UNIVERSITAT POLITÈCNICA DE CATALUNYA BARCELONATECH

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Universitat Politècnica de Catalunya
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PhD dissertation

Ariadna Giné Blasco

PhD Supervisor: **Dr. Francesc Xavier Sorribas Royo (UPC, DEAB)**
PhD Co-Supervisor: **Dr. César Ornat Longarón (UPC, DEAB)**

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“Tell me and I forget,

teach me and I may remember,

involve me and I learn.”

-Benjamin Franklin

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Index

Resum

L'objectiu general de la tesi va ser determinar la dinàmica de població de *Meloidogyne* spp. en tomàquet i cogombre, les pèrdues de producció que causa, i l'efecte de diversos mètodes de gestió, de base biològica, per a reduir la taxa de creixement de la població del nematode i el dany que causen als cultius. Els objectius específics i els resultats més rellevants són els següents: i) Elaborar models fenològics de *Meloidogyne* spp. en cucurbitàcies. Els requeriments tèrmics de *M. incognita* i *M. javanica* en cogombre i carbassó van ser similars, però diferents dels de síndria. En síndria, els requeriments tèrmics de *M. incognita* i *M. javanica* per completar el cicle de vida van diferir. ii) Caracteritzar la dinàmica de població de *M. incognita* en cogombre i tomàquet i les pèrdues de producció ocasionades pel nematode. La taxa de creixement de la població de *M. incognita* en cultiu de cogombre va ser major en el cultiu d'estiu-tardor que en el de primavera-estiu, però el límit de tolerància i la mínima producció relativa no van diferir entre períodes de cultiu. En tomàquet, la taxa de creixement de la població en el cultivar resistent va ser menor que en el susceptible. El límit de tolerància no va diferir entre cultivars, però la producció relativa mínima va ser major en el cultivar resistent. La taxa de creixement de la població en el cultivar resistent va incrementar amb els anys de cultiu reiterat. L'evolució dels paràmetres poblacionals al llarg del temps permet detectar precoçment la selecció de virulència. iii) Caracteritzar l'efecte del patró híbrid de carabassa RS841 sobre la dinàmica de població de *M. incognita* i la producció de cogombre en hivernacle. La taxa de creixement de la població del nematode en el cogombre empeltat va ser major que en el no empeltat, sense mostrar diferències en el límit de tolerància i mínima producció relativa. En les nostres condicions, el patró RS841 no es resistent al nematode ni aporta més tolerància al cultiu. iv) Detecció de fongs paràsits d'ous de *Meloidogyne* spp. en parcel·les de producció comercial d'hortalisses. Es van mostrejar un total de 40 parcel·les de producció hortícola, 10 en producció ecològica i 30 en producció

integrada. Es van detectar fongs paràsits d'ous de *Meloidogyne* spp. en totes les parcel·les ecològiques i en la majoria d'integrades. El nivell de parasitisme superava el 40% en algunes de elles. *Pochonia chlamydosporia, Fusarium* sp. i *Plectosphaerella cucumerina*, van ser els fongs més freqüents, però solament la freqüència relativa de *P. chlamydosporia* es relacionava amb el percentatge de parasitisme. v) Caracteritzar sòls supressius a *Meloidogyne* spp. en parcel·les de producció ecològica d'hortalisses. Es va realitzar un estudi del seguiment de la fluctuació de les densitats de *Meloidogyne* spp. i del parasitisme d'ous en dues parcel·les de producció ecològica en hivernacle. A més, es van realitzar bioassajos en contenidor per a certificar la supressivitat d'aquests sòls. Paral·lelament, es van obtenir els perfils microbians mitjançant DGGE i es van comparar amb un sòl conductiu. Els resultats van demostrar la supressivitat d'ambdós sòls. Els perfils microbians van revelar patrons diferents en els sòls supressius i el conductiu. vi) Determinar l'efecte de la resistència vegetal i l'aplicació de BioAct WG (*Purpureocillium lilacinus* soca 251) sobre *M. incognita* en una rotació tomàquet-cogombre en hivernacle. La resistència vegetal va ser el factor que va disminuir la reproducció del nematode i va augmentar el rendiment de cultiu, sense que s'apreciés cap efecte de BioAct WG. No obstant, en els assajos *in vitro*, el fong va mostrar gran capacitat de control. Les condicions ambientals durant el cultiu, la composició del formulat comercial, i/o les característiques fisicoquímiques del sòl podrien haver afectat la seva activitat.

Resumen

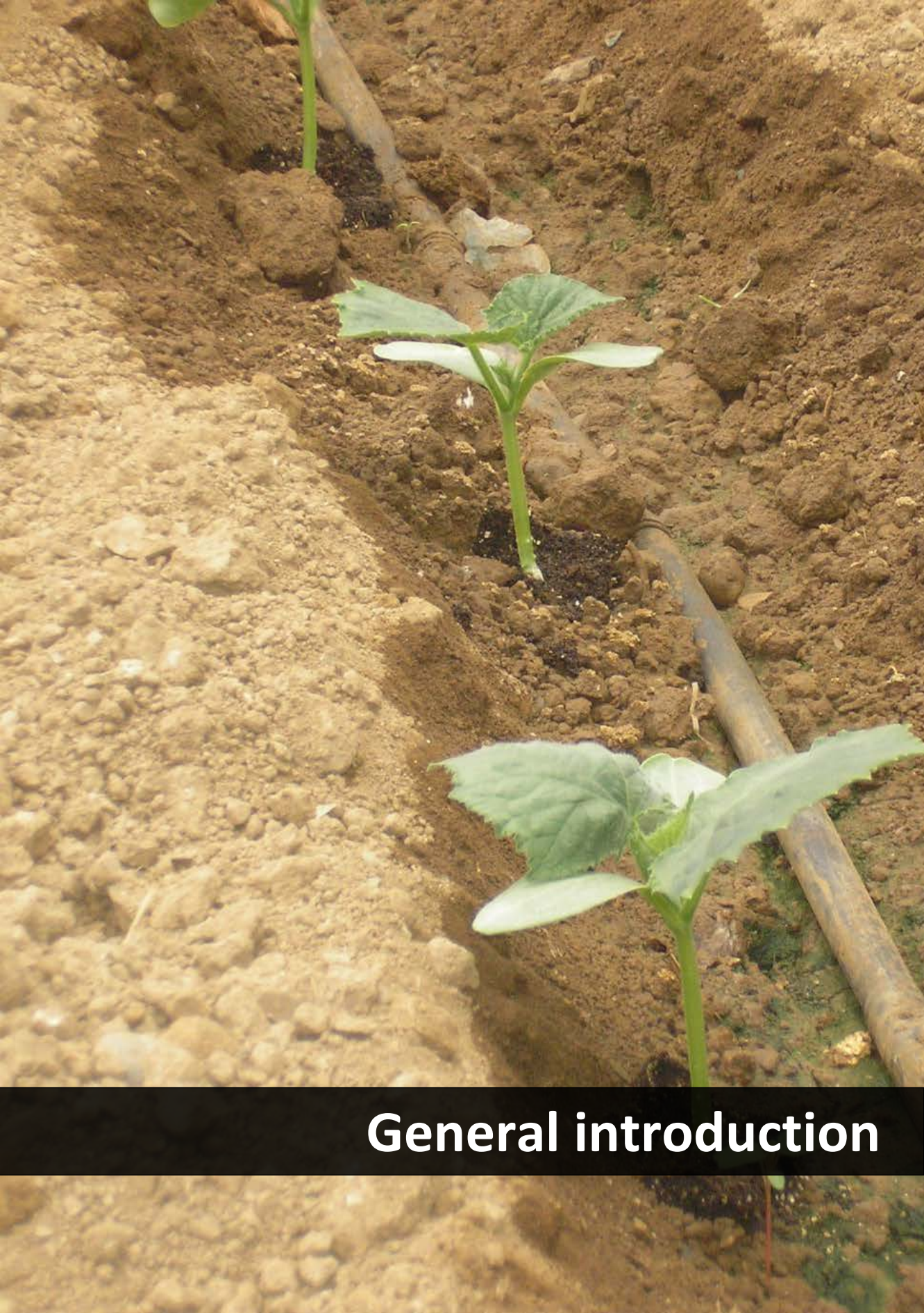
El objetivo general de la tesis fue determinar la dinámica de población de *Meloidogyne* spp. en tomate y pepino, las pérdidas de producción que causa, y el efecto de diversos métodos de gestión, de base biológica, para reducir la tasa de crecimiento de la población del nematodo y el daño que causa a los cultivos. Los objetivos específicos y los resultados más relevantes son los siguientes: i) Elaborar modelos fenológicos de *Meloidogyne* spp. en cucurbitáceas. Los requerimientos térmicos de *M. incognita* y *M. javanica* en pepino y calabacín fueron similares, pero diferentes de los de sandía. En sandía, los requerimientos térmicos de *M. incognita* y *M. javanica* para completar el ciclo de vida difirieron. ii) Caracterizar la dinámica de población de *M. incognita* en pepino y tomate y las pérdidas de producción ocasionadas por el nematodo. La tasa de crecimiento de la población de *M. incognita* en cultivo de pepino fue mayor en el cultivo de verano-otoño que en el de primavera-verano, pero el límite de tolerancia y la mínima producción relativa no difirieron entre periodos de cultivo. En tomate, la tasa de crecimiento de la población en el cultivar resistente fue menor que en el susceptible. El límite de tolerancia no difirió entre cultivares, pero la mínima producción relativa fue mayor en el cultivar resistente. La tasa de crecimiento de la población en el cultivar resistente incrementó con los años de cultivo reiterado. La evolución de los parámetros poblacionales a lo largo del tiempo permitió detectar precozmente la selección de virulencia. iii) Caracteritzar el efecto del patrón híbrido de calabaza RS841 sobre la dinámica de población de *M. incognita* y la producción de pepino en invernadero. La tasa de crecimiento de la población del nematodo en el pepino injertado fue mayor que en el no injertado, sin mostrar diferencias en el límite de tolerancia y la mínima producción relativa. En nuestras condiciones, el patrón RS841 no es resistente al nematodo ni aporta más tolerancia al cultivo. iv) Detección de hongos parasitos de huevos de *Meloidogyne* spp. en parcelas de producción comercial de hortalizas. Se muestrearon un total de 40 parcelas de

producción hortícola, 10 en producción ecológica y 30 en producción integrada. Se detectaron hongos parásitos de huevos de *Meloidogyne* spp. en todas las parcelas ecológicas y en la mayoría de integradas. El nivel de parasitismo superaba el 40% en algunas de ellas. *Pochonia chlamydosporia, Fusarium* sp. y *Plectosphaerella cucumerina*, fueron los hongos más frecuentes, pero solo la frecuencia relativa de *P. chlamydosporia* estuvo relacionada con el porcentaje de parasitismo. v) Caracterizar suelos supresivos a *Meloidogyne* spp. en parcelas de producción ecológica de hortalizas. Se realizó un estudio del seguimiento de la fluctuación de las densidades de *Meloidogyne* spp. y del parasitismo de huevos en dos parcelas de producción ecológica en invernadero. Además, se realizaron bioensayos en contenedor para certificar la supresividad de estos suelos. Paralelamente, se obtuvieron perfiles microbianos mediante DGGE y se compararon con un suelo conductivo. Los resultados demostraron la supresividad de los suelos. Los perfiles microbianos revelaron patrones diferentes en los suelos supresivos y el conductivo. vi) Determinar el efecto de la resistencia vegetal y la aplicación de BioAct WG (*Purpureocillium lilacinus* cepa 251) sobre *M. incognita* en una rotación tomate-pepino en invernadero. La resistencia vegetal fue el único factor que disminuyó la reproducción del nematodo y aumentó el rendimiento del cultivo, sin apreciarse ningún efecto de BioAct WG. No obstante, en los ensayos *in vitro*, el hongo mostró gran capacidad de control. Las condiciones ambientales durante el cultivo, la composición del formulado comercial, y/o las características fisicoquímicas del suelo podrían haber afectado a su actividad.

Abstract

The general objective of the thesis was to determinate the population dynamics of *Meloidogyne* spp. on tomato and cucumber, the yield losses caused by the nematode and the effect of biologically-based management strategies to reduce the nematode population growth rate and the damage that it causes to the crop. The specific objectives and the main results are: i) To elaborate phenology models of *Meloidogyne* in cucurbit crops. The thermal requirements of *M. incognita* and *M. javanica* on cucumber and zucchini were similar, but different than those on watermelon. On said crop, thermal requirements to complete the life cycle of *M. incognita* and *M. javanica* differed. ii) To characterize the population dynamics of *M. incognita* on cucumber and tomato and the yield losses caused by the nematode. The population growth rate of *M. incognita* on cucumber was higher in summer-autumn than in spring-summer, but the tolerance limit and the minimum relative yield were similar in both cropping periods. On tomato, population growth rate on the resistant cultivar was lower than on the susceptible one. The tolerance limit was similar between cultivars, but the minimum relative production was higher on the resistant. The population growth rate on the resistant cultivar increased progressively each year of repeated cultivation. The evolution of the parameters of the nematode population along years allows for early detection of the selection of virulence. iii) To characterize the effect of the hybrid rootstock RS841 on the population dynamics of *M. incognita* and the cucumber production in a plastic greenhouse. The population growth rate of the nematode on grafted cucumber was higher than on the ungafted crop, to very similar tolerance limits and minimum relative yields. In our conditions, the rootstock RS841 is neither resistant nor tolerant. iv) To detect fungal egg parasites of *Meloidogyne* spp. in vegetable production systems**.** Forty sites were sampled at the end of the cropping cycle, 30 under integrated systems and ten under organic production. Fungal egg parasites of *Meloidogyne* spp. were detected in all organic sites

and in the majority of the integrated ones. The level of parasitism was greater than 40% in some of them. *Pochonia chlamydosporia*, *Fusarium* sp. and *Plectosphaerella cucumerina*, were the most frequent fungi, but only the relative frequency of *P. chlamydosporia* was positively related to the percentage of parasitism in both production systems. v) To characterize suppressive soils to *Meloidogyne* spp. in organic horticulture. A study was carried out to determine the fluctuation of the population of *Meloidogyne* with regards to its density and to the percentage of fungal egg parasitism in two organic production sites under greenhouse. In addition, pot experiments were carried out to certify the soil supressiveness of both soils. Concurrently, genetic microbial profiles were obtained and compared with a non-suppressive soil by means of DGGE. Furthermore, genetic patterns differentiated suppressive from non-suppressive soils. vi) To determine the effect of plant resistance and of Bioact WG (*Purpureocillium lilacinum* strain 251) on *Meloidogyne incognita* in a tomato-cucumber rotation. Plant resistance was the only factor able to suppress nematode densities, disease severity and yield losses, with BioAct WG showing no effect. However, in in-vitro experiments, the fungus showed high capacity of control. Environmental factors during the crop, such as the composition of the formulation, and/or the soil's physicochemical characteristics could have affected its activity.



General introduction

General introduction

Vegetables are one of the most important components of the human diet, especially concerning fresh consumption, and are one of the highest valuable groups of crops for small and large growers (Sikora and Fernández, 2005). Vegetables cover a 1.1 % of the whole agricultural surface of the world, China being the largest producer. Globally, vegetable production has increased by 60 % over the last 20 years, particularly in developing countries (FAO, 2013). In 2013, the European Union's production of vegetables accounted for 5.68 % of the world production on 3.9 % of the total world crop surface (EU-28) (FAOSTAT website), with tomatoes, onions and carrots being the most important crops (EUROSTAT, 2014). Its importance becomes higher in the southern area of the EU-28 countries, where Italy and Spain account for nearly 50% of the EU's production of vegetables. Over the last ten years, the consumption of vegetables in the European Union in general, and in Spain in particular, has increased, even though production has remained stable (FAO, 2013; MAGRAMA, 2013) (Figure 1).

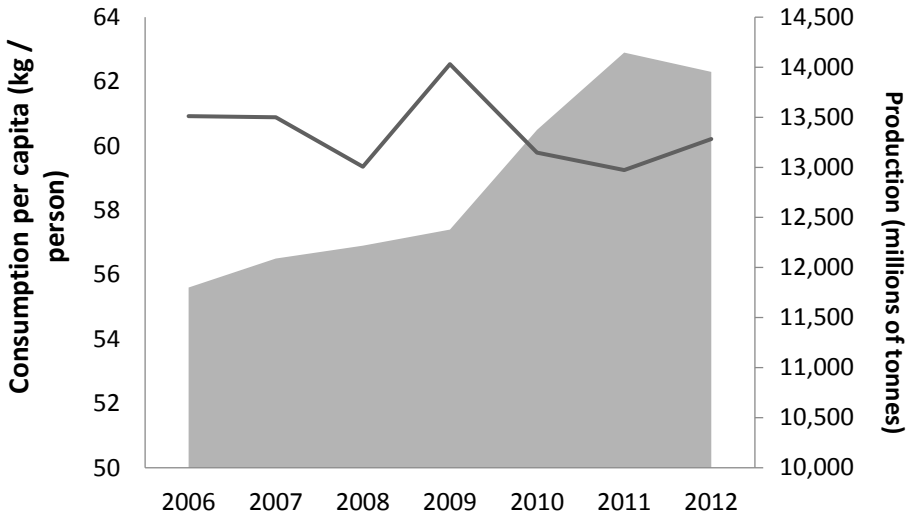


Figure 1. Vegetable consumption and production in Spain from 2006 to 2012. (Source: Representation based on data from MAGRAMA)

The production of vegetables under protected cultivation has expanded significantly throughout the world, the Mediterranean basin being the largest production area. Within the EU-28 Italy is the largest producer, followed by Spain, (Sikora and Fernández, 2005). In Spain the main cultivated crops under protected cultivation belong to the Solanaceae and Cucurbitaceae family (Table 1; FAO, 2002).

Table 1. Annual production, total surface, and surface on protected cultivation for the main solanaceous and cucurbitaceous crops in Spain (MAGRAMA, 2013).

Crop	Annual production (t)	Total surface (ha)	Surface under protected cultivation (%)
Tomato	4046413	48617	38.1
Pepper	970296	17440	61.6
Melon	882869	28130	18.7
Watermelon	871324	18942	32.7
Cucumber	748500	8811	88.2
Zucchini	465039	8879	71.2

Protected cultivation usually comes hand in hand with the latest technology, and is used primarily for intense cropping of high value crops belonging to a few plant species. Thus, monocropping or near monocropping is very common, which brings as a consequence an increased pressure from pests and diseases. In this scenario, plant parasitic nematodes of genus *Meloidogyne* have become one of the most important limiting pathogens to vegetable production (Sikora and Fernández, 2005; Ornat and Sorribas, 2008).

The genus *Meloidogyne* Göldi, 1892

Root-knot nematodes (RKN) were firstly described by Berkeley in 1855, who reported an enormous development of the vascular tissues of cucumber roots (galls), and observed cyst-like bodies (females) filled with eggs and juveniles, both free and inside eggs (egg masses). Subsequent studies included this first described nematode in the genus *Anguillula*, *Heterodera* or *Oxyuris*. In 1887, Göldi proposed the actual name of *Meloidogyne* spp. (apple-shaped female) when he described *Meloidogyne exigua* in coffee roots in Rio de Janeiro, Brazil. However, it was broadly considered to be *Heterodera* until 1949 when Chitwood separated the genera *Meloidogyne* based on morphological differences. *Meloidogyne* was carefully described by Chitwood and the following species were included: *M. arenaria* (Neal) Chitwood, *M. exigua* Göldi, *M. incognita* (Kofoid and White) Chitwood, *M. javanica* (Treub) Chitwood and *M. hapla* Chitwood.

The genus *Meloidogyne* spp. comprises around one hundred species (Hunt and Handoo, 2009), of which 23 have been reported in Europe (Wesemael et al., 2011). Amongst them, *M. chitwoodi*, *M. enterolobii,* and *M. fallax* are included on the A2 list of the European and Mediterranean Plant Protection Organization Alert List (EPPO, 2008). In Spain, nine RKN species have been reported, *M. arenaria*, *M. artiella*, *M. baetica*, *M. dunensis, M. hapla*, *M. hispanica*, *M. incognita*, *M. javanica* and *M. silvestris* (Palomares-Rius et al., 2007; Castillo et al., 2009; Melgarejo et al., 2010).

The most common RKN species, *M. arenaria*, *M. incognita* and *M. javanica* (Sikora and Fernández, 2005) have a wide geographic distribution, mainly in areas with warm summers and short and mild winters. In the Mediterranean basin area these three species are the most abundant, and in Spain are widely distributed within the most of the cropping areas (Ibrahim, 1985; Ornat and Sorribas, 2008; Talavera et al., 2009; Melgarejo et al., 2010).

Meloidogyne spp. can parasitize more than 2000 different hosts plants (Hussey, 1985) including horticultural, fruit and extensive crops. Yield losses caused by the three most widely distributed species reach 60% in cucumber and tomato and 30% in lettuce in Spain (Verdejo-Lucas et al., 1994; Ornat et al., 1997; Sorribas et al., 2005).

Plants infected by *Meloidogyne* spp. showed specific symptoms on their root system (Figure 2) developing galls in roots, which vary in appearance and size depending on the level of infection and on the host plant. The galls affect plant nutrient and water uptake. Thus, aerial symptoms such as leaf chlorosis, stunting and wilting may manifest, although these are similar to those produced by nutrient and water deficiency or by other soil-borne pathogens. The infection also affects flowering, fruit setting, and insufficient growth for marketable standards (Figure 3). Severely affected plants can die.

Figure 2. Severely galled roots of the squash hybrid *Cucurbita maxima* x *C. moschata* (up) and the susceptible tomato cv. Durinta (down) caused by *Meloidogyne incognita*

Figure 3. Aerial symptoms caused by *Meloidogyne incognita*

(a) At the front, susceptible tomato cv. Durinta showing chlorosis, reduced growth and dead plants. At the back, resistant tomato cv. Monika.

(b) On the left, French beans plants cultivated after a susceptible tomato cultivar showing chlorosis and reduced growth compared to the plants on the right, cultivated after a resistant tomato cultivar, with no observable aerial symptoms.

Life cycle

Meloidogyne spp. are obligate sedentary endoparasitic nematodes. The three main species of RKN, *M. arenaria*, *M. incognita* and *M. javanica,* reproduce parthenogenetically (Tyler, 1944). The life cycle of *Meloidogyne* (Figure 4) comprises 6 development stages: egg, 4 juveniles stages (J1 – J4) and the adult.

The life cycle begins when the second-stage juvenile (J2), the only infective and mobile stage, moves into the soil water phase searching for a plant host or attracted by root exudates. The J2 penetrates the roots just behind the root tips by mechanical (stylet thrusts) and possible chemical means (cellulase and pectinase), and it migrates through intercellular spaces of the cortex to the root tip to turn at the meristem. From there it migrates back to the zone of cellular differentiation of the vascular cylinder to establish a permanent feeding site. The establishment of the feeding site in the cells of protoxylem and protophloem induces the differentiation of five to seven cells adjacent to its head in a characteristic way. These cells, named giant cells, suffer morphological, physiological and molecular changes caused by the J2 infection (Karsen and Moens, 2006). Giant cells are formed by karyokineses without cytokinesis containing more than 80 nuclei. Therefore, every cell has a high number of copies of each gene (more than 600) (Wiggers et al., 1990) and are genetically active with a high amount of organelles, ribosomes, and mitochondria, which will supply food to the nematode. The root tissue becomes distorted due to the hyperplasia (intense cell multiplication) around the cell, forming the root gall. Once the nematode establishes the feeding site, it will become sedentary and will growth in length and width adopting a sausage-like shape. At this stage sex differentiation occurs (Taylor and Sasser, 1978). Juveniles molt three more times before reaching the adult stage. Males are vermiform, mobile and without evidence of feeding on plants. Males are scarce, and they appear under

unfavorable conditions, being a density regulation mechanism to avoid intraspecific competition. In favorable conditions, juveniles develop to females.

Females have a pear-shaped body and are sedentary. They lay the eggs into a gelatinous matrix (egg mass) composed of glycoproteins produced by the rectal glands. The egg mass is generally found outside of the gall, but it can also be inside depending on the host plant species, the nematode density at planting, and the distribution of the nematodes in the root. The egg mass is originally soft, sticky and hyaline, but it eventually becomes hard and dark in color (Moens et al., 2009). The egg mass protects eggs against unfavorable environmental conditions and against predation and parasitism because it possesses antimicrobial proprieties (Orion and Kritzman, 1991). A female can lay from 500 to 1,500 eggs in a single egg mass (Ornat and Sorribas, 2008).

Embryogenesis will lead to a J1 developing inside the egg which molds on to the J2. This juvenile will emerge and migrate into the soil in favorable conditions. Hatching depends on soil moisture and temperature, the availability of oxygen, and root diffusates (Curtis et al., 2009). Without a host plant, *Meloidogyne* survives as an egg (undifferentiated, J1 or J2) inside the egg mass, or as a J2 in the soil.

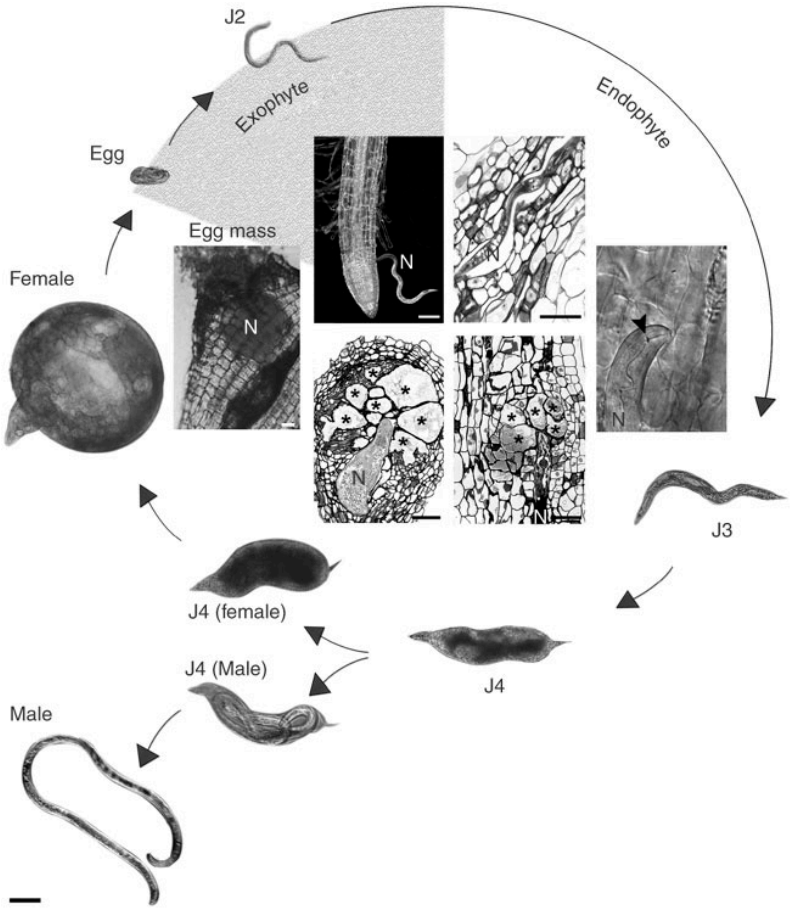


Figure 4. The life cycle of *Meloidogyne* spp. N: Nematode. * :Giant cells. Scale bars, 50 mm. Reprinted with permission from Nature Publishing Group (Abad et al., 2008).

Epidemiology

Meloidogyne spp. are poikilothermic animals, therefore, soil temperature influences the length of their life cycle (Tyler, 1933) and is the main abiotic factor which determines their development, and survival in the absence of a host plant. The optimum temperature for eggs and J2 survival is around 10 °C (Sorribas et al., 2005). Egg hatching occurs at temperatures above 10 ^{o}C (Goodel and Ferris, 1989), and so does nematode development (Ferris et al., 1985). Cardinal temperatures for the completion of the life cycle of the most frequent RKN in vegetable crops are around 10

°C, 28-30 °C and 35 °C (Evans and Perry, 2009). For the development of *Meloidogyne* spp. a constant number of accumulated degree-days (S) above a base temperature (Tb) is required to complete the life cycle of a specific RKN species – plant host pathosystem.

Phenology models of several RKN species have been developed to establish the life cycle completion rate of RKN species on specific hosts, mainly on tomato (Ferris et al., 1978; Lahtinen et al., 1988; Madulu and Trudgill, 1994; Ploeg and Maris, 1999; Tzortzakakis and Trudgill, 2005; Sorribas et al., 2006; Evans and Perry, 2009; Maleita et al., 2012). In this crop, *Meloidogyne* spp. requires between 600 and 700 accumulated degree-days over 10 °C to complete its life cycle (Ferris et al., 1985). In Spain, *Meloidogyne* spp. can complete up to three generations in tomato in spring (Sorribas et al., 2005; Talavera et al., 2009; Verdejo-Lucas et al., 2009) but only one in autumn (Talavera et al., 2009).

Besides the temperature, the fluctuation of nematode densities over time, defined as population dynamics (Greco and Di Vito, 2009), is conditioned by biotic factors, such as, food availability, crop status, nematode population density at the beginning of the crop (*Pi*), intraspecific and interspecific competition, and the presence of antagonists.

The relationship between the final population density (*Pf*) and *Pi* is represented in Figure 5 by the Nicholson model (1935):

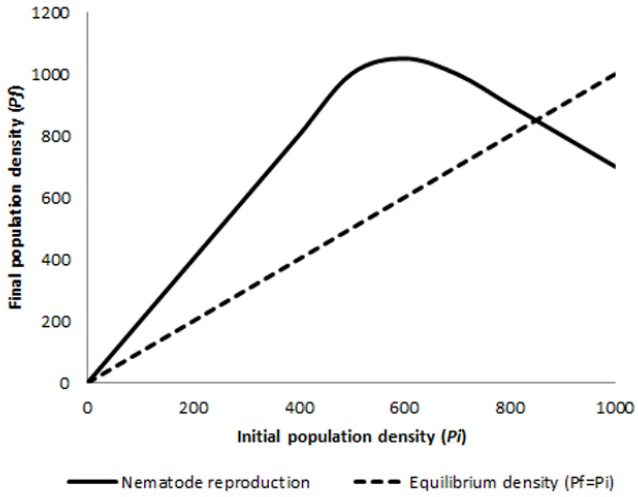


Figure 5. The relationship between initial (*Pi*) and final (*Pf*) population densities of *Meloidogyne* spp. and equilibrium density (*Pf* = *Pi*).

Under favorable conditions to the development of the RKN populations, when there is enough food and low nematode inoculum pressure at planting, the nematode density at the end of the crop will be proportionally related to *Pi*. That is, the relationship between *Pf* and *Pi* will be linear (Seinhorst, 1970):

$$Pf = a\ Pi$$

being *a* the maximum multiplication rate. The *a* value is determined be linear regression at lowest *Pi* (Greco and Di Vito, 2009). As *Pi* increases, *Pf* increases too but at lower rate than *a* up until the ceiling level is achieved (Oostenbrink, 1966); that is, *Pi* at which the *Pf* is maximum. At *Pi* higher than that at ceiling level, *Pf* decreases due to intraspecific competition for food and by increasing root damage. *Pi* at which *Pf* remains unchanged is called equilibrium density (*E: Pf = Pi; Pf/Pi=1*) at which there is enough food to maintain the nematode density at planting. The nematode population will tend to *E* in specific environmental conditions and plant genotype (Seinhorst, 1967).

These parameters (*a* and *E*) are indicators of the host status and the tolerance of a plant (Seinhorst, 1967). Higher values of *a* and *E* are indicators of a good host and lower values determine poor host or resistant host of the nematode.

As previously stated, the damage caused by RKN influences their population dynamics, and also plant growth and yield. To estimate crop yield losses it is necessary to know the relationship between *Pi* and the relative yield by the Seinhorst's damage function model (Figure 6) (Seinhorst, 1965):

$$y = m + (1 - m)\, z^{(Pi-T)}$$

where y = relative yield ($Pi \geq T$, $y = 1$ when $Pi < T$), m = the minimum yield, z = a constant ≤ 1, Pi = initial population density; T = tolerance.

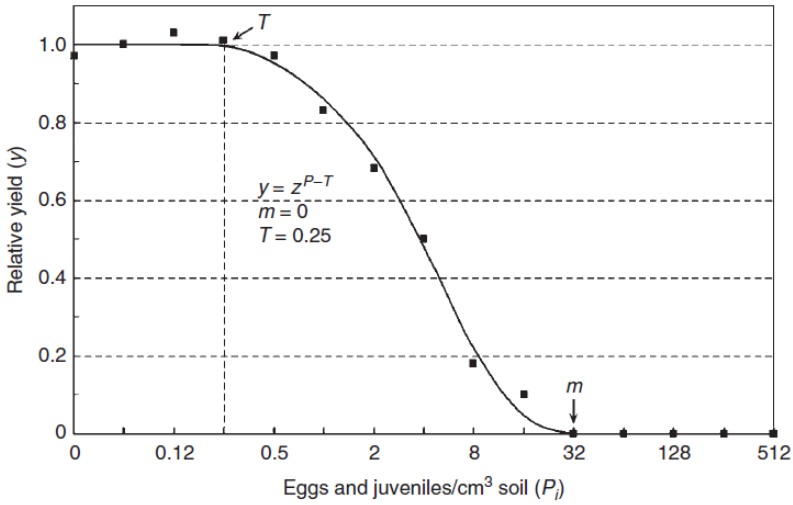


Figure 6. An example of the relationship between initial population densities (*Pi*) of *Meloidogyne incognita* and relative yield (*y*). Reprinted with permission from CABI (Greco and Di Vito, 2009).

This model provides some indicators of the plant tolerance (*T*), the maximum population density that can support a plant without yield losses; the minimum relative yield (*m*) produced by high nematode densities, and the *z* value, the damage produced by a single nematode.

The three models previously described: phenology, population dynamics and damage function models are objective tools that can be used to inform decisions on how to manage nematode densities in an integrated nematode management basis. The phenology models will be useful to decide the crop planting date and / or harvest; to reduce the number of life cycles of the nematode and the damage to the present crop; to reduce *Pi* that can affect the next crop in a rotation sequence; to use trap crops uprooting plants before the female starts laying eggs causing a reduction of the remaining nematode density in soil at the end of the crop; or to decide when to intervene to delay or interrupt the nematode cycle by using biological or chemical nematicides. Population dynamics models are used to choose the most suitable crops to include in a rotation sequence according to their host status and the prediction of the final population density at the end of the crop that could infect the next crop. The damage function models serve to determine the tolerance limit and economic thresholds for a given pathosystem, as well the effect of control measures on crop yield. The combination of data from all three models enables us to better understand the evolution and the impact of the nematode populations on any vegetable crop.

Control methods

The main objective of RKN management is to maintain nematode densities below the economic threshold by reducing initial population densities before sowing or transplanting, or by reducing the rate of growth of the population densities during the crop.

Methods to reduce the initial population density

Quarantine prevents nematode introduction in a country or local production area with inspection and certification of plant material (Karssen and Moens, 2006). Also, using sanitation measures such as cleaning the equipment and the machinery prevents the introduction and / or the spread of *Meloidogyne* in a farm (Wesemael et al., 2011).

Chemical control by fumigant and non-fumigant nematicides is the most common control method (Talavera et al., 2012). Fumigants are volatile chemical compounds which have to be applied before transplanting or sowing, and they are active against a broad-spectrum of pests and pathogens presents in the soil. Methyl bromide was the most used fumigant due to its efficacy (Zasada et al., 2010). However, it is no longer available (UNEP, 2006) as it has been proven to contribute to the depletion of the ozone layer (Zasada et al., 2010). Non-fumigants nematicides are liquid or solid formulates that can be applied before and/or after planting the crop. Although these products have shown high effectiveness against nematodes, the repeated use of some active compounds can boost the populations of some microbial organisms able to degrade them quickly which can lead to a loss of the product's effectiveness (Mojtahedi et al., 1991). Nowadays, in Spain, 1,3 dichloropropene and metam-sodium are the most commonly used fumigants against RKN (Talavera et al., 2012) and are more effective than non-fumigants (Ornat and Sorribas, 2008). Currently, the European Regulation 1107/2009 aims to improve safety on human health and on the environment by restricting the authorization of new active substances and products; which have to be more specific, less persistent and less hazardous. Moreover, nematicide banning as well as restrictions on their use by the Directive 2009/128/CE and the increasing consumer's concern about their toxicity and

environmental impact have promoted the research on this field and the use of more sustainable alternatives for RKN control.

Organic amendments and *biofumigation* use organic matter to enhance microbial activity which can antagonize with some nematodes. Green or fresh manure is also used to that end, as the increased temperature as well as some toxic compounds linked to its decomposition can be effective against RKN and other plant pathogens, pests and weeds (FAO, 2016). The incorporation of these products improves physicochemical and biological properties of soil (Sorribas and Ornat, 2008). The efficacy of these methods against RKN depends on the material composition, the dosage and the application system (Bello et al., 2002; Matthiessen and Kirkegaard, 2006). Higher efficacy has been obtained in combination with solarization (Ploeg, 2008).

Steaming and *solarization* consists on warming the soil by steam or by solar radiation, respectively. Steaming is effective if soil temperature is increased up to 70 °C for at least 30 minutes (Runia, 2000). This method requires a high amount of water and energy (Crump, 2001) making it difficult to use in some cropping areas and economic conditions. Solarization consists on covering a plot of moist land with polyethylene plastic and sealing it. Solar radiation then increases the temperature of the top 30cm of soil to levels that are lethal to nematodes which is 45 °C (Hidalgo-Diaz and Kerry, 2008). To significantly reduce nematode populations, a minimum of 6-8 weeks is required (Ornat and Sorribas, 2008). In Mediterranean conditions, with warm and dry summers, the effectiveness of solarization may reach 99% (Greco et al., 1992).

Soil-less cultivation replaces the indigenous soil by other natural or artificial substrate free of pathogens. However, the most commonly used substrates are suitable for nematode infestation (Stapel and Amsing, 2004) through infested plant material, water, equipment etc. (Hallmann et al., 2005).

Tillage and *black fallow.* Tillage at the end of the crop reduces the rate of nematode survival and consequently the nematode densities at transplanting for the following crop (Ornat et al., 1999). Black fallow between crops will cause *Meloidogyne* to use their own reserves and die from starvation (Ornat and Sorribas, 2008), especially if weather conditions between crops are warm and dry. Its efficacy depends on the length of the fallow period and on the accumulated soil temperature. In Mediterranean summer, eight weeks of black fallow can reduce nematode populations up to 50% and, when combined with tillage, it can increase the mortality rate and reduce the nematode density up to 80% (Ornat et al., 1999). Soil tillage between crops will also remove possible host weeds in which the nematode could reproduce (Barceló et al., 1997; Sikora and Fernandez, 2005).

Planting date and *trap crops.* The duration of the life cycle of *Meloidogyne* depends on soil temperature because is a poikilothermic animal. Therefore, advancing or delaying the planting date may reduce the population of *Meloidogyne* if the crop finishes its cycle before females start laying eggs, just like with trap crops. Similarly, this practice can reduce the rate of infection and reproduction, and the number of generations during a given crop's cycle by reducing the time of interaction plant-RKN. Trap cropping to control RKN consists on planting highly sensitive host plants for a period of time long enough to allow infection, after which the root is booted, removed and destroyed with the nematodes inside (Ornat and Sorribas, 2008). Some of the trap crops can be profitable for growers, such as lettuce (Ornat et al., 2001). Many weeds are hosts of *Meloidogyne* (Barceló et al., 1997; Ornat and Verdejo-Lucas, 1999; Castillo et al., 2008) and contribute to the prevalence of the nematode during a crop's cycle and between crops (Ponce et al., 1995). Removing roots from the soil at the end of the crop may also decrease population density for the following crop.

Methods to reduce the initial population density and the multiplication rate.

Crop rotation and *plant resistance* are the main control measures against *Meloidogyne* spp. (Ornat and Sorribas, 2008). A rotation that includes non-host, poor host or resistant host will reduce the multiplication rate of the nematode population and, consequently, the initial population density for the next crop (Sorribas et al., 2005; Talavera et al., 2009) which will reduce crop yield losses. However, in an intensive vegetable production system, only the most economically profitable crops are cultivated (van der Putten et al., 2006), which often leads to monocropping. However, commercial resistant cultivars or rootstocks are available that limit the reproductive capacity of plant parasitic nematodes (Roberts, 2002). Their use does not adversely affect the environment and their economic cost is not high in comparison to the costs of conventional nematicides (Miguel, 1997). One of the most widely used resistant cultivars is tomato carrying the *Mi* gene, which is effective against the most common species of RKN present in vegetable production areas in Spain (Sorribas and Verdejo-Lucas, 1994; Verdejo-Lucas et al., 2002). Resistant cultivars and rootstocks can be grown in RKN infested soil without significant tomato yield losses (Rich and Olson, 1999; Sorribas et al., 2005; Verdejo-Lucas et al., 2009), suppressing the rate of RKN multiplication and consequently reducing the *Pi* for the following crop (Hanna et al., 1993; Ornat et al., 1997). Nevertheless, the expression of the *Mi* gene can be limited by soil temperatures above 28°C over a period of time (Dropkins, 1969), by the genetic background of the tomato cultivar (Cortada et al., 2009), by some RKN species (Brown et al., 1997; Kiewnick et al., 2009; Silva et al., 2008), by virulent RKN populations which can occur suddenly (Ornat et al., 2001) or by the repeated cultivation of cultivars or rootstocks carrying the *Mi*-gene (Verdejo-Lucas et al., 2009). Regarding cucurbits, several cultivars and rootstocks have been tested against RKN (Sigüenza et al., 2005; Davis et al., 2008, Lee and Oda, 2010; Kokalis-Burelle and Rosskopf, 2011; Liu et al.,

2015; Thies et al., 2015; López-Gómez et al., 2016), and although some of them have proved to be effective to control RKN, they are not widely available or used nowadays.

Biological control. Several biological agents have been characterized as antagonist of nematodes. They can decimate their populations directly by parasitism (bacteria and fungi) or predation (protozoa, nematodes, arthropods), or indirectly (bacteria, fungi) by inducing plant defense mechanisms or interfering with the host recognition process (Stirling, 2011; Schulz and Boyle, 2006).

The detection, characterization, and selection of potential biological control agents have remarkably increased since the 70's (Hallmann et al., 2009). In Spain, several antagonists have been identified, including some fungal egg parasites (Olivares-Bernabeu and López-Llorca, 2002; Verdejo-Lucas et al., 2002 and 2013; Gené et al., 2005). The frequency of detection of fungal parasites of nematodes fluctuate between 13% and 82.4% (Olivares-Bernabeu and López-Llorca, 2002; Gené et al., 2005; Verdejo-Lucas et al., 2002 and 2013), and in some soils, several fungal species coexisted, but fungal egg parasitism was too low (up to 10.9%) to be considered an effective natural control method against RKN (Verdejo-Lucas et al., 2002 and 2013). Nevertheless, the antagonist potential of the soil could be enhanced to achieve soil suppressiveness by implementing a combination of farming practices (Sikora, 1992) or by applying biological nematicides.

Suppressive soils are defined as soils in which soil-borne pathogens do not establish or persist, establish but cause little or no damage, or establish and cause disease for a limited period of time (Baker and Cook, 1974). Suppressive soils have been described for many soil-borne pathogens including nematodes (Weller et al., 2002), mainly of cyst nematodes (Kerry et al., 1988; Westphal and Becker, 1999, 2000 and 2001), and a few of *Meloidogyne* spp. (Pyrowolakis et al., 2002). All of these soils contained a variety of nematode antagonists, but the mechanisms involved in

suppression are still poorly understood because of the great number of biological processes and interactions in the soil. Nowadays, growers are changing their horticultural production system from conventional to integrated or organic systems. They are increasing their use of organic matter as a soil amendment and fertilizer, and they are also reducing the use of pesticides. This change could result in an enhanced antagonistic potential of the soil against soil-borne pathogens, including plant-parasitic nematodes.

Biological control agents can also be applied by commercial formulates of nematode antagonists when natural antagonism does not occur or happens at a rate that does not provide enough level of control. At present, *Purpureocillium lilacinum* strain 251 (Pl251) is the only biological control agent registered against plant parasitic nematodes that is included in Annex 1 of the Directive 91/414/CE. Some studies have proven the effectiveness of this isolate against *Meloidogyne spp.*, irrespective of the formulation, in controlled conditions and pot test (Kiewnick and Sikora, 2003 and 2006). However, in field conditions, the effectiveness of a commercial formulation of Pl251, was not satisfactory (Anastasiadis et al., 2008; Kaşkavalci et al., 2009), but the causes for these contradicting results remain unknown.

Integrated nematode management (INM)

The Directive 2009/128/EC establishes the framework to achieve the sustainable use of pesticide by reducing the risks they pose and their effects on human health and the environment. It does so by promoting integrated pest management, thus prioritizing the use non-chemical methods.

In a nutshell, the integrated nematode management is based on understanding the biology of the different species of nematode, their population dynamics considering the biotic and abiotic factors that can influence them, their effect on crop

yields, and the effectiveness and the cost of the available control methods. This basic information can be used to assess damage, to establish the economic and the intervention thresholds with an aim to develop predictive models as tools for decision making. The number of orchards conducted under integrated and organic production systems is increasing with a reduction of fumigant and non-fumigant nematicides by regulatory restriction or banning. Therefore, it is necessary to design strategies for nematode management. This can be done using a combination of compatible control methods, seen as the use of a single method may not always achieve the expected effectiveness and / or could not be durable.

The knowledge of the epidemiology of *Meloidogyne* spp., the prediction of the yield losses and the combination of different control methods such as biological control, plant resistance and crop rotation is essential to provide useful information to growers in a specific growing area in order to achieve a successful and durable management of nematode populations within the framework of an integrated nematode management program.

Objectives

Objectives

The main objective of this PhD thesis was to generate basic information on population dynamics of *Meloidogyne* spp. in tomato and cucumber crops. Our aim was to develop integrated management programs that included plant resistance (cultivars and rootstocks) and biological control by conservation and augmentation of native microorganisms or by introducing foreign microorganism using commercial formulates. This objective was divided into the following specific objectives:

1. To elaborate phenology models of *Meloidogyne* spp. on cucurbit crops. (Chapter 1).

Meloidogyne spp. are poikilothermic animals. Consequently, soil temperature will influence their rate of development. A constant amount of accumulated degree-days over a base temperature (temperature below which no development occurs) are required for *Meloidogyne* to change from one developmental stage to the next and to complete its life cycle. The thermal requirements of *Meloidogyne incognita* and *M. javanica* on tomato have been reported, but there is no information on cucurbit crops. Thus, the thermal requirements of *Meloidogyne incognita* and *M. javanica* on cucumber, watermelon and zucchini were determined to construct their respective phenology models.

2. To estimate the population dynamics of *Meloidogyne incognita* in cucumber and tomato and to determine the relationship between the initial population density and the crop yield in plastic greenhouse conditions. (Chapter 2 and 3).

The population density of *Meloidogyne* spp. increases proportionally to that at planting in the absence of limiting factors. The maximum multiplication rate, achieved at lowest population densities when there is no food scarcity or competition, as well

as, the ceiling point, which is the maximum *Pf*, and, the equilibrium density, the population density at planting at which the plant can supply enough food to maintain it at the end of the crop, are indicators of the host status under particular conditions. In addition, the relationship between initial population density and relative crop yield provides indicators of plant tolerance and minimum relative yield by the Seinhorst's damage function model. All these indicators are the basis to design strategies to manage RKN, and none of them are known for two of the most important vegetable crops in northeastern of Spain. Thus, the maximum multiplication rate, the ceiling level, the equilibrium density, the tolerance limit, and the minimum relative crop yield were determined for the *M. incognita*-cucumber and *M. incognita*-tomato pathosystems in plastic greenhouse condition over several cropping cycles.

3. To characterize the influence of the cucurbit rootstock, *Cucurbita maxima* x *C. moschata* RS841, on population dynamics of *Meloidogyne incognita* and yield of cucumber in plastic greenhouse. (Chapter 4).

The squash interspecific hybrid RS841 (*Cucurbita maxima* x *C. moschata*) is the most widely used rootstock for cucumber, melon, and watermelon in Europe owing to its resistance against limiting soil-borne diseases affecting these crops. It is also most valued for its tolerance to abiotic stresses and crop yield improvement. However, little is known about its influence on population dynamics of *M. incognita* and cucumber yield. Thus, the maximum multiplication rate, the ceiling level, the equilibrium density, the tolerance limit, and the minimum relative crop yield were determined for the *M. incognita*-cucumber ungrafted or grafted pathosystems in plastic greenhouse condition over two cropping cycles. In addition, the relationship between the initial population densities and ecophysiological parameters of grafted or ungrafted cucumber as well as crop yield were assessed.

4. To determine the occurrence of fungal egg parasites of *Meloidogyne* and the level of parasitism in organic and integrated vegetable production systems in Spain. (Chapter 5).

Soils managed under sustainable production systems may have enhanced microbial diversity and activity partly due to the use of organic amendments and cover crops as fertilizers, as well as to a very limited use of chemical nematicides. Nematode antagonists, such as fungal egg parasites, could be found in the soil's microbial community. Some studies have been conducted to list the fungal egg parasites of RKN, but little is known about the biodiversity of fungal egg parasites of *Meloidogyne* and the percentage of fungal egg parasitism in commercial sites destined to vegetable production under organic and integrated standards. Similarly, not enough was known on the relationship between fungal egg parasitism of *Meloidogyne* and the physicochemical and enzymatic properties of the soil. Thus, a study was conducted to increase knowledge in this area.

5. To characterize suppressive soils to *Meloidogyne* spp. in organic horticulture in plastic greenhouse. (Chapter 6).

In suppressive soils, pathogens do not persist, or establish but cause little or no damage. Suppressive soils to plant parasitic nematodes have been mostly found in sustainable production fields which use environmentally friendly alternatives to preserve and enhance beneficial organisms. Regarding RKN in vegetable crops, little information has been reported. Thus, a study was carried out to determine the fluctuation of the population of *Meloidogyne* with regards to its density and to the percentage of fungal egg parasitism in two organic commercial production sites and in pot tests, to determine the level of suppressiveness. In addition, bacterial and fungal profiles were obtained and compared with a conductive soil to determine the putative microbes involved in RKN suppression.

6. To determinate the effect of plant resistance and the application of BIOACT WG (commercial formulate based of *Purpureocillium lilacinum* strain 251) on *Meloidogyne incognita* in a tomato-cucumber rotation in a greenhouse. (Chapter 7).

Combining control methods could benefit the management of *Meloidogyne* by reducing *Pi,* the multiplication rate and consequently increasing crop yield for the current and the following crop in a rotation sequence. Nowadays, BioAct WG is the only bionematicide included in Annex 1 of the Directive 91/414/EEC, and little information is available on the efficacy of formulates of *Purpureocillium lilacinus* strain 251 in Mediterranean conditions, and nothing is known on the extent to which combining this biological control agent with plant resistance is effective or otherwise. Thus, the effectiveness of combining resistant tomato cultivar with BioAct WG against *M. incognita* was assessed in a tomato-cucumber rotation in plastic greenhouse during two consecutive years. In addition, *in vitro* experiments were carried out to understand the performance of BioAct WG in plastic greenhouse conditions.

Chapters

Relationship of chapters and publications

Chapter 1: Thermal requirements of *Meloidogyne* spp. in cucurbit crops.

Giné, A., López-Gómez, M., Vela, M. D., Ornat, C., Talavera, M., Verdejo-Lucas, S., Sorribas, F. J. 2014. Thermal requirements and population dynamics of root-knot nematodes on cucumber and yield losses under protected cultivation. *Plant pathology* 63, 1446-1453.

López-Gómez, M., Gine, A., Vela, M. D., Ornat, C., Sorribas, F. J., Talavera, M., Verdejo-Lucas, S. 2014. Damage functions and thermal requirements of *Meloidogyne javanica* and *Meloidogyne incognita* on watermelon. *Annals of Applied Biology* 165, 466-473.

Vela, M. D., Giné, A., López-Gómez, M., Sorribas, F. J., Ornat, C., Verdejo-Lucas, S., Talavera, M. 2014. Thermal time requirements of root-knot nematodes on zucchini-squash and population dynamics with associated yield losses on spring and autumn cropping cycles. *European journal of plant pathology* 140, 481-490.

Chapter 2: Population dynamics of root-knot nematodes on cucumber and yield losses under protected cultivation

Giné, A., López-Gómez, M., Vela, M. D., Ornat, C., Talavera, M., Verdejo-Lucas, S., Sorribas, F. J. 2014. Thermal requirements and population dynamics of root-knot nematodes on cucumber and yield losses under protected cultivation. *Plant pathology* 63, 1446-1453.

Chapter 3: Population dynamics of *Meloidogyne incognita* and yield losses of susceptible and resistant tomato cultivars in Mediterranean conditions.

Giné A., Sorribas, F. J.. Population growth of *Meloidogyne incognita* and yield losses of susceptible and resistant tomato cultivars in Mediterranean conditions. *Plant pathology.* Summited

Chapter 4: Population dynamics of *Meloidogyne incognita* on cucumber grafted onto the Cucurbita hybrid RS841 or ungrafted and yield losses under protected cultivation.

Giné, A., González, C., Serrano, L., Sorribas, F. J. Population dynamics of *Meloidogyne incognita* on cucumber grafted onto the Cucurbita hybrid RS841 or ungrafted and yield losses under protected cultivation. *European Journal of Plant pathology.* Summited

Chapter 5: Natural occurrence of fungal egg parasites of root-knot nematodes, *Meloidogyne* spp. in organic and integrated vegetable production systems in Spain.

Giné, A., Bonmatí, M., Sarro, A., Stchiegel, A., Valero, J., Ornat, C., Fernández, C., Sorribas, F. J. 2013. Natural occurrence of fungal egg parasites of root-knot nematodes, *Meloidogyne* spp. in organic and integrated vegetable production systems in Spain. *BioControl* 58, 407-416.

Chapter 6: Characterization of soil suppressiveness to root-knot nematodes in organic horticulture in plastic greenhouse.

Giné, A., Carrasquilla, M., Martínez-Alonso, M., Gaju, N., Sorribas, F. J. (2016). Characterization of soil suppressiveness to root-knot nematodes in organic horticulture in plastic greenhouse. *Frontiers in Plant Science*, *7*, 164.

Chapter 7: Effect of plant resistance and BioActWG (*Purpureocillium lilacinum*s strain 251) on *Meloidogyne incognita* in a tomato–cucumber rotation in a greenhouse.

Giné, A., Sorribas, F. J. 2016. Effect of plant resistance and BioAct WG (*Purpureocillium lilacinum* strain 251) on *Meloidogyne incognita* in a tomato–cucumber rotation in a greenhouse. *Pest Management Science*. DOI**:** 10.1002/ps.4357

Chapter 1

Thermal requirements of *Meloidogyne* spp. in cucurbit crops

Thermal requirements of *Meloidogyne* spp. in cucurbit crops.

Thermal requirements of *Meloidogyne incognita* and *M. javanica* were determined for development (from inoculation to females starting laying eggs (FLE)), egg production and emergence of J2 (EH: from FLE to starting egg hatching), and completion of the life cycle (LCC) on cucumber cv. Dasher II, watermelon cv. Sugar Baby, and zucchini-squash cv. Amalthee HF1. Plants were cultivated in pots inoculated with 1 second stage juveniles (J2) per 1 cm^{-3} of soil of each *Meloidogyne* species at constant temperatures of 17, 21, 25, and 28 °C. Plants were assessed periodically to follow the process, and daily when the biological stage was expected to occur. Base temperature (*Tb*) and the accumulated degree days (*S*) were then calculated. Thermal requirements of *M. incognita* and *M. javanica* on cucumber did not differ, irrespective of the biological stage (FLE: *Tb* = 12.1 °C*, S* = 294DD; EH: *Tb* = 8.0 °C, S = 213 DD; LCC: Tb =11.4 °C, *S* = 500DD), as well as on zucchini-squash (FLE: *Tb* = 12.1°C, *S* =277.8DD; EH: *Tb* = 8.7 °C, S = 217.4 DD; LCC: *Tb* = 11°C, S =476.2DD). On watermelon, thermal requirements of both nematode species were similar for development (*Tb* = 14.9°C S = 243.9DD) but did not for the completion of the life cycle (*Tb* = 14°C, *S* = 500DD for *M. incognita* and *Tb* = 17.2°C, S = 357.1DD for *M. javanica*).

Keywords: Base temperature, *Citrullus lanatus, Cucurbita pepo***,** *Cucumis sativus,* Degree-days, *M. incognita, M. javanica***.**

Introduction

Root-knot nematodes (RKN), *Meloidogyne* spp., are obligate parasites of plants, including vegetables, with *Meloidogyne arenaria*, *M. incognita* and *M. javanica* being the major ones responsible for yield losses (Sikora and Fernández, 2005). The amount of yield loss varies according to the host status, agronomic practices and environmental conditions (mainly temperature) in irrigated agriculture (Ferris, 1986).

Meloidogyne spp. are poikilothermic animals, and therefore soil temperature will influence the rate of development (Tyler, 1933), and hence the number of generations, that the nematode will complete in a cropping cycle, the rate of population growth, and consequently the crop yield losses (Ehwaeti et al., 1998). A constant number of accumulated degree-days (*S*) over a base temperature (*Tb*: temperature below which no development occurs) is required for a crop to develop (Elnesr et al., 2013), as well as for RKN to complete the life cycle on a given plant or to change from one developmental stage to the next. Phenology models have been developed for life cycle completion of several RKN species, and for embryogenesis and emergence of second-stage juveniles (J2) (Ferris et al., 1978; Lahtinen et al., 1988; Madulu and Trudgill, 1994; Ploeg and Maris, 1999; Tzortzakakis and Trudgill, 2005; Maleita et al., 2012). However, from an epidemiological point of view, knowledge of thermal requirements for specific processes such as infection, development, and production and emergence of new inoculum (J2) can help with making decisions to reduce RKN population growth and crop yield losses; for example, the use of trap crops, changes of planting date to escape infection, or to reduce the number of generations and population densities for the following crop. The life cycle of RKN comprises (i) infection: infective juveniles penetrate roots of a plant host establishing a permanent feeding site, become sedentary and the width of juveniles increases (swollen J2); (ii) development: under favorable conditions, J2 will moult three times to achieve the mature adult female stage; (iii) production and emergence of new inoculum: females reproducing parthenogenetically lay a large number of eggs in a gelatinous matrix, the egg mass, onto the surface and/or into the galled roots. Most eggs develop to fully formed first-stage juveniles that moult once within the egg. The J2 will emerge from the egg and leave the egg mass to search for a root (Taylor and Sasser, 1978).

Cucumber (*Cucumis sativus*) is cropped in many countries around the world, and in some areas is usually included in rotation with species of Solanaceae in double-

cropping systems (Sorribas and Verdejo-Lucas, 1994; Ornat et al., 1997; Thies et al., 2004). In Spain, cucumber is cropped in 8148 ha with an annual production of 664 975 tonnes, of which 63.5% are grown under protected cultivation (Ministerio de Medio Ambiente, Medio Rural y Marino, 2012). RKN are present in all vegetable production areas (Melgarejo et al., 2010), in which 20–40% of fields are infested (Verdejo-Lucas et al., 2002; Ornat and Sorribas, 2008; Talavera et al., 2012). Cucumber is susceptible to most common RKN species, which can reduce cucumber yield up to 60% in commercial protected cultivation (Ornat et al., 1997) and the economic losses in south-eastern Spain were estimated at € 918293 (Talavera et al., 2012) .

Watermelon, (*Citrullus lanatus*) is severely damaged by RKN (Thies and Levi, 2003; Pofu et al., 2011). In Spain, is cultivated in about 18600 ha with an annual production of 782000 tonnes, of which 48% are produced under protected cultivation (Ministerio de Medio Ambiente, Medio Rural y Marino, 2012). The economic losses due to RKN on watermelon under protected cultivation in south-eastern Spain were estimated around €451940 (Talavera et al., 2012).

Zucchini-squash is an important crop in Southern Spain, where it is cultivated either as a spring or autumn crop in growing periods of three to five months. Most of the production is concentrated in the provinces of Almería, Cádiz and Granada, which account for one third of the total production in Spain (Ministerio de Medio Ambiente, Medio Rural y Marino, 2012). In 2010, 7618 ha were dedicated to zucchini-squash cultivation in Spain with a production of 366498 tonnes. Economic losses caused by RKN on zucchini-squash under protected cultivation in south-eastern Spain were estimated about €649504 (Talavera et al., 2012).

At present, there is little information available on thermal requirements of the nematode on cucumber, watermelon and zucchini-squash. Therefore it is important to standardize conditions in order to compare population growth and crop yield losses

according to the number of generations which RKN species can complete during the cropping cycle in a given area. Thus, the objectives of this study were to determine the thermal requirements of infection, development, eggs production and emergence of J2 from them, and completion of the life cycle of *M. incognita* and *M. javanica* on cucumber, watermelon and zucchini-squash.

Materials and methods

The development of two RKN isolates of *M. incognita* and *M. javanica* on cucumber cv. Dasher II (Seminis), watermelon cv. Sugar Baby (Intersemillas) and zucchini-squash cv. Amalthee HF1 (Gautier) was determined at constant temperatures of 17, 21, 25 or 28°C in climatic growth chambers with a 16:8 h light:dark photoperiod. The inoculum was produced on the susceptible tomato cv. Durinta (Western Seed). Nematode eggs were extracted from infected roots in a 0.5% NaOCl solution for 5 min (Hussey and Barker, 1973). The egg suspension was then passed through a 74 µm aperture sieve to remove root debris, and the dispersed eggs were collected on a 25 µm sieve and placed on Baermann trays (Whitehead and Hemming, 1965) at 25°C to obtain J2 as inoculum. Juveniles were collected daily on a 25 µm sieve but those collected after the first 24 h of incubation were discarded, and those emerged from day 2 to 5 were stored at 9°C until we used as inoculum. Aliquots of the J2 suspensions were placed in the respective growth chambers for 8 h before inoculation to acclimatize J2 to the tested temperatures.

Cucumber, watermelon and zucchini-squash seeds were germinated in plastic trays containing vermiculite and 2-week-old seedlings were transplanted singly to 200 cm^3 pots containing sterilized river sand. Plants were allowed to grow in the respective growth chamber for 1 week prior to inoculation with 1 J2 per cm^3 of soil. The inoculum was added into opposite holes at 1 cm apart from the stem and 3 cm deep. Plants were irrigated as needed with distilled water at the same temperature to the respective

growth chambers, preventing temperature variations in the soil while watering. Plants were fertilized with a slow release fertilizer (15% N + 9% P2O5 + 12% K2O + 2% MgO2 + microelements; Osmocote Plus) at the time of transplanting.

Assessments were made of the number of days until (i) infection of roots by J2, e.g. first swelling J2 was observed, (ii) development from swelling J2 to female starting laying eggs, (iii) from egg production to egg hatching commencing, as indicated by the presence of empty egg shells, and iv) life cycle completion, from J2 inoculation to first egg hatch of J2. Three plants per incubation temperature were removed daily until infection occurred. Thereafter, plants were assessed periodically to follow the process, and daily when the biological stage was expected to occur. Nematodes inside roots were stained with 0.05% acid fuchsine (Panreac Quimica SA; Bridge and Page, 1982), cleared and observed the same day under a stereoscopic microscope. To assess both, egg production and J2 emergence (empty eggs), the entire root system was submerged in a 0.1 g L^{-1} erioglaucine (Acros Organics) solution for 2 h to stain the gelatinous matrix of the egg masses blue (Omwega et al., 1988). Egg masses were handpicked and dispersed in a 1% NaOCl solution to determine the start of egg production and empty eggs to record the beginning of the hatching process.

Soil temperatures were recorded daily at 30-min intervals with a probe PT100 (Campbell Scientific Ltd) placed 4 cm deep into the potted soil in each growth chamber. At the end of the experiment, the average soil temperatures recorded in each growth chamber were calculated. The number of days required for infection, development, production of eggs and emergence of J2, was expressed as the reciprocal of the time elapsed between biological stages (per day) to obtain a linear relationship with soil temperatures. The base temperature (*Tb*) was calculated from the regression equation considering the reciprocal of time = 0 (reciprocal of time = aT-b; *Tb* = b/a), and *S* (thermal constant: accumulated degree days (DD) over *Tb*) as the reciprocal of the

slope (1/a; Trudgill, 1995). Regression lines for each biological stage and plant species were compared between RKN species by the general lineal model procedure (proc glm) of SAS software v.9. When no differences ($P < 0.05$) were found, a single general model was then obtained from pooled data. In addition, the regression lines for each biological stage were compared between plant species to obtain a general model when no differences ($P < 0.05$) were found.

Results

Mean soil temperatures in each growth chamber for the *M. incognita* experiment were 16.8 ± 0.6°C, 21.6 ± 0.8°C, 24.3 ± 0.7°C and 27.8 ± 0.8°C, and 17.2 ± 0.4°C, 21.4 ± 0.7°C, 24.4 ± 0.7°C and 27.6 ± 0.8°C for the *M. javanica* experiment.

The reciprocal of time until infection occurred did not increase linearly with soil temperatures, probably as a result of the time elapsed between assessments (daily interval) (data not shown). Thus, development was assessed from inoculation to egg production.

Thermal requirements and values of *Tb* and *S* for development, egg hatching, and the completion of life cycle of *M. incognita* and *M. javanica* on cucumber, watermelon and zucchini-squash are shown in Table 1. The reciprocal of time for development, did not differ ($P < 0.05$) between RKN species, irrespective of plant species (Figure 1), either for egg hatching (Figure 2) and the life cycle completion on cucumber or zucchini-squash, but did for life cycle completion on watermelon (Figure 3). In watermelon, the reciprocal of time from female of *M. javanica* starting laying eggs to J2 emergence did not increase linearly with soil temperatures, because of egg hatching did not occur at the lowest temperature of incubation assessed (17 ^{o}C; data not shown).

Thermal requirements for FLE of both RKN species on cucumber were *Tb* = 12.1°C and *S* = 294 DD, from EH of J2 were *Tb* = 8.0 ^{o}C and *S* = 213 DD, and for the LCC were *Tb* = 11.4°C and *S* = 500 DD. For watermelon, thermal requirements for FLE were *Tb* = 14.9 °C was and *S* = 243.9 DD. The *Tb* for *M. incognita* for EH was 11.1°C and *S*= 256.4 DD and for LCC *Tb* was 14.0°C and *S* = 500 DD. The thermal requirements of *M. javanica* life cycle completion were estimated from the three highest temperatures. At 17°C, hatching did not occur 180 days after inoculation when the experiment was concluded. The estimated values for LCC for *M. javanica* were *Tb* = 17.2°C and *S* = 357.1 DD. For zucchini-squash, thermal requirements for FLE were *Tb* = 12.1°C and *S* = 277.8 DD, for EH were *Tb* = 8.7 ^{o}C and *S* = 217.4 DD, and for LCC were *Tb* = 11°C and *S* = 476.2 DD.

Thermal requirements for each biological stage of both RKN species on cucumber and zucchini-squash did not differ ($P < 0.05$). Thus, a single phenology model per each biological stage was constructed including both RKN species and cucurbit crops (FLE: *Tb* =12.1°C; *S* =285.7 DD, EH: *Tb* = 8.3 °C; *S*= 212.8DD and LCC: *Tb* = 11.5 °C; *S* = 500DD).

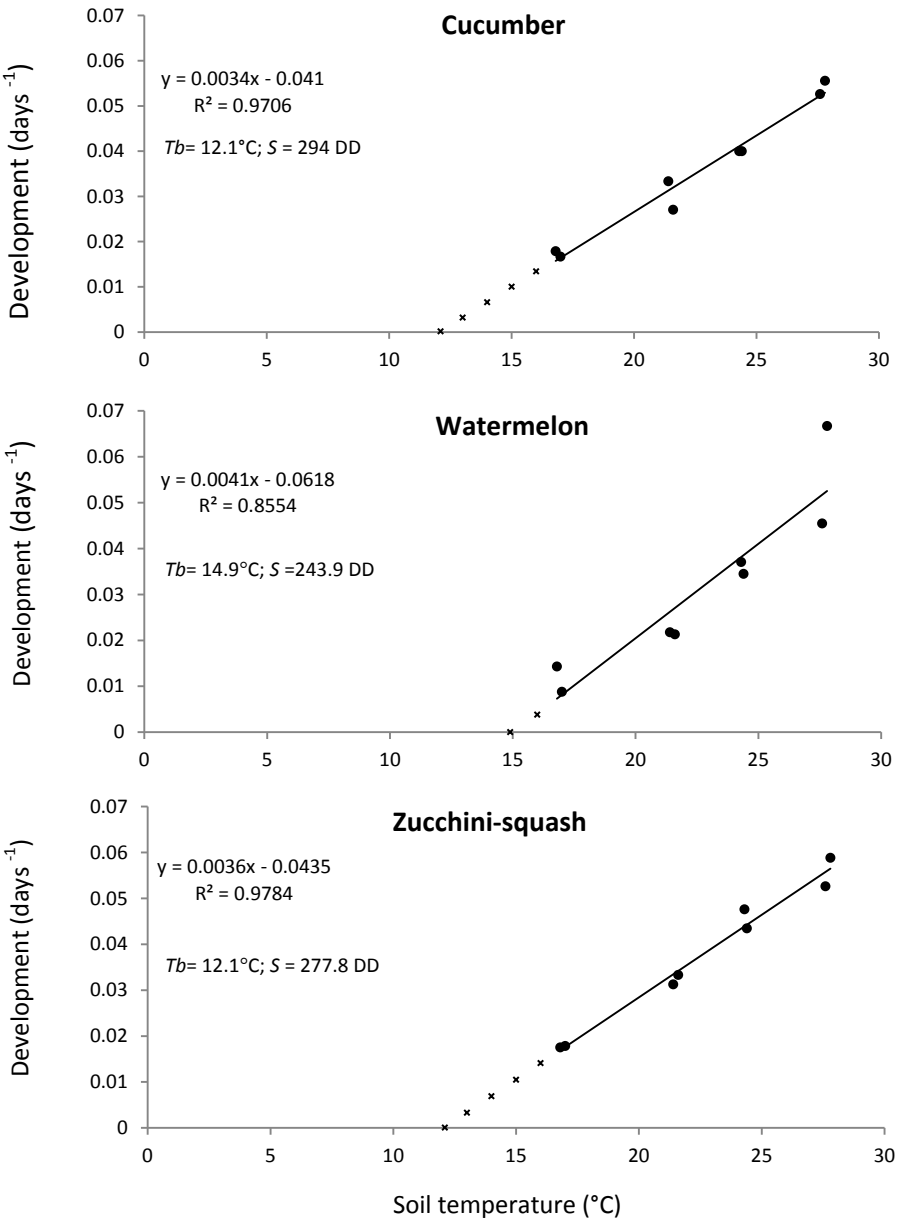


Figure 1. Relationship between soil temperature (°C) and reciprocal of time (days^{-1}) for development of *Meloidogyne incognita* and *M. javanica* on cucumber cv. Dasher II, watermelon cv. Sugarbaby and zucchini-squash cv. Amalthee HF1.

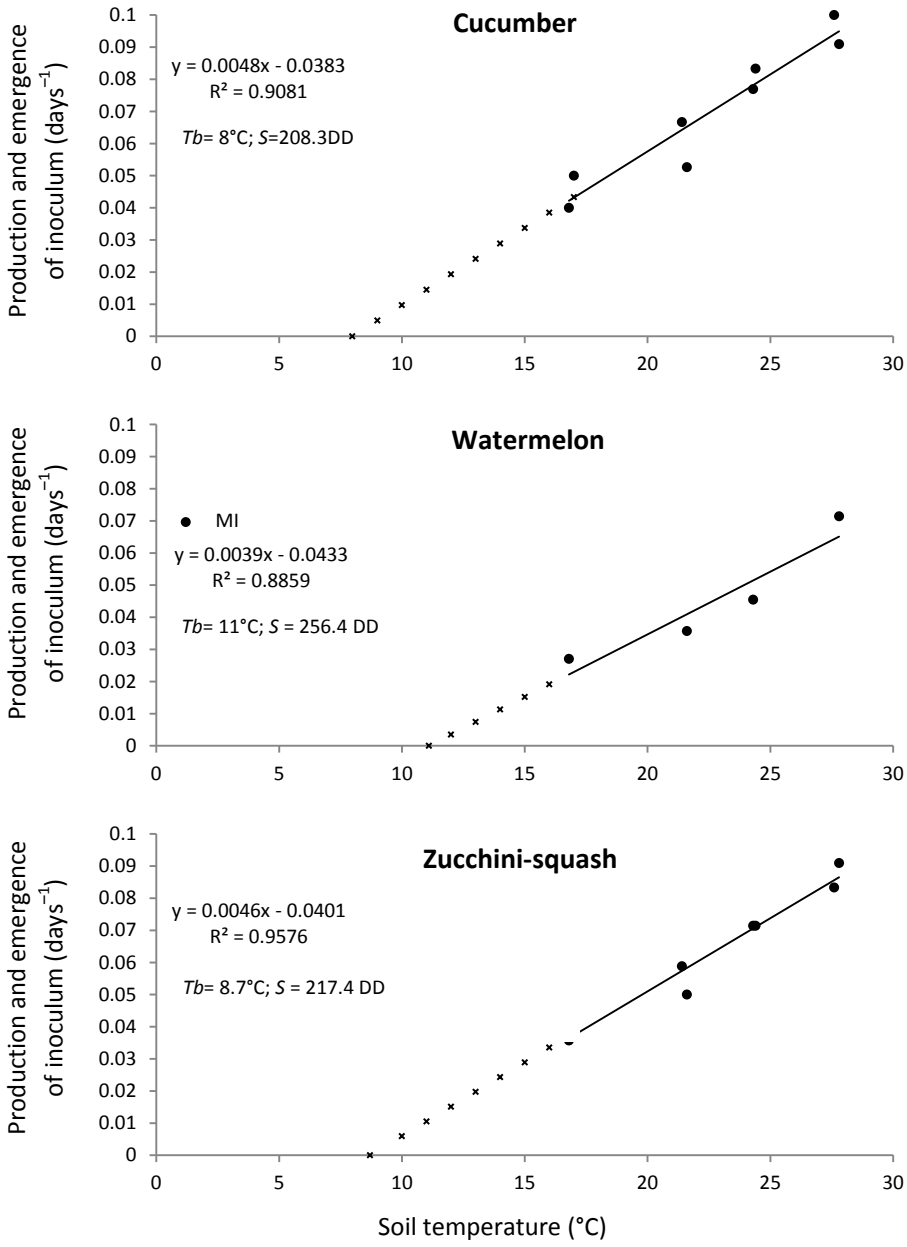


Figure 2. Relationship between soil temperature (°C) and reciprocal of time (days^{-1}) from egg production to egg hatching commencing of *Meloidogyne incognita* (MI) and *M. javanica* on cucumber cv. Dasher II and zucchini-squash cv. Amalthee HF1 and MI on watermelon cv. Sugarbaby.

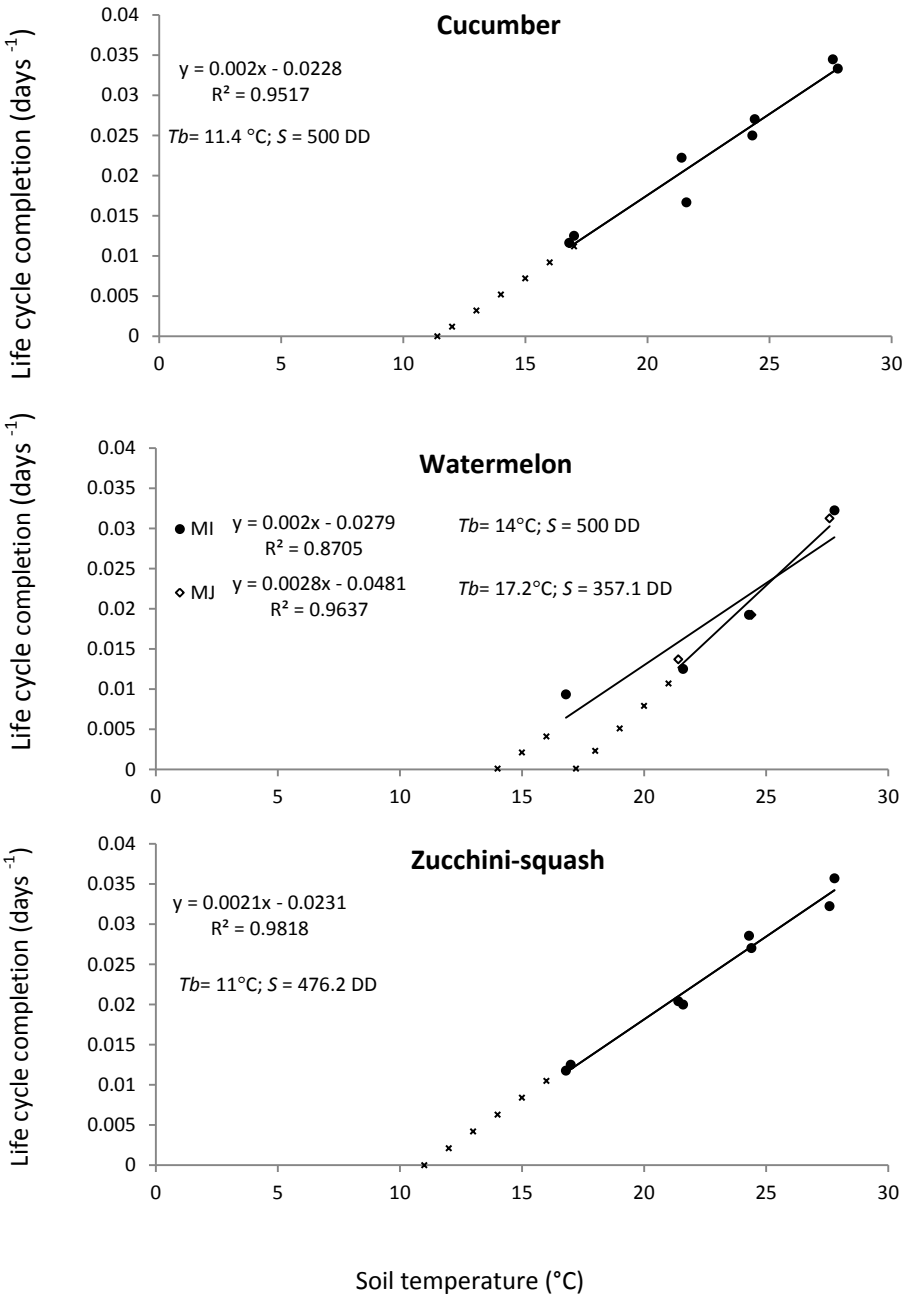


Figure 3. Relationship between soil temperature (°C) and reciprocal of time (days^{-1}) for the completion of the life cycle of *Meloidogyne incognita* (MI) and *M. javanica* (MJ) on cucumber cv. Dasher II, watermelon cv. Sugarbaby and zucchini-squash cv. Amalthee HF1.

Table 1. Number of days, base temperatutre (*Tb*) and accumulated degree-days (*DD*) above *Tb* (*S*) from inoculation of 1 J2 cm^{-3} soil until completion of several deveopment stages in the life cycle of *Meloidogyne incognita* or *M. javanica* on cucumber cv. Dasher II, wastermelon cv. Sugar Baby , and zucchini-squash cv. Amalthee HF1 in growth chambers.

	Soil	***M. incognita***			**Soil**	***M. javanica***		
Plant	Temperature (°C)	FLE	EH	LCC	Temperature (°C)	FLE	EH	LCC
Cucumber	16.8	56	25	86	17.0	60	20	80
	21.6	37	19	60	21.4	30	15	45
	24.3	25	13	40	24.4	25	12	37
	27.8	18	11	30	27.6	19	10	29
	Tb (°C)	12.3	9.2	11.9	*Tb* (°C)	11.8	6.8	11.0
	S (DD)	285.7	208.3	500.0	*S* (DD)	303.0	208.3	500.0
Watermelon	16.8	70	37	107	17.0	114	No	No
	21.6	52	28	80	21.4	46	Ns	73
	24.3	30	22	52	24.4	29	Ns	52
	27.8	17	14	31	27.6	22	Ns	32
	Tb (°C)	14.7	11.1	14.0	*Tb* (°C)	14.8	Ns	17.2
	S (DD)	250.0	256.4	500.0	*S* (DD)	277.8	Ns	357.1
Zucchini	16.8	57	28	85	17.0	56	24	80
	21.6	30	20	50	21.4	32	17	49
	24.3	21	14	35	24.4	23	14	37
	27.8	17	11	28	27.6	19	12	31
	Tb (°C)	12.0	10.6	12.0	*Tb* (°C)	11.9	6.4	10.8
	S (DD)	250.0	196.1	454.5	*S* (DD)	303.0	250.0	526.3

FLE: female laying eggs; EH: Egg hatching; LCC: Life cycle completion; no: not observed 114 days after inoculation

Discussion

In this study, thermal time requirements of *M. incognita* and *M. javanica* on cucumber, watermelon and zucchini-squash were determined by considering key processes of epidemics: infection, development and production and emergence of new inoculum. Splitting thermal requirements by biological stages can be useful to change planting dates in order to prevent root infection, to reduce the number of generations and prevent yield losses, or to reduce soil infestation by trap crops. In addition, it could

be useful to improve efficacy of antagonists or nematicides by applying them when females start laying eggs or J2 start emerging from eggs. However, the determination of the thermal requirements for some biological process, such as infection, is difficult to assess because the difficulty to observe the change from the vermiform J2 to the J2 sausage-like form. Thus, this kind of observation requires shorter assessment intervals than daily. Otherwise, the shape of the relationship between reciprocal of time and soil temperatures is not closed to a lineal relationship and the precision of *Tb* and *S* are affected. Consequently, in this study, the thermal requirements for infection were discarded.

The rate of RKN development is linearly related to the reciprocal of time between *Tb* and optimum temperature; although less frequently, nonlinear functions have also been found (Tyler, 1933; Trudgill, 1995). In this study, the estimation of *Tb* was done by projecting the straight segment of the curve according to Wilson and Barnett (1983). This may result in an overestimation of *Tb*, although the authors considered it of minor concern because for most crops temperatures are above *Tb* during the cropping season.

Differences in thermal requirements to complete specific biological stages of the life cycle have been demonstrated for several RKN species, e.g. embryonic development and hatching for *M. hispanica* and *M. arenaria* (Maleita et al., 2012), *M. javanica* and *M. incognita* (Tzortzakakis and Trudgill, 2005) and *M. chitwoodi* and *M. hapla* (Charchar and Santo, 2009). Most studies on thermal requirements of RKN used tomato as the host plant but little is known about thermal requirements of RKN in other economically important vegetable crops such as cucurbits. In this study, the thermal requirements of *M. incognita* and *M. javanica* on cucumber were similar for the biological stages considered. In fact, egg masses and egg production on cucumber cv. Dasher II did not differ between these *Meloidogyne* species after one generation in

pot experiments (López-Gómez and Verdejo-Lucas, 2014). Also, on zucchini-squash cv. Amalthee HF1 were similar for the biological stages, the results also agree with those reported by López-Gómez and Verdejo-Lucas (2014), who observed the presence of the same biological stages of these RKN species from 4–11 days post inoculation. Both RKN species showed similar thermal requirements to those on tomato (*Tb* = 10.1°C, *S* = 400 DD) (Ploeg and Maris, 1999) or clover (*Tb* = 10.1°C, *S* = 410 DD) (Vrain et al., 1978). Davila et al., (2005) reported similar degree day (DD) requirements for egg mass formation with the three widespread RKN on squash under black polythene mulch, that is, *M. arenaria* (*Tb* = 8.8; *S*= 343 DD), *M. incognita* (*Tb* = 9.3°C; *S*= 307 DD) and *M. javanica* (*Tb* = 10.2°C; S= 320 DD). In the present study, the thermal requirements from soil inoculation to females starting laying eggs of both RKN species investigated, , on cucumber and zucchini-squash (*Tb* =12.1°C; *S* =285.7 DD) were quite similar to those reported for egg mass formation of *M. arenaria* on oriental melon (*Tb* = 12.2°C; *S*= 313 DD; Yeon et al., 2003). On watermelon cv. Sugar Baby, both *M. incognita* and *M. javanica* showed similar thermal requirements for development, but not for the life cycle completion. Results are in agreement with López-Gómez and Verdejo-Lucas (2014), which watermelon cv. Sugar Baby was different between these *Meloidogyne* species after one generation in pot experiments.

As far as the authors are aware, there is no information on thermal requirements of RKN species for life cycle completion on cucurbit crops. The calculated values of *M. incognita* and *M. javanica* on cucumber and zucchini-squash (*Tb* = 11.5 °C; *S* = 500 DD) lie between those of *M. hispanica* (*Tb* = 10.2°C; *S* = 515.5 DD), *M. incognita* (*Tb* = 10.1°C; *S* = 400 DD) and *M. javanica* (*Tb* = 12.9°C; *S* = 350 DD; *Tb* = 13.1°C; *S* = 343 DD) on tomato (Madulu and Trudgill, 1994; Trudgill, 1995; Ploeg and Maris, 1999; Maleita et al., 2012) but *Tb* on watermelon was higher (*Tb* = 14°C; *S* = 500 DD for *M. incognita* and *Tb* = 17.2°C; *S* = 357.1 DD for *M. javanica*). Plant host influences thermal requirements, as reported for *M. incognita* on tomato or *Tagetes* (Ploeg and Maris,

1999), for *M. hispanica* on resistant or susceptible tomato cultivars (Maleita et al., 2012) and for development of *M. konaensis* on coffee and tomato (Zhang and Schmittm, 1995).

Therefore, knowledge of thermal requirements of RKN species on vegetable crops, or other plants to be cultivated in a given area, could be useful to manage nematode species prevalence and population growth by methods based on climatic conditions, and cropping sequences. Thermal time requirements have to be validated in field conditions to develop models as simple as possible for use by advisors and growers, as done for RKN on tomato in California (*Tb* = 10°C and *S*= 600–700 DD; Ferris et al., 1985).

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Chapter 2

Population dynamics of root-knot nematodes on cucumber and yield losses under protected cultivation

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Several studies were carried out to determine the maximum multiplication rate and the equilibrium density of root-knot nematodes on cucumber and yield losses in plastic greenhouse. The experiments were conducted from 2009 to 2012 where *M. incognita* completed three generations. The values for a and E were 1147 and 625 second stage juveniles (J2) per 250 cm^3 soil, respectively. The tolerance limit was below zero, and the minimum relative yield ranged from 0.12 to 0.34.

Keywords: *Cucumis sativus*, Equilibrium density, *Meloidogyne* spp., Multiplication rate, Tolerance limit

Introduction

Root-knot nematodes (RKN), *Meloidogyne* spp., are obligate parasites of plants, including vegetables, with *Meloidogyne arenaria*, *M. incognita* and *M. javanica* being the major ones responsible for yield losses (Sikora and Fernández, 2005). The amount of yield loss varies according to the host status, agronomic practices and environmental conditions (mainly temperature) in irrigated agriculture (Ferris, 1986).

In the absence of limiting factors, the RKN population density will increase proportionally to that at planting (*Pi*). The multiplication rate (*Pf/Pi*, where *Pf* is the population density at the end of the crop) is at a maximum with low *Pi* values. However, it decreases as *Pi* increases as a result of scarcity of food and competition (Seinhorst, 1970), tending to stabilize around an equilibrium density (*E*) at which the plant can supply enough food to maintain the population density at planting (*Pf* = *Pi*; *Pf/Pi* =1). Both constants are indicators of the host status for a given set of conditions (Seinhorst, 1967). Plant growth and yield are also related to *Pi*, and the relationship

was mathematically described by Seinhorst's damage function model (Seinhorst, 1965) that provides indicators of plant tolerance. Thus, for any nematode–plant combination, the damage caused by nematodes depends on the soil nematode densities at planting, its reproductive potential, the plant tolerance (Greco and Di Vito, 2009) and the extent of the cropping period (related to accumulated degree-days (DD)) (Ehwaeti et al., 1998). Therefore, estimation of these factors is basic to designing integrated nematode management programmes.

Cucumber (*Cucumis sativus*) is cropped in many countries around the world, and in some areas is usually included in rotation with species of Solanaceae in double-cropping systems (Sorribas and Verdejo-Lucas, 1994; Ornat et al., 1997; Thies et al., 2004). In Spain, cucumber is cropped in 8148 ha with an annual production of 664975 tonnes, of which 63.5% are grown under protected cultivation (Ministerio de Medio Ambiente, Medio Rural y Marino, 2012). RKN are present in all vegetable production areas (Melgarejo et al., 2010), in which 20–40% of fields are infested (Verdejo-Lucas et al., 2002; Ornat and Sorribas, 2008; Talavera et al., 2012). Cucumber is susceptible to most common RKN species, which can reduce cucumber yield up to 60% in commercial protected cultivation (Ornat et al., 1997).

At present, there is little information available on thermal requirements of the nematode on cucumber. Therefore it is important to standardize conditions in order to compare population growth and crop yield losses according to the number of generations which RKN species can complete during the cropping cycle in a given area. Thus, the objectives of this study were to determine the maximum multiplication rate and equilibrium density of the nematode and cucumber yield losses in response to increasing initial population densities.

Materials and methods

Experiments were carried out in a 700 m^2 plastic greenhouse located at Viladecans (Barcelona, Spain) during four consecutive cropping seasons. The soil was artificially infested with *M. incognita* in 2007. The soil texture was sandy loam with 83.8% sand, 6.7% loam and 9.5% clay; pH 8.7; 1.8% of organic matter (w/w) and 0.5 dS m^{-1} electrical conductivity.

Individual plots of 9.6 m^2 consisted of four rows 55 cm apart with six plants per row spaced at 50 cm intervals (24 plants/ plot). Cucumber was rotated with resistant or susceptible tomato cultivars, cv. Monika and Durinta, respectively, or black fallow to achieve gradients of nematode densities. Soil was prepared individually per plot to prevent cross-contamination. Cucumber cv. Dasher II was cropped from July to November from 2009 to 2012. Plants were irrigated as needed through a drip irrigation system and weekly fertilized with a solution consisting of NPK (15-5-30) at 31 kg ha^{-1} and iron chelate and micronutrients at 0.9 kg ha^{-1}. Cucumber plants were vertically trained. Weeds were removed manually during and between crops. Fruits from the eight central plants of each plot were harvested when they reached the standard commercial size. At the end of the cropping season, plants were removed from the ground with a pitchfork.

Soil temperatures were recorded daily at 30 min intervals with temperature probes 5TM (Decagon devices, Inc.) placed at a soil depth of 15 cm. Accumulated soil temperatures were 2862, 2674, 2499 and 2642°C (*Tb* = 0) from 2009 to 2012.

Composite soil samples were taken from individual plots at the beginning and the end of each cucumber crop to estimate initial (*Pi*) and final (*Pf*) nematode population densities. Individual soil samples consisted of eight soil cores taken from the first 30 cm of soil with a soil auger of 2.5 cm diameter. Samples were homogenized

and sieved through a 4 mm aperture screen to remove stones and separate roots from soil. Nematodes were extracted from a 500 cm^3 soil subsample using Baermann trays (Whitehead and Hemming, 1965). Nematode densities are expressed as J2 per 250 cm^3 of soil.

The relative yield of cucumber was plotted against the Pi values and submitted to a non-linear regression analysis to determine if they fit the Seinhorst damage function model. If no differences were found in values of minimum relative yield, tolerance limit and z between cropping seasons (considering confidence intervals at 95%), data were pooled to construct a single general model.

The multiplication rate (*Pf/Pi*) was calculated, transformed to log10(x) to linearize, and the relationship between *Pf*/*Pi* and *Pi* per cropping season was submitted to analysis of variance to compare regression lines. When no differences (P <0 .05) were found, data were pooled to construct a single general model to estimate the maximum multiplication rate (*a*) and the equilibrium density (*E*).

Statistical analysis

Statistical analyses were done using SAS v. 9 (SAS Institute Inc.). Analysis of variance was carried out by the general lineal model (PROC GLM). Data were transformed to log10(x + 1) when needed to linearize the relationships and submitted to regression analysis by the PROC REG procedure. Contrast of linear regressions was done by the general lineal model procedure (PROC GLM). Non-linear regressions were used to determine if the relationship between Pi and the relative cucumber yield from the greenhouse experiment fitted the Seinhorst damage function model ($y= m + (1- m) z^{(Pi-T)}$). This is an iterative process starting with values of the parameters provided by the user, as well as the bounds between which they can vary. The values of m and t used to start the iteration were estimated from cucumber yield against log10Pi. The

goodness of fit was estimated by the coefficient of determination. Contrasts with the Seinhorst damage function model were done considering confidence intervals at 95% of *m*, *T* and *z*. When no differences were found, a single general model was constructed from pooled data.

Results

Mean soil temperatures ranged from 16.8 to 33.5°C during the four cropping cycles. *Meloidogyne incognita* completed three generations (*Tb* = 11.4°C) in 2009 (1596 DD) and 2012 (1524 DD), and two in 2010 (1465 DD) and 2011 (1473 DD). The *Pi* values for all cropping cycles ranged from 0 to 11 802 J2 per 250 cm^3 soil. The relationship between *Pi* and *Pf*/*Pi* fitted to a negative potential function each cropping cycle (R^2 ranged between 0.5924 and 0.9987). The linearized functions did not differ ($P < 0.05$), and therefore data were pooled and submitted to a new regression analysis that was statistically significant ($R^2 = 0.610$; $P < 0.0001$) (Figure 1). The maximum multiplication rate (*a*) of *M. incognita* on cucumber was 1147, and the equilibrium density (*E*) was 625 J2 per 250 cm^3 soil.

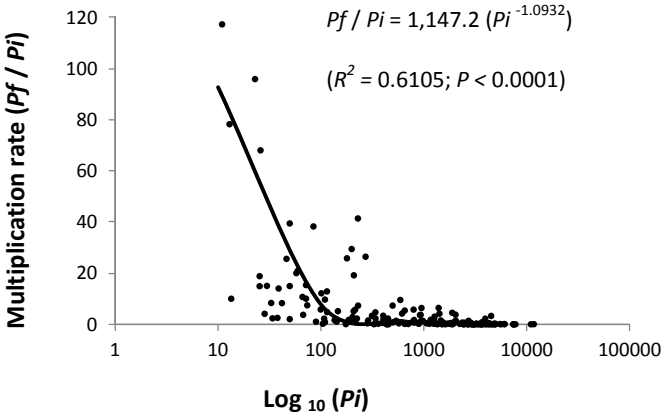

Figure 1. Relationship between initial population density (*Pi*) of *Meloidogyne incognita* and multiplication rate (*Pf*/*Pi*) on cucumber cv. Dasher II cropped in a greenhouse from July to November 2009, 2010, 2011 and 2012. Accumulated soil temperature (15 cm depth) was 2862, 2674, 2499 and 2642°C (*Tb* = 0°C) in 2009–2012, respectively.

The average yield of cucumber in plots with no nematodes (*Pi* = 0 J2 per 250 cm^3 soil) ranged from 5.4 to 4.3 kg m^{-2} in 2009, 2010 and 2012. In 2011, cucumber plants did not yield any commercial fruit as a result of blossom abortion and crooking fruits.

The relationship between the relative yield of cucumber and *Pi* fitted the Seinhorst damage function model in 2009, 2010 and 2012. Minimum relative yield ranged from 0.12 to 0.34, and the tolerance limit was under detectable levels each year (Table 1).

Table 1. Estimated parameters of the Seinhorst damage function model for cucumber cv. Dasher II cropped in soil infested by *Meloidogyne incognita* in a field greenhouse from July to November in 2009, 2010 and 2012

Cropping cycle	*m*	*T* (J2 250 cm^{-3} soil)	*z*	R^2	*P*	Accumulated soil temperature (^oC)
2009	0.34 ±0.08	0.0004	0.92 ±0.13	0.662	< 0.0001	2862
2010	0.14 ±0.06	0.4	0.90 ± 0.07	0.685	< 0.0001	2674
2012	0.12 ± 0.06	0.0003	0.96 ± 0.03	0.489	< 0.0001	2642

Data are mean ± confidence interval (95%); *m*: minimum relative yield; *T*: tolerance limit; *z*: a constant related to the effect of a single nematode.

Discussion

The maximum multiplication rate and equilibrium density of *M. incognita* on cucumber were 31.5 and 27% respectively those of *M. incognita*, and 36.3 and 21.4% those of *M. javanica* on the susceptible tomato cv. Durinta in which the nematode completed three generations (Talavera et al., 2009; Giné et al., 2012). Thus, root-knot nematode densities can increase much more and much higher densities can be tolerated on tomato than cucumber. Furthermore, the tolerance limit on cucumber was under detectable levels, that is, cucumber can experience yield losses at very low nematode densities reaching relative minimum yields of 0.12. Thus, the host status of

cucumber cv. Dasher II is susceptible–intolerant compared to the susceptible–tolerant tomato cv. Durinta. Similar results have been reported for cantaloupe melon (Ferris, 1985; Ploeg and Phillips, 2001; Kim and Ferris, 2002). However, not all cucurbit crops respond equally to RKN, e.g. watermelon is a poor-host-tolerant to *M. incognita* because the multiplication rate was very low despite moderately severe root galling (Davis, 2007), with a high tolerance limit (9 J2 per 250 cm^3 of soil; Xing and Westphal, 2012).

Few studies have been conducted to obtain epidemiologic information on *Meloidogyne* on cucumber for specific growing areas, in order to design sustainable management strategies that consider the environmental conditions. This work provides relevant information on how the RKN population will grow on cucumber considering the population density around which it will tend to fluctuate, and the effect on cucumber yield.

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Chapter 3

Population dynamics of *Meloidogyne incognita* and yield losses of susceptible and resistant tomato cultivars in Mediterranean conditions

Population growth rate of *Meloidogyne incognita* on susceptible and resistant tomato cultivars and yield losses in plastic greenhouse.

Meloidogyne spp. are the nematodes that cause the major yield losses in tomato. In the present study, the susceptible tomato cv. Durinta and the resistant cv. Monika (with *Mi*-gene) were cultivated from March to July in a plastic greenhouse for 2010, 2011 and 2012 to determine the population growth rate of *M. incognita* and the yield losses. The values of population growth increased year by year and were higher when the resistant tomato was repeatedly cultivated in the same plot than when it was rotated with the susceptible cultivar. After three years of repeated cultivation of the resistant tomato, the level of resistance decreased from very to moderately resistant. The relationship between the nematode density at transplanting (*Pi*) and the relative yield of susceptible tomato fitted to the Seinhorst damage model in 2010 and 2012, but not in the resistant one. Tolerance limit (*T*) and the relative minimum yield (*m*) were 2-4 J2 250 cm^{-3} of soil and 0.44-0.48, respectively. Production did not differ between cultivars at low *Pi*. At the highest *Pi*, the resistant cultivar produced 41 and 80% more than the susceptible each year, respectively. This study demonstrates the effectiveness of the resistant cultivar to suppress *M. incognita* and to reduce yield losses. Also, the utility of the population dynamics parameters was pointed out as an early detection indicator of virulence selection, which was progressively selected, but three years were not enough to complete it.

Keywords: Damage function, Equilibrium density, Maximum multiplication rate, Root-knot nematodes*, Solanum lycopersicum.*

Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most important crops in Europe, being cultivated mostly in the Mediterranean region where about two-thirds

of the production comes from Italy and Spain (EUROSTAT, 2008). In Spain, the annual production exceeds 4 million of tonnes in 48617 ha, of which a 38.05% is conducted under protected cultivation (MAGRAMA, 2013), mainly as monoculture (Talavera et al., 2012).

Root-knot nematodes (RKN), *Meloidogyne* spp., are the major responsible of yield losses caused by plant-parasitic nematodes on horticultural crops (Sikora and Fernández, 2005), mainly under protected cultivation (Greco and Esmenjaud, 2004). In Spain, the maximum vegetable yield losses under protected cultivation due to *M. incognita* or *M. javanica,* the most frequent RKN species in vegetable growing areas, reached 88% on cucumber, 30% on lettuce, 60% on tomato, 37% on watermelon, and 39% on zucchini (Verdejo-Lucas et al., 1994, Sorribas et al., 2005, Talavera et al., 2009; Giné et al., 2014, López-Gómez et al., 2014, Vela et al., 2014). Among all available control methods to manage RKN, plant resistance is the key control method to be used in integrated nematode management strategies due to its cost-effectiveness relationship, its compatibility with other control methods, and its low environmental impact (Starr et al., 2002). Resistant plants are able to suppress the development and reproduction of plant-parasitic nematodes (Roberts, 2002). In tomato, resistance is conferred by the *Mi*-gene introgressed from *S. peruvianum* (Smith, 1944) which is active against *M. arenaria*, *M. incognita* and *M. javanica* (Roberts, 1992; Williamson, 1998). In addition, it provides resistance to *Macrosiphum euphorbiae* (Rossi et al., 1998) and *Bemisia tabaci* biotypes B and Q (Nombela et al., 2003). The expression of the *Mi*-gene has some limitations, such as, constant soil temperature above 28 °C (Dropkin, 1969); RKN species, such as *M. hapla*, *M. chitwoodi* race 3 (Brown et al., 1997), *M. enterolobi* (Kiewnick et al., 2009), or *M. exigua* (Silva et al., 2008); the genetic background of the plant (Cortada et al., 2008; 2009; 2012); or against virulent RKN populations. Despite the fact that resistant tomato cultivars or rootstocks are widely used, the repeated cultivation of resistant genotypes can select virulent

population which could overcome the *Mi*-gene (Williamson, 1998; Verdejo-Lucas et al., 2009). Thus, some rotation sequences have been proposed to avoid the selection for virulence (Talavera et al*.,* 2009).

The selection of virulent nematode populations can be detected by an increase of population density at the end of the resistant crop (*Pf*), which tends to be similar to that achieved on the susceptible one for a given density at transplanting (*Pi*). That is, the maximum multiplication rate (*a,* defined as the multiplication rate in absence of limiting factors), the ceiling level (*M*, maximum *Pf* achieved by a nematode population on a plant host under particular conditions), and the equilibrium density (*E,* nematode density at transplanting at which the plant can supply enough food to maintain the population density at end of the crop*: Pf*=*Pi*; *Pf*/*Pi*=1) (Seinhorst, 1967) on the resistant genotype will be close to those on the susceptible one. Another useful indicator of the population growth rate is the relationship between the multiplication rate (*Pf*/*Pi*) and *Pi.* It allows comparing nematode population dynamics on different plant species or genotypes, the efficacy of control methods, or between cropping seasons for a given pathosystem (Talavera et al., 2009; Vela et al., 2014). All these relationships are focused on the nematode reproduction and population growth, but not on plant tolerance, which is provided by the relationship between *Pi* and crop yield, defined by the Seinhorst damage function model (Seinhorst, 1965). This relationship will estimate the tolerance limit (*T*) and the minimum relative crop yield (*m*) for specific agronomic conditions (Seinhorst, 1965).

The purpose of this study was: (i) to determine the maximum multiplication rate, the ceiling level and the equilibrium density, and population growth rate of *Meloidogyne incognita* on resistant and susceptible tomato cultivars cropped during three growing seasons in a plastic greenhouse as putative indicator of selection for virulence, (ii) or after repeated cultivation of susceptible or resistant tomato cultivars

over three cropping seasons; and ii) to know the effect of increasing *Pi* on relative crop yield of resistant and susceptible tomato cultivars in plastic greenhouse conditions.

Materials and methods

Experiments were carried out over three growing seasons (2010, 2011 and 2012) in a 700 m^2 plastic greenhouse located in Viladecans (Barcelona, Spain). The soil was artificially infested with a population of *M. incognita* identified by the morphology of perineal pattern, esterase pattern, and SCAR markers, in 2007. The soil texture was sandy loam with 83.8% sand, 6.7% loam and 9.5% clay; pH 8.7; 1.8% of organic matter (w/w) and 0.5 dS m^{-1} electrical conductivity.

Sixty gross plots of 9.6 m^2 were cultivated with each of the resistant tomato cv. Monika or the susceptible cv. Durinta. Individual gross plots comprised four rows, with six plants per row. Plants were spaced 50 cm between rows and 55 cm within rows. The distance between individual plots was 110 cm between rows and 100 cm along rows. The net plots of 3.2 m^2 comprised the central part of the gross plot in which 8 plants were cropped and from which soil and roots samples were taken and crop yield was assessed. The soil was prepared individually to prevent cross contamination.

Tomato plants were cultivated from 12th April to 15th July (95 days) in 2010, from 31st March to 6th July in 2011 (98 days) and from 5th March to 17th July (135 days) in 2012. Tomato crops were followed by black fallow or cucumber crop to achieve a gradient of nematode densities. Plants were irrigated by drip irrigation system as needed and fertilized with a solution consisting of NPK (15-5-30) at 31 kg ha^{-1} and iron chelate and micronutrients at 0.9 kg ha^{-1}. Plants were vertically trained and weeds were managed by hand pulling. Fruits were harvested when they reached the standard commercial size. Accumulated tomato yield was expressed as kilograms of fruit per

plant. Soil temperatures were recorded daily at 30-minute intervals with temperature probes (Campbell Scientific, Logan, USA) placed at a depth of 15 cm.

Nematode population densities were determined at transplanting (*Pi*) and at the end (*Pf*) of each crop. Samples consisted by eight cores taken from the first 30 cm of soil with an auger (2.5 cm diameter). Then, samples were mixed and passed through a 4mm-pore sieve to remove stones and roots. Nematodes were extracted from 500 cm^3 of soil by the Baermann trays (Whitehead and Hemming, 1965) incubated at 27 ^oC. After one week, second-stage juveniles (J2) were collected and concentrated from the water suspension by a 25 µm-pore sieve, counted and expressed as J2 250 cm^{-3} of soil. The nematode multiplication rate was calculated dividing *Pf* by *Pi*.

At the end of the cropping season, plants were removed from the ground with a pitchfork. Disease severity was rated by Zeck's scale from 0 to 10 (Zeck, 1971), where 0=complete and healthy root system and 10=plants and roots dead. Afterwards, the roots were cleaned with tap water, weighed and chopped in 1-cm-long segments, and two 20-g subsamples used to extract eggs by blender maceration in a 1% NaOCl solution for 10 min (Hussey and Barker, 1973). The number of eggs was expressed per gram of fresh root weight. The reproduction index (RI) was calculated as the percentage of eggs per gram of root on the resistant tomato respect to those on the susceptible one to determine the level of resistance. This value allowed the categorization of the response of the resistant cultivar as highly resistant (RI < 1%), very resistant (RI < 10%), moderately resistant (10% ≤ RI < 25%), slightly resistant (25% ≤ RI < 50%) or susceptible (RI ≥ 50%) (Hadisoeganda and Sasser, 1982).

The estimation of the maximum multiplication rate (*a*) was carried out considering the small values of *Pi*, according to $Pf = aPi$ (Seinhorst, 1970), and the maximum population density at the end of the crop (*M*) from the experimental data.

The equilibrium density (*E*) was calculated according to the expression $M = aE/(a\text{-}1)$ (Schomaker and Been, 2006).

Statistical analysis

All data were analyzed by SAS v.9 program. Values of *Pi* and *Pf* /*Pi* were transformed to log10(x) to linearize and submitted to regression analysis (proc reg) per tomato cultivar and year to determine the population growth rate. The contrast of linear regressions between years per each tomato cultivar was done by the general lineal model procedure (proc glm). In addition, contrasts of the relationship between *Pi* and *Pf/Pi* from plots in which resistant tomato was cropped one (only one out the three years), two (2010 and 2011, or 2011 and 2012) or three consecutive years (2010, 2011 and 2012) along with those cropped with susceptible tomato were carried out to determine the putative selection for virulence according to its population growth rate. Both galling index and number of eggs per gram of root registered on the resistant or susceptible cultivar were compared per cropping season by analysis of variance, and also, between years of repeated cultivation. Means were separated by the last significant difference ($P < 0.05$).

Tomato yield was compared between cultivars per each of the six *Pi* ranges. In 2010, the *Pi* ranges were: 0, 1-10, 11-100, 101-300, 301-500 and 501-1448 J2 250 cm^{-3} of soil. In 2012, the *Pi* ranges were: 0, 10-100, 101-300, 301-500, 501-1000 and 1001-3322 J2 250 cm^{-3} of soil. Tomato yield was compared between tomato cultivars per year and *Pi* range by the t-Student test. In 2011, tomato yield was not included for comparison because plant suffered blossom abortion irrespective of the cultivar. In addition, the relative yield of each tomato cultivar and the *Pi* values were submitted to a non-linear regression analysis by the non-linear procedure (proc nlin) to determine if they fit the Seinhorst damage function model ($y = m + (1 - m)\, 0.95^{(Pi/T - 1)}$). The values of *m* and *T* used to start the iteration were estimated plotting experimental values of

tomato yield against log10 *Pi*. Contrasts with the Seinhorst damage function model were done considering confidence intervals at 95% of *m* and *T.*

Results

Soil temperatures ranged from 17.2 to 30.9 °C (mean 26.0 °C) in 2010, from 19.7 to 31.4 °C (mean 25.4 °C) in 2011, and from 17.0 to 31.5 °C (mean 24.5 °C) in 2012. The number of days with soil temperatures above 28 °C without intermittent peaks during tomato crops was 18, 22 and 27 in 2010, 2011 and 2012, respectively. Fluctuation of soil temperatures during the three years of study are presented in Figure 1.

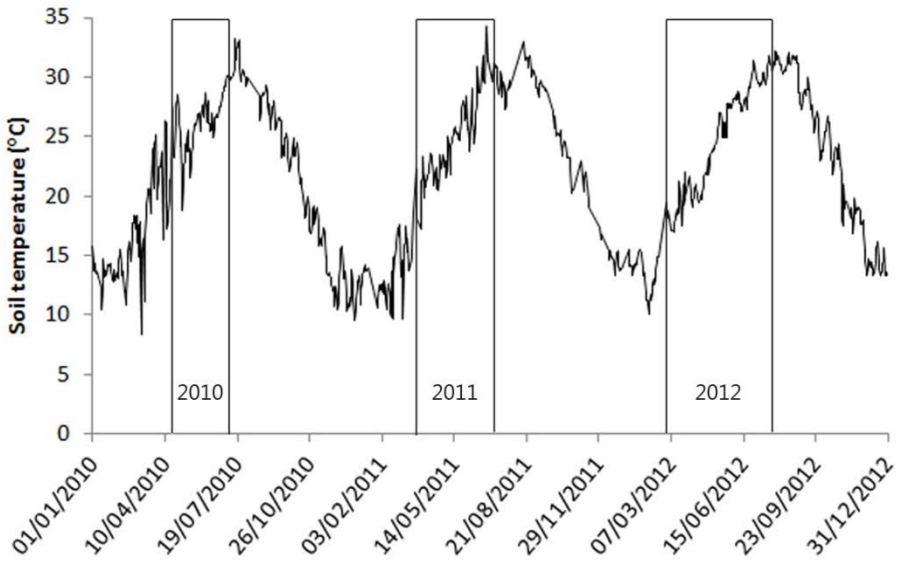

Figure 1. Fluctuation of mean daily soil temperatures in the plastic greenhouse located in Viladecans (Spain) infested by *Meloidogyne incognita* and cultivated with susceptible tomato cv. Durinta and resistant tomato cv. Monika, Soil temperatures took at 15-cm of depth. The cropping period is indicated by the shaded areas.

The nematode was able to complete two generations in each cropping season according to the accumulated soil temperature (1521, 1504 and 1959 °C DD (degree days; over a base temperature (*Tb)* of 10 °C) in 2010, 2011 and 2012, respectively) and its thermal requirements on tomato (thermal constant (*S*) = 600-700 degree days over *Tb* = 10 °C, Ferris et al., 1985).

Nematode population densities at the beginning of tomato crops ranged from 0 to 1448 J2 250 cm^{-3} of soil in 2010, from 0 to 3749 J2 250 cm^{-3} of soil in 2011, and from 0 to 3322 J2 250 cm^{-3} of soil in 2012. The maximum multiplication rate (*a*) of *M. incognita* on the susceptible cv. Durinta during the three growing seasons ranged from 3312 to 4754, the maximum population density (*M*) from 4149 to 4711 J2 250 cm^{-3} of soil, and the equilibrium density (*E*) from 4148 to 4710 J2 250 cm^{-3} of soil. On the resistant cv. Monika, *a* ranged from 6 to 780, *M* from 164 and 1315 J2 250 cm^{-3} of soil, and *E* from 137 to 1313 J2 250 cm^{-3} of soil. The values of *a*, *E* and *M* of *M. incognita* on the susceptible tomato cv. Durinta after considering years of repeated cultivation, ranged from 3312 to 4691, 3390 to 4508 J2 250 cm^{-3} of soil and 3389 to 4507 J2 250 cm^{-3} of soil, respectively. On the resistant cv. Monika the values of *a, M,* and *E* increased until 1470, 1473 J2 250 cm^{-3} of soil, and 1472 J2 250 cm^{-3} of soil, respectively after the repeated cultivation (Table 1).

Table 1. Maximum multiplication rate (*a*), ceiling point (*M*) and equilibrium density (*E*) of *Meloidogyne incognita* on resistant tomato cv. Monika and susceptible tomato cv. Durinta after each year of cultivation (2010, 2011 and 2012) and after one, two or three consecutive years of cultivation and rates of *a*, *M* and *E* of resistant *versus* susceptible

	Susceptible			**Resistant**					
	a	***M*** (J2 250cm^3 soil)	***E*** (J2 250cm^3 soil)	***a***	***M*** (J2 250cm^3 soil)	***E*** (J2 250cm^3 soil)	***a* R/S**	***M* R/S**	***E* R/S**
2010	3312	4154	4153	6	164	137	0.2	3.9	3.3
2011	3700	4149	4148	102	446	442	2.8	10.7	10.6
2012	4754	4711	4710	780	1315	1313	16.4	27.9	27.9
1 year	3312	3390	3389	6	383	319	0.2	11.3	9.4
2 years	3696	4455	4454	116	946	938	3.1	21.2	21.1
3 years	4691	4508	4507	1470	1473	1472	31.3	32.7	32.7

The relationship between *Pi* and *Pf/Pi* on the susceptible tomato did not differ between the three growing seasons (intercept *P* = 0.9280; slope *P* =0.6089) and data was pooled to obtain a general regression (Figure 2). On the resistant tomato, the relationship between *Pi* and *Pf/Pi* differed between cropping seasons (intercept *P* = 0.0247; slope *P* = 0.5084) and also from that of the susceptible cultivar (Figure 2a). The galling index and the number of eggs per gram of root on the resistant tomato during the cropping season ranged from 14.1 to 30.7 %, and from 3.6 to 9.4 % of those registered on the susceptible one. The level of resistance of the cultivar Monika according to its reproduction index (RI < 10%) was very resistant over the three cropping seasons (Table 2).

The relationship between *Pi* and *Pf/Pi* after cultivation of the susceptible tomato did not differ between one, two and three years of repeated cultivation, and data was pooled to obtain a general regression (intercept *P* = 0.7469; slope *P* = 0.9808). The relationship on the resistant tomato one, two or three consecutive years differed (intercept *P* = 0.0486; slope *P* = 0.2848) (Figure 2b) and also differed from the susceptible cultivar. The galling index and the number of eggs per gram of root registered on the resistant tomato after one, two and three years of repeated cultivation were 17.1, 21.4 and 35.3% and 3.9, 8.6 and 10.1% those on the susceptible one, respectively (Table 2). Both galling index and number of eggs per gram of root registered after three years of cultivation were higher ($P < 0.05$) than those recorded the first and the second year. After three years of repeated cultivation of the resistant tomato, the level of resistance decreased from very to moderately resistant (Table 2).

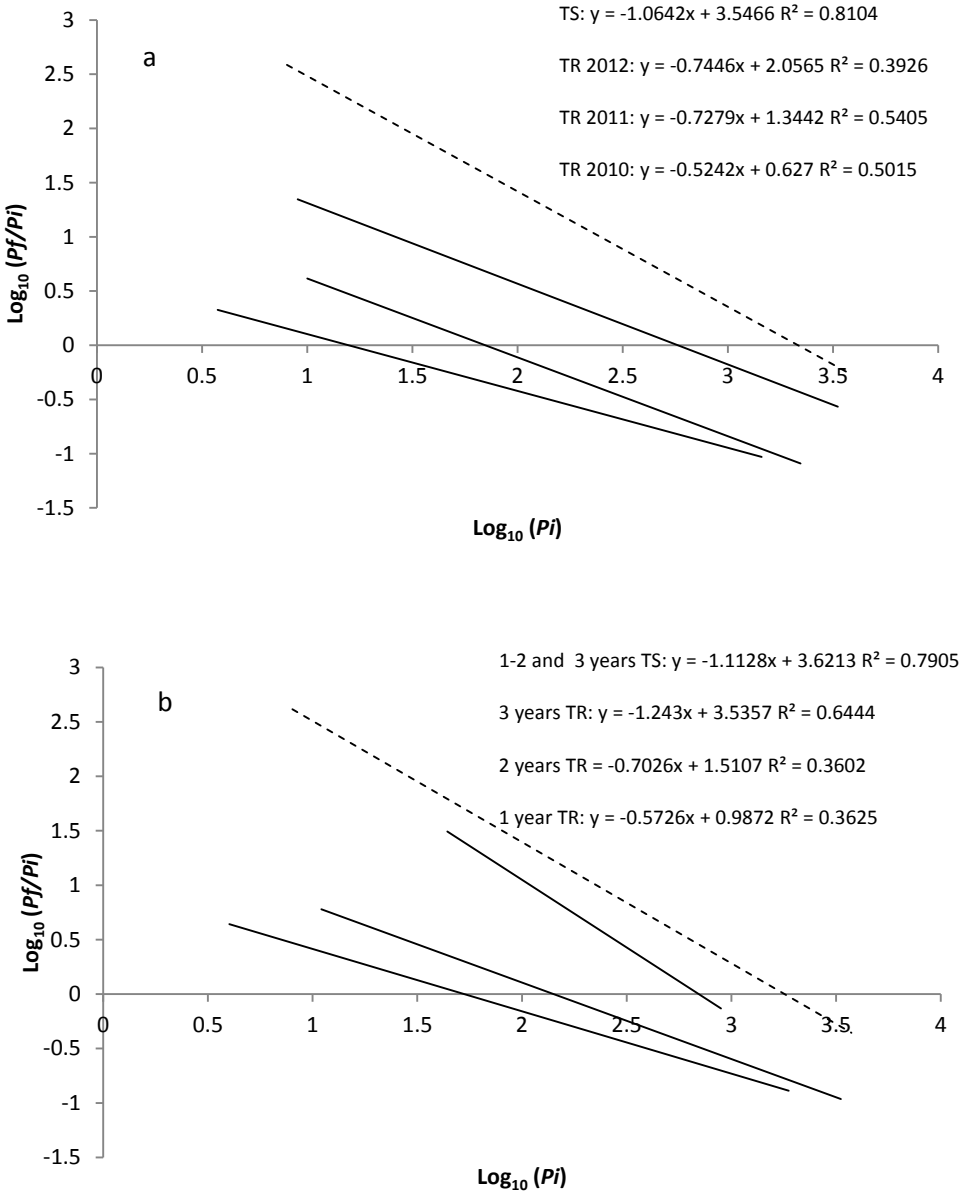

Figure 2. The relationship between initial population (*Pi*) and multiplication rate (*Pf*/*Pi*) of *Meloidogyne incognita* (J2 250 cm^3 of soil) in susceptible tomato cv. Durinta and cv. Monika over three cropping seasons (2010-2012) (a) one, two or three consecutive years (b) in a plastic greenhouse in Viladecans (Spain).

Table 2. Galling index, eggs per gram of root and reproduction index (RI) of *Meloidogyne incognita*, on tomato cultivar Durinta (Susceptible) and tomato cultivar Monika (Resistant) in 2010, 2011 and 2012 and in repeated cultivation (after 1, 2 and 3 years) in a plastic greenhouse in Viladecans (Spain).

	Galling index[a]		Eggs per gram of root		RI[b]	Category
	Susceptible	*Resistant*	*Susceptible*	*Resistant*		
2010	6.5±0.4b	1.3±0.1b	5147±546b	183±90b	3.1	VR
2011	7.1±0.3ab	1±0.1b	7228±251ab	378±62b	5.2	VR
2012	7.5±0.3a	2.3±0.1a	8329±832a	780±143a	9.4	VR
After 1 year	7.0±0.3a	1.2±0.1b	6637±395 b	261±57b	3.9	VR
After 2 year	7.0±0.2a	1.5±0.2b	7392±390ab	632±136b	8.5	VR
After 3 year	6.8±0.3a	2.4±0.4a	9574±2220a	969±264a	10.1	MR

Data are mean ± standard error. Data within the same column followed by the same letter did not differ ($P < 0.05$) between 2010, 2011 and 2012 and in repeated cultivation according to the LSD test. [a]Galling index on a scale from 0 to 10, where 0 = complete and healthy root system and 10 = plants and roots dead (Zeck, 1971). [b] RI (reproduction index) calculated as the number of eggs per gram of root on the resistant cv. Monika and divided by the number of eggs per gram of root on the susceptible cv. Durinta×100. Categories: VR: very resistant (RI < 10%), MR: moderately resistant (10% ≤ RI < 25) (Hadisoeganda and Sasser, 1982).

Tomato yield did not differ between cultivars for the first three *Pi* ranges in 2010 but did for *Pi* higher than 100 J2 250 cm^{-3} of soil. In 2012, tomato yield differed between cultivars at *Pi* higher than 10 J2 250 cm^{-3} of soil (Table 3). At the highest *Pi*, the resistant cv. Monika yielded 41 and 80% more ($P < 0.05$) than the susceptible cv. Durinta in 2010 and 2012, respectively.

Table 3. Yield (kg plant^{-1}) of resistant tomato cultivar Monika and susceptible tomato cultivar Durinta in soil infested by *Meloidogyne incognita* in a plastic greenhouse in Viladecans (Spain) in 2010 and 2012 with increasing initial population (*Pi*) range.

Year	*Pi* range	Yield (kg plant^{-1})	
		Monika	Durinta
2010	0	2.3±0.4	2.6±0.3
	1-10	1.9±0.04	1.8±0.2
	11-100	2.1±0.3	1.7±0.2
	101-300	2.3±0.2	1.1±0.1*
	301-500	1.9±0.1	1.2±0.1*
	501-1448	2.2±0.2	1.3±0.2*
2012	0-10	2.0±0.5	1.3±0.4
	11-100	2.2±0.2	0.9±0.1*
	101-300	2.2±0.3	0.9±0.2*
	301-500	2.2±0.2	0.5±0.1*
	501-1000	2.3±0.2	0.5±0.02*
	1001-3322	2.5±0.3	0.5±0.3*

Values are means ± standard deviations per each *Pi* range. Data within the same row with * are significantly different according to the Student t-test ($P < 0.05$).

The relationship between *Pi* and the relative susceptible tomato yield fitted to the Seinhorst damage model in 2010 and 2012 (Table 4), but not the resistant. The tolerance limit (*T*) and the relative minimum yield (*m*) in 2010 were 2 J2 per 250 cm^{-3} of soil and 0.48, respectively, and 4 J2 per 250 cm^{-3} of soil and 0.44 in 2012, respectively.

Table 4. Parameters of the Seinhorst damage function model for tomato cv. Durinta cropped in soil infested by *Meloidogyne incognita* in a plastic greenhouse in Viladecans (Spain) in 2010 and 2012.

Year	*m*	*T* (J2 250 cm^{-3} of soil)	R^2	*P*
2010	0.48±0.09	2.02±0.98	0.94	<0.001
2012	0.44±0.18	4.43±4.26	0.82	<0.001

Data are mean ± confidence interval (95%); Seinhorst damage function model: $y = m + (1-m)\ 0.95^{(Pi/T-1)}$; where *m*: minimum relative yield and *T*: tolerance limit.

Discussion

The results of the present study confirm the efficacy of resistant tomato suppressing *M. incognita* reproduction and disease severity without significant crop yield losses compared with the susceptible one (Ornat et al., 1997; Rich and Olson, 2004; Sorribas et al., 2005; Verdejo-Lucas and Sorribas, 2008). However, the resistant tomato did not confer a complete protection against *M. incognita* since a proportion of nematodes were able to infect, to develop and to reproduce on it. Thus, a low frequency of nematodes of the population was virulent to the *Mi*-gene when it was exposed to the resistant cultivar for the first time, being able to overcome the plant defense mechanisms. But the frequency increased along the years of repeated cultivation of resistant tomato, as it has been pointed out in this study, without achieving the condition of virulent population (RI > 50%). In this study, population dynamic parameters (*a*, *M* and *E*) were used to estimate the selection of virulent populations. On the resistant cultivar *a, M,* and *E* values increased year after year until around 31% of the values on the susceptible one, indicating that the selection for virulence was underway. This selection was also supported by the contrast of the population growth rate (relationship between *Pf/Pi* and *Pi*). These parameters were higher when the resistant tomato was repeatedly cultivated in the same plot than when it was rotated with the susceptible cultivar. That is, the maximum multiplication

rate, as well as, the equilibrium density, increased from 0.23 to 82.1 % and from 2.9 to 38.9% those achieved on the susceptible one, respectively. Disease severity and eggs per gram of root also increased after the repeated cultivation of the resistant tomato (2 and 3.7 times, respectively), but did not reach the values registered on the susceptible one, that were 2.8 and 9.9 times higher, respectively.

The tomato cv. Monika responded as very resistant to *M. incognita* when it was rotated with the susceptible one, but performed as moderately resistant (RI = 10.1) the third year of repeated cultivation, which indicates that despite the virulence selection it was not enough time to completely select for virulence. Some reports have been pointed out an increase of the reproduction index after three years of repeated cultivation of the resistant cv. Monika in plastic greenhouse, which responded as moderately (Sorribas et al., 2005) to slight resistant (Verdejo-Lucas et al*.,* 2009) at the end of these studies. However, the results of pot experiments conducted with the nematode population obtained at the end of the third resistant tomato crop to determine the putative selection for virulence showed that selection was underway because the resistant genotype responded as slight resistant (RI = 26) (Sorribas et al., 2005) or susceptible (RI = 108) (Verdejo-Lucas et al., 2009). Some RKN populations can be naturally virulent without previous exposure to resistant tomato cultivars (Ornat et al., 2001), can be suddenly selected (Williamson, 1998), or progressively selected by repeated cultivation of resistant genotypes; i.e. after 3 to 8 cropping seasons (Eddaoudi et al., 1997; Noling, 2000). Thus, the values resulting from the population dynamic studies, as well as, of those coming from the contrast of the population growth rates between cropping season for a plant genotype o between susceptible and resistant genotypes can be helpful for the early detection of the selection of virulent populations.

The tolerance limit of both tomato cultivars did not differ, but the resistant tomato yielded about 50% more than the susceptible one in both cropping seasons confirming previous results in Mediterranean conditions (Di Vito et al., 1991; Sorribas et al., 2005; Talavera et al., 2009).

Including plant resistance in rotation sequences is useful to manage the build up of RKN densities and to prevent yield losses to the following susceptible crop as it has previously been reported (Hanna et al., 1994; Ornat et al., 1997; Rich and Olson 2004). Nonetheless, when the selection for virulence begins, both virulent and avirulent components of the population coexist in soil and, as our results showed, it is maintained regardless the following susceptible crop. The fitness of the *Mi*-virulent components of the nematode population is unclear. In some cases, it has been associated to a reduction of the infective capacity and/or fecundity on susceptible genotypes or other susceptible plant host (Roberts, 1995; Huang et al., 2004; Castagnone-Sereno et al., 2007; Tzortzakakis and Blok, 2007; Dijan-Caporalino et al., 2011). Other reports do not identify any adverse cost of fitness (Castagnone-Sereno et al., 1994; Tzortzakakis et al., 1998) or both adverse and no effect, depending on the virulent nematode lines from the same isolates (Petrillo and Roberts, 2005). For this reason, is essential to prevent the increasing frequency of virulent individuals in the population to maintain the efficacy of resistant genes through time. Otherwise, if fitness of virulent populations is affected, several years of cropping a susceptible host will be needed to decrease its frequency and the damage potential (Petrillo et al., 2006). As the virulence is highly specific to a given resistant gene, the rotation with other single resistant genes and non-host crops will promote the durability of the resistance.

This study demonstrates the effectiveness of the resistant tomato cv. Monika to reduce the *M. incognita* population growth rate and to reduce yield losses

compared to cv. Durinta in our particular agronomic conditions. For the first time, the utility of the population dynamics parameters to the early detection of virulence selection has been stated, and confirm that three successive crops of the resistant cv. Monika were not enough to completely select a virulent population.

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Chapter 4

Population dynamics of *Meloidogyne incognita* on cucumber grafter onto the Cucurbita hybrid RS841 or ungrafted and yield losses under protected cultivation

Population dynamics of *Meloidogyne incognita* on cucumber grafted onto the Cucurbita hybrid RS841 or ungrafted and yield losses under protected cultivation.

The influence of the squash hybrid RS841 rootstock (*Cucurbita maxima* x *C. moschata*) on population dynamics of *Meloidogyne incognita* and yield of cucumber cv. Dasher II was assessed during 2013 and 2014 in a plastic greenhouse. In addition, the relationship between ecophysiological parameters (plant water status, gas exchange, and leaf reflectance) and *Pi* and cucumber yield were also estimated in 2013. Nematode densities were determined at the beginning (*Pi*) and at the end (*Pf*) of each crop, and the relationship between these parameters was used to estimate the maximum multiplication rate (*a*), the ceiling level (*M*) and the equilibrium density (*E*) per grafted and ungrafted cucumber and cropping season. Moreover, the relationship between the multiplication rate (*Pf*/*Pi*) and *Pi* was compared between grafted and ungrafted cucumber per cropping season. Finally, the relative yield of grafted or ungrafted cucumber was plotted against *Pi* to determine the tolerance limit (*T*) and the minimum relative yield (*m*) by the Seinhorst damage function model. Values of *a, M* and *E* in grafted cucumber were higher than in ungrafted one irrespective of the cropping season. These results were supported by comparing the relationship between *Pf/Pi* and *Pi* between grafted and ungrafted cucumber. The relationship between *Pi* and yield fitted the Seinhorst damage function. The values of *T* and *m* did not differ between grafted and ungrafted each year. Predawn water potential, net photosynthetic rate, and leaf chlorophyll index decreased with increasing *Pi*. In addition, relative yield was related to variation in net photosynthetic rate and the leaf chlorophyll index. Under the conditions of this study, RS841 rootstock was neither resistant nor tolerant to *M. incognita*.

Keywords: *Cucumis sativus, Cucurbita maxima* x *Cucurbita moschata,* Equilibrium density, Multiplication rate, Root-knot nematode, Tolerance limit.

Introduction

Cucumber (*Cucumis sativus* L.) is one of the most important vegetable crops worldwide, producing 65 million tons per year (FAOSTAT 2016). Spain produces 748500 tons of cucumbers per year in 8811 ha, of which, 88% are under protected cultivation (MAGRAMA ,2013). Cucumber is susceptible-intolerant to the most common root-knot nematodes (RKN) species, which can cause yield losses up to 88% under protected cultivation (Giné et al., 2014). Despite the chemical control is the most used control method (Talavera et al., 2012), non-chemical alternatives are needed in order to implement the European Directive 2009/128/EC.

Horticultural grafting is one of the most promising techniques which consists of the union of the aerial part (scion) of a susceptible plant with a root system (rootstock) of a resistant one. The main purpose of this technique is to control plant pathogens causing soil-borne diseases, including plant parasitic nematodes, to enhance tolerance to abiotic stresses, and to improve yield (Edelstein, 2004; Davis et al., 2008; King et al., 2010; Lee and Oda, 2010; Lee et al., 2010). Several cucurbit rootstocks have been tested against RKN (Sigüenza et al., 2005; Davis et al., 2008, Lee and Oda, 2010; Kokalis-Burelle and Rosskopf, 2011; Liu et al., 2015; Thies et al., 2015; López-Gómez et al., 2016). Among them, the squash interspecific hybrid (*Cucurbita maxima* x *C. moschata*) is the most widely used rootstock for cucumber, melon and watermelon in Europe (Lee et al., 2010). This rootstock is resistant to *Fusarium oxysporum* f. sp. *melonis*, *F. oxysporum* f. sp. *cucumerinum*, *F. oxysporum* f. sp. *lagenariae*., *F. oxysporum* f. sp. *niveum*, *F. oxysporum* f. sp. *radicis-cucumerinum*, *Rhizoctonia solani* and *Verticillium dahliae* (Davis et al., 2008; Pavlou et al., 2002; USDA, 2015). Regarding RKN, the level of resistance reported is not consistent, being either susceptible or

resistant (Davis et al., 2008; Cansev and Ozgut, 2010; Kokalis-Burelle and Rosskopf, 2011; Goreta Ban et al., 2014; USDA, 2015), but no information about its tolerance when cucumber is grafted as a scion is available. In order to know the host status of the rootstock under particular agro-environmental conditions, the relationship between nematode density at planting (*Pi*) and at the end of the crop (*Pf*) has to be determined to estimate the maximum multiplication rate (in absence of limiting factors) (*a*), the ceiling level (the maximum *Pf* achieved by a nematode population on a plant host under particular conditions) (*M*), and the equilibrium density (when the plant can supply enough food to maintain the population density at planting; *Pf* =*Pi*; *Pf*/*Pi*=1) (*E*) (Seinhorst, 1967). These parameters are higher on susceptible hosts than on resistant or poor hosts. Another useful indicator of the population growth rate is the relationship between the multiplication rate (*Pf*/*Pi*) and *Pi.* It allows comparing nematode population dynamics on different plant species or germplasms or between cropping seasons for a given pathosystem and the efficacy of control methods, (Talavera et al., 2009; Vela et al., 2014). Those parameters refer to nematode population growth but do not provide valuable information on marketable crop yield, which is provided by the relationship between *Pi* and relative crop yield by the Seinhorst's damage function model, the tolerance limit and the minimum relative yield (Seinhorst, 1965). It is known that plant growth and yield are reduced at increasing *Pi* above the tolerance limit due to a reduction of water and nutrient intakes, and consequently, a reduction of plant conductivity and transpiration, as well as leaf chlorophyll content and photosynthesis rate (Loveys and Bird, 1973; Melakeberhan, 2003; Agrios, 2005; Strajnar et al., 2012). Leaf chlorophyll content, photosynthesis rate, and plant biomass have previously been related to *Pi* (Melakeberhan et al., 1985; Giné et al., 2014; López-Gómez et al., 2015). Therefore, these ecophysiological parameters might be potential indicators of tolerance and yield losses, but it needs to be evaluated under field conditions.

The aim of this study was to determine the influence of the rootstock RS841 (*Cucurbita maxima* x *C. moschata*) on the population dynamics of the RKN, *Meloidogyne incognita* and yield of cucumber cv. Dasher II cultivated in a plastic greenhouse during two cropping seasons. In addition, the relationship between several ecophysiological parameters and *Pi* and cucumber yield was also assessed.

Materials and Methods

Experiments were carried out in spring 2013 and 2014 in a 700 m^2 plastic greenhouse located at Viladecans (Barcelona, Spain) which had been artificially infested with *M. incognita* in 2007. The soil texture was sandy loam with 83.8% sand, 6.7% loam and 9.5% clay; pH 8.7; 1.8% organic matter (w/w), and 0.5 dS m^{-1} electrical conductivity. From 2007 until the beginning of the experiments, rotations with resistant or susceptible tomato cultivars and cucumber or fallow were done.

Forty-four gross plots of 9.6 m^2 were cultivated with each of grafted or ungrafted cucumber. Individual gross plots consisted of 24 plants spaced 50 cm within each of four rows and 55 cm between rows. Net plots of 3.2 m^2 comprised the central part of the plot in which 8 plants were cropped and from which soil and roots samples were taken and crop yield was assessed. The soil of each plot was prepared individually to prevent cross contamination. In 2013, the cucumber crop was preceded by resistant or susceptible tomato cultivars or fallow to achieve gradients of nematode densities. In 2014, the cucumber crop was preceded by fallow. Cucumber cv. Dasher II was grafted onto *Cucurbita maxima* x *C. moschata* hybrid RS841 by tongue approach, because it is easy to use, successful, and provides a uniform growth rate (Davis et al., 2008). The squash hybrid RS841 has been proven to develop a longer root system than cucumber and to improve crop yield (Maršić and Jakše, 2010). Grafted and ungrafted cucumber was cropped from April 14th to July 16th in 2013 (78 days) and in 2014 from March 3rd to June 19th (109 days). Plants were irrigated as needed through a drip irrigation system

and weekly fertilized with a solution consisting of NPK (15-5-30) at 31 kg ha^{-1} and iron chelate and micronutrients at 0.9 kg ha^{-1}. Cucumber plants were vertically trained. Weeds were removed manually during and between crops. Fruits from the eight central plants of each plot were harvested when they reached the standard commercial size and were expressed as kg per m^2. Soil temperatures were recorded daily at 30 min intervals with temperature probes 5TM (Decagon devices, Inc) placed at a soil depth of 15 cm.

At the end of the cropping season, plants were removed from the ground with a pitchfork. Disease severity was rated by Zeck's scale from 0 to 10 (Zeck, 1971), where 0=complete and healthy root system and 10=plants and roots dead. After that, eggs were extracted from two root subsamples of 20 grams by maceration in a blender containing a 1 % NaOCl solution for 10 min (Hussey and Barker, 1973). The suspension was sieved through a 74 µm aperture screen, to remove root debris, and through a 25 µm aperture screen to retain the eggs. The number of eggs were counted and expressed per gram of root. Nematode population densities in soil were estimated at the beginning (*Pi*) and at the end (*Pf*) of each crop. Soil samples were taken from each of the forty-four net plots. Composite soil samples consisted of eight soil cores taken from the first 30 cm of soil with a soil auger of 2.5 cm diameter. Soil samples of about 1.2 L were homogenized and sieved through a 4 mm aperture screen to remove stones and separate roots from the soil. Nematodes were extracted from a 500 cm^3 of soil subsample using Baermann trays (Whitehead and Hemming, 1965) incubated at 27°C for a week. Second-stage juveniles (J2) that migrated to the water were then collected sieving it thorough a 25 µm aperture screen. Nematode densities are expressed as J2 per 250 cm^3 of soil.

The estimation of the maximum multiplication rate (*a*) was carried out considering the small values of *Pi*, according to $Pf = aPi$ (Seinhorst, 1970), and the

maximum population density at the end of the crop (*M*) from the experimental data. The equilibrium density (*E*) was calculated according to the expression $M = aE/(a-1)$ (Schomaker and Been, 2006).

In addition, in 2013, plant water status, gas exchange and leaf reflectance were measured 60 days after transplanting. Measurements were carried out in grafted and ungrafted plants growing in plots with *Pi* ranging from 0 to 750 J2 per 250 cm^3 of soil. Predawn water potential (Ψ_p) was determined using a Scholander pressure chamber (Soilmoisture 3005, Soil Moisture Corp., Santa Barbara CA, USA) early in the morning (around 7:00 a.m. solar time) on three leaves that were covered with both aluminum foil and plastic bags the night before. Net photosynthetic rate (A), stomatal conductance (g_s) and transpiration (E) were measured between 11:00 and 12:30 a.m. (solar time) with a portable gas exchange system CIRAS-2 (PP Systems Ltd., Havervill MA, USA) in three fully expanded leaves (one leaf/plant). Gas exchange parameters were determined at a CO_2 concentration in the cuvette of 400 ppm whereas temperature and water vapor concentration were not controlled. Afterwards, leaf reflectance was determined using a spectroradiometer (Unispec, PP Systems, Ltd., Harvervill, MA, USA) with a 2.3 mm diameter bifurcated fiber optic (model UNI410, PP Systems, Havervill, MA, USA). The detector samples 256 bands at roughly even intervals (average band-to-band spacing 3.3 nm) within a 400-1100 nm effective spectral range. Among several chlorophyll reflectance-based indices tested, the chlorophyll index proposed by Datt (1998) ($R_{672} / [R_{550} \times R_{708}]$) was selected.

Statistical analyses

Statistical analyses were carried out using the SAS system V9 (SAS Institute Inc., Cary, NC). The values of *Pi* and *Pf/Pi* were transformed to log10 to linearize them and the relationship between *Pi* and *Pf/Pi* for grafted or ungrafted cucumber was determined per cropping season by regression analysis (proc reg). The resulting

regressions per grafted or ungrafted cucumber between cropping seasons were compared by the general lineal model procedure (proc glm). When no differences ($P < 0.05$) were found, data were pooled to construct a single general model and a new comparison of regression was carried out between grafted and ungrafted cucumber.

The relative yield of grafted or ungrafted cucumber was plotted against the *Pi* values and submitted to a non-linear regression analysis (proc nlin) to determine if they fitted the Seinhorst damage function model ($y = m + (1-m)\ 0.95^{(Pi/T-1)}$; when $Pi \geq T$, and $y = 1$ when $Pi < T$, where y is the relative yield (the yield at a given *Pi* divided by the yield at $Pi \leq T$, with $y = a$ at $Pi \leq 1$), m is the minimum relative yield (the lowest value of the relative yield at higher *Pi*), and T is the tolerance limit (the nematode density (*Pi*) up to which no yield losses occurs). Values of minimum relative yield (m), and tolerance limit (T) were compared between grafted and ungrafted cucumber per cropping season considering confidence intervals at 95% (Giné et al., 2014).

Correlation analysis (proc corr) was used to determine the relationship among ecophysiological parameters (Ψ_p, A), the chlorophyll reflectance based index $R_{672}/[R_{550} \times R_{708}]$ and both *Pi* and relative yield on both grafted and ungrafted cucumber plants.

Results

Absolute soil temperatures ranged from 17.5°C and 31.6°C in 2013 (accumulated degree days 1946, Tb = 0°C) and from 17.7°C to 28.8°C in 2014 (accumulated degree days 2546, Tb = 0°C). The nematode was able to complete two generations in both cropping seasons according to its thermal requirements (Tb= 11.4°C and thermal constant= 500 degree days over *Tb;* Giné et al., 2014).

In 2013, *Pi* ranged from 0 to 6180 J2 per 250 cm^3 of soil in plots cultivated with grafted cucumber, and from 0 to 894 J2 per 250 cm^3 of soil in those cultivated with the

ungrafted one. In 2014, *Pi* ranged from 0 to 220 J2 per 250 cm^3 of soil in plots cultivated with either grafted or ungrafted cucumber.

The maximum multiplication rate (*a*), the maximum population density (*M*) and the equilibrium density (*E*) on grafted cucumber in 2013 were 754, 1125 and 1123 J2 250 cm^{-3} of soil, respectively, and 112, 696 and 690 J2 250 cm^{-3} of soil, respectively in the ungrafted one (Figure 1). In 2014, *a*, *M* and *E* on grafted cucumber were 825, 1012 and 1011 J2 250 cm^{-3} of soil, respectively, and in the ungrafted cucumber were 163, 207 and 206 J2 250 cm^{-3} of soil, respectively (Figure 1). The relationship between *Pi* and *Pf/Pi* on grafted or ungrafted cucumber did not differ between cropping seasons (in 2013: intercept $P = 0.8072$ and slope $P = 0.763$; in 2014: intercept $P = 0.0919$ and slope $P = 0.1547$), but it did when comparison was carried out between grafted and ungrafted cucumber with pooled data (intercept $P = 0.005$ and slope $P = 0.3123$) (Figure 2). *Meloidogyne incognita* produced more ($P < 0.05$) eggs per gram of root, and less ($P < 0.05$) galling index on the grafted than ungrafted cucumber in both cropping seasons (Table 1). Grafted cucumber had a higher frequency of plants with galling index between 3 and 7 than the ungrafted ones (Figure 3).

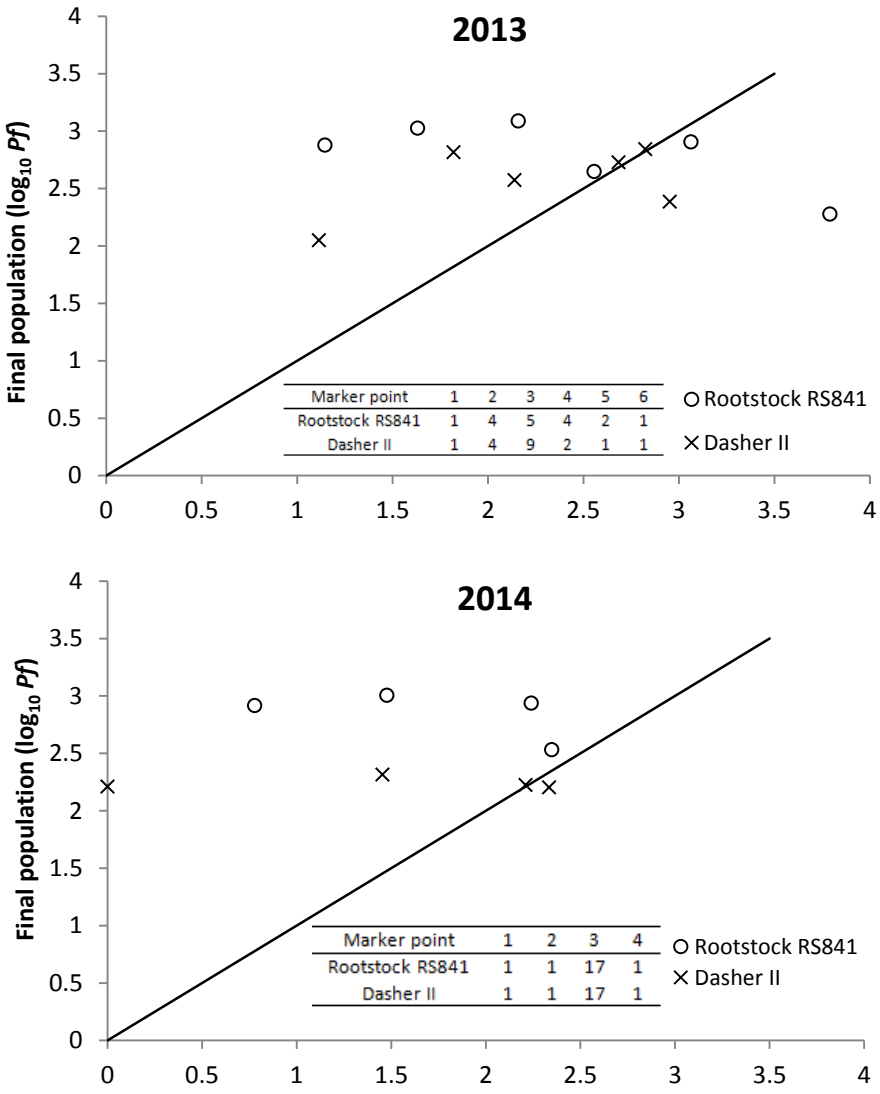

Figure 1. Relation between final and initial populaton density of *Meloidogyne incognita* on cucumber cv. Dasher II grafted onto the cucurbit rootstock RS841 (*Cucurbita maxima* x *C. moschata*) and ungrafted cucumber cv. Dasher II in 2013 and 2014. The numbers of observations were 17 and 20 of rootstock RS841 in 2013 and 2014 respectively, and 20 in cucumber cv. Dasher II for both years. Numbers of observations of each point are in the table inside the figure.

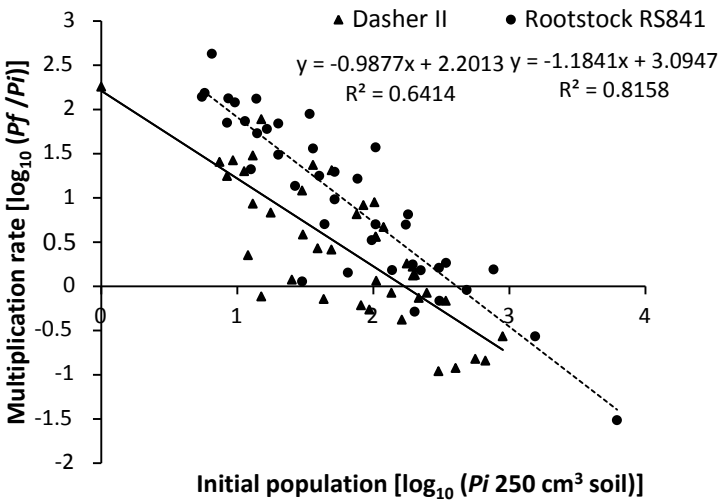

Figure 2. General model of the relationship between initial population density (*Pi*) and multiplication rate (*Pf/Pi*) of *Meloidogyne incognita* on grafted cucumber cv. Dasher II onto the cucurbit hybrid RS841 (*Cucurbita maxima* x *C. moschata*) and ungrafted cucumber cv. Dasher II cropped in plastic greenhouse from April to July in 2013 and from March to June in 2014.

Table 1. Number of eggs of *Meloidogyne incognita* per gram of root and galling index on grafted cucumber cv. Dasher II onto the cucurbit hybrid RS841 (*Cucurbita maxima* x *C. moschata*) and on ungrafted cucumber cv. Dasher II in 2013 and 2014.

Year	Treatment	Eggs per g root	Galling index[a]
2013	Rootstock RS841	19054±2591*	7.4±0.21*
	Dasher II	3822±788	8.5±0.21
2014	Rootstock RS841	9675±123*	7.3±0.25*
	Dasher II	4490±551	8.1±0.27

Values are means ± standard deviations of 22 replicated plots in all treatments. Data within the same column and year followed by *indicates a significant difference between soil treatment at $P < 0.05$ according to the Student's t -test.

[a] Galling index on a Zeck's (1971) scale from 0 to 10, where 0 = complete and healthy root system and 10 = plants and roots dead

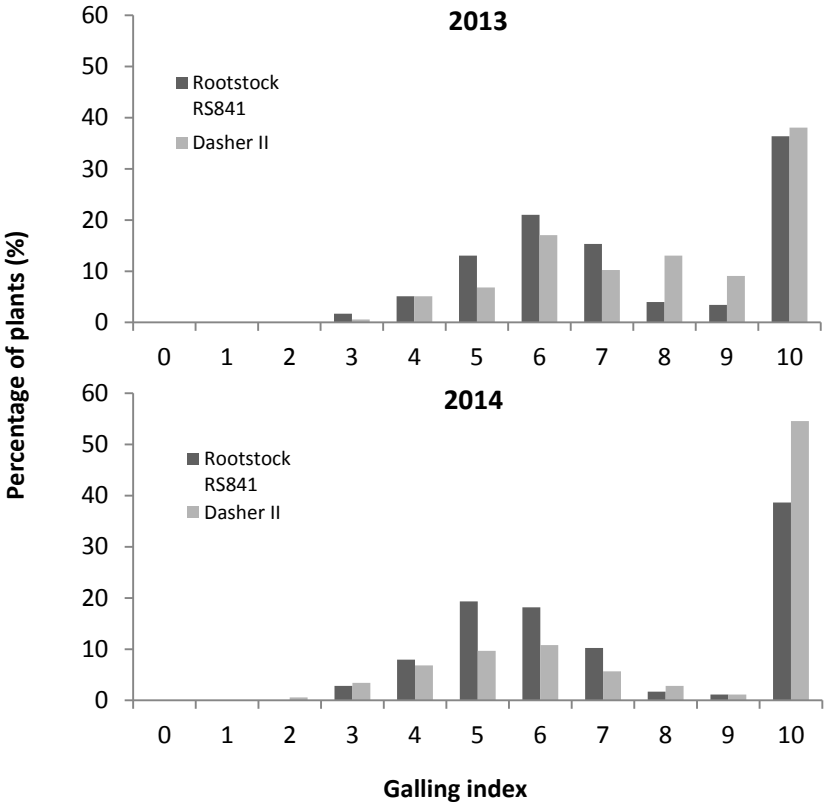

Figure 3. Desease severity (galling index) of *Meloidogyne incognita* on the grafted cucumber cv. Dasher II onto the cucurbit hybrid RS841 (*Cucurbita maxima* x *C. moschata*) and on the ungrafted cucumber cv. Dasher II cropped in plastic greenhouse from April to July in 2013 and from March to June in 2014 in plots with *Pi*>0.

Grafted cucumber yielded 4.2 and 3.7 kg m^{-2} in 2013 and 2014, respectively, and 5.3 and 3.7 kg m^{-2} the ungrafted one in uninfested plots. The relationship between *Pi* and grafted or ungrafted relative cucumber yield fitted the Seinhorst damage function model. The tolerance limit and the minimum relative yield of both grafted and ungrafted cucumber did not differ per cropping season (Figure 4).

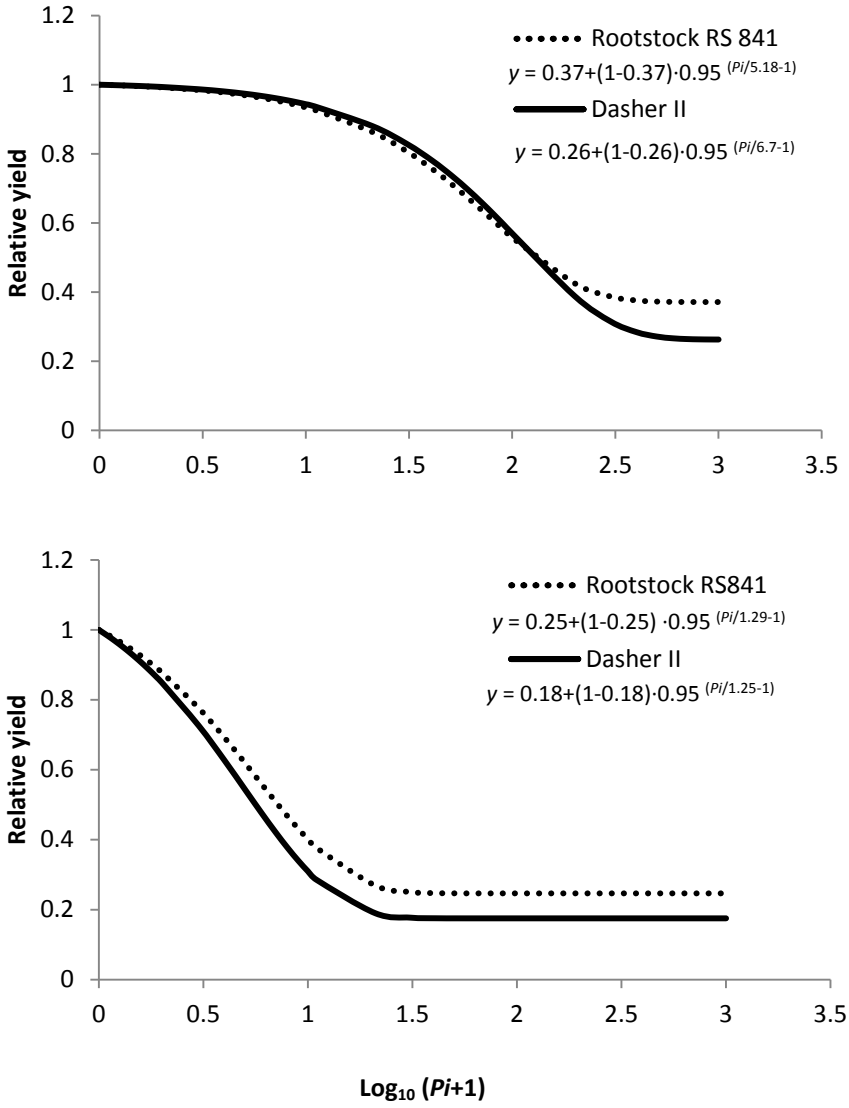

Figure 4. Seinhorst damage function model of *Meloidogyne incognita* of grafted cucumber cv. Dasher II onto the hybrid RS841 (*Cucurbita maxima* x *C. moschata*) and ungrafted cucumber cv. Dasher II cropped in plastic greenhouse from April to July in 2013 and from March to June in 2014.

Predawn water potential (Ψ_p) decreased with increasing *Pi* (r = -0.76; $P < 0.01$) for both grafted and ungrafted cucumber, ranging from -0.38± 0.02 MPa (average ± standard error) at *Pi* = 0 to -0.62 ± 0.04 MPa at *Pi* = 765 (Table 2). Similarly, A and R_{672} / [R_{550} x R_{708}] (Table 2) showed significant correlation against *Pi* with $r = -0.66$ ($P < 0.01$) and $r = -0.48$ ($P < 0.05$), respectively. Moreover, for both grafted and ungrafted plants, Ψ_p showed significant correlation against net photosynthetic rate (A) ($r = 0.53$; $P < 0.05$) and R_{672} / [R_{550} x R_{708}] ($r = -0.76$; $P < 0.01$). In addition, the relative variation in R_{672} / [R_{550} x R_{708}] was found to be significantly related to relative decreases in A ($R^2 = 0.91$, $P < 0.01$). Furthermore, decreases in relative cucumber yield were significantly related to both the relative variation in A ($R^2 = 0.88$, $P < 0.01$) and the relative variation in R_{672} / [R_{550} x R_{708}] ($R^2 = 0.91$, $P < 0.01$).

Table 2. Predawn water potential (Ψ_p), net photosynthetic rate (A) and chlorophyll index (R_{672} /[R_{550} x R_{708}]) of grafted cucumber cv. Dasher II onto the cucurbit hybrid RS841 (*Cucurbita maxima* x *C. moschata*) and ungrafted cucumber cv. Dasher II cultivated in soil infested by increasing *Meloidogyne incognita* densities at transplanting (*Pi*).

Treatment	***Pi*** (J2 250cm^{-3} of soil)	**Ψ_p** (MPa)	**A** (µmol CO_2 m^{-2} s^{-1})	**R_{672} /[R_{550} x R_{708}]**
Rootstock RS841	0	-0.38 ± 0.02	11.37 ± 0.86	0.912 ± 0.052
	342	-0.47 ± 0.02	8.60 ± 2.64	0.827 ± 0.110
	765	-0.62 ± 0.07	4.90 ± 0.75	0.680 ± 0.031
Dasher II	0	-0.47 ± 0.02	8.60 ± 2.17	0.827 ± 0.093
	301	-0.47 ± 0.03	5.57 ± 4.64	0.578 ± 0.022
	666	-0.58 ± 0.07	1.63 ± 0.78	0.524 ± 0.033

Data are mean of three replications ± standard error.

Discussion

Grafting susceptible crops onto resistant-tolerant rootstocks is a promising non-chemical alternative to manage RKN because nematode population will be suppressed and the crop will suffer less yield losses than cropping susceptible-intolerant cultivars. Several commercial resistant tomato and pepper rootstocks have been shown their effectiveness against RKN (Oka et al., 2004; Ros et al., 2006; Cortada et al., 2008; Verdejo-Lucas and Sorribas, 2008; Kokallis-Burelle et al., 2009; Verdejo-Lucas et al., 2009). However, despite the fact that the effectiveness of cucurbit rootstocks against RKN has been studied (Thies et al., 2010; Lee et al., 2010; Liu et al., 2015; Thies et al., 2015; López-Gómez et al., 2016), there are not available resistant commercial rootstocks for cucumber and melon. Nevertheless, some promising results have been reported with *Cucumis metuliferus*, which has been proven to be highly resistant to root-knot nematodes (Lee et al., 2010; Kokalis-Burelle and Rosskopf, 2011; Thies et al., 2012; Picó et al., 2013; Munera et al., 2014), and is compatible with some melon cultivars (Sigüenza et al., 2005; Gisbert et al., 2014; Guan et al., 2014).

Although the squash hybrid rootstock *Cucurbita maxima* x *C. moschata* is widely used, little information is available about its performance against increasing *Pi* of RKN in terms of host status, crop yield, and ecophysiological parameters. The results of this study showed that RKN population dynamics on ungrafted or grafted cucumber onto RS 841 differed, being the former less good host than the grafted according to *a,* and *E* values. These parameters are indicators of the host status in a given agro-environmental conditions (Seinhorst, 1967). High values of *a* and *E* are indicators of good plant hosts and low values of poor or resistant ones. In this study, *a* values of *M. incognita* on grafted cucumber were closer between cropping season and around 82% higher than on the ungrafted one. The *E* values on grafted cucumber were also higher (48% to 82%; mean 65%) than in the ungrafted one. These results were also supported

by the estimation of the population growth rate on grafted and ungrafted cucumber by the relationship between *Pf/Pi* and *Pi*. The population growth rate on grafted cucumber was higher than on the ungrafted one. That is, the *a* and *E* value were 87% and 59% higher on grafted than on ungrafted cucumber. These results could be explained because the root system of the rootstock RS841 is larger than the cucumber cv Edona F1 (Maršić and Jakše, 2010), but as far we know, there is no information comparing root systems of both the rootstock RS841 and cucumber cv. Dasher II. Thus, *Cucurbita maxima* x *C. moschata* RS481 is not resistant neither poor host to RKN considering the low values of *a* and *E* reported for *M. incognita* on resistant tomato (158 and 50 J2 per 250 cm^3 of soil, respectively; Talavera et al., 2009) or for *M. javanica* on a poor host such as watermelon (near 70 and 80 J2 per 250 cm^3 of soil, respectively) (López-Gómez et al., 2014).

The agro-environmental conditions in which the crop is cultivated can play an important role in population dynamics (Seinhorst, 1967). The results of this study show that values of *a* and *E* of *M. incognita* on ungrafted cucumber cv. Dasher II cropped in spring-summer (April to July) were lower than those reported by Giné et al., (2014) when cucumber was cropped in summer-autumn (July to November). Similar results were reported for *M. incognita* on zucchini-squash (Vela et al., 2014) and for *M. artiellia* on chickpea (Di Vito and Greco, 1988).

The relationship between *Pi* and relative yield of both grafted and ungrafted cucumber fitted the Seinhorst damage function model. The tolerance limit did not differ between cropping season. Estimated values of *T* were between 1 and 7 J2 per 250 cm^3 of soil, being similar to those reported for grafted melon onto the squash hybrid Shintozoa (Kim and Ferris, 2002) or ungrafted melon (Ploeg and Phillips, 2001), but lower than for watermelon (50 J2 per 250 cm^3 of soil) (López-Gómez et al., 2014). Cucumber yield losses ranged from 63 to 83% irrespective of grafting, similar to those

reported for ungrafted or grafted melon (Ploeg and Phillips, 2001; Kim and Ferris, 2002) and cucumber (Giné et al., 2014) but highest than for watermelon (37%) (López-Gómez et al., 2014). Thus, the results of this study suggest that grafting cucumber onto RS 841 does not provide more tolerance to RKN, as it has been reported for watermelon grafted onto RS841 which suffered 18% and 45% higher yield losses than the ungrafted cultivar at *Pi* around 67 and *Pi* < 1 J2 250 cm^{-3} of soil, respectively (López-Gómez et al., 2016).

In relation to the ecophysiological status, both grafted and ungrafted cucumber showed reduced predawn water potential along with increases in *Pi*, suggesting that increased levels of nematode population densities caused physiological drought (Audebert et al., 2000), probably as a result of impaired root functioning. Increased levels of nematode population also led to a decrease in chlorophyll content (as suggested by the negative relationship between *Pi* and the spectral based chlorophyll index R_{672} / [R_{550} x R_{708}]). As a result, nematode-induced changes in both plant water and nutrient status led to a decrease in net photosynthetic rate. Therefore, in agreement with previous studies, nematode infection caused a reduction in leaf chlorophyll content (Audebert et al., 2000; Ahmed et al., 2009; Khan and Haque, 2011; Giné et al., 2014; López-Gómez et al., 2015) as well as in photosynthetic activity (Loveys and Bird, 1973; Melakeberhan et al., 1985) as suggested by the negative relationship between *Pi* and net photosynthetic rate. In addition, for both grafted or ungrafted cucumber plants, relative changes in yield were also related to the relative variation in both R_{672} / [R_{550} x R_{708}] and net photosynthetic rate, confirming previous results in which relative dry top weight of cucumber (Giné et al., 2014) or zucchini-squash biomass (López-Gómez et al., 2015) was related to relative leaf chlorophyll content. These results suggest that reflectance based chlorophyll indices might provide an estimation of the effects of *Pi* on photosynthetic capacity and their effects, thereof, on yield in situations in which RKN is the main biotic stressful agent.

There is little information on the effect of grafted cucurbits, in general, and cucumber, in particular, on *Meloidogyne* population dynamics and crop yield losses for a given agro-environmental area. This work provides new information on these parameters to aid growers to take decisions to manage RKN. In addition, the possibility of using the reflectance-based chlorophyll as an indicator of relative crop yield losses is pointed out although some more field studies are needed to be used for advisor purpose.

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Chapter 5

Natural occurrence of fungal egg parasites of root-knot nematodes, *Meloidogyne* spp. in organic and integrated vegetable production systems in Spain

Natural occurrence of fungal egg parasites of root-knot nematodes, *Meloidogyne* spp. in organic and integrated vegetable production systems in Spain

A survey was conducted to assess the biodiversity and frequency of infection of fungal egg parasites of *Meloidogyne* spp. and relate results to soil properties in organic and integrated vegetable production in Spain. Forty sites were sampled at the end of the cropping cycle, 30 under integrated and ten under organic production. Fungal egg parasites were isolated from all organically managed sites and from 73 % sites under integrated production. Species richness and Shannon–Wiener index did not differ between production systems but the percentage of fungal egg parasitism did, as well as soil properties. Percentages of egg parasitism higher than 40 %were found in five and three sites under organic and integrated production, respectively. In all these sites, *Pochonia chlamydosporia* was present alone or co-occurring with other fungal species. The relative frequency of *P. chlamydosporia* was positively related to the percentage of parasitism in both production systems.

Keywords: Biological control, Fungal egg parasites, Integrated production, *Meloidogyne* spp., Organic farming, Vegetable crops.

Introduction

Root-knot nematodes (RKN), *Meloidogyne* Göldi (Rhabditida: Meloidogynidae), are a major limiting factor for vegetable production worldwide. In Spain, these nematodes cause yield losses of up to 60 % in cucumber and tomato and 30 % in lettuce (Verdejo- Lucas et al., 1994; Ornat et al., 1997; Sorribas et al., 2005). In conventional agriculture, control of RKN has been mainly based on the use of soil fumigants and nematicides. However, the need to reduce dependence on some agro-chemicals due to their negative impact on human health and the environment has

impelled researchers to explore non-chemical alternative methods for nematode control. The European Commission Directive 2009/128/EC on the sustainable use of pesticides established a framework for Community action to promote the use of integrated pest management and non-chemical alternatives to pesticides. One of these approaches is the conservation and enhancement of beneficial organisms. Sustainable production systems (i.e. integrated and organic farming) are good candidates for developing biologically based control methods because microbial diversity and activity could be enhanced by promoting soil fertility and restricting (integrated production) or excluding (organic farming) the use of synthetic pesticides. In Spain, the agricultural land under organic and integrated production of vegetable crops in 2010 was 10,156.06 ha and 29,210 ha, respectively, and has steadily increased during the last decade (Generalitat de Catalunya, 2012; Ministerio de Medio Ambiente y Medio Rural y Marino, 2011).

Nematode antagonists, including fungi, bacteria, and invertebrates are commonly associated to plant-parasitic nematodes, fungal parasites being the most abundant group (Stirling, 1991). All soils have an inhabitant microbial community able to restrict disease progression, and some possess specific microorganisms able to act against the pathogen, leading to specific suppressiveness (Cook and Baker, 1983). The density or biomass and functionality of the soil's microbiota can be estimated by measuring the enzymatic activity of the soil. Thus, fluorescein diacetate hydrolysis (FDA) has been related to total microbial activity (Swisher and Carroll, 1980), β-glucosaminidase to fungal biomass (Miller et al., 1998), protease to density of culturable bacteria (Asmar et al., 1992), and urease to bacterial and fungal biomass (Nannipieri et al., 1978). Furthermore, some specific enzymatic activities of soil can be of interest as indicators of the occurrence and functionality of putative biological control agents, as these enzymes affect key components of the structure of the nematode. Among them, protease and β-glucosaminidase activities could be indicators

of microbial egg antagonists because the main components of the external egg shell layer are proteins and chitin, respectively. In addition, environmental factors can influence the presence of selected antagonists (Kim et al., 1998) as well as the level of suppressiveness (Kerry et al., 1980; Gené et al., 2005). Therefore, it is important to identify key factors in growing areas, with similar environmental and agronomic practices, in order to enhance biological control by managing the antagonistic potential of the soil, which is known as ''the capacity of a soil ecosystem, through biotic factors, to prevent or reduce the spread of a pathogen, parasite, or other deleterious agent'' (Sikora, 1992).

The objectives of this study were (1) to determine and compare the biodiversity of naturally occurring fungal egg parasites of *Meloidogyne* in vegetable crops under integrated and organic production; (2) to assess the percentage of fungal egg parasitism and (3) to investigate the relationship between percentage of fungal egg parasitism, soil properties, and relative frequency of fungal egg parasites for each production system.

Materials and methods

Sampling and isolation of *Meloidogyne* spp. eggs

Forty vegetable growing sites, 30 under integrated and ten under organic production, with a history of RKN infestation, were sampled at the end of the cropping season from May to September 2010. The number of sampled sites under each production system corresponded to the actual proportion (3:1; integrated: organic production) in Spain. Sites located in the provinces of Barcelona, Tarragona (north-eastern) and Valencia (eastern Spain) were selected in collaboration with agricultural field advisors who provided information on site characteristics and history. None of the 30 sites under integrated production had been treated with chemical nematicides

before planting the chosen crop although three sites had been treated the year before, 20 sites two years before, and the rest three years before. Fertilization in integrated production was based on pellets of composted manure combined with chemical fertilizers, whereas, in organic production, it was done by annual applications of composted manure and cover crops that are incorporated into the soil every four years. Weeds were managed by mulching, mechanically, and to a lesser extent by herbicides (integrated production). The annual air temperatures in the sampled area ranged from 9 to 26°C.

At each site, 20 plants were removed from the ground at the end of the cropping season with a pitchfork to take roots and surrounding soil. Soil was sieved through a 4-mm aperture screen to remove stones and separate roots from soil, and carefully mixed to extract nematodes from two 250-cm^3 soil subsample using the sieving and centrifugation-flotation method (Jenkins, 1964).

Roots were carefully washed free of soil, chopped, and root-knot nematode eggs extracted from two 10 g-subsamples by macerating them for 10 min in a blender containing a 5 % solution of commercial bleach (40 g l^{-1} of NaOCl) (Hussey and Barker, 1973). Eggs were counted and expressed per g root.

Plant-parasitic nematodes were identified at the genus level. Meloidogyne species were identified according to the morphology of the perineal patern of the females, and by SCAR-PCR markers (Zijlstra et al., 2000).

Isolation of egg parasites and identification

Fungal egg parasites of RKN were isolated according to the de Leij and Kerry, (1991) procedure modified by Verdejo-Lucas et al., (2002). Briefly, per each site, 25–30 egg masses were handpicked from roots and placed in a watchglass containing sterile,

distilled water. The outer part of the gelatinous matrix was removed from the egg masses with tweezers to eliminate potential surface colonizers. Egg masses were then placed in an Eppendorf microcentrifuge tube containing 1 ml of sterile distilled water. Eggs were dispersed from the egg masses using a pestle and 333 µl-aliquots of the eggs' suspension were spread onto each of three replicated Petri dishes (9-cm diam) containing a growth restricting medium (streptomycin, 50 mg l^{-1}; chloramphenicol, 50 mg l^{-1}; chlortetracycline, 50 mg l^{-1}; Rose Bengal, 50 mg l^{-1}; triton, 1 ml l^{-1}; and 1 % agar) (Lopez-Llorca and Duncan, 1986). In all cases, more than 100 eggs per plate were dispensed. Plates were incubated at 25 ± 0.5°C. The number of parasitized eggs was recorded after 24 h and 48 h under a dissecting microscope and percentage of parasitism was then calculated as the number of parasitized eggs per plate/ number of eggs per plate. Eggs were considered parasitized if fungal hyphae grew from inside. The parasitized eggs were then individually transferred to corn meal agar (CMA) to establish pure cultures of the fungi. Cultures were stored in 1 % (w/v) water-agar slants, as well as lyophilized and stored at 4°C.

A single spore culture was established per each isolate and they were identified by cultural and morphological characteristics and/or molecular analyses by PCR amplification and sequencing of the internal transcribed spacers (ITSs) of the rDNA regions. DNA was extracted from 50 mg of mycelium collected from single spore cultures on potato dextrose agar (PDA). The extraction was carried out using the E.Z.N.A kit Plant MiniPrep (Omega Bio-Tek) following the protocol described by the manufacturer. The PCR reaction was performed in 25 µl mix that contained 1 µl of the DNA extraction, 10.5 µl MiliQ water (Qiagen), 12.5 µl Taq PCR Master Mix (Qiagen) and 0.5 µl of each primer (5 pmol), ITS1F (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns, 1993) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al., 1990). The amplification consisted of an initial 5 min denaturation step at 94°C, five amplification cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 60 s, followed by 33 amplification

cycles at 94°C for 30 s, 48°C for 30 s, 72°C for 60 s, and a final extension step of 72°C for 10 min (White et al., 1990). PCR products were cleaned using MinElute PCR Purification Kit (Qiagen) and sequenced by Secugen (Madrid, Spain). DNA sequences were analysed using the BLAST database (September 2011) and assigned to the reference isolate sequences with the highest bit score.

Assessment of species diversity of egg parasites

Fungal diversity at each site was estimated according to the species richness (S), Shannon–Wiener diversity index, and species evenness. The Shannon–Wiener diversity index (*H'*) was calculated according to the formula $H' = -\sum (ni/N) \times \log2 (ni/N)$, where *N* is the number of eggs parasitized and *ni* is the number of eggs parasitized by the species *i*. The species evenness (relative abundance of each fungal egg parasite species in a given site) was calculated according to the expression $J' = H'/H'max$, where H0 is the index of Shannon–Wiener and *H'* max is the maximum value of *H'* (*H'*max = log2 S, where S is the species richness).

Soil properties

A 1 kg soil sample from each surveyed site was sent to AGQ Agroalimentaria y Medio Ambiente (Sevilla, Spain) to determine the physical and chemical characteristics that are described in Table 2. Four soil enzymes associated with microbial activity, β-glucosaminidase (Parham and Deng, 2000), urease (Sastre-Conde and Lobos, 2003), fluorescein diacetate hydrolysis and protease (Fernández et al., 2001) were analyzed following the above listed protocols.

Statistical analysis

Statistical analysis was carried out with the SAS system software V9. Variables were transformed when required to log10 (x + 0.1), or log10 (x). An analysis of variance

using the general linear model (proc glm) was carried out to compare soil properties, richness of fungal species, biodiversity index, and percentage of parasitized eggs between integrated and organic production. Correlation analysis (proc corr) was performed separately per production system to determine single relationships between variables including soil properties, percentage of egg parasitism, relative frequency of fungal species, and RKN population densities in soil and in roots. In addition, the variable time elapsed since the last chemical soil treatment was also included in the correlation analysis for sites under integrated production. If no single strong relationships were found between soil properties and percentage of fungal egg parasitism, then a multiple regression analysis (proc reg) was performed with the stepwise model selection at a significance level of P = 0.05 to determine if any combination of variables could explain the variability.

Results

Survey of plant parasitic nematodes

Seven genera of plant-parasitic nematodes were common to both production system: *Meloidogyne* (40 sites), *Tylenchorhynchus* Cobb (20), *Aphelenchus* Bastian (10), *Telotylenchus* Siddiqi (8), *Aphelenchoides* Fischer (7), *Pratylenchus* Filipjev (6), and *Paratylenchus* Micoletzky (2). Three additional genera were only identified in organic production: *Helicotylenchus* Steiner (6 sites), *Ditylenchus* Filipjev (3) and *Heterodera* Schmidt (2). The species of *Meloidogyne*, in order of occurrence, were *M. incognita* (Kofoid and White) Chitwood (42.4 %), *M. javanica* (Treub) Chitwood (30.3 %), and *M. arenaria* (Neal) Chitwood (27.3 %). Population densities of *Meloidogyne* were higher than those of the other plant parasitic nematodes irrespective of the production system. In sites under organic production, RKN densities ranged from 124 to 4,000 J2 250 cm^{-3} of soil (1,197 ±1,230; mean ± SD), and from 1,330 to 22,455 eggs per gram of root (10,056 ± 8,811), and those of the other nematodes from 14 (*Helicotylenchus*) to

528 nematodes 250 cm^{-3} of soil (*Tylenchorhynchus*). In integrated production, RKN population densities ranged from 57 to 13,247 J2 250 cm^{-3} of soil (2,826 ± 7,651), and from 122 to 39,592 eggs per gram of root (6,154 ± 8,250), and the densities of the other nematodes from 11 (*Telotylenchus*) to 4,300 nematodes 250 cm^{-3} of soil (*Pratylenchus*).

Fungal parasites of RKN egg were found in 100 % of the sites under organic, and in 73 % of the sites under integrated production. Fungal species common to both production systems were *Fusarium* sp., *F. oxysporum* Schlecht, *F. solani* (Mart.) Sacc., *Paecilomyces lilacinus* (Thom) Samson, *Plectosphaerella cucumerina* (Lindfors) Gams, *Pochonia chlamydosporia* (Goddard) Zare and Gams, and *Thielavia* sp. Fungi only found in organic production sites were *Cladosporium tenuissimum* Cooke, *Colletotrichum coccodes* (Wallr.) Hughes, and *F. equiseti* (Corda) Sacc. In contrast, *Chaetomium* sp. *Cladosporium sphaerospermum* Penz., *Cylindrocarpon olidum* (Wollenw.) Wollenw., *Dactylella oviparasitica* Stirling and Mankau, *F. verticillioides*, *Monacrosporium thaumasium* (Drechsler) de Hoog and Oorschot, *Myrothecium verrucaria* (Alb. & Schwein.) Ditmar, *Penicillium citrinum* Thom, *P. olsonii* Bainier and Sartory, and *Verticillium* sp. were only found in sites under integrated production. The most frequent fungal species isolated from RKN eggs in both production systems were *P. chlamydosporia*, *Fusarium* spp. and *P. cucumerina* (Table 1).

Species richness (F = 3.31; df = 1, 38; P = 0.077) and the biodiversity index of Shannon–Wiener (F = 0.26; df = 1, 31; P = 0.3996) did not differ between production systems. In organic production, species richness ranged from 1 to 3, the biodiversity index from 0 (sites with a single fungal species) to 1, and the evenness from 0.41 to 1. In integrated production, species richness ranged from 1 to 5, the biodiversity index from 0 to 1.74, and the evenness from 0.44 to 1. In the majority of the sites with more

than one fungal species, *P. chlamydosporia* was the dominant species (evenness lower than 1) irrespective of the production system (Table 1).

Percentage of fungal egg parasitism, soil properties and their relationships

The percentage of fungal egg parasitism was higher in organic (36.2 ± 8.9; mean ± SE) than in integrated production (9.2 ± 3.3) (F = 13.25; df = 1, 38; P = 0.0008) (Table 1). The relative frequency of *P. chlamydosporia* was positively related to the percentage of fungal egg parasitism both in organic (r = 0.838; P = 0.0024) and integrated production (r = 0.575; P<0.0001).

Table 1. Vegetable crop, percentage of fungal egg parasitism of *Meloidogyne* spp., species richness, Shannon–Wiener diversity index (*H'*) and evenness (*J'*) in vegetable production sites under organic and integrated production.

Production system	**Site**	**Crop**	**Parasitism (%)[a]**	**Fungal species (richness)**	***H'***	***J'***
Organic	M10.2	Tomato	24.4 ± 7.8	*Cladosporium tenuissimum*, *Fusarium oxysporum*, *Paecilomyces lilacinus* (3)	0.92	0.58
	M10.3	Tomato	56.9 ± 8.9	*Plectosphaerella cucumerina*, *P. chlamydosporia*. (2)	1	1
	M10.4	Tomato	4.0 ± 4.0	*Plectosphaerella cucumerina*, *Thielavia* sp. (2)	1	1
	M10.16	Tomato	65.4 ± 28.2	*Fusarium equiseti*, *P. chlamydosporia* (2)	1	1
	M10.19	Eggplant	4.5 ± 2.3	Unidentified (1)	0	-
	M10.23	Eggplant	14.4 ± 5.1	*Plectosphaerella cucumerina, P. chlamydosporia* (2)	0.76	0.76
	M10.41	Tomato	80.2 ± 3.5	*Colletotrichum coccodes, Fusarium solani, P. chlamydosporia* (3)	0.65	0.41
	M10.55	Eggplant	61.0 ± 7.8	*P. chlamydosporia* (1)	0	-
	M10.56	Pepper	9.4 ± 2.5	*Fusarium oxysporum, Paecilomyces lilacinus* (2)	1	1
	M10.58	Eggplant	41.7 ± 7.3	*Fusarium* sp., *P. chlamydosporia* (2)	0.92	0.92
Integrated	M10.13	Faba bean	3.3 ± 3.3	*Plectosphaerella cucumerina* (1)	0	-
	M10.21	Carrot	1.0 ± 0.5	*Thielavia* sp. (1)	0	-
	M10.24	Pepper	15.0 ± 0.0	*Penicillium olsonii, P. chlamydosporia* (2)	0.50	0.50
	M10.25	Cucumber	15.0 ± 1.7	*Plectosphaerella cucumerina, P. chlamydosporia* (2)	0.44	0.44
	M10.26	Tomato	0.9 ± 0.9	*Plectosphaerella cucumerina* (1)	0	-
	M10.27	Cucumber	1.4 ± 0.8	*Chaetomium* sp., *Cladosporium sphaerospermum* (2)	1	1
	M10.28	Courgette	0.7 ± 0.7	Unidentified (1)	0	-
	M10.29	Tomato	0.4 ± 0.4	Unidentified (1)	0	-
	M10.32	Cucumber	4.8 ± 2.9	Unidentified (1)	0	-
	M10.33	Cucumber	4.1 ± 2.4	*Plectosphaerella cucumerina, Monacrosporium thaumasium* Unidentified (3)	1.58	1
	M10.35	Cucumber	4.0 ± 0.7	*Fusarium oxysporum* (1)	0	-

Production system	Site	Crop	Parasitism (%)[a]	Fungal species (richness)	*H'*	*J'*
	M10.36	Tomato	13.5 ± 7.1	*Fusarium verticillioides, Fusarium oxysporum, Fusarium solani*. (3)	1.54	0.77
	M10.37	Tomato	1.5 ± 0.7	*P. chlamydosporia, Verticillium* sp. (2)	1	1
	M10.39	Tomato	2.6 ± 1.3	*Fusarium* sp., *Myrothecium verrucaria* (2)	1	1
	M10.43	Tomato	69.3 ± 4.2	*Cylindrocarpon olidum, Dactylella oviparasitica, Paecilomyces lilacinus, Penicillium citrinum, P. chlamydosporia* (5)	1.16	0.5
	M10.44	Tomato	2.3 ± 1.6	*Fusarium solani, Fusarium* sp., Unidentified (3)	1.15	0.72
	M10.45	Tomato	6.5 ± 1.0	*Chaetomium* sp., *Paecilomyces lilacinus, P. chlamydosporia* (3)	1.37	0.86
	M10.46	Eggplant	31.2 ± 7.4	*Fusarium* sp., *Paecilomyces lilacinus, P. chlamydosporia,* Unidentified (4)	1.74	0.87
	M10.51	Pepper	49.4 ± 8.0	*P. chlamydosporia*, Unidentified (2)	1	1
	M10.52	Tomato	2.1 ± 2.1	*Fusarium oxysporum* (1)	0	-
	M10.53	Cucumber	3.0 ± 49.7	Unidentified (1)	0	-
	M10.54	Pepper	12.1 ± 3.0	*P. chlamydosporia* (1)	0	-
Organic[b]			36.2 ± 8.9	2.0 ± 0.21	0.7 ± 0.13	0.8 ± 0.08
Integrated[c]			9.4 ± 3.2	1.4 ± 0.24	0.6 ± 0.13	0.8 ± 0.06
Organic versus Integrated[d]			*F* = 13.25 df = 1, 38 *P* = 0.0008	*F* = 3.31 df = 1, 38 *P* = 0.077	*F* = 0.26 df = 1, 31 *P* = 0.399	*F* = 0.08 df = 1, 18 *P* = 0.780

Richness: number of fungal species in a given site; *H'*: diversity index of Shannon–Wiener (-∑ (*ni*/*N*) x log2 (*ni*/*N*), where *N* is the density of all the species and *n*i is the density of species *i*; *J'*: evenness index when number of species >1 (*H'*/*H'* max; where *H'* is the index of Shannon–Wiener and *H'* max = log2(*S*) where *S* is the total number of species.[a] Data per each site are mean ± SE of three replicates. [b] Data for organic production are mean ± SE of ten sites for parasitism, richness, and *H'* , and eight for *J'*. [c] Data for integrated production are mean ± SE of 30 sites for parasitism, and richness, 22 for *H'* , and 12 for *J'* . [d] *F*-values, degrees of freedom, and *P* values to compare parasitism, richness, *H'* , and *J'* between organic and integrated production

Soils under organic production had higher content of clay (F = 11.78; df = 1, 38; P = 0.0015), silt (F = 6.67; df = 1, 38; P = 0.014), organic matter (F = 9.16; df = 1, 38; P = 0.004), exchangeable Mg (F = 8.86; df = 1, 38; P = 0.005), exchangeable K (F = 19.48; df = 1, 38; P\0.0001), available K (F = 14.09; df = 1, 38; P = 0.0006), cationic exchange capacity (F = 32.30; df = 1, 38; P\0.0001), lime (F = 15.96; df = 1, 38; P<0.0003), exchangeable Ca (F = 17.66; df = 1, 38; P<0.0002), pH (F = 4.54; df = 1, 38; P = 0.040) and FDA (F = 9.63; df = 1, 38; P = 0.004), urease (F = 6.36; df = 1, 38; P = 0.016) and β-glucosaminidase (F = 5.64; df = 1, 38; P = 0.023) activities, and lower content of sand (F = 11.06; df = 1, 38; P = 0.002), Fe (F = 19.25; df = 1, 38; P\0.0001), Mn (F = 5.28; df = 1, 38; P = 0.027), Cu (F = 8.98; df = 1, 38; P = 0.005), Zn (F = 20.83; df = 1, 38; P<0.0001), as well as P/N (F = 5.14; df = 1, 38; P = 0.029), and (Ca/Mg)/K (F = 7.20; df = 1, 38; P = 0.011) ratios than those under integrated production (Table 2).

In the organic production sites, the percentage of fungal egg parasitism ranged from 4.0 to 80.2 %, and was correlated with the clay content of soil (r = 0.694; P = 0.026). Multiple regression analysis indicated that the percentage of parasitism was positively related to the clay content of soil and the P/N relationship (percentage parasitism (log10 (x + 0.1)) = -0.38 + 0.0596 clay (%) + 3.897 P/N; R^2 = 0.689; P = 0.0168). In sites conducted under integrated production, the percentage of fungal egg parasitism ranged from 0.0 to 69.3 %, and did not correlate with any soil parameter nor with the time elapsed from the last nematicide treatment. In this system, multiple regression analysis did not reveal any relationship between fungal parasitism and soil properties.

Table 2. Physicochemical properties and enzymatic activity of soils conducted under integrated and organic vegetable production systems surveyed to detect fungal egg parasitism of *Meloidogyne* spp.

Variable	Integrated production	Organic production
B (ppm)	2.58 ± 0.25	2.63 ± 0.43
Exchangeable Ca (meq 100 g^{-1})	9.71 ± 1.11	15.43 ± 0.69 *
Lime (% $CaCO_3$)	2.97 ± 0.29	5.06 ± 0.36 *
Cation exchange capacity (meq 100 g^{-1})	13.12 ± 1.11	25.95 ± 2.32 *
Cu (ppm)	11.03 ± 3.41	2.61 ± 0.11 *
Electric conductivity (µmhos cm^1)	656.71 ± 150.8	397.77 ± 32.55
Available P (ppm)	157.94 ± 18.5	129.21 ± 3.24
Sand (%)	69.52 ± 4.15	44.8 ± 3.24 *
Clay (%)	11.65 ± 1.73	23.6 ± 2.34 *
Silt (%)	18.84 ± 2.62	31.6 ± 3.81 *
Fe (ppm)	101.23 ± 27.22	5.64 ± 0.64 *
Exchangeable Mg (meq 100 g^{-1})	1.74 ± 0.14	2.67 ± 0.33 *
Available Mg (meq 100 g^{-1})	2.29 ± 0.25	2.92 ± 0.36
Mn (ppm)	74.36 ± 8 41.	51 ± 11.97 *
Organic matter (%)	2.08 ± 0.16	3.12 ± 0.45 *
N (ppm)	1229.69 ± 113.78	1639.35 ± 210.76
pH (1:2.5 in water)	8.08 ± 0.05	8.28 ± 0.04 *
Exchangeable K (meq 100 g^{-1})	0.33 ± 0.05	0.78 ± 0.10 *
Available K (meq 100 g^{-1})	0.52 ± 0.08	1.08 ± 0.14 *
C/N	10.59 ± 0.47	11.11 ± 0.51
Exchangeable Na (meq 100 g^{-1})	0.35 ± 0.02	0.41 ± 0.03
Available Na (meq 100 g^{-1})	1.07 ± 0.25	1.04 ± 0.27
Zn (ppm)	35.04 ± 8.62 *	5.88 ± 1.91
Ca/Mg	5.9 ± 0.48	6.47 ± 0.71
Ca + Mg/K	49.54 ± 4.93	25.32 ± 2.03 *
P/N	0.14 ± 0.01	0.09 ± 0.02 *
Fluorescein diacetate hydrolysis (µg fluorescein h^{-1} x g soil)	2.67 ± 0.25	4.4 ± 0.71 *
b±glucosaminidase (µmols p±nitrophenol h^{-1} x g soil)	0.19 ± 0.03	0.31 ± 0.05 *
Urease (µmols N–NH4 h^{-1} x g soil)	0.91± 0.22	1.24 ± 0.15 *
Protease (µg tyrosine h^{-1} x g soil)	7.97 ± 0.32	6.85 ± 0.90

Each data are mean ± SE of 30 replicates (integrated production) or ten replicates (organic production). Values in bold and followed by * in the same row are different statistically according to the analysis of variance ($P<0.05$)

Discussion

Twenty fungal species belonging to 15 genera, and nine unidentified fungi, were isolated from RKN eggs. Most of the isolated fungi have the capacity to parasitize or be antagonists of RKN or cyst nematode eggs in vitro, pot or field experiments like *Cladosporium sphaerospermum* (Meyer et al., 2004), *Dactylella oviparasitica*, *Fusarium equiseti* (Nitao et al., 2001), *F. oxysporum* (Olatinwo et al., 2006), *F. solani* (Zareen et al., 2001), *Monacrosporium thaumasium*, *Myrothecium verrucaria*, *Chaetomium* sp., *Thielavia* sp., (Sun et al., 2006), *Paecilomyces lilacinus* (Kiewnick and Sikora, 2006; Kiewnick et al., 2011), *Plectosphaerella cucumerina* (Atkins et al., 2003), and *P. chlamydosporia* (Sorribas et al., 2003; Kerry and Hidalgo- Diaz, 2004). *Colletotrichum coccodes* was reported associated to potato cyst nematode by van der Laan (cited in Jones et al., 1986) although it was not consider a parasite. Three out the four remaining fungi, *Cladosporium tenuissimum*, *Penicillium citrinum*, and *P. olsonii*, have been reported as endophytes (Fisher and Petrini, 1992; Vega et al., 2006; Maciá-Vicente et al., 2008), and *F. verticillioides* as a plant pathogen, but as far as we know, their capacity to parasitize nematode eggs has not been investigated.

Higher levels of parasitism were found in organic than integrated production. The former had more content of organic matter, finer textured particles, FDA, β-glucosaminidase, and urease activities of soil, which suggest that these factors may play a key role in increasing the antagonistic potential of the soils. It is widely accepted that organic matter improves soil structure, fertility, and enhances biological control because it acts as a substrate to microbial inhabitants of soil, including nematode antagonists (Akhtar and Malik, 2000; Oka, 2010). This idea is also supported by this study, because soils under organic production showed higher microbial activity (FDA), probably due to fungi (urease activity) which in turn had chitinolitic activity (β-glucosaminidase). Furthermore, clay and silt content of soil influences pore size (30–90

µm), and affects the habitability of nematodes in the soil (Hassink et al., 1993). Eggs' hatching of Meloidogyne in soils with large pore size occurs earlier and in greater numbers than in soils with smaller pores (Evans and Perry, 2009). Thus, in fine textured rather than sandy soils, unhatched eggs in the egg masses could be exposed to fungal egg parasites for a longer time, which would increase the likelihood of being parasitized. Also, fine-textured soils have a higher water holding capacity than coarse soils, which could maintain soil's humidity at sufficiently high levels to allow the activity of the fungal-egg parasites. For instance, parasitism of *Heterodera avenae* Wollenweber by *Nematophora gynophila* Kerry and Crump decreased at drier conditions (Kerry et al., 1980).

Fungal species co-occurred (Shannon–Wiener index [0) more frequently in sites under organic (80 %) than under integrated production (54.5 %). However, the biodiversity index was not related to percentage of parasitism irrespective of the production system. This suggests that the success of biological control might be more influenced by the functionality than by the diversity of fungal species. Thus, *P. chlamydosporia* was the only fungal species present alone or co-occurring with other fungal species that positively related to the percentage of fungal egg parasitism in both organic and integrated production, which suggest that *P. chlamydosporia* may be the main fungal species involved in the antagonistic potential of the surveyed soils. In California, Borneman and Becker (2007) reported that *D. oviparasitica* was the main biotic factor responsible for the suppressiveness against *H. schachtii* in sugar beet, despite the fact that *F. oxysporum* and other egg parasitic fungi co-occurred in the same field. Soil suppressiveness might be achieved in undisturbed systems, such as perennial crops, or when the nematode resistance form remains in the soil for long periods, as is the case of cyst nematodes. Such conditionsmay favour long-term associations of the nematode with microbial parasites that require direct contact with their hosts. On the contrary, soil suppressivenessmight be less likely to occur in

cropping systems with high soil disturbances, as in intensive vegetable production, or where nematode eggs are surrounded by a gelatinous matrix (egg mass) or remain unexposed to microbial parasites within the root. Nonetheless, suppressive soils have been reported in agricultural fields in California, USA (Pyrowolakis et al., 2002).

The sites sampled in this survey were selected for their previous history on RKN infestation. However, a diversity of plant parasitic nematodes genera were found, being higher under organic (ten genera) than in integrated production (7), and with similar levels of infestation for a given crop. Studies to compare nematode impact on organic production in comparison with integrated or conventional production have not been done in the country although yield losses caused by Meloidogyne can reach up to 60 % depending on the crop (Verdejo-Lucas et al., 1994; Ornat et al., 1997; Sorribas et al., 2005). In Germany, plant parasitic nematodes are widely spread in organic vegetable production and can cause severe damage (Hallmann et al., 2007). Despite yield losses due to plant parasitic nematodes are related to the population densities at planting and the plant tolerance (Seinhorst, 1965), the cropping system could also influence the population dynamics of the nematodes, due to changes in the antagonistic potential of soils, and can thus affect the relationship between yield losses and nematode population densities. Long-term studies should be done to determine the effect of native fungal egg parasites on the population dynamics of RKN and crop yield of the most common rotation sequences, and to improve agronomical practices that enhance the level of suppression of plant parasitic nematodes.

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Chapter 6

Characterization of soil suppressiveness to root-knot nematodes in organic horticulture in plastic greenhouse

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The fluctuation of *Meloidogyne* population density and the percentage of fungal egg parasitism were determined from July 2011 to July 2013 in two commercial organic vegetable production sites (M10.23 and M10.55) in plastic greenhouses, located in northeastern Spain, in order to know the level of soil suppressiveness. Fungal parasites were identified by molecular methods. In parallel, pot tests characterized the level of soil suppressiveness and the fungal species growing from the eggs. In addition, the egg parasitic ability of 10 fungal isolates per site was also assessed. The genetic profiles of fungal and bacterial populations from M10.23 and M10.55 soils were obtained by Denaturing Gradient Gel Electrophoresis (DGGE), and compared with a non-suppressive soil (M10.33). In M10.23, *Meloidogyne* population in soil decreased progressively throughout the rotation zucchini, tomato, and radish or spinach. The percentage of egg parasitism was 54.7% in zucchini crop, the only one in which eggs were detected. *Pochonia chlamydosporia* was the only fungal species isolated. In M10.55, nematode densities peaked at the end of the spring-summer crops (tomato, zucchini, and cucumber), but disease severity was lower than expected (0.2–6.3). The percentage of fungal egg parasitism ranged from 3 to 84.5% in these crops. The results in pot tests confirmed the suppressiveness of the M10.23 and M10.55 soils against *Meloidogyne*. The number of eggs per plant and the reproduction factor of the population were reduced ($P < 0.05$) in both non-sterilized soils compared to the sterilized ones after one nematode generation. *P. chlamydosporia* was the only fungus isolated from *Meloidogyne* eggs. In in vitro tests*, P. chlamydosporia* isolates were able to parasitize *Meloidogyne* eggs from 50 to 97% irrespective of the site. DGGE fingerprints revealed a high diversity in the microbial populations analyzed. Furthermore, both bacterial and fungal genetic patterns differentiated suppressive

from non-suppressive soils, but the former showed a higher degree of similarity between both suppressive soils than the later.

Keywords: Antagonistic potential of soil, Biological control, Biodiversity, DGGE fingerprints, *Meloidogyne* spp., *Pochonia chlamydosporia*, Vegetable crops

Introduction

Root-knot nematode (RKN), *Meloidogyne* spp., is the most harmful plant-parasitic nematode on vegetable crops in the world (Sasser and Freckman, 1987). In Spain, RKN are present in all horticulture production areas (Melgarejo et al., 2010) causing economical losses. Estimation of the maximum yield losses on important vegetable crops include: 88% for cucumber, 60% for tomato, 39% for zucchini, 37% for watermelon, and 30% for lettuce (Verdejo-Lucas et al., 1994; Sorribas et al., 2005; Talavera et al., 2009; Giné et al., 2014; López-Gómez et al., 2014; Vela et al., 2014).

Soil fumigants and nematicides are the most popular control methods (Talavera et al., 2012). However, the Directive 2009/128/EC from the European Commission promotes the use of non-chemical methods based on integrated pest management strategies in order to reduce the use of pesticides.

A sustainable production system uses environmentally friendly alternatives to preserve and enhance beneficial organisms, which represents the antagonistic potential. Soils with high antagonistic potential lead to suppression of soil borne pathogens. In a suppressive soil, pathogens do not establish, persist, or establish but cause little or no damage (Baker and Cook, 1974). Suppressive soils have already been described for many soil pathogens (Weller et al., 2002) including plant parasitic nematodes (Timper, 2011). Suppressive soils to cyst nematodes and RKN have been intensively studied. In such soils, fungal parasites were responsible of suppression in

cereal (Kerry, 1980), sugar beet (Westphal and Becker, 1999, 2001), and soybean cyst nematodes (Chen, 2007), as well as RKN (Pyrowolakis et al., 2002; Adam et al., 2014). However, the suppression mechanisms are not well understood. Janvier et al. (2007) summarized the biotic and abiotic factors related to soil suppressiveness. Among them, the soil microbiota plays an important role (Weller et al., 2002), being essential to sustain biological productivity (Garbeva et al., 2004). Soil microbial diversity changes depending on the type of plant, soil, and management, and the interaction of microorganisms with those other factors can lead to the soil's disease suppressiveness (Garbeva et al., 2004). The study of microbial communities can be done by culture-independent methods, such as denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 2004), which allows the analysis of the total microbial structure of the soil, including the microorganisms that cannot be recovered by cultivation (Smalla and Heuer, 2006).

In Spain there are no reports of soils suppressive to RKN, despite the occurrence of antagonists of the nematode (VerdejoLucas et al., 1997, 2002, 2013; Olivares-Bernabeu and LópezLlorca, 2002). In 2010, 10 commercial organic production sites were sampled in the northeastern horticultural growing area of Spain to assess the occurrence of fungal egg parasites of RKN and the percentage of parasitized eggs. Fungal egg parasites occurred in all sampled sites, and mean percent of parasitized eggs was 36.2 (Giné et al., 2012). In some of those sites, growers did not use any specific control measures against *Meloidogyne*, although attenuated disease symptoms were observed, mainly at the end of the spring-summer crops. Then, could be considered that some of those soils could be suppressive to RKN despite be intensively perturbed agrosystems, this is, with several crops per season and favorable climatic conditions that enable nematodes' development. Furthermore, as far as we know, there is little knowledge about the fluctuation of soil suppressiveness during the cropping sequences in commercial farms or the microbial profiles of RKN suppressive

soils. Thus, two sites were selected in order to (i) determine the fluctuation of the RKN population's densities and the percentage of fungal egg parasitism along 2 years, (ii) assess soil suppressiveness in pot test, (iii) know the parasitic ability to RKN eggs of the fungal isolates from each soil, and (iv) compare microbial profiles between these two soils and a conducive one.

Materials and methods

Sites

Two commercial organic horticultural production sites, M10.23 and M10.55, cropped in plastic greenhouses were selected from a previous study (Giné et al., 2012) considering that the percentage of fungal egg parasitism was similar to the average obtained from all organic production sites sampled (36.2%). Both sites are located at the Tarragona province (northeastern Spain). Physicochemical properties and enzymatic activity of soils are presented in Table 1, and the rotation sequences conducted for both sites appear in Table 2. Soil at M10.23 was infested with *Meloidogyne javanica*; fertilization was done using a mixture of composted sheep and chicken manure at a rate of 2 kg m^{-2} that was incorporated into the soil just before transplanting each crop. Weed management was done by flaming and mechanically. Soil at M10.55 was infested with *M. javanica* and *Meloidogyne incognita* at a rate 10:1; fertilization was done with composted sheep manure at a rate of 1.7 kg m^{-2} that was also incorporated just before transplanting each crop. Mustard was grown as a cover crop planted in summer, just at the end of the spring crop, and incorporated as green manure 2 weeks before transplanting the autumn crop. Weeds were managed mechanically.

The commercial production site M10.33 was selected as nonsuppressive soil due to its history on *Meloidogyne* infestation and disease severity on cucumber, pea,

and tomato. At the end of those crops, fungal egg parasites were recovered at low percentage, 4.1% after cucumber crop (Giné et al., 2012), and 0% after the pea and tomato crops (data not shown). The grower managed RKN by biosolarization after the spring-summer crop. The site was conducted under integrated production in plastic greenhouse located in the province of Barcelona (northeastern Spain). Physicochemical properties and enzymatic activity of soils are also presented in Table 1. Fertilization was based on pellets of composted manure combined with chemical fertilizers. Weeds were managed mechanically. The soil of this site was used in the DGGE analysis for comparison between microbial communities of soils.

Table 1. Physicochemical properties and enzymatic activity of soil of two vegetable production sites managed organically (M10.23 and M10.55) and an integrated production site (M10.33) in plastic greenhouses at the beginning of the study.

	Sites		
Variable	**M10.23**	**M10.55**	**M10.33**
Sand (%)	45	68	50
Silt (%)	40	0	20
Clay (%)	15	32	30
Soil texture (USDA)	loam	sandy clay loam	sandy clay loam
pH	8.3	8.1	7.9
Organic matter (w/w)	5.8	2.5	1.6
Electric conductivity (µS/cm)	276	1069	2030
B (ppm)	2.8	1.1	4.6
Exchangeable Ca (meq 100 g^{-1})	17.3	18.2	8.8
Available Ca (meq 100 g^{-1})	17.2	19.0	14.6
Lime	3.8	4.1	4.4
Cation exchange capacity (meq 100 g^{-1})	41.2	25.7	13.7
Cu (ppm)	3.6	2.5	3.5
Available P (ppm)	379.4	75.8	107.6
Fe (ppm)	11.4	5.0	5.0
Exchangeable Mg (meq 100 g^{-1})	4.0	3.0	2.3
Available Mg (meq 100 g^{-1})	5.0	3.7	4.7
Mn (ppm)	64.0	2.5	148.0
N (ppm)	2329	1498	865
Exchangeable K (meq 100 g^{-1})	1.2	0. 7	0.5
Available K (meq 100 g^{-1})	1.9	0.7	1.0
C/N	14.4	9.7	10.5
Exchangeable Na (meq 100 g^{-1})	0.3	0.5	0.3
Available Na (meq 100 g^{-1})	1.0	3.2	3.0
Zn (ppm)	20.6	2.5	81.0
Ca + Mg/K	18.0	31.5	21.4
P/N	0.2	0.1	0.1
Fluorescein diacetate hydrolysis (µg fluorescein h^{-1} x g soil)	5.5	1.0	2.0
b-glucosaminidase (µmols *p*-nitrophenol h^{-1} x g soil)	0.4	0.1	0.1
Urease (µmols $N–NH_4$ h^{-1} x g soil)	1.6	0.9	0.1
Protease (µg tyrosine h^{-1} x g soil)	4.5	12.4	8.7

Fluctuation of RKN Population Densities

Composite soil samples were collected at the beginning and at the end of each crop to determine initial (*Pi*) and final (*Pf*) nematode population densities. Each plastic greenhouse was divided in four plots of 75 and 82 m2 at the M10.23 and the M10.55 sites, respectively. Individual samples consisting of 20 soil cores were taken from the first 30 cm of soil with a soil auger (2.5 cm diameter) from each plot. Soil cores were mixed thoroughly and sieved through a 4-mm aperture screen to remove stones and separate roots from soil. RKN juveniles (J2) were extracted from two 250-cm^3 soil subsamples using the sieving and centrifugation-flotation method (Jenkins, 1964). J2 were counted and expressed as J2 per 250 cm^3 of soil. The reproduction rate of RKN in each crop was calculated as *Pf*/*Pi* ratio. At the end of each crop, eight plants per plot were randomly collected and removed from the ground with a pitchfork; damage caused by RKN in the root system was rated for galling based on a scale from 0 to 10, where 0 = complete and healthy root system and 10 = plants and roots dead (Zeck, 1971). Roots were carefully washed free of soil, mixed, chopped, and root-knot nematode eggs extracted from two10 g-subsamples by macerating them for 10 min in a blender containing a 1% NaOCl solution (Hussey and Barker, 1973). Eggs were counted and expressed per g of root.

Soil temperature and soil water content from each site were recorded at 60 min intervals with temperature probes (5TM, Decagon devices, Inc., Pullman, WA, USA) placed at 15 cm depth.

Fungal egg parasitism

At the end of each crop, fungal egg parasites of RKN were isolated according to the de Leij and Kerry (1991) procedure modified by Verdejo-Lucas et al. (2002). Briefly, per each plot, 10–20 egg masses were handpicked from roots and placed in a

watchglass containing sterile distilled water. The outer part of the gelatinous matrix was removed from the egg masses with tweezers to eliminate potential surface colonizers. Egg masses were then placed in an Eppendorf microcentrifuge tube containing 1 ml of sterile distilled water. Eggs were dispersed from the egg masses using a pestle and 333µl-aliquots of the eggs' suspension were spread onto each of three replicated Petri dishes (9-cm diameter) containing a growth restricting medium (streptomycin, 50 mg l^{-1} ; chloramphenicol, 50 mg l^{-1} ; chlortetracycline, 50 mg l^{-1} ; Rose Bengal, 50 mg l^{-1} ; triton, 1 ml l^{-1} ; and 1% agar) (LopezLlorca and Duncan, 1986). Plates were incubated at 25 ± 0.5◦C. Number of parasitized eggs was recorded after 24 and 48 h under a dissecting microscope and percentage of parasitism was then calculated as the number of parasitized eggs per plate/number of eggs per plate. Eggs were considered parasitized if fungal hyphae grew from inside. At least, 20 parasitized eggs per plot and crop were individually transferred to corn meal agar (CMA) to establish pure cultures of the fungi. Fungal isolates were stored in 1% (w/v) water-agar slants, as well as lyophilized and stored at 4°C.

Fungal parasites characterization

Identification of fungal species isolated at the end of the first crop was carried out by PCR amplification and sequencing of the internal transcribed spacers (ITSs) of the rDNA regions. DNA was extracted from 50 mg of mycelium collected from single spore cultures on potato dextrose agar (PDA) using the E.Z.N.A kit R Plant MiniPrep (Omega Bio-Tek) according to the protocol described by the manufacturer. The PCR reaction was performed in 25µl mix that contained 1µl of the DNA extraction, 10.5µl MiliQ water (Qiagen), 12.5µl Taq PCR Master Mix (Qiagen) and 0.5µl of each primer (5 pmol), ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990). PCR conditions were the same as those described in the original studies (White et al., 1990). PCR products were cleaned using MinElute PCR Purification Kit (Qiagen) and sequenced by

Secugen (Madrid, Spain). DNA sequences were analyzed using the BLAST database (July 2013) and assigned to the reference isolate sequences with the highest bit score. Identification of fungal isolates from eggs produced on the rest of crops was carried out according morphological characters (Gams, 1988).

Soil suppressiveness against RKN in pot tests

Two experiments were carried out in 2012. Experiment 1 was conducted from March 27 to June 10 [907 degree-day (DD), 10°C basal temperature and thermal constant between 600 and 700 DD over the basal temperature; (Ferris et al., 1985)] with soil samples taken in January 2012. Experiment 2 was carried out from August 9 to October 23 (1092 DD, 10°C basal temperature and thermal constant between 600 and 700 DD over the basal temperature) with soil samples taken in July 2012. Both experiments were conducted using the same procedure. A soil sample was taken from the first 30 cm of soil with a hoe. Sample consisted of 48 soil cores (12 per plot). Soil was mixed thoroughly and passed through a 5-mesh sieve to remove stones and separate roots from soil. A part of soil was sterilized at 121°C during 1 h and the procedure was repeated after 1 day. The rest of soil was stored at 4°C until the experiment was carried out. Sterilized soil was mixed with steam-sterilized sand at a ratio 1:1 (dry w: dry w) to avoid soil compaction and improve plant growth. The same procedure was carried out with non-sterilized soil. After that, RKN juveniles were extracted from two 500-cm^3 subsamples of both sterilized and non-sterilized soil mixtures using Baermann trays (Whitehead and Hemming, 1965) maintained at 27 ± 2 °C for a week to determine the level of nematode inoculum at the beginning of the experiments. Thereafter, soil was placed in 3-L pots and a susceptible tomato cv. Durinta was transplanted into each pot at three true developed leaves stage. Nematode inoculum consisted of juveniles emerged from eggs that were extracted from tomato roots by the Hussey and Barker (1973) procedure and placed in Baermann

trays (Whitehead and Hemming, 1965) for a week at 27 ± 2 °C. Soil was inoculated with *M. incognita* J2 to achieve a total of 3000 J2 per plant, which was added in two opposite holes, 3 cm deep, made in the soil at 2 cm from the stem of the plants.

Ten replicate pots were prepared per each soil mixture, site, and experiment. Plants were arranged at random on a greenhouse bench, were irrigated as needed and fertilized with a slow-release fertilizer (15N + 10P + 12K + 2MgO + microelements). Soil temperatures and soil water content at 8 cm depth was recorded at 30 min interval during the experiments.

At the end of the experiments, plants were removed from pots. Roots were washed with tap water to remove soil particles and gently dry before determine fresh weight. Galling index was estimated according to Zeck scale (1971). To determine percentage of egg parasitism, three egg masses were handpicked from individual plants growth in both sterilized and nonsterilized soils and processed according to the method described previously. Fungi growing from eggs were isolated and identified as previously described. Eggs were extracted from roots by Hussey and Barker (1973) method, and reproduction factor was calculated considering *Pi* as number of juveniles inoculated, and *Pf* number of non-parasitized eggs per plant (Sorribas et al., 2003).

Parasitism of fungal isolates against RKN eggs

Five single-spore culture isolates of *Pochonia chlamydosporia* coming from each pot test and site were assessed for fungal egg parasitism. Single 5 mm-diameter plugs from the margin of the colony growing on PDA were transferred to the center of plates containing 1% water agar (WA) and incubated at 25°C in the dark for 2 weeks. Sterilized RKN eggs used as inoculum were obtained according to the procedure of Verdejo et al. (1988) modified. Briefly, 30 *M. incognita* eggs masses coming from tomato roots were handpicked and placed in a sterile conical centrifuge tube

containing 1 ml of 4% NaOCl solution. The egg suspension was shaken during 4 min at 30 s intervals, and finally diluted 10 times with sterile distilled water. Egg suspension was left undisturbed for 30 min to allow deposition. After that, sterilized nematode eggs were spread axenically around 1-cm apart from the margin of the colony using a Pasteur pipette. Plates were incubated at 25°C in the dark for 1 week. Eggs surrounded by a dense fungal colony were considered as parasitized and validated by observation under the light microscope (Lopez-Llorca et al., 2002). Percentage of egg parasitism was calculated as described previously. Three replicate plates were prepared per each fungal isolate and experiment.

DGGE analysis of fungal and bacterial soil community

DNA Fungal and bacterial profiles from M10.23 and M10.55 soils were obtained by DGGE, and compared to a commercial vegetable production site, M10.33, managed under integrated production, but with low percentage of fungal egg parasitism (4.1%) (Giné et al., 2013). Soil samples used for this study were taken in February 2013.

DNA extraction of soil samples was carried out using the Ultraclean Soil DNA Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol, using 0.25 g of soil. DNA extractions were performed from each composite soil sample. The quantity and quality of the extracted DNA was checked by agarose gel electrophoresis and by spectrophotometer measurement at wavelength 260 and 280 nm. All DNA samples were stored at −20°C for further analyses.

Fungal DNA was amplified using a nested approach, where primers EF4/ITS4 (Gardes and Bruns, 1993) amplifies 18S rDNA and ribosomal ITS regions in a first PCR, and this product is then used as template in a second PCR applying primers ITS1fGC/ITS2 (White et al., 1990). Bacterial DNA was amplified using the universal

bacterial primers 341F-GC and 907R (Muyzer et al., 2004). The PCR mixture and conditions were the same as those described in the original studies. PCR products were analyzed for size and quantity by agarose gel electrophoresis and stained with ethidium bromide. DGGE analyses were carried out using a D-Code Universal Detection System (Bio-Rad Laboratories, Richmond, CA, USA).

Nine hundred nanograms of PCR product were loaded onto 8% (w/v) polyacrylamide gels (40% acrylamide/bis solution, 37.5:1, Bio-Rad) with denaturing gradients ranging from 10 to 50% for the fungal DNA and 20 to 70% for the bacterial DNA (100% denaturants defined like 7 M urea and 40% v/v deionized formamide) (Schäfer and Muyzer, 2001). Electrophoresis was performed in 1× Tris-acetate-EDTA (TAE) buffer, at 60°C. The gel with fungal DNA was run for 16 h at 75 V, while the gel with bacterial DNA was run for 16 h at 80 V. Gels were stained with ethidium bromide (0.5µg/ml), and inspected under UV illumination and photographed. Prominent bands were excised from the gels, reamplified, and then purified using the PCR Clean up Kit (MoBio Laboratories) for subsequent sequencing.

Sequencing reactions were performed by Macrogen (South Korea) using the Big Dye Terminator v3.1 sequencing kit; reactions were run in an automatic capillary type ABI 3730XL analyzer-96. Sequences were first screened to detect potential chimeric artifacts using the Chimera.uchime program in Mothur 1.33.3 (http://www.mothur.org/wiki/Download_mothur) (Edgar et al., 2011) and then compared to those deposited in the GenBank nucleotide database using the BLAST program (Tatusova and Madden, 1999; Maidak et al., 2001).

Statistical Analyses

Statistical analyses were carried out with the SAS system software V9.2 (SAS Institute Inc., Cary, NC, USA). Variables were transformed when required to log10 (x +

1) or arcsine square root (x + 0.5). Data from pot experiments to assess soil suppressiveness were compared between experiments and site by t-Student test, using the t-test procedure, and were pooled together as replications of a single experiment because no differences ($P > 0.05$) were found. Then, data were submitted to t-Student test to compare between sterilized and non-sterilized mixture soil per each site. Data from experiments conducted to determine the ability of fungal isolates to parasitize RKN eggs were submitted to analysis of variance using the general linear model (proc glm) to compare the parasitic capability between isolates per site. When the analyses were significant ($P \leq 0.05$), the means were separated according to the least significant difference (LSD) test. DGGE images were analyzed using the InfoQuest™FP 4.5 software (Bio-Rad Laboratories, Richmond, CA, USA). Similarities of the DGGE profiles were calculated based on the Dice coefficient and dendrograms were obtained using the UPGMA clustering algorithm. A band position tolerance of 0.5% was used. Band patterns were normalized using the marker lanes as reference, allowing the comparison among samples loaded on different DGGE gels. The number of DGGE bands in each fingerprint was used as a measure of the apparent fungal and bacterial richness (S). Shannon Index was used as a measure of genetic diversity, and was calculated as $H = pi \ln pi$, where pi is the relative intensity of each DGGE band. Evenness (E) was calculated as $E = \ln (S)$. Diversity variables were submitted to nonparametric analysis of variance (proc npar1way) using Wilcoxon rank sum test.

Results

Fluctuation of RKN Population Densities and Percentage of Fungal Egg Parasitism

Daily soil temperature and water content of soil, as well as crop rotation sequences in sites M10.23 and M10.55, are presented in Figures 1, 2, respectively.

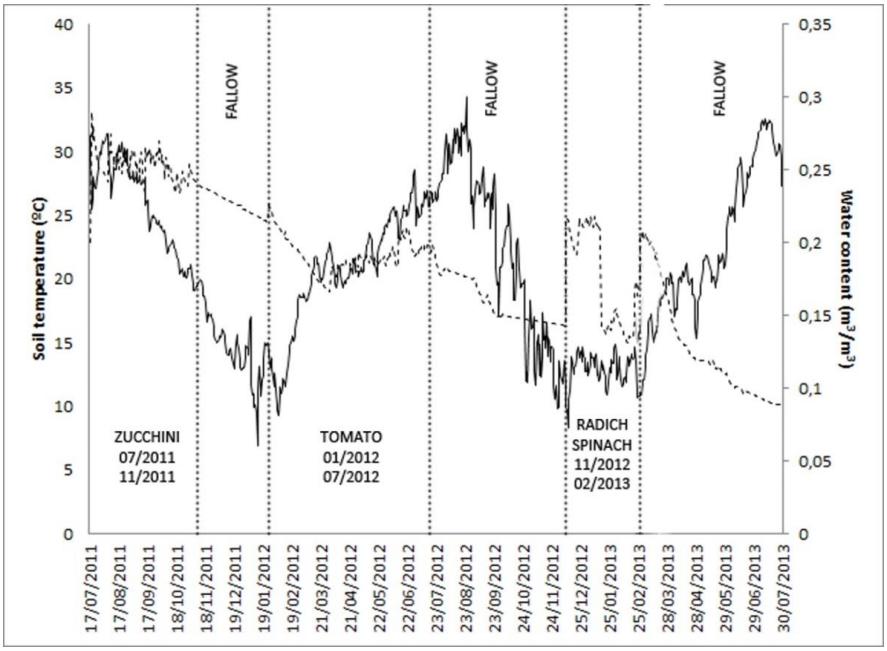

Figure 1 Fluctuation of mean daily soil temperature, soil water content, and crop rotation sequence in site M10.23.

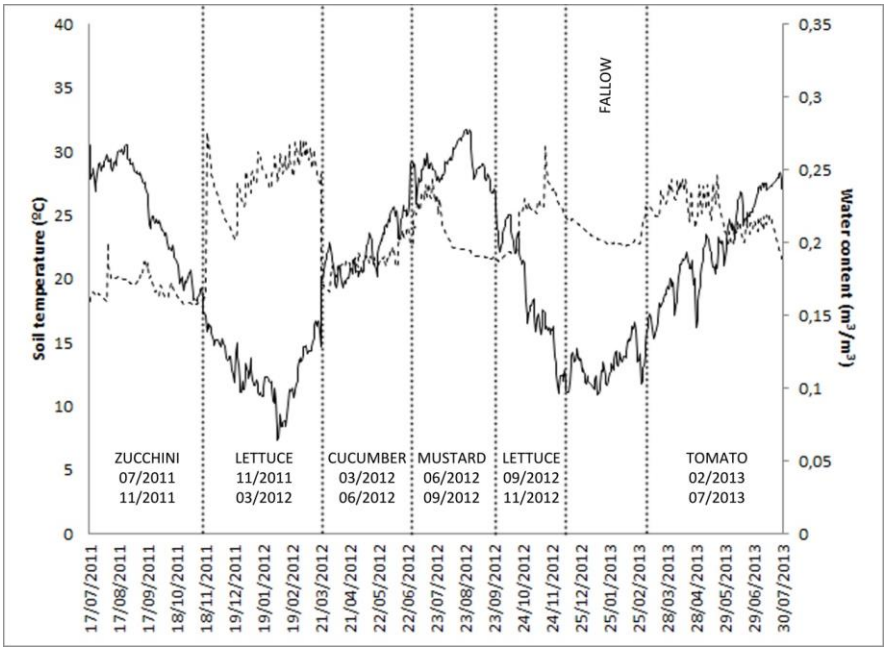

Figure 2. Fluctuation of mean daily soil temperature, soil water content, and crop rotation sequence in site M10.55

In M10.23, nematode population in soil decreased progressively from 2951 J2 250 cm^{-3} of soil in July 2011 to 15 J2 250 cm^{-3} of soil in July 2013, all throughout the rotation zucchini-resistant tomato-fallow-radish/spinach-fallow. At planting the winter crops (radish and spinach), nematode densities were below 10 J2 250 cm^{-3} of soil, and did not increase at the end of the crop. For these two crops, no galls were observed in the roots and no eggs were recovered (Table 2). Maximum densities of eggs per g root were recovered from the RKN susceptible zucchini cv. Dundoo cropped during summer, but not from the resistant tomato cv. Royesta. Disease severity in zucchini ranged from 0 to 4, while no galls were observed on the tomato roots. Fungal egg parasitism was only detected in zucchini (54.7%) in which root infection occurred and egg masses were produced (Table 2). *P. chlamydosporia* was the only fungal species isolated.

In M10.55, densities of nematodes in soil peaked at the end of spring-summer crops. Population densities at planting of the susceptible cucumber cv. Dasher II and the zucchini cv. Dundoo crops were 81 and 1013 J2 250 cm^{-3} of soil, respectively, but disease severity was less than expected [galling index (GI) of 6.3 and 3.0, respectively (Table 2)]. High percentage of fungal egg parasitism was recorded after cultivation of the susceptible cucurbit crops (84.5 and 71.7%), it was low after the cropping of resistant tomatoes (16%), and not apparent in the winter lettuce crop (0%), in which no egg masses were produced (Table 2). Nematode densities decreased after incorporation of the mustard cover crop into the soil, but survivors were able to infect roots (GI = 2.0) of the following crop of lettuce cultivated from September to November 2013, and to produce eggs, some of which were parasitized (0.2%). Again, *P. chlamydosporia* was the only fungal egg parasite recovered.

Table 2 *Meloidogyne* population densities in soil at planting (*Pi*) and at the end of the crop (*Pf*), galling index, number of eggs on roots, and percentage of egg parasitism in two commercial organic vegetable productions sites in plastic greenhouse during three consecutive years (2011-2013)

Site	Crop [a]	Date	Nº of juveniles 250 cm^{-3} soil		Galling index	Nº of eggs g $root^{-1}$	Parasitized eggs (%)
			Pi	*Pf*			
M10.23	Zucchini cv. Dundoo	07/2011-11/2011	2951 ± 487	61 ± 15	1.6 ± 0.3	1301 ± 530	54.7 ± 13.9
	Tomato cv. Royesta (R)	01/2012-07/2012	61 ± 15	0 ± 0	0 ± 0	0 ± 0	nem
	Radish cv. Saxa	11/2012-02/2013	9 ± 9	3 ± 3	0 ± 0	0 ± 0	nem
	Spinach cv. Gigante de invierno	11/2012-02/2013	4 ± 4	9 ± 3	0 ± 0	0 ± 0	nem
	Fallow	02/2013-07/2013	6 ± 3	15 ± 12	na	na	na
M10.55	Tomato cv. Lladó (R)	02/2011-07/2011	238 ± 62	1013 ± 883	0.2 ± 0.1	41 ± 26	3.0 ± 0.04
	Zucchini cv. Dundoo	07/2011-11/2011	1013 ± 883	1351 ± 238	3.0 ± 0.4	1870 ± 478	84.5 ± 3.6
	Lettuce cv. Maravilla	11/2011-03/2012	1351 ± 238	81 ± 17	0.1 ± 0.1	0 ± 0	nem
	Cucumber cv. Dasher II	03/2012-06/2012	81 ± 17	1329 ± 505	6.3 ± 1.0	6026 ± 1165	71.7 ± 2.7
	Mustard cv. Caliente 109	06/2012-09/2012	1329 ± 505	40 ± 18	na	na	na
	Lettuce cv. Maravilla	09/2012-11/2012	40 ± 18	56 ± 5	2.2 ± 0.2	999 ± 645	0.2 ± 0.2
	Tomato cv. Caramba (R)	02/2013-07/2013	19 ± 6	126 ± 30	1.3 ± 0.8	206 ± 115	16.0 ± 10.6

Data are mean ± standard error of 4 replications. Galling index on a scale from 0 to 10, where 0 = complete and healthy root system and 10 = plants and roots dead (Zeck, 1971)

[a] R: Resistant cultivar Na: Not available; Nem: Not egg masses

Soil suppressiveness against RKN in pot test

Minimum and maximum soil temperatures ranged from 18.6 to 25.7°C (21.9 ± 1.8°C, mean ± standard deviation) in experiment 1, and from 17.9 to 30.7°C (24.7°C ± 3.4°C) in experiment 2. Water content of soil ranged from 0.14 to 0.31 w^3 /w^3 (0.22 ± 0.04 w^3 /w^3) and from 0.15 to 0.26 w^3 /w^3 (0.21 ± 0.03 w^3 /w^3) in experiment 1 and 2, respectively.

Fungal egg parasites were recovered only from non-sterilized soils, being *P. chlamydosporia* the only fungal species identified. Eggs were parasitized at a rate of 24.8% in non-sterilized soil from site M10.23, and 70.9% from site M10.55 (Table 3). In nonsterilized M10.23 soil, fewer ($P < 0.05$) eggs per plant (73.30%), lower reproduction factor (73.91%), and less disease severity (17.07%) were recorded compared to the sterilized soil according to the Abbott's formula. In addition, less ($P < 0.05$) tomato fresh root weight (61.96%) was also recorded. Similar results were obtained with the non-sterilized M10.55 soil, in which the number of eggs per plant, reproduction factor and tomato fresh root weight were 61.43, 66.67, and 45.07% less ($P < 0.05$) than in the sterilized one, although disease severity did not differ ($P > 0.05$) (Table 3).

Table 3. Effect of soil sterilization or not- sterilization of sites M10.23 and M10.55 on *Meloidogyne* densities on roots, reproduction factor, galling index, percentage of egg parasitism and fresh root weight of tomato cv. Durinta inoculated with 3000 juveniles per pot after completion of one nematode generation.

Site	Soil mixture [a]	Fresh root weight (g)	Nº of eggs (x 10^3) / plant [b]	Reproduction factor[c]	Galling index[d]	Parasitized eggs (%)
M10.23	Sterilized	16.3 ± 1.3 *	41.2 ± 13.4 *	13.8 ± 4.5 *	4.1 ± 0.2 *	0
	Non-sterilized	6.2 ± 0.5	11.0 ± 3.0	3.6 ± 1.0	3.4 ± 0.2	24.8 ± 1.7
M10.55	Sterilized	14.2 ± 1.5 *	40.7 ± 8.8 *	12.6 ± 2.9 *	4.3 ± 0.2	0
	Non-sterilized	7.8 ± 0.6	15.7 ± 2.8	4.2 ± 0.7	3.8 ± 0.1	70.9 ± 2.0

Data are mean ± standard error of 20 replications. Data within the same column and site followed by * indicates a significant difference between soil treatment at $P < 0.05$ according to the Student's *t* -test.

[a] Sterilized soil mixture: 50% sterilized soil + 50% sterilized sand; Non-sterilized soil mixture: 50% non-sterilized soil + 50% sterilized sand.

[b] Parasitized eggs excluded

[c] Number of non-parasitized eggs per plant / initial population density

[d] Galling index on a scale from 0 to 10, where 0 = complete and healthy root system and 10 = plants and roots dead (Zeck, 1971)

Parasitism of fungal isolates against RKN eggs

P. chlamydosporia isolates from site M10.23 parasitized between 55.5 and 97.4% of the RKN eggs, and those from site M10.55 between 56.5 and 93.7%. In both sites, 3 out 10 fungal isolates parasitized more than 90% of the RKN eggs (Table 4).

Table 4. Percentage of parasitized eggs of *Meloidogyne* spp. by isolates of *P. chlamydosporia* in *in vitro* test.

Site	Assay. Isolate [a]	Parasitized eggs (%)
M10.23	C1.1	55.5±1.0 d
	C1.2	66.5±18.7 bcd
	C1.3	83.7±7.3 abcd
	C1.4	60.6±10.7 cd
	C1.5	86.5±3.9 abc
	C2.1	82.9±2.7 abcd
	C2.2	97.4±1.8 a
	C2.3	88.0±4.8 abc
	C2.4	90.7±5.6ab
	C2.5	93.9±2.1 ab
M10.55	H1.1	56.5±6.5 f
	H1.2	64.0±5.6 ef
	H1.3	65.1±5.9 ef
	H1.4	76.7±1.7 de
	H1.5	82.6±4.0 cd
	H2.1	93.7±1.7 ab
	H2.2	89.0±0.7 abc
	H2.3	91.1±0.3 abc
	H2.2	93.3±4.3 abc
	H2.5	86.3±2.4 bcd

Data are mean ± standard error of three replications. Data within the same column and site followed by the same letter did not differ ($P < 0.05$) according to the LSD test.

[a] Single-spore isolates of *P. chlamydosporia* isolated at the end of both pot assay, in June 10 (1), and October 23 (2) 2012.

DGGE Analysis of Fungal and Bacterial Communities

Band profiles obtained by the DGGE of bacterial and fungal rDNA amplified fragments and the DGGE fingerprints cluster analysis are shown in Figure 3. The 16S rRNA-DGGE analysis (Figure 3A) revealed composite banding patterns reflecting a high microbial diversity. Conversely, the ITS rDNA-DGGE analysis (Figure 3C) showed a lower diversity in the fungal communities. Two first-order clusters were clearly differentiated by the UPGMA analysis of the DGGE fingerprints, both in the bacterial and the fungal communities of the soils. These first-order clusters were identified at a similarity score of 53 and 65% for the fungal and the bacterial communities, respectively. Regarding the bacterial community, the first-order cluster differentiated non-suppressive M10.33 soil from M10.23 and M10.55 suppressive soils, and the second-order subcluster (75.5% similarity) differentiated between both suppressive soils (Figure 3B). Concerning the fungal communities, M10.23 soil was clearly differentiated from the rest in the first-order clusters, and M10.55 and M10.33 soils were grouped in a second-order subcluster (56.5% similarity) (Figure 3D). The bacterial and fungal genetic diversity was evaluated based on the number of DGGE bands and their relative intensity. Diversity variables for the bacterial communities did not differ between soils (Shannon–Wiener $P = 0.12$; richness $P = 0.73$; evenness $P = 0.09$), but some of them did for the fungal communities. The Shannon–Wiener index and the evenness in soil M10.55 differed ($P = 0.05$ and $P = 0.03$, respectively) from M10.33 but not from M10.23 soils. However, richness was similar between soils ($P = 0.45$).

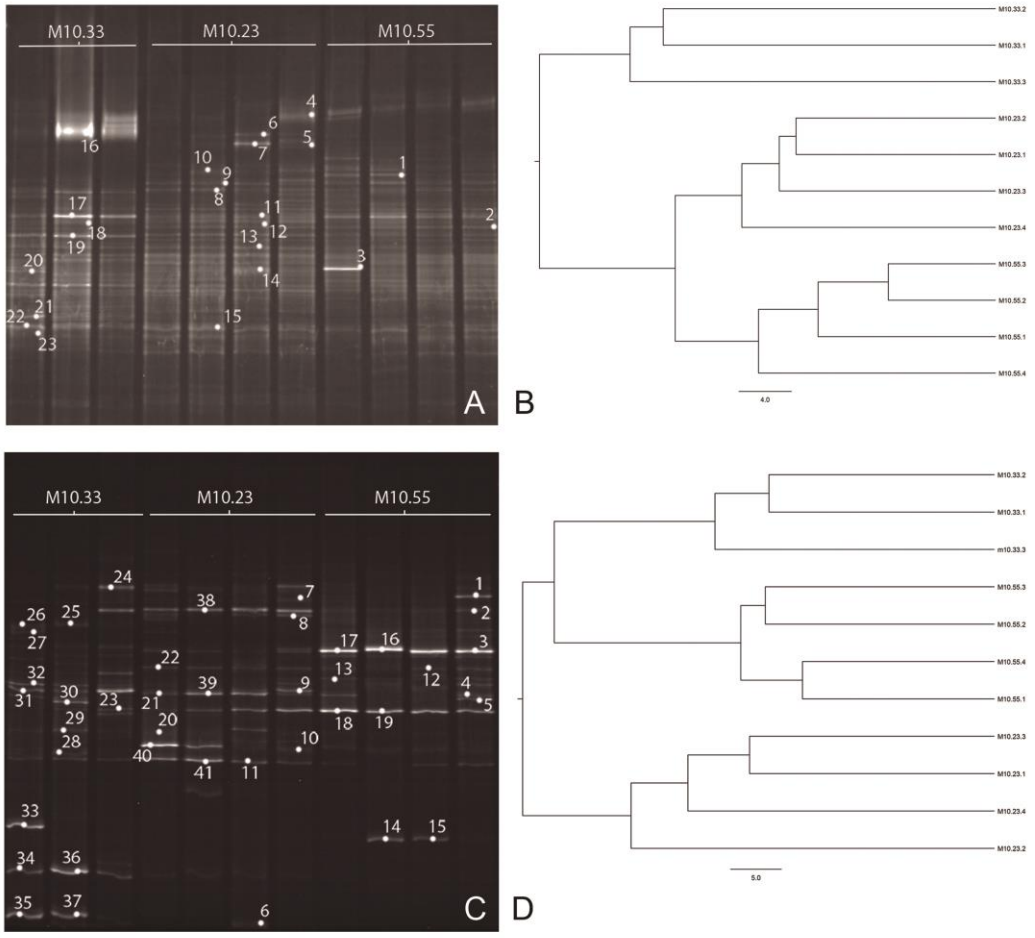

Figure 3. DGGE profiles of 16S rRNA fragments of bacteria (A) and partial fungal ITS sequences (C) from DNA sample replicates of one non-suppressive (M10.33) and two suppressive (M10.23 and M10.55) soils. Bands marked with numbers correspond to the bands excised from the gel and sequenced. Cluster dendrograms based on UPGMA algorithm show similarity among DGGE band patterns of bacteria (B) and fungi (D). Bar indicates percentage of divergence.

In Figures 3A,C, the bands marked with numbers correspond to the dominant bands that were extracted from the DGGE gels and sequenced. Tables 5, 6 show the sequenced bands, their similarity values compared to the closest related GenBank

sequences, and their phylogenetic affiliations. Sequence similarity values compared to previously reported sequences were more than 93.5% in all cases. The majority of the 23 bacterial sequences belonged to the phylum Bacteroidetes (65.2%) followed by Proteobacteria (17.4%) (Table 5). Regarding fungi, the 41 sequences fell into three taxonomic groups. On average, Ascomycota (56.1%) was the most abundant phylogenetic groups, followed by Basidiomycota (31.7%) (Table 6).

The bacterial and fungal rRNA sequences determined in this study are available at the GenBank under accession numbers KT991569 through KT991632. Each band designation includes a code specifying its origin (ASS, Agricultural Soil Suppressiveness) followed by a number indicating the order in which the sequence was isolated from the gel.

Table 5. DGGE bands sequenced, band length, associated GenBank accession numbers, and phylogenetic affiliation from profiles of bacterial populations.

Phylotype			Phylogenetic affiliation		
Band code[a]	**Sequence length (bp)**	**Accession code**	**Taxonomic linage[b] (Phylum, Class, Order, Family, Genus)**	**Closest match[c] (accession no.)**	**Similarity (%)[d]**
B1	336	KT991569	Bacteroidetes (100), Sphingobcteria (83), Sphingobacteriales (83)	Uncultured bacterium clone (KM155241)	94,4
B2	368	KT991570	Bacteroidetes (100), Sphingobcteria (100), Sphingobacteriales (100)	Uncultured Sphingobacterium sp clone (KM155241)	98.9
B3	536	KT991571	Cyanobacteria/Chloroplast (100)	Uncultured Streptophyta clone (JF703638)	99.8
B4	551	KT991572	Bacteroidetes (100), Flavobacteria (100), Flavobacteriales (100), Flavobacteriaceae (100), *Flavobacterium* (100)	Flavobacterium sp. (JN650574)	100
B5	519	KT991573	Bacteroidetes (100), Flavobacteria (100), Flavobacteriales (100), Flavobacteriaceae (100), *Chryseobacterium* (100)	*Chryseobacterium* sp. (KJ482798)	100
B6	570	KT991574	Bacteroidetes (100), Sphingobcteria (100), Sphingobacteriales (100), KD3-93 (100)	Uncultured Bacteroidetes (AM116744)	98.5
B7	556	KT991575	Bacteroidetes (100), Sphingobcteria (100), Sphingobacteriales (100), KD3-93 (100)	Uncultured bacterium clone (JF176318)	97.9
B8	547	KT991576	Bacteroidetes (100), Sphingobcteria (100), Sphingobacteriales (100), Cytophagaceae (100), *Flexibacter* (100)	Uncultured bacterium clone (KJ909017)	99.6
B9	541	KT991577	Bacteroidetes (100), Sphingobcteria (100), Sphingobacteriales (100), Cytophagaceae (100), *Flexibacter* (100)	Uncultured Flexibacteriaceae bacterium (FM209167)	99.4
B10	442	KT991578	Bacteroidetes, Sphingobcteria, Sphingobacteriales, Cytophagaceae (100), *Flexibacter* (100)	Uncultured Bacteroidetes bacterium (KJ024617)	98.0

B11	510	KT991579	Bacteroidetes (100), Sphingobcteria (100), Sphingobacteriales (100), Cytophagaceae (96), *Flexibacter* (89)	Uncultured Bacteroidetes bacterium (HF564268)	99.0
B12	511	KT991580	Bacteroidetes (100), Sphingobcteria (100), Sphingobacteriales (100), Cytophagaceae (100), *Flexibacter* (94)	Uncultured Bacteroidetes bacterium (HF564268)	99.8
B13	449	KT991581	Proteobacteria (100), Gammaproteobacteria (100), Xanthomonadales (98). Sinobacteriaceae (98), *Steroidobacter* (96)	Uncultured bacterium clone (GQ263704)	95,8
B14	519	KT991582	Proteobacteria (100), Gammaproteobacteria (100), Xanthomonadales (100), Xanthomonadaceae (100), *Lysobacter* (99)	*Lysobacter* sp. MHS036 (DQ993327)	97.5
B15	485	KT991583	Proteobacteria (100), Alphaproteobacteria (100), Rhizobiales (100), Methylobacteriaceae (100), *Methylobacterium* (100)	*Methylobacterium radiotolerans* (LC026013)	100
B16	490	KT991584	Bacteriodetes (100), Flavobacteria (100), Flavobacteriales (100); Flavobacteriaceae (100)	*Winogradskyella rapida* (KF009869)	93.5
B17	518	KT991585	Bacteroidetes (100), Sphingobcteria (100), Sphingobacteriales (100), Cytophagaceae (100), *Flexibacter* (100)	Uncultured Sphingobacteriales bacterium (KF733506)	99.6
B18	484	KT991586	Bacteroidetes (100), Sphingobcteria (100), Sphingobacteriales (100), Cytophagaceae (99), *Flexibacter* (99)	Uncultured Bacteroidetes bacterium (HF564295)	98.6
B19	513	KT991587	Bacteroidetes (100), Sphingobcteria (97), Sphingobacteriales (97), Cytophagaceae (94)	Uncultured Sphingobacteriales bacterium (AM936482)	99.8

B20	514	KT991588	Firmicutes (100), Bacilli (100), Bacillales (96), Bacillaceae (82)	*Marinococcus halophilus* (HF678777)	99.8
B21	472	KT991589	Unclassified Chloroflexi	Uncultured bacterium clone (HQ697759)	100
B22	494	KT991590	Proteobacteria (100), Alphaproteobacteria (100), Rhizobiales (100), Methylobacteriaceae (100), *Methylobacterium* (100)	*Methylobacterium mesophilicum* (KP293855)	100
B23	487	KT991591	Acidobacteria (100), Acidobacteria (100), Acidobacteriales (100), Acidobacteriaceae (100), Candidatus *Solibacter* (100)	Uncultured bacterium clone (JQ654947)	99.6

[a] band numbers correspond to those presented in figure 3A for bacterial samples. [b] taxonomic string with bootstrap values (in parentheses), generated in mothur using SILVA database reference file release 119. [c] closest relative according to INSA (International Nucleotide Sequence Database)
[d] percentage sequence similarity with closest INSA using BLAST tool

Table 6. DGGE bands sequenced, band length, associated GenBank accession numbers, and phylogenetic affiliation from profiles of fungal populations.

Phylotype			Phylogenetic affiliation		
Band code[a]	**Sequence length (bp)**	**Accession code**	**Taxonomic linage[b] (Phylum, Class, Order, Family, Genus)**	**Closest match[c] (accession no.)**	**Similarity (%)[d]**
F1	231	KT991592	Ascomycota (100), Pezizomycetes (100), Pezizales (100), Pyronemataceae (100), *Pseudaleuria* (100)	Uncultured fungus clone (JF432996)	100
F2	219	KT991593	Ascomycota (100), Pezizomycetes (98), unclassified_Pezizomycetes_order (69)	Uncultured fungus clone (JX323746)	97,72
F3	173	KT991594	Ascomycota (100), Pezizomycetes (99), unclassified_Pezizomycetes_order (75)	Uncultured fungus clone (JX323746)	97,70
F4	189	KT991595	Ascomycota (100), Sordariomycetes (100), Hypocreales (99), Hypocreales_family_incertae_sedis (60), *Fusarium* (60)	*Fusarium equiseti* (KM246255)	98,4
F5	165	KT991596	Ascomycota (100), Dothideomycetes (89), Pleosporales (89), Sporormiaceae (84), *Preussia* (74)	Uncultured fungus clone (JX340328)	100
F6	251	KT991597	Ascomycota (100), Eurotiomycetes (100), Onigenales (100), Arthrodermataceae (100), *Ctenomyces* (100)	Uncultured fungus clone (JX349691)	99,2
F7	186	KT991598	Fungi_phylum_incertae_sedis (100), Fungi_class_incertae_sedis (100), Mortierellales (87), Mortierellaceae (85), *Mortierella* (74)	Uncultured fungus clone (JX377362)	97,85
F8	192	KT991599	Fungi_phylum_incertae_sedis (100), Fungi_class_incertae_sedis (100), Mortierellales (99), Mortierellaceae (98), *Mortierella* (98)	Uncultured fungus clone (JX345268)	99,48
F9	169	KT991600	Ascomycota (100), Dothideomycetes (100), Capnodiales (100), Capnodiales_family_incertae_sedis (100), *Cladosporium* (100)	*Graphiopsis chlorocephala* (JN116693)	100
F10	180	KT991601	Fungi_phylum_incertae_sedis (100), Fungi_class_incertae_sedis (100), Mortierellales (99), Mortierellaceae (94), *Mortierella* (94)	Uncultured fungus clone (JX387233)	98.9
F11	210	KT991602	Ascomycota (100), Sordariomycetes (100), Hypocreales (100), Hypocreales_family_incertae_sedis (100), *Stachybotrys* (100)	Uncultured Stachybotrys clone (KF493978)	100

F12	201	KT991603	Ascomycota (99), Sordariomycetes (96), Microascales (73), Microascaceae (73), *Pseudallescheria* (69)	Uncultured *Pseudallescheria* clone (KM108739)	99,5
F13	173	KT991604	Ascomycota (100), Sordariomycetes (98), Microascales (74), Microascaceae (74), *Pseudallescheria* (66)	Uncultured *Pseudallescheria* clone (KM108739)	100
F14	290	KT991605	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (94), *Psathyrella* (60)	Uncultured fungus clone (GQ225128)	100
F15	292	KT991606	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (100), *Psathyrella* (64)	Uncultured fungus clone (GQ225128)	100
F16	249	KT991607	Ascomycota (87), Pezizomycetes (78), Pezizales (76), Pyronemataceae (74), *Heydenia* (50)	*Pezizaceae* sp (JQ775581)	98,35
F17	220	KT991608	Ascomycota (100), Pezizomycetes (96), Unclassified Pezizomycetes genus (53)	Uncultured fungus clone (JX323746)	97,27
F18	193	KT991609	Ascomycota (100), Sordariomycetes (100), Microascales (75), Microascaceae (75), *Pseudallescheria* (71)	Uncultured *Pseudallescheria* clone (KM108739)	100
F19	193	KT991610	Ascomycota (100), Sordariomycetes (97), Microascales (81), Microascaceae (80), *Pseudallescheria* (79)	Uncultured *Pseudallescheria* clone (KM108739)	100
F20	212	KT991611	Ascomycota (100), Sordariomycetes (100), Hypocreales (100), Hypocreales_family_incertae_sedis (100), *Stachybotrys* (100)	Uncultured fungus clone (JX348029)	100
F21	202	KT991612	Ascomycota (100), Sordariomycetes (100), Hypocreales (100), Hypocreales_family_incertae_sedis (93), *Fusarium* (93)	*Fusarium equiseti* isolate (KM246255)	100
F22	194	KT991613	Ascomycota (100), Sordariomycetes (99), Hypocreales (99) Hypocreales_family_incertae_sedis (84), *Fusarium* (83)	Uncultured *Fusarium* clone (KP235758)	97,94
F23	193	KT991614	Ascomycota (100), Sordariomycetes (98), Microascales (85), Microascaceae (85), *Pseudallescheria* (82)	Uncultured *Pseudallescheria* clone (KM108739)	100
F24	221	KT991615	Fungi_phylum_incertae_sedis (100), Fungi_class_incertae_sedis (100),	Uncultured fungus clone	97,26

			Mortierellales (100), Mortierellaceae (99), *Mortierella* (99)	(GQ866183)	
F25	305	KT991616	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (100), *Coprinopsis* (100)	*Hormographiella aspergillata* (KP132299)	100
F26	304	KT991617	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (100), *Coprinopsis* (100)	Uncultured Coprinopsis clone (GQ219811)	100
F27	298	KT991618	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (99), *Psathyrella* (61)	Uncultured fungus clone (GQ225128)	100
F28	287	KT991619	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (100), *Coprinopsis* (100)	Uncultured *Coprinopsis* clone (GQ219811)	100
F29	307	KT991620	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (100), *Coprinopsis* (100)	*Hormographiella aspergillata* (KP132299)	100
F30	204	KT991621	Ascomycota (100) Sordariomycetes (100), Microascales (99), Microascaceae (99), *Pseudallescheria* (99)	Uncultured fungus clone (JX383001)	94,2
F31	204	KT991622	Ascomycota (100), Dothideomycetes (100), Capnodiales (100), Capnodiales_family_incertae_sedis (100), *Davidiella* (53)	*Cladosporium sphaerospermum* (KP174687)	100
F32	245	KT991623	Ascomycota (96), Sordariomycetes (90), Microascales (61), Microascaceae (60), *Scedosporium* (47)	Uncultured fungus clone (JQ989314)	99,59
F33	298	KT991624	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (99), *Psathyrella* (58)	Uncultured fungus clone (GQ225128)	100
F34	297	KT991625	Basidiomycota (100), Agaricomycetes (100), Agaricales (100) , Psathyrellaceae (100), *Coprinopsis* (100)	*Hormographiella aspergillata* (KP132299)	100
F35	308	KT991626	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (100), *Coprinopsis* (100)	Uncultured *Coprinopsis* clone (GQ219811)	100
F36	305	KT991627	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (100), *Coprinopsis* (100)	*Hormographiella aspergillata* (KP132299)	100

F37	279	KT991628	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (100), *Coprinopsis* (100)	*Coprinopsis* sp. (AB499044)	100
F38	206	KT991629	Fungi_phylum_incertae_sedis (99), Fungi_class_incertae_sedis (99), Mortierellales (99), Mortierellaceae (95), *Mortierella* (95)	Uncultured soil fungus clone (JX489813)	100
F39	209	KT991630	Ascomycota (100), Sordariomycetes (100), Hypocreales (100), Hypocreales_family_incertae_sedis (95), *Fusarium* (95)	*Fusarium equiseti* isolate (KM246255)	100
F40	313	KT991631	Basidiomycota (100), Agaricomycetes (100), Agaricales (100), Psathyrellaceae (100), *Coprinellus* (100)	Uncultured fungus clone (JX353314)	100
F41	211	KT991632	Ascomycota (100), Sordariomycetes (100), Hypocreales (100), Hypocreales_family_incertae_sedis (100), *Stachybotrys* (100)	Uncultured *Stachybotrys* clone (KF493978)	100

[a] band numbers correspond to those presented in figure 3C for fungal samples
[b] taxonomic string with bootstrap values (in parentheses), generated in mothur using Findley database
[c] closest relative according to INSA (International Nucleotide Sequence Database)
[d] percentage sequence similarity with closest INSA using BLAST tool

Discussion

In this study, two suppressive soils to RKN were identified, increasing the list of previous studies reporting this kind of agricultural soil (Stirling et al., 1979; Gaspard et al., 1990; Pyrowolakis et al., 2002; Timper, 2011; Adam et al., 2014). However, as far as the authors know, this is the first report of suppressive soils to RKN in which vegetables are cultivated organically in plastic greenhouses. In addition, despite several studies to identify suppressive soils to RKN, none reported the fluctuation of both nematode densities and fungal egg parasites during the rotation sequences. This is the first comparison of microbial profiles of both suppressive and non-suppressive soils to be published.

The antagonistic potential of agricultural soils, defined as its capacity to prevent or reduce the spread of pathogens by biotic factors, is a product of the capacity of the microbial antagonists to survive the agronomic practices and their ability to limit the damage caused by the pathogens (Sikora, 1992). It is widely accepted that high levels of suppressiveness to plant parasitic nematodes are only achieved under perennial crops or monoculture in which soil perturbation practices are low (Baker and Cook, 1974). However, this study shows that high levels of soil suppressiveness can be also achieved in highly perturbed crop systems, probably due to the confluence of favorable interactions between plant-RKN-antagonists, cultural practices and abiotic factors. Both sites, M10.23 and M10.55, were located in the same cropping area, with similar agro-climatic conditions, but differing in crop management. In both sites, RKN were detected in soil and in roots at the beginning of the study. However, nematode densities decreased to near and below detectable levels in soil and roots, respectively, at the end of the study in site M10.23, but not in site M10.55, in which RKN was always detected. Agricultural practices such as crop rotation, tillage and organic amendments have been proved to influence the antagonistic potential of

soil (Sikora, 1992; Kerry and Bourne, 1996; Westphal and Becker, 2001; Janvier et al., 2007; Timper, 2011), and could be the reason for the results of this study. For instance, site M10.23 was fertilized with a mixture of sheep and chicken manure but only sheep manure was used at M10.55. Chicken manure has been reported to suppress RKN infection and reproduction on several crops (Kaplan and Noe, 1993; Riegel and Noe, 2007), but there is still limited information about the suppressive capacity of sheep manure.

In site M10.23, the nematode was able to reproduce on susceptible crops cultivated during spring-summer, in which fungal RKN egg parasites were isolated, mainly *P. chlamydosporia*. The highest percentage of fungal egg parasitism was recorded on zucchini, which ranged from 30 to 78% in the four plots (mean of 54.7%). At the end of this crop, galling index ranged from 0 to 4, less than expected considering a *Pi* of 2951 J2 250 cm^{-3} of soil, and in which the nematode completed three generations according to thermal requirements of *M. incognita* (Vela et al., 2014). Vela et al. (2014) recorded galling indexes of 2.6 and 5.1 on zucchini cultivated in plastic greenhouse, with *Pi* of 222 and 594 J2 250 cm^{-3} of soil, respectively, and in which nematodes completed two generations. *P. chlamydosporia* is a fungal egg parasite that affects the increase of nematode inoculum (J2) and consequently reduces disease severity when more than one generation occurs, because emerged J2 from non-parasitized eggs are able to invade roots (Bailey et al., 2008). Results from the pot test conducted for just one nematode generation showed differences in disease severity between sterilized and non-sterilized soils, indicating that other microorganisms could be involved in soil suppressiveness. Fungal and bacterial DNA sequenced from soil DGGE revealed the presence of several species that can affect nematodes by the production of active toxins against RKN J2 such *Stachybotrys* spp. *Cladosporium* spp. (Qureshi et al., 2012), and *Flavobacterium* spp. (McClure, 1989); by inducing the activity of other nematode antagonists, such *Chryseobacterium* spp. that induce trap

formation in *Arthobotrys oligospora* (Li et al., 2011); by suppressing disease severity, such *Chryseobacterium* spp. (Liu et al., 2014); or by parasitizing RKN eggs, such *Fusarium equiseti* and *Cladosporium* spp. (Giné et al., 2012). The growing media used in this study did not allow bacterial isolation. Thus, the use of culture independent methods is necessary to complement the information obtained by traditional culture dependent ones in order to know the composition and function of microbial communities and their putative contribution to soil suppressiveness.

At the end of autumn-winter crops, no galls were observed and no eggs were extracted from roots. Soil temperatures influence the movement of J2 in soil, root penetration and infection, development and reproduction of RKN. Minimum and maximum soil temperatures at planting autumn-winter crops were 8.3 and 17.3°C (mean 12.8°C), temperatures below the minimum activity threshold of J2 (Roberts et al., 1981). Thus, roots could escape infection.

In site M10.55, *Meloidogyne* was detected in soil and roots of each crop. Nematode densities fluctuated during the cropping season as well as *P. chlamydosporia*, the only fungal species isolated from eggs. Highest nematode densities and levels of egg parasitism were recorded at the end of cucumber and zucchini cultivated in spring-summer and summer-winter, respectively. *Meloidogyne* completed two generations on cucumber and three on zucchini according to RKN thermal requirements on these crops (Giné et al., 2014; Vela et al., 2014). However, disease severity was less than expected, as occurred in M10.23. Soil microbial profiles showed the occurrence of Cyanobacteria, able to suppress RKN densities and disease severity (Khan et al., 2007), and the fungi, *F. equiseti*, and *Preussia* spp, which have been reported as egg parasite of *Heterodera schachtii* (Saleh and Qadri, 1990). Results of pot experiments suggest that the only active antagonist of RKN was *P. chlamydosporia* because despite high percentage of egg parasitism was recorded,

there was no reduction on disease severity after completion of one nematode generation, but it did in field conditions in which the nematode completed more than one.

Despite resistant tomato cultivars suppressed nematode densities and disease severity, as previously reported in plastic greenhouses in Spain (Sorribas et al., 2005; Talavera et al., 2009), *P. chlamydosporia* was also isolated, but the percentage of egg parasitism decreased compared to those on susceptible crops. A positive relation ($r = 0.89$, $P = 0.042$) between egg density on roots (logarithm) and percentage of egg parasitism was found demonstrating the density dependent relationship, as previously stated (Bourne and Kerry, 1998).

Lettuce cultivated from November to March or September to November reduced or maintained nematode densities in soil, but the number of eggs on roots was fewer when planted in November than in September. Absolute minimum and maximum soil temperatures from November to March were 5.1°C and 21.0°C, and 16.5°C and 29.1°C from September to November. Thus, in lettuce planted in September, RKN was able to accumulate enough degree days to complete its life cycle, to produce more eggs and to maintain densities in soil than when cultivated from November to March in which no eggs were produced and fewer nematodes were recovered from soil. The date of planting has an important repercussion in the life cycle of *Meloidogyne* because after root penetration, the nematode needs to accumulate a minimum number of degree days over a specific temperature threshold to complete its life cycle, otherwise, the crop will act as a trap crop. Some crops as lettuce, radish, and arugula have been used as trap crops (Cuadra et al., 2000; Melakeberhan et al., 2006). In north-eastern Spain, lettuce acted as a trap crop when it was transplanted in middle October or November, but not in September when the nematode was able to accumulate enough degree days to produce eggs (Ornat and Sorribas, 2008).

The cover crop of mustard cv. Caliente 119 (a blend of white mustard, *Sinapis alba*, and Indian mustard, *Brassica juncea*) was used as green manure. After its incorporation into the soil, nematode densities dropped considerably as well as percentage of fungal egg parasitism at the end of the following lettuce crop. Mustard cv. Caliente 119 has been shown effective against plantparasitic nematodes and soil-borne fungi (Potter et al., 1998; Charron and Sams, 1999; Friberg et al., 2009). Nevertheless, *P. chlamydosporia* survived, being recovered after the following resistant tomato crop in 2013.

DGGE fingerprints revealed the occurrence of fungal and bacterial species that have been reported associated with the cuticle of RKN J2 or egg masses, or *Heterodera glycines* cysts (Nour et al., 2003; Adam et al., 2014; Cao et al., 2015), but the effect of the majority of them on viability of the nematode is unknown. Some of them such as *Mortierella* spp., Sphingobacteriales, and *Methylobacterium* spp. (reported associated with the J2 cuticle), and *Flexibacter* spp. (associated with the cysts of *H. glycines*) were identified in nonsuppressive and one or both suppressive soils. *Davidiella* spp. reported associated with the J2 cuticle was only identified in non-suppressive soil M10.33; *Sphingobacterium* spp., reported associated with *H. glycines* cysts was only identified in M10.55. *Steroidobacter* spp. and *Lysobacter* spp. were reported associated with RKN egg masses and were only identified in M10.23. Several species of *Lysobacter* spp., affect egg hatching of *Meloidogyne* sp. (Chen et al., 2006; Lee et al., 2014) and reduced disease severity in pot tests (Lee et al., 2013).

Diversity indices were similar for both suppressive and nonsuppressive soils. In fact, suppressiveness is more related to microbial functionality than diversity. In both suppressive soils, *P. chlamydosporia* was the only and most prevalent fungal egg parasites recovered from RKN eggs throughout the study and deemed to be one of the factors responsible for soil suppressiveness in M10.23, and the most responsible in

M10.55. In this study, a density dependent relationship between percent of egg parasitism and density of eggs in roots was found, according to that reported by Bourne and Kerry (1998). Moreover, great variability in virulence of several isolates coming from the same soil was also found. It is known that isolates of *P. chlamydosporia* from the same or different soils differ greatly in growth, development and virulence, in their saprophytic and parasitic ability, and in their ability to colonize the plant rhizosphere (Kerry and Hirsch, 2011). Thus, the environmental plasticity and variability in the virulence showed in this study could be a strategy to persist in a given site, even at low densities. *P. chlamydosporia* was fully adapted to these soil environments and agronomical management practices. It was recovered from eggs in field and pot experiments in site M10.55, or in pot experiments from non-sterilized soils despite no eggs being produced in the majority of crops in field conditions in site M10.23. This plasticity could explain why *P. chlamydosporia* has been found more frequently in the last years in north-eastern Spain, since integrated and organic production systems have been increasingly implemented by growers (Verdejo-Lucas et al., 2002; Giné et al., 2014).

This research provides new information about the antagonistic potential of soils against RKN in two sites used for commercial production of vegetables under organic standards in plastic greenhouse during two growing seasons. *P. chlamydosporia* was the main biotic factor responsible of suppressiveness in site M10.55, because it was the only fungal species recovered from RKN eggs in the field study and pot experiments, and no other antagonist species or effects on RKN were identified by DNA sequencing from DGGE or in pot experiments. However, in M10.23, RKN suppressiveness could be attributed to a combination of microbes, because despite *P. chlamydosporia* was isolated from eggs, some other microorganism with antagonistic effect against the nematode were identified by DGGE and results from pot test agree with their mode of action. Besides the biotic factors identified in both sites,

a combination of several agronomic practices such as crop rotation, including RKN resistant cultivars and cover crop as green manure, the addition of organic amendments, and date of planting, can contribute to prevent nematode build-up. These findings will lead to further studies deep in the knowledge of the relations between microbial communities and crop management that achieve soil suppressiveness, in order to design strategies to improve the antagonistic potential of soil.

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Chapter 7

Effect of plant resistance and BioAct WG (*Purpureocillium lilacinum* strain 251) on *Meloidogyne incognita* in a tomato–cucumber rotation in a greenhouse

Effect of plant resistance and BioAct WG (*Purpureocillium lilacinum* strain 251) on *Meloidogyne incognita* in a tomato–cucumber rotation in a greenhouse

The effectiveness of combining resistant tomato with BioAct WG (*Purpureocillium lilacinum* strain 251) (Pl251) against *Meloidogyne incognita* was assessed in a tomato-cucumber rotation in a greenhouse over 2 years. Additionally, the enzymatic activity of the fungus, the percentage of fungal egg and juvenile parasitism, cardinal temperatures and the effect of water potential on mycelial growth and the soil receptivity to Pl251 were determined *in vitro*. Plant resistance was the only factor that suppressed nematode and crop yield losses. Percentage of egg parasitism in plots treated with BioAct WG was less than 2.6%.However, under *in vitro* conditions, Pl251 showed protease, lipase and chitinase activities and parasitised 94.5% of eggs, but no juveniles. Cardinal temperatures were 14.2, 24–26 and 35.4 °C. The maximum Pl251 mycelial growth was at −0.25 MPa and 25 °C. Soil temperatures and water potential in the greenhouse were in the range of the fungus. However, soil receptivity was lower in greenhouse soil, irrespective of sterilisation, than in sterilised sand.Plant resistance was the only factor able to suppress nematode densities, disease severity and yield losses, and to protect the following cucumber crop. Environmental factors involved in soil receptivity could have negatively affected fungus effectiveness.

Keywords: Biological control; *Cucumis sativus*; Double-cropping system; Integrated management; Root-knot nematodes; *Solanum lycopersicum*

Introduction

Spain is the main producer of vegetables under protected cultivation in the Mediterranean area, with 71 003 ha (Anuario de estadística agraria, 2013). The major crops are tomato (*Solanum lycopersicum* L.) and cucumber (*Cucumis sativus* L.), frequently cultivated in a double-cropping system (Ornat et al., 1997). Greenhouse

tomato is grown on 18 501 ha, with an annual production of 1 881 922 tonnes, which represents 38% of the tomato production area and 47% of the total yield. Greenhouse cucumber is grown on 7768 ha, with an annual yield of 717 693 tonnes, representing 88% of the total production area and 95% of the total yield (Anuario de estadística agraria, 2013).

Root-knot nematodes (RKNs), *Meloidogyne* spp., are one of the most important soil pathogens limiting horticulture production worldwide, especially under protected cultivation (Sikora et al., 2005). *Meloidogyne* spp. are widespread in all vegetable production areas in Spain. Yield losses caused by RKNs can reach 60% in tomato and 88% in cucumber (Sorribas et al., 2005; Giné et al., 2014). RKNs are mainly managed by fumigant and non-fumigant nematicides (Talavera et al., 2012). However, the limitations imposed by Directive 2009/128/EC have encouraged research to find alternatives to chemical pesticides, as well as to design effective and durable strategies to manage RKNs.

There are a large number of non-chemical alternatives for the control of RKNs (Sikora et al., 2005), including crop rotation with resistant cultivars and biological control. In nematology, resistance is defined as the ability of a plant to suppress infection, development and/or reproduction of plant-parasitic nematodes (Roberts, 2002). Therefore, resistant tomato cultivars or rootstocks carrying the *Mi* gene are widely used because of their effectiveness in suppressing *M. arenaria*, *M. incognita* and *M. javanica* (Sorribas et al., 2005; Roberts, 2002). However, its expression can be limited by (i) constant soil temperatures above 28 °C (Dropkin, 1969), (ii) the genetic background of the tomato cultivar or rootstock (Cortada et al., 2009), (iii) the RKN species (not effective against *M. hapla*, *M. chitwoodi* race 3 (Brown et a., 1997), *M. enterolobi* (Kiewnick et al., 2009) or *M. exigua* (Silva et al., 2008) and (iv) virulent populations, which can occur suddenly or be selected by repeated cultivation of

resistant cultivars (Ornat et al., 2001; Verdejo-Lucas et al., 2009). Inclusion of resistant tomato cultivars in a cropping sequence helps to suppress the reproduction of RKNs and to reduce yield losses of the following susceptible crop (Talavera et al., 2009), such as cucumber (Ornat et al., 1997), because yield losses are related to nematode densities in soil at transplanting (Seinhorst, 1965).

Several microorganisms have been evaluated for biological control of plant-parasitic nematodes (Sikora, 1992). The use of biological control agents able to suppress the build-up of RKNs can be of interest to reduce the pressure on the resistant genes avoiding the selection of virulent populations and to contribute to maintaining nematode densities below the economic threshold level. Out of these, *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*) strain 251 (Pl251) is the only biological nematicide listed in annex 1 of the European register of active substances (EUROPEAN UNION, 2009). *P. lilacinum* is a common soil hyphomycete able to parasitise RKN sedentary stages by direct hyphal penetration and by using hydrolytic enzymes (Holland et al., 1999; Khan et al., 2004 and 2006a). Moreover, the effectiveness of Pl251 in controlling RKNs has been widely reported under controlled conditions and in pot tests (Kiewnick et al., 2006 a and b and 2011), although few reports are available on its effectiveness under field conditions (Anastasiadis et al., 2008; Kaskavalci et al., 2009).

The aim of the present study was to evaluate the effectiveness of combining the resistant tomato cv. Monika with *P. Lilacinum* strain 251 (BioAct WG®; Belchim Crop Protection, Londerzeel, Belgium) over two consecutive growing seasons in a greenhouse to manage RKNs in a tomato–cucumber rotation. Additionally, *in vitro* experiments were carried out to determine extracellular enzymes produced by Pl251 and its capability to parasitise eggs and second-stage juveniles of RKNs, as well as to

establish the cardinal temperatures and the effect of temperature and water potential on mycelial growth and soil receptivity to Pl251.

Materials and methods

Greenhouse trial

The greenhouse trial was conducted in a 700m^2 greenhouse infested with *M. incognita* in Viladecans (41° 17′ 18′′ N, 2° 2′ 39′′ E, Barcelona, Spain) during 2011 and 2012. The soil was a sandy loam with 83.8% sand, 6.7% silt and 9.5% clay; pH 8.7; 1.8% of organic matter (w/w) and 0.5 dS m^{-1} of electrical conductivity. Soil was inoculated with *M. incognita*, identified by morphology of perineal pattern, esterase pattern and SCAR markers, in 2007, and rotations with resistant or susceptible tomato cultivars and cucumber or black fallow were carried out from 2008 to 2010. The rotation sequence consisted of resistant tomato cv. Monika (bearing the *Mi* gene) or the susceptible cv. Durinta from March to July, followed by cucumber cv. Dasher II cultivated from July to October–November. Individual plots of 9.6m^2 comprised four rows, with six plants per row. Plant spacing was 50 cm between rows and 55 cm within rows. The distance between individual plots was 110 cm between rows and 100 cm along rows.

In 2011, combinations of resistant (TR) or susceptible tomato (TS) with or without BioAct WG preceding cucumber with or without BioAct WG were assessed. Each combination was replicated 10 times according to a stratified randomised block design. In 2012, the combinations were located on the same plots as in 2011, with slight modifications. Plots cultivated with TR did not receive any treatment with BioAct WG because of results obtained in 2011. Then, combinations including TR or TS without BioAct WG were replicated 10 times, and 20 times for the combination of TS with BioAct WG. Cucumber crop grown after TS with or without application of BioActWG was replicated 15 times, and 5 times each combination after TR.

Dates and rates of application of BioActWG to soil and seedlings were as recommended by the manufacturer. Briefly, the first soil application of the commercial product BioAct WG (1×10^{10} viable spores g^{-1} of *P. lilacinum* strain 251 dried on glucose) was carried out 14 days before transplanting by drip irrigation at a rate of 0.4 g m^{-1} linear and 10 cm width. The following fungal applications to soil were repeated at the same rate at 6 week intervals: two applications during the tomato and cucumber crops in 2011 and cucumber crop in 2012, and three during the tomato crop in 2012. Seedlings were watered with a suspension of 0.l g BioAct WG 1 L^{-1} just before transplanting.

Tomato was cultivated from 31 March to 6 July (98 days), and cucumber from 29 July to 26 October (90 days) in 2011. In 2012, tomato was cultivated from 5 March to 17 July (135 days), and cucumber from 31 July to 5 November (98 days). The plants were irrigated by drip irrigation and fertilised weekly with an NPK solution (15–5–30) at 31 kg ha^{-1} and iron chelate and micronutrients at 0.9 kg ha^{-1}. Crops were vertically trellised. Weeds were removed manually during and between cropping cycles.

Tomato yield was assessed from the first six fruit sets produced from the eight central plants in each plot. Fruits were harvested according to commercial standards as they reached maturity. Similarly, cucumber fruits were harvested from the eight central plants of each plot when they reached the standard commercial size. Total yield per crop cycle was expressed as kg $plant^{-1}$. Soil temperatures were recorded at 30min intervals in order to estimate the number of nematode generations and the effect of soil temperature on fungus growth. In 2011, soil temperatures were recorded with soil probes 107 (Campbell Scientific, Logan, UT) placed at a depth of 15 cm. In 2012, soil temperatures and water potential were recorded at the same interval and soil depth with 5TM and MPS-1 probes (Decagon Devices, Inc., Pullman,WA).

Composite soil samples were collected from each plot at the beginning and at the end of each crop to estimate initial (*Pi*) and final (*Pf*) nematode population densities respectively. Each soil sample consisted of eight subsamples from the top 30 cm taken with an auger (2.5 cm diameter). Soil samples were sieved through a 4mm aperture screen to remove stones, and carefully homogenised to extract nematodes from 500 cm^3 by Baermann trays (Whitehead and Hemming, 1965). Second-stage juveniles (J2) that migrated to the water were collected 1 week later, concentrated on a 25 µm sieve, counted and then expressed as number of J2 per 250 cm^3 of soil. At the end of each crop, plants were removed with a pitchfork and cut at ground level, and the disease severity was assessed using Zeck's galling index (Zeck, 1971) on a scale of 0 to 10, where 0 means complete and healthy root system (no galls observed) and 10 means plants and roots dead. Afterwards, the roots were weighted and chopped into 2 cm long segments, and two 10 g subsamples were used to extract eggs by blender maceration in a 1% NaOCl solution (Hussey and Barker, 1973). After 10 min of maceration, the egg suspension was passed through a 75 µm sieve to retain the plant material, and through a 25 µm sieve to retain the eggs. The number of eggs was counted and expressed per gram of fresh root weight.

To assess fungal egg parasitism, the procedure described in Giné et al., (2013) was used. In brief, 20 egg masses per individual plot were handpicked from the remaining roots and placed in a watchglass containing sterile demineralised water. The outer part of the gelatinous matrix was removed, and eggs were dispersed in an Eppendorf microcentrifuge tube containing 1mL of sterile demineralised water with a pestle. Then, 333 µL aliquots of egg suspension were spread onto each of three replicated petri plates containing a growth-restricting medium (Lopez-Llorca and Duncan, 1986) and incubated at 25±0.5 °C in the dark. The number of parasitised eggs was recorded after 24 and 48 h under a dissecting microscope, and the percentage of egg parasitism was then calculated as the number of parasitised eggs per plate per

total eggs per plate. Eggs were considered to be parasitised if fungal hyphae grew from inside unhatched eggs. Parasitised eggs were individually transferred to the growth-restricting medium to establish pure cultures, and fungal species were identified by cultural and morphological characteristics (Samson, 1974).

Extracellular enzyme production

Extracellular enzyme production by Pl251 was evaluated using a semi-quantitative API ZYM® (BioMérieux, Marcy l'Etoile, France) system, which identifies 19 cellular enzymes, i.e. alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, nacetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

Conidia of Pl251 were collected from three-week-old cultures of the fungus growing on potato dextrose agar (PDA) (39 g L^{-1}) at 25 °C in the dark. The colony was washed with 5 mL of sterile distilled water. The number of conidia was then counted with a haemocytometer and adjusted to 1×10^6 conidia mL^{-1}. The API ZYM system was used according to the manufacturer's instructions.

Briefly, 65 µL of the conidia suspension was placed into each cupule and incubated at 25 °C for 6 h in the dark (Castellá et al., 1999). Enzyme activity was observed after the addition of a drop each of ZYM A and ZYM B reagents and exposed to sunlight for 1 h to eliminate yellowing from the reagents. Enzyme production was assessed to a scale from 0 (no enzyme production) to 5 (maximum enzyme production) according to the colour chart provided by the manufacturer. The experiment was carried out once.

Capability of Pl251 to parasitise *M. incognita* eggs and J2 in vitro

Individual plugs of 9mm diameter from the edge of a Pl251 colony growing on PDA were placed in the centre of a total of six petri dishes (90 mm) containing water agar (agar 12 g L^{-1}) and incubated at 25±0.5 °C in the dark for 3 days.

Surface-sterilised eggs or J2 of the same *M. incognita* population used to inoculate the soil of the greenhouse in 2007 and maintained in tomato in pots were used to determine the parasitic ability of Pl251. *M. incognita* was identified by morphology of perineal pattern, esterase pattern and SCAR markers. Eggs from 30 egg masses handpicked from tomato roots were surface sterilised following the protocol of Verdejo et al., (1998) with some modifications. Egg masses were placed into a conical sterile tube with 1mL of a 0.5% NaOCl solution for 4 min, and were shaken at 30 s intervals for 10 s. After that, the solution was diluted 10 times with sterile distilled water and left undisturbed for 30 min to allow the eggs to settle at the bottom of the tube. Then, eggs were taken and spread into three petri dishes at 1 cm from the edge of the colony of the Pl251 using a sterile Pasteur pipette, and incubated at 25±0.5 °C in the dark for 1 week. The assessment of egg parasitism was carried out as described previously.

Second-stage juveniles of *M. incognita* were obtained by extraction of eggs from tomato roots by the Hussey and Barker et al., (1973) method and left to emerge on Baermann trays (Whitehead and Hemming, 1965). Then, J2 were surface sterilised following the Mountain et al., (1955) procedure, with some modifications. Briefly, a suspension of 100 J2 in 0.5mL of water was placed in a conical sterile tube containing streptomycin 0.1% for 4 h. The suspension was shaken at 1 h intervals for 10 s. The solution was then diluted 20 times with sterile distilled water and left undisturbed for 30min for J2 to settle at the bottom of the tube. Then, J2 were taken and placed in three petri dishes at 1 cm from the edge of the Pl251 colony using a sterile Pasteur.

pipette, and incubated at 25 ± 0.5 °C in the dark for 1 week. The assessment of J2 parasitism was carried out as described previously for egg parasitism. The percentage of parasitism was then calculated as the number of unhatched parasitised eggs or J2 per petri dish divided by the number of unhatched eggs or J2 per petri dish. Experiments were conducted once.

Cardinal temperatures and the effect of temperature and water potential on Pl251mycelial growth *in vitro*

Cardinal temperatures of Pl251 were determined by placing individual 9mm diameter plugs of Pl251 in the centre of each of 24 petri dishes containing water agar (WA) (12 g L^{-1}). The petri dishes were incubated at 4, 10, 15, 20, 25, 30, 35 or 40 °C in the dark (three dishes per temperature). Minimum and maximum diameters (mm) of fungal colonies were measured every 24 h until the colonies occupied 80% of the surface of the petri plate. The mycelial growth rate (mm day^{-1}) was calculated as the relation between mean colony diameter (mm) and growth time (day). Concurrently, the effect of water potential (Ψ) and temperature on Pl251 growth was assessed. Water agar media with different concentrations of polyethylene glycol 8000 (PEG8000) was prepared according to Michel's equation36 to achieve Ψ between 1.25, −1, −0.75, −0.5 and −0.25 MPa. Mycelial plugs of 9mm diameter from the edge of a fungal colony were placed in the centre of the petri plates and then incubated at 15, 20, 25 and 30 °C in the dark. Each combination of temperature–PEG8000 concentration was repeated 3 times. Minimum and maximum diameters of fungal colonies were measured daily until the colonies occupied 80% of the surface of the petri plate. For each water potential, a linear regression was calculated for the relationship between mycelial growth rate and temperature, and the slopes were used to construct the regressions to determine the effect of temperature and water potential on mycelial growth. Experiments were repeated once.

Soil receptivity

Soil collected from BioAct WG non-treated plots of the greenhouse trial was assessed for receptivity to the fungal isolate. A part of the soil was 2× sterilised at 121 °C for 1 h within 24 h. The other part remained non-sterilised. The experiment was carried out following the procedure described by Monfort et al., (2006). Briefly, 40 g of sterilised or non-sterilised air-dried soil was placed in petri dishes and saturated with sterile distilled water. Soils included in the experiment were: (i) sterilised greenhouse soil, (ii) non-sterilised greenhouse soil and (iii) sterilised sand. A polyvinylidene difluoride (PVDF) membrane (0.22 µm pore size and 45mm diameter) was sterilised at 121 °C for 20 min and placed on top of the soil, ensuring full contact. A 4mm diameter plug of Pl251 was placed in the middle of each membrane. Petri dishes were then sealed and incubated at 25 ± 0.5 °C in the dark. After 3 weeks, the membranes were washed with sterile distilled water and then dried in a laminar air flow cabinet. Then, the membranes were incubated in a solution of 1% trypan blue in lactic acid for 12 h at room temperature to stain the mycelia. After that, the excess of the stain was removed with sterile distilled water, and minimum and maximum colony diameters were measured. The experiment was repeated once.

Statistical analysis

Statistical analyses were done using SAS v.9 (SAS Institute Inc., Cary,NC).Data from experiments were transformed when required to log10 (x + 1) or √ (x + 0.5) to normalise. Analysis of variance was done by the general linear model procedure (PROC GLM). A factorial ANOVA was used for the greenhouse trial to determine the effect of the tomato cultivar, the application of BioAct WG and the interaction (except for tomato 2012) on nematode densities in soil, eggs in roots, disease severity and crop yield per cropping season. Analysis of variance was also carried out to compare the

effect of temperature and water potential on the growth of Pl251, as well as soil receptivity to Pl251.

Results

Greenhouse trial

Minimum, maximum and average soil temperatures during the cultivation of each crop and year are provided in Table 1.

Table 1. Cropping dates of the rotation sequence tomato-cucumber and soil temperatures in the greenhouse during two consecutive growing seasons.

Year	Crop	Cropping dates	Average soil temperature (°C) [a]	Minimum and maximum temperatures (°C) [b]
2011	Tomato	31 March-6 July	25.4	19.8-34.3
	Cucumber	29 July-26 October	27.8	20.2-32.9
2012	Tomato	5 March-17 July	24.4	17.0-31.4
	Cucumber	31 July-5 November	27.0	17.5-31.2

[a] Average soil temperature at 15cm depth: mean of daily mean temperatures during the cropping period. [b] Absolute minimum or maximum soil temperature at 15cm depth during the cropping period.

Accumulated soil temperatures during the tomato and cucumber crops in 2011 were 1504 degree-days (DD) (base temperature *Tb* =10 °C) and 1473 DD (*Tb* =11.4 °C) respectively. According to the thermal requirements for nematode development on tomato and cucumber (Ferris et al., 1985; Giné et al., 2014) *M. incognita* completed two generations in tomato (thermal constant *S* = 600–700 DD over *Tb* =10 °C) (Ferris et al., 1985) and in cucumber (*S* = 500 DD over *Tb* =11.4 °C) (Giné et al., 2014). In 2011, the tomato cultivar was the only factor that explained differences ($P < 0.05$) in nematode densities in the soil and roots, galling index and crop yield, both in tomato and cucumber (Tables 2 and 3). Nematode densities in the soil and roots and the galling index at the end of the resistant tomato crop were 8, 6 and 18 % of those

recorded at the end of the susceptible cultivar, which yielded 78% less than the resistant cultivar. The percentage of fungal egg parasitism at the end of the crop was less than 0.1% (Table 2). At cucumber transplanting, higher ($P < 0.05$) soil nematode densities occurred in plots preceded by the susceptible cultivar than in plots preceded by the resistant tomato cultivar.

Galling indices were lower ($P < 0.05$) after the resistant tomato cultivar than after the susceptible cultivar. Eggs from cucumber plants were only recovered from plants preceded by the resistant tomato because cucumber following susceptible tomato died (data not shown). Egg parasitism by the fungus was less than 0.5% (Table 3).

Table 2. Initial (*Pi*) and final (*Pf*) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal egg parasitism, and yield of the resistant tomato cv. Monika (TR) and susceptible cv. Durinta (TS) alone or combined with the application of BioAct WG cultivated from 31st March to 6th July of 2011.

BioAct WG [a]	Tomato cultivar	*Pi* (J2 250 cm^{-3} soil)	*Pf* (J2 250 cm^{-3} soil)	Galling index [b]	Eggs g^{-1} root	Egg parasitism (%)	Yield (kg $plant^{-1}$)
No application	TR	663±241 a	334±113 b	1.2±0.2 b	421±1100 b	0±0	1.5±0.2 a
	TS	612±182 a	2347±331 a	6.8±0.2 a	7499±347 a	0±0	0.3±0.2 b
Application	TR	579±210 a	100±24 b	1.3±0.2 b	482±110 b	0.04±0.02	1.3±0.1 a
	TS	576±161 a	3300±649 a	7.4±0.2 a	6957±441 a	0.02±0.01	0.3±0.1 b
BioAct [c] *vs* no BioAct		NS	NS	NS	NS		NS
TR *vs* TS		NS	S	S	S		S
T x BioAct		NS	NS	NS	NS		NS

Data are mean ± standard error of 10 replicates.

[a] BioAct WG (1 x 1010 viable spores g-1) Treatments: At soil, 14 days before transplanting and every six weeks after transplanting at rate of 0.4 g m-1 linear and 10 cm width: in seedling before transplanting at 0.1g L-1 rate.

[b] Galling index based on the Zeck'45 scale; from 0 (healthy plants) to 10 (dead plants).

Different letters in the same column indicate differences ($P < 0.05$) according to Tukey's test.

Table 3. Initial (*Pi*) and final (*Pf*) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal egg parasitism, and yield of the cucumber cv. Dasher II alone or combined with the application of BioAct WG cultivated after resistant tomato cv. Monika (TR) or susceptible cv. Durinta (TS) from 29th July to 26th October of 2011.

BioAct WG [a]	Previous Crop	*Pi* (J2 250 cm^{-3}soil)	*Pf* (J2 250 cm^{-3} soil)	Galling index [b]	Eggs g^{-1} root	Egg parasitism (%)	Yield (kg $plant^{-1}$)
No application	TR	241±99	357.85±91	6.6 ±0.6	1352±561	0±0	0
	TS	3202±700	234.60±99	9.7±0.3	179±121	0±0	0
Application	TR	193±80	684.30±183	6.9±0.7	3094±956	0.44±0.27	0
	TS	2446±243	185.05±64	10.0±0	0±0	0.09±0.09	0
BioAct [c] *vs* no BioAct		NS	NS	NS	NS		
TR *vs* TS		S	S	S	S		
T x BioAct		NS	NS	NS	NS		

Data are mean ± standard error of 10 replicates.

[a] BioAct WG (1 x 10^{10} viable spores g^{-1}) Treatments: At soil, 14 days before transplanting and every six weeks after transplanting at rate of 0.4 g m^{-1} linear and 10 cm width: , in seedling before transplanting at 0.1g L^{-1} rate.

[b] Galling index based on the Zeck' scale; from 0 (healthy plants) to 10 (dead plants). S and NS indicate significant or non-significant differences between factors, respectively, according to Tukey's test ($P < 0.05$). Different letters in the same column indicate differences ($P < 0.05$) according to Tukey's test.

In 2012, accumulated temperatures during the tomato and cucumber crops were 1959 (*Tb* = 10 °C) and 1524 DD (*Tb* = 11.4 °C) respectively. Hence, *M. incognita* completed three generations in both crops according to its thermal requirements. In this cropping season, the tomato cultivar was also the factor responsible for the differences ($P < 0.05$) in nematode densities in the soil and roots, disease severity, as well as crop yield in both tomato and cucumber crops (Tables 4 and 5). As in the previous season, cucumber following susceptible tomato had higher ($P < 0.05$) nematode levels at transplanting, and all plants died at the end of the experiment. The percentage of fungal egg parasitism at the end of tomato and cucumber crops was 2.4 and 2.6% respectively (Table 5).

Table 4. Initial (*Pi*) and final (*Pf*) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal egg parasitism, and yield of the susceptible cv. Durinta (TS) alone or combined with the application of BioAct WG, and the resistant tomato cv. Monika (TR) alone, cultivated from 5th March to 17th July of 2012.

BioAct WG [a]	Tomato cultivar	*Pi* (J2 250 cm^{-3}soil)	*Pf* (J2 250 cm^{-3}soil)	Galling index [b]	Eggs g^{-1} root	Egg parasitism (%)	Yield (kg $plant^{-1}$)
No application	TR	358±91	1009±232 b	2.9±0.3 b	811±250 b	0	2.2±0.1 a
	TS	185±80	4498±705 a	7.2±0.3 a	6406±1695 a	0	0.9±0.2 b
Application	TS	363±106	4010±513 a	7.7±0.3 a	8586±989 a	2.39±1.23	0.8±0.1 b
TR *vs* TS		NS	NS	NS	NS		NS
T x BioAct		NS	S	S	S		S

Data are mean ± standard error of 10 replicates of the combination of "no BioAct application with TR or TS" and 20 replications of the combination of "BioAct application with TS".

[a] BioAct WG (1 x 1010 viable spores g-1) Treatments: At soil, 14 days before transplanting and every six weeks after transplanting at rate of 0.4 g m-1 linear and 10 cm width: , in seedling before transplanting at 0.1g L-1 rate.

[b] Galling index based on the Zeck'45 scale; from 0 (healthy plants) to 10 (dead plants).

Different letters in the same column indicate differences ($P < 0.05$) according to Tukey's test.

Table 5. Initial (*Pi*) and final (*Pf*) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal egg parasitism, and yield of the cucumber cv. Dasher II alone or combined with the application of BioAct WG cultivated after resistant tomato cv. Monika (TR) or susceptible cv. Durinta (TS) from 31st July to 5th November of 2012.

BioAct WG [a]	Previous Crop	*Pi* (J2 250 cm^{-3} soil)	*Pf* (J2 250 cm^{-3} soil)	Galling index [b]	Eggs g^{-1} root	Egg parasitism (%)	Yield (kg $plant^{-1}$)
No application	TR	1187±400 b	1379±253 a	7.6±1.1 b	1083±381	0±0	0.2±0.1 a
	TS	4319±464 a	659±162 ab	10.0±0 a	na	na	0.03±0.002 b
Application	TR	801±199 b	768±184 ab	8.7±0.5 b	3646±1482	2.60±1.01	0.10±0.04 a
	TS	3968±695 a	522±217 b	10.0±0 a	na	na	0.02±0.002 b
BioAct [c] *vs* no BioAct		NS	NS	NS			NS
TR *vs* TS		S	S	S			S
T x BioAct		NS	NS	NS			NS

Data are mean ± standard error of 15 replicates of the combination each combination with TS and 5 replicates of each combination with TR.

[a] BioAct WG (1 x 1010 viable spores g-1) Treatments: At soil, 14 days before transplanting and every six weeks after transplanting at rate of 0.4 g m-1 linear and 10 cm width: in seedling before transplanting at 0.1g L-1 rate.

[b] Galling index based on the Zeck'45 scale; from 0 (healthy plants) to 10 (dead plants).

Different letters in the same column indicate differences ($P < 0.05$) according to Tukey's test.

Na: no available

Extracellular enzyme production

Six extracellular enzymes were produced by Pl251. The enzyme produced in highest amounts by the fungus was leucine arylamidase (5 on the colour scale), followed by esterase and acid phosphatase (4 on the colour scale) and esterase-lipase (3 on the colour scale), and the lowestwere naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase (2 on the colour scale).

Capability of Pl251 to parasitise RKN eggs and J2 *in vitro*

Pl251 parasitised 94.91±2.88% (mean±standard error) of unhatched *M. incognita* eggs. However, no J2 were parasitised.

Cardinal temperatures and the effect of temperature and water potential on Pl251mycelial growth *in vitro*

Mycelial growth of Pl251 occurred between 14.2 °C (minimum) and 35.4 °C (maximum), with 24–26 °C as the optimal growth temperature range (Figure 1). No growth was detected at 4, 10 and 40 °C.

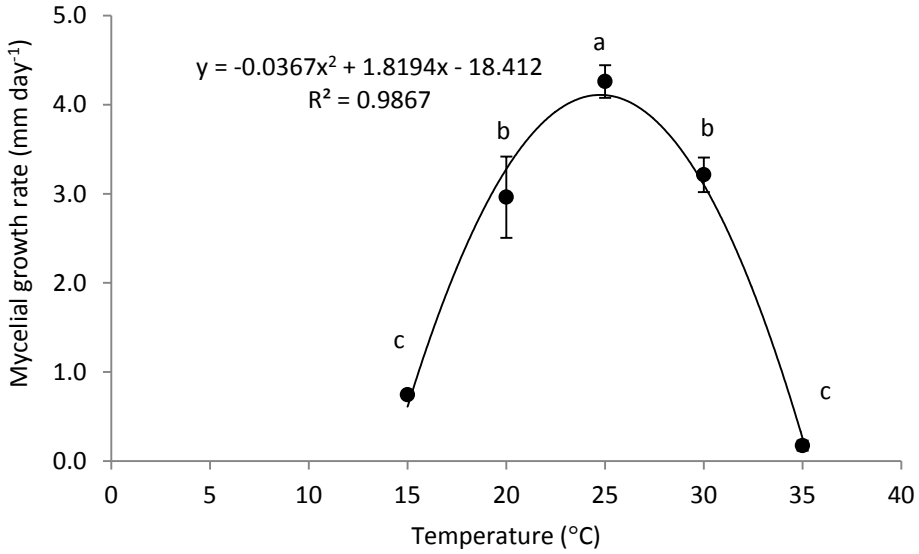

Figure 1. Mycelial growth rate (mm day^{-1}) of *Purpureocillium lilacinum* strain 251 in water agar (12 g L^{-1}) at 15, 20, 25, 30 and 35°C. Values are the means of six replicates. Bars represent the standard error (n=6) Different letters indicate differences at $P < 0.05$ according to LSD's test.

The water potential (−1.25 to −0.25 MPa) directly influenced the mycelial growth of Pl251. The mycelial growth was higher at optimal temperatures (25 °C) and highest water potential (−0.25 MPa), followed by 30, 20 and 15 °C (Figure 2).

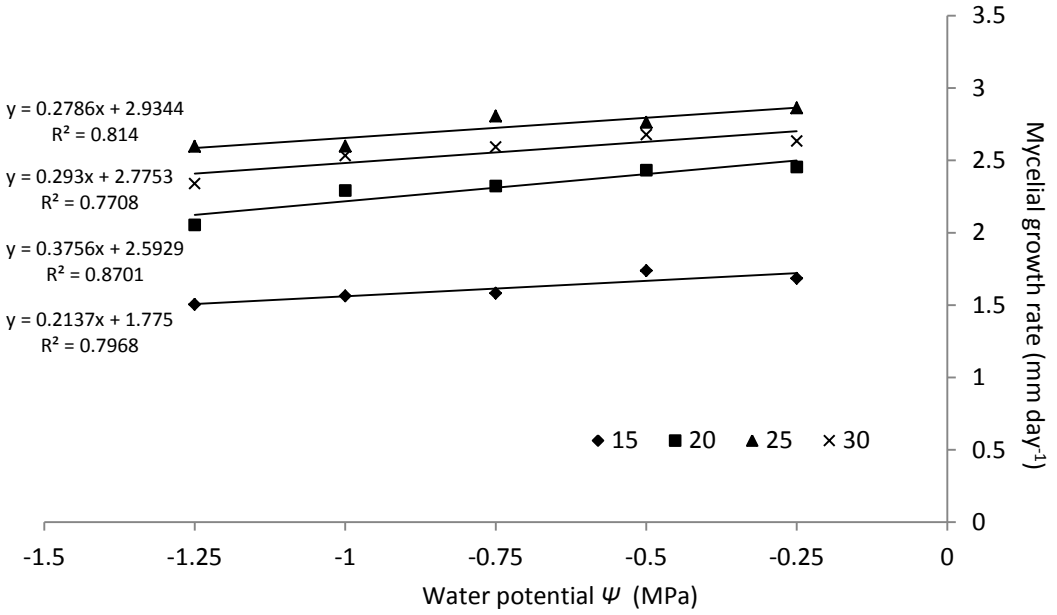

Figure 2. Mycelial growth rate (mm dia^{-1}) of *Purpureocillium lilacinum* strain 251 at 15, 20, 25, 30 °C and water potentials from -1,25 to -0,25 MPa. Values are the means of six replicates.

Soil receptivity

The mycelial growth of Pl251 in sterile sand (2.55 ± 0.06 cm, mean ±standard error) was 91.83% higher ($P < 0.05$) compared with non-sterile (0.18 ± 0.06 cm) or sterile greenhouse soil (0.23 ± 0.07 cm). However, no differences ($P > 0.05$) were found between sterile and non-sterile greenhouse soil.

Discussion

The effectiveness of combining a tomato cultivar carrying the *Mi* gene for resistance to *M. incognita* and BioAct WG based on the nematode antagonist *P. lilacinum* strain 251 against RKNs has been assessed in a tomato–cucumber rotation under greenhouse conditions. The initial hypothesis considered an additive effect of

both methods to suppress nematode densities. The first one, mediated by plant resistance, should suppress nematode infection, development and reproduction. The second one, due to Pl251, should parasitise eggs produced by nematodes that escaped plant resistance. In the following cucumber crop, fewer RKNs at transplanting after resistant tomato should mean a greater percentage of egg parasitism, because most egg masses should be on the root surface, favouring egg infection, as well as reducing yield losses owing to fewer J2 at transplanting. However, the hypothesis was not confirmed in our trial, with plant resistance being the only factor that consistently suppressed RKNs. The effectiveness of tomato cultivars or rootstocks carrying the *Mi* gene against RKNs in greenhouses in Spain was consistent with previous reports (Sorribas et al., 2005; Cortada et al., 2009; Verdejo-Lucas et al., 2009; Talavera et al., 2009). In this study, resistant tomato suppressed disease severity and reproduction by 82–91% and 87–95% compared with the susceptible cultivar, each year, respectively. The effect of intermittent peaks of soil temperatures over 28 °C did not affect the effectiveness of the *Mi* gene, as previously reported (Verdejo-Lucas et al., 2013). During the tomato crop in 2011, the number of days with maximum soil temperatures over 28 °C was 23 after 35 days of transplanting, and 38 after 62 days of transplanting in 2012. In addition, the benefit of cropping a resistant tomato cultivar on yield losses of the following susceptible crop was also observed, as previously stated (Ornat et al., 1997; Talavera et al., 2009).

Unlike plant resistance, there are few reports about the effectiveness of BioAct WG alone and/or in combination with other control methods against RKNs under Mediterranean conditions (Anastasiadis et al., 2008; Kaskavalci et al., 2009). In studies conducted in Greece and Turkey, BioAct WG did not provide satisfactory RKN control. However, in several *in vitro* and pot tests, the control capability of Pl251 against several plant-parasitic nematode species was reported (Khan et al., 2004 and 2006 b; Kiewnick and Sikora 2006a and b; Kiewnick et al., 2009 and 2011; Kiewnick, 2006; Rumbos et al.,

2008). The ability of Pl251 to penetrate eggs and cuticles of sedentary stages of RKNs by mechanical and chemical mechanisms has been reported (Khan et al., 2004 and 2006a). The results obtained by the API ZYM method showed high protease and lipase activity and low chitinase activity able to degrade the main components of egg shell and nematode cuticle (Bird et al., 1991). Therefore, high proportion of egg parasitism was expected, as in our *in vitro* experiment (94.9%). In addition, Kahn et al., (2006a) pointed out the parasitic ability of Pl251 on all stationary stages of *M. javanica*, that is: eggs, juveniles contained in eggs, post-infective juvenile stages, from swollen J2 to J4, and females, but they did not assess the effect on the mobile infective J2. The results of this study showed that Pl251 was not able to parasitise the infective J2 of *M. incognita* nor the sedentary stages of RKNs because Pl251 is not a root endophyte of tomato or cucumber plants (Holland et al., 2003; Rumbos, 2006). Thus, Pl251 could exert its parasitic potential only on eggs and juveniles contained in eggs that remained in soil at the end of the crop, or on those produced on roots and exposed to the soil. In fact, in greenhouse conditions, the percentage of egg parasitism was less than 2.6% in both crops and years. Moreover, microorganisms associated with the gelatinous matrix of the egg masses can inhibit fungal egg parasites such as *Pochonia chlamydosporia* (Kok and Papert, 2001). Thus, fungal application did not affect nematode development, in spite of four applications per crop and year.

Environmental factors can play an important role in nematode biocontrol (Stirling, 2014). Rumbos et al., (2008) reported a negative correlation between the persistence of Pl251 in soil and the sand content of soil. Thus, sandy soils, as in this study (83.8% sand), could not be suitable for the fungus. However, testing of soil receptivity showed that mycelial growth was better in sterilised sand than in sterilised sandy loam soil from the greenhouse experiments. This indicates that other factors different to microbial communities or thermosensitive chemicals in soil could limit the

effectiveness of Pl251 because mycelial growth was equally poor in sterile and non-sterile sandy loam soil from the greenhouse.

Soil temperatures during the cropping period or time of application could also affect BioAct WG effectiveness. In this study, cardinal temperatures of mycelial growth were determined. The maximum temperature of Pl251 mycelial growth in water agar was similar to that reported by Kiewnick (2006) but not the optimal range, which was less wide (24–26 °C versus 24–30 °C). Moreover, in this study, minimum temperature was also estimated (14.2 °C) because low soil temperatures at transplanting tomato in spring (17–19 °C) could affect Pl251 and thus its effectiveness. Soil temperatures during the cultivation of tomato and cucumber were in the range of fungus development according to cardinal temperatures, but 37 out of 98 days and 60 out of 90 days during each tomato crop were over optimal temperatures, and 63 out of 135 and 59 out of 98 days during each cucumber crop. At soil temperatures between the optimal for fungal growth (24–26 °C) and the optimal for *M. incognita* development (30 °C) (Ploeg and Maris, 1999), the nematode could take advantage over Pl251, which could reduce its effectiveness. In fact, at temperatures of 28±1.5 °C, no dose–response relationship was observed, but it was at 25±1 °C (Kiewnick et al., 2011). The soil water potential recorded during the cropping period should not affect fungal growth because it was near field capacity (−0.033 MPa), and according to the results of *in vitro* testing, Pl251 mycelia grow more at a higher water potential.

Another putative explanation for the lack of efficacy of BioAct WG in the greenhouse trial could be the content of glucose in the formulation, which inhibits the protease activity and consequently the capability to parasitise RKN eggs (Bonants et al., 2005).

The present study aimed to demonstrate the usefulness of combining plant resistance with BioActWG to manage RKNs. However, Pl251 was not able to parasitise

eggs in the greenhouse owing to suboptimal soil temperatures for several days during the cropping period and/or inhibition of enzymes produced by the fungus by the components of the formulation and/or non-thermosensitive chemical factors in the soil. Therefore, no additive effect was observed. Resistant tomato suppressed nematode reproduction and yield losses of tomato as well as yield losses of the following cucumber crop, but nematode populations increased at the end of the crop. *P. lilacinum* is found naturally worldwide (Stirling, 2014), and it has been isolated from RKNs in Spain (Giné et al., 2013). Pl251 was isolated from the Philippines (Timm, 1987), and, despite its effectiveness in parasitizing eggs *in vitro*, it is well known that native isolates can be more suitable and can provide better control than foreign isolates in field conditions (Sorribas et al., 2003). More studies are needed to optimise BioAct WG usage and to improve knowledge on optimal environmental conditions to improve its effectiveness.

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General discussion

General discussion

The present thesis focuses on the study of the epidemiology of *Meloidogyne* spp. on tomato and cucumber crops, and on the effect of biologically-based management strategies such as plant resistance, crop rotation, and biological control, in order to suppress nematode population growth rates and crop yield losses.

Several abiotic factors can influence the rate of development of *Meloidogyne* and that of completion of its life cycle, soil temperature being the most important one in irrigated crops. This also influences the nematode population growth rate along with the plant host status. That is, how the nematode-plant interaction affects nematode population growth and plant productivity. Knowledge of the thermal requirements along with the parameters of the population dynamics and the yield losses caused by the nematode will be helpful to design nematode management strategies. In this thesis, thermal requirements of *M. incognita* and *M. javanica* for cucumber, zucchini-squash and watermelon were determined and phenology models were constructed (Chapter 1). Alongside this, the population dynamics of *M. incognita* on grafted and ungrafted cucumber and on susceptible and resistant tomato cultivars cultivated in a plastic greenhouse were determined, as well as, the damage function models (Chapter 2, 3 and 4). Finally, different biologically-based management tactics were evaluated. First, indigenous fungal egg parasites of root-knot nematodes (RKN) were isolated and identified from commercial fields conducted under integrated and organic standards in the north-east of Spain (Chapter 5). After that, two of the organic fields in which fungal egg parasites were detected in the previous survey (Chapter 5), were selected to determine if they were suppressive to RKN (Chapter 6). As a final point, the efficacy of *Purpureocillium lilacinum* strain 251, the only biological nematicide included in annex 1 of the European register, was assessed in combination

with plant resistance in a tomato-cucumber rotation in a plastic greenhouse (Chapter 7).

In the next sections, the main results and conclusions of the different studies will be discussed.

Epidemiology of *Meloidogyne* spp.

Soil temperature is considered the most important abiotic factor affecting the development, and consequently, the epidemiology of *Meloidogyne*. The thermal requirements of *M. incognita* and *M. javanica* were established for cucumber cv**.** Dasher II (Giné et al., 2014), watermelon cv. Sugar Baby (López-Gómez et al., 2014) and zucchini cv. Amalthee HF1 (Vela et al., 2014) (Chapter 1). Thermal requirements of *Meloidogyne* spp. have been intensively studied on tomato (Madulu and Trudgill, 1994; Trudgill, 1995; Ploeg and Maris 1999; Maleita et al., 2012) but information on cucurbits is scarce. In this thesis, thermal requirements were determined splitting the life cycle in different epidemiological processes: from inoculation to females starting laying eggs (FLE) (called development), from egg production and emergence of J2 (EH: from FLE to starting egg hatching) and for the life cycle completion (LCC). Both *Meloidogyne* species had similar thermal requirements on cucumber and zucchini-squash at each of the biological stages (FLE: *Tb* =12.1°C; *S* =285.7DD, EH: *Tb* = 8.3 °C; *S* = 212.8DD and LCC: *Tb* = 11.5 °C; *S* = 500DD), supporting the results found by López-Gómez et al., (2014) who reported similar rates of root penetration of both *Meloidogyne* species on both plant species. However, in watermelon all the *Meloidogyne* species had the slowest rate of development and the highest base temperature to complete their life cycle. These results also agree with those of López-Gómez et al., (2014a) who reported a delay of the development on watermelon compared with cucumber and zucchini-squash. Cross-referencing its thermal requirements on cucumber, which we established in this thesis, with the soil temperatures registered throughout the cycle of

the cucumber crops, we were able to estimate the number of generations of *M. incognita* per crop cycle (Chapter 2, 4 and 7). To the same end, the thermal requirements of *Meloidogyne* spp. were used to determine the number of generation of *Meloidogyne* on watermelon (López-Gómez et al., 2014b; 2016), as well as, on zucchini-squash (Vela et al., 2014; López-Gómez et al., 2015) in pots and on field under protected cultivation.

The number of generations that RKN can complete during the cropping period will depend on the soil temperatures rather than on the length of the crop cycle. For example, on the same number of days *M. incognita* completed two generations during the spring crop but only one in the autumn (Vela et al., 2014). In the agronomic conditions of the Mediterranean area, the three cucurbits evaluated are planted in spring-summer and/or summer-autumn periods when soil temperatures range from 15 to 33 °C (Chapters 2, 4, 6 and 7) (Vela et al., 2014, López-Gómez et al., 2016). These temperatures are appropriate for the development of the two most common *Meloidogyne* species in this area. In fact, it is know that *Meloidogyne* develops between 10 and 35 °C (Evans and Perry, 2009).

An unsolved issue in RKN management is the prediction of the population growth rate and the crop yield losses related to population densities at planting (*Pi*), under local agronomic conditions. In a plastic greenhouse experiments located in Viladecans (Spain), the population dynamics of *M. incognita* were assessed in different plant species and cultivars in order to determinate their host status (Seinhorst, 1970). Also, the plant tolerance and the minimum relative yield were estimated by the relationship between *Pi* and the relative crop yield using the Seinhorst damage function model (Seinhorst, 1965). Seinhorst (1967) stated that agro-environmental factors, such as the cropping period, can affect the population dynamics of *Meloidogyne* spp. on a given plant species. The population growth rate of *M. incognita*

on cucumber cv. Dasher II cultivated in summer-autumn was higher than when cultivated in spring-summer but this had no effect on the tolerance limit (< 1 to 7 J2 250 cm^{-3} soil) or the maximum cucumber yield losses (89%) (Chapter 2 and 4). Similar results of population dynamics were reported for *M. incognita* on zucchini cv. Amalthee HF1 in which population growth rate was higher in the autumn-winter than in the winter-spring, but with slight effects on plant tolerance and crop yield (Vela et al., 2014).

The study of population dynamics of *M. incognita* on resistant and susceptible tomato cultivars and crop yield provided some important information (Chapter 3). It is known that resistant tomato cultivars suppress nematode reproduction, disease severity and, in addition, prevent crop yield losses compared to susceptible ones. The maximum multiplication rates, the ceiling levels, and the equilibrium densities on the resistant cultivar were lower than on the susceptible one, and yielded more, as previously stated (Sorribas et al., 2005; Talavera et al., 2009), but no differences were found regarding the tolerance limit, in agreement with the results of Di Vito et al. (1991). The suppression of the build-up of the RKN population densities by the resistant cultivar will reduce yield losses on the following crop by the reduction of *Pi* (Seinhorts, 1970) as discussed in chapter 7, in which disease severity and yield losses of cucumber cv. Dasher II were lower when it was preceded by the resistant tomato cv. Monika. In other studies, cucumber crop yielded 60% more when cultivated after resistant tomato cultivars than when it followed a susceptible one (Ornat et al., 1997). Similar findings were reported for melon (Rich and Olson, 2004) and cucumber and zucchini (Thies et al., 2004). However, the repeated cultivation of resistant cultivars may select virulent populations (Chapter 3). In this thesis, the comparison of population dynamics parameters of *M. incognita* on the resistant tomato cv. Monika and the susceptible cv. Durinta over three cropping seasons (2010-2011-2012) were used to estimate the extent to which this is true. In the resistant cultivar, the maximum

reproduction rates, ceiling levels and equilibrium densities, as well as population growth rates increased every year, suggesting a selection of more virulent populations. Thus, data from population dynamics studies could be used to determine the early selection of virulent populations. It is worth noting that selection for virulent populations appears to be lower when the resistant tomato is cropped every three years, but the selection pressure increases when it is cropped more often. Some reports have suggested that reproductive fitness of virulent individuals in the population is reduced on susceptible crops (Castagnone-Sereno et al., 2007). If this was the case, from an agronomical point of view the extra damage caused by virulent individuals could be brought close to zero. That is, no damage, no yield reduction and low levels of galled roots. Otherwise, the selection for virulence would be widespread in most of the vegetable growing areas in which tomato is usually cultivated, while this is not the case at present. A plausible explanation could be the use of control methods against soil-borne diseases including those caused by plant parasitic nematodes. These methods may be bringing the population densities at or near undetectable levels. It is known that the virulence is genetically stable and is transmitted to the progeny (Castagnone-Sereno et al., 1993). Therefore, a rotation sequence with other available resistant cultivars carrying other resistant gens, such as pepper, can suppress this selection for virulence (Castagnone-Sereno et al., 1996; Djian-Caporalino et al., 2011).

The effect of grafting cucumber onto RS841 against *M. incognita* was evaluated. Grafting vegetables is an old practice and is widely used to overcome abiotic stresses and pests. The results in Chapter 4 showed that the squash hybrid RS841 (*Cucurbita maxima* x *C. moschata*), performs better as a host than cucumber cv. Dasher II, leading to higher *Pi* for the following crop in the rotation, which compromises the development and yields of the following crop. The susceptibility of the squash hybrid RS841 was also reported by López-Gómez et al. (2016) who observed a significant increase of the nematode densities at the end of a cycle of watermelon cv. Sugar Baby

grafted onto RS841 compared with the ungrafted one, which proved to be a poor host. Regarding cucumber crop yields, no differences on the minimum relative yield and the tolerance limit were found. Thus, this rootstock is not an alternative control method to *M. incognita* and provides no additional tolerance.

The damage caused by RKN reduces the ability of the roots to intake water and nutrients, which weakens the plant. It was assumed that, seen as greater *Pi* results in greater yield losses, ecophysiological parameters would also be affected by that. In chapter 4, predawn water potential, the chlorophyll index and the net photosynthetic rate in cucumber, grafted and ungrafted, was found to be lower at higher *Pi*. This results are in line with previous studies that established that nematodes can cause a reduction in leaf chlorophyll content (Audebert et al., 2000; Ahmed et al., 2009; Khan and Haque, 2011; Giné et al., 2014; López-Gómez et al., 2015) as well as in the photosynthetic activity (Loveys and Bird 1973; Melakeberhan et al., 1985). Thus, some ecophysiological parameters could be early indicators to predict crop yield losses, but more studies are needed to validate this theory, because other factors can contribute to cause stress on plants.

Biological control of *Meloidogyne* spp.

The present thesis has studied different approaches to the biological control of RKN, from the natural biological control to the application of a commercial biological nematicides. Regarding natural biological control, a survey was carried out to determine the occurrence of fungal egg parasites of RKN, the levels of egg parasitism in field conditions and the link between that and the physicochemical and enzymatic soil properties. Numerous antagonist of *Meloidogyne* spp. have been detected in Spain (Sorribas and Ornat, 2005) and the list has been enlarged with the results of this thesis (Chapter 5). In our case of study, in which 40 sites were sampled (10 under organic and 30 under integrated production systems), fungal egg parasites were isolated from soil

from all the organic sites and from 73% of the sites under integrated management. The percentage of fungal egg parasitism was higher (36.2%) in organic than in integrated (9.2%) production. Also, egg parasitism was greater than 50%, in 40% of the organic plots and 3% of the integrated sites. *Pochonia chlamydosporia*, *Fusarium* species, and *Plectosphaerella cucumerina* were the most common fungal species, *P. chlamydosporia* being the most common parasite, and the only one related to percent of egg parasitism. In 62.5% of the fields that we studied, more than one species was detected, although in most cases, one species was predominant, in line with Verdejo-Lucas et al., (2002 and 2013). Thus, fungal egg parasites in soil were found to be most common in the organic production fields. In fields where intensive agriculture is carried out, where practices such as the use of fumigant or non-fumigant nematicides and inorganic fertilizers are widespread, fungal egg parasites were less frequent and/or the percentage of egg parasitism was lower (Verdejo-Lucas et al., 2002 and 2013). In organic managed sites, no chemical nematicides are used and soil is organically amended. Similarly, cover crops are incorporated and a greater variety of crops are included in the rotation (Chapter 6). In these sites, the enzymatic activity in the soil, linked to microbial biomass, was greater than in integrated managed sites (Chapter 5). A lot has been said about how crop management can improve the soil's physicochemical proprieties (Magdoff and Weill, 2004), enhance its suppressiveness (Stirling, 2011) and preserve and increase the diversity of microorganisms (Chapter 5 and 6), as it was evidenced by the high amount of soil bacteria and fungi detected by DGGE profiles in two sites managed under organic production compared to a site managed under integrated production (Chapter 6).

The proportion of parasitized eggs in several organic production sites suggested that some of those soils could be suppressive to *Meloidogyne*. Then, two organic production sites were selected to assess their suppressiveness against RKN (Chapter 6). The natural suppressiveness of both soils was corroborated in pot

experiments, and during two cropping seasons during which the fluctuation on nematode density was assessed along with the percentage of fungal egg parasitism. In one of these sites, the RKN population density decreased to undetectable levels, but the percentage of fungal egg parasitism due to *P. chlamydosporia,* the only fungal egg parasite that was isolated, was not high enough to explain this phenomenon in plastic greenhouse cultivation. The results in pot test were in line with those of the field study. Thus, other microorganisms could be involved in the soil suppressiveness to RKN, as it was found by the DGGE analysis, in which several bacterial and fungal species were previously reported as active nematode antagonists by different modes of action. In the other site, RKN were detected in field at the end of each crop with high percentage of fungal egg parasitism due to the only fungal egg parasite isolated, *P. chlamydosporia.* In pot test, the lower nematode reproduction and the high egg parasitism by *P. chlamydosporia* suggested that *P. chlamydosporia* was the only responsible for the soil's supressiveness, in spite of the several microorganisms detected in DGGE. Similar results were reported by Borneman and Becker (2007) who reported *Dactylella oviparasitica* as the fungal species responsible for suppressiveness of *Heterodera shachtii* despite the presence of other fungal species. The results of Chapter 6 represent the first report of suppressive soils against *Meloidogyne* spp. in commercial vegetable growing sites in Spain. It covers the two main types of soil suppressiveness: general suppressiveness due to a variety of microorganisms, and specific suppressiveness due to a single species of microorganism. Both suppressive soils were conducted under organic production in which the agronomic practices could enhance and/or maintain the existing antagonistic potential of the soil.

The last approach in biological control was the introduction of a mass-produced antagonism (Chapter 7). The experiments were carried out in a plastic greenhouse over two seasons in which the biological-based nematicide BioAct WG (*Purpureocillium lilacinum* strain 251) was applied on a tomato (resistant - susceptible)

- cucumber sequence to assess the effect on nematode reproduction and disease severity. In addition, the plastic greenhouse experiment was complemented with *in vitro* experiments to assess the effectiveness of *P. lilacinum* strain 251 to parasitize RKN eggs and J2, the production of enzymes able to degrade the egg layer, the effect of temperatures and available water on mycelia growth, and soil receptivity. *Purpureocillium lilacinum* strain 251 was able to parasitize high proportion of eggs (94.5%) but not J2, and protease, lipase, and chitinase activities were detected by API-ZYM. So, it was expected that the biological control agent could maintain the levels of nematode population below the damage threshold. However, the effectiveness in plastic greenhouse conditions of BioAct WG was quite low. The application of BioAct WG four times per cropping season did not provide any RKN control neither improved yields, along the lines of the finding of Anastasiadis et al., (2008) and Kaskavalci et al., (2009). In our study, while soil temperature and soil water content were in the range of fungal development, only during half of the cropping period were soil temperatures in the optimum range for fungal growth. In addition, some non-thermo-sensitive chemicals in soil could affect fungal development as showed by the soil receptivity test. This inconsistent relationship between results from experiments conducted in controlled conditions and those undertaken in the field had been identified before by Kerry and Evans (1996). One disadvantage of the introduction of foreign biological control agents is that, once an organism is applied, it is subjected to rigorous and different agro-environments which could affect its effectiveness (Sirtling, 2011). Previous research has evidenced that foreign fungal isolates to control nematodes are not as effective as indigenous ones (Sorribas et al., 2005) due to their lower adaptation to local conditions (Alabouvette et al., 2006). More information is needed in areas such as physicochemical and biochemical soil properties, soil microbiome, and agronomical practices related to environmental conditions to improve the effectiveness of biological control. This information can help make decisions to enhance natural antagonism or to make soil receptive to foreign antagonists. The growing area under

sustainable production systems with friendly control practices against nematodes can lead to improved soil conditions and therefore increase the level of suppressiveness against *Meloidogyne*. Further studies may consider new strategies to improve the soil's natural suppressiveness and to stimulate the naturally occurring antagonisms.

Conclusions

Conclusions

I. The thermal requirements for development, egg production, and hatching, and life cycle completion of *Meloidogyne incognita* and *M. javanica* on cucumber, watermelon and zucchini-squash have been determined, and phenology models have been constructed. A single phenology model per biological process is enough for both *Meloidogyne* species on cucumber and zucchini-squash, but not on watermelon, on which the thermal requirements of both species differed. These models have to be validated in field conditions before they can be used as a predictive tool for epidemiological studies and management purposes.

II. The parameters which define the population dynamics of *M. incognita* on cucumber have been determined for the two main cropping periods. The population growth rate of *M. incognita* on cucumber cropped in spring-summer is lower than when it is cropped in summer-autumn, but there is no effect on plant tolerance because both the tolerance limit and the relative cucumber yield do not differ between cropping periods.

III. The cucurbit hybrid rootstock RS841 is neither resistant against *M. incognita* nor any more tolerant to the nematode than cucumber cv. Dasher II, since the population growth rate of *M. incognita* is higher on RS841 than on cucumber but suffers the same amount of yield losses.

IV. Resistant tomato cultivars suppress *M. incognita* reproduction rate, disease severity and crop yield losses compared to susceptible cultivars. However, the prevalence of virulent nematodes in the populations increases with the number of years of cultivation of resistant tomatoes. It is worth noting that, however, after three consecutive crops of the resistant tomato cv. Monika the population did not completely acquire the virulent condition. For the first time, the parameters which define the population dynamics of plant parasitic

nematodes have been used for early detection of selection of virulent nematodes.

V. Several fungal egg parasites of *Meloidogyne* spp. are present in commercial vegetable growing areas under organic and integrated production systems in the north-east of Spain and, in some cases, a high percentage of egg parasitism occurs. Among fungal egg parasites, *Pochonia chlamydosporia* is the most frequent and its relative frequency is positively related to the percentage of fungal egg parasitism.

VI. Suppressive soils to *Meloidogyne* spp. have been identified and characterized for the first time in Spain. Various causes of soil supressiveness have been identified. In one soil, the cause has been attributed to a complex of soil microorganisms with a different mode of action against the nematode, and in the other, the most plausible responsible is the fungal egg parasite *P. chlamydosporia.*

VII. *Purpureocillium lilacinum* strain 251, in *in vitro* conditions, parasitize a high proportion of *Meloidogyne* eggs and produces proteolytic, lipolytic and chitinolytic enzymes able to degrade the nematode eggshell. Also, the mycelium develops at temperatures and water potentials which are in the range that usually occurs in the soil during the cropping cycle. However, the biological based nematicide BioAct WG (*Purpureocillium lilacinum* strain 251) is not effective to control *M. incognita* on a tomato (resistant or susceptible) and cucumber rotation in a plastic greenhouse. Several factors could affect its effectiveness such as large periods with suboptimal soil temperatures, inhibition of enzymatic activity due to components of the formulation, or existing physicochemical and/or biological properties of the soil not conductive for the establishment and the development of the fungus. More studies are needed to understand the lower performance of this product in field conditions.

References

General introduction

General discussion

References: general introduction and general discussion

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List of tables and figures

List of tables

General Introduction

Chapter 1

Chapter 2

Chapter 3

yield of the cucumber cv. Dasher II alone or combined with the application of BioAct WG cultivated after resistant tomato cv. Monika (TR) or susceptible cv. Durinta (TS) from 31st July to 5th November of 2012.

List of figures

General Introduction

Chapter 1

Chapter 2

Chapter 3

Chapter 4

List of publications

List of publications related to this thesis

Publications included in SCR

Giné, A., Sorribas, F. J. 2016. Population dynamics of *Meloidogyne incognita* and yield losses of susceptible and resistant tomato cultivars in Mediterranean conditions**.** *Plant Pathology* Summited

Giné, A., González, C., Serrano, L., Sorribas, F. J. 2016. Population dynamics of *Meloidogyne incognita* on cucumber grafted onto the Cucurbita hybrid RS841 or ungrafted and yield losses under protected cultivation. Accepted. *European Journal of Plant Pathology.* Summited

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