is a by-product with dark brown to black color of a liquid formulation. Increasing concentrations (0.25, 0.5, and 1.0%) of this solution were prepared in distilled water.

Fungi isolates were taken from Prof. Ahmed A. Dawabah - Nematode Diseases Research Department, Plant Pathology Research Institute, Egypt. *T. harzianum* was isolated from galled roots of guava trees in the Riyadh region (Elad and Chet, 1983). The *T. harzianum* isolate was molecularly identified using Internal Transcribed Spacer of ribosomal DNA (Maymon et al., 2004; Hermosa et al., 2000). While *P. lilacinus* (ATCC® MYA2107™) was obtained from the American Type Culture Collection, USA.

All tests were repeated with five replicates for each. Combined data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test at P values ≤ 0.05 (SAS, 2013).

2.1 Effect of different concentrations of humic acid on nematode reproduction

In vitro, two tests were elaborated to examine the fatal effects of increasing concentrations of humic acid (0.25, 0.5, and 1.0%) on the egg hatch and J2 mortality of *M. javanica*. Twenty milliliters of each concentration was poured into separate 35-mm Petri plates. Then, 100 eggs of

M. javanica suspended in 1 ml of sterile water were added to each plate in the egg hatch test while 100 of newly hatched juveniles were placed in the J2 mortality test. The control set-up had 20 ml of sterile water instead of the humic acid solution. Test control set-up had sterile water instead of the humic acid solution. In a completely randomized design (CRD), treatments were located on a bench at room temperature ($24 \pm 2^\circ\text{C}$).

At the egg hatched test (Table 1), two weeks after incubation, the newly hatched eggs were computed, whereas, at the J2 mortality test (Table 2), the immobile juveniles were counted after 4, 7, and 10 days of exposure.

Table 1- Effect of humic acid (ha) at increasing concentrations on egg hatch (mean \pm standard error) of *Meloidogyne javanica*.

Treatment	Hatched eggs*	% inhibition
Distilled Water	92.8 \pm 3.2 a	7.2
ha 0.25%	83.2 \pm 1.5 b	16.8
ha 0.5%	71.4 \pm 2.3 c	28.6
ha 1%	40.2 \pm 1.6 d	59.8

There aren't any significant differences at Duncan test ($P \leq 0.05$) between mean \pm standard error in each column followed by the same letter.

*Out of 100 eggs.

Table 2- Effect of humic acid (ha) at increasing concentrations on mortality of the second stage juveniles (mean \pm standard error) of *Meloidogyne javanica*.

Treatment	Days after exposure		
	4 days	7 days	10 days
Distilled Water	10 \pm 0.32 *b	27.4 \pm 1.74 b	48.8 \pm 0.54 d
ha 0.25%	17.4 \pm 0.57 a	45.7 \pm 3.54 a	63.4 \pm 1.98 c
ha 0.5%	18.2 \pm 0.59 a	46 \pm 1.93 a	78.1 \pm 2.96 b
ha 1%	19 \pm 0.64 a	51.1 \pm 1.86 a	89.5 \pm 0.47 a

Means \pm standard error in each column followed by the same letter are not significantly different at Duncan test ($P \leq 0.05$).

*No. of dead J2, out of 100 J2.

2.2 Effect of humic acid with *T. harzianum* or *P. lilacinus* on nematode reproduction

In these two *in vitro* tests, water agar (WA) plates were used. Humic acid (1%) was inserted to designated water agar plates before the agar solidified. Subsequently, a block of a one-week-old culture on potato dextrose agar (PDA) of either of *T. harzianum* or *P. lilacinus* was placed onto the center of each plate. Other agar plates with humic acid were left without any fungus inoculation. Plain water agar plates were had to be controls. At the egg hatched test, a concentrated 100 eggs of *M. javanica* was added up to all plates, and 100 freshly hatched J2 were put into each plate in the J2 mortality tests. Treatments were located on a bench at room temperature ($24 \pm 2^\circ\text{C}$) in a CRD.

Two weeks after incubation, the newly hatched eggs were counted in the egg hatched test (Table 3). And after 4, 7, and 10 days of exposure, the immobile juveniles were computed at the J2 mortality test (Table 4).

Table 3- Effects of humic acid (ha), *Trichoderma harzianum* (Th), and *Paecilomyces lilacinus* (Pl) on egg hatching (mean \pm standard error) of *Meloidogyne javanica*.

Treatment	Hatched eggs*	% inhibition
Control (water agar only)	87 \pm 5.4 a	13
Humic acid (ha)	41.2 \pm 1.6 b	59
<i>Trichoderma harzianum</i> (Th)	22.6 \pm 2.0 cd	77
<i>Paecilomyces lilacinus</i> (Pl)	27.4 \pm 1.3 c	73
Th + ha	19.8 \pm 1.5 cd	80
Pl + ha	15.4 \pm 1.7 d	85

There aren't any significant differences at Duncan test ($P \leq 0.05$) between means \pm standard error in each column followed by the same letter.

*Out of 100 eggs.

2.3 Effects of humic acid, *T. harzianum*, and *P. lilacinus*, either separately or in combination, on plant growth, root galling, and nematode reproduction

In this greenhouse test, humic acid, *T. harzianum*, and *P. lilacinus* (Table 5), were comparatively evaluated separately or in combination for their capability to control *M. javanica* growth on tomato.

Table 4- Effects of humic acid (ha), *Trichoderma harzianum* (Th), and *Paecilomyces lilacinus* (Pl) on juvenile (J2) mortality (mean \pm standard error) of *Meloidogyne javanica*.

Treatment	Days after exposure		
	4 days	7 days	10 days
Control (water agar only)	25.1 \pm 1.37 *c	38.2 \pm 0.79 d	46.8 \pm 0.94 d
Humic acid (ha)	14.1 \pm 1.17 d	49.4 \pm 0.94 c	69.4 \pm 2.0 c
<i>Trichoderma harzianum</i> (Th)	57.4 \pm 1.90 a	72.3 \pm 0.48 a	75.2 \pm 0.78 b
<i>Paecilomyces lilacinus</i> (Pl)	62.1 \pm 0.50 a	73.9 \pm 0.78 a	75 \pm 1.12 b
Th + ha	35.4 \pm 0.75 b	63.9 \pm 0.56 b	74.6 \pm 1.13 b
Pl + ha	36.9 \pm 1.06 b	64.5 \pm 1.06 b	78.1 \pm 1.18 a

Means \pm standard error in each column followed by the same letter are not significantly different at Duncan test ($P \leq 0.05$).

*No. of dead J2, out of 100 J2.

For fungi inocula, isolates of *T. harzianum* or *P. lilacinus* were cultured according to Jatala (1986). During soil inoculation, 2.0 g of humic acid, as well as, 10.5 g (0.7%) of the two fungi cultures, were mingled in thoroughly plastic bags with the autoclaved potting soil mixture (2 sand: 1 clay: 1 peat moss). Designated plastic pots of 15 cm diameter were filled by the infested soil samples and transplanted with four-week-old tomato seedlings (cv. Forester). A week later, 10 ml water suspension contain 5,000 eggs of *M. javanica* was poured into three holes made around the seedling base. Non-inoculated seedlings were also considered as controls. Pots were sorted in the greenhouse ($26 \pm 2^\circ\text{C}$) in a CRD. Irrigation and fertilization with Hogland's solution were added when needed (Hoagland and Arnon, 1950). 55 days after nematode inoculation, root galling and nematode reproduction were recorded (Oostenenbrink, 1966; Sasser et al., 1984).

3. RESULTS

3.1 Effect of different concentrations of humic acid on nematode reproduction

Concentration-dependent egg hatch inhibition was observed with the exposure of *M. javanica* eggs to humic acid (Table 1). While the percent of J2 mortality was increased along with the rise of either the concentrations of humic acid or the exposure periods (Table 2). Significantly, the highest percent of J2 mortality (89.5%) was recorded at 1% concentration of humic acid exposure after 10 days. On the other hand, no significant differences were shown between humic acid concentrations at an exposure period of 4 or 7 days.

3.2 Effect of humic acid with *T. harzianum* or *P. lilacinus* on nematode reproduction

Generally, all treatments in the egg hatch were significantly decreased (Table 3). Although the treatment of *P. lilacinus* with humic acid was had the lowest number of hatched eggs, there aren't significant differences with treatments of *T. harzianum* with humic acid or *T. harzianum* alone. Moreover, the effect of each factor of the *P. lilacinus* and humic acid independently was significantly less than the effect of their combination (Table 3).

Apparently, all treatments with periods had an influence on the mortality rates of *M. javanica* (Table 4). After 10 days of treated of *P. lilacinus* combined with humic acid, the J2 mortality was the highest point, but their effect was less than *P. lilacinus* alone at 4 and 7 days (Table 4).

3.3 Effects of humic acid, *T. harzianum*, and *P. lilacinus*, either separately or in combination, on root galling and nematode reproduction

T. harzianum, *P. lilacinus*, and humic acid suppressed the number of root galls, egg masses, and eggs of *M. javanica* (Table 5). However, plant growth parameters (total fresh weight) among the treatments were inconclusive.

Depend on the factor of nematode reproductive with the indexes of galls and egg masses, treatments were divided into three classes (Table 6): 1) the combinations *T. harzianum* and *P. lilacinus*, *T. harzianum* and humic acid, *P. lilacinus* and humic acid, and *P. lilacinus*, *T. harzianum*, and humic acid (GI=2.9–3.2, EMI=2.4–2.7, Rf = 0.09–0.11), 2) the individual treatments with *T. harzianum* and *P. lilacinus* (GI=3.3, EMI=3–3.1, Rf=0.21–0.27), and 3) the treatment with humic acid alone.

Table 5- Effects of humic acid (ha), *Trichoderma harzianum* (Th), and *Paecilomyces lilacinus* (Pl), separately or in combination, on root galling and *Meloidogyne javanica* reproduction (mean \pm standard error) on tomato, 55 days after inoculation (greenhouse 26 \pm 2°C).

Treatment	No. of galls per g of root	No. of egg masses per g of root	Reproduction factor (Rf)
<i>M. javanica</i> (N)	10.4 \pm 2.105 a	2.81 \pm 0.612 a	4.33 \pm 0.239 a
N + <i>Trichoderma harzianum</i> (Th)	0.46 \pm 0.002 bc	0.31 \pm 0.002 c	0.27 \pm 0.011 c
N + <i>Paecilomyces lilacinus</i> (Pl)	0.38 \pm 0.020 bc	0.28 \pm 0.011 c	0.21 \pm 0.043 c
N + humic acid (ha)	2.74 \pm 0.855 b	1.07 \pm 0.263 b	0.94 \pm 0.282 b
N + Th + Pl	0.42 \pm 0.053 bc	0.23 \pm 0.041 c	0.1 \pm 0.002 c
N + Th + ha	0.26 \pm 0.008 c	0.1 \pm 0.002 c	0.09 \pm 0.011 c
N + Pl + ha	0.48 \pm 0.020 bc	0.2 \pm 0.002c	0.11 \pm 0.002 c
N + Th+ Pl + ha	0.37 \pm 0.016 bc	0.19 \pm 0.031c	0.09 \pm 0.011 c

Values were come from means of twice tests of five replicates.

According to Duncan test ($P \leq 0.05$), there aren't any significant differences between means \pm standard error within the same column that had the same letter(s).

Reproduction factor (Rf) = final nematode population (Pf) / initial inoculum (Pi).

Table 6- Management categories of treated *Meloidogyne javanica* by humic acid (ha), *Trichoderma harzianum* (Th), and *Paecilomyces lilacinus* (Pl).

Treatment	Root galling index (0–5)	Egg masses index (0–5)	Reproduction factor (Rf)
High efficacious category			
Th+ Pl + ha	2.9	2.4	0.09
Th + ha	3.1	2.5	0.09
Th + Pl	3.1	2.7	0.1
Pl + ha	3.2	2.6	0.11
Moderate efficacious category			
Th	3.3	3	0.27
Pl	3.3	3.1	0.21
Low efficacious category			
ha	4.3	3.8	0.94

4. DISCUSSIONS

Depending on concentrations of humic acids, all tests demonstrated that high levels of humic acid were reduced egg hatching and increased J2 mortality of *M. javanica*. The humic acid is one of the organic acids group that detected to have lethal effects against nematode species such as *Aphelenchus avenae* (Elmiligy and Norton, 1973), *Aphelenchoides goodey* (Elmiligy and Norton, 1973), *Heterodera glycines* (Dias and Ferraz, 2001), *Helicotylenchus pseudorobustus* (Elmiligy and Norton, 1973), *Meloidogyne arenaria* (Mian and Rodriguez-Kabana, 1982), *Meloidogyne incognita* (McBride et al., 2000; Zaki et al., 2004; Saravanapriya and Subramanian, 2007; Jothi

et al., 2009; Saravanapriya and Subramanian, 2017), *Meloidogyne hapla* (Elmiligy and Norton, 1973), *Pratylenchus penetrans* (Min et al., 2007), and *Xiphinema americanum* (Elmiligy and Norton, 1973). Our results on testing the effects of humic acid on egg hatch and J2 mortality support previous reports of studies done on *M. incognita* (Jothi et al., 2009; Saravanapriya and Subramanian, 2007, 2017). The direct toxicity of humic acid toward nematodes came from organic amendments decomposition (Khan et al., 1974). Dias and Ferraz (2001) reported that 90% mortality in *H. glycines* was related to humic acid that released from poultry manure biodegradation. The active principles of humic acid could be observed as an effective nematode management (Chitwood, 2002).

Furthermore, our study showed the indirect function of humic acid toward controlling *M. javanica*, by enhancing the growth of *T. harzianum*, and *P. lilacinus*. Related studies on humic acid showed exist of enough amount of humic acid in the media will improve the development of soil microorganisms (Vissera, 1985; Gryndler et al., 2005; Pouneva, 2005; Burkowska and Donderski, 2007; Lodhi et al., 2013). Vissera (1985) found that 30 mg of humic acids is sufficient for increasing the population of soil microorganisms. More specifically reports (Gryndler et al., 2005; Pouneva, 2005; Burkowska and Donderski, 2007) were showed that humic acid may act as growth stimulator in the soil to fungi, algae, and bacteria. Recently, three fungi; *T. harzianum*, *T. hamatum*, and *Alternaria alternata* were stimulated by humic acid (Lodhi et al., 2013).

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Compliance with ethical standards

Conflict of interest all authors declare that they have no conflict of interest.

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