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Orijinal araştırma (Original article)

Pasteuria penetrans suppression of root-knot nematode *Meloidogyne arenaria* race 1 in vegetables[1]

Sebzelerde zararlı *Meloidogyne arenaria* ırk 1'in *Pasteuria penetrans* tarafından baskı altına alınması

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Summary

This study was conducted to determine the suppressiveness of obligat parasite *P. penetrans* against the peanut root-knot nematode *Meloidogyne arenaria* race 1 in vegetable growing site and to find out whether the field site have infested with other root-knot nematode species, causing the suppression to break down. The study were carried out at four vegetable crops grown sites tomato *Lycopersicon lycopersicum* cv. Bella Rosa, cucumber *Cucumis sativus* L. cv. Cobra, okra *Abelmoschus* (=*Hibiscus*) *esculentus* (L.) Moench cv. Clemson Spineless and squash *Cucurbita pepo* L. cv. Golden Summer at the Plant Science Research and Education Unit-Citra, University of Florida, US. The field site was arranged in a split plot design with eight replicates. To determine the effect of 1,3-dichloropropene (1,3-D) on *P. penetrans*, the split plot was fumigated with 1,3-D (Telone II)at dose of 112 L/ha.. Plant and soil samples were collected from each plot at harvest time. The percentages of *M. arenaria* race 1 infective juvenile (J2) with endospores attached ranged from 40%to 10%.. Only a very low incidence of *M. incognita* was extracted from tomato roots galls.. The results indicate that *P. penetrans* is likely to be one of the important effective agent in the nematode suppression and the reduction *M. arenaria* race 1 damage..

Key words: Bacterium, biological control, *Meloidogyne arenaria, Pasteuria penetrans,* suppressive soil

Özet

Bu çalışmanın amacı, obligat parazit olan *Pasteuria penetrans* bakterisinin sebze yetiştirilen alanlarda kök-ur nematodu *Meloidogyne arenaria* ırk 1'e karşı baskınlığını ortaya çıkarmak, bu baskınlığın etkisinin kırılmasına neden olan diğer kök-ur nematod türlerinin alanda bulunup bulunmadığını tespit etmektir. Bu amaçla 2011 yılının Mart ayında, daha önceden *P. penetrans* bulaştırılmış ve *M. arenaria* race 1'e karşı baskın olduğu bilinen Florida Üniversitesi'nin araştırma arazisinde, domates *Lycopersicon lycopersicum* cv. Bella Rosa, salatalık *Cucumis sativus* L. cv. Cobra, bamya *Abelmoschus* (=*Hibiscus*) *esculentus* (L.) cv. Clemson Spineless ve kabak *Cucurbita pepo* L. cv. Golden Summer gibi dört farklı sebze çeşidi yetiştirilmeye başlanmıştır. Çalışma bölünmüş parseller deneme deseninde 8 tekerrürlü olarak yürütülmüştür. Her bir sebze çeşidi için biri ilaçlı diğeri ilaçsız olacak şekilde parseller hazırlanmıştır. Ilaçlı parseller 112 lt/ha oranında 1,3-dichloropropene (1,3-D) (Telone II) ile ilaçlanmıştır. Tüm deneme parsellerinden hasat sonunda bitki kökleri ve toprak örnekleri alınarak inceleme yapılmıştır. Alınan toprak örneklerinden elde edilen *M. arenaria* ırk 1 juvenillerinin *P. penetrans*'ın endosporları ile bulaşıklık oranı en yüksek %40 olarak kimyasal uygulanmış kabak parselinde, en düşük endospor bulaşıklık oranı ise kimyasal uygulanmayan bamya parselinde yaklaşık %10 olarak gözlenmiştir. Deneme alanlarında yetiştirilen sebzelerden yalnızca domates bitkisi köklerinde çok düşük oranda diğer nematode türlerinden *M. incognita* tespit edilmiştir. Çalışmadan elde edilen sonuçlar, sebzelerde *M. arenaria* race 1 tarafından meydana gelen zararın azaltılmasında ve bu nematodun baskı altında tutulmasında *P. penetrans*'ın önemli bir etmen olduğunu ortaya koymuştur.

Anahtar sözcükler: Bakterium, biolojik mücadele, *Meloidogyne arenaria, Pasteuria penetrans,* baskılayıcı toprak

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Introduction

Plant-parasitic nematodes are recognized as major agricultural pathogens and known to attack plants and cause great economic losses in agricultural crops worldwide (Taylor & Sasser, 1978; Sasser, 1990; Lambert & Bekal, 2002; Sikora & Fernandez, 2005). Among the nematode pathogens, root-knot nematodes (*Meloidogyne* spp.) are global threats in crop production (Sasser, 1990) and cause annual losses of about US $100 billion worldwide (Brand et al*.,* 2010). In most tropical countries, root-knot nematodes are one of the major problems of vegetable production. Vegetable crops usually are among the most susceptible and worst affected by root-knot nematodes (Sharma et al., 2006; Anwar et al., 2007; Singh & Khurma, 2007). Sikora & Fernandez (2005) reported yield losses of over 30% in highly susceptible vegetable crops egg-plant, tomato and melon.

Since the 1950s, control of plant parasitic nematodes has relied on chemical nematicides (Fernandez et al., 2001). Environmental side effects associated with chemical control and the recent loss of methyl bromide as a multi-purpose soil fumigant have spurred research into nematode control alternatives (Hutchinson et al. 1999). Different approaches were tested to understand the roles of soil microorganisms in induced suppressiveness to plant parasitic nematodes (Fernandez et al., 2001). Soil is a complex ecosystem, one that harbours many different organisms with a complex network of interactions. These organisms that have adverse effect on nematode populations are called nematode antagonists. Nematode antagonists are a wide range of organisms including fungi, bacteria, viruses, richettseae, protozoans, turbellarians, tardigrades, enchytraeides, mites, insects and nematodes (Chen, et al., 2004). Among these, bacterial antagonist is one of the most important organisms in regulating nematode population in soil. A wide range of bacteria (Hallmann et al., 2001) have been used to reduce a range of plant-parasitic nematodes. *Pasteuria* spp. have been reported from more than 116 genera of nematodes distributed worldwide (Chen et al., 2004). *Pasteuria penetrans* is considered as the primary microorganism responsible for soil suppressiveness to root-knoot nematodes in many fields. Dickson et al., (1992), Dickson et al*.,* (1994), Chen & Dickson, (1998), Freitas et al., (2000) reviewed the potential of *P. penetrans* as a biological control agent for root-knot nematodes. Also Chen et al., (1996) and Cetintas & Dickson (2004), reported reducition in root galls on peanut by *Meloidogyne arenaria* race 1 when *P. penetrans* was present. In most studies, the suppressiveness of soils to *Meloidogyne* spp. was observed, but there are few data on the soil suppressiveness of vegetable growing fields. Due to the importance of *Meloidogyne* spp. it is necessary having more data with biological control agents such as *P. penetrans*.

The objectives of this study were to determine whether the soil suppressiveness induced by *P. penetrans* would prevail when vegetables were planted in the former peanut growing site and to determine if the former peanut-growing site would become infested with other root-knot nematode species with vegetable production.

Materials and Methods

Nematode isolate

The origin of *M. arenaria* race 1, used in this study, was from peanut (*Arachis hypogaea* L.), Levy County, Florida. A single egg-mass isolate of *M. arenaria* race 1 was used in the study. The nematode was cultured in a greenhouse at the University of Florida on tomato (*Solanum lycopersicum* cv. Rutgers).

The Field Site

The field site was located at the Plant Science Research and Education Unit-Citra, University of Florida (29°.24'19.11" N; 82°.8'32.19" W). In 2001, the *Pasteuria* isolate specific to *M. arenaria* race 1 was transferred from a known suppressive field site to a site that was clean of the organism. High

densities of *M. arenaria* race 1 were added and peanut (*A. hypogaea* L. cv. Georgia Green (susceptible to Ma)) was grown in spring-summer months and common hairy vetch was grown as a winter cover crop. *Pasteuria penetrans* reached suppressive numbers within 3 years. Peanut was grown in a monoculture on the site for 9 years.

In March 2011, the field was arranged in a split-plot design with eight replicates. Four vegetables crops susceptible to *Meloidogyne* spp., include tomato *Lycopersicon lycopersicum* cv. Bella Rosa, cucumber *Cucumis sativus* L. cv. Cobra, okra *Abelmoschus* (=*Hibiscus*) *esculentus* (L.) Moench cv. Clemson Spineless and squash *Cucurbita pepo* L. cv. Golden Summer, served as the main plots. The split plots were fumigated with 1,3- dichloropropene (1,3-D) (Telone II) applied at 112 L/ha. Plot size was 6 m in length with 1.8 m centers. Each plot was covered with aluminized metallic polyethylene film. Irrigation and liquid fertilizer were applied via a single drip tubes per plot. Seedlings were transplanted to the plots on March 2011. At the end of the growing season (18 weeks later after planting) six plants were chosen from each plot. The root systems were dug out and rated for root galling on a scale of 0 to 10, in which 0 = 0%, 10= 100% of roots with galls (Barker et al*.,* 1986).

Soil samples

Soil samples were collected from each plot on July. Six cores (2.5-cm-diam., 20 cm deep) of soil were taken from each plot with a cone-shaped sampling tube. Soil cores from each plot were combined and mixed thoroughly. The samples were kept in plastic bags and labeled. Before processing, the samples were stored at 10 °C in an incubator.

Laboratory study

Extraction of Second-stage juveniles

Second-stage juveniles (J2) of *M. arenaria* were extracted from 250 cm^3 of soil collected from each plot by centrifugal-flotation (Jenkins, 1964) and counted using a compound inverted light microscope.

Soil Bioassay

To determine the presence of *P. penetrans* in the soil, a portion of soil from the samples was taken from each plot at harvest time on 6th July. The soil samples were air-dried 2 weeks at room temperature and 40 g from each was placed in a 50 ml sterile polyethylene centrifuge tube. Soil water contend was adjusted to 100% field capacity (saturated with 7.5 ml water) to increase the endospore attachment. About 100, 1- to 4-day old J2, *M. arenaria* race 1 were added and the tubes left at room temperature (27 ^oC). After 4 days, the J2s were extracted by centrifugal-flotation method (454 g of sugar dissolved in water and made up to 1 L) (Jenkins, 1964) from the each plot of the four vegetable sites. The first 20 J2s were observed from each tube and the number of endospores attached to J2 was counted using an inverted light microscope at 40X magnification. In order to estimate the number of endospores attached per J2, the following scale, 0= none, 1= 1-2, 2= 3-5, 3= 6-15, 4= 16-100, 5= >100, was used.

Identification of other root-knot nematodes

Galled roots from each plant plots were collected at harvest in July and the infected roots were put into polythene bags, labeled and brought to the laboratory of the Nematology, University of Florida for identification of root-knot nematode species. *Meloidogyne* females were dissected by hand from infected plant with the aid of a stereo-binocular microscope. Each female was identified based on their esterase (EST) and malate dehydrogenase profiles derived by polyacrylamide gel electrophoresis (Esbenshade and Triantaphyllou, 1985).

A Bio-Rad Mini-Protein II (Bio-Rad, Philadelphia, PA) electrophoretic unit was used. Before electrophoresis, the females were thawed and homogenized individually in a microhaematocrite plastic tube in a 5-µl of extraction buffer. Each sample then was loaded into a well on the polyacrylamide gel. Each gel contained 15 wells. The standard was a *M. javanica* female extract and it was placed into wells 1 and 14. The remaining 13 wells were loaded with the protein extract from unknown female samples. Electrophoresis was carried out in a discontinuous buffer system with 8% acrylamide running gel, pH 8.8 and 4% acrylamide stacking gel, pH 6.8 (BioRad). The voltage was maintained at 80 volts for the first 15 minutes and then increased to 200 volts for 35 minutes. Following electrophoresis, the gels were removed and placed in an enzyme reaction mixture to determine esterase and malate dehydrogenase activity (Harris and Hopkinson, 1976; Esbenshade and Triantaphyllou, 1985).

Statistical analysis

The data was analzed with Mann-Whitney U-Test for differences between groups while Kruskal Wallis H-Test was used for comparison between more than two groups. The data analysis was performed using SPSS 21,0 (SPSS Inc. Chicago, Il, USA).

Results

At the end of the growing season, the root systems of vegetable plants from each plot were observed and rated for root galling. *Meloidogyne* spp. produced variable low number of galls on roots of all vegetables cultivars except squash cv. Golden Summer. Data showing the galling indices recorded on four vegetable cultivars is presented in figure 1. The root-knot nematode galling indices were low among all treatments ranging from 0,3 on tomato to 0 for squash. Maximum number of gall were observed in tomato cv. Bella Rosa from untreated soil with gall index of 0,3, which was not significantly different from treated soil with gall index of 0,1 (Figure 1). There were no differences in galling indices between 1,3-dichloropropene treated vs. nontreated plots ($P \leq 0.05$). There were also no differences between galling indices among vegetable cultivars ($P \leq 0.05$).

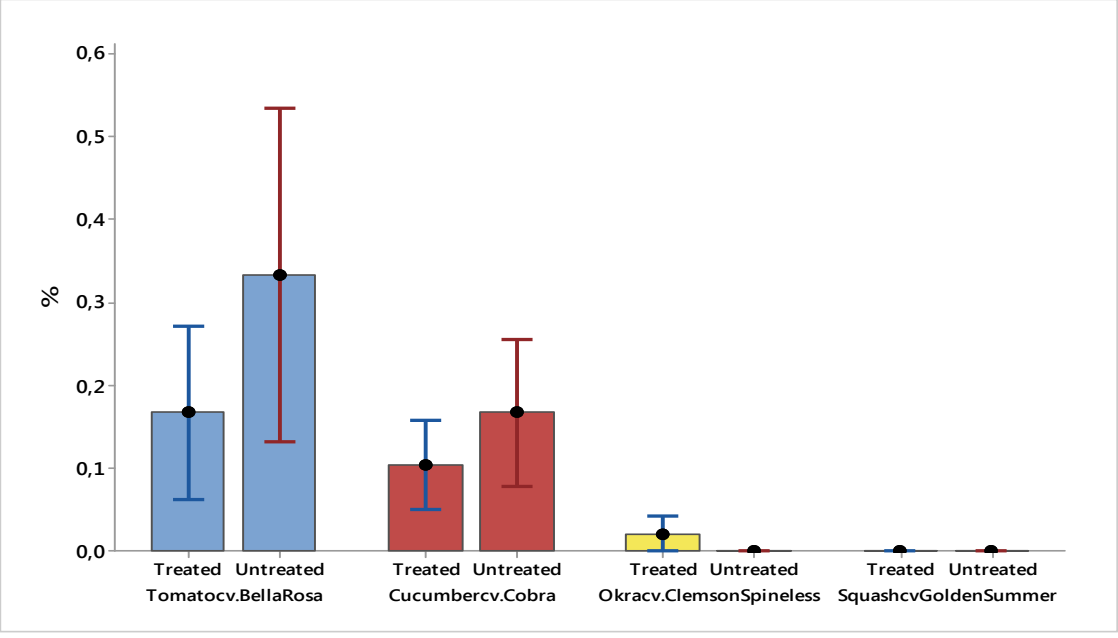

Figure 1. Root galling indices on tomato cv. Bella Rosa, cucumber cv. Cobra, okra cv. Clemson Spinless and squash cv. Golden Summer infected with *Meloidogyne arenaria* race 1 in 1,3-D treated and untreated soils.

Soil samples were collected from each plot on July after the harvest time , and counted the number of J2 per 250 cm^3 of soil. There was no difference between 1,3-D treated plots and the non-treated plots with respect to the number of J2 per 250 cm^3 of soil. The greatest number of J2s were found in the cucumber untreated plots with 17, and the least number in the 1,3-D-treated okra plot ($P \leq 0.05$) (Figure 2). The attached bacterial endospores were also counted on the nematode cuticle extracted from each plot using light microscope. There was no difference among treatments (treated and nontreated) for tomato and squash regarding the percentage of J2 with endospores attached. The percentages for treated and nontreated plots were 36% and 37% for tomato and 40% and 37% for squash, respectively. These results indicated that there was also no difference between tomato and squash cultivars for all treatments. When considered the results of cucumber and okra for nontreated plots, the percentages of J2 with endospores attached were 34% and 10%, respectively. The highest and lowest the percentages of J2 with endospores attached ranged from a high of 40% for treated squash to a low of 10% for nontreated okra (Figure 2).

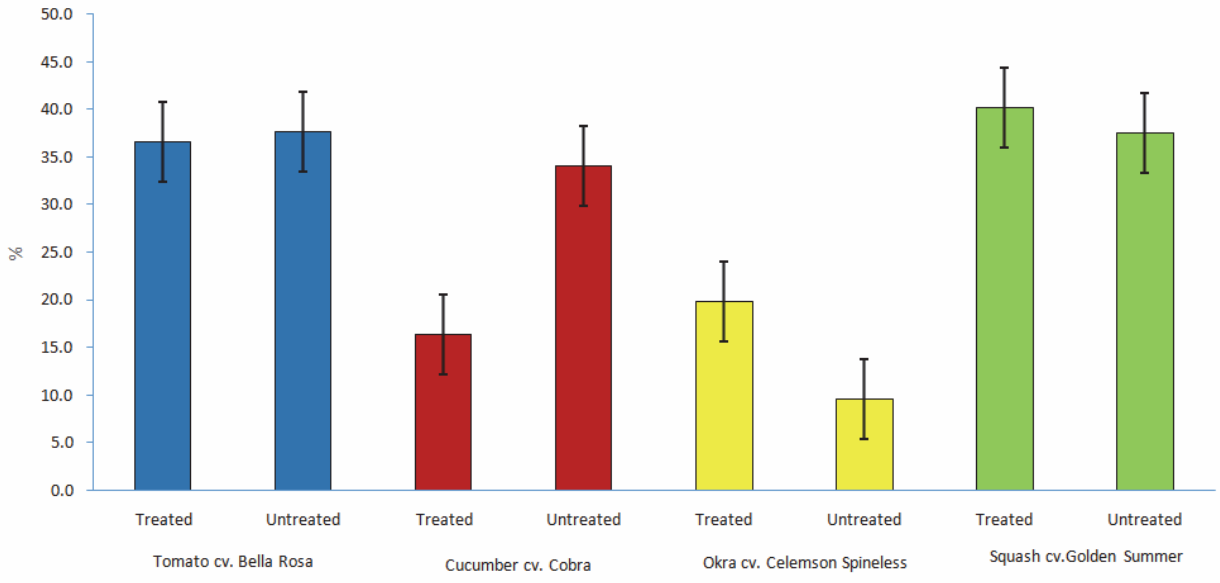

Figure 2. Percentage of juvenile (J2) with endospores attached to *Meloidogyne arenaria* race 1 per 250 cm^3 1,3-D treated and untreated soils collected from each vegetable plots.

Laboratory data

The field soil bioassay demonstrated that there was a high rate of survival of *P. penetrans* endospores in the various treatments. The J2 was observed under light microscope and counted *P. penetrans* endospores on the cuticle.The cuticle of second-stage juveniles of *M. arenaria* was found with a heavy burden of attached *Pasteuria* endospores (Figure 3). *Pasteuria penetrans* endospores were attached to nearly all J2 from all plots. The percentages of J2 with endospores extracted from the vegetables soils were similar ranging from 72% to 78%. The highest percentage of endospore attachment was recorded for soils collected from okra (78%), followed by cucumber (77%), tomato (75%), and squash (72%). There was no endospore attachment number greater than 100 endospores/J2 (Table 1).

To identification of other root-knot nematodes biochemical analysis were used. *Meloidogyne* spp. Females, extracted tomato roots, showed the typical esterase pattern for *M. incognita* phenotype. From only a low incidence of *M. incognita* females were extracted from the galls taken from tomato cv.Bella Rosa roots. *M. arenaria* remains the dominant root-knot nematode species in the field site albeit at low densities.

Table 1. Bioassay[a] of soil collected at harvest of vegetable crops to determine the percentage of second-stage juveniles (J2) of *Meloidogyne arenaria* race 1 with endospores of *Pasteuria penetrans* attached.

Plants cultivars	Percentage[b] of J2 with endospores attached	Scale (endospores/J2)					
		0 (None)	1 (1-2)	2 (3-5)	3 (6-15)	4 (16-100)	5 (>100)
Tomato cv. Bella Rosa	75.0	5.0*	4.3	2.6	3.5	4.6	0
Cucumber cv. Cobra	77.0	4.6	2.6	3.5	4.4	4.9	0
Okra cv. Clemson Spineless	78.0	4.4	2.9	3.5	3.3	6.0	0
Squash cv. Golden Summer	72.0	5.6	2.9	3.8	2.9	4.9	0

* Data are means of eight replicates.

[a] Forty grams of air-dried soil was placed in a 50 ml polyethylene centrifuge tube. One hundred 1-to 4- day old *Meloidogyne arenaria* race 1 J2 were added. Four days later the J2 were extracted via centrifuge-flotation method and the number of endospores attached per J2 was counted based on the first 20 J2 observed per sample.

[b] Percentage of J2 with endospores attached based on 20 nematodes per plot divided by six categories (category 0 = no endospores attached/J2, category 1 = 1 to 2 endospores attached/J2, 2 = 3 to 5, 3 = 6 to 15, 4 = 16 to 100, and category 5 = >100 endospores attached/J2).

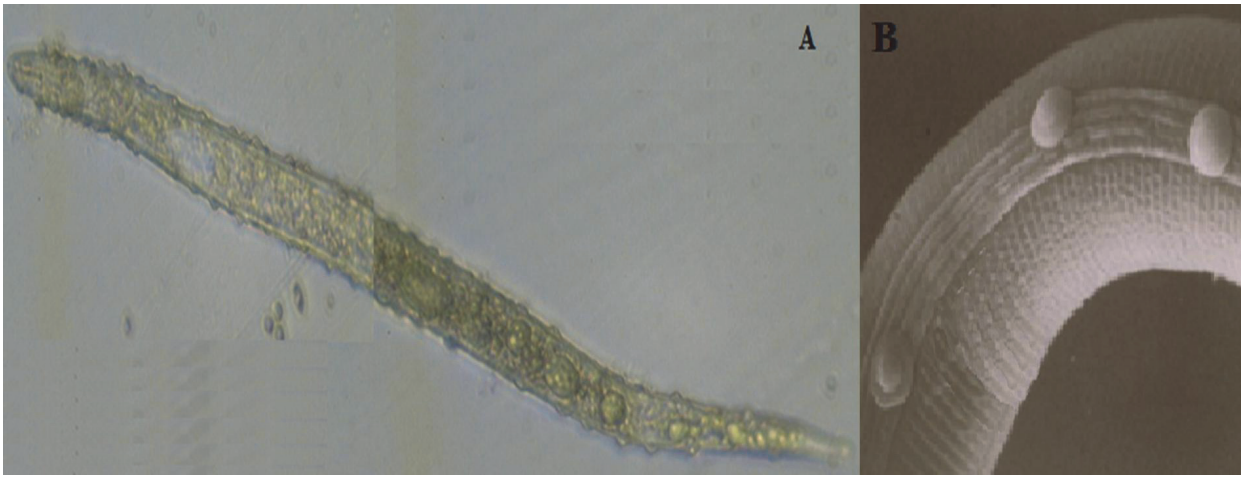

Figure 3. A) Second-stage juvenile of *Meloidogyne arenaria* with a heavy burden of attached *Pasteuria* endospores under light microscope, B) Scanning electron micrograph of *Pasteuria penetrans* endospores attached along the lateral field of a second-stage juvenile of *Meloidogyne arenaria.*

Discussion

The data suggests that *P. penetrans* played an important role in the nematode suppression and the reduction of plant damage by *M. arenaria* race 1. Chen et al., (1996), Oostendorp et al., (1990; 1991) and Weibelzahl-Fulton, (1998) reported that *P. penetrans was* a successful example of a biological control agent that suppressed *M. arenaria* on peanut. Cetintas & Dickson (2004) also reported that *P. penetrans was* suppressive on *M. arenaria* race 1 on peanut. A high degree of soil suppressiveness was maintained in the field even the transition from peanut to vegetables. The ability of the biocontrol agent to reduce numbers of root-knot nematodes was dependent on the densities of the nematode and *P. penetrans* spores in the soil (Giannakou & Gowen, 2004). Stirling (1985) noted that *P. penetrans* attached to *Meloidogyne incognita* and other root-knot nematode species, but **a** very low incidence of *M. incognita* females was extracted from galls on tomato. Our result indicates that even *M. arenaria* remained the dominant root-knot nematode species in the field site*, P. penetrans* was present in the soil, some species like *M. incognita* might break down suppressiveness.

P. penetrans has played an important role in suppressing root-knot nematodes in the field. *P. penetrans* has been reported to suppress *Meloidogyne* spp. on many vegetable crops. Cho et al., (2000) reported that *P. penetrans was* suppressive on *M. arenaria* on tomato. It is a very promising biological control agent against root-knot nematodes. It has many advantages such as the host specificity, the resistance to various nematicides, adverse environment, the longevity of endospores in soil, the capacity of amplifying itself and maintaining suppressiveness for years. Unfortunately, their narrow host range limits their wide use, and mass endospore production is currently hard to achieve. Commercial use of the *P. penetrans* requires an in vitro method for mass production. Many media were used for artificial cultivation, but none of them was successful. The *Pasteuria Biosciences* is the only company able to produce enough endospores in a bioreactor to accommodate small field trials.

In conlusion, this study provides evidence that soil suppressiveness with the *P. penetrans* effectively reduced the number of galls of *M. arenaria* race 1 on vegetables.

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