Biological control approaches of *Meloidogyne* spp. in vegetable crops: from application of selected antagonists to suppressive soils

Zahra Ghahremani

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Zahra Ghahremani

Universitat Politècnica de Catalunya
Departament d'Enginyeria Agroalimentària i Biotecnologia
Programa de Doctorat Tecnologia Agroalimentària i Biotecnologia

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PhD dissertation:

Zahra Ghahremani

PhD Supervisor: **Dr. Francesc Xavier Sorribas Royo**

PhD Co-Supervisor: **Dr. Nuria Escudero Benito**

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'The secret of success is constancy to purpose'.

-Benjamin Disraeil

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Resum

Els nematodes formadors de gal·les, *Meloidogyne* spp., és el gènere més important nematodes fitoparàsits que causen danys considerables i generen pèrdues econòmiques en cultius hortícoles arreu del món. Les estratègies actuals de gestió de *Meloidogyne* solen reduir l'ús dels nematicides químics fomentant mètodes de control alternatius com l'ús de plantes amb gens de resistència (gens *R*) i/o l'ús de la resistència vegetal induïda per microorganismes, i el potencial antagonista dels sòls. En la present tesis, dos aproximacions al control biològic de *Meloidogyne* spp. van ser estudiades: 1) l'aplicació d'antagonistes dels nematodes: el fong *Pochonia chlamydosporia* i el bacteri *Bacillus firmus* aïllat I-1582 i es a avaluar la seva capacitat per induir resistència vegetal, i 2) el nivell de supressivitat de sòls de producció vegetal orgànica o integrada.

Respecte a la capacitat de *P. chlamydosporia* i *B. firmus* I-1582 (Bf I-1582) a induir resistència vegetal, els resultats d'aquesta tesis van donar evidències que dos de cinc aïllats de *P. chlamydosporia* (M10.43.21 i M10.55.6) i Bf I-1582 induien resistència sistèmica enfront *M. incognita* en tomàquet susceptible (*Solanum lycopersicum*) cv. Durinta però no en cogombre (*Cucumis sativus*) cv. Dasher II en experiments "split-root". A més, les temperatures cardinals de creixement i de formació de biofilm de Bf I-1582 van ser determinades per tal de millorar el seu ús en condicions de camp. A més, el bacteri va ser transformat amb GFP per estudiar el seu efecte sobre els ous del nematode i la seva colonització sobre les arrels de tomàquet i cogombre per microscopia de rastreig laser confocal. En tomàquet, tant el nombre de masses d'ou com el nombre d'ous per planta es va veure reduït quan s'aplicaven tant els aïllats fúngics com el bacteri. Els aïllats de *P. chlamydosporia* colonitzaven les arrels de tomàquet i cogombre, però diferien en el nivell de colonització. L'aïllat M10.43.21 va ser el millor colonitzador de les arrels de tomàquet mentre que l'aïllat M10.55.6 ho va ser per cogombre. En el cas de Bf I-1582, el bacteri va ser capaç de colonitzar endofíticament les arrels de les dues plantes, però es va trobar un 61% més de densitat d'ADN de bacteri en arrels de tomàquet. La regulació dinàmica dels gens relacionats amb l'àcid jasmònic (JA) i l'àcid salicílic (SA) a tres temps diferents van ser avaluats: 7 dies després de la inoculació de l'antagonista i just després de la inoculació del nematode (0 dani), 7 dies després de la inoculació del nematode (7 dani) i 40 dies després de la inoculació del nematode (40 dani). Les dues vies SA (gen *PR-1*) i JA (gen *Lox D*) van ser sobre-expressades plantes de tomàquet a 0 dani, reduint el nombre de masses d'ou al final de l'experiment "split-root" quan es va inocular amb Bf I-1582. No obstant, no hi va haver diferencies en l'expressió dels gens relacionats SA (*PR 1*) i JA (*Lox D*) en cogombre inoculat amb el bacteri com tampoc en el nombre de masses d'ou produïdes en les arrels de cogombre. A 7 dani, el gen relacionat amb el JA (*Lox D*) estava sobre-expressat en tomàquet i podria afectar el desenvolupament del nematode i la seva reproducció. En cogombre, la via del SA (*Pal I*) estava sobre-expressada tant en les plantes inoculades amb *M. incognita* com en les co-inoculades amb el bacteri i el nematode. A 40 dani, quan va començar l'eclosió dels ous i es van produir noves infeccions a l'arrel, les plantes de tomàquet co-inoculades amb els nematode

i Bf I-1582 tenia reprimit el gen relacionat amb el JA (*Lox D*), mentre que el gen relacionat amb la via del SA (*PR 1*) estava sobre-expressat en plantes co-inoculades i també amb només Bf I-1582, però va ser reprimit en plantes inoculades només amb el nematode. En cogombre, les dues vies, JA i SA, van ser reprimides en plantes inoculades amb *M. incognita* però només la JA en plantes co-inoculades. Respecte l'aïllat de *P. chlamydosporia* M10.43.21, va induir l'expressió de la via del SA en arrels de tomàquet a 0, 7 i 42 dani. La via del JA va ser també sobre-expressada a 7 dani. Per tant, alguns aïllats de *P. chlamydosporia* i l'aïllat Bf I-1582 poden induir resistència sistèmica envers al nematode, encara que depèn de l'espècie vegetal. Aquests resultats han demostrat el model similar de regulació dinàmica d'aquestes vies d'hormones vegetals relacionades amb mecanismes de defensa de les plantes contra el nematode.

El bacteri Bf I-1582 va créixer en el rang de temperatures des de 15 ºC a 45 ºC, sent 35 ºC la temperatura òptima de creixement tant en medi sòlid com en líquid, però no a 10 ˚C i 50 ˚C. Igualment, es va observar la formació de biofilm entre 15 ºC i 45 ºC però tampoc a 10 ºC ni a 50 ºC, sent més gruixut i uniforme a 35 ºC. La degradació de la closca del nematode i la colonització dels ous per Bf I-1582 GFP va mostrar que a 3 dies després de la seva inoculació (dai) el bacteri estava envoltant i degradant l'ou del nematode; a 5 dai, colònies de bacteri es van adherir a la closca de l'ou i es van trobar alguns bacteris dins de l'ou; a 10 dai, el bacteri era completament adherit a la closca de l'ou i dins de l'ou. A més, Bf I-1582GFP va colonitzar les pèls radiculars i cèl·lules epidèrmiques a 5 dai; es van observar colònies de bacteris en pèls radiculars de tomàquet i alguns bacteris dins de l'arrel a 10 dai. En cogombre, es van observar pocs bacteris a les cèl·lules epidèrmiques a 5 dai i no es va trobar el bacteri dins de l'arrel a 10 dai.

En relació al nivell de supressivitat del sòl, es va realitzar un estudi a sis parcel·les de producció d'hortalisses localitzades al nord-est d'Espanya. Quatre realitzaven producció orgànica (M10. 16, M10.41, M10.55, i M10.56) i dues (M10.43 i M10.45) producció integrada. La fluctuació de la densitat de població de *Meloidogyne* i el parasitisme d'ous per part de fongs van ser determinats durant la seqüència de rotació de cultius durant dos anys (2015-2016). Cinc dels sols estudiats eren sòls supressius a *Meloidogyne* spp. El percentatge de parasitisme d'ous va variar de 11.2 a 55 % i *P. chlamydosporia* va ser l'única espècie fúngica aïllada dels ous. En paral·lel, dos experiments es van dur a terme utilitzant sòl de cada parcel·la. Una part de cada sòl es va esterilitzar i es va barrejar amb sorra estèril, i una altre part no es va esterilitzar i es va barrejar també amb sorra estèril amb una relació 1:1 i es va col·locar en testos de 3-l. El cultivar susceptible de tomàquet Durinta es va trasplantar en cada test i es va inocular amb juvenils de segon estadi (J2) amb un nivell de 1 J2 cm^{-3} de sòl. En els dos experiments en testos, els nombre d'ous per planta es va reduir (P<0.05) en tots els sòls no esterilitzats comparats amb els estèrils, excepte en el M10.45. També, *P. chlamydosporia* va la única espècie fúngica aïllada d'ous parasitats de nematodes. *P. chlamydosporia* és el fong més freqüent i més prevalent amb una alta plasticitat capaç d'adaptar-se a les pràctiques agronòmiques en un sistema de producció vegetal molt pertorbat.

Paraules claus: *Meloidogyne* spp., control biològic, resistència sistèmica, *Pochonia chlamydosporia*, *Bacillus firmus*, tomàquet cv. *Durinta*, cogombre cv. *Dasher II*, sòls supressius.

Resumen

Los nematodos formadores de agallas, *Meloidogyne* spp., es el género de nematodos fitoparásitos más importante económicamente que causa daños considerables y genera pérdidas en cultivos hortícolas en todo el mundo. Las estrategias actuales de gestión de *Meloidogyne* suelen reducir el uso de los nematicidas químicos fomentando métodos de control alternativos como el uso de plantas con genes de resistencia (genes R) y / o el uso de la resistencia vegetal inducida por microorganismos, y el potencial antagonista de los suelos. En la presente tesis, dos aproximaciones al control biológico de *Meloidogyne* spp. fueron estudiadas: 1) la aplicación de antagonistas del nematodo: el hongo *Pochonia chlamydosporia* y la bacteria *Bacillus firmus* aislado I-1582 y evaluar su capacidad para inducir la resistencia vegetal, y 2) el nivel de supresividad de suelos de producción vegetal orgánica y integrada.

Respecto a la capacidad de *P. chlamydosporia* y *B. firmus* I-1582 (Bf I-1582) a inducir resistencia vegetal, los resultados de esta tesis dan evidencias de que dos de cinco aislados de *P. chlamydosporia* (M10.43.21 y M10. 55.6) y Bf I-1582 inducen resistencia sistémica frente *M. incognita* en tomate susceptible (*Solanum lycopersicum*) cv. Durinta pero no en pepino (*Cucumis sativus*) cv. Dasher II en experimentos "split-root". Además, las temperaturas cardinales crecimiento y de formación de biofilm de Bf I-1.582 fueron determinadas para mejorar su uso en condiciones de campo. Además, la bacteria fue transformada con GFP para estudiar su efecto sobre los huevos del nematodo y su colonización sobre las raíces de tomate y pepino por microscopía de barrido láser confocal. En tomate, tanto el número de masas de huevo como el número de huevos por planta se vio reducido cuando se aplicaban tanto los aislados fúngicos como la bacteria. Los aislados de *P. chlamydosporia* colonizaban las raíces de tomate y pepino, pero diferían en el nivel de colonización. El aislado M10.43.21 fue el mejor colonizador de las raíces de tomate mientras que el aislado M10.55.6 lo fue para pepino. En el caso de Bf I-1 582, la bacteria fue capaz de colonizar endofíticamente las raíces de las dos plantas, pero se encontró un 61% más de densidad de ADN de bacteria en raíces de tomate. La regulación dinámica de los genes relacionados con el ácido jasmónico (JA) y el ácido salicílico (SA) a tres tiempos diferentes fueron evaluados: 7 días después de la inoculación del antagonista y justo después de la inoculación del nematodo (0 dani), 7 días después de la inoculación del nematodo (7 dani) y 40 días después de la inoculación del nematodo (40 dani). Las dos vías SA (gen *PR-1*) y JA (gen *Lox D*) fueron sobre-expresadas en plantas de tomate en a 0 dani, reduciendo el número de masas de huevo al final del experimento "split-root" cuando se inoculó con Bf I-1.582. Sin embargo, no hay diferencias en la expresión de los genes relacionados SA (*PR 1*) y JA (*Lox D*) en pepino inoculado con la bacteria como tampoco en el número de masas de huevo producidas en las raíces de pepino. A 7 dani, el gen relacionado con el JA (*Lox D*) estaba sobre-expresado en tomate y podría afectar al desarrollo del nematodo y su reproducción. En pepino, la vía del SA (*Pal I*) estaba sobre-expresada tanto en las plantas inoculadas con *M. incognita* como en las co-inoculadas con la bacteria y el nematodo. A 40 dani, cuando comenzó la eclosión de los huevos y se produjeron nuevas infecciones en la raíz, las plantas

de tomate co-inoculadas con los nematodos y Bf I-1582 tenían reprimido el gen relacionado con el JA (*Lox D*), mientras que el gen relacionado con la vía del SA (*PR 1*) estaba sobre-expresado en plantas co-inoculadas y también con sólo Bf I-1582, pero fue reprimido en plantas inoculadas sólo con el nematodo. En pepino, las dos vías, JA y SA, fueron reprimidas en plantas inoculadas con *M. incognita* pero sólo la JA en plantas co-inoculadas. Respecto el aislado de *P. chlamydosporia* M10.43.21, indujo la expresión de la vía del SA en raíces de tomate a 0, 7 y 42 dani. La vía del JA fue también sobre-expresada a 7 dani. Por lo tanto, algunos aislados de *P. chlamydosporia* y el aislado Bf I-1582 pueden inducir resistencia sistémica al nematodo, pero depende de la especie vegetal. Estos resultados han demostrado el modelo similar de regulación dinámica de estas vías de hormonas vegetales relacionadas con mecanismos de defensa de las plantas contra el nematodo.

La bacteria Bf I-1582 creció en el rango de temperaturas desde 15 ºC a 45 ºC, siendo 35 ºC la temperatura óptima de crecimiento tanto en medio sólido como en líquido, pero no a 10 ºC y 50 ºC. Igualmente, se observó la formación de biofilm entre 15 ºC y 45 ºC pero tampoco a 10 ºC ni a 50 ºC, siendo más grueso y uniforme a 35 ºC. La degradación de la cáscara del nematodo y la colonización de los huevos para Bf I-1582 GFP mostró que a 3 días después de su inoculación (dai) la bacteria envolvía y degradaba el huevo del nematodo; a 5 dai, colonias de bacteria se adhirieron a la cáscara del huevo y se encontraron algunas bacterias dentro del huevo; a 10 dai, la bacteria se encontraba completamente adherida a la cáscara del huevo y dentro del huevo. Además, Bf I-1582GFP colonizó los pelos radiculares y las células epidérmicas a 5 dai; se observaron colonias de bacterias en pelos radiculares de tomate y algunas bacterias dentro de la raíz a 10 dai. En pepino, se observaron pocas bacterias a las células epidérmicas a 5 dai y no se encontró la bacteria dentro de la raíz a 10 dai.

En relación al nivel de supressividad del suelo, se realizó un estudio en seis parcelas de producción hortícola localizadas en el noreste de España. Cuatro realizaban producción orgánica (M10.16, M10.41, M10.55, y M10.56) y dos (M10.43 y M10.45) producción integrada. La fluctuación de la densidad de población de *Meloidogyne* y el parasitismo de huevos por parte de hongos fueron determinados durante la secuencia de rotación de cultivos durante dos años (2015-2016). Cinco de los suelos estudiados eran suelos supresivos a *Meloidogyne* spp. El porcentaje de parasitismo de huevos varió de 11.2 a 55% y *P. chlamydospori*a fue la única especie fúngica aislada de los huevos. En paralelo, dos experimentos se llevaron a cabo utilizando suelo de cada parcela. Una parte de cada suelo se esterilizó y se mezcló con arena estéril, y otra parte no se esterilizó y se mezcló también con arena estéril con una relación 1: 1 y se colocaron en macetas de 3-l. El cultivar susceptible de tomate Durinta se trasplantó en cada maceta y se inoculó con juveniles de segundo estadio (J2) con un nivel de 1 J2 cm^{-3} de suelo. En los dos experimentos en macetas, el número de huevos por planta se redujo ($P < 0.05$) en todos los suelos no esterilizados comparados con los estériles, excepto en el M10.45. También, *P. chlamydosporia* fue la única especie fúngica aislada de huevos parasitados de nematodos. *P. chlamydosporia* es el hongo más frecuente y más prevalente con una alta

plasticidad capaz de adaptarse a las prácticas agronómicas en un sistema de producción vegetal muy perturbado.

Palabras claves: *Meloidogyne* spp., Control biológico, resistencia sistémica, *Pochonia chlamydosporia*, *Bacillus firmus*, tomate cv. Durinta, pepino cv. Dasher II, suelos supresivos.

Abstract

Root-knot nematodes (RKN), *Meloidogyne* spp., are the most economically important genus of plant parasitic nematodes that cause considerable damage and yield loss of horticultural crops worldwide. RKN management strategies tend to reduce chemical nematicides in agriculture by encouraging alternative control methods like the use of plants bearing resistance genes (*R*-genes) and/or by microbe-inducing plant resistance, and the antagonistic potential of soils. In the thesis, two biological control approaches of *Meloidogyne* spp. were evaluated: 1) the application of selected nematode antagonists, the fungus *Pochonia chlamydosporia* and the bacteria *Bacillus firmus* I-1582, to know its ability to induce plant resistance, and 2) the level of soil suppressiveness of vegetable production sites conducted under organic or integrated standards.

Regarding the ability of *P. chlamydosporia* and the bacteria *Bacillus firmus* I-1582 (Bf I-1582) to induce plant resistance, the results of this thesis provide evidence that two out of five *P. chlamydosporia* isolates (M10.43.21 and M10.55.6) and Bf I-1582 to induce systemically resistance against *M. incognita* in the susceptible tomato (*Solanum lycopersicum*) cv. Durinta but not in the cucumber (*Cucumis sativus*) cv. Dasher II in split-root experiments. In addition, the cardinal temperatures for the Bf I-1582 growth and biofilm formation were determined in order to improve its use in field conditions. Moreover, the bacterial was transformed with GFP to study its effect on nematode eggs and root colonization of tomato and cucumber by laser-scanning confocal microscopy. In tomato, both number of egg masses and number of eggs per plant were reduced by both fungal isolates and by the bacteria. *P. chlamydosporia* isolates colonized both tomato and cucumber roots, but they differed in the level of root colonization. Isolate M10.43.21 was the best tomato root colonizer whilst isolate M10.55.6 was in cucumber. In the case of *B. firmus* I-1582, the bacteria colonized endophytically roots of both plants, but ca. 61% more density of bacterial DNA was recorded in tomato roots. The dynamic regulation of genes related to jasmonic acid (JA) and salicylic acid (SA) at three different times: 7 days after nematode antagoniust inoculation and just after nematode inoculation (0 dani), 7 days after nematode inoculation (7 dani) and 40 days after nematode inoculation (40 dani) were evaluated. Bf I-1582 primed tomato plants by both SA (*PR-1* gene) and JA (*Lox D* gene) at 0 dani, reducing the number of egg masses at the end of the split-root experiment. However, no differences in the expression of SA (*PR 1*) and JA (*Lox D*) related genes were found in cucumber plants inoculated with the bacteria as well in the number of egg masses produced in cucumber roots. At 7 dani, the JA (*Lox D*) related gene was up-regulated in tomato and could affect nematode development and reproduction. In cucumber, the SA pathway (*Pal I*) was up-regulated both in the *M. incognita* inoculated plants and those co-inoculated with the bacteria and the nematode in cucumber. At 40 dani, when egg hatching began and new root infections occurred, tomato plants co-inoculated with the nematode and Bf I-1582 had repressed the JA related gen (*Lox D*), while the gene related to the SA pathway (*PR 1*) was up-regulated in plants co-inoculated and also inoculated with Bf I-1582 alone, but was repressed in plants

inoculated only with the nematode. In cucumber, both JA and SA pathways were repressed in plants inoculated with *M. incognita* but only JA in the co-inoculated plants. Regarding *P. chlamydosporia* isolate M10.43.21, it induced the expression of the SA pathway in tomato roots at 0, 7 and 42 dani. The JA pathway was also upregulated at 7 dani. Thus, some isolates of *P. chlamydosporia* and Bf I-1582 can induce systemic resistance against root-knot nematodes but this is plant species dependent. These results have pointed out the similar model of dynamic regulation of these plant hormone pathways related to plant defense mechanisms against the nematode.

The bacteria grew in the range of temperatures from 15 ºC to 45 ºC, being 35 ºC the optimal growth temperature in both solid and liquid media, but did not at 10 ˚C or 50 ˚C. Similarly, biofilm formation was observed between 15 ºC and 45 ºC but neither at 10 ºC nor 50 ºC, being thicker and uniform at 35 ºC. The nematode egg shall degradation and egg colonization by Bf I-1582 GFP showed that at 3 days after inoculation (dai) the bacteria were surrounding and degrading the nematode egg; at 5 dai, bacteria colonies were adhered to the egg shell and some bacteria were found inside the egg; at 10 dai, adhered to the egg shell and inside the egg. In addition, Bf I-1582GFP was colonized on root hairs and epidermal cells at 5 dai; bacterial colonies were observed in tomato root hairs and some bacteria were found inside the root at 10 dai. In cucumber, few bacteria were observed on epidermal cells at 5 dai and were no bacteria found inside the root at 10 dai.

In relation to the level of soil suppressiveness of vegetable production sites it was carried out a study in six vegetables production sites located in northeastern Spain. Four were conducted under organic (M10. 16, M10.41, M10.55, and M10.56) and two (M10.43 and M10.45) under integrated production standards. The fluctuation both of *Meloidogyne* population density and fungal egg parasitism were determined during the rotation sequences in two years (2015-2016). Five of these sites were suppressive soils to *Meloidogyne* spp. The percentage of fungal egg parasitism ranged from 11.2 to 55 % and P. *chlamydosporia* was the only fungal species isolated from the eggs. In parallel, two experiments were carried out using soil from each site. A part of each soil was sterilized and sterilized and non-sterilized soils were mixed with sterile sand 1:1 (dry w: dry w) and placed in 3-l pots. The susceptible tomato cv. Durinta was transplanted into each pot and inoculated with second-stage juveniles (J2) to achieve a rate of 1 J2 cm^{-3} of soil. In both pot experiments, the number of nematode eggs per plant was reduced (P<0.05) in all non-sterilized soils compared to the sterilizes ones, except M10.45. Also, *P. chlamydosporia* was the only fungal species isolated from parasizided nematode eggs. *P. chlamydosporia* is the most frequent and prevalent fungal species with a high plasticity able to be adapted to the agronomical practices in highly perturbed vegetable production system.

Key words: *Meloidogyne* spp., Biological control, systemic resistance, *Pochonia chlamydosporia*, *Bacillus firmus*, tomato cv. *Durinta*, cucumber cv. *Dasher II*, Suppressive soil.

General Introduction

General Introduction

Plant Parasitic Nematodes (PPNs)

The nematodes have been existed for more than 600 million years ago. Although, there are described less than 25,000 species within this phylum, the estimation of the total number nematodes species ranges between 500,000 to more than 10 million (Bird et al., 2015). There are two nematode classes, the Chromadorea (order: Rhabditida) and the Enoplea (orders: Dorylaimida and Triplonchida). The order Tylenchida includes the majority of plant-parasitic nematodes that affect crops (Lambert & Bekal, 2002), which their worldwide crops losses have been estimated by $157 billion annually (Abad et al,. 2008). The most important plant parasitic nematodes are the root-knot nematodes (RKN) (*Meloidogyne* spp.) and the cyst nematodes (*Heterodera* spp. and *Globodera* spp.) (Jones et al, 2013).

Root Knot Nematodes (*Meloidogyne* spp.)

RKNs belonging to *Meloidogyne* spp. genus, are sedentary nematodes and a distractive factor on vegetable crops causing important yield losses in the world (Ornat & Sorribas, 2008). The first root-knot disease was reported by Berkeley (1855) on glasshouse in cucumber roots. In 1887, Göldi proposed the name *Meloidogyne* spp. (apple-shaped female) to described *M. exigua* in coffee plants in Brazil. However, it was included into the genus *Heterodera* (cyst nematodes) until 1949 when Chitwood separated the RKN from the *Heterodera* genus because they differed from cyst nematodes, and described the new genus named *Meloidogyne* that included *M. arernaria* (Neal) Chitwood, *M. exigua* Göldi, *M. incognita* (Kofoid & White) Chitwood, *M. javanica* (Treub) Chitwood and *M. hapla* Chitwood (Moens et al., 2009).

The genus *Meloidogyne* contains nearly one hundred species (Hunt & Handoo, 2009) and can affect ca. 5500 different plant species, which include most of the economically important crops, ornamental plants, and several weeds (Trudgill & Blok, 2001). The tropical and sub-tropical *M. arenaria*, *M. incognita* and *M. javanica*, and the temperate *M. hapla* are commonly widespread (Moens et al., 2009). In Spain, *M. arenaria*, *M. incognita* and *M. javanica* are commonly distributed (Ornat & Sorribas, 2008). In figures 1 and 2 there are shown the main aerial symptoms (plant growth, flowering and fruit setting) and in figure 3 tomato galled roots can be observed.

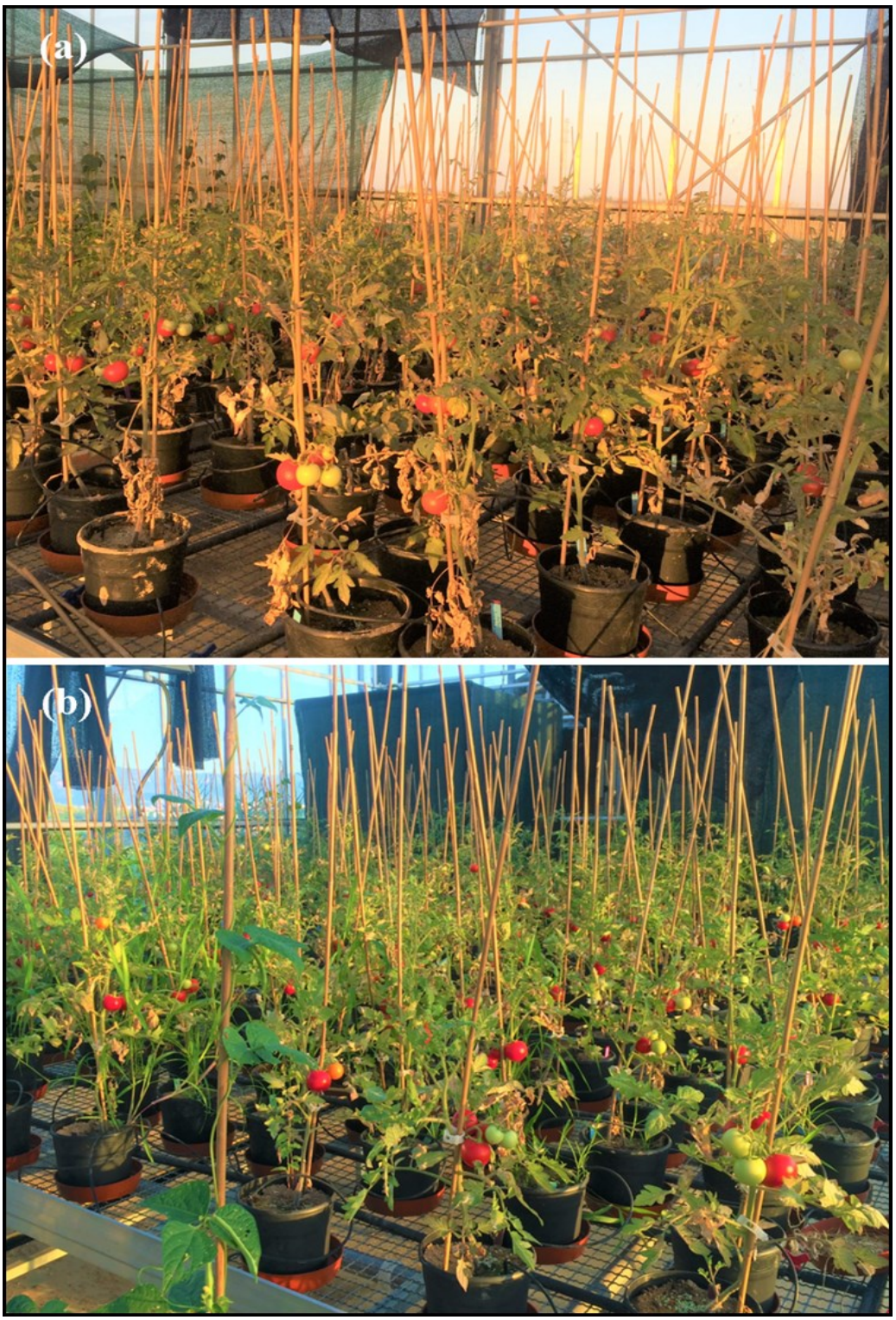


Figure 1. Aerial symptoms caused by *M. incognita* on susceptible tomato cv. Durinta showing chlorosis, reduced plants growth (a) compared to healthy plants (b).

Figure 2. Aerial symptoms caused by *M. incognita* on susceptible tomato cv. Durinta (a) and Cucumber cv. Dasher II (b) showing chlorosis and reduced plants growth.

Figure 3. Severely galled root of the susceptible tomato cv. Durinta caused by *Meloidogyne spp*.

The life cycle of *Meloidogyne spp.*

Most species of RKN have a life cycle consisting of the egg, four larval stages (J1–J4) and the adult (male and female) (Abad et al., 2008), in Figure 4 is represented the *M. incognita* life cycle. J2 are the only infectivity stage of the root-knot nematode. The J2 migrate into the soil, penetrates the growing root tips just behind the root cap thanks to the stylet (Bengtsson, 2015). The stylet is located on the nematode head and is used to pierce, draw food and inoculating plant cells via secrete effectors (Lambert & Bekal, 2002). During infection, the establishment of the feeding site in the cells of protoxylem or protophloem and chemical components excreted by the nematode induces the differentiation of four to nine root cells known as giant-cells (Bengtsson, 2015; Hewezi & Baum, 2017). At the same time of giant-cell induction, other surrounding cortical cells quickly gain and form gall-like organs (Figure 4), giving rise of the term root-knot nematodes (Hewezi & Baum, 2017). After feeding has started, the J2 will growth and transform into a 'sausage-shape' and under favourable conditions, molts into the J3, J4 and adult stage (Bengtsson, 2015). The nematode differentiates into female under favourable environmental conditions, but on the contrary, the nematode differentiates into male (Moens et al., 2009). Males do not play an important role in reproduction. At the end of the life cycle, the female lays a gelatinous matrix and the process is repeated by depending on host species and environmental conditions. (Bengtsson et al, 2015). Each female may lay from 500 to 1500 eggs in a gelatinous matrix denominated egg mass (Ornat & Sorribas, 2008). Egg masses are composed of glycoproteins produced by the rectal glands. Gelatinous matrix surrounds and holds the eggs to protect them from environmental stress and against predation and parasitism and located on the root surface, inside or outside of the gall depending on the host plant species (Moens et al., 2009). The eggshell of typically consists of three layers vitelline, chitinous and lipid from outer to inner,

respectively. Under favourable conditions, eggs rapidly develop into first-stage juvenile (J1). Then, molts into a second-stage juvenile (J2).

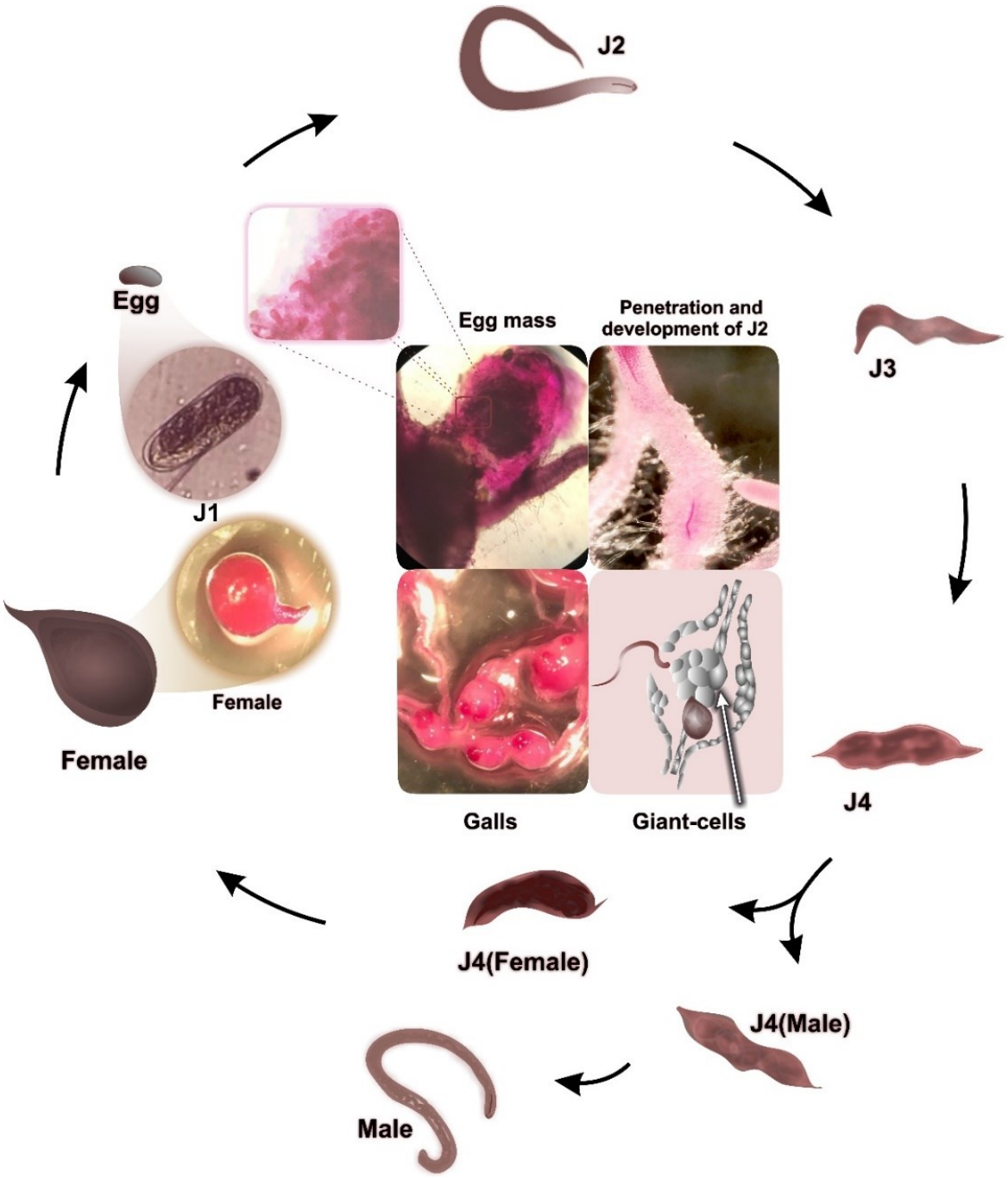


Figure 4. The parasitic life cycle of *Meloidogyne incognita*. Infective second stage juveniles (J2) penetrate the root, and migrate between cells to reach the plant vascular cylinder. The stylet connected to the esophagus is used to pierce plant cell walls, to release esophageal secretions and to take-up nutrients. Each J2 induces the differentiation of five to seven root cells into multinucleate and hypertrophied feeding cells that called giant cells. These cells supply nutrients to the nematode. At the same time of giant-cell induction, other surrounding cortical cells quickly gain and produce forming of gall-like organs. The nematode becomes sedentary and goes through three molts (J3, J4, adult). Occasionally, males develop and migrate out of the roots. However, it is believed that they play no role in reproduction. The pear-shaped female produces eggs that are released on the root surface. Embryogenesis within the egg is followed by the first molt, generating second-stage juveniles (J2) (Modified from Abad et al., 2008).

Epidemiology

Meloidogyne spp., are poikilothermic organisms, which their life cycle depends on soil temperatures (Tyler, 1933). The nematode development occurs when soil temperatures are between 10ºC and 35ºC and needs a constant number of accumulated degree-days (S) above basal temperature (Tb) to complete their life cycle (Trudgill, 1995; Evans & Perry, 2009). For example, *M. incognita* requires S = 350 DD (degree days); Tb = 12.9°C on tomato and S = 500 DD; Tb = 11.4°C on cucumber plants to complete its life cycle (Madulu & Trudgill, 1994; Giné et al., 2014). In tomato, the *M. incognita* life cycle needs 63, 44, 27, and 20 days at temperatures of 16.2, 20, 25 and 30°C, respectively (Ploeg & Paul, 1999). The population dynamics is described as "the variation in the number of nematodes over time" that affecting by several factors such as temperature, crop status and nematode species. The amount of nematode in the soil before transplantation is the initial population density (*Pi*). The relation between the *Pi* with the final population density (the nematode population at the end of the cultivation period; *Pf*) is studied by population dynamics (Greco & Di Vito, 2009). The relationship between *Pi* and *Pf* is described in Figure 5 following the model described by Seinhorst (1970):

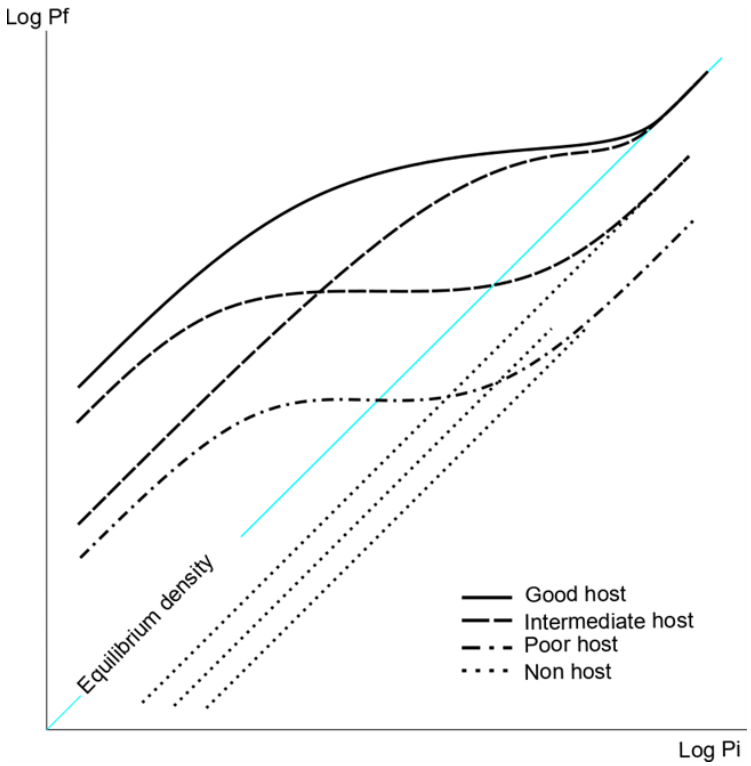


Figure 5. Relationship between initial (*Pi*) and final (*Pf*) populations of *Meloidogyne spp*. with good, intermediate, poor and non-hosts. The equilibrium density is defined as *Pf = Pi* and *Pi* and *Pf*: initial and final densities on logarithmic scales (adapted from Seinhorst, 1970).

There are three parameters that serve as indicators of host status a potential crop to nematode: i) The maximum reproduction rate (a) of the nematode occur at lowest nematode density and determined by linear regression at lowest *Pi* ii) the maximum population density achieved by the nematode, and iii) the equilibrium density (E) that show there is enough food to maintain a *Pf* value similar to the *Pi*, being the *Rf* = 1. These three parameters are usually less in resistant or poor host than susceptible host. The knowledge of the relationship between *Pi* and *Pf* is necessary for estimating these parameters (Ferris, 1985; Greco and Di Vito, 2009). The maximum reproduction rate of nematode (a) and the equilibrium density (E) can be used to assess the host status that high values of a and E determine good host plants and low values indicate poor host or resistance plants and are 0 for non host plants (Seinhorst, 1970; Figure 5).

Host plants

More than 2,000 plant species, which include nearly all plant families, are affected by *Meloidogyne* spp. The most economically important crops are also affected by at least one species of this genus (Jenkins & Taylor, 1967; Sasser & Carter, 1985).

Solanaceae

The Solanaceae family (order Solanales) contains more than 90 genera and 3000- 4000 species. Some of these species are the main food sources such as potato (*Solanum tuberosum*), eggplant (*S. melongena*); tomato (*S. lycopersicum*); peppers (*Capsicum* sp), as well as, ornamentals e.g. petunia (*P. hibrida*). The tomato represents the second importance solanaceous crop after potato. The cultivated tomato (*S. lycopersicum*) was domesticated in Mexico or Peru and was introduce into Europe around the middle of the fifteenth century (Gebhardt, 2016). The worldwide tomato production in 2017 was 182 million tonnes (mt) in 4.8 million hectares according to the United Nations Food and Agriculture Organization data (FAOSTAT, 2019). The largest producer of tomato was China with 59 mt in 2017. The European Union tomato cultivation area is 270,000 ha, and produces 18 mt. The 13.5% of the tomato production come to the European Union (EU) that 29% Europe tomato is cultivated in Spain. The maximum yield losses caused by *Meloidogyne* spp. can reach a 60% on tomato (Giné & Sorribas, 2017).

Cucurbitaceae

The Cucurbitaceae family (Order Cucurbitales) includes 96 genera and 1000 species with two subfamilies *Cucurbitoideae* and *Zanonoideae* (Renner & Schaefer, 2016). The *Cucurbitoideae,* includes important agronomical plants like those belonging to the following genera, *Cucumis*: cucumber (*C. sativus* L.) and melon (*C. melo* L.), *Citrullus*: watermelon (*C. lanatus*) and *Cucurbita*: squash (*Cucurbita pepo*), winter squash (*C. maxima*), and bitter melon (*Momordica charantia*)] (Ezura, 2016).

The cucurbits origin happened in different locations in worldwide, from India and Himalayan mountain, north Africa and southern Europe (Staub et al., 1997). The worldwide cultivation area of cucumber and gherkins in 2017 was is 2.3 million hectares, and the production is 8.3 mt. Also, in that year was reported that the largest producer of cucurbits was China with 6.4 mt. In Europa the cultivation area is 52000 ha which produces 2.8 mt. The 23% was cultivated in Spain (FAOSTAT, 2019). The maximum yield losses caused by *Meloidogyne* spp. have been estimated in a 88% on cucumber and a 39% on zucchini (Giné et al., 2014; Vela et al., 2014).

Root Knot Nematodes management and control methods

There are different strategies such as chemical substances, physical, cultural and biological methods for the management and control of RKN. Integrated pest management (IPM) minimizes the application of chemical pesticides using a combination of several environmentally friendly practices to control RKN such as crop rotation, resistant varieties and the use of biological control agents (Ornat & Sorribas, 2008).

Chemical control

Fumigant and non-fumigant nematicides are chemical substances and the most used method to control RKN (Talavera et al., 2012). Methyl Bromide due its negative impact on the environment by depleting the ozone layer and its toxicity has been restricted. Fumigants such as 1,3 dichloropropene and metam-sodium are mostly using toward nematode that have more effect than non-fumigants (Ornat & Sorribas, 2008), although 1,3-dichloropropene and chloropicrin were banned in Europe following the Europe Regulation 1107/2009 in 2010 and 2013, respectively (López-Aranda et al., 2016). As a result of the problems associated with chemical control, identify alternative management approaches are one of the main focus in the RKN management.

Physical control

Steam and Soil Solarization. Soil Solarization and steam are two techniques that increase soil temperature for protecting plants toward RKN. In solarization, the soil surface is cover with polyethylene mulch for trapping solar energy into the soil to increase the soil temperature ca. 10 ºC. This rate is enough to reach 45 ºC, a lethal temperature to RKN (Sikora & Fernández, 2005; Hallmann & Meressa, 2018). In steam heat, soil is sterilized with steam water that increases soil temperatures to control RKN. However, dry or steam heat methods has been limited because of their high economically costs (Sikora & Fernández, 2005), in addition, repeated treatments have negatively affected on organic matter and in soil microorganisms (Scopa et al., 2009).

Flooding. Root-knot nematode densities reduce significantly when soils are flooded for prolonged periods of time. However, water availability and the ability to control water levels are as limiting factor in most areas where vegetables are cultivated (Sikora & Fernández, 2005). Flooding alternatived 15 to 20 days cycles with drying to be more effective than long, continuous flooding cycles (Noling, 2003). Padgham et al. (2003) stated that reproduction of *M. graminicola* in rice roots decreased more than 70% comparative to its reproduction at the beginning of the experiment after twelve weeks incubation in flooded soil.

Cultural control

Crop rotation. Crop rotation is the practise of growing different crops in the same field in sequenced seasons. A rotation must be designed with non-host, susceptible or resistant host to control *Meloidogyne* species to reduce RKN population (Ornat & Sorribas, 2008). Also, in Spain, crop rotation including solanaceous and cucurbit crops are very common (Talavera et al., 2012). Moreover, RKN have hundreds of different host plant species fact that difficult to use crop rotation for controlling this nematode genus (Ornat & Sorribas, 2008).

Cover crops. A cover crop is a non-host RKN that is mainly planted to manage the soil erosion, soil quality, suppress weeds and control nematode (Hallmann & Meressa, 2018). Cover crops may be planted after harvesting the crop used by the farmers. Brassica crops (Fourie et al., 2016), velvet bean (*Mucuna pruriens*), joint vetch (*Aeschynomene americana*) and sorghum-sudangrass (McSorely et al., 1994) were effectively to control the population densities of *Meloidogyne* spp.

Trap crops. A good host crop to RKN is planting for a suitable period of time to ensure good infection by the nematode, and then the roots are harvested to eliminate from the soil the developing sedentary juveniles (Hallmann & Meressa, 2018). Lettuce (*Lactuca sativa*) and radish (*Raphanus sativus*) have been described as RKN trap crop (Cuadra et al., 2000).

Antagonistic crops. There are plants able to produce nematicidal compounds. One of the most used are species from the genus *Tagetes* spp. The cultivation of selected marigold varieties *T. patula* and *T. hybrid* consistently successfully suppressed *Meloidogyne* spp. and increased 50 % of tomato yields after marigold higher than after fallow and increased 45-95 % melon yields during spring and summer of year fallowing cultivation of marigold varieties (Ploeg, 2002).

Planting Date. Due to root-knot nematodes are poikilothermic organisms, changing the planting dates to months with lower temperatures would provide a delay in the root invasion (Roberts, 1987), in addition, the crops can be harvested before females start to lay egg masses.

Organic amendment and Biofumigation. The incorporation of organic material into soil improves physicochemical and biological properties of soil and reduces RKN densities. This method is efficient against RKN through enhancement of nematode antagonists or can release toxic compounds present in these products or toxic metabolites produced during microbial degradation. However, there is not much information about economic and practical impact of this method (Hallmann & Meressa, 2018). Also, biofumigation is a sustainable approach to control soil-borne pathogens; in this case, volatile compounds from the biodegradation of organic matter are released into the soil to improve soil characteristics. Microbial activity on the organic matter during its decomposition can produce the best biofumigation that aid in the control of soilborne pathogens (Bello et al., 2000).

Root destruction. At the end of the crop the roots should be removed and destroyed because RKN can survive on the roots left in the soil. This method reduced population density of nematode for the following crop (Hallmann & Meressa, 2018).

Plant resistance

Resistance is defined as a plant that has one or more effective resistance genes to infection or suppress nematode development or reproduction. There are known just a few resistance genes toward RKN. The most studied are the *Mi*-1 to *Mi*-9 genes, all targeting three common root-knot nematodes *M. incognita*, *M. javanica*, and *M. arenaria* in tomato (Williamson & Roberts, 2009; Sorribas et al., 2005). However, the gene *Mi*-1 is not effective against *M. hapla* on tomato (Liu & Williamson, 2006). Among Cucumis species, *Cucumis metuliferus* is known to be resistant to *Meloidogyne incognita* infections. The lack of RKN resistance genes in Cucurbitaceae family have serious problems associated to RKN infections (Ling et al., 2017).

Biological control

Biological control includes a range of control strategies such as crop rotations, host plant resistance, and the introduction of a natural enemies to RKN management. Among this natural enemies, they have different mode of action: (i) predators such as protozoa, nematodes and arthropods that kill and eat nematode, (ii) parasites such as bacteria and fungi that grow within or on their host (RKN), and these microorganisms can also act indirectly inducing plant defence mechanisms, (iii) several organisms that effect on nematode abundance with other mechanisms of action than parasitism and predation (Stirling, 1988). For example, symptoms such as Lysed, shrivelled, coagulated, or decaying eggs found in *Heterodera* were through apparently non-specific causes. Although the most of nematode females are killed by fungal pathogens, more than 25 % of diseased cysts are destroyed through non-specific causes (Tribe, 1977).

Effective abiotic factors on biological control

Some abiotic factors such as, soil type, temperature, soil moisture, and pH can affect the effectiveness of biological control. For most soil microorganisms the optimum pH ranges between 6-7 and they can grow between 4 and 9 values (Stirling, 2014). Regarding the soil properties, eggs hatching of *Meloidogyne* in soils with small pore size occurs later and in least numbers than in soils with larger pores (Evens & Perry, 2009). Some studies relate the efficiency of biological control agents with the texture of the soil, for example the bacteria *Pasteuria penetrans* against *M. incognita* on coffee plants was more effective in a sandy than in a sandy clay soils that measured with the reduction of nematode reproduction factor (Cardeiro et al., 2007). Also, the percentage of parasitism was related to the soil texture and the P/N relationship (Giné et al., 2013). Nasu et al., (2018) observed that the nematophagous fungus *P. chlamydosporia* successfully control *M. incognita* in soybean plants in both sandy and clay soils.

Antagonist Microorganisms of RKN

Some of better-studied fungal antagonists against RKN are *Purpureocillium lilacinus*, *Pochonia chlamydosporia* and *Trichoderma* spp. and the bacteria antagonists are *Pasteuria penetrans* and *Bacillus* spp (Hallmann & Meressa, 2018). Specifically, this PhD thesis is focused on the use of the nematophagous fungus *Pochonia chlamydosporia* and the bacteria *Bacillus firmus* on tomato cv. Durinta and cucumber cv. Dasher II to management of *M. incognita*.

Nematophagous Fungi

Nematophagous fungi are a group of soil microorganisms that can be used as biological control agents. There are described more than 200 fungal species able to capture, kill and digest plant parasitic nematodes (eggs, juveniles, and females). They are included in different taxonomical levels: Ascomycetes, Basidiomycetes, Zygomycetes, Chytridiomycetes and Oomycetes such as *Haptoglossa*, *Myzocytiopsis*, *Nematophthora* of the Lagenidiaceae taxa. The nematophagous fungi are classified into four major groups depending on their mode of action: Nematode-trapping fungi (NTF) (predacious or predatory fungi), endoparasitic (endozoic), Female and egg parasitic fungi and toxic fungi (Nordbring-Hertz et al., 2011; Stirling, 2014).

Nematode-trapping fungi

Nematode-trapping fungi (NTF) are a heterogeneous group that contained adhesive or mechanical structures to trap the nematodes. These trapping structures can be hyphae, nets, knobs, branches, networks or rings. (Jiang et al., 2016). The majority of nematode-trapping fungi belong to Ascomycota, Order Orbiliales, genus *Arthrobotrys* spp. appear commonly in most soils, highlighting *A. oligospora* (Nordbring-

Hertz et al., 2011; Presmark & Jansson, 1997). The formation of traps of nematode-trapping fungi is important factor for their lifestyle switch from saprophytic to predacious stages (Yang et al., 2011). Several biotic and abiotic factors in soil such as nematode density, soil factors such as pH and moisture importantly affect the nematophagous fungi distribution (Gray ,1985).

Endoparasitic fungi

Endoparasitic fungi infect juvenile or adult nematodes through their spores (conidia or zoospores) that be swallowed by the host or injected spore contents into the nematode. Most of endoparasites are obligate parasites with a wide host range (Lopez-Llorca et al., 2008). Endoparasites such as *Drechmeria coniospora*, *Hirsutella rhossiliensis*, *Haptoglossa dickii* and *Catenaria anguillulae* complete their vegetative stages inside of infected plant parasitic nematodes. *Drechmeria coniospora* and *Hirsutella rhossiliensis* produce about 10,000 and 1000 conidia per infected nematode, respectively (Nordbring-Hertz et al., 2011).

Eggs and females' parasites

A third group of nematophagous fungi are parasites of nematode females, eggs, or cysts and developed specific structures named appressoria or zoospores. *Pochonia* spp., *Purpureocillium* spp., *Lecanicillium* spp. and *Nematophthora* spp. are egg-parasites of nematodes. *P. chlamydosporia* will be explained in detail in the next section of this general introduction. The mode of action of the fungal parasite of nematode eggs *Pochonia rubescens* showed that the fungus adhered to nematode egg and then the fungus developed the appressoria and adhesive secretions to penetrate on the eggshell and degrade eggshell from *Heterodora schachtii* with enzymatic activities (Lopez-Llorca et al., 2008).

Toxin-producing fungi

Finally, the toxin-producing fungi produce toxins to immobilize the nematodes, with prior hyphae penetration through the cuticle and colonize the nematode. Basidiomycetous fungus *Pleurotus* produces nematotoxic microdroplets by hyphae and *Coprinus* produces an unusual structure "spiny ball" are two main genera of this group (de Freitas Soares et al., 2018).

Pochonia chlamydosporia

The nematophagous fungus *Pochonia chlamydosporia* (syn. *Metacordyceps chlamydosporia*) has a worldwide distribution and has been reported as a nematode eggs parasite, females of cyst and root-knot nematodes of the several genera such as *Heterodera*, *Globodera*, *Meloidogyne*, *Nacobbus* and *Rotylenchulus*. *P. chlamydosporia* belongs to the family Clavicipitaceae (Ascomycota: Pezizomycotina: Sordariomycetes: Hypocreales) (Manzanilla-López et al., 2017; Medina-Canales & Rodríguez-Tovar,

2017). *P. chlamydosporia* was found in 1974 by for first time as a parasite of *Heterodera* spp. eggs (Wilcox & Tribe, 1974).

Life styles of P. chlamydosporia

P. chlamydosporia, is one of the most studied fungus with potential as biological control agent of plant parasitic nematodes. The fungus can colonize endophytically roots of Gramineae and Solanaceae species such as barley (Maciá-Vicente et al., 2009a; Larriba et al., 2015), tomato (Bordallo et al., 2002; Escudero & Lopez-Llorca. 2012) or potato (Manzanilla-López et al., 2011) The fungus can remain saprophytically in soil both in the absence of nematodes and host plants. There is a balanced relationship between the plant and the fungus related to nutrients released by the plant into the rhizosphere and switch from the saprophytic to the parasitic phase (Manzanilla-López et al., 2011). *P. chlamydosporia* has multitrophic lifestyles (saprotrophtic, endophyte, parasite) that according to available substrate, plants host, and environmental conditions (Manzanilla-López et al., 2017). Giné et al. (2013) reported the egg parasite *P. chlamydosporia* alone or co-occurring with other fungal species that was associated with suppressiveness against *Meloidogyne* spp. in Spain.

Nematophagous Bacteria

Bacteria are a group of natural enemies than have been frequently use to biological control of sedentary and migratory endoparasitic nematodes. Nematophagous bacteria have different modes of action including: parasitism; competing for nutrients uptake; toxins or antibiotics production (Sikora et al., 2007).

Obligate bacterial parasite, Pasteuria penetrans

Pasteuria penetrans is an obligate, endospore-forming bacteria, which parasitize to a number of important plant parasitic nematodes. Three species from *Pasteuria* genus are able to parasitize plant parasitic nematodes: *P. penetrans*, *P. thornei* and *P. nishizawae*, which is a parasite of *Meloidogyne* spp., *Pratylenchus* spp. and cyst nematodes of the genera *Heterodera* and *Globodera* spp., respectively. (Daives et al., 2011). *P. penetrans* is commonly parasite of *Meloidogyne* and spores adhere to with the cuticle of second stage nematode juveniles and they prevent of producing nematode eggs by infection females. *P. penetrans* completed its life cycle inside the female by degrading of the females, mature spores are released (Darban et al., 2015).

Bacillus firmus, Strain I-1582

Bacillus firmus (Bf) is a gram-positive and spore-forming bacterium, which was first recognized by Werner in 1933. Bf is Facultative anaerobic, with 0.8-0.9 µm in diameter, with an optimal range of temperatures from 30 to 40 ºC and an optimal pH of 7.0-9.0 (Logan & Vos, 2015). *B. firmus* used as

active ingredient of commercially available seed treatment products, VOTiVO® or powder, Nortica® (Castillo et al., 2013).

B. firmus effectively controls different PPNs by affecting on egg hatching, paralysing of second-stages juveniles, reducing number of galls, population size and mortality of various species of nematodes among them are the root-knot nematode *Meloidogyne* spp (Terefe et al., 2009; Xiong et al., 2015), the soybean cyst nematode (Geng et al., 2016); sting nematode (Crow, 2014) and reniform nematode (Castillo et al., 2013). In addition, Mendoza et al., 2008 reported that *B. firmus* reduces significantly hatching of root-knot nematode *M. incognita* with a low concentration of pure culture filtrates, also reduces mortality of *M. incognita*, the stem nematode (*Ditylenchus dipsaci*), burrowing nematode (*Radopholus similis*) in *in-vitro* system. The nematode *M. incognita* paralysis and mortality was related to bioactive secondary metabolites produced by *B. firmus* (Mendoza et al., 2008; Xiong et al., 2015). This nematicidal activity is also related to DS-1, a recent serine protease that degrades *M. incognita* cuticle eggs (Geng et al., 2016).

The role of fungi and bacteria in the induction of plant resistance

Beneficial microbes such as bacteria and fungi enhance defense against pathogens, insect and herbivores. Plants can develop induced resistance through tissue colonization with these specific beneficial microbes (Pieterse et al., 2014). A wide induced resistance consists of two main pathways: the systemic acquired resistance (SAR) and the induced systemic resistance (ISR) pathway. "SAR when the induced resistance is triggered by a pathogen or demonstrated to be SA dependent and to ISR when the induced resistance is triggered by a beneficial microbe or demonstrated to be SA independent". SAR cause hypersensitive response by salicylic acid (SA) and ISR enhances the defence plant systems by jasmonic acid (JA) and ethylene (ET) pathways, PR proteins is related to SAR pathway. Indirectly mechanism of resistance protects plants of pathogens that SA pathway is generally involved defence against biotrophic pathogens while JA/ET pathways defence against necrotrophic pathogens (Pieterse et al., 2014). However, sedentary nematodes are obligate biotrophs, the JA/ET pathway is a key defence pathway against *M. graminicola* as well, SA only plays a minor role in rice plant (Nahar et al., 2011).

Colonization of *Arabidopsis thaliana* by *P. chlamydosporia* promotes root growth, reduces flowering time and enhances yield. They strongly suggest that the role of JA signalling in modulating *A. thaliana* are related to colonization by *P. chlamydosporia* (Zavala-Gonzalez et al., 2017). Also, the barley root transcriptomic analysis to response fungal root endophytically colonization showed that *P. chlamydosporia* induces the up-regulation of several genes related with the biosynthesis of the plant hormones auxin, ethylene and jasmonic acid and of other genes involved a moderate induced resistance and promote plant growth (Larriba et al., 2015). The ability of the fungus *Trichoderma harzianum* T-78 to regulate JA and SA-inducible defences in tomato cultivars against *M. incognita* reported by Martínez -

Medina et al. (2017). In addition, Schrimsher (2013) found *Bacillus firmus* GB-126 significantly reduced egg hatching of *H. glycines* and *M. incognita* as well as induced systemic resistance was evident in the *H. glycines* but not in *M. incognita* in split-root experiments in the greenhouse. Siddiqui and Shauket (2004) suggested that *Pseudomonads fluoresecent* induced systemic resistance against *M. javanica* via a transduction pathway, which is independent of SA production in tomato roots. Moreover, the combination of the two modes of action will result in a decrease of the nematode population growth rate, and consequently lower crop yield losses. For example, *P. chlamydosporia* was combined with the application of cis-jasmone reduced RKN reproduction in potato (Vieira Dos Santos et al., 2014) found a reduction in when, as well as an increase in fungal egg parasitism.

Suppressive Soil

Suppressive soils are defined by Baker and Cook (1974) as "soils in which soil-borne pathogens not establish or persist, they can be establishment but cause little or no damage". Disease suppressive soils protect plants due to the presence microorganisms antagonistic in soils against soilborne pathogens such as nematodes (Gómez Expósito et al., 2017). There are two classical type of suppressiveness: General suppression activity related to the total microbial community in soil resulting in suppression of the disease and is not transferable between soils. Specific suppression is specific microorganism suppress pathogen that can transferable. In contrast, conducive are non-suppressive soils that disease easily occurs (Weller et al., 2002).

The mechanism of suppressive soils is not exactly known, but the mechanism might be due to various microbiota activities such as antibiosis, competition, parasitism, predation, organic amendments and physico-chemical properties of soil (Devi & Meetei, 2018). In fact, natural suppressive soils by a balance biotic and abiotic conditions in soil prevent or reduce pathogens/disease. The knowledge of these soils laid to which components are responsible for their natural suppressiveness. Hence, besides biological factors, have to be attention to either physico-chemical soil properties, involved in suppressiveness. Microorganisms have important role into these soils and among them egg parasites are one of the most studied groups involved in suppressiveness against PPNs (Silva et al., 2018). Several nematode antagonists can occur naturally in agricultural soils providing some extent of suppressiveness to RKN (Giné et al., 2016; Elhady et al., 2017). *P. chlamydosporia* has a worldwide distribution and has been found in nematode suppressive soils to parasitize eggs (Manzanilla-López et al., 2013). *P. chlamydosporia* has been reported as a main biotic factor responsible for soil suppressiveness to RKN in horticultural crops. The relative frequency of *P. chlamydosporia* was positively related to the percentage of parasitism in both organic and integrated production systems that were found to be most common in the organic production (Giné et al., 2013; 2016). In addition, managing physical and chemical soil properties improve health and quality of soil by suppressing some pathogen. Organic amendments can improve soil quality by altering soil parameters and increase the diversity of antagonistic microbiota which can environmentally

alternative chemical pesticides (Devi & Meetei, 2018). Giné et al., (2016) reported the first comparison of microbial profiles of both suppressive and non-suppressive soils. They clearly showed that combination of biotic and abiotic factors consists of microbiota activities, a combination of several farming practices such as crop rotation, including RKN resistant cultivars, green manure, the using of organic amendments, and planting date, can prevent nematode build-up.

Objectives

Objectives

Solanaceae and Cucurbitaceae families are the main cultivated vegetables in the world, being the tomato and cucumber two of the most important crops produced in greenhouses. Many plants from both families are host of *Meloidogyne* spp., which produces large yield losses. Plants can develop induced resistance against diseases through the colonization with beneficial microbes. However, for most disease-suppressive soils, the microbes and mechanisms of action responsible for pathogen control are still unknown. The knowledge of this ability of antagonists to induce plant responses against RKN will be helpful to design nematode management strategies. The general objective of the thesis is the biological control approaches of *Meloidogyne* spp. in vegetable crops (tomato and cucumber) by the application of selected antagonists, five *Pochonia chlamydosporia* isolated and *Bacillus firmus* I–1582 (Bf I–1582), and the evaluation of the level of soil suppressiveness of agricultural soil conducted under organic and integrated vegetable production standards. This main objective can be divided into the following specific objectives:

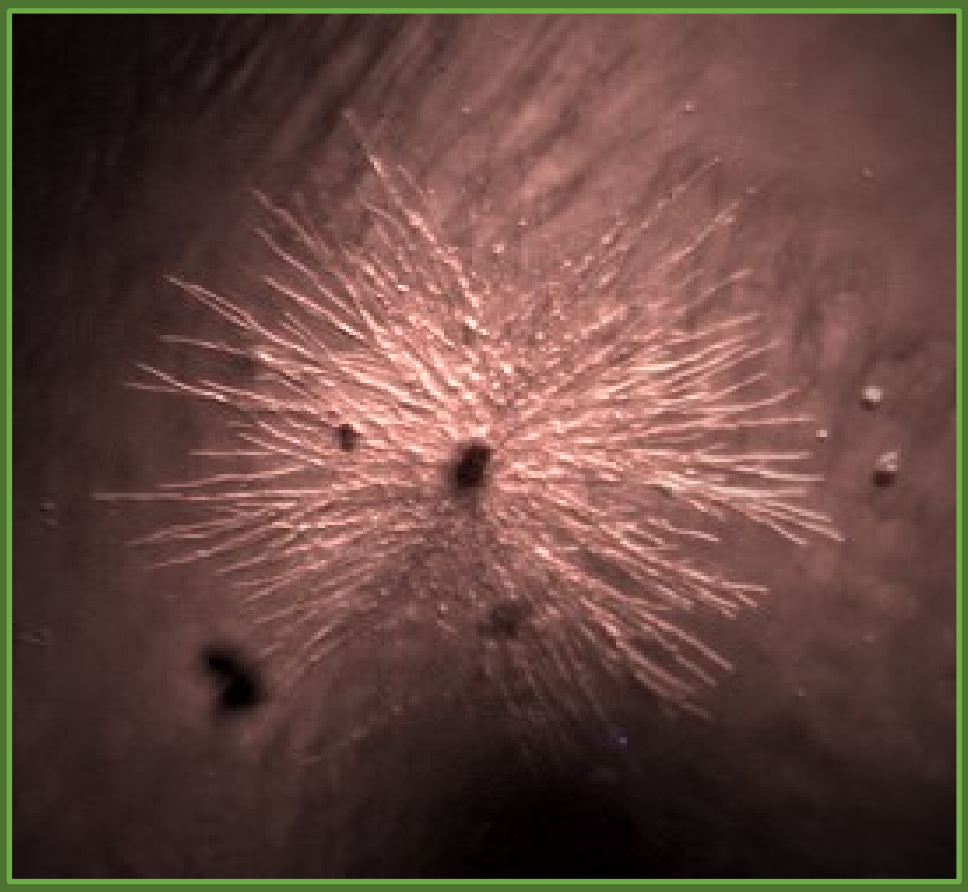
Objective 1: to determine the capability of *P. chlamydosporia* isolates to induce plant resistance against *M. incognita* in tomato and cucumber as representatives of solanaceous and cucurbit crops frequently including in rotation schemes (Chapter 1).

Objective 2: to determine the: i) the cardinal temperatures for *B. firmus* I-1582 growth and biofilm formation; ii) the effect of *B. firmus* I-1582 transformed with the green fluorescent protein gene (*B. firmus* I-1582-GFP) on RKN eggs; iii) the colonization of tomato and cucumber roots by *B. firmus* I-1582-GFP; and iv) the putative induction of systemic resistance and the dynamic regulation of genes related to the jasmonic acid (JA) and salicylic acid (SA) pathways in tomato and cucumber (Chapter 2).

Objective 3: to determine the fluctuation of RKN densities in field conditions during two years in six sites, four conducted under organic and two under integrated vegetable production as well as the level of soil suppresiveness in pot test (Chapter 3).

Chapter 1

Pochonia chlamydosporia induces plant-dependent systemic resistance to *Meloidogyne incognita*



Chapter 1: *Pochonia chlamydosporia* induces plant-dependent systemic resistance to *Meloidogyne incognita*

Zahra Ghahremani, Nuria Escudero, Ester Saus, Toni Gabaldón and F. Javier Sorribas

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Abstract

Meloidogyne spp. are the most damaging plant parasitic nematodes for horticultural crops worldwide. *Pochonia chlamydosporia* is a fungal egg parasite of root-knot and cyst nematodes able to colonize the roots of several plant species and shown to induce plant defense mechanisms in fungal-plant interaction studies, and local resistance in fungal-nematode-plant interactions. This work demonstrates the differential ability of two out of five *P. chlamydosporia* isolates, M10.43.21 and M10.55.6, to induce systemic resistance against *M. incognita* in tomato but not in cucumber in split-root experiments. The M10.43.21 isolate reduced infection (32–43%), reproduction (44–59%), and female fecundity (14.7–27.6%), while the isolate M10.55.6 only reduced consistently nematode reproduction (35–47.5%) in the two experiments carried out. The isolate M10.43.21 induced the expression of the salicylic acid pathway (*PR-1* gene) in tomato roots 7 days after being inoculated with the fungal isolate and just after nematode inoculation, and at 7 and 42 days after nematode inoculation too. The jasmonate signaling pathway (*Lox D* gene) was also upregulated at 7 days after nematode inoculation. Thus, some isolates of *P. chlamydosporia* can induce systemic resistance against root-knot nematodes but this is plant species dependent.

Keywords: *Cucumis sativus*, induced resistance, root endophytes, root-knot nematodes, *Solanum lycopersicum*

Introduction

The root-knot nematodes (RKN), *Meloidogyne* spp., are obligate parasites of plants. The genus comprises more than 100 species, but only four of them are considered the most damaging plant parasitic nematodes due to its wide range of plant hosts, worldwide distribution, and high reproductive capacity (Jones et al., 2013). The RKN infective juveniles (J2) enter the root near the elongation zone and migrate intercellularly to establish a permanent feeding site into the vascular cylinder, inducing the formation of giant cells and root galls by affecting cell wall architecture, plant development, defenses, and metabolism (Shukla et al., 2018). Once the infection occurs, J2 become sedentary, and molt three times to achieve the mature adult female stage. The most frequent and damaging tropical species, *M. arenaria*, *M. incognita*, and *M. javanica*, reproduce parthenogenetically. The female lays a large number of eggs in a gelatinous matrix, the egg mass, located on the surface or within the galled roots.

The damage potential of some *Meloidogyne* species has been summarized (Greco & Di Vito, 2009; Wesemael et al., 2011). *M. arenaria*, *M. incognita* and *M. javanica* are responsible for the majority of vegetable yield losses caused by plant parasitic nematodes (Sikora & Fernández, 2005). Among vegetables, those belonging to the solanaceae and cucurbitaceae families are commonly included in rotation schemes because they are economically important for growers. The estimation of maximum crop yield losses caused by the nematode in field and plastic greenhouse cultivation varies according to the plant germplasm, environmental conditions and agronomic practices. For instances, maximum yield losses from 62 to 100% have been reported in susceptible tomato cultivars (Seid et al., 2015; Giné & Sorribas, 2017), 30 to 60% in aubergine (Sikora & Fernández, 2005), 50% in cantaloupe (Sikora & Fernández, 2005), 37 to 50% in watermelon (Sikora & Fernández, 2005; López-Gómez et al., 2014), and 88% in non-grafted or grafted cucumber on *Cucurbita* hybrid rootstocks (Giné et al., 2014, 2017).

Control of RKN is conducted mainly with fumigant and non-fumigant nematicides (Djian-Caporalino, 2012; Talavera et al., 2012). However, due to environmental and toxicological concerns, some legislative regulations, such as the European Directive 2009/128/EC, aim to reduce the use of pesticides by promoting alternative methods such as biological control and plant resistance.

Several nematode antagonists belonging to different taxonomic groups have been described (Stirling, 2014). They can act in three ways: (1) directly parasitizing several or specific RKN development stages, such as *Pasteuria penetrans* (Davies et al., 2011); (2) indirectly by repelling, immobilizing and/or killing them by means of metabolites, and/or inducing plant response, such as *Fusarium oxysporum* strain Fo162 (Dababat & Sikora, 2007a,b); or (3) both directly and indirectly, such as *Trichoderma atroviride* strain T11 or *Trichoderma harzianum* strain T-78 (de Medeiros et al., 2017; Martínez-Medina et al., 2017).

Pochonia chlamydosporia (syn. *Metacordyceps chlamydosporia*) is a fungal antagonist of RKN and cyst nematodes that acts directly by parasitizing eggs, and could also acts indirectly. This fungal species

colonizes endophytically the root of several plants, including barley (Maciá-Vicente et al., 2009), tomato (Bordallo et al., 2002), potato (Manzanilla-López et al., 2011), or *Arabidopsis* (Zavala-Gonzalez et al., 2017), inducing plant defense mechanisms, such as the formation of papillae (Bordallo et al., 2002) and the modulation of miRNA in tomato (Pentimone et al., 2018) or plant defense genes related to salicylic acid and jasmonic acid pathways in barley and *Arabidopsis* (Larriba et al., 2015; Zavala-Gonzalez et al., 2017).

It is assumed that some of these defense mechanisms could suppress root infection, development and/or reproduction of RKN (Escudero & Lopez- Llorca, 2012), but as far we know, only one study has proven the induction of local resistance (de Medeiros et al., 2015), and none to elucidate the capability of this fungal species to induce systemic resistance. As *P. chlamydosporia* parasitizes RKN eggs, a split-root system is required to determine the capability of this nematophagous fungus to induce plant resistance avoiding the direct interaction with the nematode. Therefore, in the present study, the capability of five *P. chlamydosporia* isolates to induce plant resistance against *M. incognita* was assessed in a split-root system. To assess whether the response was plant dependent, tomato and cucumber were used as representatives of solanaceous and cucurbit crops frequently including in rotation schemes.

Materials and methods

Plant material, nematode and fungi

Tomato cv. Durinta and cucumber cv. Dasher II were used in this study. For all the experiments, seeds were surface-sterilized in a 50% sterilized bleach solution (35 g L^{-1} active chlorine) for 2 min, washed three times in sterilized distilled water for 10 s each, sown in a tray containing sterile vermiculite, and maintained in a growth chamber at 25°C ± 2°C with a 16 h:8 h (light:dark) photoperiod.

Five *P. chlamydosporia* isolates M10.41.42, M10.43.21, M10.51.3, M10.55.6, and M10.62.2 were used. The fungal isolates were obtained from horticultural commercial growing sites in northeastern Spain from RKN eggs (Giné et al., 2012) and maintained as single-spore isolates at the Universitat Politècnica de Catalunya. Fungal chlamydospores were produced in barley seeds following the procedure of Becerra and collaborators (Becerra Lopez-Lavalle et al., 2012) with some modifications. Briefly, for each isolate, three 200 g batches of barley seeds were soaked for 18 h and each batch sterilized in an Erlenmeyer flask at 121°C for 22 min over two consecutive days, then were incubated at 25°C ± 2°C in the dark. Afterward, 10 5-mm plugs from the edge of each *P. chlamydosporia* isolate grown in CMA were added to each Erlenmeyer flask and they were shaken at 5-day intervals to homogenize fungal growth. After a month, the number of chlamydospores produced on barley was determined following the procedure of Kerry & Bourne (2002). Three 10-seed subsamples per Erlenmeyer were plated onto CMA and incubated at 25 ± 2°C in the dark for 2 weeks to assess putative contaminations prior to being used. The viability of the chlamydospores was assessed as in Escudero et al. (2017).

J2 of the isolate Agropolis of *Meloidogyne incognita* were used as inoculum. Eggs were extracted from tomato roots by blender maceration in a 5% commercial bleach (40 g L^{-1} NaOCl) solution for 5 min (Hussey & Barker, 1973). The egg suspension was passed through a 74-µm aperture sieve to remove root debris, and eggs were collected on a 25-µm sieve and placed on Baermann trays (Whitehead & Hemming, 1965) at 25 ± 2°C. Nematodes were collected daily using a 25-µm sieve for 7 days and stored at 9°C until their use.

Induction of systemic plant resistance by P. chlamydosporia isolates against root-knot nematodes

Tomato and cucumber were grown in a split-root system as described in previous studies (de Medeiros et al., 2017; Martínez- Medina et al., 2017). In this system, the root is divided into two halves transplanted in two adjacent pots: the inducer, inoculated with the antagonist, and the responder, inoculated with the nematode. Briefly, the main root of 5-day-old seedlings was excised and plantlets were individually transplanted in seedling trays containing sterile vermiculite and maintained under the same conditions for 2 weeks for cucumber, and 3 weeks for tomato plants. Afterward, plantlets were transferred to the split-root system by splitting roots into two halves planted in two adjacent 200 cm^3 pots filled with sterilized sand. Four treatments were assessed for each fungal isolate: Fungi-RKN and None-RKN, to assess the capability of each fungal isolate to induce plant response against RKN, and Fungi-None and None-None, to assess the effect of each fungal isolate on plant growth. Each treatment was replicated 10 times, and the experiment was conducted two times. The inducer part of the root of the treatments containing Fungi was inoculated with 10^5 viable chlamydospores of *P. chlamydosporia* just before transplanting. One week later, the responder part of the root of the treatments containing RKN was inoculated at a rate of 1 J2 per cm^3 of soil. The treatment None-None, with no inoculation with either fungi or RKN received the same volume of water. The plants were maintained in a growth chamber in the same conditions described previously in a completely randomized design for 40 days. The plants were irrigated as needed and fertilized with Hoagland solution twice per week. Soil temperatures were recorded daily at 30-min intervals with a PT100 probe (Campbell Scientific Ltd) placed in the pots at a depth of 4 cm. At the end of the experiments, both inducer and responder root fresh weight and the shoot dry weight of each single plant were measured. Roots from the RKN-inoculated responder were immersed in a 0.01% erioglaucine solution for 45 min to stain the egg masses (Omwega et al., 1988) before counting them. Afterward, the eggs were extracted from the roots as in Hussey and Barker (1973)'s method and counted. The number of egg masses was considered as the infective capability of the nematode because it indicates the number of J2 able to penetrate, to infect the root, and to develop into egg-laying females. The number of eggs was considered the reproductive capability of the nematode, and the female fecundity was calculated as the number of eggs per egg mass.

The tomato and cucumber root colonization by each fungal isolate were estimated by quantifying the fungal DNA by qPCR at the end of the second experiment. The inducer part of the root was washed three times in sterilized distilled water for 10 s each and then blotted onto sterile paper. Per each fungal isolate three biological replicates were assessed. Each biological replicate consisted of the inducer part of the roots from three plants pooled together. The DNA was extracted from each biological replicate following the Lopez-Llorca et al. (2010)'s procedure. qPCR reactions were performed using the FastStart Universal SYBR Green Master (Roche) mix in a final volume of 25 µl containing 50 ng of total DNA and 0.3 µM of each primer (5′ to 3′ direction) VCP1-1F (CGCTGGCTCTCTC ACTAAGG) and VCP1-2R (TGCCAGTGTCAAGGACGTAG) (Escudero & Lopez-Llorca, 2012). Negative controls containing sterile water instead of DNA were included. Reactions were performed in duplicate in a Stratagene Mx3005P thermocycler (Agilent Technologies) using the following thermal cycling conditions: initial denaturation step at 95°C for 2 min, then 40 cycles at 95°C for 30 s, and 62°C for 30 s. Genomic DNA dilutions of the fungal isolate M10.43.21 were used to define a calibration curve from 5 pg to 50 ng. After each run, the specificity of the PCR amplicons was verified by melting curve analysis and agarose gel electrophoresis. The fungal DNA biomass of each isolate was referred to the total DNA biomass (50 ng) and expressed as a proportion.

Dynamic regulation of the jasmonic and salicylic acid pathways by P. chlamydosporia and M. incognita

Tomato seeds were sterilized as previously described. Three-week-old tomato seedlings were transferred to 200 cm^3 pots with sterilized sand and maintained in a growth chamber as previously described. The fungal isolate M10.43.21 was selected for this experiment because it reduced nematode infectivity and reproduction in the split-root system experiments. The experiment consisted of two treatments: non-inoculated and co-inoculated to determine the expression of genes related to the salicylic acid and jasmonic acid pathways. In the co-inoculated treatment, the soil was inoculated with 10^5 viable chlamydospores just before transplanting and with 1 J2 of *M. incognita* per cm^3 1 week after transplanting. Each treatment was replicated 40 times. An additional treatment only inoculated with the nematode was included to determine the effect of the fungal isolate on nematode reproduction. The fungus and nematode inoculation procedure were as previously stated.

The expression of the pathogenesis-related protein 1 (*PR-1*) gene and the lipoxygenase (*Lox D*) gene the from the salicylic acid and jasmonic acid pathways, respectively, was evaluated at three time points: just after nematode inoculation, that is, at 0 days after nematode inoculation (dani), at 7 dani, and at 42 dani. At each assessment time, roots were washed three times with sterile distilled water, placed onto sterilized filter paper, frozen in liquid nitrogen and stored at −80°C until being used. At 7 dani, the J2 were stained inside roots with acid Fuchsin following the Byrd et al. (1983) procedure to confirm that nematode

had penetrated and infected. At the end of the experiment (42 dani), the number of eggs per plant from three plants for each treatment was determined by extracting them as described previously.

Total RNA from roots was isolated using the PureLink RNA Mini Kit (Invitrogen), according to the manufacturer's instructions. Afterward, the DNA-free kit (Invitrogen) was used to remove the remaining DNA from the sample. Total RNA integrity and quantity of the samples were assessed by means of agarose gel, NanoDrop 1000 Spectrophotometer (Thermo Scientific) and Qubit RNA BR assay kit (Thermo Fisher Scientific). To assure that the sample was DNA free, a PCR was carried out. Then, the RNA was retro-transcribed with the SuperScript II (Invitrogen) according to manufacturer's instructions. The relative gene expression was estimated with the ΔΔCt methodology (Livak & Schmittgen, 2001), using the ubiquitin (*UBI*) gene as a reference gene (Song et al., 2015). Primers used in the RT-qPCR were (5′ to 3′ direction): LeUbi3-F (TCCATCTCGTGCTCCGTCT), LeUbi3-R (GAACCTTTCCA GTGTCATCAACC) Song et al. (2015), LoxD-F (GACTGGTCCA AGTTCACGATCC), LoxD-R (ATGTGCTGCCAATATAAAT GGTTCC) Fujimoto et al. (2011), LEPR1F (GCAACAC TCTGGTGGACCTT), and LEPR1R (ATGGACGTTGTCCTC TCCAG) Gayoso et al. (2007). qPCR reactions were performed in a final volume of 20 µl with 1 µl of cDNA, 0.3 mM of the corresponding primers, and 1X Fast SYBR Green Master Mix (Applied Biosystems). The qPCR was performed in a 7900HT Fast Real Time PCR System thermocycler (Applied Biosystems) using: 20 s at 95°C followed by 40 cycles of 30 s at 95°C and 60 s at 60°C (Gayoso et al., 2007). The specificity of PCR amplicons was verified as described previously. Reactions were performed with three biological replicates per treatment. Each biological replicate consisted of the roots from three plants pooled together. Two technical replicates per biological replicate were assessed.

Statistical analysis

Statistical analyses were performed using the JMP software v8 (SAS institute Inc., Cary, NC, USA). Both data normality and homogeneity of variances were assessed. When confirmed, a paired comparison using the Student's t-test was done. Otherwise, paired comparison was done using the non-parametric Wilcoxon test, or multiple comparison using the Kruskal-Wallis test and groups separated by Dunn's test ($P \leq 0.05$). The repetitions of the split-root experiments for each crop were compared using the non-parametric Wilcoxon test, and considered as one experiment when no differences ($P \leq 0.05$) were found.

Results

Induction of systemic plant resistance by P. chlamydosporia isolates against root-knot nematodes

The split-root system experiments with tomato differed ($P < 0.05$) between them and were treated separately. But no differences were found between the two split-root experiments with cucumber and thus

we considered them as replicates of a single experiment. Both tomato and cucumber fresh root weight of the two halves of the split-root system of the None-None treatment did not differ ($P < 0.05$) (data not shown), showing that the split-root system did not influence root development. Shoot dry biomass did not differ in any fungal isolate-plant species combination ($P < 0.05$) (data not shown). Two of the five *P. chlamyodosporia* isolates induced resistance in tomato plants in both experiments, but none of them did in cucumber (Table 1). The fungal isolate M10.43.21 reduced both the number of egg masses per plant (32–43%), the number of eggs per plant (44–59%), and the female fecundity (14.7–27.6%), while the isolate M10.55.6 reduced the number of eggs per plant in both experiments (35–47.5%) but the number of egg masses or the female fecundity in only one.

P. chlamydosporia isolates differed in the level of root colonization estimated by qPCR irrespective of the plant species (Figure 1). The standard curves for qPCR obtained by representing the cycle thresholds (Ct) against the log of 10-fold serial dilution of DNA from isolate M10.43.21 were accurate and reproducible to estimate the DNA concentration of the different treatments (tomato=-3.66x + 19.36; R^2=0.9736 and cucumber y=-3.4937x + 21.29; R^2=0.9947). Regarding tomato, isolate M10.43.21 was the best root colonizer followed by M10.55.6 (Figure1. a). In relation to cucumber, isolate M10.55.6 was the best root colonizer followed by M10.43.21 (Fiqure1. b). In the inducer part of the root from treatments None-None and None-RKN the fungus was not detected.

Table1. Capability of *P. chlamydosporia* isolates to induce systemic resistance in tomato cv. Durinta or cucumber cv. Dasher II against *Meloidogyne incognita* in two split root experiments.

Crop-experiment	Fungal isolate	Egg masses per plant	Eggs (x 10^3) per plant	Eggs per egg masse
Tomato - 1	M10.41.42	110±14	56.6±8.3	517±41
	M10.43.21	67±16 *	29.5±7.3 *	445±41 *
	M10.51.3	91±5	45.1±3.2 *	500±27 *
	M10.55.6	82±9 *	38.0±6.1 *	506±102
	M10.62.2	103±8	44.3±2.4 *	434±16 *
	Non-inoculated	118±5	72.4±3.3	615±31
Tomato - 2	M10.41.42	71±4	62.1±4.4	876±37
	M10.43.21	57±7 *	44.4±5.3 *	812±68 *
	M10.51.3	65±7	58.9±8.1	881±40
	M10.55.6	62±9	51.3±7.6 *	831±28 *
	M10.62.2	68±5	60.4±3.8	897±38
	Non-inoculated	84±7	79.3±7.1	952±41
Cucumber - 1 and 2	M10.41.42	52±6	24.9±4.3	459±42
	M10.43.21	52±5	24.7±3.0	464±22
	M10.51.3	54±6	26.1±3.6	466±28
	M10.55.6	53±7	25.4±5.2	463±40
	M10.62.2	51±6	24.8±4.4	458±39
	Non-inoculated	46±6	20.5±4.2	422±45

The inducer part of the root was inoculated with 10^5 chlamydospores of the fungus just before transplanting and the responder part of the root was inoculated with 200 nematode juveniles a week later. The parameters assessed in the responder part of the root at the end of the experiments, 40 days after being inoculated with the nematode, were: the number of egg masses per plant (infectivity), the number of eggs per plant (reproduction), and the number of eggs per egg mass (fecundity).
Data of each tomato experiment are mean ± SE of 10 replicates. Data of the cucumber experiments 1 and 2 are mean ± SE of 20 replicates because no differences ($P < 0.05$) were found between experiments and data were considered as a single experiment. Data within the same column per crop and experiment followed by *differ ($P< 0.05$) from the non-inoculated treatment according to the non-parametric Wilcoxon test.

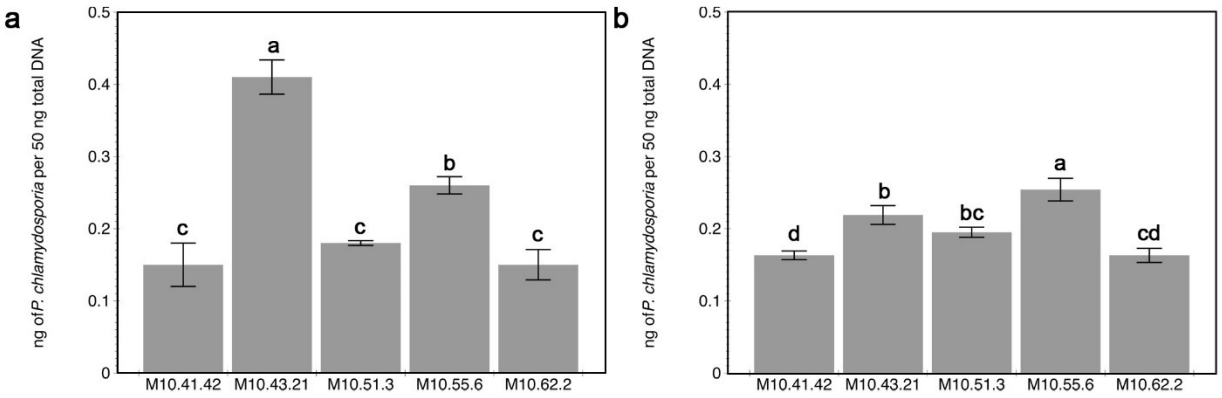

Figure 1. Capability of five *P. chlamydosporia* isolates to colonize roots of tomato cv. Durinta (a) and cucumber cv. Dasher II (b). The root colonization is expressed as the proportion of fungal DNA biomass (isolates) per 50 ng of the total DNA biomass extracted from the responder part of the root of the split-root experiment 2. Each value is mean ± SE of three biological samples with two technical replicates each. Different letters indicate statistical differences between isolates ($P < 0.05$) according to the Dunn's test.

Dynamic regulation of the jasmonic and salicylic acid pathways by P. chlamydosporia and M. incognita in tomato

Changes in the expression of genes *PR-1* and *Lox D* from the salicylic acid and jasmonic acid pathways at 0, 7, and 42 dani are shown in Figure 2. The expression of the *PR-1* gene in roots inoculated with the fungal isolate M10.43.21 was upregulated at 0, 7, and 42 dani compared to the non-inoculated plants (Figure 2. a-c). Regarding the jasmonic acid pathway (Figure 2. d-f), the gene *Lox D* was only upregulated at 7 dani. The nematode reproduction in plants co-inoculated with the fungal isolate was suppressed by 60% compared to plants only inoculated with the nematode (Figure 3).

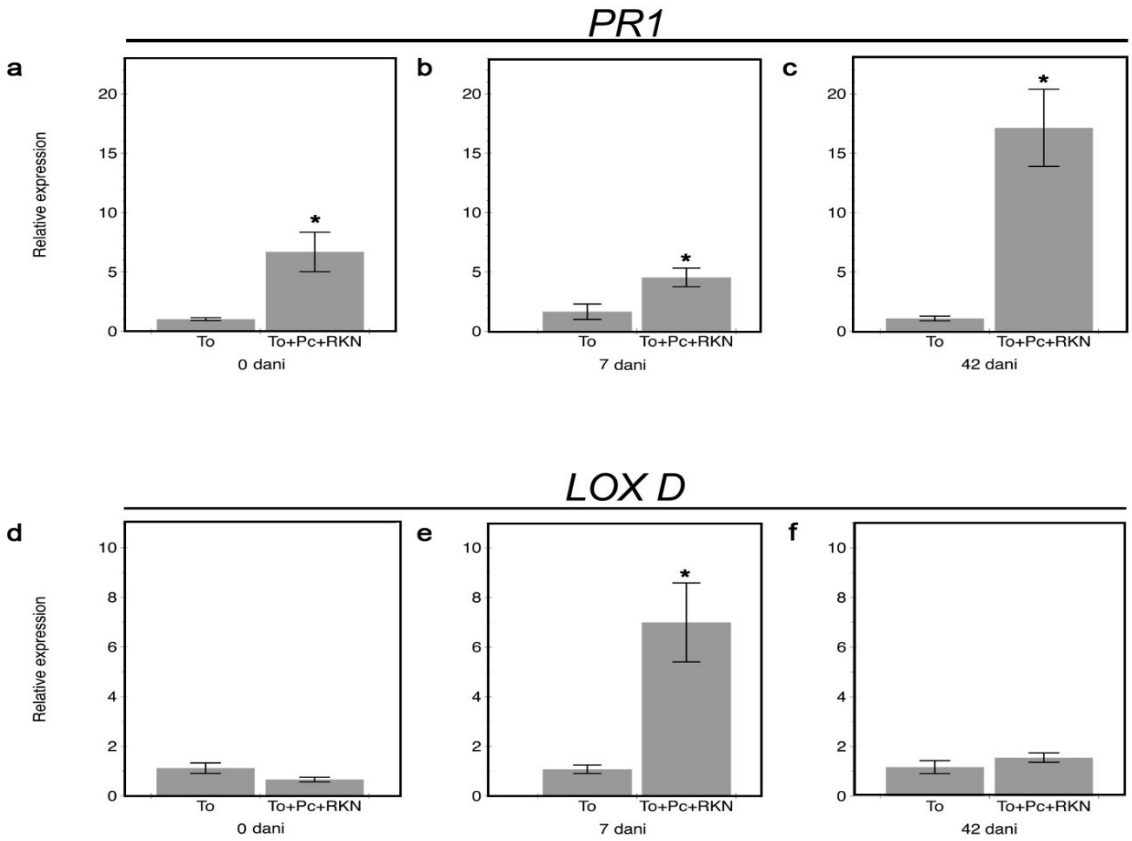

Figure 2. Relative expression of genes related to the salicylic acid and jasmonic acid pathways. The expression of genes *Pr1* (a-c) and *Lox D* (d-f) in roots of the tomato cv. Durinta non inoculated (To) or inoculated with 10^5 chlamydospores of *P. chlamydosporia* isolate M10.43.21 just before transplanting and with 200 J2 of *M. incognita* per plant a week after transplanting (To+Pc+RKN) at three time points: just after nematode inoculation (0 dani), at 7 dani, and at 42 dani. Each value is mean ± SE of three biological samples with two technical replicates each. Asterisks indicate significant differences ($P < 0.05$) according to the non-parametric Wilcoxon test.

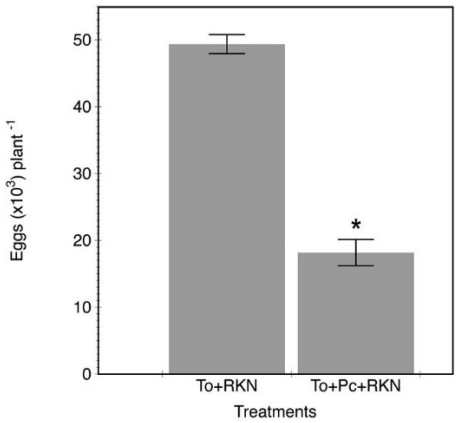

Figure 3. Effect of primed tomato plants by *P. chamydosporia* on *M. incognita* reproduction. Number of eggs produced in the tomato cv. Durinta after 42 days of being inoculated with 200 J2 of *M. incognita* per plant a week after transplanting (To+RKN) or inoculated with 10^5 chlamydospores of *P. chlamydosporia* isolate M10.43.21 just before transplanting and with the nematode at the same rate and time mentioned before (To+Pc+RKN). Each value is mean ± SE of three replications. Asterisk indicates significant differences ($P < 0.05$) according to the non-parametric Wilcoxon test.

Discussion

The results of this study provide evidence for the ability of some *P. chlamydosporia* isolates to induce systemically resistance against *M. incognita*, and that this induction is dependent on the plant species. The isolate M10.43.21 showed the most consistent response in both split-root experiments with tomato, and was the reason for selecting it to determine the hormone modulation in this plant species. The mechanisms responsible for the endophyte-induced resistance are unclear (Schouten, 2016). Both salicylic acid- and jasmonic acid-dependent signaling pathways have been proposed as responsible for the systemically induced resistance to *Meloidogyne* spp. in tomato in split-root experiments (Selim, 2010; de Medeiros et al., 2017; Martínez-Medina et al., 2017). Martínez-Medina et al. (2017) reported that *Trichoderma harzianum* T-78 induced the upregulation of genes related to salicylic acid at early stage of nematode infection, whereas those related to jasmonic acid were upregulated from 3 to 21 days after nematode inoculation. In our study, the *P. chlamydosporia* isolate primed salicylic acid from the first assessment time (7 days after fungal inoculation and just after nematode inoculation) until the end of the experiment (42 dani). This effect could be responsible for the suppression of nematode infection at early stages, as well as the infection by the J2 produced by the primary inoculum that were able to overcome the plant defense mechanisms. In addition, the upregulation of the *Lox D* gene, related to jasmonic acid, at 7 dani could affect nematode reproduction and fecundity. Thus, the induction of the salicylic acid and jasmonic acid signaling pathways by the fungal isolate M10.43.21 in tomato counteract the suppression of these phytohormones by the nematode described in the susceptible tomato-nematode interaction (Shukla et al., 2018). The three-phase model proposed to explain the induced protection to RKN by *Trichoderma harzianum* T-78 in tomato consisting of salicylic acid induction suppressing RKN infection followed by jasmonic acid induction suppressing RKN reproduction and fecundity and finally salicylic acid induction affecting root infection by the next J2 generation (Martínez-Medina et al., 2017) is valid for *P. chlamydosporia.* Other local plant defense mechanisms induced by *P. chlamydosporia* against RKN have been reported, including the increase of the peroxidases (*POX*) and poliphenoloxidases (*PPO*) enzymes activity at root nematode invasion stage (24–96 h after nematode inoculation) (de Medeiros et al., 2015). However, considering that *P. chlamydosporia* does not extensively colonize the root (Maciá-Vicente et al., 2009; Escudero & Lopez-Llorca, 2012), even being improved by chitosan irrigation (Escudero et al., 2017), the effect of local defense mechanisms alone may be insufficient to achieve significant nematode suppression.

Not all *P. chlamydosporia* isolates induced systemic resistance in tomato. The variability of this attribute among fungal isolates can be added to other observations previously reported, such as the production of chlamydospores, root colonization, plant growth promotion, or egg parasitism (Kerry & Bourne, 2002; Zavala- Gonzalez et al., 2015). In fact, egg parasitism can even differ between single-fungal spore isolates originating from the same field population (Giné et al., 2016). The frequency of occurrence of *P. chlamydosporia* in horticultural production sites under integrated and organic standards has increased

since the 1990s in northeastern Spain (Giné et al., 2012), showing that this fungal species is adapted to environmental characteristics and agronomic practices. Field populations of *P. chlamydosporia* can contain individuals representing a diversity of functions that are highly beneficial to plants, such as plant growth promoters which enhance plant tolerance; inducers of plant defense mechanisms suppressing infection, development and reproduction of RKN; efficient egg parasites suppressing the RKN inoculum; or saprophytic behaviour contributing to the organic matter cycle and plant nutrition. A given proportion of *P. chlamydosporia* representing some or all of these functions could be present in a given soil and adapted to the plant species involved in the rotation scheme and contributing to their health status. It seems that most of these functions are not interlinked. In fact, none of the five fungal isolates assessed in our study was a plant growth promoter. Thus, molecular tools must be developed to facilitate knowledge of the functional composition of the fungal field population in a given soil.

P. chlamydosporia has been reported as the main biotic factor responsible for soil suppressiveness to RKN in horticultural crops (Giné et al., 2016). In soils with low antagonistic potential, the use of fungal isolates with both direct and indirect action mechanisms could suppress RKN. Indeed, primed plants along with egg parasitism will protect against infection and reproduction of RKN and decrease the inoculum viability. The combination of the two modes of action will result in a decrease of the nematode population growth rate, and consequently lower crop yield losses. Alternatively, combining the use of *P. chlamydosporia* with plant defense activators can produce a similar effect. Vieira Dos Santos et al. (2014) found a reduction in RKN reproduction when *P. chlamydosporia* was combined with the application of cis-jasmone, as well as an increase in fungal egg parasitism.

In conclusion, this study proves that some *P. chlamydosporia* isolates induce systemic resistance to *M. incognita* in tomato but none of them in cucumber. Thus, this response is plant species dependent. In future studies, the interaction between *P. chlamydosporia* isolates and selected economically important crops should be characterized to elucidate the mechanisms and genes involved in inducing plant resistance in order to maximize the efficacy of control.

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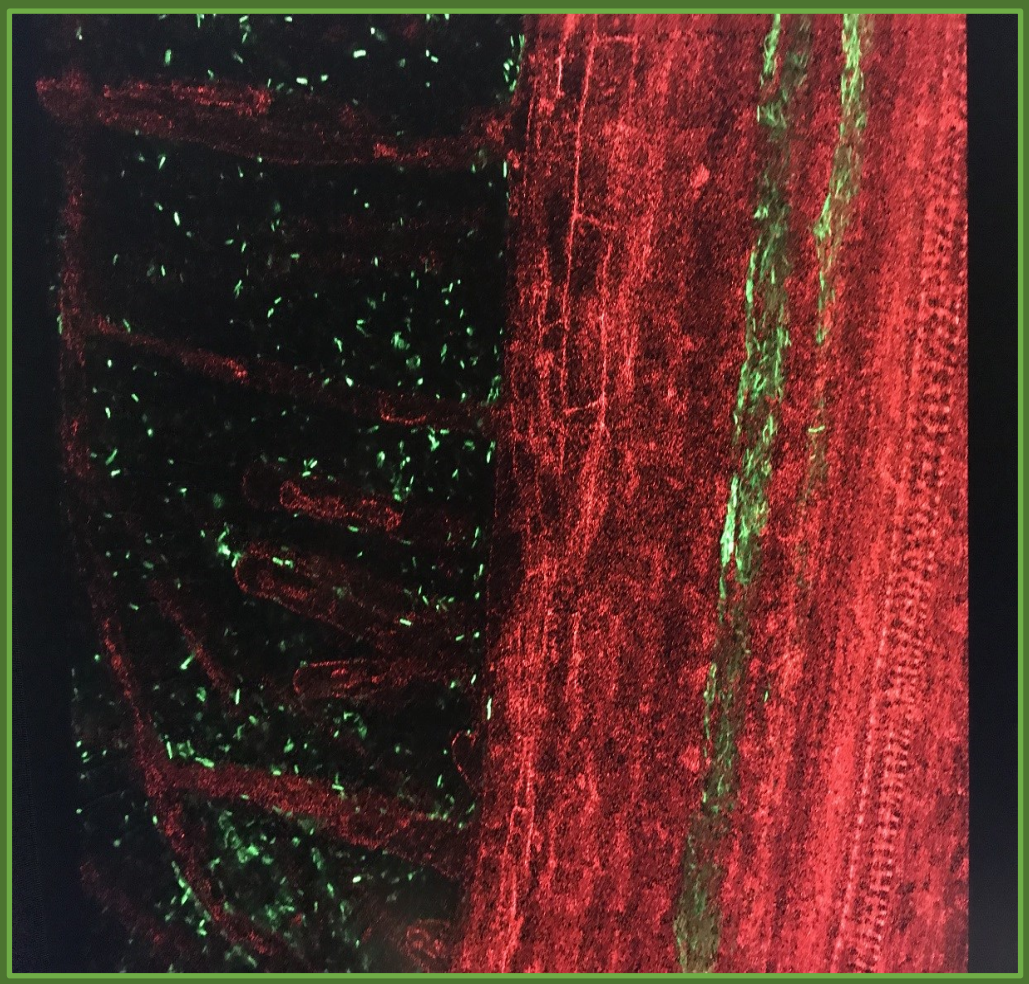
Chapter 2

Bacillus firmus strain I-1582, a nematode antagonist by itself and through the plant



Chapter 2: *Bacillus firmus* strain I-1582, a nematode antagonist by itself and through the plant

Zahra Ghahremani, Nuria Escudero, Daniel Beltrán-Anadón, Ester Saus, Marina Cunquero, Jordi Andilla, Pablo Loza-Alvarez, Toni Gabaldón and F. Javier Sorribas

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Abstract

Bacillus firmus I-1582 is approved in Europe for managing *Meloidogyne* on vegetable crops. However, little information about its modes of action and temperature requirements is available, despite this is needed for maximizing its efficacy. The cardinal temperatures for bacterial growth and biofilm formation were determined. The bacterial was transformed with GFP to study its effect on nematode eggs and root colonization of tomato (*Solanum lycopersicum*) and cucumber (*Cucumis sativus*) by laser-scanning confocal microscopy. Induction of plant resistance was determined in split-root experiments and the dynamic regulation of genes related to jasmonic acid (JA) and salicylic acid (SA) by RT-qPCR at three different times after nematode inoculation (dani). The bacteria grew and formed biofilm between 15 and 45 ºC; degraded the egg-shell and colonized the egg; colonized more extensively tomato than cucumber roots; induced systemic resistance in tomato, but did not in cucumber; SA and JA related genes were primed at different dani in tomato, but only SA at 7 dani in cucumber. In conclusion, *B. firmus* I-1582 is active in a wide range of temperatures being optimal at 35 ºC; is able to degrade *Meloidogyne* eggs, and to colonize plant roots inducing systemic resistance in a plant dependent species manner.

Keywords: *Cucumis sativus*, induced resistance, nematode antagonist, *Meloidogyne incognita*, root-knot nematodes, *Solanum lycopersicum*

Introduction

Plant parasitic nematode (PPN) management is a challenge for reducing crop yield losses all over the world. These parasites are responsible for annual yield losses reaching ca. 10% of life-sustaining crops and 14% of economically-important crops (Sasser & Freekmann, 1987), varying greatly between areas due to specific nematode-plant-environment interactions. Among PPN, root-knot nematodes (RKN) belonging to the genus *Meloidogyne* are the most damaging worldwide due to their wide range of plant hosts, worldwide distribution and high reproductive capacity (Jones et al., 2013). The potential damage of some *Meloidogyne*-crop combinations has been summarized by Greco and Di Vito, (2009). Currently, RKN management strategies tend to reduce dependence on chemical nematocides by encouraging alternative control methods promoting both the incompatible plant-RKN relationship by the use of plants bearing resistance genes (*R*-genes) and/or by microbe-inducing plant resistance, and the antagonistic potential of soils.

In a compatible plant-RKN relationship leading to a susceptible response, the second-stage juveniles (J2) of *Meloidogyne* enter the root near the elongation zone and migrate intercellularly to circumvent the endodermal barriers to establish a permanent feeding site into the vascular cylinder inducing the formation of giant cells and root galls. The J2 secrete effectors affecting cell wall architecture, plant development, plant defences and metabolism to complete their life cycle successfully (Shukla et al., 2018). Subsequently, J2 become sedentary and moult three times to achieve the mature adult female stage. The most frequent and damaging tropical species, *M. arenaria, M. incognita* and *M. javanica*, reproduce parthenogenetically. The female lays a large number of eggs in a gelatinous matrix, the egg mass, located on the surface or into the galled roots. In an incompatible plant-RKN relationship due to plant *R*-genes leading to a resistant response, the expression of plant genes related to phytohormone-mediated resistance responses, signal transduction pathways and secondary metabolite-mediated resistance responses are activated leading to a suppression of the nematode development and reproduction (Shukla et al., 2018). Plant hormones play a regulatory role in the induction of plant resistance by microorganisms (Pieterse et al., 2014). Primed plants can improve resistance to stressing agents along their life cycle and the effects can be maintained over generations (Mauch-Mani et al., 2017).

The antagonistic potential of a soil is the capacity to reduce the spread of deleterious agents to plants through biotic factors (Sikora, 1992). Several nematode antagonists can occur naturally in agricultural soils providing some extent of suppressiveness (Giné et al., 2016; Elhady et al., 2017). High levels of soil suppressiveness can be achieved under favourable interactions between plant-RKN-antagonists, cultural practices and abiotic factors (Giné et al., 2016). In soils with no or low levels of soil suppressiveness, nematode antagonists can be introduced by the application of biological-based nematicides. Currently, three biological-based nematicides are approved in Europe: *Bacillus firmus* I-1582, *Purpurocillium lilacinum* (=*Paecilomyces lilacinus*) strain 251 and *Pasteuria nishizawae* Pn1. Among

them, only *B. firmus* I-1582 and *P. lilacinum* strain 251 are approved to be used against RKN in vegetable crops.

Vegetables are important components of the diet that are cultivated worldwide in both open field and under protected conditions. Rotation sequences including fruiting solanaceous and cucurbit crops are very common because they represent the main source of income for growers but all of them are affected by RKN (Hallmann & Meressa, 2018). Some attempts to manage RKN by *Purpurocillium lilacinum* (=*Paecilomyces lilacinus*) strain 251 have shown a high percentage of fungal egg parasitism in both *in vitro* and pot experiments (Khan et al., 2004 and 2006; Kiewnick & Sikora, 2006a, b; Kiewnick et al, 2011). However, limited efficacy has been reported in field experiments under Mediterranean conditions (Anastasiadis et al., 2008; Kaşkavalci et al., 2009; Giné & Sorribas, 2017), probably due to sustained sub-optimal soil temperatures during the cropping seasons and/or the inhibition of fungal enzymes by components of the formulation (Giné & Sorribas, 2017). Regarding *Bacillus firmus* I-1582, some reports have shown its effectivity against several plant parasitic nematodes (PPNs) including RKN under different experimental conditions, from *in vitro* to field experiments on different crops (Giannakou et al., 2004 and 2007; Mendoza et al., 2008; Terefe et al., 2009; Castillo et al., 2013). This bacterial strain reduced egg hatching and the viability of nematodes in *in vitro* experiments, suppressed nematode reproduction and disease severity. Moreover, triggered systemic resistance (ISR) in soybean (*Glycine max*) to *Heterodera glycines,* and in cotton to RKN in split-root experiments, but did not in corn (*Zea mays*) against *M. incognita* (Schrimsher, 2013; Gattoni et al., 2018). The interactions of *Bacillus* species and plants with special reference to ISR have been reviewed (Choudhary & Johri, 2009). It seems that the specific transduction pathway promoted during ISR by *Bacillus* spp. depends on the bacterial strain, the host plant and the plant pathogen (Kloepper et al, 2004). Extensive work has been done with several *Bacillus s*pecies but a few with *B. firmus* (Wilson & Jackson, 2013).

The optimal and successful use of microbial antagonists against RKN on vegetable irrigated crops has to consider the cardinal temperatures of microbial growth, the effect on the most abundant and vulnerable nematode development stages, and the plant-microbe interaction, especially the putative induction of plant resistance against nematodes. In this manuscript, all these aspects are presented: i) the cardinal temperatures for *B. firmus* I-1582 growth and biofilm formation; ii) the effect of *B. firmus* I-1582 transformed with the green fluorescent protein gene (*B. firmus* I-1582-GFP) on RKN eggs; iii) the colonization of tomato and cucumber roots by *B. firmus* I-1582-GFP; and iv) the putative induction of systemic resistance and the dynamic regulation of genes related to the jasmonic acid (JA) and salicylic acid (SA) pathways in tomato (*Solanum lycopersicum*) and cucumber (*Cucumis sativus*).

Materials and methods

Plants, nematode and bacteria

Tomato cv. Durinta and cucumber cv. Dasher II, both susceptible to root-knot nematodes, were used in this study. For all the experiments seeds were surface-sterilized in a 50% bleach solution (40 g l^{-1} NaOCl) for 2 min, washed three times in sterilized distilled water for 10 seconds each, sown in a tray containing sterile vermiculite and maintained in a growth chamber at 25 °C ± 2 ºC with a 16h:8h (light:dark) photoperiod. Second-stage juveniles (J2s) of the *Meloidogyne incognita* isolate Agropolis were used as inoculum. Eggs were extracted from tomato roots by blender maceration in a 5% commercial bleach (40 g l^{-1} NaOCl) solution for 10 min (Hussey & Barker, 1973). The egg suspension was passed through a 74 µm aperture sieve to remove root debris, and eggs were collected on a 25 µm sieve and placed on Baermann trays (Whitehead & Hemming, 1965) at 25 °C ± 2 ºC. J2s were collected daily using a 25 µm sieve during 7 d and stored at 9 ºC until their use.

Bacillus firmus strain I-1582 (Bf I-1582) isolated from the VOTiVO® formulation (Bayer CropScience) was used in the cardinal temperatures and biofilm formation experiments, as well as for the bacterial transformation with the green fluorescent protein (GFP) gene to study root and nematode eggs interactions. The commercial formulates VOTiVO® was used in the induction of plant resistance experiments and gene expression analysis.

Bf I-1582 GFP transformation

Bf I-1582 was transformed with the GFP gene using the pAD43-25 plasmid (Dunn & Handelsman, 1999) kindly provided by the *Bacillus* Genetic Stock Center. Bacterial protoplasts were prepared according to Aono et al. (1992) with slight modifications. Three ml of *B. firmus* I-1582 cells grown overnight in Penassay medium (1.5 g l^{-1} beef extract, 1.5 g l^{-1} yeast extract, 5 g l^{-1} peptone, 1 g l^{-1} glucose, 3.5 g l^{-1} NaCl, 3.6 g l^{-1} dipotassium phosphate and 1.32 g l^{-1} monopotassium phosphate) were harvested by centrifugation at 3000 g for 10 min at 4°C. The pellet was suspended in 0.1 ml of SMMP medium consisting of SMM (0.5M sucrose, 0.02 M maleic acid and 0.02 M $MgCl_2$) in double-strength Penassay media pH 6.4, supplemented with 40 mg of lysozyme, and incubated at 37 °C with gently shaking for 75 min. The *B. firmus* protoplasts were recovered by centrifugation at 1000 g for 30 min at 10 ºC, washed twice with SMMP medium and finally suspended in 0.1 ml of SMMP media.

The *B. firmus* transformation was performed according to Chang and Cohen (1979). Briefly, 150 ng of purified plasmid pAD43-25 were mixed with 5µl of 2X strength SMM buffer and 50 µl of *B. firmus* protoplasts. Then, 150 µl of 40% Polyethylene Glycol (PEG) were added, and 0.5 ml of SMMP media were

added after 2 min of incubation and protoplasts were recovered by centrifugation at 2500 rpm for 10 min. Protoplasts were suspended in 0.1 ml of SMMP media and kept at 30 °C with gently shaking for 4 h. Finally, cells were plated in solid LB supplemented with 20 µg ml^{-1} chloramphenicol and incubated at 37 ºC. After 2 d, the GFP expression of pAD43-25 of the transformed *B. firmus* colonies was assessed by fluorescence microscopy.

To corroborate that the species transformed with the pAD43-25 plasmid was *B. firmus,* one stable colony transformant was selected. The colony was grown O/N in LB before performing a genomic DNA extraction and a PCR with BLS342F (5' CAGCAGTAGGGAATCTTC 3') and 1392R (5' ACGGGCGGTGTGTACA 3') primers following the conditions described in Blackwood et al., (2005). The 1050 bp PCR product obtained was sequenced.

Cardinal temperatures of Bf I-1582 and biofilm formation

The cardinal temperatures of Bf I-1582 were determined. Sets of three Petri dishes containing nutrient agar (3 g l^{-1} Beef extract, 5 g l^{-1} Peptone, 5 g l^{-1} NaCl, 15 g l^{-1} agar, pH 7) were inoculated with 200 bacteria colony-forming units (CFU) and incubated in the dark at 4, 9, 20, 25, 30, 35, 40, 45 and 50 ºC for 96 h before counting.

Growth kinetics in liquid media were determined inoculating 10^6 CFU in Erlenmeyer flasks containing 200 ml of LB. Sets of three Erlenmeyer flasks were incubated at 10, 15, 20, 25, 30, 35, 40, 45, and 50 ºC. Cultures were maintained with shaking and one aliquot of three ml was taken at 0, 2, 4, 6, 8, 10, 12, 24, 30, 36 and 48 h to measure the optical density at 590 nm (OD_{590nm}) in an UV-Vis Evolution 300 spectrophotometer (ThermoFisher Scientific, USA).

Sets of three Petri dishes (40 mm diameter) with 10 ml of SGG medium (5 g l^{-1} Beef extract, 10 g l^{-1} Peptone, 2 g l^{-1} KCl, 0.5 g l^{-1} $MgSO_4$ $7H_2O$, 1 mM $Ca(NO_3)_2$, 0.1 mM $MnCl_2 \cdot 4\ H_2O$, 1 µM $FeSO_4$, 1 g l^{-1} glucose, and 1% glycerol) were inoculated with 10^6 CFU and incubated at 10, 15, 20, 25, 30, 35, 40, 45, and 50 ºC in the dark for 2 d to determine the effect of the temperature on the Bf I-1582 biofilm formation (Hsueh et al., 2015).

Live-cell imaging of tomato and cucumber roots and M. incognita eggs colonized by Bf I-1582-GFP

Tomato and cucumber seeds were surface-sterilized, as described previously, and were placed on a moist chamber at 25 ºC until the main root was ca. 2 cm (5-7 d). Seedlings were transferred to an Erlenmeyer flask filled with 80 ml of 0.5X Murashige and Skoog (MS) semisolid agar medium (0.07 % agar) and inoculated with 20 ml of the Bf I-1582-GFP overnight culture grown at 35 ºC in liquid LB supplemented with 20 µl ml^{-1} chloramphenicol (Sigma). The seedlings were incubated at 25 ºC for 5 and 10 d (Hao & Chen, 2017). Afterwards, roots were washed with distilled water to eliminate non-adhered

bacteria and ten fragments of the root system (ca. 1 cm long) were examined with laser-scanning confocal microscopy. To determine the spatial pattern of root colonization by Bf I-1582-GFP, roots were imaged in a Leica TCS 5 STED CW microscope (Leica Microsystem) equipped with hybrid detectors and with a 40x 1.25NA HCX Pl Apo CS Leica objective. A 488 nm laser was used for fluorescence excitation. GFP fluorescence was detected at 505-530 nm and autofluorescence of root cell walls at 580-620 nm, as described in Macià-Vicente et al. (2009). Stacks of 8 - 13 µm, step size of 0.2 - 0.3 µm, were acquired. Z projection-images and Z stack movies are shown at Figure 2. and Video S2, respectively. All image analysis was performed using Fiji (Schindelin et al., 2012).

Nematode eggs were surface-sterilized as in McClure et al. (1973). A 200 µl suspension containing ca. 100 eggs were dispensed in a 1.5 ml microcentrifuge tube containing 50 µl of the bacteria culture grown on LB supplemented with 20 µg ml^{-1} chloramphenicol (Sigma) at 35 ºC for 2 d. After 3, 5 and 10 d of incubation at 35 ºC ± 2 ºC eggs were examined with laser-scanning confocal microscopy in a Leica TCS 5 STED CW microscope (Leica Microsystem) equipped with hybrid detectors and with a 63x 1.4NA HCX Pl Apo CS Leica objective. A 488 nm laser was used for fluorescence excitation and transmission-light detection. GFP fluorescence was detected at 505-530 nm and eggs autofluorescence was detected at 580-620 nm (Escudero & Lopez-Llorca, 2012). Stacks of 8 - 13 µm, step size of 0.2 - 0.3 µm, were acquired. Z projection-images are shown at Figure 3. A three-dimensional (3D) reconstructed Z-stack (Video S1) of a *M. incognita* egg after 10 days from bacterial inoculation were done with Huygens software (Huygens SVI, Netherlands).

Induction of plant resistance by Bf I-1582 against M. incognita

Tomato and cucumber were grown in a split-root system as described in previous studies (Ghahremani et al., 2019). In this system, the root is divided into two halves transplanted in two adjacent pots: the inducer, inoculated with Bf I-1582 and the responder inoculated with *M. incognita*. Briefly, the main root of 5-d-old seedlings was excised and plantlets were individually transplanted in seedling trays containing sterile vermiculite and maintained in a growth chamber at 25 ± 2 ºC and 16 h:8 h photoperiod for 3 wk for tomato and 2 wk for cucumber. Afterwards, plantlets were transferred to the split-root system by splitting roots into two halves planted into two adjacent 200 cm^3 pots filled with sterilized sand. Three treatments were assessed: Bf I-1582/*M. incognita* (Bf-Mi) and None/*M. incognita* (None-Mi) to assess the capability of Bf I-1582 to induce plant resistance against *M. incognita*, and None/None to assess the effect of the split root system on the development of each root half. Each treatment was replicated 10 times, and the experiment with tomato was repeated once.

The inducer pot was inoculated with a liquid suspension of 10^9 CFU of BF I-1582 one wk after transplanting. One wk after bacterial inoculation, the responder pot was inoculated with a liquid suspension to achieve a rate of 1 J2 of *M. incognita* per cm^3 of soil. The None treatment received the same volume of water. The plants were maintained in a growth chamber under the same conditions described previously in a completely randomized design for 43 d. The plants were irrigated as needed and fertilized with Hoagland solution twice per wk. Soil temperatures were recorded daily at 30-min intervals with a PT100 probe (Campbell Scientific Ltd) placed in the pots at a depth of 4 cm. At the end of the experiment, plants were uprooted and the fresh root weight of both inducer and responder halves from the None-None treatment were measured. Roots from the *M. incognita* inoculated responder pots were immersed in a 0.01% erioglaucine solution for 45 min to stain the egg masses (Omwega et al., 1988) before counting them. Afterwards, the nematode eggs were extracted from the roots in a 10% commercial bleach solution (40 g l^{-1} NaOCl) for 10 min (Hussey & Barker, 1973) and counted.

The endophytic root colonization by Bf I-1585 was estimated at the end of the first and second experiment with cucumber and tomato, respectively. The bacterial DNA was quantified by qPCR from three individual biological samples from the Bf-*Mi* treatment. Each biological sample consisted on the inducer part of the roots from three plants. The inducer part of the root was surface sterilized using 50% commercial sodium hypochlorite (40 g l^{-1} NaOCl) for 2 min and washed three times with sterile distilled water for 10 s each, and then blotted onto sterile paper. The DNA was extracted from each biological replicate following the López-Llorca et al. (2010) procedure. qPCR reactions were performed using the Brilliant Multiplex QPCR Master Mix in a final volume of 25 µl containing 50 ng of total DNA and 0.5 µM of each primer (5' to 3' direction): Votivo-2F (forward) CTCCAATTCCTAATATCCTGCAAAG, Votivo-2R (reverse) GGAAAGTCACGGGACAGTTAT (Mendis et al., 2018). Negative controls containing sterile water instead of DNA were included. Reactions were performed in duplicate in a Stratagene Mx3005P thermocycler (Agilent Technologies) using the following thermal cycling conditions: initial denaturation step at 95 ºC for 15 min, then 39 cycles at 95 ºC for 30 s, and 58 ºC for 1 min. DNA of *B. firmus* I-1582 was used to define a calibration curve ranging from 5 pg to 50 ng. PCR specificity was verified by means of melting curve analysis and agarose gel electrophoresis. The Bf DNA was referred to the total DNA biomass (50 ng) and expressed as a proportion.

Dynamic expression of JA and SA related genes by Bf I-1582 and M. incognita in tomato and cucumber

Two-wk-old cucumber seedling and three-wk-old tomato seedlings were transferred to 200 cm^3 pots with sterilized sand, and maintained in a growth chamber as previously described. The assessed treatments were: non-inoculated plants (Control), plants inoculated with Bf I-1582 (Bf), plants inoculated with *M. incognita* (Mi) and plants co-inoculated with both organisms (Mi+Bf). Bf I-1582 treatments were

inoculated with 10^9 CFU one wk after transplanting, and those *M. incognita* treatments were inoculated with 200 J2 two wk after transplanting. The expression of the genes related to JA and SA pathways was evaluated at 0 days after nematode inoculation (dani), at 7 dani, when the nematode infected the roots, and at 40 dani. Root infection was determined by staining the nematode into the root with acid fuchsin (Byrd et al., 1983). At 40 dani, the nematode eggs were extracted by the Hussey and Barker's method (1973) from roots of three individual tomato or cucumber plants from all treatments inoculated with the nematode. The tomato and cucumber total root colonization by Bf I-1585 was estimated from three different plants of each Bf treatment by qPCR at 0 and 40 dani. The roots were washed three times in sterilized distilled water for 10 s each and then blotted onto sterile paper. The DNA extraction and qPCR were conducted as previously described.

For the expression study, three biological replicates were assessed at each sampling time. Each biological replicate consisted on the roots from three plants pooled together. Roots excised from the aboveground were washed three times with sterile distilled water, blotted on sterile filter paper, and immediately frozen on liquid nitrogen. Samples were stored at -80 °C until their use. RNA isolation and retrotranscription were carried out as in Ghahremani et al. (2019). The dynamic regulation in the JA pathway was determined by the expression of the lipoxygenase D (*Lox D*) gene for tomato (Fujimoto *et al*., 2011) and lipoxygenase 1 (*Lox 1*) gene for cucumber (Shoresh et al., 2004). In the SA pathway we evaluated the expression of the pathogenesis-related 1 (*PR 1*) gene for tomato (Gayoso et al., 2007) and phenylalanine ammonia-lyase (*PAL*) gene for cucumber (Shoresh et al., 2004). The ubiquitin (*UBI*) gene was used as a reference gene for both plant species (Song et al., 2015, Yang et al., 2012). The relative gene expression was estimated with the ΔΔCt methodology (Livak & Schmittgen 2001). The sequences of the primers used in the RT-qPCR are shown in supplementary Table 1. qPCR reactions were performed in a final volume of 20 µl with 1 µL of cDNA, 0.3 mM primers and 1X Fast SYBR Green Master Mix (Applied Biosystems). The qPCR was performed in a 7900HT Fast Real Time PCR System thermocycler (Applied Biosystems). Reactions were performed with two technical replicates per each biological replicate using the following conditions: 20 s at 95 °C followed by 40 cycles of 30 s at 95 °C and 1 min at 60 °C for tomato (Gayoso et al., 2007) and 20 s at 95 ° C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C for cucumber (Shoresh et al., 2004). PCR specificity was verified by means of melting curve analysis and agarose gel electrophoresis.

Table 1. Primers used to quantify expression of genes related with jasmonic acid and salicylic acid pathways using qRT-PCR.

Gene	Plant	Primers	Primer secuence (5'-3')	PCR product size (pb)	Reference
UBI	Tomato	LeUbi3-F LeUbi3-R	TCCATCTCGTGCTCCGTCT GAACCTTTCCAGTGTCATCAACC	144	Song, et. al. (2015)
	Cucumber	UBQ-F UBR-R	GGTGCCAAGAAGCGTAAGAA CACCAGCTTTGTTGTAAACGT	260	Yang, et al. (2012)
LOX	Tomato	LoxD-F LoxD-R	GACTGGTCCAAGTTCACGATCC ATGTGCTGCCAATATAAATGGTTCC	178	Fujimoto, et al. (2011)
	Cucumber	Lox1-F Lox1-R	AAGGTTTGCCTGTCCCAAGA TGAGTACTGGATTAACTCCAGCCAA	201	Shoresh, et.al. (2004)
PAL	Tomato	LEPR1F LEPR1R	GCAACACTCTGGTGGACCTT ATGGACGTTGTCCTCTCCAG	272	Gayoso et. al. (2007)
	Cucumber	Pal1-R Pal1-F	CCATGGCAATCTCAGCACCT ATGGAGGCAACTTCCAA	201	Shoresh, et.al. (2004)

Statistical analysis

Statistical analyses were performed using the JMP software v8 (SAS institute Inc., Cary, NC, USA). Both data normality and homogeneity of variances were assessed. When confirmed, a paired comparison using the Student's t-test was done. Otherwise, paired comparison was done using the non-parametric Wilcoxon test ($P < 0.05$).

Results

Cardinal temperatures of Bf I-1582 and biofilm formation

Bf I-1582 grew in the range of temperatures from 15 ºC to 45 ºC, being 35 ºC the optimal growth temperature in both solid (Figre 1. a) and liquid media (Figure 1. b), but did not at 10 ºC or 50 ºC. Similarly, biofilm formation was observed between 15 ºC and 45 ºC but neither at 10 ºC nor 50 ºC, being thicker and uniform at 35 ºC (Figure 1. c).

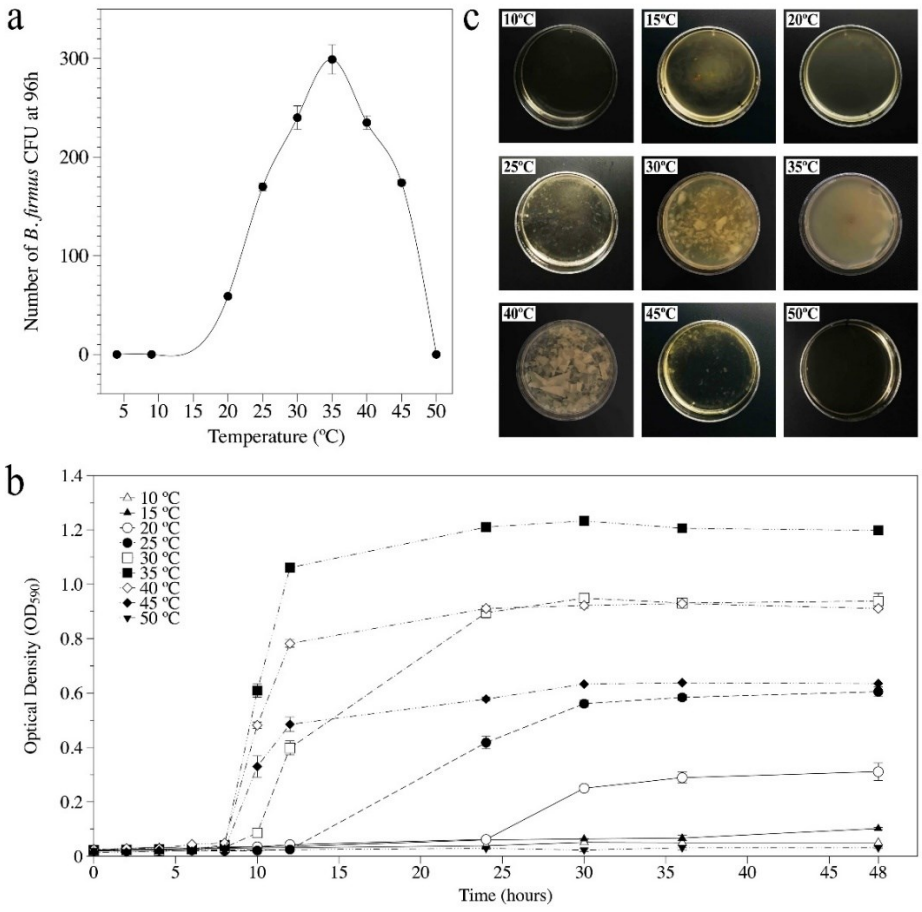

Figure 1. Cardinal temperatures for *B. firmus* I-1582 growth and biofilm formation. (a) Number of bacterial CFU after 96 h of incubation in solid nutrient agar at different temperatures. Each value is mean ± standard error of three replicates. (b) Bacterial growth kinetics incubated in LB liquid medium at different temperatures. Each value is mean ± standard error of three replicates. (c) Biofilm formation in SGG medium 48 h after being inoculated with 10^6 bacterial CFU and incubated at different temperatures.

Tomato and cucumber roots colonization and M. incognita eggs degradation by Bf I-1582-GFP

Both tomato and cucumber roots were colonized by *B. firmus* (Figure 2). In tomato, Bf I-1582GFP was observed on root hairs and epidermal cells at 5 d after inoculation (dai) (Figure 2. a). At 10 dai bacterial colonies were observed in root hairs and some bacteria were found inside the root (Figure 2. c). In cucumber, few bacteria were observed on epidermal cells at 5 dai (Figure 2. b). There were no bacteria found inside the root at 10 dai (Figure 2. d).

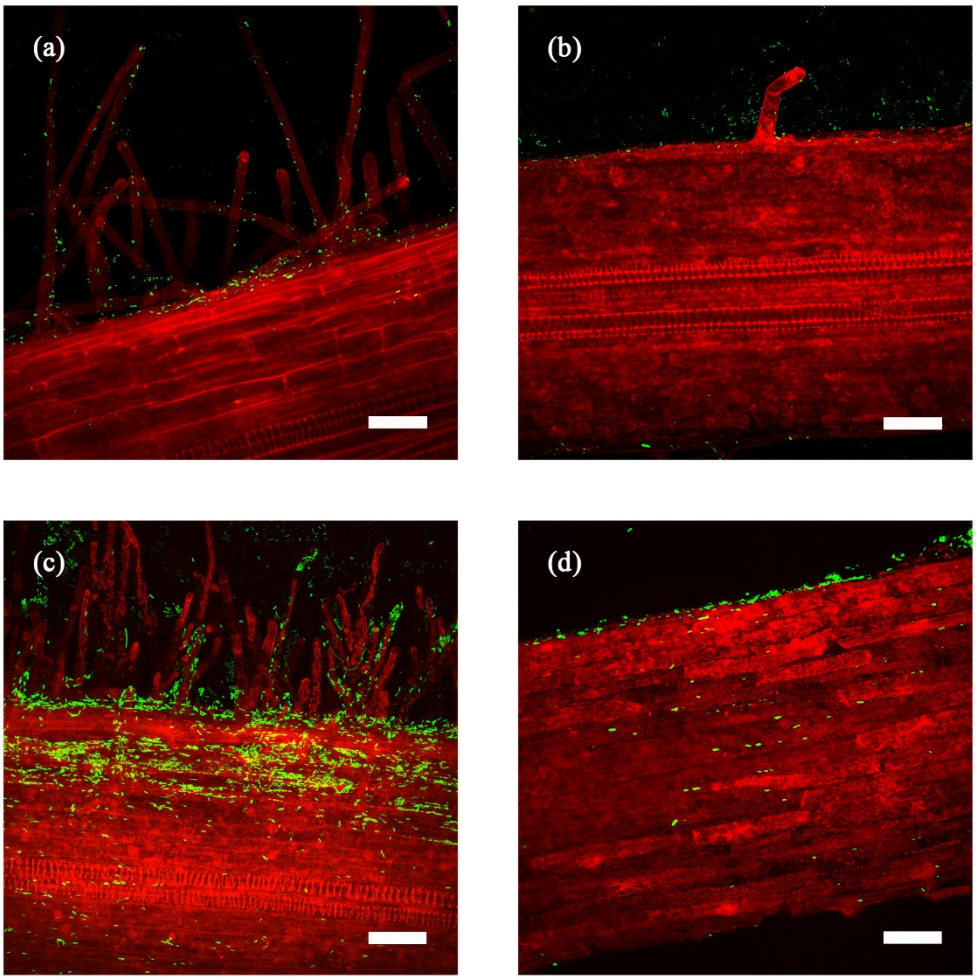

Figure 2. Z projection of laser-scanning confocal microscopy images of *B. firmus* I-1582 transformed with the green fluorescent protein gene (Bf I-1582GFP) colonizing tomato (a, c) and cucumber (b, d) roots after 5 (a, b) and 10 (c, d) d from bacterial inoculation and incubation at 25 ºC. Scale bar: 50 µm.

The *M. incognita* egg-shell degradation and egg colonization by Bf I-1582-GFP were studied with confocal-scanning laser microscopy (Figure 3). At 3 dai, bacteria were surrounding and degrading the nematode egg and embryo (Figure 3. a); at 5 dai, bacterial colonies adhered to the egg-shell and some bacteria were found inside the egg (Figure 3. b); at 10 dai bacterial biofilms adhered to the egg-shell and inside the egg were observed (Figure 3. c).

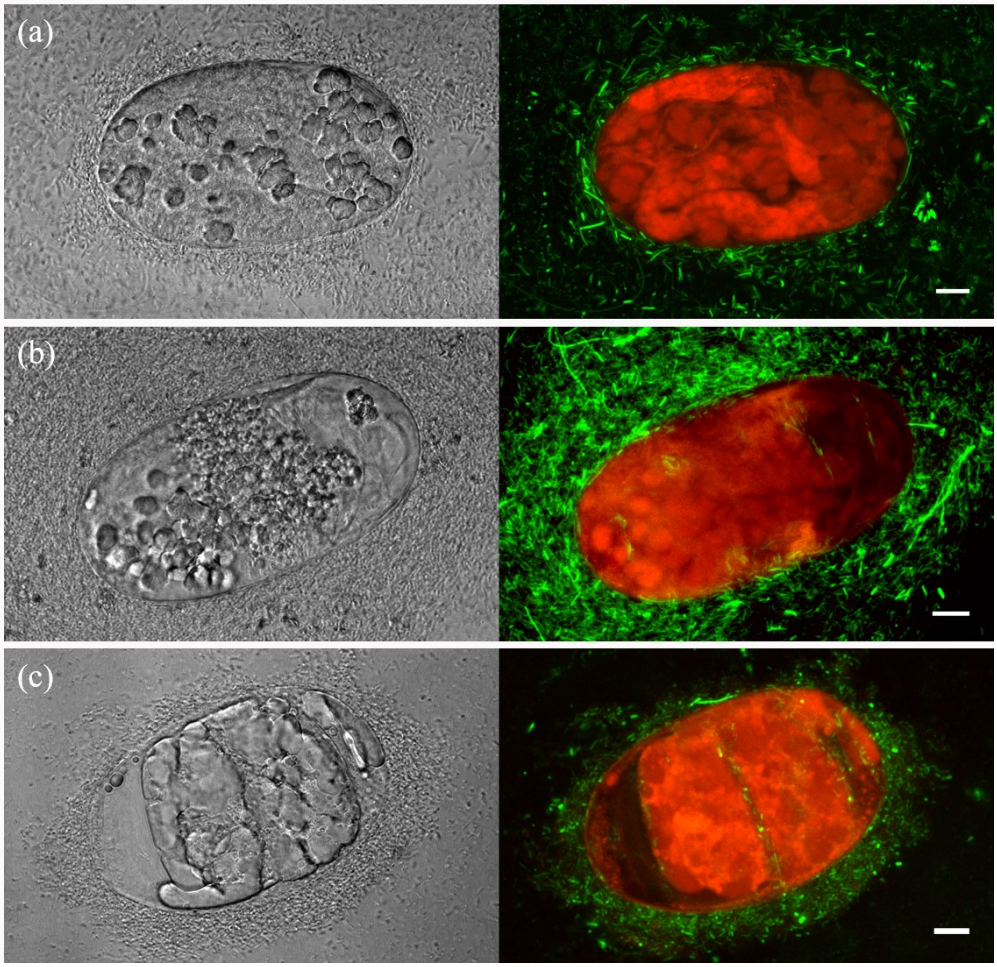

Figure 3. Laser-scanning confocal microscopy images of *B. firmus* I-1582 transformed with the green fluorescent protein gene (Bf I-1582GFP) damaging *M. incognita* eggs after 3 (a), 5 (b) and 10 (c) d of incubation at 35 ºC. Z projection images of transmitted (left) and fluorescence (right) channels. Scale bar: 10 µm.

Bf I-1582 induces plant resistance to M. incognita in tomato but not in cucumber

The split-root system did not influence the root development because the tomato and cucumber fresh root weight of the two halves of the split-root system from the None-None treatment did not differ ($P < 0.05$; data not shown).

Fewer ($P < 0.05$) egg masses and eggs per plant were recorded in the responder part of the root of tomato of the Bf-Mi treatment compared to the None-Mi, irrespective of the experiment (Figure 4 a, b, d, e). However, no effect was detected in cucumber (Figure 4 c, e).

The standard curve for qPCR used for estimating the bacterial DNA density was: Ct= -3.1413 * log10 DNA concentration + 24.522 (R^2 = 0.9591). *B. firmus* colonized roots of both plant species endophytically, but ca. 61% more ($P < 0.05$) density of bacterial DNA was recorded in tomato than

cucumber roots (Figure 5.a). The bacterial colonization of tomato roots were ca. 65% higher in tomato than cucumber at 0 and 40 dani (Figure 5. b, c).

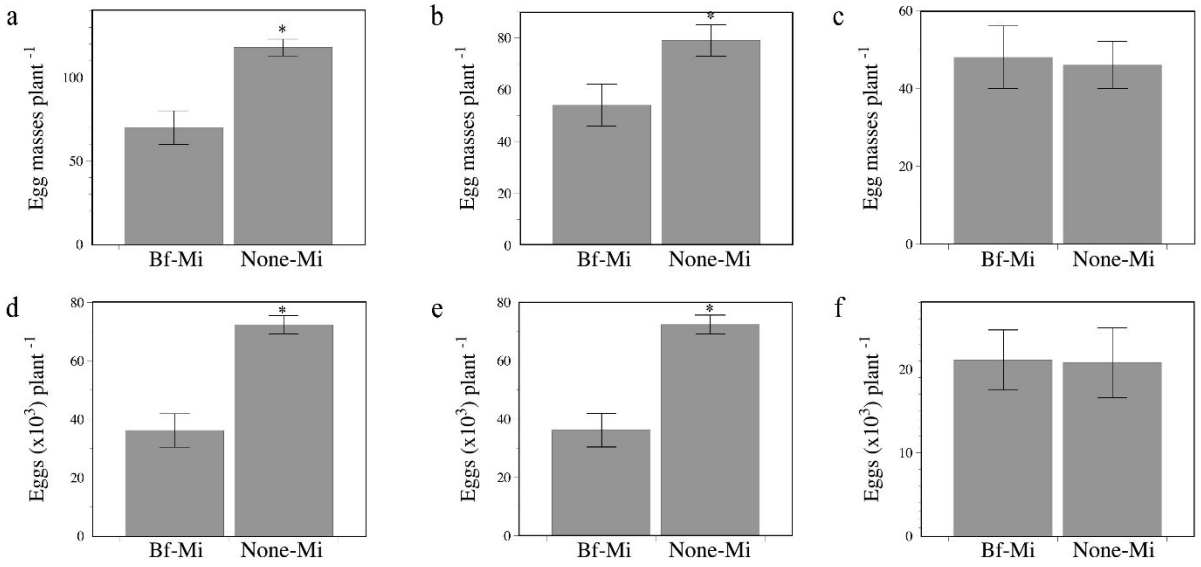

Figure 4. Capability of *B. firmus* I-1582 to induce systemic resistance in tomato cv. Durinta and cucumber cv. Dasher II against *Meloidogyne incognita* in two split root experiments. The inducer part of the root was inoculated with 10^9 CFU of *B. firmus* I-1582 and the responder part of the root was inoculated with 200 J2 of *M. incognita*. In the responder part of the root the number of egg masses per plant (infectivity) and the number of eggs per plant (reproduction) were assessed 43 d after nematode inoculation. Number of egg masses per plant (a-c) and total nematode eggs per plant (d-f). Tomato experiment 1 (a, d); Tomato experiment 2 (b, e); Cucumber experiment (c, f). Data are mean ± standard error of 10 replicates. Data within the same column and experiment followed by * are different ($P < 0.05$) according to the non-parametric Wilcoxon test.

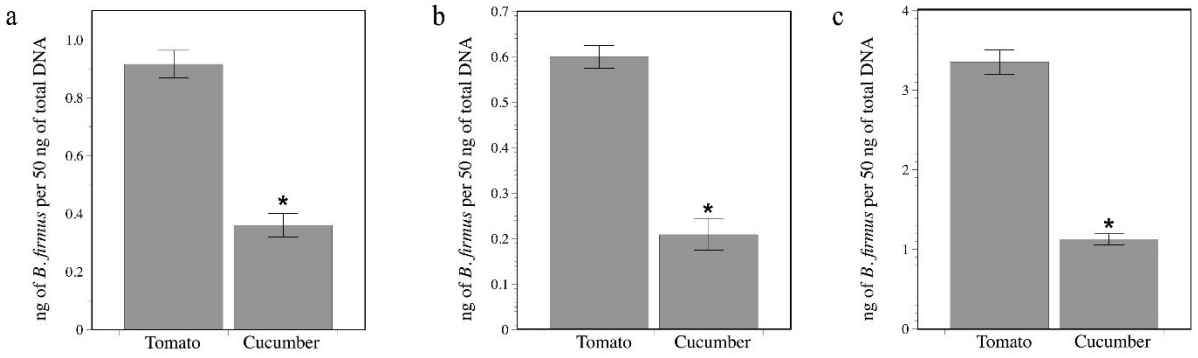

Figure 5. *B. firmus* I-1582 colonizes tomato and cucumber roots. (a) Endophytic bacterial DNA biomass in relation to the total DNA biomass of the sample from the inducer half of the root 50 days after being inoculated with 10^9 CFU and 43 d after nematode inoculation whit 200 J2 in the responder half of the root from the split-root experiment. The inducer part of the root was surface sterilized with 50% commercial sodium hypochlorite for 2 min and washed three times with sterile distilled water for 10 s each previous to the DNA extraction. Each value is mean ± standard error of three biological replicates composed of three plant roots each. (b) Bacterial DNA biomass in relation to the total DNA biomass of the sample 7 d after bacterial inoculation at a rate of 10^9 CFU per plant and just after nematode inoculation with 200 J2 (0 dani) and (c) at 40 dani. Each value is mean ± standard error of three biological replicates composed of one plant root each. Asterisk indicates significant difference between plant species according to the non-parametric Wilcoxon test ($P < 0.05$)

Dynamic regulation of JA and SA genes by Bf I-1582 is plant-dependent

The dynamic regulation of genes related to JA and SA pathways varied between tomato and cucumber (Figure 6). In tomato, at 0 d after nematode inoculation (dani), which corresponds to 7 d after Bf I-1582 inoculation and just after nematode inoculation, both JA (*Lox D*) and SA (*Pr 1*) pathways were up-regulated in plants inoculated with the bacteria in comparison to non-inoculated plants (Figure 6. a). At 7 dani, when nematode infection was established, only the plants co-inoculated with nematode and bacteria showed an up-regulation of the JA related gen (*Lox D*) (Figure 6. b). At 40 dani, when egg hatching began and new root infections occurred, tomato plants co-inoculated with the nematode and Bf I-1582 had repressed the JA related gen (*Lox D*), while the gene related to the SA pathway (*Pr 1*) was up-regulated in plants co-inoculated and also inoculated with Bf I-1582 alone, but was repressed in plants inoculated only with the nematode (Figure 6. c).

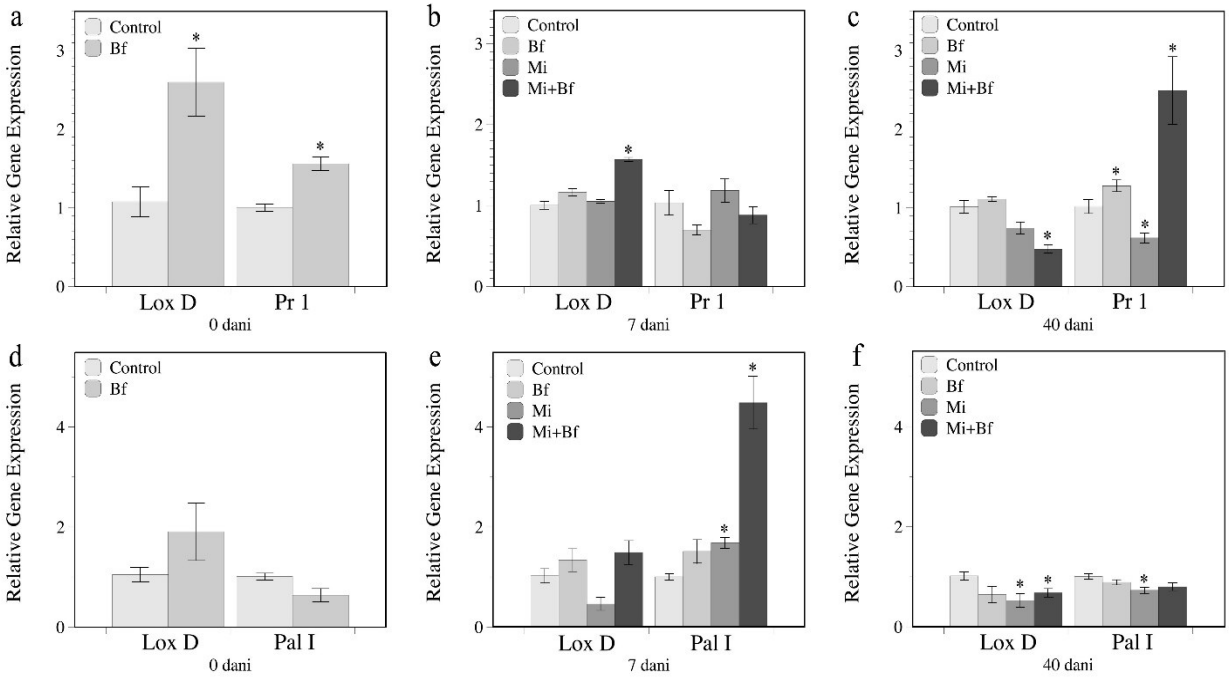

Figure 6. Dynamics of the relative gene expression of genes related to the jasmonic acid (*Lox D*) and the salicylic acid (*Pr1/Pal I*) pathways in tomato (a-c) and cucumber (d-f) plants non-inoculated (Control), plants inoculated with Bf I-1582 (Bf), plants inoculated with *M. incognita* (Mi) and plants co-inoculated with both nematode and bacteria (Mi+Bf). Bf I-1582 treatments were inoculated with 10^9 CFU one wk after transplanting, and those *M. incognita* treatments were inoculated with 200 J2 two wk after transplanting. The relative expression of the genes was evaluated at 0 d after nematode inoculation (dani), at 7 and at 40 dani. The ubiquitin (*UBI*) gene was used as a reference gene for both plant species. Each value is mean ± standard error of three biological samples with two technical replicates each. Each biological replicate consisted on the roots from three plants pooled together. Asterisks indicate significant differences with respect to the Control using the non-parametric Wilcoxon test ($P < 0.05$).

In cucumber, no differences between treatments were found at 0 dani (Figure 6. d). At 7 dani the SA pathway (*Pal I*) was up-regulated both in the *M. incognita* inoculated plants and those co-inoculated with the bacteria and the nematode (Figure 6. e). At 40 dani, both JA and SA pathways were repressed in plants inoculated with *M. incognita* but only JA in the co-inoculated plants (Figure 6. f).

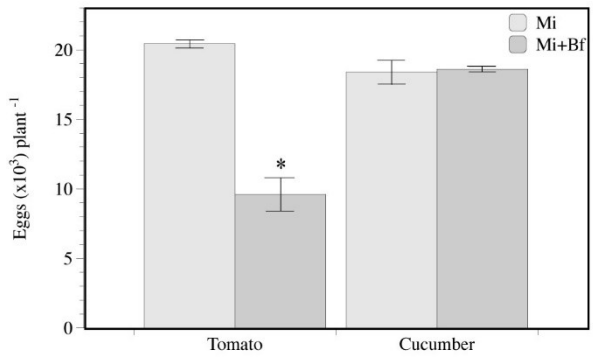

Figure 7. *Meloidogyne incognita* reproduction is affected in Bf I-1582 primed tomato plants but not in cucumber. Number of eggs in tomato and cucumber plants 40 d after inoculation with *M. incognita* (Mi) or co-inoculated with 10^9 CFU of Bf I-1582 one wk after transplanting and with 200 J2 of *M. incognita* two wk after transplanting (Mi+Bf). Each value is mean ± standard error of three replicates. Asterisks indicate significant differences between treatments per each plant species according to the non-parametric Wilcoxon test in tomato or the Student's t-test in cucumber ($P < 0.05$).

The nematode reproduction in tomato co-inoculated with the bacteria was reduced ($P < 0.05$) in a 53%, but it did not differ between treatments in cucumber (Figure 7).

Discussion

The results of this study contribute to a better understanding of the interactions between *B. firmus* with tomato and cucumber, two economically important fruiting vegetables cultivated worldwide; as well as with nematode eggs, which is the most abundant nematode developmental stage in soil and in roots. In addition, the determination of the cardinal temperatures of bacterial growth and biofilm formation provide valuable information to optimize the use of *B. firmus*–based formulations and to maximize the nematode control efficacy.

B. firmus was able to colonize both tomato and cucumber roots, but more extensively those of tomato. The GFP-transformed bacteria colonized the rhizoplane of both plant species, and the observation of attenuated fluorescence indicates that some bacterial could colonize tomato roots endophytically. This finding was corroborated by qPCR, showing that the proportion of bacteria inside or on tomato roots was ca. 65% higher than that found inside or on cucumber roots. It is known that *B. firmus* is able to colonize roots of other economically important crops such as corn, soybean and cotton (Mendis et al., 2018; Gattoni et al., 2019). Root colonization by bacteria could prevent nematode infection and production of viable

inoculum by affecting the nematode cuticle and the egg-shell. Some reports pointed out the capability of *B. firmus* to inhibit egg hatching and J2 motility and viability (Mendoza et al., 2008; Terefe et al., 2009; Xiong et al., 2015; Gattoni et al., 2018). In this study, the degradation of nematode eggs by the GFP-transformed bacteria was observed by laser-scanning confocal microscopy. Serine proteases have been reported in several fungal and bacterial nematode antagonists as a key factor affecting directly the PPN host physical barriers (Bonants et al., 1995; Segers et al., 1996; Lian et al., 2007; Iqbal et al., 2018). Recently, the serine protease Sep 1 from *B. firmus* DS-1 able to degrade multiple cuticle and intestinal-associated proteins has been identified (Geng et al., 2016). Then, bacterial colonies growing on or inside the roots could affect the nearest infective or sedentary nematodes and eggs by some of the nematicidal virulence factors in *B. firmus* (Geng et al., 2016; Zheng et al., 2016; Marin-Bruzos & Grayston, 2019). This aspect should be investigated.

B. firmus I-1582 induced systemic resistance against *M. incognita* in tomato but not in cucumber in split-root experiments. These results support the hypothesis that this phenomenon is plant species dependent. Indeed, systemic resistance to *M. incognita* was induced in cotton (Gattoni et al., 2018) but not in corn (Schrimsher, 2013). In this study, the relative expression of genes related to SA and JA pathways were assessed in tomato and cucumber at 7 d after bacterial inoculation and just after nematode inoculation (0 dani), after root infection by the nematode (7 dani), and when the offspring reinfected plant roots (40 dani), and some differences were observed between plant species. Tomato plants were primed by SA and JA at 0 dani, and the infection of the nematode was reduced as showed the fewer egg masses recorded at the end of the split-root experiment. However, no differences in the expression of SA and JA related genes were found in cucumber plants inoculated with the bacteria as well in the number of egg masses produced in cucumber roots. Martinez-Medina et al. (2017) and Ghahremani et al. (2019) considered that SA primed plants affect nematode infection, corroborating the results of this study. At 7 dani, the JA related gene was up-regulated in tomato and could affect nematode development and reproduction, as proposed by Martínez-Medina et al. (2017) and Ghahremani et al. (2019). Nonetheless in cucumber, the SA related gene was up-regulated at that time but no effect on nematode infection and/or nematode reproduction was observed at the end of the split-root or co-inoculation experiments. The time elapsed between the bacterial and nematode inoculations could be the responsible of this delay in the plant response because less bacterial colonization was found in cucumber than in tomato roots, as assessed by laser-scanning confocal images and qPCR measuring of bacterial DNA. Volatile and non-volatile bacterial compounds have been reported as elicitors of plant resistance. Among them, surfactin production was strongly correlated with defence-inducing activity in the *Bacillus subtilis*/*B. amyloliquefaciens* complex (Cawoy et al., 2014). Then, lower production levels of these compounds in cucumber rhizosphere could lead to a lack of expression of systemic resistance. In addition, some exudates produced by cucumber roots, such as *p*-coumaric acid, can decrease the bacterial diversity in the rhizosphere, affecting the relative abundance of bacteria belonging to the Firmicutes class among others (Zhou et al., 2018). At 40 dani, the SA related genes were down-regulated in both tomato and cucumber plants inoculated only with the nematode, in agreement with previous results

reported by Shukla et al. (2018) in compatible nematode-plant interaction at nematode infection. However, it was up-regulated in tomato plants co-inoculated with the bacteria and the nematode, according to the incompatible nematode-plant interaction (Shukla et al., 2018), but not effect was observed in cucumber. Regarding the JA related gene, it was down-regulated in both plant species co-inoculated with the bacterium and the nematode. Recently, the dynamic regulation of genes related to SA and JA induced by *B. firmus* I-1582 and *B. amyloliquefaciens* QST713 in cotton against *M. incognita* has been studied (Gattoni et al., 2019). At 1 wk after inoculation, the gene related to SA was up-regulated by both bacterial strains, concluding that a long-term SA-dependent systemic response was the responsible of nematode suppression. According to our results, *B. firmus* I-1582 induce response in plants but it varies depending on the plant species affecting differentially the nematode. In plant species in which the nematode is suppressed, shifting from SA to JA regulation genes affects nematode infection and reproduction in primed plants, as it has been reported for *Trichoderma harzianum*-tomato (Martinez-Medina et al., 2017) and *Pochonia chlamydosporia*-tomato interactions (Ghahremani et al., 2019). Conversely, regulation of plant defence genes can also occur but only in those times in which no effect on nematode is observed, as it was found in cucumber.

The results of this study have shown that *B. firmus* I-1582 is a nematode antagonist that can act by itself and/or through the plant by induction of plant defence mechanisms in a plant species dependent manner. These findings are particularly interesting for developing strategies to maximize the efficacy of *B. firmus* I-1582-based formulations against RKN. Indeed, the application of formulates to the substrate of tomato plants 7 days before transplanting will allow root colonization and induction of resistance to nematode infection and reproduction, and thus, the bacteria will act through the plant. Moreover, the bacteria could also act by itself degrading nematode eggs and reducing the potential of inoculum. However, it will not have any effect against RKN through cucumber plants when applied to the substrate before transplanting, but bacteria could act by itself when applied to the soil. In Greece, the broadcast application of the *B. firmus* formulate significantly suppressed the number of females in root and disease severity and/or juveniles in soil after cropping cucumber in greenhouses from May to September at environmental temperatures ranging from 20 to 45 ºC (Giannakou et al., 2004). Giannakou et al. (2007) assessed the efficacy of the bacterial formulate alone or combined with soil solarisation against *M. incognita*. The number of nematodes in roots and the galling index of the following cucumber crop were reduced in *B. firmus* I-1582 treated plots compared to the control but were higher than those registered in plots treated with the chemical nematicide Basamid. Nonetheless, no differences were found between plots treated with the chemical nematicide and the application of the bacterial strain just after soil solarisation for 30 days with soil temperatures ranging from 32 to 40.5 ºC at 15 cm depth. In a pot experiment, the viability of J2 and eggs were suppressed after daily exposure at 35 and 40 ºC for 4 h followed by 20 h at 27-30 ºC for 2 and 4 weeks. In our study, the optimal temperature for bacterial growth and biofilm formation was registered at 35 ºC. Then, the application of the bacteria in summer when soil temperatures are around the

optimal for bacterial growth and biofilm formation, will provide the best environmental conditions for nematode management in soil, reducing nematode densities and consequently the disease severity and crop yield losses.

In conclusion, *B. firmus* I-1582 is active in a wide range of temperatures; is able to degrade and colonize *Meloidogyne* eggs; is able to colonize roots and to induce systemic resistance in tomato but not in cucumber. Field studies should be conducted to design successful strategies to achieve optimal nematode management using *B. firmus* I-1582-based formulations.

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Chapter3

Pochonia chlamydosporia is the most prevalent fungal species responsible for *Meloidogyne* suppression in sustainable vegetable production systems

Chapter 3: *Pochonia chlamydosporia* is the most prevalent fungal species responsible for *Meloidogyne* suppression in sustainable vegetable production systems.

Zahra Ghahremani, Nuria Escudero, Ismael Marín, Anna Sanz, Sergi García, and F. Javier Sorribas

*Correspondence: francesc.xavier.sorribas@upc.es

Abstract

The fluctuation of *Meloidogyne* population densities and the percentage of fungal egg parasitism were determined from February 2015 to July 2016 in six sites, four conducted under organic and two under integrated production standards. Moreover, the percentage of fungal egg parasitism was also assessed in pot experiments using soil samples taken every year. The nematode population densities in soil at transplanting and at the end of each crop were determined. At the end of the crop, galling index was estimated and the density of eggs in roots was determined along with the percentage of fungal egg parasitism and the fungal species involved. In parallel, two experiments were carried out using soil from each site taken in February 2015 and 2016. A part of each soil was sterilized and sterilized and non-sterilized soils were mixed with sterile sand 1:1 (dry w: dry w) and placed in 3-l pots. The susceptible tomato cv. Durinta was transplanted into each pot and inoculated with second-stage juveniles (J2) to achieve a rate of 1 J2 cm^{-3} of soil. The experiment 1 consisted of 12 replicates per each soil mixture and site and was conducted from May 15 to July 20 2015. The experiment 2 consisted of 10 replicates per each soil mixture and site and was conducted from April 19 to July 14 2016. At the end of the experiments, the fresh root weight was measured, the number of eggs per plant, as well the percentage of fungal egg parasitism and the fungal species involved were determined. In the organic production sites, the nematode population densities in soil decreased in general. The nematode was able to infect all crops (galling index > 0) except spinach (M10.41). The maximum number of eggs in roots was registered at the end of the spring-summer crops. The percentage of fungal egg parasitism ranged from 11.2 to 55 %. *P. chlamydosporia* was always the only fungal species isolated from parasitized eggs. In the integrated production sites, the nematode densities in soil increased at the end of the first crop and high number of eggs in roots was registered although a 16.5% of them were parasitized by *P. chlamydosporia.* In site M10.45, a low nematode density in soil was registered at transplanting

zucchini and was under undetectable levels at the end of the crop. Few galls in roots were observed and few eggs in roots were registered, of which a 0.8% were parasitized by *Fusarium* spp. In both pot experiments, fresh root weight of tomato plants was lower when cultivated in non-sterilized than sterilized soils, irrespective of the site ($P < 0.05$). Fungal egg parasites were not recovered from nematode eggs produced in plants cultivated in sterilized soils, and also in non-sterilized M10.45 soil. *P. chlamydosporia* was the only fungal species isolated from parasitized *Meloidogyne* eggs at rates varying between 11 to 74%. Percentages of fungal egg parasitism varied between years in two out of the six sites: M10.16 (52% to 11%) and M10.56 (14% to 74%). In conclusion, the antagonistic potential of these vegetable production soils to root-knot nematodes is highly variable, being *P. chlamydosporia* the only fungal species isolated from *Meloidogyne* eggs produced in crops cultivated in both field conditions and pots. This fungal egg parasite species is the most frequent and prevalent in these soils, showing a high plasticity able to be adapted to the agronomical practices in highly perturbed vegetable production system.

Keywords: biological control, *Meloidogyne* spp., *Pochonia chlamydosporia*, vegetable crops.

Introduction

Plant parasitic nematodes are responsible for annual yield losses reaching ca. 10% of life-sustaining crops and 14% of economically-important crops (Sasser & Freekmann 1987), varying greatly between areas due to specific nematode-plant-environment interactions. Among them, root-knot nematodes (RKN), *Meloidogyne* spp., are the most damaging worldwide (Jones et al., 2013).

Vegetables are important components of the diet that are cultivated worldwide in both open field and under protected condition, and all of them are host of the most widespread and damaging RKN species: *M. arenaria, M. incognita* and *M. javanica* (Hallmann & Meressa, 2018). Currently, RKN management strategies tend to reduce the dependence on chemical nematicides by encouraging alternative control methods promoting the conservation and enhancement of beneficial organisms improving the antagonistic potential of soil to achieve high levels of soil suppressiveness. The antagonistic potential of a soil is "the capacity to reduce the spread of deleterious agents to plants through biotic factors" (Sikora, 1992). Several nematode antagonists can occur naturally in agricultural soils providing some extent of suppressiveness to RKN (Stirling, 1991). High levels of soil suppressiveness can be achieved under favourable plant-RKN-antagonist interactions, cultural practices and abiotic factors (Giné et al., 2016). Suppressive soils, 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 & Cook, 1974), can exercise a general or a specific suppression if it is conferred by several or a narrow number of antagonists, respectively

(Stirling, 2011). A few suppressive soils to RKN conferred by fungal antagonists have been described (Pyrowolakis et al., 2002; Adam et al., 2014; Giné et al., 2016). Recently, Topalović et al., (2019b) demonstrated the *M. hapla* suppression by the total microbiome extracted from a soil, but the microbial specie or species responsible for that was not elucidated. The majority of studies conducted to demonstrate the level of soil suppression are carried out in pot test with soils samples taken at a single time (Pyrowolakis et al., 2002; Adam et al., 2014; Topalović et al., 2019b) and few of them study the fluctuation of nematode densities in field conditions and the level of soil suppresiveness in pot test using soil samples taken in different years in order to know the level of soil suppressiveness along the time (Giné et al., 2016). The latter approach is especially interesting for agricultural production systems highly perturbed such as vegetable production, and much more under protected cultivation, in which different plant species are succeeded in rotation each cropping season, soil tillage is done between crops, and in some cases organic matter is incorporated into the ground previous sowing or transplanting. In the study conducted by Giné et al. (2016) during two years to determine the fluctuation of RKN densities and the level of soil suppressiveness in two sites where vegetables were cropped organically under protected cultivation, the nematode densities decreased progressively until undetectable levels in one site and was maintained but with low levels of disease severity in the other site, but in both of them, the level of egg parasitism was maintained in pot experiments with soil samples taken at two different times. In both sites, *Pochonia chlamydosporia* was the only fungal isolated from RKN eggs. Some studies have been carried out to determine the presence of fungal RKN antagonists in different agricultural production areas from Spain, *Fusarium* spp. and *P. chlamydosporia* being the most frequent out of more than 20 fungal species isolated (Verdejo-Lucas et al., 1997, 2002 and 2013; Olivares-Bernabeu & López-Llorca, 2002; Giné et al., 2013). In 2010, a survey conducted to detect fungal RKN egg parasites in 40 vegetable growing sites, 10 conducted under organic and 30 under integrated production, showed that fungal egg parasitism was recorded in all organic production sites at percentages ranging from 4% to 80%, and in 22 integrated production sites at percentages ranging from 2 to 69% (Giné et al., 2013).

In the present study, the fluctuation of RKN densities in field conditions was determined during two years in 6 sites out of the 40 previously studied: 4 conducted under organic production with percentages of fungal egg parasitism between 9% and 80%; and 2 conducted under integrated production with percentages of fungal egg parasitism of 6% and 69%. Moreover, the level of soil suppresiveness in pot test using soil samples taken every year was also determined.

Materials and methods

Fluctuation of RKN densities and egg parasitism in field conditions

The study was carried out from winter-spring 2015 to summer 2016 in six vegetable production sites, located in the Tarragona and Barcelona provinces in northeastern Spain. Four sites (M10.16, M10.41, M10.55, and M10.56) were conducted under organic and two (M10.43 and M10.45) under integrated production standards. These sites were selected because fungal egg parasites of *Meloidogyne* were detected, isolated and identified, and the microbiome associated to J2 RKN cuticle was also determined in previous studies (Giné et al., 2013; Elhadi et al., 2017). Moreover, site M10.55 was also characterized as suppressive to RKN (Giné et al., 2016). The physicochemical properties of these soils are shown in Table 1.

In sites conducted under organic production crop residues were incorporated as green manure at the end of each crop and fertilized with composted sheep or cow manure alone or combined with chicken manure at a total rate of 1.7 - 2 kg m^{-2} just before transplanting each crop. The cropping sequences were diverse including Brassicaceae, Chenopodiaceae, Compositae, Cucurbitaceae, Fabaceae, and Solanaceae. In both sites under integrated production, fertilization was done with humus combined with chemical fertilizers, and in site M10.45, crop residues were occasionally incorporated into the soil. The rotation sequences included crops belonging to the Cucurbitaceae, Fabaceae, Solanaceae and Umbelliferae families.

The nematode population densities in soil at transplanting and at the end of each crop were determined. Composite soil samples were taken from four plots of 50 - 85 m^2 each, depending on the site. Ten soil subsamples were taken from the first 30 cm of soil of each plot with a soil auger of 2.5 cm of diameter. Soil subsamples were carefully mixed and sieved through a 4 mm sieve screen to remove stones. Nematodes were extracted from two 250 cm^3 soil subsamples by the Jenkins's method (1964). At the end of the crop, the ten plants closest to the sampled soil were uprooted and the galling index was estimated using the Zeck's index scale from 0 to 10, where 0 = no galls and 10 = plants and roots dead (Zeck, 1971). After that, roots were washed with tap water and 2 egg masses per plant (20 egg masses per plot) were handpicked to evaluate the percentage of fungal egg parasites and to identify the fungal species according to the method described in Giné et al. (2016). The outer part of gelatinous matrix of the egg masses were removed with forceps and were placed in a 1.5 ml microcentrifuge tube containing 1 ml of sterile distilled water.

The eggs were scattered from the egg masses with a sterile pestle and a 0.33 ml aliquot of the egg suspension was spread onto each of three replicated Petri dishes (9-cm diameter) containing a restrictive growing media (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 & Duncan, 1986). The plates were incubated at 25 °C ± 0.5 °C. The number of parasitized eggs was counted at 24 and 48 hours using a dissecting microscope. The eggs were considered parasitized when the hyphae grew from the inside. The percentage

of parasitism in each Petri dish was calculated as the number of parasitized eggs in relation to the total number of eggs per plate. The parasitized eggs (maximum 20 per plate) were individually transferred to corn meal agar (CMA) to establish pure cultures of the fungi and incubated at the same conditions described previously. Fungal species identification was done according to morphological characteristics. Fungal isolates were stored in 1% (w/v) water-agar slants, as well as lyophilized and stored at 4 °C.

Table 1. Physicochemical properties of four soils cultivated with vegetables under organic standards (M10.16, M10.41, M10.55 and M10.56) and two soils cultivated under integrated production (M10.43 and M10.45).

	Soils					
	Organic farming				Integrated production	
Variable	M10.16	M10.41	M10.55	M10.56	M10.43	M10.45
Sand (%)	50	33	68	53	75	67
Silt (%)	30	38	0	29	15	14
Clay (%)	20	29	32	18	10	19
Soil texture (USDA)	loam	clay loam	sandy clay loam	sandy loam	sandy loam	sandy loam
pH	8.25	8.17	8.12	8.32	8.13	7.77
Organic matter (w/w)	1.20	4.38	2.50	4.29	1.51	2.5
Electric conductivity (µS/cm)	300	516	1069	415	156	332
B (ppm)	2.62	5.33	1.12	1.21	2.82	0.75
Exchangeable Ca (meq 100 g^{-1})	14.15	14.88	18.16	15.89	7.18	9.98
Available Ca (meq 100 g^{-1})	15.74	14.7	19.0	16.96	8.14	10.74
Lime	6.0	4.0	4.1	5.1	1.0	3.8
Cation exchange capacity (meq 100 g^{-1})	26.84	14.06	25.70	22.89	9.12	12.22
Cu (ppm)	2.5	2.5	2.5	2.5	28.31	28.8
Available P (ppm)	118.25	247.47	75.79	80.19	57.94	86.92
Fe (ppm)	5.0	5.0	5.0	5.0	239.1	79.72
Exchangeable Mg (meq 100 g^{-1})	1.37	4.8	3.0	2.21	0.91	1.41
Available Mg (meq 100 g^{-1})	1.43	4.43	3.66	2.72	1.06	1.58
Mn (ppm)	55.54	92.4	2.5	2.5	92.23	54.3
N (ppm)	522.9	2388.7	1497.7	2209.8	629.8	1824.9
Exchangeable K (meq 100 g^{-1})	0.59	1.47	0. 67	0.62	0.15	0.35
Available K (meq 100 g^{-1})	0.79	1.67	0.69	1.05	0.18	0.43
C/N	13.36	10.64	9.68	11.26	13.87	7.94
Exchangeable Na (meq 100 g^{-1})	0.46	0.57	0.54	0.37	0.34	0.36
Available Na (meq 100 g^{-1})	0.38	1.85	3.17	0.62	0.36	0.54
Zn (ppm)	2.64	7.19	2.50	2.50	24.63	24.52

Finally, plant roots from each plot were chopped, mixed, and eggs extracted from three 10 g-subsamples by blender maceration in a 10% commercial bleach solution (43 g l^{-1} NaOCl) for 10 min (Hussey & Barker, 1973) and sieved through a 74 µm-aperture sieve to retain root debris, collected in a 20 µm sieve, counted and expressed as eggs per g of root.

Soil suppressiveness to RKN in pot experiments

Two experiments were carried out using soil from each site taken in February 2015 and 2016. Soil samples consisting of 12 soil subsamples per plot and site were taken from the first 30 cm with a hoe. Per each site, soil samples were mixed and sieved through a 4 mm-sieve screen to remove stones and roots. A part of each soil was sterilized at 121 ºC during 1h and it was repeated after 1day. Soil samples were then conserved at 4 ºC until the experiments were conducted following the same procedure described in Giné et al. (2016). Sterilized and non-sterilized soils were mixed with sterile sand 1:1 (dry w: dry w) to avoid soil compaction and improve root development. Afterwards, the RKN density in both sterilized and non-sterilized soil mixtures were determined to adjust the level of inoculum at 1 J2 cm^{-3} of soil. RKN juveniles were extracted from two 500-cm^3 subsamples using Baermann trays (Whitehead & Hemming, 1965) maintained at 27 ± 2 ºC for a week. After that, soil was placed in 3L pots and a susceptible tomato cv. Durinta was transplanted into each pot at three true developed leaves stage. Nematode inoculum consisted of *M. inocognita* J2 emerged from eggs extracted from tomato roots by the Hussey & Barker (1973) procedure and placed in Baermann trays (Whitehead & Hemming, 1965) for a week at 27 ± 2 ºC. The nematode inoculum was added to the soil in two opposite holes (3 cm deep) at 2 cm apart from the stem of the plants. The experiments consisted of 12 and 10 replications per each soil mixture and site in experiment 1 and 2, respectively. The plants placed at random on greenhouse benches and were irrigated as needed. Soil temperatures and soil water content at 8 cm depth was recorded at 1 h interval during the experiments with temperature probes (5TM, Decagon devices, Inc., Pullman, WA, USA). The first experiment was conducted from May 15 to July 20 2015 (1085 degree-day (DD); 10 ºC basal temperature and thermal constant between 600-700 DD over the basal temperature; Ferris et al., 1985) and the second one from April 19 to July 14 2016 (1050 DD).

At the end of the experiments, roots were washed and gently dry before determine fresh weight. Afterwards, two egg masses were handpicked from individual plants growth in both sterilized and non-sterilized soils to determine the percentage of fungal egg parasitism, according to the method described previously, and fungal species were identified by morphological characteristics. Finally, the number of eggs per plant was determined extracting them from roots by the Hussey & Barker (1973) method.

Statistical analyses

Statistical analyses were performed using the JMP software v8 (SAS institute Inc., Cary, NC, USA). The normal distribution of the data and homogeneity of variances were assessed. Paired comparison between sterilized and non-sterilized mixed soil was conducted per each site by the Student's t-test or by the non-parametric Wilcoxon test ($P \leq 0.05$).

Results

Fluctuation of RKN densities and egg parasitism in field conditions

In the organic production sites cultivated under protected, the nematode population densities in soil decreased in sites M10.41 and M10.56 or did not change in site M10.55 at the end of the study. The nematode was able to infect all crops (galling index > 0) except spinach (M10.41). The maximum number of eggs in roots were registered at the end of the spring-summer crops of zucchini (1490 and 5613 eggs g^{-1} root, site M10.41), eggplant (306 eggs g^{-1} root, site M10.55) and tomato (4137 eggs g^{-1} root, site M10.55; 3097 eggs g^{-1} root, site M10.56). The percentage of fungal egg parasitism ranged from 11.2 to 35.9 % (Table 2). *P. chlamydosporia* was the only fungal species isolated. The lowest values of galling index and less number of eggs in roots were registered in the summer-autumn chard, spinach and radish crops and in the autumn-winter lettuce crop. The percentage of fungal egg parasitism was not assessed because few or not egg masses were observed. In site M10.56, the watermelon crop transplanted in March 2016 died two months after being transplanted due to technical problems, and the nematode densities in soil decreased from March to July 2016 (Table 2). In the open field site M10.16, conducted under organic standards, the nematode density in soil at transplanting tomato in May 2015 was 34 J2 250 $cm^{-3}$of soil and the nematode was under undetectable levels six months later. Few galls in roots were observed and few eggs in roots were registered (828 eggs g^{-1} root). The percentage of fungal egg parasitism was 55%, being *P. chlamydosporia* the only fungal species isolated (Table 2).

In the open field sites M10.43 and M10.45, conducted under integrated production, the rotation sequences were less diverse. In site M10.43 two French beans crops were conducted during 2015. The nematode densities in soil increased at the end of the first crop and high number of eggs in roots was registered (25466 eggs g^{-1} root) although a 16.5% of them were parasitized by *P. chlamydosporia.* In site M10.45, a low nematode density was registered at transplanting zucchini in April 2015 (6 J2 250 cm^{-3} soil) and was under undetectable levels at the end of the crop in August 2015. Few galls in roots were observed and few eggs in roots were registered (250 eggs g^{-1} root) of which a 0.8% were parasitized by *Fusarium* spp. The nematode density at sowing the following faba bean crop in November 2015 was 54 J2 250 cm^{-3} of soil. No information on galling index, density of eggs in roots and percentage of fungal egg parasitism was obtained because the crop had been incorporated into the ground previously to take samples in June 2016 (Table 2).

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 fungal egg parasitism in four organic vegetable production sites (M10.16, M10.41, M10.55 and M10.56) and two integrated vegetable production sites (M10.43 and M10.45).

Site	Crop	Date	Juveniles 250cm^{-3}soil		GI	Eggs (x10^2) g root^{-1}	Parasitized eggs (%)
			Pi	*Pf*			
Organic							
M10.16	Tomato	05/2015-11/2015	34±21	0±0	1.9±0.5	8.3±1.7	55.0±13
M10.41	Zucchini	02/2015-07/2015	402±59	16±9	3±0.2	56.1±7.8	17.0±3.2
	Chard	07/2015-11/2015	16±9	9±2	2.4±0.2	0.14±0.03	Na
	Spinach	07/2015-11/2015	16±9	4±4	2±0.2	0±0	Na
	Zucchini	03/2016-07/2016	8±4	7±6	2.8±0.1	14.9±0.9	11.2±2.4
M10.55	Eggplant	03/2015-07/2015	16±6	20±11	0.5±0	3.1±0.4	35.9±4.4
	Lettuce	11/2015-02/2016	239±63	118±39	2.2±0.2	0.5±0.2	Na
	Tomato	02/2016-07/2016	118±39	35±11	3.9±0.1	41.4±2.7	21.7±2.8
M10.56	Tomato	02/2015-07/2015	55±18	42±17	8.3±0.2	31±6.9	24.8±5.5
	Radish	08/2015-11/2015	42±17	24±14	0.47±0.1	1.5±0.2	Na
	Watermelon	03/2016-07/2016	24±14	3±2	Np	Np	Na
Integrated							
M10.43	French bean	05/2015-08/2015	123±30	795±182	4.5±0.3	255±22	16.5±3.9
	French bean	08/2015-12/2015	795±182	11±5.4	Np	Np	Na
M10.45	Zucchini	04/2015-08/2015	6±2	0±0	1.3±0.2	2.5±1	0.8±0.7
	Faba bean	11/2015-06/2016	54±18	24±13	Np	Np	Na

Data are mean ± standard error of 4 replications. Galling index (GI) on a scale from 0 to 10, where 0 = complete and healthy root system and 10 = plants and roots dead (Zeck, 1971), Na: Not assessed, Np: No-plant.

Table 3. *Meloidogyne* reproduction in the susceptible tomato cv. Durinta cultivated in sterilized or non-sterilized soil from four organic (M10.16, M10.41, M10.55 and M10.56) and two integrated vegetable production sites (M10.43 and M10.45) in 3 L-pot experiments after 66 days (experiment 1) or 86 days (experiment 2) of being inoculated with nematode juveniles to achieve 1 J2 cm^{-3} of soil, and percentage of fungal egg parasitism.

	Production	Site	Soil treatment	Fresh root weight (g)	Eggs (x 10^3) / plant [a]	Parasitized eggs (%)
1	**Organic**	M10.16	Sterilized	19.9 ± 2.5 *	323 ± 53 *	0
			Non-sterilized	8.3 ± 1.2	28 ± 4	52 ± 7
		M10.41	Sterilized	44.1 ± 3.6 *	371 ± 28 *	0
			Non-sterilized	26.5 ± 2.8	193 ± 20	24 ± 4
		M10.55	Sterilized	36.5 ± 3.3 *	241 ± 44 *	0
			Non-sterilized	25.3 ± 2.4	47 ± 9	44 ± 4
		M10.56	Sterilized	24.3 ± 2.3 *	249 ± 33 *	0
			Non-sterilized	11.7 ± 2.4	42 ± 4	14 ± 6
	Integrated	M10.43	Sterilized	39.4 ± 3.2 *	246 ± 27 *	0
			Non-sterilized	21.9 ± 1.9	138 ± 17	17 ± 4
		M10.45	Sterilized	45.9 ± 3.2 *	262 ± 23	0
			Non-sterilized	20.5 ± 1.5	226 ± 30	0
2	**Organic**	M10.16	Sterilized	25.9 ± 3.2 *	161 ± 26 *	0
			Non-sterilized	15.0 ± 2.0	104 ± 14	11 ± 2
		M10.41	Sterilized	33.2 ± 1.5 *	195 ± 20 *	0
			Non-sterilized	10.2 ± 1.5	117 ± 24	16 ± 4
		M10.55	Sterilized	28.0 ± 3.4 *	62 ± 9 *	0
			Non-sterilized	12.3 ± 1.6	34 ± 5	33 ± 9
		M10.56	Sterilized	30.7 ± 2.3 *	163 ± 31 *	0
			Non-sterilized	9.9 ± 1.2	21 ± 3	74 ± 3
	Integrated	M10.43	Sterilized	24.6 ± 2.4 *	59 ± 6 *	0
			Non-sterilized	8.1 ± 0.7	27 ± 4	15 ± 6
		M10.45	Sterilized	19.6 ± 3.0 *	68 ± 9	0
			Non-sterilized	10.6 ± 1.1	47 ± 6	0

Data are mean ± standard error of 12 (experiment 1) or 10 replicates (experiment 2). 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 or the non-parametric Wilcoxon test. [a] Parasitized egg excluded

Soil suppressiveness against RKN in pot test

In both experiments, fresh root weight of tomato plants cultivated in non-sterilized soils was lower compared to those plants cultivated in sterilized soils, irrespective of the site ($P < 0.05$). Moreover, no fungal egg parasites were recovered from nematode eggs produced in plants cultivated in sterilized soils (Table 3).

In experiment 1 conducted with soil samples taken in February 2015, fungal parasites were recovered from *Meloidogyne* eggs produced in tomato roots cultivated in almost all non-sterilized soils, except in those plants grown in M10.45 soil. *P. chlamydosporia* was the only fungal species identified. The percentage of fungal egg parasitism ranged from 14% (M10.56) to 52% (M10.16). A fewer number of eggs per plant cultivated in non-sterilized respect to sterilized soils were recorded for all the soils ($P < 0.05$), except for soil M10.45 where no differences were found (Table 3). Similar results were obtained in experiment 2, conducted with soil samples taken in February 2016, fungal egg parasites were recovered from roots grown in all non-sterilized soils, except from soil site M10.45. *P. chlamydosporia* was the only fungal species identified. The percentage of fungal egg parasitism ranged from 11% (M10.16) to 74% (M10.56). Fewer ($P < 0.05$) eggs per plant cultivated in non-sterilized than sterilized soils were recorded, except from those of soil M10.45 (Table 3).

Discussion

The results obtained in this study have shown the variable antagonistic potential of six agricultural soils, four conducted under organic and two under integrated vegetable production standards in north-eastern Spain. Fungal parasites were recovered from *Meloidogyne* eggs in almost all sites, being *P. chlamydosporia* the most frequent and abundant fungal species isolated from RKN eggs produced in different crops in field conditions. This result was confirmed in pot experiments since *P. chlamydosporia* was the only fungal species isolated from nematode eggs produced in tomato. Giné et al. (2013) reported a great diversity of fungal egg parasites in a previous study conducted in 2010 in 40 vegetable growing sites. Regarding the six sites included in this study, Giné et al. (2013) isolated 11 fungal RKN egg parasites species including *Chaetonium*, *Colletotrichum coccodes*, *Cylindrocarpon olidum*, *Dactylella oviparasitica*, *Fusarium equiseti*, *F. oxysporum*, *F. solani*, *Fusarium* spp, *Penicillium citrinum*, *P. chlamydosporia*, and *Purpureocillium lilacinum*, at percentages of parasitism ranging from 6.5% (M10.45) to 80.2% (M10.41). Five years later, less diversity of fungal egg parasites was observed, being *P. chlamydosporia* the only fungal species isolated from RKN eggs produced in 5 out of the 6 sampled sites. Then, it confirms the hypothesis of Giné et al. (2013, 2016) suggesting that this fungal species has a key role in the RKN-antagonistic potential of these soils and that it is greatly resilient in highly perturbed agricultural systems.

In addition to *P. chlamydosporia*, other components of the soil microbiome could affect RKN infection and/or reproduction. Recently, some bacterial isolates from the cuticle of *M. hapla* J2 have been reported affecting nematode invasion into tomato roots (Topalović et al., 2019a). Elhady et al. (2017) used a fraction of the soil samples taken in February 2015 from the same 6 sites in order to determine which fraction of the soil microbiome were able to attach to RKN J2-cuticle. They found that the most microbial J2-attached species had a minor relative abundance in soil and were not related to the vegetable production system. Interestingly, Topalović et al., (2019 b) have also shown that the microbiome extracted from a suppressive soil to plant parasitic nematodes affected the capability of *M. hapla* to invade and to reproduce in tomato plants by inducing plant resistance. No information about the ability of the RKN J2-attached microbial species identified by Elhady et al. (2017) to induce plant resistance is unknown. Nonetheless, according to our results, egg parasitism seems to be the main factor responsible for reducing nematode reproduction since none of the identified attached microorganisms have been reported inducing plant resistance against RKN. On the other hand, regarding some dominant fungal species detected in one of these soils, such as *Trichoderma asperellum* (Elhady et al., 2017), and *P. chlamydosporia*, isolated from RKN eggs in 5 out of the 6 sampled sites, both fungal species are able to induce resistance in tomato when applied 7 days before nematode inoculation (Gharemani et al., 2019; Pocurull et al., 2020). In our experimental conditions, in which the infective RKN juveniles were inoculated just after transplanting, the induction of plant resistance could not occur due to the short time elapsed for favouring the fungal-plant interaction against the nematode.

Other factors influencing the antagonistic potential of soils such as crop rotation can improve soil-borne diseases suppression. Peralta et al. (2018) reported a positive relationship between diversity of crops in rotation and disease suppression in agricultural soils by bacteria. The disease suppressiveness was increased if cover crops were included in a three-crops rotation sequence, but it did not when only one crop was cultivated. In addition, fallowing decreased disease suppressiveness. The effect that crop rotation and the plant species included could have on the microbiome responsible for root-knot nematode suppression is unknown. In fact, the rhizosphere microbial community is more affected by the plant species than the management soil practices (Harkes et al., 2020). Even more, the endosphere microbiome is affected by nematode infection as pointed out by Wolfgang et al. (2020) in tomato roots infected by RKN, irrespective of the disease severity, compared to healthy roots. Previous studies conducted with the soils included in this study showed that several components of the soil microbiome could affect the nematode behaviour and viability (Giné et al., 2016; Elhady et al., 2017). Other agronomical practices such as the cultivation of RKN resistant cultivars, the fertilization with organic amendments, the incorporation of crop wastes and data of transplanting or sowing can affect the survival and nematode build-up. Some more field studies complemented with pot experiments are needed to understand the main components of soil suppressiveness to RKN at agroecosystem, microcosm and molecular levels to transfer this knowledge to growers in order to improve the sustainability of vegetable production systems.

In conclusion, the antagonistic potential of vegetable production soils to RKN is highly variable, *P. chlamydosporia* being the only fungal species isolated from RKN parasitized eggs from plants cultivated in field conditions and also in pot experiments. This fungal species is the most frequent and prevalent with a high plasticity, adaptable to agronomical practices in highly perturbed vegetable production system.

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General Discussion

General Discussion

Root-knot nematodes are the most damaging plant parasitic nematodes worldwide (Jones et al., 2013) that cause economical losses on a considerable number of crops including those belonging to Solanaceae and Cucurbitaceae families. The chemical control is the method most used but due to environmental and toxicological concern, the implementation and combination of alternative management methods are required. Biological control agents are one of the most studied sustainable alternatives because they can reduce the use of chemical pesticides. There are commercial products available containing microorganisms, selected for thier capacity to control plant parasitic nematodes for example BioYieldTM, (Gustafson LLC) which contains a mix of two bacteria, *Paenobacillus macerans* and *Bacillus amyloliquefaciens*; VOTiVO® (Bayer CropScience), which contains *Bacillus firmus*, Nemaxxion Biol® (Green corp, 2014) which is composed of a consortium of microorganisms (*Bacillus subtilis*, *Trichoderma* spp., *Paecilomyces* spp.) and plant extracts (tagetes); BIO-ACT® (Bayer CropScience) and BIOSTAT® (EPA approved) contain the fungus *Purpureocillium lilacinum*; Trianum® is a product marketed by Koppert based on *Trichoderma harzianum* T-22 (Trainer et al., 2014). Some commercial products based on *P. chlamydosporia* are available worldwide, such as KlamiC® (agricultureal bionematicide) (Hernández et al., 2008) and Rizotec® (Stoller Brazil) (Bontempo et al., 2017). There are other non-commercial biological control agents that have the capacity to parasitize or be antagonists of RKN or cyst nematode eggs *in-vitro* among them *P. chlamydosporia*, *Fusarium* spp. and *Plectosphaerella cucumerina* are common fungal species isolated from RKN eggs (Gine et al., 2013).

P. chlamydosporia is one of the most studied biological control agents against RKN and cyst nematodes that parasite the nematode eggs (Stirling, 2014). The fungus can also colonise endophytically roots of several crops, such as, tomato (Bordallo et al., 2002), barley (Macia Vicente et al., 2009a) and potato (Manzanilla-López et al., 2011), and also, can induce plant defence mechanisms, such as the formation of papillae (Bordallo et al., 2002) or plant defence genes related to salicylic acid and jasmonic acid pathways in *Arabidopsis* (Zavala-Gonzalez et al., 2017). Moreover, *P. chlamydosporia* induces the local resistance in tomato roots against *M. javanica* mediated by increasing polyphenoloxidases and peroxidases activities at root nematode invasion stage (de Medeiros et al., 2015). We have used five different isolates of *P. chlamydosporia* obtained from horticultural commercial growing sites in north eastern Spain from RKN eggs (Giné et al., 2012) to study the ability of these isolates to induce systemic resistance in tomato and cucumber in a split root system. Two of them, isolate M10.43.21 and M10.51.6, induced systemically resistance against *M. incognita* on tomato but not in cucumber determining that this effect is plant species dependent. In tomato, the fungal isolates reduced the number of egg masses per plant, the number of eggs per plant, and the female fecundity. Other fungal species belonging to the *Trichoderma* genera have also been reported as inductor of plant defense mechanisms in tomato against root-knot

nematodes (de Medeiros et al., 2017; Martínez-Medina et al., 2017; Pocurull et al., 2020). This fact can contribute to the management of *Meloidogyne* in field conditions by using primed plants, increasing the available tools for managing the most damaging plant parasitic species group. In addition, Pocurull et al. (2020) have demonstrated the additive effect of priming plants with *Trichoderma* with the *Mi 1.2* resistance gene in tomato against a virulent *Meloidogyne* isolate. Some field work has to be conducted to determine the effect of primed plants on the population's dynamics and yield crop.

In order to know the hormonal pathways related with the induction of plant resistance, we evaluated the dynamic regulation of the salicylic acid (*PR-1* gene) and jasmonic acid (*Lox D* gene) pathways by *P. chlamydosporia* isolated M10.43.21 and *M. incognita* at 0, 7, and 42 days after nematode inoculation (dani) in tomato. The isolate M10.43.21 induced the expression of the salicylic acid pathway in tomato roots from the first assessment time (7 days after fungal inoculation and just after nematode inoculation) until the end of the experiment (42 dani), however, the jasmonate signalling pathway was only upregulated at 7 days dani. So, salicylic acid and jasmonic acid signalling pathways have been proposed as responsible for the systemically induced resistance to *Meloidogyne* spp. in tomato. Martinez-Medina et al, (2017) suggested that *Trichoderma harzianum* T-78-induced systemic resistance with and up-regulation of the salicylic acid pathway in the infection of the nematode followed by jasmonic acid induction suppressing RKN reproduction and fecundity and finally salicylic acid induction affecting root infection by new J2. Selim, (2010) showed that induced systemic resistance (ISR) and systemic acquired resistance (SAR) signalling pathways can be stimulated biologically by using the biocontrol agent *Fusarium oxysporum* (Fo162) or chemically through the chemical elicitors SA and JA in tomato plants that are responsible for reducting in *M. incognita* infection in spilt-root experiment.

In addition, some bacterial species have been reported as biocontrol agents to reduce nematode populations. Several studies informed that *Bacillus* spp. have shown the capacity to reduce RKN. In chapter 2, we determine the induced systemic resistance against *M. incognita* by *B. firmus* I-1582 in tomato but not in cucumber by means of split-root experiments. The number egg masses and eggs per plant were reduced in the Bf I-1582/*M. incognita* treatment compared to the None/*M. incognita* treatment. Different species of *Bacillus* spp. reduced egg hatching, motility second-stage juveniles (J2), the reproduction rates or population sizes of *Meloidogyne* spp. such as *Bacillus thuringiensis* (Ravai & Moghaddam et al., 2015), *B. cereus* (Gao, 2016), and *B. firmus* (Giannakou et al., 2007; Terefe et al., 2009; Xiong et al., 2015). *Bacillus firmus* has also the capability to promote host plant growth, inducing systematic response by secondary metabolites production (Mendoza et al. 2008; Xiong et al. 2015). Furthermore, *B. firmus* was able to colonize both tomato and cucumber roots, but more extensively those of tomato. The colonisation of bacteria measured by qPCR that *B. firmus* was colonized roots of both plant species endophytically, but ca. 61% more density of bacterial DNA was recorded in tomato than cucumber roots. In addition, the GFP-transformed bacteria colonized the rhizoplane of both plant species but those of tomato more extensively. It is known, some exudates produced by cucumber roots such as Phenolics, p-coumaric, acid from cucumber

root exudates into the environment can decrease the bacterial diversity of rhizosphere and inhibit the relative abundances of species can have pathogen-antagonistic and/or plant-growth-promoting effects (Zhou et al., 2018). Moreover, egg-shell erosion and egg colonization by Bf I-1582-GFP was observed by the GFP-transformed bacteria by laser-scanning confocal microscopy. Geng et al., (2016) reported that serine proteases, *Sep1*, from that *B. firmus* strain DS-1 has high toxicity against *M. incognita* and soybean cyst nematode and affecting directly the PPN host physical barriers and degrade intestinal multiple and cuticle-associated proteins.

In addition, we evaluated dynamic regulation of the salicylic acid (*PR-1* gene) and jasmonic acid (*Lox D* gene) pathways, because as explained earlier, both plant hormones can be activated by microorganisms. In our study, at 0 day after nematode inoculation (dani) (7 days after Bf I-1582 inoculation and just after nematode inoculation), both SA (*Pr1*) and JA (*LoxD*) pathways were up-regulated in tomato inoculated with the bacteria in compared to control but no differences between treatments in cucumber. At 7 dani, when nematode infected roots, only the plants co-inoculated with nematode and bacteria showed an up-regulation of the JA related gen (*Lox D*) in tomato, while, observed SA pathway (*Pal I*) was up-regulated both in the nematode inoculated plants and those co-inoculated with the bacteria and the nematode in cucumber. At 40 dani, the SA related genes were down-regulated in both tomato and cucumber plants inoculated only with the nematode. Similarly, Shukla et al 2018 reported that in compatible nematode-plant interaction at nematode infection. However, it was up-regulated in tomato plants co-inoculated with the bacteria and the nematode, not effect was reported in cucumber. Regarding the JA related gene, it was down-regulated in both plant species co-inoculated with the bacterium and the nematode in 40 dani. In a similar way, Gattoni et al., (2019) reported that two *B. amyloliquefaciens* QST713 and *B. firmus* I-1582 significantly decreased ca. 85% the *M. incognita* population on cotton in split root experiment. In addition, they found that 1 week after bacterial inoculation, the gene related to SA was up-regulated by both bacterial strains. According to our results, Bf I-1582 can induce response in plants but it depends on the plant species that have different affects on the nematodes.

Moreover, in chapter 2, we determined that the optimal growth temperature of Bf I-1582 is 35 ºC. Similarly, biofilm formation was observed between 15 ºC and 45 ºC, being thicker and uniform at 35 ºC. Gonzalez et al., 1999 reported the sporulation of two strains (ATCC 4342 and 9818) of *Bacillus cereus* was increased in the range 20 to 45 ºC of temperature with the other strain (ATCC 7004), the most heat resistance spores were obtained at 35 ºC. All these findings in chapter 2 will be able to develop strategies to maximize the efficacy of Bf I-1582-based formulations against RKN. Thus, the application of the bacteria in summer will can improve to reduce nematode densities and crop yield losses.

The results of chapter 1 and 2 showed that both nematode antagonists, *P. chlamydosporia* and *B. firmus* strain I-1582, were able to induce systematic resistance reducing the *M. incognita* eggs and egg masses in tomato. In addition, *P. chlamydosporia* was found naturally parasitizing root-knot nematode eggs

in agricultural soils. Giné et al., 2013 showed that *P. chlamydosporia* alone or co-occurring with other fungal species that was responsible for some levels of soil suppressiveness in both organic and integrated production systems. However, the characterization of soil suppressiveness was only carried out in two of these soils, being *P. chlamydosporia* the main fungal species responsible for that. The microbiome profiles of these soils showed that other putative microorganisms could exercise some action against plant parasitic nematodes (Giné et al., 2016). Nematophagous fungi and bacteria can act directly or/and indirectly through mechanisms of competition, parasitism and the production of toxic compounds or by induce resistance in plants (Silva et al., 2018) that is one reason why we have selected them in our study.

In chapter 3, we determined the variable antagonistic potential of six agricultural soils, four conducted under organic and two under integrated vegetable production standards in northeastern Spain, in order to identify patterns in the fluctuation of the population densities of *Meloidogyne* spp. with regards to both its density, to the percentage of fungal egg parasitism and the level of soil suppressiveness in pots experiments. In our study, the proportion of parasitized eggs, the amount of nematodes and the health of the crops in almost all the production sites suggested that some of those soils could be suppressive to *Meloidogyne* spp. *P. chlamydosporia* was the only fungal species isolated from RKN eggs produced in 5 out of the 6 sampled sites at percentages of fungal egg parasitism ranging from 11 to 74 % in field conditions, and from 11.2 to 55% in tomato cv. Durinta in pot experiments. The highest nematode densities and percentage of egg parasitism was registered in the spring-summer crops, demonstrating a denso-dependent relationship *Meloidogyne*-antagonist. A wide range of factors such as plant, soil properties and agricultural management (crop rotation, soil tillage, fertilizers, pesticides, irrigation, etc.) significantly affects soil microbial community (Garbeva et al., 2004). The physical and chemical soil properties, which can directly affect nematode populations or indirectly to the development of PPN antagonists, are involved in suppressiveness. For instance, organic amendments can improve the soil physicochemical properties and increase the diversity of antagonistic microorganisms. Plant residues as a green manure, biofumigation, animal manures and organic soil fertilisers become important tools for PPNs control (Silva et al., 2018). This information can help in making decisions to enhance natural antagonism or to make soil receptive to foreign antagonists, and further to increase the level of supressiveness. So, farmers should reduce intensity of tillage and use practices that increases the soil supressiveness, such as crop rotation, cover cropping and fertilization with organic amendments to improve and enhance the physiochemical and biological properties of soils, thus improving and enhancing the soil microbiome diversity and functions.

Further studies in the plant-nematode-antagonists'interactions are needed to elucidate the mechanisms and genes involved in inducing plant resistance in order to maximize RKN control. In addition, more field studies should be conducted to elucidate the relations between microbial communities and crop management to improve soils suppressiveness in order to design successful strategies to achieve optimal nematode management by the antagonistic potential of soils.

Conclusions

Conclusions

I. The *P. chlamydosporia* isolates M10.43.21 and M10.55.6 induce systemical resistance against *M. incognita* in tomato but not in cucumber. Thus, this response is plant species dependent. These fungal isolates reduced the number of egg masses per plant, the number of eggs per plant, and the female fecundity in tomato. *P. chlamydosporia* isolates differed in the level of total root colonization irrespective of the plant species that the best root colonizer was isolate M10.43.21 in tomato and isolate M10.55.6 in cucumber. In addition, *PR-1* gene (related with Salicilic acid pathway) and *Lox D* (related with Jasmonic pathway) were up-regulated in tomato roots inoculated with Pc M10.43.21 just after nematode inoculation. *PR-1* continued up-pregulated 7 and 42 days after nematode inoculation.

II. *B. firmus* I-1582 is active in a wide range of temperatures between 15 to 45 ºC. The optimal growth temperature and the biofilm fluctuation production were observed at 35 ºC. This bacteria isolate is able to degrade the egg-shell and parasitized the nematode egg. Colonization tomato roots was significantly higher than cucumber. Bf I-1582 primed tomato plants by both SA and JA at all the times in tomato, but only SA at 7 dani in cucumber roots. Moreover, Bf I-1582 reduced the number of egg masses and eggs per plant on tomato roots and this effect was no detected in cucumber.

III. The induction of plant resistance by some isolates of *P. chlamydosporia* and *B. firmus* I-1582 was plant dependent and showed a similar model of dynamic regulation of those genes related with Salicylic acid and Jasmonic acid in tomato.

IV. The antagonistic potential of vegetable production soils to RKN is highly variable, *P. chlamydosporia* being the only fungal species isolated from RKN parasitized eggs from plants cultivated in field conditions and also in pot experiments. This fungal species is the most frequent and prevalent with a high plasticity able to be adapted to the agronomical practices in highly perturbed vegetable production system.

References of

General Introduction and General Discussion

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General Introduction

Chapter1

Chapter 2

Root-knot nematodes (RKN), *Meloidogyne* spp., are the most economically important genus of plant parasitic nematodes that cause considerable damage and yield losses of horticultural crops worldwide. RKN management strategies tend to reduce chemical nematicides by encouraging alternative control methods like the use of plants bearing resistance genes (R-genes) and/or by microbe-inducing plant resistance, and the antagonistic potential of soils. In the thesis, two biological control approaches of *Meloidogyne* spp. were evaluated: 1) the application of selected nematode antagonists, the fungus *Pochonia chlamydosporia* and the bacteria *Bacillus firmus* I-1582 (Bf I-1582), to know its ability to induce plant resistance, and 2) the level of soil suppressiveness of vegetable production sites conducted under organic or integrated standards.

Zahra Ghahremani

UPC