Interaction of *Tagetes patula cv*. Single Gold with the life cycle of the plant-parasitic nematodes *Meloidogyne chitwoodi* and *Pratylenchus penetrans*

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Summary. The influence of marigold, *Tagetes patula* cv. Single Gold, and host plants on hatching, migration, penetration and multiplication of *Meloidogyne chitwoodi* and *Pratylenchus penetrans* was determined. Root diffusate of marigold and tomato, soil leachate and distilled water did not influence the hatching pattern of *M. chitwoodi*. The hatching pattern of *P. penetrans* did not differ between 4- and 8-week-old marigold and maize root diffusate. However, the number of second-stage juveniles (J2) that hatched in diffusate of 12-week-old marigolds, soil leachate or distilled water was significantly lower than in diffusate of 4-week-old marigolds and maize. The migration rate of J2 of *M. chitwoodi* was not influenced by any of the root diffusates. However, the age of the marigold from which diffusate was collected affected the migration rate of *P. penetrans*. J2 of *M. chitwoodi* penetrated marigold roots at the same rate as they penetrated tomato. *Pratylenchus penetrans* penetrated marigold roots in higher numbers than maize. In a 10-week pot experiment with marigold, the multiplication coefficients of *M. chitwoodi* and *P. penetrans* were 0.05 and 0.00, respectively.

Key words: hatching, migration, multiplication, penetration, root diffusate.

Marigolds (*Tagetes* spp.) are known for their suppressive effect on plant-parasitic nematodes, particularly on root-knot (*Meloidogyne* spp.) and root lesion (*Pratylenchus* spp.) nematodes (Tyler 1938; Oostenbrink *et al.*, 1957). Growing marigolds for 65 to 105 days in fields heavily infested with root-knot (Hackney & Dickerson, 1975; Motsinger *et al.*, 1977; Ploeg, 2002) or root lesion nematodes (Kimpinski *et al.*, 2000; Machado & Inomoto, 2001; Pudasaini *et al.*, 2006) resulted in a significant decrease of the nematode populations and a yield increase of the subsequent crop of up to 98%. The effect of *Tagetes* on populations of *P. penetrans* lasted for two (Reynolds *et al*., 2000) or more (Evenhuis *et al.*, 2004) years. Marigolds even generated a better control than soil fumigation with Vorlex Plus CP (68% dichloropropene and related chlorinated hydrocarbons, 17% methyisothiocyanate and 15% chloropicrin) and metam sodium. Also soil amendments with roots or tops, cold aqueous extracts (Ploeg, 2000; Natarajan *et al.*, 2006) or seed exudates (Riga *et al.*, 2005) of *Tagetes* were reported to be toxic to plant-parasitic nematodes.

Tagetes spp. kill or inhibit the development of plant-parasitic nematodes by producing allelopatic compounds. Gommers & Bakker (1988) hypothesised that alpha-terthienyl was a major component of the allelopathic compounds in marigold responsible for nematode suppression. The component is triggered by nematode penetration in plant tissue (Bakker *et al.*, 1979). Next to this allelopathic mechanism, marigolds may reduce plant-parasitic nematodes by acting as a non-host or a poor host, creating an environment that favours nematode antagonistic flora or fauna, or behaving as a trap crop (Hooks *et al*., 2010).

Most research on the effects marigolds on plant-parasitic nematodes has focused on the outcome of the relationship between the host and the nematode, *viz*. the nematode population density observed at the end of the crop. Little is known about the nematode suppressive mechanisms of *Tagetes* spp. in conjunction with the biology of the target nematode. Different phases in the development of the nematode, *i.e*., hatching of second-stage juveniles (J2), migration to, penetration into, and

multiplication in the plant, might be affected by marigolds and eventually influence the final nematode population density.

The objective of the study reported here was to examine the effect of *Tagetes patula* cv. Single Gold on the hatching, migration, penetration and multiplication of the sedentary endoparasitic nematode, *M. chitwoodi*, and the migratory endoparasitic nematode, *P. penetrans*.

MATERIALS AND METHODS

Nematodes. All experiments with *M. chitwoodi* were done with a population originating from The Netherlands. In the hatching, penetration and multiplication experiments with *P. penetrans* a population from maize (Kerkom, Belgium) was used. The migration experiment was conducted with a population of HZPC research (Joure, The Netherlands). The population of *M. chitwoodi* was maintained and increased on tomato (*Solanum lycopersicum*, cv. Moneymaker) grown in pots filled with heat-sterilised sandy soil (sand 87%, loam 9%, clay 4%) in a temperature controlled glasshouse (20 ± 5°C) with a daily 14 h light period. *P. penetrans* was multiplied on carrot discs (Speijer & De Waele, 1997).

Collecting eggs and vermiform nematodes. To obtain eggs of *M. chitwoodi*, infected tomato roots were first washed with water. Egg masses were then removed manually from the roots and stirred in 1% NaOCl in a Waring blender at high speed for 40 s (McClure *et al*., 1973). Immediately after stirring, the egg suspension was poured on a 20-µm sieve and washed thoroughly with water to avoid effects of the chemical on the eggs. J2 of *M. chitwoodi* were obtained by extraction from chopped tomato roots on a Baermann funnel (Southey, 1986) over 7 days. J2 were stored at 4°C before use. Eggs of *P. penetrans* were separated from the carrot discs by zonal centrifugation (Hendrickx, 1995). Vermiform *P. penetrans* were separated from eggs by sieving the nematode suspension five times through a 50-µm sieve.

Collecting root diffusate. Root diffusate was obtained from plants grown in pots filled with heat-sterilised sandy soil (sand 87%, loam 9%, clay 4%) and maintained in a glasshouse (20 ± 5°C). Diffusates were collected from 4-(T1), 8-(T2), and 12-week (T3) old plants of *T. patula* cv. Single Gold, 2-week old plants of tomato (T) cv. Moneymaker, and maize plants (M) cv. Ronaldino, of the same age. For tomatoes and maize, one plant was grown in a 200 ml pot; *T. patula* was grown in groups of five plants in 1 l pots.

Pots were watered at 2-day intervals maintaining soil moisture at 15% by weight. Watering was stopped 4 days before collecting the diffusate. This was obtained by watering the pots with 300 ml (marigold) or 70 ml (tomato and maize) tap water. The root diffusate was pooled, filtered (Ederol Rundfilter, 40 g m^{-2}; Munktell Filter AB, Falun, Sweden), diluted four times and stored at 2-4°C. Soil leachate (SL) was collected from pots filled with soil (see above) and kept in the same conditions for 12 weeks. For *T. patula* and soil leachate, ten pots per treatment were used. For tomato and maize, each treatment consisted of three pots.

Hatching test. Hatching of both nematode species was monitored in glass vials (28 mm diam.) in which 20-µm micro-sieves (8 mm diam.) were fitted. At the start of the assay, the vials were filled with 4 ml of the test solution, and a 1 ml suspension of 1000 eggs was transferred onto the micro-sieves. The vials were covered with a perforated plastic lid and incubated in the dark at 20 ± 1°C. At weekly intervals, the micro-sieves with eggs were transferred to a new set of vials filled with fresh test solutions. The hatched J2 in the first vial were counted using a dissection microscope. For *M. chitwoodi*, the effect of T1, T2, T3, SL, M and distilled water (DW) were tested. For *P. penetrans*, the influence of treatments T1, T2, T3, SL, T and W was examined. All treatments were repeated five times.

Migration test. A vertical migration assay was set up in 12-cm tubes composed of six PVC rings (8 mm diam., 2 cm long) taped together. Part of the tubes (10 cm from top) was filled with heat-sterilised sandy soil (see above) adjusted to 15% moisture by weight. Section 10-11 cm of the tube was filled with dry soil, which received a 150 ml suspension of 1000 nematodes (J2 of *M. chitwoodi* or vermiform stages of *P. penetrans*). After inoculation of the nematodes, section 11-12 cm was filled with soil at 15% moisture. The bottom of the tube was closed to avoid evaporation. The top of the tube was extended with an additional 2 cm segment to provide space where 500 µl of the test solutions could be added. Finally the top of the tube was covered with perforated tape. The tubes were incubated at 16°C for 2 weeks in a vertical position before they were disassembled for observation. Nematodes were extracted from the individual rings by centrifugal flotation (Coolen & D'Herde, 1972). The treatments were repeated five times.

Table 1. Coefficients of the logistic curve y = c/(1+exp(-bx(time – m))) describing hatching of *Meloidogyne chitwoodi* second-stage juveniles in the presence of root diffusate of four-(T1), eight-(T2) and 12-(T3) week old marigold plants, tomato root diffusate (T), soil leachate (SL), distilled water (DW) and corresponding R^2-values. Mean ± the standard deviation of the time at which 50% hatch is reached (m), the hatching rate at the time at which 50% hatch is reached (b) and the final hatching percentage (c). Per column, significant differences between treatments are marked with a different letter (Duncan's multiple range test, $P < 0.05$).

Treatments	Coefficients of logistic curve			R^2
	m	b	c	
T1	2.05±0.46 a	1.6±0.29 a	53.6±13.5 a	0.97
T2	1.68±0.39 ab	1.4±0.16 a	55.3±8.9 a	0.97
T3	1.51±0.09 b	1.7±1.25 a	58.5±11.2 a	0.97
T	1.76±0.43 ab	1.6±0.34 a	51.4±14.3 a	0.98
SL	1.92±0.14 a	1.4±0.16 a	57.9±30.6 a	0.98
DW	1.91±0.33 a	1.5±0.36 a	58.5±19.3 a	0.98

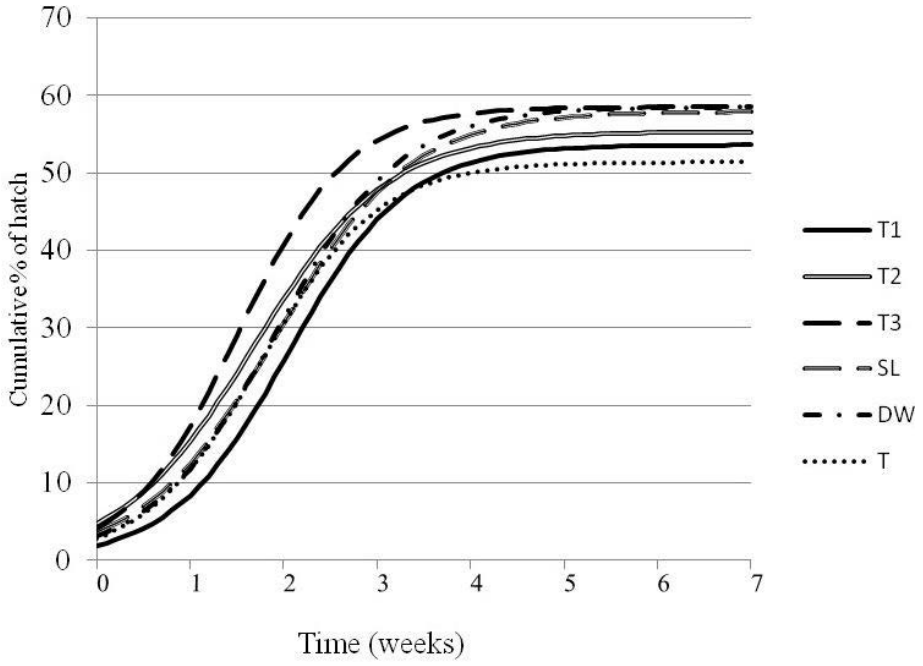

Fig. 1. The dynamics of hatching of *Meloidogyne chitwoodi* second-stage juveniles in root diffusate of four- (T1), eight- (T2) and 12- (T3) week old marigold plants, soil leachate (SL), distilled water (DW), and tomato root diffusate (T).

Penetration and multiplication tests. Root penetration and multiplication abilities of the nematodes were assayed in 200-ml plastic plots containing either 10-day old seedlings of tomato or marigold (*M. chitwoodi*), or maize or marigold (*P. penetrans*). Each pot was inoculated with 200 J2 of *M. chitwoodi* or 200 vermiforms of *P. penetrans*. The penetration was terminated after 2 weeks. Plants were uprooted and roots were washed free of soil before the nematodes present inside the roots were stained with acid-fuchsin (Byrd *et al.*, 1983). Observation and counting were done under a dissecting microscope. The multiplication test was terminated after 10 weeks. *M. chitwoodi* egg masses were then stained with McCormick Schilling red food colouring stain (Thies *et al.*, 2002), whereas individuals of *P. penetrans* inside the roots were stained with acid-fuchsin. For both species, the entire quantity of soil was processed by zonal centrifuge to collect the soil inhabiting nematodes. Both penetration and multiplication assays were replicated five times.

Statistical analyses. The hatching data obtained for both *M. chitwoodi* and *P. penetrans* were fitted to the logistic model y=c/(1 + exp(-bx (time – m))), where y is the cumulative theoretical hatch (%). The model is described by three parameters: the time at which 50% hatch is reached (m), the hatching rate at

Table 2. Coefficients of the logistic curve y = c/(1+exp(-bx(time – m))) describing hatching of *Pratylenchus penetrans* second-stage juveniles in presence of root diffusate of four- (T1), eight- (T2) and 12- (T3) week old marigold plants, maize root diffusate (M), soil leachate (SL), distilled water (DW) and corresponding R^2-values. Mean ± the standard deviation of the time at which 50% hatch is reached (m), the hatching rate at the time at which 50% hatch is reached (b) and the final hatching percentage (c). Per column, significant differences between treatments are marked with a different letter (Duncan's multiple range test, $P < 0.05$).

Treatments	Coefficients of logistic curve			R^2
	m	b	c	
T1	1.01±0.30 a	3.2±1.19 a	47.4±6.9 a	0.96
T2	0.83±0.06 a	4.28±0.13 a	38.8±8.5 ab	0.96
T3	1.04±0.28 a	3.58±1.16 a	34.8±8.5 bc	0.92
M	0.88±0.13 a	4.2±0.19 a	47.5±6.7 a	0.85
SL	0.99±0.07 a	3.74±0.55 a	27.7±6.5 c	0.86
DW	0.83±0.12 a	4.3±0.19 a	26.0±9.2 c	0.89

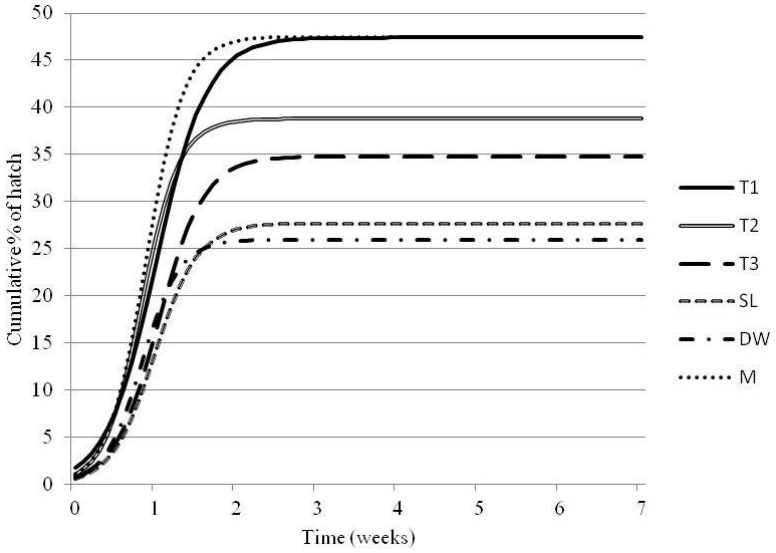

Fig. 2. The dynamics of hatching of *Pratylenchus penetrans* second-stage juveniles in root diffusate of four- (T1), eight- (T2) and 12- (T3) week old marigold plants, soil leachate (SL), distilled water (DW) and maize root diffusate (M).

the time at which 50% hatch is reached (b) and the final hatching percentage (c). Statistical analyses were performed with Statistica. All data were subject to analysis of variance (ANOVA). Whenever necessary, the data were transformed to log10 values to satisfy ANOVA assumptions. Differences are reported as significant or not significant according to Duncan's multiple range test ($P < 0.05$).

RESULTS

Hatching. The hatching rate of *M. chitwoodi* (b) varied between 1.4 (SL) and 1.7 (T3), whereas the final hatching percentage (c) ranged between 51.4% (T) and 58.5% (DW). There was no difference between the treatments on these parameters (Table 1; Fig. 1). However, the time at which 50% of the final number of hatched J2 had hatched (m) was significantly influenced by treatments. The 50% hatch of T3 was significantly smaller than T1 ($P < 0.014$) and SL ($P < 0.037$) hatch.

For *P. penetrans*, the treatments had no significant effect on the 50% hatch and the hatching rate, which varied between 0.83 (DW)-1.04 (T3) and 3.2 (T1)-4.3 (DW), respectively (Fig. 2, Table 2). However, the final hatching rate significantly differed between the treatments. The highest percentage hatch was observed in treatment M (47.5%) and the lowest in DW (26.0%). J2 hatched in significantly higher numbers from eggs exposed to T1 or M than when exposed to T3 ($P < 0.02$; $P < 0.017$), SL ($P < 0.0005$; $P < 0.0005$) and DW ($P < 0.0001$; $P < 0.0002$). Hatch from eggs exposed to T2 was greater than in SL ($P < 0.033$) and DW ($P < 0.016$).

Migration. The movement in the sandy soil columns of J2 *M. chitwoodi* and all stages of *P. penetrans* was not influenced by any of the treatments (Fig. 3) but decreased gradually with the distance from the point of inoculation. Between 61%

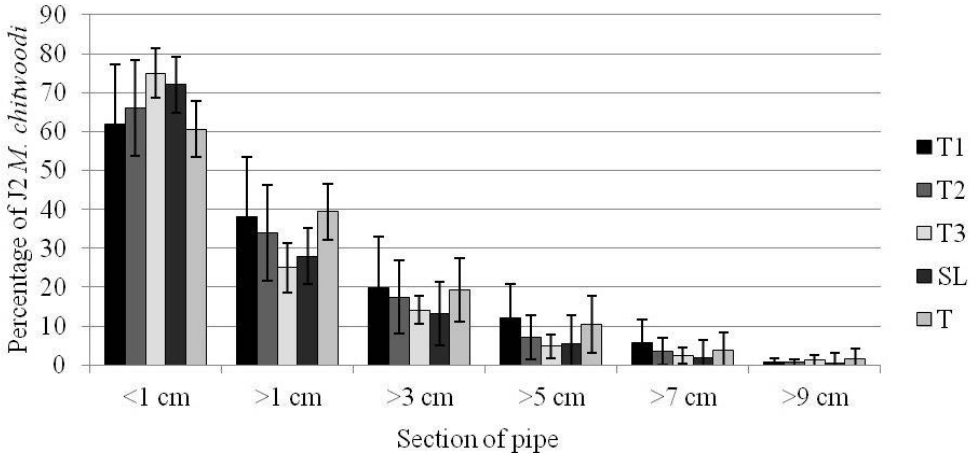

Fig. 3. Movement of second-stage juveniles (J2) of *Meloidogyne chitwoodi* in sand columns. Root diffusate of four- (T1), eight- (T2) and 12- (T3) week old marigold plants, soil leachate (SL) and root diffusate of tomato (T) were introduced at the end of the tube opposite to the point of inoculation of the nematodes. The bars represent the cumulative percentage (mean ± sd) of J2 found passing different sections of the pipes after 14 days of exposure.

Table 3. Penetration of second-stage juveniles of *Meloidogyne chitwoodi* in roots of tomato and *Tagetes patula* cv. Single Gold and of vermiforms of *Pratylenchus penetrans* in maize and *T. patula* cv. Single Gold. Penetration (means ± sd) was evaluated by the number of nematodes found in the roots 2 after weeks after inoculation. Significant differences between treatments are marked with different letter ($P < 0.05$).

Nematodes (g root)$^{-1}$	Plants		
	Tomato	Maize	*Tagetes*
Meloidogyne chitwoodi	42.8±12.9 a	–	34.2±48.9 a
Pratylenchus penetrans	–	22.5±10.1 a	75.3±54.0 b

Table 4. Multiplication of *Meloidogyne chitwoodi* in roots of tomato and *Tagetes patula* cv. Single Gold and of *Pratylenchus penetrans* in roots of tomato and *Tagetes patula* cv. Single Gold. Multiplication (Pf/Pi ± sd) was observed 10 weeks after inoculation of the nematodes. Significant differences between treatments are marked with different letter ($P < 0.05$).

Multiplication rate	Host		
	Tomato	Maize	*Tagetes*
Meloidogyne chitwoodi	20.97±10.01 a	–	0.05±0.02 b
Pratylenchus penetrans	–	4.84±5.01 a	0.00±0.00 b

(T) and 75% (T3) of the *M. chitwoodi* J2 and between 49% (T1 and M) and 65% of *P. penetrans* remained at the section where they were introduced.

Penetration. J2 of *M. chitwoodi* penetrated the roots of tomato and marigold at a similar rate (Table 3). Penetration of *P. penetrans* was significantly higher per g of root mass in marigold than in maize roots.

Multiplication. The number of *M. chitwoodi* J2 and eggs that were extracted 10 weeks after inoculation in soil planted with marigold was only 5% of the initial inoculum density (Table 4). No egg masses were observed on the roots. On tomato, the multiplication factor (Pf/Pi) was 20.1. No *P. penetrans* was recovered from soil and roots of marigold after 10 weeks, but on maize the population increased five-fold.

DISCUSSION

Most plant-parasitic nematodes are obligate parasites of higher plants. To complete their life cycle, endoparasitic nematodes must survive the time between two hosts. They further need to be able to locate, penetrate and multiply on host plants. Failure in any of these steps results in an incompleted life cycle followed by dying out of the nematode population.

Hatching can be influenced by the host (Perry, 2002), temperature (Robinson *et al.*, 1987; Pudasaini *et al.*, 2008), soil moisture (Gaur & Haque, 1987) and soil type (Devine & Jones, 2001). In our study, hatching of J2 of *M. chitwoodi* from egg masses collected on 20-24-week old tomato plants was only partly influenced by diffusates obtained from marigold of 4-, 8- and 12-week old or 2-week old tomato roots. Compared to soil leachate, distilled water and diffusate of 4-week old marigolds, the diffusate from 12-week old marigold plants shortened the time needed for 50% hatch. However, both the percentage of hatched J2 after 6 weeks of exposure and the hatching rate at the time

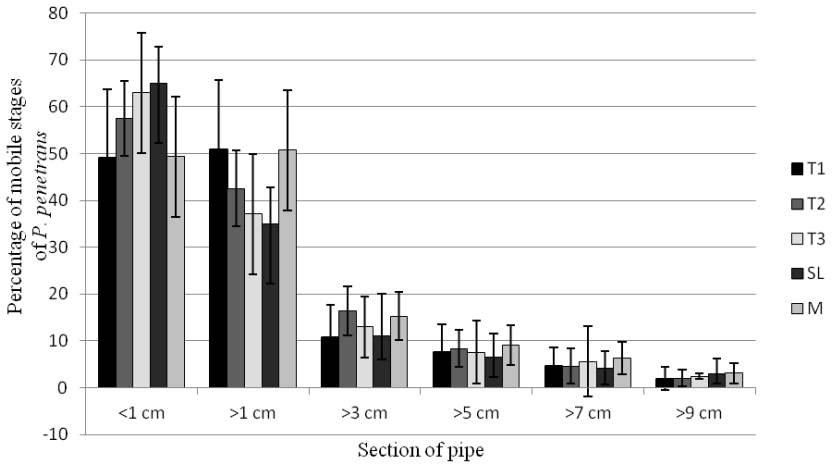

Fig. 4. Movement of *Pratylenchus penetrans* in sand columns. Root diffusate of four- (T1), eight- (T2) and 12- (T3) week old marigold plants, soil leachate (SL) and root diffusate of maize (M) were introduced at the end of the tube opposite to point of inoculation of the nematodes (vermiform stages). The bars represent the cumulative percentage (mean ± sd) of J2 found passing different sections of the pipes after 14 days of exposure.

of 50% hatch was reached, were similar after all treatments. This confirms the results obtained by Wesemael *et al*. (2006) who found no effect of host root diffusate on hatching of J2 from eggs masses of *M. chitwoodi* taken from 13-week old tomato plants. However, the time at which 50% hatch was reached and the final hatching percentage observed in our experiments (1.5-2.1 weeks and 51.4-58.5%) differed distinctly from those observed by Wesemael *et al*. (2006) (3.7-4.2 weeks and 93.6-95.9%). This difference might be explained by the difference in condition of the eggs in both experiments. The latter authors used entire egg masses, whereas in our experiments the eggs of *M. chitwoodi* had been separated from each other with NaOCl. Obviously, the egg mass matrix slows down the speed of hatching. However, any effect of the NaOCl treatment on hatching can be eliminated since McClure *et al.* (1973) found no effect of NaOCl on eggshell permeability. Unlike *M. chitwoodi*, hatching of J2 of *P. penetrans* differed with the root diffusate. Compared to soil leachate or distilled water, exposure of eggs to diffusate of maize significantly increased hatching of J2. The effect of *Tagetes* diffusate varied with the age of the plant. Diffusates of younger plants stimulated hatching, whereas diffusates of 12-week did not. These results confirm the work of Pudasaini *et al*. (2008).

Second-stage juveniles of plant-parasitic nematodes search for a suitable host immediately after hatching. The ability to orientate towards a host relies primarily on gradients of chemicals released by the host's root system (Perry, 1997). The root diffusates studied in our experiments did not influence the movement of J2 of *M. chitwoodi.* Similarly, Pinkerton *et al*. (1987) did not observe any dependence of *M. chitwoodi* on host diffusates of tomato for migration. Our study did also not reveal any influence of host diffusates and SL on migration of *P. penetrans*. However, Pudasaini *et al*. (2007) using a living plant to study the influence of root diffusates on J2 of *P. penetrans* over a period of 14 days, did find effects between hosts. This difference suggests the importance of a continuous supply of fresh root diffusate over time. Bioassays using small quantities of artificially extracted root diffusate may underestimate the effect of the diffusate. The fact that the population of *P. penetrans* migrated at higher rate (34-51%) than the population of *M. chitwoodi* (26-38%) can be explained by the differences in composition of both populations. The population of *P. penetrans* consisted of all mobile stages, which have higher energy content than the J2 that composed the population of *Meloidogyne*.

In our experiments, the proportion of *M. chitwoodi* that penetrated the roots of tomato and *Tagetes* was not different. However, unlike *M. chitwoodi*, *P. penetrans* penetrated both hosts in different proportions. The lesion nematode penetrated *Tagetes* in much greater numbers than maize. Evidently, marigold is as attractive as tomato and maize, which are reputed hosts for *M. chitwoodi* and *P. penetrans*, respectively. Yet, in resistant cultivars, high levels of penetration do not result in massive multiplication (Schomaker & Been, 2013).

Both nematode species reproduced to high numbers on their host, tomato and maize for *M. chitwoodi* and *P. penetrans*, respectively. However, no reproduction of *P. penetrans* was observed on marigold, whereas for *M. chitwoodi* the

multiplication factor was only 0.05. Obviously, marigold has a negative effect on both nematode species and can be used as a control strategy leading to an important reduction of the population.

The results of this series of bioassays do not support the idea that allelopathic compounds produced by marigolds could reduce endoparasitic nematodes. Hatching and migration of both *M. chitwoodi* and *P. penetrans* were not affected by root diffusates of *T. patula* cv. Single Gold. For early generations of endoparasitic nematodes, these two phases in the life cycle happen in the rhizosphere, where allelopathic compounds might play a role. Other authors came to a similar conclusion by observing soil microorganisms. Topp *et al.* (1998) compared the microbial activity in soil cropped to *T. erecta* cv. CrackerJack or *T. patula* cv. Creole with bare soil and soil cropped to rye. The authors observed that both *Tagetes* species did not suppress microorganisms in the soils and concluded that nematode control is not achieved by releasing a biocidal agent into the soil.

Pratylenchus penetrans entered the roots of *T. patula* in larger quantities than maize. Maize is an excellent host and attracts *P. penetrans* better than many other hosts (Pudasaini *et al*., 2007). Although marigold appears to be attractive it did not allow the nematode to reproduce. Unlike the lesion nematode, marigolds attracted *M. chitwoodi* at the same level, as did tomatoes. The root-knot nematode, however, hardly survived on marigold. Obviously the nematode control mechanism of marigold resides for both nematode species in the non-host behaviour. *Tagetes patula* cv. Single Gold herewith confirms its behaviour as a trap crop as suggested by Pudasaini *et al*. (2008).

The different rates of root penetration of *T. patula* cv. Single Gold between *M. chitwoodi* and *P. penetrans* demonstrate that even though the final effect of marigold is similar for both species, *viz*. no reproduction, phases in the development of the nematodes can be affected differently by the plant. Suatmadji *et al*. (1969) reported *M. hapla* to be suppressed by *T. patula* but not by *T. erecta*. Observations of the effect of marigold on the different phases in the life cycle of these nematodes may provide explanation for these differences.

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B. Nježić, N. de Sutter and M. Moens. Воздействие бархатцев *Tagetes patula* cv. Single Gold на жизненный цикл фитопаразитических нематод *Meloidogyne chitwoodi* и *Pratylenchus penetrans*.

Резюме. Исследовали воздействие бархатцев *Tagetes patula* cv. Single Gold и растений-хозяев на вылупление, расселение и размножение *Meloidogyne chitwoodi* и *Pratylenchus penetrans*. Корневые диффузаты бархатцев и томатов, экстракты почвы и дистиллированная вода не влияли на вылупление у *M. chitwoodi*. Характер вылупления *P. penetrans* не различался при обработке корневыми диффузатами 4-х и 8-недельных бархатцев и кукурузы. Однако количество личинок 2-й стадии (J2), вылупившихся в корневых диффузатах 12-недельных бархатцев, почвенных экстрактах и дистиллированной воде было достоверно ниже, чем в диффузатах 4-недельных бархатцев и кукурузы. На коэффициент миграции личинок 2-й стадии *M. chitwoodi* не повлиял ни один из диффузатов. Тем не менее, возраст бархатцев, от которых получали корневой диффузат, достоверно влиял на способность к расселению у *P. penetrans*. Личинки 2-й стадии *M. chitwoodi* проникали в корни бархатцев столь же успешно, что и в корни томатов. *P. penetrans* проникали в корни бархатцев в больших количествах, чем в корни кукурузы. В 10-недельном горшечном эксперименте с бархатцами коэффициент размножения *M. chitwoodi* и *P. penetrans* составлял 0,05 и 0,00, соответственно.