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Effects of *Tagetes patula* on Active and Inactive Stages of Root-Knot Nematodes

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Abstract: Although marigold (*Tagetes patula*) is known to produce allelopathic compounds toxic to plant-parasitic nematodes, suppression of *Meloidogyne incognita* can be inconsistent. Two greenhouse experiments were conducted to test whether marigold is more effective in suppressing *Meloidogyne* spp. when it is active rather than dormant. Soils infested with *Meloidogyne* spp. were collected and conditioned in the greenhouse either by 1) keeping the soil dry (DRY), 2) irrigating with water (IRR), or 3) drenching with cucumber (*Cucumis sativus*) leachate (CL) for 5 wk. These soils were then either planted with cucumber, marigold or remained bare for 10 wk. Suppression of nematode by marigold was then assayed using cucumber. DRY conditioning resulted in the highest number of inactive nematodes, whereas CL and IRR had higher numbers of active nematodes than DRY. At the end of the cucumber bioassay, marigold suppressed the numbers of *Meloidogyne* females in cucumber roots if the soil was conditioned in IRR or CL, but not in DRY. However, in separate laboratory assays, marigold root leachate slightly reduced *M. incognita* J2 activity but did not reduce egg hatch ($P > 0.05$). These finding suggest that marigold can only suppress *Meloidogyne* spp. when marigold is actively growing. This further suggests that marigold will more efficiently suppress *Meloidogyne* spp. if planted when these nematodes are in active stage.

Key words: cover crop, *Cucumis sativus*, dormant stage, marigold, *Meloidogyne*, nematode, survival.

Survival strategies are common in several species of root-knot nematodes, *Meloidogyne* spp. (Van Gundy, 1985). *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949 survives harsh environmental conditions by delaying egg hatch or carrying over eggs from one cropping season to the next (Van Gundy, 1985). Some *Meloidogyne* spp. delay egg development during unfavorable conditions such as cold weather or when host plants are absent (McSorley, 2003). For example, when the weather is cold or host plants are not present, *M. incognita* delays hatching a portion of the egg mass (Guiran, 1979; Starr and Jegger, 1985). Unfavorable events for *Meloidogyne* survival may include 1) host senesce (usually occurs toward crop harvest), 2) onset of low temperatures, and 3) dry seasons (Van Gundy, 1985). This survival strategy makes managing *Meloidogyne* a more challenging task.

For more than 60 yr, marigold (*Tagetes* spp.) has been known to suppress plant-parasitic nematodes especially *Meloidogyne* spp. (Suatmadji, 1969) and *Pratylenchus* spp. (Oostenbrink, 1960) (Hooks et al., 2010). Tyler (1938) reported that 29 marigold varieties were resistant against root-knot nematodes. However, French marigold (*Tagetes patula* L.) is more effective than many other species and varieties of *Tagetes* in suppressing *M. incognita* (Ploeg, 2002). Alpha-terthienyl is believed to be the main allelopathic compound in marigold responsible for nematode suppression (Gommers and Bakker, 1988). Rice (1984) defined allelopathy as a plant-plant or plant-microorganism biochemical interaction. The nematode suppressive effect of marigold is most prominent in actively growing marigold roots (Jagdale et al., 1999). However, in some instances, French marigold failed to suppress plant-parasitic nematodes (Dao, 1972; Marahatta et al., 2010). French marigold did not suppress *M. incognita* when marigold was planted after a fallow (Marahatta et al., 2010). In contrast, when French marigold was planted immediately after the termination of a *Meloidogyne* susceptible host, bitter melon (*Momordica charantia* L.), marigold suppressed approximately 50% of *M. incognita* compared to the bare ground treatment (Marahatta et al., 2010). One hypothesis is that marigold suppresses *Meloidogyne* more effectively if it is grown when nematodes are present in active stages (mobile J2) vs inactive stages (such as eggs and anhydrobiotes).

To examine this hypothesis, a study was conducted to determine if 1) leachate from a *Meloidogyne* susceptible host or irrigation could keep *Meloidogyne* spp. in an active stage in the absence of host, 2) marigold could suppress *Meloidogyne* spp. more efficiently when nematodes are active, and 3) marigold could suppress vermiform stage of *Meloidogyne* spp. more effectively than the egg stage.

Materials and Methods

Greenhouse Experiment: Two greenhouse experiments were conducted at the University of Hawaii at Manoa greenhouse facilities between 2009 and 2010. In Trial I, soils were collected from a *M. incognita* infested tomato (*Solanum lycopersicon* L.) field on the island of Oahu in Kunia, HI on 27 May 2009. Nematodes were then extracted by elutriation and centrifugal flotation method (Jenkins, 1964; Byrd et al., 1976). The average nematode population size was 5,890±1,070/250 cm^3 soil). To verify the species of root-knot nematodes present in the field, approximately 10 fully mature *Meloidogyne* females were collected from the tomato roots and identified by

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esterase phenotype (Esbenshade and Triantaphyllou, 1985). Approximately 750 cm^3 of well mixed soil was placed in 45, 10.16-cm diam clay pots. Pots were divided into three groups and subjected to 3 conditionings: 1) dry soil (DRY), 2) irrigated at 100 ml water/pot/day (IRR), and 3) drenched with cucumber leachate at 100 ml/pot/day (CL). Cucumber leachate was collected from 1-mo old 'Sweet Slice' cucumber (*Cucumis sativus* L.) plants grown in kiln-dried beach sand (#2/16, Koll Center Parkway, CA) with the sand:soil mix at 1:1 ratio (v/v) by placing the cucumber planted pot on top of a 11-cm diam. transparent plastic cup and drenching with 100 ml water each day until experiment termination. Cucumber leachate was collected from 20 pots and composited.

One month after conditioning initiation, soils were either planted with 1) one 'Sweet Slice' cucumber plant, 2) five 'Single Gold' French marigold plants, or 3) remained bare for 10 wk. All pots were irrigated with 100 ml water per pot per day. At completion of 10 wk, all plants were cut at the soil line and removed. Afterwards, one 2-wk old cucumber seedling was transplanted into each pot as a bioassay plant and maintained for 3 wk. This was a 3 × 3 (conditioning × treatment) factorial design experiment arranged in randomized complete blocks with 5 replications. A similar experimental design was used in Trial II except that soil was collected from a cucumber field infested with mixed populations of *M. incognita* and *M. javanica* at the University of Hawaii, Poamoho Research Station, on the island of Oahu, in Wailua HI on 12 September 2009 (nematode population density was 480/250 cm^3 soil). Soil nematodes were extracted from 50 cm^3 soil by elutriation and centrifugal flotation as described earlier at termination of each conditioning. Soil conditioning period for all the treatments was one month as described before. Extracted *Meloidogyne* were counted using an inverted microscope (Fluovert, Leitz Wetzlar, Germany). *Meloidogyne* were categorized into active and non-active stages by probing. Nematodes that moved in response to probing were categorized as active, and those did not move were considered non-active. At termination of the cucumber bioassay, 0.3 g of air dried roots were subsampled from each cucumber bioassay plant and stained with acid fuchsin (Daykin and Hussey, 1985). Stained *Meloidogyne* were categorized as infective juveniles, sausage, and mature females (Eisenback and Triantaphyllou, 1991; Perry et al., 2009).

Laboratory assay: Two laboratory trials were conducted to examine the allelopathic effect of marigold root leachate on eggs or J2s of *M. incognita*. Seedlings of 'Sweet Slice' cucumber and 'Single Gold' French marigold were grown in 11-cm diam. plastic pots filled with 1:1 (v/v) mixture of steam sterilized soil and kiln dried beach sand. Root leachate from French marigold (MG) and cucumber (CU) were compared to leachate collected from sand:soil mix (SA), and from distilled water (DW). Each leachate was collected from 5 replicated pots and composited. In Trial I, CU and MG were 3-wk old, whereas in Trial II, CU and MG were 3- and 9-wk old, respectively. Leachate was collected by placing each pot on top of an 11-cm diam. transparent plastic cup and drenched with 50 ml of water. Collected leachate was then filtered through a 0.2 μm micro-syringe (MCE, Fisher Scientific, Ireland) prior to *M. incognita* egg hatch or J2 activity assays. However, the egg hatch assay in Trial I was initiated through non-filtered leachate, but was replaced with filtered leachate after 1 wk. Only filtered leachate was used in Trial II.

For the egg hatch assay, *M. incognita* eggs were extracted from greenhouse on 'Pixie' tomato culture using NaOCl and centrifugal flotation methods (Hussey and Barker, 1973). Freshly extracted *M. incognita* eggs suspended in 1-ml water were imbibed in a 3-cm diam. glass dish containing the designated leachate or DW. The number of nematode eggs/ml suspension in Trial I and II were 61±6 and 110±9, respectively. Four replicated transparent glass dishes (3 cm ht; 6 cm diam.) containing 10-ml filtered leachates of MG, CU, SA or DW were prepared for the assay. A total of 16 glass dishes, were prepared for each trial. Each glass dish contained a tube (~2.5-cm ht; 1-cm diam.) with nylon fiber mesh (60.33 μm in size). The egg suspension was placed into the mesh. Hatched J2 would swim through screen into the glass dish. Leachate was replaced with fresh leachate weekly during the duration of the experiment, and hatching was continued to be observed for 1 wk.

For the J2 activity assay, 20 ml leachate of MG, CU, or SA were filtered as described above, and kept in 100-ml sized beakers (Pyrex, USA, no. 1000). DW was used as the control. Four replicated beakers were prepared for each treatment. Newly hatched *M. incognita* J2 were added in 1 ml suspension to each beaker. The J2 activity assay was repeated in 3 trials. Number of freshly hatched J2 used in Trial I, II, and III were 652±26, 184±14 and 95±3, respectively. Beakers were covered by aluminum foil and kept at room temperature. Juvenile activity was determined by counting mobile and immobile nematodes at 48 hr after placement of J2 into the leachate as described by Meyer et al. (2006) and Zasada et al. (2006). To make sure the suppression was not due to nematostatic compounds (nematostatic compounds only immobilize nematodes temporarily and will allow the nematode to resume movement after it is replaced by water), leachate in each beaker was replaced by distilled water after the nematodes were counted. In Trial I and II, viability of J2s was counted again at 72 hr (24 hr after changing into distilled water). In Trial III, the viability of *M. incognita* J2 was assessed by dental pick probing at 48 hr instead of 72 hr after exposure to leachates. Nematodes that did not respond to probing were considered dead. The age of cucumber used to collect leachate for the J2 activity assay was 2-, 3-, and 4-wk old at the initiation of Trial I, II, and III, respectively. Marigold plants used to collect leachate were

7-, 8-, and 9-wk old at the initiation of Trial I, II, and III, respectively.

Statistical analysis: For the greenhouse experiment, data were subjected to one-way analysis of variance (ANOVA) for soil nematodes counted at termination of conditioning, and two-way ANOVA for root nematodes counted at termination of cucumber bioassay. Data were analyzed using the general linear model (GLM) procedure in Statistical Analysis System (SAS Institute, Cary, NC). For the egg hatching and J2 activity assays, data were subjected to one-way ANOVA. Numbers of nematodes were log-transformed [log $(x+1)$] prior to ANOVA to normalize the data. Only untransformed arithmetic means were presented. Means for each trial in each assay were separated by Waller-Duncan k-ratio (k=100) t-test wherever appropriate.

Results

Greenhouse experiment: In Trial I of the greenhouse experiment, DRY conditioning resulted in higher number of non-active *Meloidogyne* as compared to IRR and CL at termination of soil conditioning ($P < 0.05$, Table 1). However, more active *Meloidogyne* were detected in IRR and CL as compared to DRY conditioning ($P < 0.05$). Similarly in Trial II, DRY conditioning had a higher number of non-active *Meloidogyne* than in IRR and CL, whereas CL had a higher number of active *Meloidogyne* ($P < 0.05$) compared to DRY and IRR (Table 1).

Based on results at termination of the cucumber bioassay, numbers of infective, sausage and mature female *Meloidogyne* in bioassay cucumber roots were affected by crop treatments but not by conditioning in both Trials I and II ($P < 0.01$, Table 2). Significant interaction ($P < 0.05$) between conditioning and crop treatment was only detected for mature female numbers. Planting of marigold suppressed all stages of *Meloidogyne* stained in the bioassay cucumber roots as compared to planting of cucumber in both trials (Table 3). Fallow treatment only suppressed mature females in Trial I and sausage and mature females in Trial II (Table 3). However, Fallow treatment did not suppress sausage and mature females as effectively as marigold in Trial II.

Table 1. Number of non-active and active *Meloidogyne* in soil at termination of soil conditioning.

Conditionings	Trial I		Trial II	
	Non-active	Active	Non-active	Active
	---------- *Meloidogyne* number/250 cm^3 soil---------			
DRY[a]	4900[b] a	40 b	485 a	105 b
IRR	35 b	390 a	0 b	120 b
CL	0 b	460 a	0 b	245 a

[a] Pre-plant soil were conditioned either by keeping the soil dry (DRY), irrigating with water (IRR), or drenching with cucumber leachate (CL) for one mo.
[b] Means are average of five replications. Means in columns under each trial followed by same letter do not differ according to the Waller-Duncan k-ratio (k= 100) t-test based on log(x+1) transformed values.

Table 2. Analysis of variance (ANOVA) for effects of soil conditioning and nematode management treatment on infective, sausage, and mature female stage *Meloidogyne* numbers per g cucumber roots in Trial I and Trial II.

Factors	Trial I			Trial II		
	Infective juvenile	Sausage female	Mature female	Infective juvenile	Sausage female	Mature female
Conditioning[a]	NS	NS	NS	NS	NS	NS
Treatment[b]	**	**	**	**	**	**
Conditioning × Treatment	NS	NS	*	NS	NS	*

[a] Soil conditioned either by keeping the soil dry, irrigating with water, or drenching with cucumber leachate.
[b] Conditioned soils were either planted with cucumber, marigold or remained bare for 10 wk.
NS = non-significantly differ; *, ** indicate significantly different at $P < 0.05$ and $P < 0.01$, respectively based on a 3 × 3 (conditioning × treatment) factorial analysis of variance.

Since interaction between conditioning and plant treatment was significant (Table 2) for numbers of mature females, plant treatments were compared under each conditioning (Table 4). Planting of marigold only suppressed ($P < 0.05$) mature females under IRR and CL conditioning but not with DRY conditioning in either trial (Table 4). Although fallow treatment suppressed ($P < 0.05$) mature females compared to planting cucumber in all conditionings, lower number of mature females was detected in planting marigold than fallow in CL in Trial I. However, number of mature females was similar between marigold planting and fallow in Trial II ($P > 0.05$, Table 4).

Laboratory root leachate assay: Percentage of *M. incognita* that hatched did not differ among DW, SA, MG, and CU treatments in Trial I ($P > 0.05$) but were lower in all leachates (MG, CU, SA) than DW in Trial II (Table 5). In the J2 activity assay, although a lower percentage of active J2 was found in MG root leachate as compared to CU ($P < 0.05$, Table 6), MG leachate did not reduce the percentage of active J2 as compared to DW during all three trials at 48 hrs after incubation. MG leachate only reduced the percentage of active J2 as compared to DW and SA at 24 hrs after replacement with distilled water in Trial I.

Table 3. Numbers of infective juvenile, sausage female, and mature female stage *Meloidogyne* per g cucumber roots in Trial I and Trial II.

Factors	Trial I			Trial II		
	Infective juvenile	Sausage female	Mature female	Infective juvenile	Sausage female	Mature female
	----------Nematode number/g cucumber root----------					
Cucumber	164[a] a	131 a	42 a	32 a	21 a	44 a
Fallow	113 a	87 a	4 b	28 a	10 b	9 b
Marigold	58 b	44 b	2 b	15 b	3 c	5 c

[a] Means are average of 15 replications. Means in a column followed by the same letter do not differ according to the Waller-Duncan k-ratio (k= 100) t-test based on log(x+1) transformed values.

TABLE 4. Number of *Meloidogyne* mature females on bioassay cucumber roots grown after cucumber, fallow and marigold treatments under three conditionings.

Treatments	Trial I			Trial II		
	DRY[a]	IRR	CL	DRY	IRR	CL
	---------Nematode number/g cucumber root---------					
Cucumber	19[b] a	62 a	46 a	12 a	58 a	63 a
Fallow	1 b	4 b	8 b	8 a	6 b	12 b
Marigold	4 ab	4 b	0 c	6 a	4 b	4 b

[a] Soil were conditioned either by keeping the soil dry (DRY), irrigating with water (IRR), or drenching with cucumber leachate (CL).
[b] Means are average of 15 replications. Means in a column followed by the same letter do not differ according to the Waller-Duncan *k*-ratio (k= 100) *t*-test based on log(x+1) transformed values.

DISCUSSION

Current research results supported the theory that more *Meloidogyne* spp. will remain active under favorable soil conditions such as providing irrigation or host leachate. This is consistent with the supposition of Van Gundy (1985) that optimum soil moisture level under irrigated conditions may favor a more active *Meloidogyne* population. Most soil nematodes subjected to DRY conditioning were not-active at termination of the conditioning period. This result is consistent with the findings of Towson (1977) who found an increase in proportion of coiled J2 of *M. javanica* with a decrease in soil moisture content from 39.4 to 21.1%. Furthermore, current experimental results are also consistent with findings of Sehgal and Gaur (1995) with regard to suppression of active *M. incognita* juveniles by ethylene dibromide (EDB) or formaldehyde in moist but not in dry soil. Sehgal and Gaur (1995) suggested that non-active nematodes in dry soil were more resistant to EDB and formaldehyde than active nematodes in moist soil. In the current study, it was unclear whether non-active nematodes were alive or dead. These non-active *Meloidogyne* in the dry conditioning samples did not decompose even at 1 mo after incubation and had clear distinct body parts. Schroeder and MacGuidwin (2007) determined that stressed dauer larvae of *Caenorhabditis elegans* were actually live nematodes by using fluorescein isothiocyanate. Therefore, it is conceivable that these non-active *Meloidogyne* in the dry conditioning samples were subjected to stress and could have converted into a survival stage. Current results clearly support the theory that planting marigold or leaving the soil fallow suppresses *Meloidogyne* more efficiently compared to planting nematode hosts. Suppression of nematodes by fallow (Towson, 1977; Caswell et al., 1991) or by planting of marigold (Khan et al., 1977; Rangaswamy et al., 1993; Cannayane and Rajendran, 2002; Hooks et al., 2010) has been well documented. The novelty of the current research is that marigold could suppress *Meloidogyne* more efficiently than fallow only if the nematodes were active such as those in irrigated soil but not if the nematodes were inactive such as those dominated in dry soil. Additionally, both fallow and marigold could suppress *Meloidogyne* in moist rather than dry soil.

TABLE 5. Effects of marigold and cucumber roots, sand : soil leachates, and distilled water on *Meloidogyne incognita* egg hatching in Trial I and Trial II of laboratory leachate assay.

Treatments	Trial I	Trial II
	-----% *Meloidogyne incognita* egg hatched[a]-----	
Distilled water (DW)	20.43[b] a	79.56 a
Sand : soil (SA)	32.22 a	60.59 b
Marigold (MG)	21.61 a	45.31 b
Cucumber (CU)	30.42 a	56.48 b

[a] Number of *Meloidogyne incognita* eggs used in Trial I and Trial II were 61±6 and 110±9, respectively.
[b] Means are average of four replications. Means in a column followed by the same letter do not differ according to the Waller-Duncan *k*-ratio (k= 100) *t*-test.

TABLE 6. Effects of marigold (MG) and cucumber (CU) roots, and sand : soil (SA) leachates, and distilled water (DW) on % of *Meloidogyne incognita* remained active at 48 hr of incubation and 24 hr after transferring to distilled water in Trial I, II and III of the laboratory leachate assay.

Treatments	Trial I		Trial II		Trial III
	48 hr	72 hr	48 hr	72 hr	48 hr
	----------% *Meloidogyne incognita* activity[a]----------				
DW	93.89[b] b	87.85 b	87.75 bc	87.78 ab	88.83 ab
Sand	94.05 b	91.14 ab	90.00 ab	90.10 a	93.29 ab
MG	91.42 b	79.66 c	86.26 c	83.63 b	86.64 b
CU	97.33 a	95.36 a	91.43 a	91.81 a	95.29 a

[a] Initial numbers of *Meloidogyne incognita* introduced to each experimental units were 652±26, 184±14, and 95±3 in Trial I, II, and III, respectively.
[b] Means are average of 4 replications. Means in a column followed by the same letter(s) are not different according to Waller-Duncan *k*-ratio (k= 100) *t*-test.

The differential *Meloidogyne* suppressive effect following different conditioning might explain why marigold did not suppress *M. incognita* after a non-irrigated fallow period in one of the field trials described by Marahatta et al. (2010). In that experiment, marigold suppressed *M. incognita* when planted immediately after the completion of a *Meloidogyne* susceptible host crop. Furthermore, Gommers and Bakker (1988) have suggested that the nematicidal properties of marigolds result from a cascade of chemical reactions triggered by the penetration and movement of the nematodes through the cortex. This means that an active plant and active nematode are necessary to achieve good nematode suppression.

The current research documented the lack of a nematode suppressive effect of MG leachate on *M. incognita* egg hatching. Sesaneli and Vito (1991) also found that root leachate of *Tagetes erecta* and *T. signata* had no effect on the hatching of golden nematode, *Globodera rostochiensis*. Additionally, Pudasaini et al. (2008) did not find evidence of hatching inhibition by root leachate of 'Single Gold' *T. patula* on the root-lesion nematode, *Pratylenchus penetrans*. Jagdale et al. (1999) demonstrated that α-terthienyl is only suppressive to nematodes when the plant is actively growing.

The limited nematode suppressive effect from the MG root leachate in the current experiment was not

consistent with the findings of Franzener et al. (2007), who found an inhibition on *M. incognita* mobility and up to 68% *M. incognita* J2 mortality from an aqueous extract of *T. patula* under *in vitro* conditions. The concentrations of α-terthienyl in marigold root leachate used here could possibly be lower compared to marigold root extract in the previous study. The nematicidal effect of marigold root extract reported by Franzener et al. (2007) could also be due to a combination of several potential allelopathic compounds including biologically active essential oils and not solely α-terthienyl.

In conclusion, this research demonstrated that cucumber root leachate and irrigation can cause *Meloidogyne* to remain in an active stage and that marigold or fallow could suppress *Meloidogyne* spp. more efficiently if *Meloidogyne* remains active. However, the current research demonstrated that marigold root leachate did not suppress *Meloidogyne* spp. egg hatch and had only limited effect on vermiform motility. Future research should be conducted to test the effect of living marigold on nematode stages. Findings from this study, suggest that farmers wanting to incorporate marigold into a nematode management plan should plant it immediately after termination of the nematode-susceptible host crop or before *Meloidogyne* enters into a survival stage.

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