Management strategies of *Meloidogyne*-resistant plant germplasm to avoid virulence selection

UNIVERSITAT POLITÈCNICA DE CATALUNYA BARCELONATECH

PhD dissertation

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Castelldefels, January 2025

“Si el hombre fuera constante, sería perfecto”

- William Shakespeare

The work presented in this thesis has been developed in the Horticulture: Production, Transformation and Utilization research group (HorPTA) of the Department of Agri-Food Engineering and Biotechnology (DEAB) in Universitat Politècnica de Catalunya-BarcelonaTech (UPC), in the framework of the following research project:

"**Estrategias de gestión de germoplasma vegetal resistente a *Meloidogyne* para evitar la selección de virulencia**". Funded by Ministerio de Ciencia e Innovación and confunded by FEDER, a way of making Europe, (AGL2017-89785-R).

This project has also financed the pre-doctoral scholarship of Aïda Magdalena Fullana Pons (PRE2018-084265).

Agraïments

Crec que els agraïments és un dels textos més complicats d'escriure, no vols deixar-te a ningú, no vols ofendre a ningú, però sobretot, vols que tothom sent-hi el gran suport que ha estat per tu en aquest moment de la teva vida.

Vull donar les gràcies, primer de tot, als meus tutors, per donar-me l'oportunitat de realitzar la present tesis Doctoral amb ells. Al Dr. Xavier Sorribas, qui sempre ha estat aquí, constant, persistent i sense tirar la tovallola amb mi -encara que més d'un cop segurament ho mereixia-. Ha sabut transmetrem tota la seva passió i coneixement. Ell és qui m'ha fet estimar la nematologia i voler endinsar-me en el coneixement d'aquest món. A la Dra. Ariadna Giné, que inclús abans d'acceptar la co-tutela ja estava disposada a ajudar-me amb tot el que calgués. Ha estat més que una tutora, una amiga amb qui poder parlar i comptar en els moments més baixos però també amb qui compartir l'emoció dels èxits.

Y a la Dr. Nuria Escudero. Se que no eres mi tutora, pero si las circunstancias hubieran sido otras, nadie se merecía este puesto más que tú. Te considero mi mentora, fuiste la primera persona quien me habló de ciencia e investigación sinceramente. Supiste transmitir-me tu pasión por el nuevo conocimiento y gracias a ti, hoy estoy aquí.

A tots els meus companys del Lab 20. A l'Isma i a l'Ana qui em van ensenyar totes les tècniques necessàries. A l'Helio i a L'Alex, qui van anar perdent temps i paciència fins que vaig saber contar. Al Roger, junts vam saber superar la pandèmia i ens vam convertir en més que companys. A tots els TFG, TFM, becaris, tècnics i gent de pràctiques, especialment a Manuel, nuestro Mejicano favorito. I a l'Irma és clar! Qui m'ha acompanyat pel món de la biologia molecular perquè no renunciés en l'intent. A la Sheila, la Maria i el Miquel per donar un cop de mà sense tenir-lo que demanar. I a tota la gent de la UPC qui ha estat aquí en algun moment (Olga(es), Cristina, Eva...) i a la Mariné, la mamà de tothom.

À Dra. Carla Maleita e à Dra. Isabel Abrantes que me receberam e acolheram no vosso laboratório. Com a vossa orientação adquiri e aprendi novas práticas e técnicas. Obrigado por me darem a oportunidade de estarem com vocês, são excelentes pessoas onde tiveram sempre tempo e disponibilidade para me ajudarem e mostrarem o trabalho que estavam a fazer. Obrigado à Dra. Ivânia e a todo o pessoal do NematoLab, a vossa hospitalidade foi enorme. Queria agradecer também e especialmente aos futuros doutores, Duarte e Mikael, onde para

além de colegas considero-vos grandes amigos! Ao restante pessoal que conheci em Portugal, obrigado também! Vocês fizeram-me sentir em casa.

Agradecer a la Dr. Meritxell Pérez y al Dr. Albero Urbaneja por invitarme en su laboratorio. Vuestro entusiasmo es contagioso y los conocimientos que adquirí con vosotros invaluables. Veros trabajar y debatir juntos es un auténtico placer. Gracias Shaima y Carolina por compartir conmigo todos los trucos de laboratorio que hacen más fácil el día a día.

I no em vull oblidar de la Marina i el Dr. Pablo Loza (i més recentment el Dr. Emilio Gualda) qui em van acollir al ICFO i em van descobrir les meravelloses imatges que es poden obtenir gràcies a la microscòpia làser.

A tots els veïns de despatx, Laura, Rein, Ruben, Jordi... ¡y la gente del CREDA! Without you, the time would have passed much more slowly.

A tots els amics de sempre, i als més nous. Jasmina, Mario, Laia, Alba, Julen, Lichet, Pito... que heu aguantat les meves anades d'olla continues estoicament. I al constant que ha estat fonamental perquè aquest projecte no s'acabés abans d'hora.

Vull agrair especialment a la meva família, la de sang i la que no. A mons pares, qui no em van deixar tirar la tovallola, gràcies a ells soc qui (lo que s'aconsegueix sent caparruda!). Al meu avi, qui sempre va estar al meu costat fins als últims moments, ojalá haguessis pogut llegir aquestes paraules...

Y finalmente a mi compañero de vida, quien ha vivido todos los momentos de una tesis sin necesidad de realizarla (¡Qué honor!). Gracias por apoyarme en todo, incluso, sin entenderlo.

En definitiva, a toda la gente que hizo posible que iniciara y terminara este proyecto...

MOLTES GRÀCIES!

Contents

Abstract

During the development of the PhD, several studies were conducted to explore the potential of plant resistance for the management of *Meloidogyne* in horticultural crops, none of which promoted the selection of virulent populations in the resistant germplasms used. Specifically: i) the effect of three growing seasons of resistant germplasm rotation; ii) the effect of *Solanum torvum* as a tomato rootstock as a method of alternating resistance sources in monoculture situations; iii) the capacity of zoophytophagous mirids to induce resistance in tomato germplasm susceptible to nematodes to reduce the frequency of use of resistant germplasm.; and iv) the response of potential rootstocks of melon and cucumber or watermelon to quarantine species in Spain as a possible management tool if they are found and not eradicated. The results obtained showed that: i) the rotation sequence with resistant germplasm (tomato grafted (grft.) onto 'Brigeor' - melon grft. onto *Cucumis metuliferus* - pepper grft. Onto 'Oscos' – watermelon grft. onto *Citrullus amarus* - resistant tomato cv. Caramba) cultivated in plots infested by an avirulent (Avi) or partially virulent (Vi) population of *M. incognita* to the *Mi1.2* resistance tomato gene reduced the Avi population density by 94% and the Vi population density to below detectable levels. Cumulative yield was 1.5 and 2.4 times higher in plots infested with Avi and Vi, respectively, compared to rotation with susceptible germplasm. The Avi population did not acquire virulence to any of the resistant germplasms used in the rotation; ii) the severity of the disease and nematode reproduction in tomatoes grafted onto *S. torvum* were reduced by 8.6 and 56 times, respectively, compared to non-grafted tomatoes, and yield was up to 87% higher at densities above 1 J2/cm^3 of soil. The resistance of *S. torvum* remained unchanged after three consecutive crops; iii) exposure of susceptible tomatoes to 15 nymphs/plant of *Nesiodiocoris tenuis* or *Macrolophus pygmaeus* for 24 hours before transplanting such as (Z)-3-hexenil propanoato volatile emitted by fed tomato plants, reduce nematode's reproduction until 60, 70 and 45% respectively; and iv) *Cucumis metuliferus* was immune to *M. chitwoodi*, highly resistant to M. enterolobii, and resistant to *M. luci. Citrullus amarus* ranged from resistant to moderately resistant to *M. chitwoodi* and *M. enterolobii*, and resistant to *M. luci*.

The results of these studies provide valuable information for nematode control, even in scenarios where species and/or populations virulent to specific resistance genes coexist, which are increasingly common in production areas.

Resumen

Durante el desarrollo de la tesis doctoral se realizaron varios estudios con objeto de conocer el potencial de la resistencia vegetal para la gestión de *Meloidogyne* en cultivos hortícolas sin favorecer la selección de poblaciones virulentas en los germoplasmas resistentes utilizados. Concretamente: i) el efecto de la rotación de germoplasma resistente durante 3 campañas agrícolas; ii) el efecto *de Solanum torvum* como portainjerto de tomate como método de alternar fuentes de resistencia en situaciones de monocultivo; iii) la capacidad de inducir resistencia por míridos zoofitófagos en germoplasma de tomate susceptible al nematodo para disminuir la frecuencia de uso de germoplasma resistente; y iv) la respuesta de potenciales portainjertos de melón y pepino o sandía frente a especies de cuarentena en España como posible herramienta de gestión en caso de que se encuentren y no sean erradicadas. Los resultados obtenidos han mostrado que: i) la secuencia de rotación con germoplasma resistente (tomate injertado (inj.) en 'Brigeor'-melón inj. en *Cucumis metuliferus*- pimiento inj. en 'Oscos'-sandía inj. en *Citrullus amarus*-tomate resistente cv. Caramba) cultivadas en parcelas infestadas por una población de *M. incognita* avirulenta (Avi) o parcialmente virulenta (Vi) al gen *Mi1.2* de resistencia en tomate redujo la densidad de la población Avi un 94% y la de Vi por debajo del nivel de detección. La producción acumulada fue de 1.5 y 2.4 veces mayor en las parcelas infestadas por Avi y Vi, respectivamente, respecto la rotación con germoplasma susceptible. La población Avi no adquirió virulencia a ninguno de los germoplasmas resistentes utilizados en la rotación; ii) la severidad de la enfermedad y la reproducción del nematodo en los tomates injertados en *S. torvum* se redujeron 8.6 y 56 veces, respectivamente, comparado con el tomate sin injertar, y la producción fue de hasta un 87% mayor a densidades superiores a 1 J2/cm^3 de suelo. La resistencia de *S. torvum* se mantuvo inalterada después de tres cultivos consecutivos; iii) la exposición del tomate susceptible a 15 ninfas/planta de *Nesiodiocoris tenuis* o *Macrolophus pygmaeus* durante 24h antes del trasplante así como el compuesto volátil (Z)-3-hexenil propanoato que emiten las plantas de tomate picadas por los mismos, reducen la capacidad reproductora del nematodo hasta un 60, 70 y 45% respectivamente; y iv*) Cucumis metuliferus* fue inmune a *M. chitwoodi*, muy resistente a *M. enterolobii* y resistente a *M. luci*. *Citrullus amarus* varió entre resistente a moderadamente resistente a *M. chitwoodi* y *M. enterolobii*, y resistente a *M. luci*.

Los resultados de estos estudios proporcionan información valiosa para el control del nematodo, incluso en escenarios en los que coexisten especies y/o poblaciones virulentas a determinados genes de resistencia, los cuales son cada vez más comunes en las áreas de producción.

Resum

Durant el desenvolupament de la tesi doctoral es van realitzar diversos estudis a fi de conèixer el potencial de la resistència vegetal per a la gestió de *Meloidogyne* en cultius hortícoles sense afavorir la selecció de poblacions virulentes en els germoplasmes resistents utilitzats. Concretament: i) l'efecte de la rotació de germoplasma resistent durant 3 campanyes agrícoles; ii) l'efecte de *Solanum torvum* com portaempelt de tomàquet com a mètode d'alternar fonts de resistència en situacions de monocultiu; iii) la capacitat d'induir resistència per mírids zoofitòfags en germoplasma de tomàquet susceptible al nematode per a disminuir la freqüència d'ús de germoplasma resistent; i iv) la resposta de potencials portaempelts de meló i cogombre o síndria enfront d'espècies de quarantena a Espanya com a possible eina de gestió en cas que es trobin i no siguin erradicades. Els resultats obtinguts han mostrat que: i) la seqüència de rotació amb germoplasma resistent (tomàquet empeltat (emp.) en 'Brigeor'-meló emp. en *Cucumis metuliferus*- pebrot emp. en 'Oscos'-síndria emp. *en Citrullus amarus*-tomàquet resistent cv. Caramba) conreades en parcel·les infestades per una població de *M .incognita* avirulenta (Avi) o parcialment virulenta (Vi) al gen *Mi1.2* de resistència en tomàquet, va reduir la densitat de la població Avi un 94% i la de Vi per sota del nivell de detecció. La producció acumulada va ser d'1.5 i 2.4 vegades major en les parcel·les infestades per Avi i Vi, respectivament, respecte la rotació amb germoplasma susceptible. La població Avi no va adquirir virulència a cap dels germoplasmes resistents utilitzats en la rotació; ii) la severitat de la malaltia i la reproducció del nematode en els tomàquets empeltats en *S. torvum* es van reduir 8.6 i 56 vegades, respectivament, comparat amb el tomàquet sense empeltar, i la producció va ser de fins a un 87% major a densitats superiors a 1 J2/cm³ de sòl. La resistència de *S. torvum* es va mantenir inalterada després de tres cultius consecutius; iii) l'exposició del tomàquet susceptible a 15 nimfes/planta de *Nesiodiocoris tenuis* o *Macrolophus pygmaeus* durant 24h abans del trasplantament així com el compost volàtil (Z)-3-hexenil propanoat emès per les plantes de tomàquet picades pels mateixos, redueixen la capacitat reproductora del nematode fins un 60, 70 y 45% respectivament; i iv) *Cucumis metuliferus* va ser immune a *M. chitwoodi*, molt resistent a *M. enterolobii* i resistent a *M. luci*. *Citrullus amarus* va oscil·lar entre resistent a moderadament resistent a *M. chitwoodi* i *M. enterolobii*, i resistent a *M. luci*.

Els resultats d'aquests estudis proporcionen informació valuosa per al control del nematode, fins i tot en escenaris en els quals coexisteixen espècies i/o poblacions virulentes a determinats gens de resistència, els quals són cada vegada més comunes en les àrees de producció.

Relationship between the chapters and the derived publications and conferences

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Publications in peer-reviewed journals

CHAPTER 1

Fullana, A. M., Exposito, A., Escudero, N., Cunquero, M., Loza-Alvarez, P., Gine, A., and Sorribas, F. J. (2023). Crop rotation with *Meloidogyne*-resistant germplasm is useful to manage and revert the (a) virulent populations of Mi1. 2 gene and reduce yield losses. Frontiers in plant science, 14, 1133095

Hamdane, Y., Gracia-Romero, A., Buchaillot, M. L., Sanchez-Bragado, R., Fullana, A. M., Sorribas, F. J., Araus J. L. and Kefauver, S. C. (2022). Comparison of proximal remote sensing devices of vegetable crops to determine the role of grafting in plant resistance to *Meloidogyne incognita*. Agronomy, 12(5), 1098

Expósito, A., Pujolà, M., Achaerandio, I., Giné, A., Escudero, N., Fullana, A. M., Cunquero, M., Loza-Alvarez, P and Sorribas, F. J. (2020). Tomato and melon *Meloidogyne* resistant rootstocks improve crop yield but melon fruit quality is influenced by the cropping season. Frontiers in Plant Science, 11, 560024

CHAPTER 2

Fullana A. M., Expósito, A., Pujolà, M, Achaerandio, I., Cunquero, M., Loza-Alvarez, P., Giné, A. and Sorribas, F. J. (2024). Effect of grafting tomato onto *Solanum torvum* on the population dynamics of *Meloidogyne incognita* and *M. javanica* and crop yield losses. Plant Pathology, 00,1–13

CHAPTER 3

Fullana, A. M., Giné, A., Urbaneja, A., Pérez-Hedo, M., Sorribas, F. J., Expósito, A. (2024). *Nesiodiocoris tenuis* and *Macrolophus pygmaeus* (Hemiptera: Miridae) induce systemic resistance in tomatoes against the root-knot nematode *Meloidogyne* spp. Summited in Biological Control

Vernet, H., Fullana, A. M., Sorribas, F. J., and Gualda, E. J. (2022). Development of microscopic techniques for the visualization of plant–root-knot nematode interaction. Plants, 11(9), 1165

CHAPTER 4

Fullana, A. M., Maleita, C., Santos, D., Abrantes, I., Sorribas, F. J. and Giné, A. (2024). Reaction of *Cucumis metuliferus* and *Citrullus amarus* to *Meloidogyne chitwoodi*, *M. enterolobii* and *M. luci*. Phytopathologia Mediterranea, 63(1), 79-90

Other publications

CHAPTER 1

Fullana, A. M., Expósito, A, García-Mendivil, H.A, Escudero, N, Giné, A and Sorribas, F. J. (2022). Bones pràctiques d'ús de la resistència vegetal per optimitzar el control de *Meloidogyne* en cultius hortícoles i evitar la selecció de virulència. Fitxa tècnica 02. Horta.net.

Oral communications at international conferences

CHAPTER 1

Hamdane Y., Gracia-Romero A., Buchaillot, M. L., Sanchez-Bragado R., Fullana, A. M., Sorribas-F. J., Araus, J. L. and Kefauver, S. C. (May, 2021). Comparison of proximal remote sensing devices of vegetable crops to determine the role of grafting in plant resistance to *Meloidogyne incognita*. 1st International Electronic Conference on Agronomy, Online

CHAPTER 2

Gualda, E.J., Fullana, A.M., Sorribas, F. J. and Vernet, H (November, 2022). Development of microscopic techniques (LSFM, OPT and Tissue Clearing) for the visualization of plant--root-knot nematode interaction. Internacional Congress of Spanish-Portuguese Meeting for Advanced Optical Microscopy, Salamanca, España

CHAPTER 3

Expósito, A., Fullana, A. M., Urbaneja, A., Pérez-Hedo, M., Urbaneja-Bernat, P., Riudavets, J., Giné, A., Sorribas, F. J. (April, 2024). *Macrolophus pygmaeus* and *Nesiodiocoris tenuis* (Heteroptera: Miridae) induce systemic resistance in tomato against *Meloidogyne* spp. 35th Symposium of the European Society of Nematologists, Córdoba, Spain

CHAPTER 4

Fullana, A. M., Maleita, C., Santos, D., Abrantes, I., Sorribas, F. J. and Giné, A. (April, 2024). Host suitability of *Cucumis metuliferus* and *Citrullus amarus* to Meloidogyne chitwoodi, *M. enterolobii* and *M. luci*. 35th Symposium of the European Society of Nematologists, Córdoba, Spain

Oral communications at national conferences

Fullana, A.M., Expósito, A., Escudero, N., Cunquero, M., Loza-Alvarez, P., Pérez-Hedo, M., Maleita, C., Abrantes, I., Giné, A. and Sorribas, F. J. (September, 2024). Manejo de germoplasma vegetal resistente a *Meloidogyne* para evitar la selección por virulencia. XXI congreso nacional de la Sociedad Española de Fitopatología, Córdoba, Spain.

CHAPTER 1

Fullana, A.M., Expósito, A., García-Mendívil, H.A., Giné, A. and Sorribas, F. J. (October, 2022). La rotación de germoplasma resistente a *Meloidogyne* es útil para gestionar poblaciones (a)virulentas al gen *Mi1.2* de tomate y reducir las pérdidas de producción. XX congreso nacional de la Sociedad Española de Fitopatología, Valencia, Spain.

CHAPTER 3

Fullana, A.M., Expósito, A., García-Mendívil, H.A., Giné, A. and Sorribas, F. J. (October, 2022). Dinámica de población de *Meloidogyne incognita* y *M. javanica* en tomate sin injertar e injertado en *Solanum torvum* y pérdidas de producción. XX congreso nacional de la Sociedad Española de Fitopatología, Valencia, Spain.

Poster communications at national conferences

CHAPTER 3

Fullana, A.M.; Vernet, H.; Gualda Manzano, Emilio Jose; Sorribas, F. J. (October, 2022). Desarrollo de técnicas microscópicas para la visualización de la infección de raíces por nematodos agalladores". En: XX Congreso nacional de la Sociedad Española de Fitopatologia, Valencia, Spain.

CHAPTER 4

Expósito, A.; Urbaneja-Bernat, P.; Boncompte, S.; Fullana, A.M.; Giné, A.; Sorribas, F.J.; Riudavets, J. (September, 2024). *Macrolophus pygmaeus* (Heteroptera: Miridae) induce resistencia sistémica en tomate frente *Meloidogyne* spp.. XXI congreso nacional de la Sociedad Española de Fitopatología, Córdoba, Spain.

General introduction

General introduction

The genus *Meloidogyne*

Meloidogyne spp., commonly known as root-knot nematodes (RKN), are obligate sedentary endoparasites responsible for approximately half of the crop yield losses attributed to plant-parasitic nematodes worldwide (Bent et al., 2008), and the most limiting for vegetable crops (Hallman and Meressa, 2018). Among the roughly 100 RKN species described so far, four of them are the most frequent globally: *M. incognita* (51%), *M. javanica* (31%), *M. arenaria* (8%), and *M. hapla* (8%) (Sasser et al., 1983; Jones et al., 2013). These four species are considered responsible for the majority of yield losses caused by RKN which are able to parasitize nearly every species of vascular plants (Jones et al., 2013). Other RKN species, such as *M. chitwoodi, M. enterolobii, M. fallax*, and *M. luci,* are gaining importance globally due to their high pathogenicity to several economically important crops (Castagnone-Sereno, 2012; Elling, 2013; Maleita et al., 2022). Despite the restricted distribution of these emerging species, Europe has implemented legislative measures prevent their introduction into non-infested regions and to eradicate them if they are detected. Recognizing their threat, *M. chitwoodi, M. enterolobii*, and *M. fallax* have been included in the EPPO A2 list of pests recommended for regulation as quarantine pests (EPPO, 2023a), and *M. luci* has been added to the EPPO Pest Alert List (EPPO, 2017).

The roots of plants infected by *Meloidogyne* show root-knots called galls (Figure 1A) impairing the absorption of water and nutrients affecting the development of the aboveground part of the plant that can show stunting, symptoms of nutritional deficiency, wilting, and plants can die depending on the nematode density at sowing or transplanting, the plant tolerance and the number of life cycles that the nematode can complete during the cropping season (Sorribas et al., 2020).

The tropical RKN species, *M. arenaria, M. incognita,* and *M. javanica,* are the most frequent RKN species found in Spain, mainly under protected cultivation due to favourable conditions for their development, such as soil temperature and humidity, and the continuous presence of host plants which favours nematode population growth and crop yield losses associated to them (Sorribas and Ornat, 2011). For instance, estimated maximum crop yield losses due to *Meloidogyne* spp. in horticultural crops under protected cultivation can reach 73% in tomato, 10% in pepper, 23% in eggplant, 88% in cucumber, 93% in melon, 39% in zucchini and 37% in watermelon (Expósito et al., 2020; Giné and Sorribas, 2017a; Khan, Mukhtar and Saeed, 2019; López-Gómez et al., 2014; Talavera-Rubia et al., 2022; Vela et al., 2014).

Figure 1. Healthy tomato plant (A) and galls formed in roots (B) caused by *M. incognita.*

Meloidogyne life cycle

Meloidogyne spp. are obligate sedentary endoparasites that have developed sophisticated interactions with their plant hosts (Abad et al., 2003). The life cycle includes six developmental stages: egg, four juvenile stages (J1-J4), and adult. The second-stage juvenile (J2) is the only infective stage. J2 penetrates the roots through the elongation zone, aided by the mechanical action of the stylet and secretions, and migrates toward the vascular cylinder to establish a permanent feeding site, where nematode secretions stimulate the growth of adjacent cells into multinucleated giant cells (GC), forming a rosette. Depending on the host status of the plant, the GC vary in number, shape and volume (Expósito et al., 2020; Phan et al., 2018). In good hosts, GC are multinucleated with a dense cytoplasm. In poor hosts, CG are less voluminous, have fewer nuclei, and have vacuoles that reduce the volume of the cell's cytoplasm. In resistant hosts, there are a greater number of GC per feeding site but are smaller; some have no nuclei and are empty (Expósito et al., 2020). Thus, the nematode development and reproduction depend on the characteristics of the feeding site (Nyczepir and Thomas, 2009). Once the feeding site is correctly established, J2 becomes sedentary, going through three molts until they reach the adult stage. Sexual differentiation occurs in the larval stages, although the final result may change depending on environmental conditions. The saccate (pyriform) females remain sedentary, producing large egg masses extruded in a gelatinous matrix. The egg mass preserves the eggs against adverse environmental conditions and predators or microbial antagonists in the soil (Orion et al., 2001). Meanwhile, males, when present, migrate out of the plant tissues and do not feed plant roots (Abad et al., 2003).

Sex chromosomes are absent in with sex determination occurring epigenetically under suboptimal environmental conditions (Snyder, 2004). In addition, males can be found in both

meiotic and mitotic parthenogenetic species. The genus exhibits three distinct types of reproduction: (i) amphimixis, where the sperm from males fertilize oocytes in females, and meiosis subsequently occurs. (ii) facultative meiotic parthenogenesis, where amphimixis occurs in the presence of males, but in their absence, while the egg nucleus undergoes classical meiosis, the first polar body degenerates, and the fusion of the second polar body with the egg pronucleus restores the somatic chromosome number, and (iii) obligate mitotic parthenogenesis, where males are not involved (apomixis or amixis). In this process, no meiosis occurs during the production of the female gamete, and the (unfertilized) eggs simply derive from unreduced oocytes. Most *Meloidogyne* species, including the four majority species, are parthenogenetic (Chitwood and Perry, 2009). *Meloidogyne arenaria, M. incognita*, and *M. javanica* are parthenogenetic mitotic species, and *M. hapla* is mostly meiotic parthenogenetic. Still, the populations of the same species may differ in their mode of reproduction (Castagnone-Sereno and Danchin, 2014). Differentiation into males in parthenogenetically RKN species is considered a population regulation mechanism to avoid intraspecific competition.

The duration of the life cycle of *Meloidogyne* spp. will depend on the soil temperature, the nematode species and the host status of the plant (Sorribas et al., 2020).

Population dynamics of *Meloidogyne*

Population dynamics refer to the variation in the number of nematodes over time, influenced by the availability of a host plant, its host status, the initial density of nematodes at sowing or transplanting (*Pi*), and the environmental conditions. The relationship between *Pi* and nematode density at the end of the crop (*Pf*) can be illustrated in the following graph (Figure 2).

Under favourable conditions, where *Pi* is low, and there are no limiting factors for the nematode development, the relationship between *Pi* and *Pf* is linear ($Pf = a \cdot Pi$), where a represents the maximum multiplication rate. As *Pi* increases, intraspecific competition begins leading to a lower multiplication rate until *Pf* reaches the maximum density that a specific host can support (*M*).

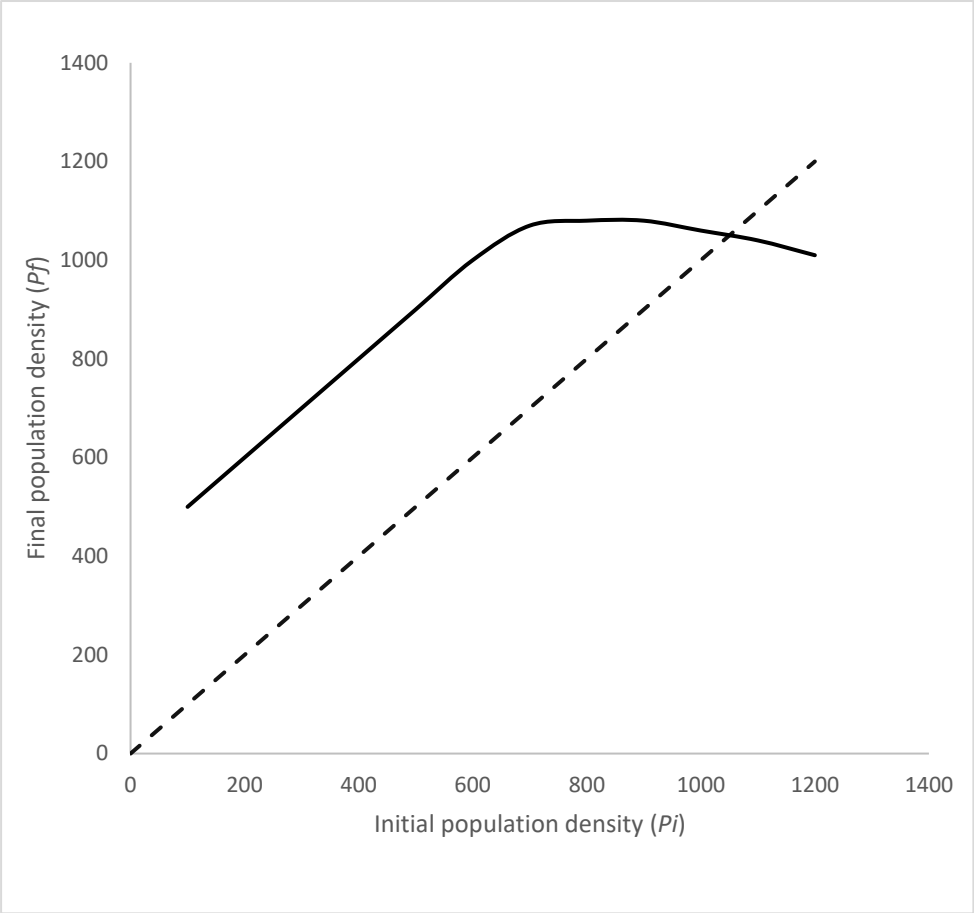


Figure 2. Relationship between initial (*Pi*) and final (*Pf*) population densities of *Meloidogyne* spp. (continue line) and equilibrium density (discontinue line).

When *Pi* exceeds *M*, a reduction in *Pf* occurs due to insufficient food resources to sustain the nematode density to achieve the equilibrium density (*E*), defined as the maximum density of nematodes at the time of sowing or transplanting that the plant can support by the end of the crop (*Pf* = *Pi*) (Schomaker and Been, 2006).

$$E = \frac{M\ (a-1)}{a}$$

E: equilibrium density; *M*: maximum population density; *a*: maximum reproduction rate

Knowing the values of *a, M* and *E* allow us to define the nematode's host plant status. Depending on the ability to feed and reproduce on a given plant species, the plant can be classified as a good host (or susceptible), poor host, resistant host, or no host (Figure 3).

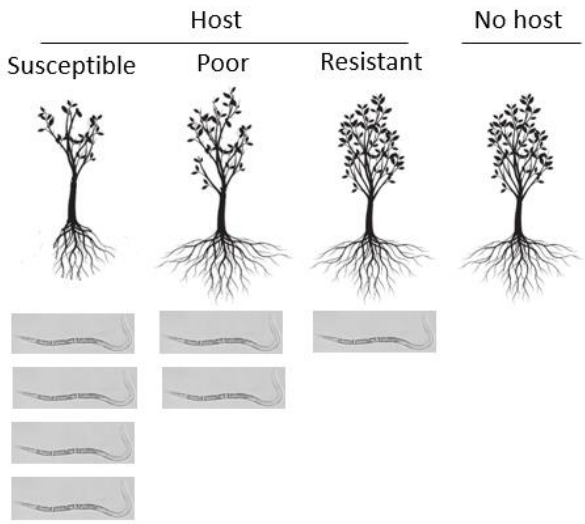


Figure. 3. The *Meloidogyne* host plant type according to its ability to feed and reproduce there.

The parameters *a, M*, and *E* indicate the rate of nematode population development in a given host but do not reflect the nematode's effect on plant productivity. The crop yield is related to the *Pi*, the plant's tolerance to support nematode densities without suffering yield losses (tolerance), and the number of generations the nematode can complete during the cropping season (Sorribas et al., 2020). The relationship between the relative crop yield at increasing *Pi* densities is described by the Seinhorst's damage function model ($y = m + (1-m) \cdot 0.95^{(Pi/T-1)}$) (Seinhorst, 1998), where *y* is the relative crop yield; *T* is the tolerance limit, that is, the nematode density below which no yield losses occur; and *m* is the minimum relative crop yield.

In the absence of a host plant, the eggs into the egg masses can survive into soil or plant debris for some time depending on the environmental conditions, but the J2 will consume their reserves in proportion to soil temperatures between the base and optima nematode temperature range (Sorribas et al., 2000).

Meloidogyne spp. management

Meloidogyne management encompasses strategies to prevent nematode infestation and suppress primary inoculum and population growth to mitigate their effects on crop production. Effective control requires an integrated approach combining multiple methods to ensure long-term efficacy and sustainability.

INFESTATION PREVENTION

Preventing nematode infestation is a fundamental strategy, particularly for regulated RKN species. This strategy involves legislative measures to restrict these species' entry,

establishment, and spread in nematode-free zones as defined by the EPPO alert list. Certified plant material free of harmful nematodes is crucial to avoid infestation.

At the farm level, it is recommended to ensure that machineries and tools are free of infested soil to prevent the spread of plant-parasitic nematodes through agricultural practices from nematode-infested to non-infested soils (Djian-Caporalino et al., 2009). Moreover, irrigation water should be controlled, as nematodes can spread through water (Hugo and Malan, 2010). When the soil conditions are not conducive to healthy crop growth, for example, for high levels of RKN density, replacing native soil with artificial substrates such as bark, fiber, vermicompost, perlite, vermiculite…, or a mixture is advisable. These pathogen-free substrates are typically placed in bags deposited in or on the soil, and depending on the crop, these bags must be changed periodically to prevent root dissemination into infected native soil due to the bag-breaking capacity of some roots.

MANAGEMENT OF PRIMARY INOCULUM

Understanding the plant-parasitic nematode community occurring in a field is essential for identify the potential nematode species that can affect the crops usually cultivated in the area to design effective management strategies to keep population densities of the limiting nematode species below the economic threshold.

The occurrence of limiting nematode species at high densities requires the use of control methods able to reduce them. Chemical nematicides, including fumigants and non-fumigants, have been widely used due to its rapid action and high efficacy. Soil fumigants are applied before sowing or transplanting due to its phytotoxic effect. Fifteen years ago, 1,3-dichloropropene and metam-sodium were the two active ingredients most commonly used against RKN in Spain (Talavera et al., 2012). However, according to EU Regulation 540/2011 regarding the list of approved active substances, the former was not approved, and the latter is subjected to restrictions through an extension until November 30, 2025, by the Commission Implementing Regulation. Non-fumigant nematicides can be applied before and/or after sowing or transplanting the crop but are less effective than the fumigants. Nowadays, only six active ingredients are authorised in Spain against RKN (Ministerio de Agricultura Pesca y Alimentación, 2024). But, given the obligation to reduce the use of pesticides under the European Directive 2009/128/EC and the Green Deal Programme, which aim to achieve sustainable use of pesticides, sustainable and economically viable alternatives are needed to reduce the population density of nematodes below the economic damage threshold.

Soil solarization is an effective alternative to chemical nematicides. It uses solar radiation to heat moist soil covered with transparent, airtight plastic mulch, and it efficiently reduces nematode densities if temperatures reach 40-60°C within the top 30 cm of soil (Katana, 1981). 32.9 and 46.2 h are enough to kill 100% of eggs and J2 at 40 °C, respectively (Wang and McKsorley, 2008). Unfortunately, this method is not selective and can alter soil microbial communities. Biosolarization is a practice that combines biofumigation, using green and or cattle manure amendments, with solarization that improves the effectiveness of the latter (Curto et al., 2014; Pikerton et al., 2000) and mitigates its negative effects (Guerrero et al., 2013; Núñez-Zofío et al., 2013; Ros et al., 2016), restoring soil structure, enhancing beneficial microorganisms, improving microbial activity, and maintaining soil fertility (Cook and Baker, 1983; Snapp et al., 2005). If biofumigant plants such as cruciferous or certain species of sorghum are used as green manure, the biocidal compounds released during its decomposition into the soil can significantly affect the viability of plant-parasitic nematodes (Argento et al., 2019; Djian-Caporalino et al., 2019; Widmer and Abawi, 2000).

SUPPRESSION OF NEMATODE POPULATION GROWTH

Maintaining and stimulating the natural soil microbiota plays an important role in preventing RKN infection and nematode population to growth. Numerous organisms commonly present in soil can regulate nematode populations directly through parasitism or predation and antibiosis or indirectly interfering with host recognition and or inducing plant defence mechanism (Stirling, 1991). In Spain, several nematode antagonists have been identified in vegetable growing areas (Giné et al., 2013; Verdejo-Lucas et al., 2002), and suppressive soils to RKN have been characterized (Giné et al., 2016). These suppressive soils are environments in which pathogens do not establish or persist; or in which they cause little or no damage for a limited period. The soil antagonistic potential could be enhanced to achieve soil suppression by combining agricultural practices (Sikora, 1992) or by applying biological nematicides.

Additionally, some microorganisms that can be present in the soil have been shown to induce systemic resistance to RKN, such as *Pochonia chlamydosporia* (Ghahremani et al., 2019), *Trichoderma* spp. (de Medeiro et al., 2017; Martinez-Medina et al., 2017; Pocurull et al., 2019) and *Bacillus firmus* strain I-1582 (Ghahremani et al., 2020). Induced resistance improves the basal resistance of plant genotypes in response to stimuli from biotic and/or abiotic agents. It is mainly regulated by three hormonal signalling pathways: salicylate (SA), jasmonate (JA), and ethylene (ET) (Grant and Jones, 2009; Pieterse et al., 2009). Signalling pathways influence each other in a complex network of synergistic and antagonistic interactions (Beckers and Spoel, 2006; Turner et al., 2002; Van Loon et al., 2006), allowing the plant to adapt its defense

mechanisms according to the inducing agent. Shukla et al., 2018 demonstrated that in the early stages of infection in the compatible *Meloidogyne*-susceptible tomato interaction, the abscisic acid (ABA) and ET pathways are induced, and the SA and JA pathways are repressed. Meanwhile, the incompatible *Meloidogyne*-tomato, tomato-resistant interaction induces the ABA and SA pathways.

The induction of plant defense is not exclusive to soil microorganisms. It is known the ability of some species of zoophytophagous predators belonging to the *Miridae* family to induce resistance in tomatoes against *Bemisia tabaci* and *Tetranichus urticae* by increasing the expression of some genes associated with the JA pathway (Pérez-Hedo et al., 2015a and b, 2018), and it could be also effective against *Meloidogyne* because the exogenous application of methyl jasmonate enhances defense in some plants against RKN (Cooper et al., 2005; Fujimoto et al., 2011; Hu et al., 2017), Moreover, abiotic factors, such as biostimulants, can also induce plant resistance to pests and pathogens (Shukla et al., 2019), some of which are commercial products authorized (Regulation (EC) No. 1107/2009) such as chitosan, fructose, willow bark, horsetail or calcium hydroxide, among others.

Plant resistance is one of the most used and effective control methods to reduce the initial population density and the multiplication rate of RKN (Ornat and Sorribas, 2011; Petrillo and Roberts, 2005). Resistance can be conferred by specific plant genes (genetic resistance) or by activating plant defense mechanisms in response to stimuli from biotic and/or abiotic agents (induced resistance) as it has been seen previously. Genetic resistance is due to single major genes or a combination of two or more genes or quantitative trait loci (QTL) that can supress one or more of the key stages of pathogenesis, such us nematode penetration, infection, development and/or reproduction. Different genes for resistance can occur in a plant species showing different responses to different RKN species or populations of the same species according to the presence or absence of avirulent factors in the nematode (Williamson and Roberts, 2009). Consequently, the expression and level of plant resistance depend on the genetic background of both plant and nematode (Cortada et al., 2008; López-Pérez et al., 2006). In fruiting vegetable crops, the commercial availability of plant resistance in cultivars and or rootstocks is scarce and restricted to solanaceous and cucurbit crops such as tomato, pepper, eggplant and watermelon but some resistant rootstocks not commercially available currently have been described and characterized for cucumber and melon (Sorribas et al., 2000; Williamson and Roberts, 2009). Among the identified and characterized resistance genes in fruiting solanaceous crops, the *Mi1.2* resistance gene of tomato is the most used worldwide. The *Mi1.2* gene was introgressed in *S. lycopersicum* from *Solanum peruvianum* by embryo

culture by Smith in the 1940's. This gene is active against *M. arenaria*, *M. incognita*, *M. javanica,* and *M. luci* (Roberts and Thomason, 1989; Santos et al., 2019). In pepper, a series of dominant resistance genes have been identified clustered on chromosome P9 in various pepper lines (Djian-Caporalino et al., 2007; Hendy et al., 1985). Commercial cultivars and/or pepper rootstocks carrying some of the *Me1, Me3*, or *N* resistance genes are available which are actives against *M. arenaria*, *M. incognita* and *M. javanica* (Djian-Caporalino et al., 1999; Fazari et al., 2012; Hare 1956; Thies et al., 2003). Regarding eggplant, there are not commercial cultivars resistant to root-knot nematodes but there is a commercial eggplant rootstock, *Solanum torvum,* resistant to *M. arenaria, M. incognita, M. javanica, M. luci,* and *M. enterolobii* (Dhivya et al., 2014; García-Mendívil et al., 2019; Murata and Uesugi, 2021; Öçal et al., 2018; Pinheiro et al., 2022; Uehara et al., 2017). Moreover, this rootstock is partially compatible with tomato (Daunay and Dalmasso, 1985), but it delays plant growth and yield (de Miguel et al., 2011) and some fruits can be affected by physiological disorders (Lee and Oda, 2003).

Concerning cucurbit crops, all commercial cultivars are susceptible to RKN, except watermelon that is a poor host (López-Gómez et al., 2014). However, some plant species that can be cucurbit rootstocks are resistant to the most widely distributed RKN, such as *Citrullus amarus* (García-Mendívil et al., 2019b; Thies et al., 2010), currently available commercially as Carolina Strongback (https://www.syngentavegetables.com/en-au/product/seed/watermelon/carolina-strongback) or *Cucumis metuliferus* that is a compatible rootstock for melon (Gisbert et al., 2015; Sigüenza et al., 2005) and is resistant to *M. arenaria*, *M. chitwoodi*, *M. enterolobii*, *M. incognita*, *M. javanica,* and *M. luci* (Expósito et al., 2018, 2019; Pinheiro et al., 2019). The Cucurbita hybrid rootstocks are not resistant to RKN (Giné and Sorribas, 2017a; López-Gómez et al., 2016).

Despite the benefits of using plant resistance, some aspects must be taken into account to maximize its effectiveness such as its sensitivity at high soil temperatures and the putative selection for nematode virulence. The first can be solved by the use of mulching material to creating a barrier between the soil and the external environment to keep the soil cooler in summer and raising it in the winter, the later by avoid monocropping of the same resistance gene, otherwise, virulence is progressively selected (Aydinli et al., 2019; Giné and Sorribas, 2017b; Expósito et al., 2019; Verdejo -Lucas et al., 2012) notwithstanding the asexual reproduction of the most widespread RKN species. This fact should not be surprising since the genome of *Meloidogyne* evolves efficiently in response to environmental changes, as demonstrated by its global distribution and high polyphagous capacity. Large chromosomal rearrangements are common in the *Meloidogyne* genome (Triantaphyllou, 1985), with transposable elements being an important source of genetic diversity, facilitating its adaptation

to environmental changes (Kidwell and Lisch, 2000). Castagnone-Sereno (2002) suggested that the ability to bypass the R genes may be located in an unstable chromosomal region. However, the acquisition of virulence remains a stable trait in the nematode lineage (Castagnone-Sereno et al., 1993). The offspring from a single female should theoretically lead to the establishment of nearly isogenic lines that differ only in their ability to overcome the R gene when exposed successively to the same R-gene. However, there is not any molecular marker of virulence available until now.

The acquisition of virulence has been widely reported for the resistant gene *Mi1.2* in tomato cultivars and rootstocks (Giné and Sorribas, 2017b; Ornat et al., 2001; Verdejo-Lucas et al., 2009, 2012), as well as some of the *Me3, Me7* and/or N genes in pepper (Djian-Caporalino et al., 2011; Ros-Ibáñez et al., 2014; Thies et al., 2011). For *S. torvum*, only one *M. arenaria* genotype in Japan has been reported overlapping with the cultivation area of grafted eggplant onto *S. torvum* (Uehara et al., 2017). Regarding the cucurbit rootstocks, *C. metuliferus* and *C. amarus,* no virulent nematode populations have been reported so far.

The acquisition of virulence represents a high biological cost for the nematode resulting in a reduction of their reproductive capacity in susceptible genotypes of the same plant species (Djian-Caporalino et al., 2011). Consequently, the inclusion of susceptible and resistant plant germplasm of the same species in rotation sequences could suppress or delay the acquisition of virulence (Nilusmas et al., 2016; Talavera et al., 2009). Another possibility is to use different R genes to preserve their durability, with pyramiding, alternating, mixture of R genes, sequential use of a R gene introgressed in a susceptible background (Djian-Caporalino et al., 2014). However, Expósito et al (2019) reported that two different R gens are insufficient to prevent the selection of virulence to one of them. So, alternating more than two different R genes is necessary to avoid virulence selection.

In summary, the control methods described against RKN have all shown their limits when used individually (Collange et al., 2011). Consequently, integrated nematode management strategies need to be designed for effective and sustainable management based on knowledge of the potentially dangerous plant-parasitic nematodes present on a farm, the biotic and abiotic factors influencing their population dynamics and their consequent impact on crop yields, together with a thorough understanding of the efficacy of the available control methods, and when and how to use them.

Objectives

Objectives

The general objective was to design management strategies to control *Meloidogyne* using resistant germplasm to prevent the selection of virulent populations through two approaches: i) characterizing resistant plant germplasm and determining the effect of its rotation on virulence selection, and ii) enhancing the durability of plant resistance through combination with resistance-inducing plant organisms.

Following the main objective, five specific objectives emerge:

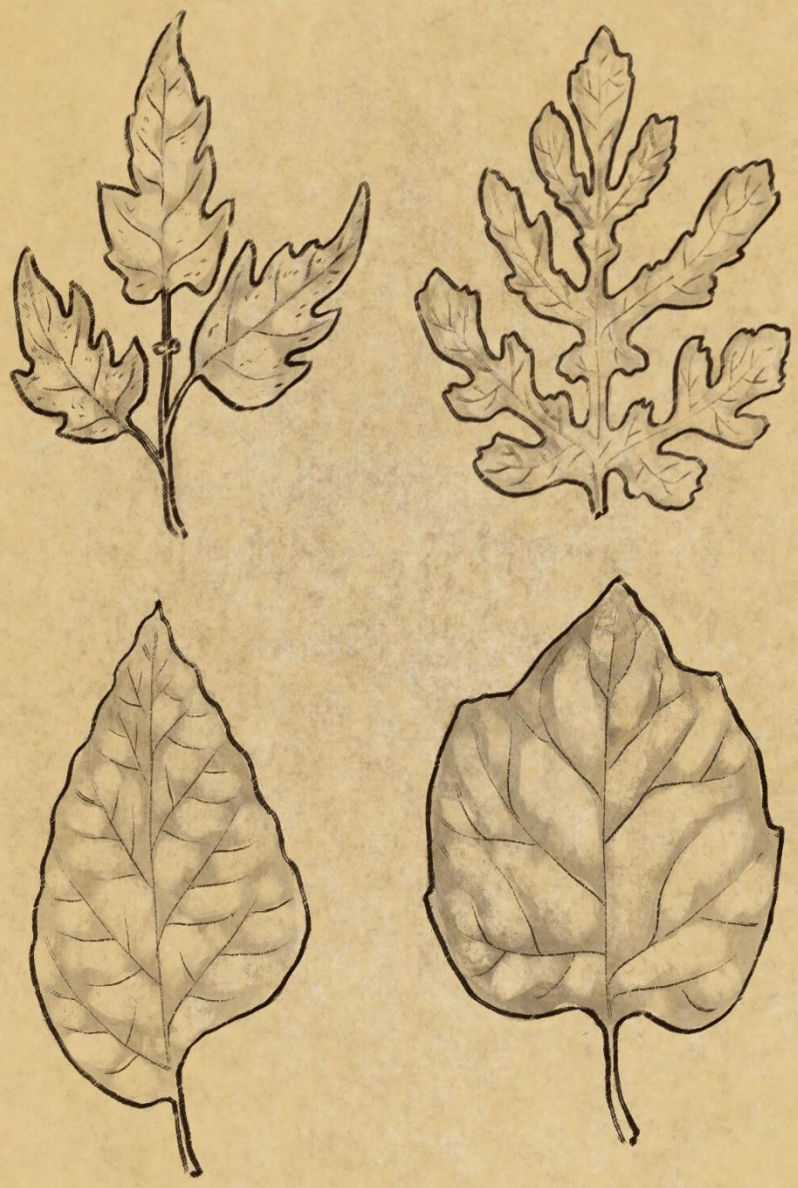
Objective 1: Manage the emergence of virulence through a crop rotation, including solanaceous-cucurbitaceous *Meloidogyne*-resistant germplasm, without compromising fruit yield (Chapter 1).

Objective 2: Assess *Solanum torvum* cv. Brutus's potential as a tomato rootstock by examining its impact on *Meloidogyne*'s population dynamics of fruit yield and quality (Chapter 2).

Objective 3: Evaluate the impact of the fitness cost induced by *S. torvum* on developing nematode populations under continuous exposure as a rootstock (Chapter 2).

Objective 4: Evaluate the effect of zoophytophagous myriad insects commonly used for tomato pest control on the induction of resistance in plants against *Meloidogyne* (Chapter 3).

Objective 5: Determine the host suitability of *Cucumis metuliferus* BGV11135 and *Citrullus amarus* BGV5167 used in the rotation of Objective 1 for European-regulated *Meloidogyne chitwoodi*, *M. enterolobii* and *M. luci* species (Chapter 4)



Chapter 1

Manage the emergence of virulence through the rotation of *Meloidogyne*-resistant germplasm in solanaceous-cucurbitaceous sequences without affecting fruit yield

Abstract

A rotation sequence of ungrafted and grafted tomato-melon-pepper-watermelon on resistant rootstocks 'Brigeor', *Cucumis metuliferus*, 'Oscos' and *Citrullus amarus*, respectively, was carried out in a plastic greenhouse, ending with a susceptible or resistant tomato crop. The rotation was conducted in plots infested by an avirulent (Avi) or a partially virulent (Vi) *Meloidogyne incognita* population to the *Mi1.2* gene. At the beginning of the study, the reproduction index (RI, relative reproduction in the resistant respect susceptible tomato) of Avi and Vi populations was 1.3% and 21.6%, respectively. Soil nematode density at transplanting (*Pi*) and at the end (*Pf*) of each crop, disease severity and crop yield were determined. Moreover, the selection of putative virulence and fitness cost were determined at the end of each crop in pot tests. In addition, a histopathological study was carried out 15 days after nematode inoculation in the pot test. The volume and number of nuclei per giant cell (GC) and the number of GC, their volume and the number of nuclei per feeding site in susceptible melon, tomato, pepper, and watermelon were compared with *Cucumis metuliferus*, resistant tomato, resistant pepper, and *Citrullus amarus*. At the beginning of the study, the *Pi* of Avi and Vi plots did not differ between susceptible and resistant germplasm. At the end of the rotation, the *Pf* of Avi was 1.2 the *Pi* in susceptible and 0.06 in resistant, the cumulative yield of grafted crops was 1.82 times higher than that of the ungrafted susceptible ones, and the RI in resistant tomato less than 10% irrespective of the rotation sequence. Concerning the Vi, *Pf* was below the detection level at the end of the rotation in resistant and 3 times *Pi* in the susceptible. The cumulative yield of grafted crops was 2.83 times higher than that of the ungrafted, and the RI in resistant tomato was 7.6%, losing the population's virulence. In the histopathological study, no differences in the number of GC per feeding site were observed in watermelon compared to *C. amarus.* In contrast, a lower number of giant cells per feeding site was observed in tomato and melon compared to the resistant ones. In all, susceptible germplasm had more voluminous GC. It contained more nuclei per GC and feeding site than the resistance one, except for the pepper Avi population, which did not penetrate resistant rootstock.

Keywords: *Citrullus*, *Cucumis*, pepper, plant resistance, root-knot nematodes, tomato

Introduction

Root-knot nematodes (RKN) *Meloidogyne* spp. are the most limiting plant-parasitic nematode genus for horticultural crop production worldwide (Hallman and Meressa, 2018). Four out of about 100 RKN described until now, *Meloidogyne arenaria, M. incognita, M. javanica,* and *M. hapla*, are the most damaging ones. These species are widely distributed around the world; they can parasitize a large number of plant species, and reproduce parthenogenetically (Subbotin et al., 2021). *Meloidogyne* spp. are sedentary endoparasitic nematodes of underground plant organs, mainly the roots. The nematode induces the formation of galls, which affect the proper absorption of water and nutrients by the roots and the plant productivity, mainly in intensive vegetable production systems under protected cultivation. Environmental and agronomical conditions favour the increase of nematode densities to be able to cause crop production losses (Sorribas et al., 2020). Maximum yield losses of vegetables due to root-knot nematodes under protected cultivation in the Mediterranean basin have been estimated at 62% in tomato, 86% in melon, 52% in pepper, and 37% in watermelon (Expósito et al., 2020; Giné and Sorribas, 2017a; Lõpez-Gõmez et al., 2014; Talavera-Rubia et al., 2022).

Plant-parasitic nematodes control has been mainly based on chemical nematicides. Nonetheless, the number of active substances available has been progressively decreased due to their harmful effects on the environment and human and animal health (Sorribas et al., 2020). In addition, the use of nematicides has been limited to strictly necessary circumstances in the application of the European Directive 2009/128/EC, for the sustainable use of pesticides. Consequently, nematode management should combine durable and sustainable control methods, prioritising the natural regulatory elements to maintain nematode densities below the economic damage thresholds in an integrated pest management framework. In this context, plant resistance and agronomic practices are fundamental tools for nematode management. The genetic resistance is an effective and economically cost-effective technique against *Meloidogyne arenaria, M. incognita and M. javanica* (Sorribas et al., 2005). Its use reduces the nematode population growth rate and the equilibrium density (Giné and Sorribas, 2017a; Talavera et al., 2009), resulting in a lower soil infestation at the end of the crop and significantly reducing the yield losses in the following crop in a rotation sequence (Giné and Sorribas, 2017a; Ornat et al., 1997; Thies et al., *1998*, n.d.). Genetic resistance for nematode control can be used through cultivars or rootstocks carrying resistance (R) gene(s). Unfortunately, resistant cultivars or rootstocks are only available for tomato (conferred by the *Mi1.2* gene), pepper (*N, Me1, Me3/Me7* genes), aubergine (*Solanum torvum*), and watermelon (*Citrullus amarus*) (García-Mendívil and Sorribas, 2019, 2021; Thies, et al., 2015a,b and 2016). Regarding melon and

cucumber crops, despite some experimental rootstocks, such as *Cucumis metuliferus*, have been characterized as resistant to the most widely distributed RKN (Expósito et al., 2019; Gisbert et al., 2017; Guan et al., 2014; Kokalis-Burelle and Rosskopf, 2011; Ling et al., 2017; Sigüenza et al., 2005), there are not any available commercially at this time. Despite being an effective control method, the expression of some R genes can be affected by the genetic background of the plant and/or the RKN species or population (Cortada et al., 2008; López-Pérez et al., 2006), and the repeated cultivation of plant germplasm carrying the same R gene. It has been proved that the frequency of virulent individuals in a population increases progressively (Giné and Sorribas, 2017a) until the resistance is overcome after 2 or 3 consecutive crops with the same R gene, as it has been reported in tomato carrying the *Mi1.2* resistant gene (Expósito et al., 2019; Verdejo-Lucas et al., 2009) and pepper carrying the *Me3* resistance gene (Ros et al., 2006). However, the biological cost of acquiring virulence against specific R genes may lead to a decrease in the reproductive capacity of the nematode in susceptible genotypes of the same plant species(Djian-Caporalino et al., 2011). Therefore, the selection of virulence could be attenuated with rotation sequences including susceptible genotypes to achieve an acceptable production by the farmer, as proposed byTalavera et al., 2009, although monoculture contravenes the principle of sustainable production systems. Then, crop rotation sequences alternating different R genes could be a sustainable technique to improve resistance durability. In case of virulence selection to any specific R gene, the acquired virulence does not compromise other different R genes(Djian-Caporalino et al., 2011). Expósito et al., 2019 demonstrated that a 3-year rotation sequence with two different resistant sources (grafted tomato onto resistant tomato rootstock 'Aligator' and grafted melon onto *C. metuliferus*) was not enough to avoid virulence selection to a specific R gene, but the level of virulence was reduced. According to that, we hypothesized that including a greater diversity of R genes in a rotation sequence could reduce the risk of virulence selection or reverse it if it occurs. Therefore, the objective of the present study was to determine the effect of a 3-year rotation sequence including tomato, melon, pepper, and watermelon, ungrafted or grafted onto RKN-resistant germplasm on the *Meloidogyne incognita* densities in soil and roots, the disease severity, the crop yield, and the putative selection for virulence in each resistant germplasm. In addition, histopathological studies were conducted to compare the volume and number of nuclei per giant cell (GC) and the number of GC, their volume, and the number of nuclei at the feeding between susceptible and resistant germplasm.

Material and methods

CROP ROTATION EXPERIMENT

The study was conducted in an experimental plastic greenhouse of 700 m2 in Viladecans (Barcelona, Spain) during three growing seasons (from 2018 to 2021). The soil texture was sandy loam with 83.8% sand, 6.7% silt, and 9.5% clay; pH 8.7; 1.8% organic matter (w/w); and 0.5 dS /m electrical conductivity. The soil in the plastic greenhouse was solarised in the summer of 2014. After that, the soil was infested with a *Mi1.2* avirulent population of *Meloidogyne incognita* and cultivated from 2015 to 2017 in two crop rotation sequences: tomato-melon or melon-tomato. The susceptible tomato cv. Durinta (Seminis Seeds, Missouri, USA), ungrafted or grafted onto the resistant tomato rootstock "Aligator" (Gautier Seeds, Eyragues, France) and the melon cv. Paloma (Fitó Seeds, Barcelona, Spain) ungrafted or grafted onto the resistant rootstock *Cucumis metuliferus* accession BGV11135 (COMAV-UPV, Valencia, Spain) were used (Expósito et al., 2019). When the experiment finished, two nematode subpopulations were characterized for their level of virulence to the *Mi1.2* gene, one avirulent (Avi; reproduction index (RI, relative nematode reproduction in the resistant respect susceptible tomato) = 1.3%) and one partially virulent (Vi; RI = 21.6%) (Expósito et al., 2019).

In this scenario, we conducted an experiment consisting of a crop rotation sequence of ungrafted and grafted tomato-melon-pepper-watermelon- tomato cultivated in both plots infested with an avirulent (Avi) and a partially virulent (Vi) nematode population (Figure 1A). The susceptibles tomato cv. Durinta, melon cv. Paloma, pepper cv. Tinsena (Enza Zaden Seeds, Enkhuizen, The Netherlands), watermelon cv. Sugar Baby (Batlle Seeds, Molins de Rei, Spain) ungrafted or grafted onto the resistant rootstocks 'Brigeor' (Gautier Seeds), *Cucumis metuliferus* accession BGV11135, 'Oscos' (Ramiro Arnedo Seeds, Calahorra, Spain), and *Citrullus amarus* accession BGV5167 (COMAV-UPV), respectively, were produced by the commercial nursery Hishtil GS (Malgrat de Mar, Spain). The resistant *Mi1.2* tomato cv. Caramba (De Ruiter Seeds, Bergschenhoek, The Netherlands) was used instead of the last grafted tomato crop of the rotation sequence due to the unavailability of the 'Brigeor' rootstock. Crops were cultivated in plots of 3.75 m^2 (2.5 m length and 1.5 width) containing four plants spaced 0.55 m between them. Plots were spaced 0.9 m within a row and 1.5 m between rows. The experimental design in each area infested by the avirulent or partially virulent nematode population was randomized. The resistant and the susceptible germplasm were distributed randomly to the plots at the beginning of the experiment, and they were maintained along the rotation sequence. The tomato was cultivated from March to September 2018, melon from March to August 2019, pepper from August 2019 to March 2020, watermelon from March to August 2020, and tomato

from August 2020 to January 2021. Each combination of susceptible or resistant crop - (a)virulent population was replicated 10 times. The soil of each plot was carefully prepared to avoid cross-contamination. Plants were irrigated as needed via drip irrigation and fertilized with an NPK solution (15-5-30) at 31 kg/ha, iron chelate, and micronutrients at 0.9 kg/ha. Fruits of each crop were harvested and weighed when they reached commercial standards according to the European Union commission regulation numbers 790/2000 (tomato fruits), 1615/2001 (melon fruits), 2147/2002 (pepper fruits) and 1862/2004 (watermelon fruits), and values were expressed as kg/plant. At the end of the rotation sequence, the cumulative yield of all grafted or ungrafted crops was calculated as the sum of the yield of each crop. Weeds were removed manually before and during the experiments. Initial nematode population densities were determined at transplanting (Pi) and each crop's end (*Pf*). Soil samples consisted of eight cores taken from the top 30 cm of soil with a 2.5 cm diameter auger. Then, they were mixed and passed through a 4 mm-pore sieve to remove stones and roots. For each plot, *Meloidogyne* juveniles (J2) were extracted from 500 cm^3 of soil using Baermann trays (Whitehead and Hemming, 1965) and incubated at 25°C ± 2°C for 2 weeks. Afterward, the J2 were collected using a 25 µm aperture screen sieve, counted, and expressed as J2 per 250 cm^3 of soil. At the end of each crop, roots were carefully removed from the ground, washed, and weighed, and then the galling index was evaluated on a scale from 0 to 10 –where 0 is a complete and healthy root system, and 10 is a dead plant (Zeck, 1971). After that, each plant root was cut into 0.5-1 cm pieces and homogenized, and two 20 g samples were used to determine the number of eggs. The eggs were extracted from roots by maceration in a 10% commercial bleach solution (40 g/L NaOCl) for 10 min (Hussey and Barker, 1973), passed through a 74 µm-aperture sieve to remove root debris, and collected on a 25 µm sieve. Eggs were counted in a Hawksley chamber under a compound microscope and expressed as eggs per plant. The remaining root samples of each nematode subpopulation-plant germplasm combination was mixed to obtain nematode inoculum to assess the selection for virulence in pot experiments (Figure 1B).

SELECTION FOR VIRULENCE EXPERIMENTS

Pot experiments were conducted in climatic chambers at the beginning of the plastic greenhouse experiment to determine the initial level of nematode (a)virulence to the resistant germplasm (experiment 1) and after each crop in the rotation sequence as an indicator of putative changes along the rotation sequence (Figure 1B). The first experiment was conducted using J2 extracted from the soil at the beginning of the field experiment to corroborate the (a)virulence status of the nematode populations to the *Mi1.2* gene observed at the end of the 2015-2017 experiment previously described in (Expósito et al., 2019). The resistant tomato cv.

Monika (Syngenta, Basel, Switzerland), and the resistant rootstocks *Cucumis metuliferus* BGV11135, *Citrullus amarus* BGV5167 and pepper 'Oscos', and the susceptibles melon cv. Paloma, pepper cv. Tinsena and watermelon cv. Sugar Baby were used in the first and last experiments if enough nematode subpopulations inoculum was available. In the remaining experiments, only the resistant and susceptible tomato were used owing to the lack of nematode inoculum (Figure 1B). Seeds of *Cucumis metuliferus* were germinated as reported in(Expósito et al., 2018) and the rest of the plant seeds were sown in sterile vermiculite and maintained in a climatic chamber at 25 °C ± 2 °C and 16:8 h photoperiod (light: dark) for two weeks. Afterward, plants were transplanted individually into pots (6.8 cm diameter and 8.2 cm high) filled with 200 cm^3 sterile river sand and maintained under the same conditions. The nematode inoculum at the beginning of the crop sequence consisted of J2 extracted from the soil of plots with the avirulent (Avi) or partial virulent (Vi) population to the *Mi1.2* gene. The inoculum for the rest of the experiments consisted of J2 hatched from eggs produced on the resistant or the susceptible plant germplasm at the end of each crop of the rotation sequence. Thus, four subpopulations were obtained – VarAvi (from an ungrafted crop grown in plots infested with an avirulent population), VarVi (from an ungrafted crop grown in plots infested with partial virulent population), RootAvi (from a grafted crop grown in plots infested with avirulent population), and RootVi (from a grafted crop grown in plots infested with partial virulent population) (Figure 1B). Eggs were extracted from roots by maceration in a 5% commercial bleach solution (40 g/L NaOCl) for 10 min (Hussey and Baker, 1973), as previously described. The egg suspension was placed in Baermann trays at 25°C ± 2°C, and nematodes were collected daily for 7 days using a 25 µm sieve and stored at 9°C until inoculation. J2 obtained during the first 24 hours were discarded to ensure that the bleach solution did not affect the J2 used as inoculum. Plants were inoculated with 200 J2 each when they had the third true leaf expanded. Plants were arranged randomly. Each plant species-subpopulation combination was replicated 15 times. Each experiment was conducted once at each time. Plants were watered as needed and fertilized with a slow-release fertilizer (15% N, 9% P2O5, 12% K2O, 2% MgO2, microelements; Osmocote® Plus). Plants were kept in the climatic chamber for 40 days. Afterward, roots were carefully washed, and infectivity was assessed as the number of J2 able to infect and develop into egg-laying females; and expressed as the number of egg masses per plant. The number of egg masses produced in each root system was counted after staining in 0.01% erioglaucine solution for 20 min (Omwega et al., 1988). Nematode eggs were extracted from the whole root of each plant and counted as described above, and expressed as the number of eggs per plant. Fecundity was estimated as the number of eggs laid by each female and expressed as the number of eggs per egg mass. The RI of each plant subpopulation was

calculated as the percentage of the number of eggs per plant produced on resistant plants relative to that on susceptible plants for the same crop. The response was classified according to RI as highly resistant (RI < 1%), resistant (1% ≤ RI < 10%), moderately resistant (10% ≤ RI < 25%), slightly resistant (25% ≤ RI < 50%), or susceptible (RI ≥ 50%) (Hadisoeganda and Sasser, 1982).

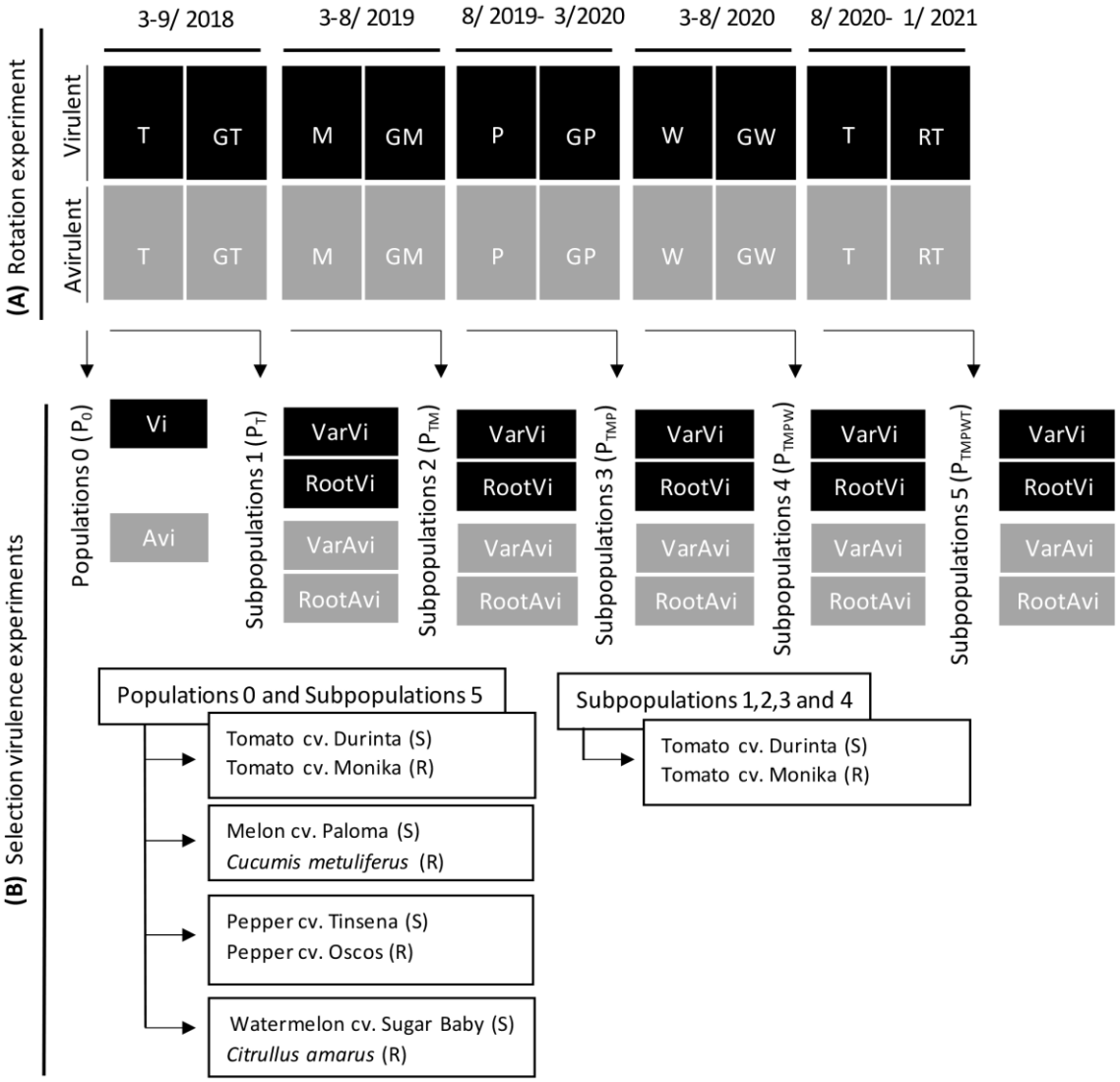


Figure 1. (A) Rotation schemes for ungrafted tomato (T) - melon (M) - pepper (P) - watermelon (W) or grafted onto the resistant rootstocks 'Brigeor'(GT)- *Cucumis metuliferus* (GM) accession BGV11135- 'Oscos' (GP)- *Citrullus amarus* (GW) accession BGV5167 followed by a susceptible tomato cv. Durinta or resistant tomato cv. Caramba respectively in a plastic greenhouse infested with *Meloidogyne incognita* avirulent (Avi) or partial virulent (Vi) to the *Mi1.2* resistance gen. (B) Pot experiments conducted with the nematode populations (Avi and Vi) extracted just before the beginning of the rotation sequence (P0) and with avirulent (VarAvi and RootAvi) and partial virulent (VariVi and RootVi) subpopulations after each crop of the rotation scheme (PT-PTM-PTMP-PTMPW-PTMPWT) on susceptible and resistant cultivars or rootstocks.

HISTOPATOLOGY

A histopathology study with laser-scanning confocal microscopy of cleared galled roots was performed. Three-leaf stage plants of the susceptible melon cv. Paloma, pepper cv. Tinsena, tomato cv. Durinta and watermelon cv. Sugar Baby, and the resistant *Cucumis metuliferus* accession BGV11135, pepper 'Oscos,' tomato cv. Monika and Citrullus amarus accession BGV5167 germinated under the conditions described above. Once the second true leaf was expanded, 5 plants of each germplasm were transplanted into pots containing 200 cm^3 of sterilized river sand. A week later, 1 or 3 *Meloidogyne incognita* J2 per cm^3 of soil were added to the pots with nematode susceptible or resistant plants, respectively, into two opposite holes of 3 cm depth and 1 cm from the stem. The nematode inoculum was obtained as previously described. The highest nematode density was used to inoculate the resistant germplasm to increase the probability of detecting the nematode inside the roots. Conversely, the susceptible germplasms were inoculated with a low density to avoid the coalescence of infection sites that could make it challenging to observe. The study was conducted once. Fifteen days after the nematode inoculation, 10 galled-root pieces per each plant were taken. Galled-root pieces were fixed, clarified, and stored following the procedure described by Cabrera et al. (2018), with some modifications. In brief, galled-root pieces were handpicked and introduced in a vial containing 1 mL sodium phosphate buffer (10 mM, pH = 7). The pieces were fixed in sodium phosphate buffer (10 mM, pH = 7) with glutaraldehyde 4% under a soft vacuum for 15 min and maintained at 4 °C overnight. Afterward, pieces were rinsed for 10 min with sodium phosphate buffer, sequentially dehydrated for 20 min in 30, 50, 70, and 90% ethanol solutions, and finally in pure ethanol for 60 min. Clarification was conducted in a solution 1:1 v/v EtOH: BABB (1:2 v/v benzyl alcohol: benzyl benzoate) for 20 min, followed by 20 min in BABB solution at room temperature. The galls were then left in an automatic tube shaker at 4 °C for 2 weeks. Afterward, the samples were stored at 4 °C. The cleared galls were imaged with laser-scanning confocal microscopy. This allowed to determine: the number of nuclei and giant cells (GC) per feeding site and the volume of each GC. The thinnest galls were selected and mounted in #1.5 bottom-glass petri dishes and fully embedded in the BABB solution. Fluorescence images were acquired with an inverted Leica TCS 5 STED CW microscope (Leica Microsystem) equipped with a 10 x 0.40NA HCX Pl Apo CS air objective. The different structures within the cleared galls produced different autofluorescence spectra, partly overlapping. Two different excitation-emission schemes were used to separate them. Thus, the root cell walls of the samples were excited with a 488 nm argon laser, and the fluorescence emission was collected with a hybrid detector in the range of 498– 550 nm. The nuclei of GC and the nematodes was visualized with 633 nm HeNe laser, and the fluorescence emission was collected with a hybrid detector in the range of 643–680 nm. Depending on the

sample, the visualized volume had a thickness ranging from 60 to 170 mm. Each volume was optically sectioned to produce a collection of Z-stack images (step size of 2–3 mm). For the GC volume measurement, images were manually segmented using the TrakEM2 ImageJ plugin (ImageJ, version 1.50i), which provides the giant cell area at each segment and calculates the volume of the structure. The volume of the feeding site was the sum of the volumes of all GC belonging to a feeding site. Representative frames of each plant germplasm are shown in Figure 2.

STATISTICAL ANALYSES

Statistical analyses were performed using the statistical software R V3.6.1 and the R Commander package (R Foundation for Statistical Computing, Vienna, Austria). Data from the field experiment concerning initial (Pi) and final (*Pf*) nematode densities in soil (number of J2 per 250 cm^3 soil), nematode reproduction (number of eggs per plant), and disease severity (galling index) were compared between resistant and susceptible plant germplasms of the same crop per each Avi and Vi nematode population. Crop yield (kg per plant) was compared between ungrafted and grafted plants per each crop and nematode population. Comparisons ($P \leq 0.05$) were done either using the Student's t-test if the data fitted a normal distribution or the nonparametric Wilcoxon test otherwise. The yield of the last tomato crop was not assessed due to the lack of information on the comparative performance and precocity between cultivars in the agri-environmental conditions in which the experiment was conducted.

Infectivity (number of egg masses per plant), reproduction (number of eggs per plant), and fecundity (number of eggs per egg mass) data from pot experiments were compared between resistant and susceptible germplasm per each nematode population (first experiment) or subpopulations (experiments 2 to 6) to determine the putative selection for virulence. The nematode infectivity, reproduction, and fecundity were also pair compared between each nematode subpopulation with the VarAvi subpopulation (which was never exposed to resistant plant germplasm) to estimate the fitness cost to acquire virulence. The Student's t-test ($P \leq 0.05$) was used when data were normally distributed otherwise the nonparametric Wilcoxon test was used.

The number of nuclei and GC per feeding site, the volume of each GC and the number of nuclei per GC were compared ($P \leq 0.05$) between resistant and susceptible germplasm per each crop. Data were compared using the Student's t-test if the data fitted a normal distribution or the nonparametric Wilcoxon test.

Results

CROP ROTATION EXPERIMENT

The *Pi* of the avirulent or the partially virulent subpopulations at the beginning of the experiment did not differ between resistant and susceptible plant germplasms (Table 1). At the end of the rotation sequences, the *Pf* of the avirulent subpopulation was 1.2 greater than the *Pi* in plots cultivated with the susceptible germplasms but was 0.06 times the *Pi* where the resistant ones were cultivated. The reproduction of the avirulent subpopulation in the resistant germplasm ranged from 2.2% (tomato) to 39.6% (melon) of that achieved in the susceptible germplasm. Regarding disease severity, it only differed between the resistant and the susceptible tomato germplasm, being, in the former 0.2 and 0.26 times that of the registered in the susceptible one. In melon, 18.8% of the ungrafted plants died due to *Monosporascus cannonballus*, and the surviving ones showed a similar level of disease severity to that registered in grafted melon. In pepper, the root system developed poorly on both ungrafted and grafted plants, and the galling index was not determined, but there were few nematodes that succeeded to reproduce. Grafted tomato and melon yielded 1.5 and 10.5 times more than the ungrafted ones, but no differences were found between the ungrafted and grafted pepper and watermelon yields. The cumulative yield of all grafted crops was 1.83 times more than that of the ungrafted at the end of the rotation sequence. Concerning the partial virulent population, the *Pf* at the end of the rotation sequence in plots cultivated with susceptible germplasms increased 3 times the *Pi*, whereas, in plots cultivated with resistant germplasm, the nematode was not detected in soil at the end of the rotation sequence (Table 1). The nematode reproduced 2.6 more times in grafted than ungrafted tomato (11164/4288). In the resistant tomato cv. Caramba the nematode reproduced 0.0012 times that achieved in the susceptible cv. Durinta (0.43/357).

The disease severity was between 3 (watermelon) and 33 (last tomato crop) times higher ($P < 0.05$) in the susceptible than in the resistant germplasm, except in the first tomato crop, which did not differ. The grafted crops yielded between 1.24 and 9.44 more in the first tomato crop and the watermelon crop, respectively, than in the ungrafted susceptible genotypes. The cumulative yield of all grafted crops was 2.83 times more than that of the ungrafted at the end of the rotation sequence.

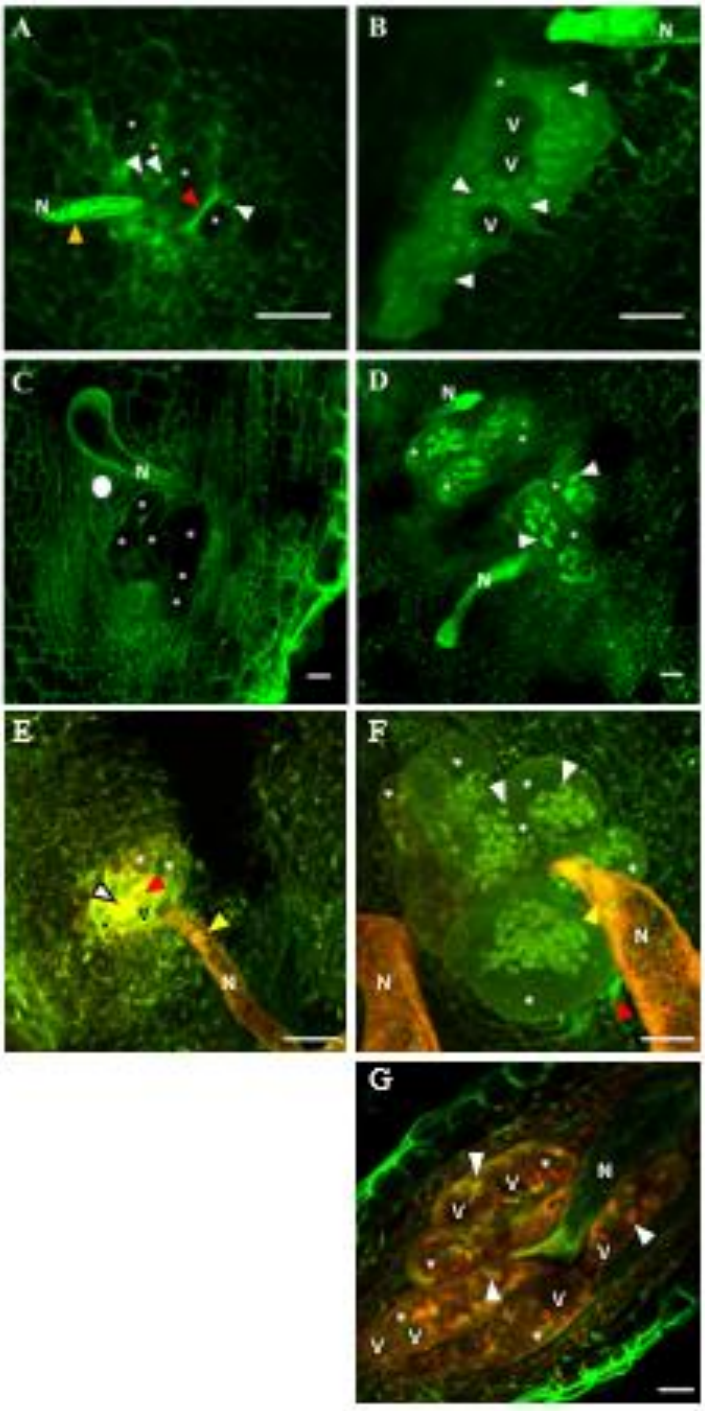


Figure 2. Laser scanning confocal microscope images of the infection site of *Meloidogyne incognita* 15 days after inoculation in the resistant *Cucumis metuliferus* BGV11135(B), tomato cv. Monika (D), *Citrullus amarus* BGV5167 (F), and the susceptible cultivars melon cv. Paloma (A), tomato cv. Durinta (C), watermelon cv. Sugar Baby (E) and pepper cv. Tinsena (G). Nematode (N), vacuoles (V), giant cells (asterisk), some nuclei (white arrowhead), necrosed area (red arrowhead), and esophageal median bulb (yellow arrowhead) are indicated. Scale bar:50 µm.

Table 1. Nematode soil densities at transplanting (*Pi*) and at the end of the crop (*Pf*), nematode reproduction (eggs per plant), galling index, and yield (kg per plant) of the rotation sequence Tomato cv. Durinta (T)-melon cv. Paloma (M)-pepper cv. Tinsena (P) watermelon cv. Sugar Baby (W), ungrafted or grafted onto the resistant rootstocks "Brigeor"(GT), *Cucumis metuliferus* (GM), "Oscos" (GP), and *Citrullus amarus* (GW), respectively, followed by a susceptible tomato cv. Durinta (T) or resistant tomato cv. Caramba (C) respectively, cultivated in a plastic greenhouse located at Viladecans (Spain) infested with a *Mi1.2* avirulent (Avi) and a partially virulent (Vi) *Meloidogyne incognita* populations from to 2021.

RKN population	Crop sequence	Cultivar/ Rootstock	*Pi* (J2 per 250 cm^3 soil)	*Pf* (J2 per 250 cm^3 soil)	Reproduction (Eggs(10^2) per plant)	GI	Yield (kg per plant)
Avi	Tomato (3-9/ 2018)	T	385 ± 116	3274 ±1316*	6451 ± 1335*	7.1±0.6*	3.1 ± 0.3*
		GT	846 ± 200	532 ± 329	1021 ± 647	1.4 ± 0.3	4.6 ± 0.3
	Melon (3-8/ 2019)	M	398 ± 104	332 ± 65	1744 ± 389*	4.3 ± 0.1	0.2±0.01*
		GM	243 ± 76	283 ± 76	690 ± 347	2.8 ± 0.6	2.1 ± 0.4
	Pepper (8/ 2019- 3/ 2020)	P	332 ± 65	79 ± 46*	8 ± 2*	nd	0.2 ± 0.05
		GP	283 ± 76	5 ± 2	0.2 ± 0.1	nd	0.4±0.002
	Watermelon (3-8/ 2020)	W	79 ± 46*	11 ± 6	20 ± 8*	2.3 ± 0.2	1.9 ± 0.5
		GW	5 ± 2	12 ± 7	3 ± 3	1.6 ± 0.3	2.8 ± 0.5
	Tomato* (8/2020- 1/ 2020)	T	11 ± 6	456 ± 124*	1024 ± 423*	3.8± 0.3*	nd
		C	12 ± 7	48 ± 24	23 ± 11	1.0 ± 0.2	nd
Vi	Tomato (3-9/ 2018)	T	154 ± 56	556 ± 285	4288 ± 1437	4.6 ± 1.2	4.2 ± 0.2*
		GT	184 ± 74	332 ± 165	11164 ± 4651	3 ± 1	5.2 ± 0.2
	Melon (3-8/ 2019)	M	166 ± 55	531 ± 67*	678 ± 289*	4.5 ± 0.5*	0.9 ± 0.3*
		GM	87 ± 45	67 ± 24	101 ± 61	1.4 ± 0.5	3.3 ± 0.4
	Pepper (8/ 2019- 3/ 2020)	P	531 ± 67*	63 ± 16*	19 ± 16	nd	0.3±0.02*
		GP	67 ± 24	1 ± 1	0.38 ± 0.08	nd	0.8 ± 0.001
	Watermelon (3-8/ 2020)	W	63 ± 16*	8 ± 6	17 ± 10*	1.5 ± 0.4*	0.9 ± 0.4*

	GW	1 ± 1	3 ± 3	0.03 ± 0.02	0.5 ± 0.2	8.5 ± 0.8
Tomato* (8/2020- 1/ 2021)	T	8 ± 6	469 ± 193*	357 ± 113*	3.3 ± 0.4*	nd
	C	3 ± 3	0 ± 0	0.43 ± 0.29	0.1 ± 0.1	nd

Data on nematode population densities in soil are the means ± standard errors of 10 replicates. Data on reproduction, Galls Index (GI), and yield are the mean ± standard error of 40 replicates. Values followed by * are different between grafted and ungrafted plants for each crop according to the Student-t Test or the non-parametrical Wilcoxon rank test ($P < 0.05$); GI: according to the scale of Zeck (1971), nd: Not determined; * Tomato cv. Caramba carrying the *Mi1.2* resistance gene was used as the last crop due to the commercial unavailability of the rootstock "Brigeor"

SELECTION FOR VIRULENCE EXPERIMENTS

In the first pot experiment conducted with J2 from the soil just before starting the rotation sequence experiment, all the plant materials were assessed against the Avi population, but only resistant and susceptible tomato cultivars against the Vi population because of the lack of nematode inoculum. The RI of the Avi and Vi populations in tomato was 1.3% and 21.6%, respectively, confirming the avirulent and partially virulent status of the nematode populations (Table 2). Both Vi and Avi subpopulations showed lower ($P < 0.05$) infective (97.8% and 70.3%, respectively) and reproductive (98.7% and 78.4, respectively) capacity in the resistant than in the susceptible tomato cultivar. In addition, the fecundity of the Vi population in the resistant tomato cultivar decreased ($P < 0.05$) (Table 2).

Table 2. Number of egg masses per plant, eggs per plant, and eggs per egg mass produced in tomato cv. Durinta (S) and Monika (R), melon cv. Paloma (S) and *Cucumis metuliferus* (R), pepper cv. Tinsena (S) and 'Oscos' (R) and watermelon cv. Sugar Baby (S) and *Citrullus amarus* (R) from the *Mi1.2* avirulent (Avi) and partially virulent (Vi) soil subpopulations of *Meloidogyne incognita* obtained before the rotation sequence in pot experiments.

Cultivar (host status)	Egg masses per plant		Eggs (10^3) per plant		Eggs per egg mass	
	Avi	Vi	Avi	Vi	Avi	Vi
Monika (R)	2 ± 0.27	27 ± 3.21	1 ± 0.24	16 ± 1.67	944 ± 169	628 ± 40
Durinta (S)	92 ± 2.98 *	91 ± 5.33 *	75 ± 3.36 *	74 ± 0.01 *	824 ± 30	831 ± 90 *
C. metuliferus (R)	2 ± 0.42	nd	0.7 ± 0.19	nd	322 ± 56	nd
Paloma (S)	50 ± 4.07 *	nd	60 ± 7.19 *	nd	1212 ± 115 *	nd
Oscos (R)	0 ± 0	nd	0 ± 0	nd	0 ± 0	nd
Tinsena (S)	34 ± 2.87 *	nd	25 ± 1.3 *	nd	754 ± 55 *	nd
C. amarus (R)	2 ± 0.42	nd	0.1 ± 0.004	nd	59 ± 15	nd
Sugar Baby (S)	6 ± 1.43 *	nd	4 ± 0.9 *	nd	568 ± 53 *	nd

Data are means ± standard errors of 15 replicates. Data followed by * are different between resistant and susceptible plants for each crop according to the Student-t Test or the non-parametrical Wilcoxon rank test ($P < 0.05$). nd, Not determined due to the low inoculum availability.

The RI of the Avi population in 'Oscos', *Cucumis metuliferus,* and *Citrullus amarus* was 0%, 1.1% and 2.5%, respectively (Figure 3A). The infective and reproductive capacity, as well as the fecundity in the resistant germplasms, were lower ($P < 0.05$) than in the susceptible ones (Table 2).

Four nematode subpopulations were differentiated after the first tomato crop: VarAvi, RootAvi, VarVi, and RootVi (Figure 1B). The VarAvi subpopulation remained avirulent to the resistance *Mi1.2* gene throughout the rotation sequence, and the RootAvi subpopulation finished the rotation with an RI of 10% after the last tomato crop (Figure 3B). The level of virulence of the VarVi subpopulation obtained at the end of the ungrafted tomato and melon crops was above 100%, decreasing progressively after the ungrafted watermelon (RI = 35%) and the last tomato crops (RI = 6.98%) (Figure 3B). The RootVi subpopulation maintained virulence levels of around 25% after the grafted tomato and melon crops (Table 3). At the end of the remaining crops in the crop sequence, not enough nematode inoculum was obtained for further evaluation. Concerning the fitness cost of acquiring virulence to the *Mi1.2* gene, less ($P < 0.05$) number of eggs per plant were produced by the VarVi subpopulation after tomato and melon crops, and the subpopulation RootAvi after the last tomato crop compared to those produced by the VarAvi subpopulation in susceptible tomato (Table 3).

HISTOPATHOLOGY

Fifteen days after *Meloidogyne incognita* inoculation, the nematode induced 1.8 more ($P < 0.05$) giant cells (GCs) in *Cucumis metuliferus* than in melon cv. Paloma, but they were less ($P < 0.05$) voluminous (94.3%), holding 92.9% fewer ($P < 0.05$) nuclei per GC. Both GCs volume and number of nuclei per feeding site were higher ($P < 0.05$) in susceptible melon than in *Cucumis metuliferus* (Table 4). Some GCs in *C. metuliferus* did not emit fluorescence, and no nuclei were observed compared to those observed in the susceptible melon cv. Paloma, which were more voluminous, multinucleated, and vacuolated (Figures 2 A, and B). Regarding tomato, 2.1 more ($P < 0.05$) GCs were induced in the resistant tomato cv. Monika than in the susceptible cv. Durinta, but they were 72.5% less ($P < 0.05$) voluminous and had 93.3% fewer ($P < 0.05$) nuclei per GC (Table 4). However, GCs volume per feeding site did not differ between tomato cultivars.

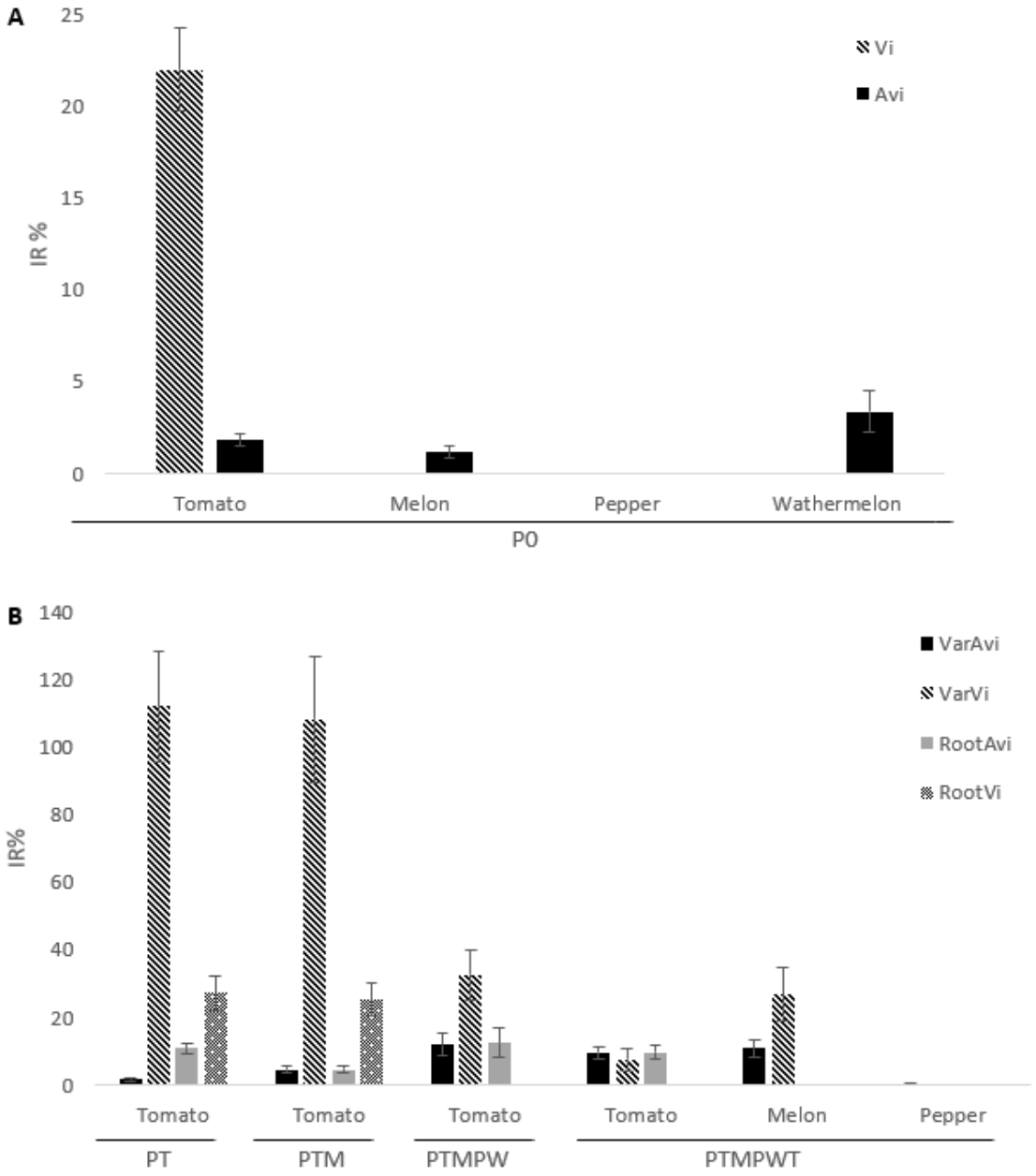


Figure 3. Reproduction Index (RI: percentage eggs per plant produced in resistant germplasm respect those produced in the susceptible germplasm) (A) of *Mi1.2* avirulent (Avi) and virulent (Vi) *Meloidogyne incognita* populations obtained from soil before the rotation sequence (P0) in resistant tomato cv. Monika, melon rootstock *Cucumis metuliferus*, pepper rootstock 'Oscos' and watermelon rootstock *Citrullus amarus* and (B) of the *Mi1.2* avirulent (VarAvi and RootAvi) and partially virulent (VarVi and RootVi) subpopulation obtained from roots of each crop of the rotation scheme tomato (PT)- melon (PTM)- watermelon (PTMPW) in resistant tomato cv. Monika, and from roots of the last tomato crop (PTMPWT) in the melon rootstock *C. metuliferus* and pepper rootstock 'Oscos' too. The columns represent the mean and the bars represent the standard error of 15 replicates.

Table 3. Number of egg masses per plant, eggs per plant, and eggs per egg mass produced in the tomato cv. Durinta (S) and Monika (R) of *Meloidogyne incognita* after each crop of the rotation scheme (tomato (PT)- melon (PTM)- watermelon (PTMPW)- tomato (PTMPWT)) on the cultivar and rootstock crop lines of the avirulent (VarAvi and RootAvi) and virulent (VariVi and RootVi) subpopulation, and those produced in the melon cv. Paloma (S) and *Cucumis metuliferus* (R) and in the pepper cv. Tinsena (S) and Oscos (R) from the root subpopulations obtained after the last tomato crop (PTMPWT) in 200cm^3 pot experiments.

Sub-population	Cultivar (host status)	Egg masses per plant				Eggs (10^3) per plant				Eggs per egg mass			
		VarAvi	**VarVi**	**RootAvi**	**RootVi**	**VarAvi**	**VarVi**	**RootAvi**	**RootVi**	**VarAvi**	**VarVi**	**RootAvi**	**RootVi**
P_T	Monika (R)	1± 0.2	11± 1.7†	19± 2.9†	16± 3.3†	0.8± 0.2	9± 1.3†	12± 1.7†	14± 2.6†	608± 133	822± 76	678 ± 39	1010 ± 136
	Durinta (S)	49± 5.7*	34± 3.6*	134±15.6*†	57± 5.7*	49± 4.1*	8± 0.7†	111±9.9*†	51± 6.5*	1067± 80*	255± 27*†	924 ± 96	907 ± 76
P_{TM}	Monika (R)	3± 0.4	8± 1.1†	3± 0.4	26± 3.5†	2± 0.5	12± 2.1†	2± 0.5	16± 3.1†	855± 117	1348± 1185	618 ± 96	606 ± 68
	Durinta (S)	59± 3.5*	28±1.9*†	48± 4.3*	44±4.9*†	49± 3.6*	11± 0.7†	48± 18.3*	64±10.8*	848± 60	429± 28*†	1086 ± 126*	1444 ± 173*
P_{TMPW}	Monika (R)	2± 0.3	2± 0.4	1± 0.1	nd	0.6± 0.2	0,7± 0.2	0.4± 0.1	nd	349± 75	270± 37	308 ± 84	nd
	Durinta (S)	10± 2.9*	10± 2.1*	5± 0.9*	nd	5± 2.0*	2± 0.6*	3± 0.4*	nd	422± 90	232± 41	541 ± 82	nd
P_{TMPWT}	Monika (R)	5± 1.2	5± 1.7	20± 3.7†	nd	4± 0.7	3± 1.5	12± 2.4†	nd	906± 67	863± 280	596 ± 56,83†	nd
	Durinta (S)	138± 5.8*	65±5.9*†	72± 6.6*†	nd	39± 5.3*	43± 3.9*	120±5.9*†	nd	898± 36	656± 19†	529 ± 36†	nd
	C.metuliferus (R)	4± 0.6	5± 1.1	nd	nd	1± 0.25	1± 0.3	nd	nd	340± 79	276± 52	nd	nd
	Paloma (S)	24± 2.4*	16± 4.6*	nd	nd	10± 1.5*	4± 1.2*†	nd	nd	431± 38	576± 206	nd	nd
	'Oscos' (R)	0,4± 0.1	0.0001±0	nd	nd	0.1± 0.0	0.004±0.003	nd	nd	195± 49	36± 2†	nd	nd
	Tinsena (S)	17± 1.8*	39±4.4*†	nd	nd	15± 1.1*	22± 2.5*†	nd	nd	952± 77*	574± 28*†	nd	nd

Data are means ± standard errors of 15 replicates. Data followed by * are different between resistant and susceptible plants for each crop according to the Student-t Test or the non-parametrical Wilcoxon rank test ($P < 0.05$). Data followed by † show significant differences ($P < 0.05$) between VarVi, RootAvi, and RootVi nematode subpopulations in compare to VarAvi subpopulation per plant according to the nonparametric Wilcoxon test ($P < 0.05$). nd, Not determined due to the low inoculum availability.

Still, the number of nuclei per feeding site did, being higher ($P < 0.05$) in susceptible than in resistant tomato (Table 4). Several GCs did not emit fluorescence in resistant tomato, and no nuclei were observed compared to the voluminous and multinucleated GCs observed in the susceptible tomato (Figures 2 C and D). *Meloidogyne incognit*a induced a similar number of GC in watermelon than in *Citrullus amarus*, but they were 8.9 times less ($P < 0.05$) voluminous in the former than in the latter. The volume of GC per feeding site was 5.7 times higher ($P < 0.05$) in watermelon than in *Citrullus amarus* (Table 4). In addition, GC in *C. amarus* had large empty vacuoles compared to watermelon. The number of nuclei per GC and per feeding site were 7.7 and 20.2 times more ($P < 0.05$) in *Citrullus amarus* than in watermelon (Table 4). Most of the induced GC in *C. amarus* presented few or no nuclei. Moreover, they were very difficult to image since the autofluorescence levels emitted were very low. The resulting images were dim compared to the resistant germplasms (Figure 2). The nematode could infect and induce GC in the susceptible pepper cv. Tinsena, no J2 were observed inside the root or GC in the resistant pepper rootstock 'Oscos' 15 days after nematode inoculation. Therefore, no comparisons between susceptible and resistant germplasm were carried out.

Table 4. Giant cell volume (GCV), GC volume per feeding site (GCV /fs), number of nucle per GC (N/GC), number of nuclei per feeding site (N/fs), and number of cells per feeding site (NC/fs) in the resistant plants (R) pepper rootstock 'Oscos', *Citrullus amarus, Cucumis metuliferus*, and tomato cv. Monika, and the susceptible plants (S)pepper cv. Tinsena, watermelon cv. Sugar Baby, melon cv. Paloma, and tomato cv. Durinta, 15 days after nematode inoculation with 3 or 1 J2 cm 3 of soil, respectively, and cultivated in 200 cm^3 pots in a growth chamber.

Cultivar (host status)	GCV (μm^3 10^5)	GCV/fs (μm^3 10^5)	N/GC	N/ fs	NC/ fs
Oscos (R)	0 ± 0*	0 ± 0*	0 ± 0*	0 ± 0 *	0 ± 0*
Tinsena (S)	11.3 ± 1.1	81.9 ± 8.8	13.3 ± 1	96.5 ± 10.7	7.5 ± 1
C. amarus (R)	0.9 ± 0.2*	9.3 ± 2.5	0.7 ± 0.6*	6.5 ± 0.9*	10 ± 1.1
Sugar Baby (S)	8.0 ± 1.1	54.2 ± 16.4	19.4 ± 2.8	131 ± 8.6	7 ± 0.8
C. metuliferus (R)	0.45 ± 0.1*	3.4 ± 0.8*	1.2 ± 0.7*	9.2 ± 5.5*	8.0 ± 1.1*
Paloma (S)	8 ± 7.5	33.2 ± 9.9	17.1 ± 1.8	72.0 ± 7.8	4.5 ± 1.0
Monika (R)	3.1 ± 0.4*	26.9 ± 3.7	0.9 ± 0.4*	7.0 ± 3.0*	8.7 ± 1.2*
Durinta (S)	11.4 ± 1.9	45.99 ± 7.3	13.7 ± 1.0	56.2 ± 7.3	4.1 ± 0.4

Data are means ± standard errors of 4 replications. Data in the same column followed by * indicates differences ($P < 0.05$) between Cucumis species or tomato or pepper cultivars according to the non-parametric Wilcoxon test or Student's t-test.

Discussion

The present work demonstrated that crop rotation, including at least four different sources of resistance to RKN, is efficient for managing avirulent and virulent Meloidogyne incognita populations to specific R genes and reducing crop yield losses. In the current study, the *Mi1.2* gene in tomato, the *Me3* gene in pepper, and the resistant rootstocks *Cucumis metuliferus* and *Citrullus amarus* were included in the rotation sequence, assuming that each resistant plant germplasm has different plant defense mechanisms against the nematode and the risk to select cross-virulent populations is very low. Previous studies have shown that the level of resistance exhibited by resistant pepper carrying the *Me1* or *Me3* genes, *Cucumis metuliferus* and *Citrullus amarus* to virulent *Mi1.2* RKN isolates did not differ from that of avirulent ones (Castagnone-Sereno et al., 1996; Djian-Caporalino et al., 2011; Expósito et al., 2018; García-Mendívil et al., 2019). Therefore, different plant defense mechanisms can be induced by the nematode in those resistant plant germplasms, avoiding the overlapping of signaling and the recognition of the resistance pathways that could result in cross-virulence selection (Petrillo et al., 2006).

In tomato, the resistant *Mi1.2* gene induces localized cell death when J2 attempts to establish a feeding site (Williamson and Hussey, 1996) by preventing the production of enzymes that degrade or modify the cell wall and up-regulating the expression of genes encoding the defensin protein and protease, leading to phytoalexin production and proteolysis (Stotz et al., 2009). In addition, it induces the up-regulation of genes involved in activating signal transduction pathways, such as, receptor like kinase and protein phosphatase. These actions result in the repression of giant cells formation, which are necessary to feed the nematode (Shukla et al., 2018). In pepper, the *Me3* resistant gene induces necrosis in cells of the root epidermis adjacent to the J2 by chlorogenic acid accumulation suppressing nematode penetration into the roots (Pegard et al., 2005). Regarding *Cucumis metuliferus*, the reduction in J2 penetration and development has been associated with greater phenylalanine ammonia-lyase and peroxidase activities along with the expression of several genes relevant for phenylpropanoid biosynthesis and plant hormone signaling compared to cucumber (Ye et al., 2017). Recently, 18 different root volatiles have been identified in *Cucumis metuliferus* accession CM3 compared to cucumber, including hydrocarbons, alcohols, aldehydes, ketones, and esters (Xie et al., 2022), which seems to be related to repelling J2 from roots. In *Citrullus amarus*, the resistance has been associated with higher root fibrosity (Thies and Levi, 2003; Thies and Levi, 2007; Thies et al., 2016) and a different root metabolic profile, compared with watermelon, including amino acids, some of them reported to have nematicide effects, such as arginine (Sayed and Thomason, 1988); carbohydrates and several organic compounds (Kantor and Amnon, 2018).

The histopathological study provided interesting information related to the number and volume of giant cells and the number of nuclei in them. Giant cell formation is a key factor for a successful plant-nematode interaction after the nematode arrives in the cortical cylinder. The induced multinucleated giant cells have a high metabolic activity necessary for nematode nutrition for its life cycle completion (Abad et al., 2009). Conversely, if giant cells are not formed or appear degenerated, holding none or few nuclei, the nematode development and/or reproduction will be suppressed, indicating a resistant response of the plant. Cabrera et al. (2015) used 3D reconstructions of GCs induced by *Meloidogyne javanica* in *Arabidopsis* roots and to compare GCs formed in the *Arabidopsis* transgenic line J0121 > > DTA, in which the GCs are genetically ablated, with a control (line J0121 > > GFP). These authors found that the GCs volume in the control was 2 fold larger. Our study's results have shown resistant *Cucumis metuliferus* and tomato cv. Monika had more number of giant cells per feeding site than melon and susceptible tomato 15 days after *Meloidogyne incognita* inoculation. However, the GCs were smaller, less voluminous, and had fewer nuclei; some were empty of cytoplasm in all resistant germplasm studied. Previous histopathological studies reported some of the observations pointed out in this study. Fassuliotis (1970) observed small GCs in *Cucumis metuliferus* accession C-701 compared with those induced by *M. incognita* in melon; the nematode developed slowly, and 20% of juveniles differentiated to males. Walters et al. (2006) observed elongated GCs conforming abnormally in shape feeding sites in *C. metuliferus* accession 482454 compared with melon. More recently, Ye et al. (2017) observed that most of the GC were empty of cytoplasm in the *Cucumis metuliferus* accession PI 482443-*Meloidogyne incognita* interaction 14 days after nematode inoculation along with a slow nematode development compared with melon. Expósito et al. (2018) reported poorly GC development with multiple vacuoles, some of them without cytoplasm and necrotic areas surrounding the nematode head in *the Cucumis metuliferus* accession BGV11135– *Meloidogyne javanica* interaction compared to cucumber. Interestingly, the major number of GCs found in resistant *Cucumis metuliferus* and tomato could be due to an attempt of the nematode to achieve enough nutrients for its life cycle completion. Conversely, in *Citrullus amarus, Meloidogyne incognita* induces a similar number of GC as in watermelon. This is possible because watermelon is considered a poor host of *Meloidogyne*. In fact, the development of small GCs holding low number of nuclei could indicate a low effective metabolic activity for nematode nourishment. This strategy to achieve nutrients can have a biological cost for the nematode resulting in a slow development rate, as it was previously reported for both C. metuliferus and *Mi1.2* resistant tomato as well as for other resistant germplasms (Fassuliotis, 1970; Pedrosa et al., 1996; Walters et al., 2006; Williamson and Roberts, 2009; Ye et al., 2017). Regrettablycomparisons were not

possible in pepper owing to J2 infecting roots were not found in the resistant pepper rootstock 'Oscos' 15 days after nematode inoculation. In fact, a low percentage of plants were infected in the pot experiments conducted in the present study, and the nematode reproduced poorly in the plastic-greenhouse experiment. The defense mechanisms induced by the *Me3* gene previously described can explain these results.

Despite the effectiveness of plant resistance to manage RKN, after 3-years of monocropping resistant tomato or pepper, the level of resistance decreases or is null (Giné and Sorribas, 2017a; Ros-Ibáñez et al., 2014; Verdejo-Lucas et al., 2009). It is known that 2-4 years of rotation, including non-host, poor-host, and resistant-host, is highly effective against *Meloidogyne* spp., but its effectiveness depends on the level of resistance of the plant germplasm (Trivedi and Barker, 1986), as well as the resistance source. Previous works have shown that a 3-year rotation with two different sources of resistance, such as tomato grafted onto 'Aligator' rootstock and melon grafted onto *Cucumis metuliferus*, decreased yield losses caused by the nematode, but it did not prevent the selection for virulence to the *Mi1.2* resistance gene in tomato although it was attenuated (Expósito et al., 2019). The 3- year rotation sequence carried out in the present study with four different resistance sources has reduced the cumulative yield losses, has prevented the selection for virulence of an avirulent *Mi1.2* population, and has reduced the nematode population density in the soil of a partially virulent population to undetectable levels. Interestingly, the VarVi population's virulence level decreased progressively after the melon crop from 100% to 7%. This subpopulation was exposed two times to resistant tomato germplasm during the period 2015-2017, but no fitness cost was detected, hypothesizing that a minimum of three resistant tomato crops would be needed to fix the trait (Expósito et al., 2019). Surprisingly, in the current study, the level of reproduction and fecundity of the females of VarVi in susceptible tomato was reduced compared to VarAvi -which was never exposed to resistant germplasm- when the inoculum produced in roots of the first tomato and melon crops were used, but not after the others. This event could be explained by a progressively decreasing proportion of virulent individuals in the population influenced by the following pepper and watermelon crops as well as the variability in infectivity, reproduction, and female fecundity in the successive nematode generations. Petrillo and Roberts (2005) reported variability in the reproductive factors between isofemale lines, single descendent lines, or isolates of virulent *Meloidogyne incognita* to the Rk gene on susceptible cowpea, even between nematode generations of the same origin. In fact, the subpopulation RootVi showed the same ability to reproduce on grafted and ungrafted tomato at the beginning of the plastic greenhouse experiment to reproduce poorly on resistant tomato cv. Caramba at the end of the rotation

sequence, resulting in an insufficient nematode inoculum to be included in the virulence selection and fitness cost experiments.

Grafting improves crop yield (Gaion et al., 2018) and constitutes one of the most effective management methods to control soil-borne plant pathogens (Davis et al., 2008; Galatti et al., 2013), including RKN (Expósito et al., 2020). In our study, the cumulative yield of grafted crops at the end of the rotation sequence was higher than of ungrafted irrespective of the virulence status of the nematode population. Regarding watermelon and pepper crops, no differences in yield were found between grafted and ungrafted ones, possibly due to the poor host status and nematode tolerance of the former (López-Gómez et al., 2016) and the cropping season of the latter. In our conditions, pepper is transplanted from February to April instead of August, as in the present study. The date of transplanting could influence the performance of the crop and the development of the nematode population, as has been reported by Vela et al. (2014) in zucchini squash.

The use of plant resistance is an effective and safe control method that has to be used properly in combination with other compatible and sustainable control methods to improve its durability. The resistance level expressed by a resistant plant germplasm depends on its background (Cortada et al., 2008; Jacquet et al., 2005). For instance, although all resistant tomato cultivars and rootstocks carry the *Mi1.2* gene, at least one additional locus is required for the expression of resistance (Martinez de Ilarduya et al., 2001). This fact could explain the differential response of some commercial tomato rootstocks and its influence in selecting virulent nematode populations (Expósito et al., 2019; Verdejo- Lucas et al., 2009). Understanding molecular plant-nematode interactions is needed to develop alternative approaches for nematode control (Abd-Elgawad, 2022). In addition to that, the use of plant resistance to a given nematode species could lead to shifts in the plant-parasitic nematode communities. For example, cropping systems, including resistant and susceptible crops and nematicidal cover crops designed for controlling RKN, led to the replacement of RKN by *Telotylenchidae* nematodes (Mateille et al., 2020). Therefore, other control methods, such as the use of cover crops, organic amendments, biological control agents, physical control methods, or plant resistance inducers, such as T*richoderma* species*, Bacillus firmus* or *Pochonia chlamydosporia* (Ghahremani et al., 2019, 2020; Pocurull et al., 2020) are necessary.

In summary, crop rotation with at least four different resistance sources is effective for the management of avirulent and partially virulent nematode populations to a given R gene and reduces crop yield losses.

Funding

UPC authors acknowledge funding from the R+D+i project AGL2017-89785-R, financed by MCIN and FEDER; and Fondo Social Europeo (PRE2018-084265, AF). ICFO authors acknowledge funding from Fundació CELLEX; Ministerio de Economía y Competitividad - Severo Ochoa program for Centres of Excellence in RandD (CEX2019-000910-S); CERCA program and Laserlab-Europe (871124); Ministerio de Ciencia e Innovació n (MCIN/AEI/10.13039/501100011033); and Fondo Social Europeo (PRE2020-095721, MC).

Acknowledgments

The authors are grateful Dr. Picó and Dr. Gisbert from Institut de Conservació i Millora de l'Agrodiversitat Valenciana from Universitat Politècnica de Valencia (COMAV-UPV), as well as the seeds companies (Seminis Seeds, Ramiro Arnedo Seeds, Gautier Seeds, Fitó Seeds, and De Ruiter Seeds) to provide us the seeds for the experiments. We also thank the commercial nursery Hishtil GS for producing most of the plant material. Further, we are grateful to Mr. Roger Nieto, Mr. Sergi Garcia, Ms. Sheila Alcala, Ms. Maria Julià, Mr. Miquel Massip, and Dr. Helio Adán García, and the SLN Facility members for their technical support in the laboratory and greenhouse, and Dr. Javier Cabrera for his advice in the root clarification protocol.

References

Abd-Elgawad, M. M. (2022). Exploiting plant–phytonematode interactions to upgrade safe and effective nematode control. Life 12 (11), 1916.

Cabrera, J., Díaz-Manzano, F. E., Barcala, M., Arganda-Carreras, I., de AlmeidaEngler, J., Engler, G., et al. (2015). Phenotyping nematode feeding sites: three-dimensional reconstruction and volumetric measurements of giant cells induced by root-knot nematodes in arabidopsis. New Phytol. 206, 868–880.

Castagnone-Sereno, P., Bongiovanni, M., Palloix, A., and Dalmasso, A. (1996). Selection for *Meloidogyne incognita* virulence against resistance genes from tomato and pepper and specificity of the virulence/resistance determinants. Eur. J. Plant Pathol. 102 (6), 585–590.

Cortada, L., Sorribas, F. J., Ornat, C., Kaloshian, I., and Verdejo-Lucas, S. (2008). Variability in infection and reproduction of *Meloidogyne javanica* on tomato rootstocks with the *Mi* resistance gene. Plant Pathol. 57 (6), 1125–1135.

Davis, A. R., Perkins-Veazie, P., Sakata, Y., López-Galarza, S., Maroto, J. V., Lee, S. G., et al. (2008). Cucurbit grafting. Crit. Rev. Plant Sci. 27, 50–74.

Djian-Caporalino, C., Molinari, S., Palloix, A., Ciancio, A., Fazari, A., Marteu, N., et al. (2011). The reproductive potential of the root-knot nematode *Meloidogyne incognita* is affected by selection for virulence against major resistance genes from tomato and pepper. Eur. J. Plant Pathol. 131, 431.

Expósito, A., García, S., Gińe, A., Escudero, N., and Sorribas, F. J. (2019). *Cucumis metuliferus* reduces *Meloidogyne incognita* virulence against the *Mi1.2* resistance gene in a tomato–melon rotation sequence. Pest Man. Sci. 75 (1), 902–1910.

Expósito, A., Munera, M., Giné, A., López-Gómez, M., Cáceres, A., Picó, B., et al. (2018). *Cucumis metuliferus* is resistant to root-knot nematode *Mi1.2* gene (a)virulent isolates and a promising melon rootstock. Plant Pathol. 67, 1161–1187.

Expósito, A., Pujolà., M., Achaerandio, I., Gińe, A., Escudero, N., Fullana, A. M., et al. (2020). Tomato and melon *Meloidogyne* resistant rootstocks improve crop yield but melon fruit quality is influenced by the cropping season. Front. Plant Sci. 11, 560024.

Gaion, L. A., Braz, L. T., and Carvalho, R. F. (2018). Grafting in vegetable crops: A great technique for agriculture. Int. J. Veg. Sci. 24, 85–102.

Galatti, F. D. S., Franco, A. J., Ito, L. A., Charlo, H. D. O., Gaion, L. A., and Braz, L. T. (2013). Rootstocks resistant to *Meloidogyne incognita* and compatibility of grafting in net melon. Rev. Ceres. 60, 432–436.

García-Mendívil, H. A., Munera, M., Giné, A., Escudero, N., Picó, M. B., Gisbert, C., et al. (2019). Response of two *Citrullus amarus* accessions to isolates of three species of *Meloidogyne* and their graft compatibility with watermelon. Crop Prot. 119, 208–213.

García-Mendívil, H. A., and Sorribas, F. J. (2021). Effect of *Citrullus amarus* accessions on the population dynamics of *Meloidogyne incognita* and *M. javanica* and watermelon yield. Sci. Hortic. 275, 109680.

Ghahremani, Z., Escudero, N., Beltrán-Anadón, D., Saus, E., Cunquero, M., Andilla, J., et al. (2020). *Bacillus firmus* strain I-1582, a nematode antagonist by itself and through the plant. Front. Plant Sci. 11, 796.

Ghahremani, Z., Escudero, N., Saus, E., Gabaldón, T., and Sorribas, F. J. (2019). *Pochonia chlamydosporia* induces plant-dependent systemic resistance to *Meloidogyne incognita*. Front. Plant Sci. 10, 945.

Giné, A., and Sorribas, F. J. (2017a). Quantitative approach for the early detection of selection for virulence of *Meloidogyne incognita* on resistant tomato in plastic greenhouses. Plant Pathol. 66, 1338–1344.

Giné, A., and Sorribas, F. J. (2017b). Effect of plant resistance and BioAct WG (Purpureocillium lilacinum strain 251) on *Meloidogyne incognita* in a tomato– cucumber rotation in a greenhouse. Pest Manage. Sci. 73 (5), 880–887.

Gisbert, C., Gammoudi, N., Munera, M., Gińe, A., Pocurull, M., Sorribas, F. J., et al. (2017). Evaluation of two potential Cucumis spp. resources for grafting melons. Acta Hortic. 1151, 157–162.

Guan, W., Zhao, X., Dickson, D. W., Mendes, M. L., and Thies, J. (2014). Root-knot nematode resistance, yield, and fruit quality of specialty melons grafted onto *Cucumis metuliferus*. Hort Science 49 (8), 1046–1051.

Hadisoeganda, W. W., and Sasser, J. N. (1982). Resistance of tomato, bean, southern pea, and garden pea cultivars to root-knot nematodes based on host suitability. Plant Dis. 66 (2), 145–150.

Hallman, J., and Meressa, B. H. (2018). "Nematode parasites of vegetables," in Plant parasitic nematodes in subtropical and tropical agriculture. Eds. R. A. Sikora, D. Coyne, J. Hallman and P. Timper (Wallingford: CABI International), 346–410.

Hussey, R. S., and Barker, K. R. (1973). Comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. Pl. Dis. Rep. 57, 1025–1028.

Jacquet, M., Bongiovanni, M., Martinez, M., Verschave, P., Wajnberg, E., and Castagnone-Sereno, P. (2005). Variation in resistance to the root-knot nematode *Meloidogyne incognita* in tomato genotypes bearing the *Mi* gene. Plant Pathol. 54, 93–99.

Kantor, M., and Amnon, L. E. V. I. (2018). Utilizing genetic resources and precision agriculture to enhance resistance to biotic and abiotic stress in watermelon. Not. Sci. Biol. 10 (1), 1–7.

Kokalis-Burelle, N., and Rosskopf, E. N. (2011). Microplot evaluation of rootstocks for control of *Meloidogyne incognita* on grafted tomato, muskmelon, and watermelon. J. Nematol. 43 (3-4), 166.

Ling, J., Mao, Z., Zhai, M., Zeng, F., Yang, Y., and Xie, B. (2017). Transcriptome profiling of *Cucumis metuliferus* infected by *Meloidogyne incognita* provides new insights into putative defense regulatory network in cucurbitaceae. Sci. Rep. 7 (1), 1– 15.

López-Gómez, M., Giné , A., Vela, M. D., Ornat, C., Sorribas, F. J., Talavera, M., et al. (2014). Damage functions and thermal requirements of *Meloidogyne javanica* and *Meloidogyne incognita* on watermelon. Ann. Appl. Biol. 165, 466–473.

López-Gómez, M., Talavera, M., and Verdejo-Lucas, S. (2016). Differential reproduction of *Meloidogyne incognita* and *M. javanica* in watermelon cultivars and cucurbit rootstocks. Plant Pathol. 65 (1), 145–153.

López-Ṕerez, J. A., Le Strange, M., Kaloshian, I., and Ploeg, A. T. (2006). Differential response of *Mi* gene-resistant tomato rootstocks to root-knot nematodes (*Meloidogyne incognita*). Crop Prot. 25 (4), 382–388.

Martinez de Ilarduya, O., Moore, A. E., and Kaloshian, I. (2001). The tomato *Rme1* locus is required for *Mi-1*-mediated resistance to root-knot nematodes and the potato aphid. Plant J. 27, 417–425.

Mateille, T., Tavoillot, J., Goillon, C., Pare, L., Lefèvre, A., ́Vedie, H., et al. (2020). Competitive interactions in plant-parasitic nematode communities affecting organic vegetable cropping systems. Crop Prot. 135, 105206.

Omwega, C. O., Thomason, I. J., and Roberts, P. A. (1988). A nondestructive technique for screening bean germ plasm for resistance to Meloidogyne incognita. Plant Dis. 72 (11), 970–972.

Ornat, C., Verdejo-Lucas, S., and Sorribas, F. J. (1997). Effect of the previous crop on population densities of *Meloidogyne javanica* and yield of cucumber. Nematropica 27, 85–90.

Pegard, A., Brizzard, G., Fazari, A., Soucaze, O., Abad, P., and Djian-Caporalino, C. (2005). Histological characterization of resistance to different root-knot nematode species related to phenolics accumulation in *Capsicum annuum*. Phytopathology 95 (2), 158–165.

Petrillo, M. D., Matthews, W. C., and Roberts, P. A. (2006). Dynamics of *Meloidogyne incognita* virulence to resistance genes *Rk* and *Rk2* in cowpea. J. Nematol 38 (1), 90–96.

Petrillo, M. D., and Roberts, P. A. (2005). Fitness of virulent *Meloidogyne incognita* isolates on susceptible and resistant cowpea. J. Nematol 37 (4), 457–466.

Pocurull, M., Fullana, A. M., Ferro, M., Valero, P., Escudero, N., Saus, E., et al. (2020). Commercial formulates of trichoderma induce systemic plant resistance to *Meloidogyne incognita* in tomato and the effect is additive to that of the *Mi1.2* resistance gene. Front. Microbiol. 10, 3042.

Ros, C., Guerrero, M. M., Martiinez, M. A., Lacasa, A., and Bello, A. (2006). Integrated management of *Meloidogyne resistance* in sweet pepper in greenhouses. IOBC WPRS Bull. 29 (4), 319.

Ros-Ibáñez, C., Robertson, L., Martínez-Lluch, M., Cano-García, A., and Lacasa Plasencia, A. (2014). Development of virulence to *Meloidogyne incognita* on resistant pepper rootstocks. Span. J. Agric. Res. 12, 225–232.

Sayed, A. A., and Thomason, I. J. (1988). *Meloidogyne incognita* and tomato response to thiamine, ascorbic acid, l-arginine, and l-glutamic acid. J. Nematol. 20 (3), 451.

Shukla, N., Yadav, R., Kaur, P., Rasmussen, S., Goel, S., Agarwal, M., et al. (2018). Transcriptome analysis of root-knot nematode (*Meloidogyne incognita*) -infected tomato (*Solanum lycopersicum*) roots reveals complex gene expression profiles and metabolic networks of both host and nematode during susceptible and resistance responses. Mol. Plant Pathol. 19 (3), 615–633.

Sigüenza, C., Schochow, M., Turini, T., and Ploeg, A. (2005). Use of *Cucumis metuliferus* as a rootstock for melon to manage *Meloidogyne incognita*. J. Nematol. 37 (3), 276.

Sorribas, F. J., Djian-Caporalino, C., and Mateille, T. (2020). "Nematodes," in Integrated pest and disease management in greenhouse crops, vol. Vol 9 . Eds. M. L. Gullino, R. Albajes and P. C. Nicot (Cham, Switzerland: Springer), 147–174.

Sorribas, F. J., Ornat, C., Verdejo-Lucas, S., Galeano, M., and Valero, J. (2005). Effectiveness and profitability of the *Mi*-resistant tomatoes to control root-knot nematodes. Eur. J. Plant Pathol. 111, 29–38.

Stotz, H. U., Spence, B., and Wang, Y. (2009). A defensin from tomato with dual function in defense and development. Plant Mol. Biol. 71 (1), 131–143.

Subbotin, S. A., Palomares-Rius, J. E., and Castillo, P. (2021). Systematics of rootknot nematodes (Nematoda: *Meloidogynidae*), nematology monographs and perspectives (Leiden: Brill).

Talavera, M., Verdejo-Lucas, S., Ornat, C., Torres, J., Vela, M. D., Macias, F. J., et al. (2009). Crop rotations with *Mi* gene resistant and susceptible tomato cultivars for management of root-knot nematodes in plastic houses. Crop Prot. 28 (8), 662–667.

Talavera-Rubia, M., Vela-Delgado, M. D., and Verdejo-Lucas, S. (2022). A cost-benefit analysis of soil disinfestation methods against root-knot nematodes in Mediterranean intensive horticulture. Plants 11 (20), 2774.

Thies, J. A., Ariss, J. J., Hassell, R. L., Buckner, S., and Levi, A. (2015a). Accessions of *Citrullus lanatus* var. citroides are valuable rootstocks for grafted watermelon in fields infested with root-knot nematodes. HortScience 50 (1), 4–8.

Thies, J. A., Ariss, J. J., Hassell, R. L., Olson, S., Kousik, C. S., and Levi, A. (2010). Grafting for management of southern root-knot nematode, *Meloidogyne incognita*, in watermelon. Plant Dis. 94 (10), 1195–1199.

Thies, J. A., Ariss, J. J., Kousik, C. S., Hassell, R. L., and Levi, A. (2016). Resistance to southern root-knot nematode (*Meloidogyne incognita*) in wild watermelon (*Citrullus lanatus* var. citroides). J. Nematol. 48, 14–19.

Thies, J. A., Buckner, S., Horry, M., Hassell, R., and Levi, A. (2015b). Influence of *Citrullus lanatus* var. citroides rootstocks and their *F1* hybrids on yield and response to root-knot nematode, *Meloidogyne incognita*, in grafted watermelon. HortScience 50 (1), 9–12.

Thies, J. A., and Levi, A. (2003). Resistance of watermelon germplasm to the peanut root-knot nematode. HortScience 38 (7), 1417–1421.

Thies, J. A., and Levi, A. (2007). Characterization of watermelon (*Citrullus lanatus* var. citroides) germplasm for resistance to root-knot nematodes. HortScience 42 (7), 1530–1533.

Thies, J. A., Mueller, J. D., and Fery, R. L. (1998). Use of a resistant pepper as a rotational crop to manage southern root-knot nematode. HortScience 33 (4), 716–718.

Trivedi, P., and Barker, K. (1986). Management of nematodes by cultural practices. Nematropica 16, 213–236.

Vela, M. D., Giné , A., López-Gómez, M., Sorribas, F. J., Ornat, C., Verdejo-Lucas, S., et al. (2014). Thermal time requirements of root-knot nematodes on zucchini-squash and population dynamics with associated yield losses on spring and autumn cropping cycles. Eur. J. Plant Pathol. 140, 481–490.

Verdejo-Lucas, S., Cortada, L., Sorribas, F. J., and Ornat, C. (2009). Selection of virulent populations of *Meloidogyne javanica* by repeated cultivation of *Mi* resistance gene tomato rootstocks under field conditions. Plant Pathol. 58, 990–998.

Whitehead, A. G., and Hemming, J. R. (1965). A comparison of some quantitative methods of extracting small vermiform nematodes from soil. Ann. App. Biol . 55, 25–38.

Chapter 2

Effect of grafting tomato onto *Solanum torvum* on the population dynamics of *Meloidogyne incognita* and *M. javanica* and crop yield losses

Abstract

Meloidogyne spp. are the most devastating plant-parasitic nematodes affecting tomato worldwide. Although resistant cultivars and rootstocks are used, selection for virulence occurs in the pathogen. Consequently, using other resistance sources, such as *Solanum torvum*, could improve resistance durability. Several experiments in microplots and plastic greenhouses were carried out to determine the potential use of *S*. *torvum* as a tomato rootstock to protect against *M*. *incognita* and *M*. *javanica*. In microplots, the relationship between nematode density at transplanting (*Pi*) and multiplication rate did not differ between *Meloidogyne* species in either ungrafted or grafted tomato. However, maximum multiplication rate and maximum density on grafted tomato were 1.27% and 2.93% those on ungrafted, respectively. The grafted tomato plants yielded between 2.9 and 7.5 more times than the ungrafted plants at *Pi* ≥ 100 eggs + J2s per 100 cm^3 of soil, but no differences were observed in plastic greenhouse where a large amount of scion-rooting occurred. In microplots, the quality of the tomato fruits of ungrafted and grafted plants was affected by the *Pi*. In parallel, some pot experiments were conducted on *S*. *torvum* and susceptible eggplant to determine the putative selection for nematode virulence to *S*. *torvum* and the nematode fitness cost. These showed that the nematode subpopulations infected and reproduced less on *S*. *torvum* than on eggplant. However, the female fertility was only reduced after development of three or four subpopulations on *S*. *torvum*. Finally, a histopathological study showed that nematode infection and development in *S*. *torvum* was delayed compared to eggplant.

Keywords: damage function model, fitness cost, resistance durability, root-knot nematodes, selection for virulence, *Solanum lycopersicum*

Introduction

Tomato, *Solanum lycopersicum*, is the most important fruiting solanaceous crop in the world, covering 4.9 million ha, with an annual production of 186.1 Mt of which 20.4 Mt were roduced in Europe in 2022 (FAOstat, 2023). Spain is the second largest tomato producer in Europe but the largest producer under protected cultivation.Several soilborne plant pathogens, including

plant-parasitic nematodes (PPNs), affect this crop. Among them, the root-knot nematodes (RKNs), *Meloidogyne* spp., are the most devastating (Jones et al., 2013), reducing tomato yield to 73% in protected cultivation (Expósito et al., 2020). RKNs are distributed worldwide due to the wide range of plant hosts they can parasitize and their high reproductive capacity enhanced by environmental conditions and agronomic practices, particularly under protected cultivation (Greco and Di Vito, 2009). The most prevalent RKN species in the Mediterranean basin are *M*. *arenaria*, *M*. *incognita* and *M*. *javanica*. All RKN species are obligate sedentary endoparasitic nematodes that reproduce parthenogenetically. The second-stage juvenile (J2) penetrates the plant root near the elongation zone, moves intercellularly and establishes a feeding site within the vascular cylinder. The plant cells adjacent to the J2 head are modified by effectors produced by the nematode, becoming hypertrophied, multinucleate and with an increased number of organelles. These giant plant cells supply nutrients to the nematode for the rest of its life cycle. After root infection, the J2 becomes sedentary and undergoes three moults until reaching the adult stage. Under favourable conditions, the juveniles develop to females that lay eggs into a gelatinous matrix. Embryogenesis gives rise to a J1, which moults inside the egg to become a J2. Under unfavourable conditions, such as plant stress, the juveniles develop to males, leaving the roots instead of feeding and live freely in the soil (Abad et al., 2009). As a result of nematode infection, plant roots become galled, interfering with the uptake of water and nutrients, which causes nonspecific symptoms in the aboveground part of the plant. The disease severity can range from asymptomatic plants to plant death, depending on the density of nematodes in the soil at sowing or transplanting, the plant's tolerance and the environmental conditions (Greco and Di Vito, 2009).

The use of resistant tomato cultivars or rootstocks has become one of the most widespread control methods included in the nematode management strategy. Plant resistance is a cost-effective control method, is environmentally friendly, benefits human health and, in addition, is also easy for farmers to use (Sorribas et al., 2005). In tomato, the *Mi1.2* gene confers resistance to *M*. *arenaria*, *M*. *incognita*, *M*. *javanica* (Roberts and Thomason, 1989), *M*. *ethiopica* and *M*. *luci* (Santos et al., 2019) but not to *M*. *enterolobii* (Castagnone-Sereno, 2012) or *M*. *hapla* (Roberts and Thomason, 1989). Unfortunately, the repeated cultivation of *Mi1.2* resistant tomato cultivars or rootstocks leads to the selection of virulent nematode populations (Giné and Sorribas, 2017; Verdejo-Lucas et al., 2009). Therefore, it is recommended to include several sources of resistance in crop rotation sequences to prevent the increase of virulent individuals in the nematode population and promote the durability of the resistance (Fullana et al., 2023). In vegetable-growing areas where monocropping of tomato is common, grafting tomato onto

compatible *Meloidogyne*-resistant rootstocks with sources of resistance different from the *Mi1.2* gene could be effective. *Solanum torvum* is a promising candidate that can be used as an eggplant rootstock and is resistant to *M. arenaria*, *M. enterolobii*, *M. incognita*, *M. javanica*, *M. luci*, as well as against some *Meloidogyne* populations virulent to the *N* and *Mi1.2* resistance genes in pepper and tomato, respectively (García-Mendívil et al., 2019; Öçal et al., 2018; Pinheiro et al., 2022; Uehara et al., 2017). Furthermore, in a previous study, two consecutive eggplant crops grafted onto *S. torvum* did not select for nematode virulence (García-Mendívil and Sorribas, 2019). Another study using *S. torvum* as a rootstock for tomato showed that tomato growth was delayed and yielded less compared to tomato grafted onto tomato rootstocks (de Miguel et al., 2011); however, to our knowledge, no information is available on the comparative performance of ungrafted tomato plants with those grafted onto *S. torvum* grown in soils with increasing nematode densities at transplanting and its effect on fruit quality. Therefore, the objective of the present study was to determine the potential use of *S. torvum* 'Brutus' as a rootstock for tomato to protect against RKN, considering its effect on (i) the population dynamics of the nematode; (ii) the tomato crop yield quantity and quality; (iii) the durability of the resistance by repeated cultivation and (iv) the fitness cost to the nematode.

Materials and methods

Effect of grafted tomato onto S. torvum 'Brutus' on nematode population dynamics and crop yield

Three experiments were conducted, one in microplots and two under normal plastic-greenhouse conditions.

Microplot experiment

The microplot experiment was carried out from March to November in a plastic greenhouse. Microplots consisted of 30-L pots containing sterile river sand, which were buried into the soil to be exposed to the natural temperature fluctuation that occurred in the soil. The experiments used two nematode isolates: *M. incognita* Agròpolis and *M. javanica* MJ05. The nematode inoculum was produced on the susceptible tomato cv. Durinta (Seminis Seeds). The infected roots were processed separately to avoid cross-contamination between RKN species. Nematode eggs were extracted from infected roots by blender maceration in a 5% commercial bleach solution (40 g/L NaOCl) for 10 min (Hussey and 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. Eggs were counted in a Hawksley chamber under an optical microscope to calculate the number

of eggs per gram of root. Afterward, the weight of roots needed to achieve different nematode densities at transplanting (*Pi*: 0, 5, 10, 50, 100, 500, 1000, 2500 and 5000 eggs + J2s per 100 cm^3 of soil) was mixed into the substrate. The susceptible tomato cv. Durinta plants ungrafted or grafted onto *S. torvum* 'Brutus' (Fitó Seeds), provided by the nursery Hishtil GS, were transplanted at the 4-leaf stage. Each combination of ungrafted and grafted tomato– nematode inoculum density–RKN species was replicated four times. The plants were irrigated as needed with a drip irrigation system and fertilized with a solution of NPK (15–5-30) at 31 kg/ha, iron chelate and micronutrients at 0.9 kg/ha. Soil temperatures were recorded daily at 1 h intervals with five TM digital probes (Decagon Devices, Inc.) placed at 15 cm depth. Tomato fruits were harvested and weighed when they reached the commercial standards according to the European Union Commission regulation 790/2000 and kept at −20 } 1°C until processing for fruit quality assessment.

Assessment of fruit quality

Chemical analyses of tomato fruits were conducted in duplicate. When available, the official nalysis methods were used (AOAC, 2019). The soluble solid content (SSC) was measured with a digital refractometer (model PR-101, Atago, Co.) at 20 ± 1°C, and the results were expressed as Brix. The pH was determined according to AOAC 981.12. The dry matter content was obtained following the gravimetric method (AOAC 931.04) and the fruit's dry weight (dw) was expressed as a percentage of the fresh fruit weight. Subsequently, dried samples were kept in a muffle furnace and incinerated at 475°C until white ashes were obtained (AOAC 940.26). Mineral content was then assessed. Sodium and potassium content were determined by flame atomic emission spectrometry using a Corning 410 C spectrophotometer. Iron, calcium and magnesium were determined by atomic absorption spectrometry using a Varian SpectrAA-110. The results were expressed as g/kg dw, except for Fe (mg/kg dw). Ascorbic acid content was measured using a titration method (AOAC 967.21) and the results were expressed in mg of ascorbic acid/kg dw. Lycopene extraction and HPLC-UV quantification was done according to the procedure stated by Vallverdú-Queralt et al. (2012). Lyophilized pure tomato samples (0.3 g) were homogenized with 5 mL of ethanol/hexane (4:3 vol/vol). The homogenate was sonicated for 5 min and centrifuged at 2140 *g* for 5 min at 20°C. The supernatant was transferred into a flask and the extraction was repeated. The two supernatants were combined and evaporated under nitrogen flow. Finally, the residue was reconstituted with methyl *tert*-butyl ether (MTBE) up to 1 mL and filtered through a 13 mm, 0.45 µm polytetrafluoroethylene (PTFE) filter (Waters) into an insert-amber vial for HPLC analysis. Samples were stored at −20°C until analysis.

Chromatographic analysis was performed using an HP 1100 HPLC system (Hewlett-Packard) equipped with a quaternary pump, an autosampler and a column oven. The analytes were separated on a 250 mm × 4.6 mm i.d., 5 µm C30 column (YMCTM; Waters) maintained at 20°C. The injection volume was 20 µL and the flow rate was 1 mL/min. The mobile phases were water (A), methanol (B) and MTBE (C). The HPLC run was carried out in 23 min under the following conditions: 0 min, 70% B; 10 min, 20% B; 20 min, 6% B; 21 min, 6% B; 23 min, 70% B. Water was kept constant at 4% throughout the analysis. The column was equilibrated for 10 min prior to each analysis. MTBE was used as a modifier to facilitate the elution of lycopene, which is strongly retained in a methanol environment. Commercially available carotenoid standard (*trans*-lycopene) was used to identify analytes by retention times and UV–visible spectra. The HPLC-UV chromatograms were acquired at 450 nm wavelength. Results were expressed in mg of lycopene/kg dw.

Assessment of disease severity

At the end of the experiment, the plants were uprooted and washed with tap water, and the disease severity was estimated using the Zeck (1971) galling index (GI) scale from 0 to 10, where 0 is a complete and healthy root system and 10 is a dead plant. Afterward, the root system was cut into 1–2 cm fragments and homogenized and two 20 g root subsamples were used to extract the eggs by macerating them in a 10% commercial bleach solution (40 g/L NaOCl) for 10 min (Hussey and Barker, 1973) as described previously and counted to determine the final nematode population density (*Pf*). The remaining root samples were used to obtain nematode inoculum to determine if the development on *S. torvum* entailed a putative fitness cost. Afterward, the reproduction rate (*Pf/Pi*) was calculated and the relationship between *Pf/Pi* and *Pi* was estimated by regression analyses, and comparisons between RKN species per ungrafted or grafted tomato plants were conducted. The maximum multiplication rate (*a*) was estimated by calculating *Pf/Pi* at small *Pi* values at which *a* is maximum (*a* = *Pf/Pi*) (Seinhorst, 1970); the maximum nematode density *M* was estimated from the experimental data, and the equilibrium density (*E*, nematode density at which *Pf = Pi; Pf/Pi* = 1) was calculated by the regression equation obtained from the relationship between log10(*Pf/Pi*) and log10(*Pi*). The relationship between the relative crop yield (kg/plant) at increasing *Pi* densities for either ungrafted or grafted tomato, was submitted to a nonlinear regression analysis to determine whether they fitted the Seinhorst's damage function model ($y = m + (1 - m) \times 0.95^{[Pi/T - 1]}$) (Seinhorst, 1998), where *y* is the relative crop yield; *T* is the tolerance limit, (i.e., the nematode density below which no yield losses occur), and *m* is the minimum relative crop yield.

Investigation of nematode fitness

The putative cost to nematode fitness of using *S. torvum* as rootstock was assessed on susceptible eggplant (*Solanum melongena*) seedlings using the four nematode subpopulations obtained at the end of the first experiment. These were obtained from tomato plants ungrafted or grafted onto *S. torvum* inoculated with *M. incognita* Agròpolis or *M. javanica* MJ05. The seeds of the susceptible eggplant c v. Cristal (Fitó Seeds) were germinated in sterile vermiculite and maintained in a climatic chamber at 25 ± 2°C and 16:8 h photoperiod (light:dark) for 2 weeks. Then, the seedlings were individually transferred to 200 cm^3 pots containing sterile river sand and maintained in the same conditions described previously. The nematode inoculum consisted of J2s obtained from eggs extracted from the roots in a commercial 5% bleach solution (40 g/L NaOCl) for 10 min (Hussey and Barker, 1973) and incubated in Baermann trays to allow egg hatching. The J2s hatched during the first 24 h were discarded, and those that emerged later were collected every other day and kept at 9°C until use. Plants of *S. melongena* were inoculated with 200 J2s per plant 1 week after transplanting. Plants were fertilized with a slow-release fertilizer (15% N, 9% P2O5, 12% K2O, 2% MgO2, microelements; Osmocote Plus, Scotts Company LLC) and watered as needed. Each combination of plant germplasm–RKN subpopulation was replicated 10 times. Soil temperatures were recorded at 1 h intervals with PT100 probes (Campbell Scientific Ltd). At the end of the experiment, 45 days after nematode inoculation, the plants were uprooted, the roots were carefully washed with tap water and the number of egg masses was counted after dyeing them in a 0.01% erioglaucine solution for 20 min (Omwega, 1988). The infectivity was expressed as the number of egg masses per plant and the number of J2s able to infect and develop into egg-laying females. The number of eggs produced in the entire root system determined the nematode reproduction. Female fertility was estimated as the number of eggs laid per female and expressed as the number of eggs per egg mass.

Plastic greenhouse experiments

The plastic greenhouse experiments were conducted in an experimental plastic greenhouse at Agròpolis belonging to the UPC (Viladecans, Spain) and in a commercial plastic greenhouse of Ametller (Mataró, Spain). At Agròpolis, the soil was infested by the Agròpolis isolate of *M. incognita*. The experiment was conducted from March to November. At the beginning of the experiment, the soil of each plot was sampled with a 2.5 cm diameter auger taking eight cores from the top 30 cm of soil. The soil cores were mixed and J2s were extracted from 500 cm^3 of soil by Baermann trays incubated at 25 ± 2°C for 2 weeks to determine the nematode population density at transplanting (*Pi*) and decide the experimental design. The J2s were collected using a 25 µm aperture screen sieve, counted and expressed as J2s per 100 cm3 of soil. The tomato cultivars Durinta and Candido (Cooperativa Agrícola Barbastro – SCLAB), ungrafted or grafted

onto *S*. *torvum* 'Brutus' provided by Hishtil GS, were used in this study. Each plot consisted of four plants spaced 0.55 m apart. The plots within a row were spaced 1 m apart. Each treatment was replicated 10 times. Plants were irrigated as needed with a drip irrigation system and fertilized with a solution of NPK (15–5-30) at 31 kg/ha, iron chelate and micronutrients at 0.9 kg/ha. Weeds were removed manually during the experiment. Soil temperatures were recorded daily at 1 h intervals with five TM digital probes (Decagon Devices, Inc.) placed at 15 cm depth. Fruits were harvested and weighed when they reached commercial standards according to the European Union Commission regulation 790/2000. At the end of the experiment, soil samples were taken, as previously described, to determine the nematode density at the end of the crop (*Pf*). The galling index and nematode reproduction were assessed following the procedure described in the microplot experiment. The putative selection for nematode virulence to *S*. *torvum* o f e ach R KN s ubpopulation (coming from ungrafted or grafted tomato onto *S*. *torvum* 'Brutus') was assessed in a pot experiment with 14 replications per treatment following the procedure described in the fitness cost experiment. In addition, the level of resistance of *S*. *torvum* to each nematode subpopulation was determined by the reproduction index (RI = (number of eggs produced in *S*. *torvum*/number of eggs produced in the susceptible eggplant) × 100) and the plant response was classified as highly resistant (RI <1%), resistant (1% ≤ RI < 10%), moderately resistant (10% ≤ RI < 25%), slightly resistant (25% ≤ RI < 50%), or susceptible (RI ≥ 50%) (Hadisoeganda and Sasser, 1982).

At the commercial plastic greenhouse Ametller, the soil was naturally infested by *M*. *incognita* and *M*. *javanica* communities. The RKN species were identified by the morphology of the perineal pattern of the females and by molecular markers (Zijlstra et al., 2000). Plots of 5 m length and 7.5 m width consisted of three rows 2.5 m apart, each with five plants separated by 0.5 m. Plots were separated by 1 m. Soil samples were taken from each plot before transplanting to determine the *Pi* and to decide the experimental design, as previously described. The tomato cv. Monterosa (Fitó Seeds) grafted onto *S*. *torvum* 'Brutus' or the resistant tomato rootstock cv. Silex (Fitó Seeds) were cultivated from July to December. The crop was irrigated and fertilized according to the farmer. Each treatment was replicated three times. At the end of the crop, *Pf*, galling index and nematode reproduction were determined as previously explained. The virulence status of the RKN community to the *Mi1.2* resistance gene and the effect of using *S*. *torvum* on nematode infection and reproduction on the susceptible tomato cv. Durinta) and the resistant tomato cv. Monika (Syngenta) was assessed, following the procedure described in the fitness cost experiment below and the level of resistance was categorized according to the RI

values (Hadisoeganda and Sasser, 1982). Each combination of plant germplasm-RKN subpopulation (from tomato rootstock Silex or *S. torvum* 'Brutus') was replicated 14 times.

DURABILITY OF THE RESISTANCE AND NEMATODE FITNESS COST AFTER REPEATED CULTIVATION OF *S. TORVUM*

We investigated the putative selection of nematode virulence to *S. torvum* and the cost to nematode fitness of using the *S. torvum* rootstock on susceptible eggplant and on a following susceptible and resistant crop. Three different subpopulations of *M. incognita* were used, differentiated by the number of times that the nematode population had developed on *S. torvum* in the plastic greenhouse at Agròpolis and/or climatic chamber. The first subpopulation (*Subpop1*) came from two consecutive eggplant crops cultivated at Agròpolis, followed by one *S. torvum* grown in a climatic chamber. The second subpopulation (*Subpop2*) came from one eggplant crop followed by one crop of eggplant grafted onto *S. torvum* cultivated at Agròpolis and one *S. torvum* grown in a climatic chamber. The third subpopulation (Subpop3) came from two consecutive crops (eggplant then tomato) grafted onto *S. torvum* cultivated at Agròpolis and one *S. torvum* grown in climatic chamber. In addition, a subpopulation (*Subpop*0) never exposed to *S. torvum* was used as a control for comparison (Figure 1A).

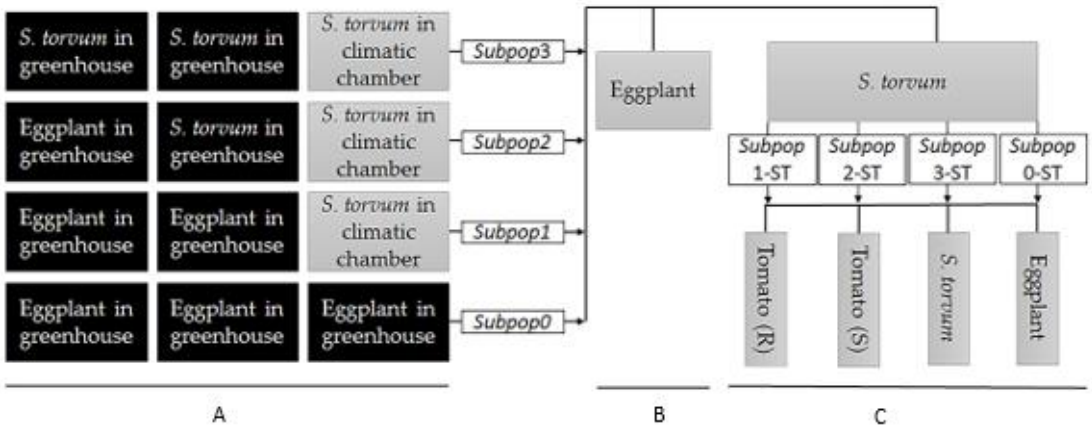


Figure 1. (A) Experiment to investigate putative selection of nematode virulence to *Solanum torvum* and the cost to nematode fitness of using the *S. torvum* 'Brutus' rootstock for successive crops. Diagram shows successive crops used for development of different nematode subpopulations. Black squares refer to crops cultivated in the plastic greenhouse at Agròpolis, while grey squares refer to plant growth in climate chambers. (B) Fitness cost experiment where subpopulations were tested on susceptible eggplant cv. Cristal. (C) Experiment of durability of the resistance of *S. torvum* after repeated cultivation and the effect on nematode growth and reproduction on following susceptible or resistant crop. Nematode subpopulations *Subpop*0 to *Subpop*3 were multiplied on another crop of *S. torvum* and then tested on reisistant tomato, susceptible tomato, *S. torvum* and eggplant.

Subsequently, the potential biological cost associated with the number of times that the nematode developed in *S. torvum*, expressed as a reduction of the ability to infect, to reproduce and the fertility of females in the susceptible eggplant, was assessed for each of the four nematode subpopulations in the eggplant cv. Cristal (Figure 1B). Simultaneously, each subpopulation was inoculated into *S. torvum* to produce enough inoculum to assess each nematode subpopulation's ability to infect and reproduce in the following susceptible tomato cv. Durinta or eggplant cv. Cristal, as well as on the resistant tomato cv. Caramba or *S. torvum* (Figure 1C). The tomato cv. Caramba was used because the resistant cv. Monika was not available.

Eggplant and tomato seeds were germinated in sterile vermiculite and maintained in a climatic chamber at 25 ± 2°C and 16:8 h photoperiod (light:dark) for 2 weeks. The seeds of *S. torvum* were pretreated with a KNO_3 solution to improve germination (Ranil et al., 2015), then transferred to vermiculite-filled trays and incubated in the same growth chamber for 4 weeks to reach the same physiological stage as the rest of the plants. Plantlets were individually transplanted into 200 cm^3 pots with sterilized sand. After 1 week, each plant was inoculated with 200 J2s and placed in a climatic chamber at 25°C and 16:8 (light: dark) photoperiod. Each treatment was replicated 10 times. The experiments lasted 55 days after nematode inoculation. At the end of the experiments, the nematode's infectivity and reproduction were determined, and the fertility was calculated using the same procedure described previously. In addition, at the end of the durability of resistance experiment, the reproduction index (RI) of each nematode subpopulation was calculated and the level of resistance was categorized (Hadisoeganda and Sasser, 1982), providing information on the virulence level of each nematode subpopulation after being developed four (*Subpop*3-ST), three (*Subpop*2-ST), two (*Subpop*1-ST) or one time (*Subpop*0-ST) on *S. torvum*.

HISTOPATHOLOGY

A histopathology study with laser-scanning confocal microscopy was carried out to compare the nematode–plant interaction between eggplant or *S. torvum* and each of the *M. incognita* subpopulations *Subpop*0 and *Subpop*1 at 15 and 70 days after inoculation (DANI). The susceptible eggplant cv. Cristal and the resistant rootstock *S. torvum* 'Brutus' were transplanted into 200 cm^3 pots containing sterilized river sand at the stage of two expanded leaves. Seven days later, eggplants were inoculated with 200 J2s of the subpopulations *Subpop*0 or *Subpop*1 of *M. incognita* and *S. torvum* was inoculated with 600 J2s to increase the probability of visualizing the nematodes inside the roots. The nematode inoculum was obtained as previously described. Plants were maintained in a climatic chamber kept at the same conditions described

above. Each plant germplasm–*Meloidogyne* subpopulation was replicated six times. At 15 DANI and 70 DANI, 10 galled root fragments of each of three plants were selected to observe the morphological changes caused by the nematode. The roots were fixed, clarified and stored following the procedure described by Expósito et al. (2020) to observe the feeding site characteristics. The cleared galls were viewed by fluorescence microscopy and images were acquired with an inverted TCS 5 STED CW microscope (Leica Microsystem) equipped with a 10× 0.40NA HCX Pl Apo CS air objective. Two different excitation-emission combinations were used. The root cell walls of the samples were excited with a 488 nm argon laser, and the fluorescence emission was collected with a hybrid detector in the range of 498–550 nm. The nuclei of giant cells and the nematodes were visualized with a 633 nm HeNe laser, and the fluorescence emission was collected with a hybrid detector in the 643–680 nm range. The visualized volume had a thickness ranging from 60 to 170 µm. Each volume was optically sectioned to produce a collection of *z*-stack images (step size of 2–3 µm).

STATISTICAL ANALYSIS

Statistical analyses were done using the SAS Studio (SAS Institute Inc.) and Statistics Kingdom. The normal distribution of the data and homogeneity of variances were checked using the proc univariate and the hovtest statement to perform Levene's test, respectively. When data accomplished these assumptions, paired comparisons between grafted and ungrafted plants or resistant and susceptible plant germplasm concerning galling index, number of egg masses, number of eggs per plant and crop yield were done by Student's *t* test ($p < 0.05$); otherwise, the nonparametric Wilcoxon test was used. For multiple comparisons between RKN subpopulations regarding nematode infectivity, reproduction and fertility on eggplant or *S. torvum*, the Kruskal–Wallis analysis followed by Dunn's test ($p < 0.05$) was carried out.

The *Pf* was relativized to 100 cm^3 of soil and the relationship between *Pf/Pi* and *Pi* of each RKN species on tomato, ungrafted or grafted onto *S. torvum*, cultivated in microplots was determined by regression analysis (proc reg) after being linearized to $\log_{10}x$, and compared using the general linear model procedure (proc glm). As no differences ($p \geq 0.05$) were found between RKN species per ungrafted or grafted tomato plants, data from different species were pooled together and compared between ungrafted and grafted tomato. The relative yields of tomato ungrafted or grafted onto *S. torvum* were submitted to a nonlinear regression (proc nlin) to determine the compliance with the Seinhorst damage function model ($y = m + (1 - m) \times 0.95^{(Pi/T - 1)}$) when $Pi \geq T$ and $y = 1$ at $Pi < T$, where m is the minimum relative yield, and T is the tolerance limit (Seinhorst, 1998). Regarding tomato fruit quality, correlation analysis (proc corr) between

each parameter and $\log_{10}(Pi + 1)$ per each RKN species and ungrafted or grafted tomato was conducted.

Results

Effect of tomato grafted onto *S. torvum* 'Brutus' on nematode population dynamics and crop yield quantity and quality in microplots

In the microplot experiment, the galling index, nematode reproduction and tomato yield for a given *Pi* did not differ ($p \geq 0.05$) between plants inoculated with *M*. *incognita* or *M*. *javanica*. Consequently, data were pooled for statistical analyses and comparisons (Table 1). The ungrafted tomato plants inoculated with nematode densities higher than 50 eggs + J2s per 100 cm^3 of soil were removed at the beginning of August due to poor growth and irregular fruit set. Subsequently, the ungrafted plants inoculated with between 5 and 50 eggs + J2s per 100 cm^3 of soil were removed at the beginning of November, while the noninoculated plants were maintained for an additional 2 weeks.

Table 1. Galling index (GI), final nematode population on infested roots (*Pf*) and yield of tomato cv. Durinta ungrafted (T) or grafted onto *Solanum torvum* 'Brutus' (GT).

Pi **(Eggs+J2/ 100 cm^3)**	**GI**		***Pf*** **(Eggs (x10^3) per plant)**		**Yield (Kg per plant)**	
	T	**GT**	**T**	**GT**	**T**	**GT**
0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	2.23 ± 0.06	1.99 ± 0.16
5	6 ± 0.4*	1 ± 0.6	1568 ± 633 *	20 ± 7	1.59 ± 0.06	1.57 ± 0.07
10	7 ± 0.0*	2 ± 0.9	702 ± 149 *	26 ± 11	1.22 ± 0.06	1.06 ± 0.10
50	7 ± 0.1*	3 ± 0.2	473 ± 210 *	46 ± 15	1.16 ± 0.08	1.61 ± 0.08
100	7 ± 0.4*	3 ± 0.2	362 ± 199 *	26 ± 5	0.68 ± 0.19 *	1.95 ± 0.25
500	7 ± 0.0*	2 ± 0.5	20 ± 15	31 ± 15	0.30 ± 0.07 *	2.25 ± 0.22
1000	7 ± 0.0*	3 ± 0.2	11 ± 4	29 ± 15	0.32 ± 0.04 *	2.02 ± 0.25
2500	7 ± 0.1*	2 ± 0.3	5 ± 2	42 ± 9	0.33 ± 0.04 *	1.87 ± 0.22
5000	7 ± 0.0*	3 ± 0.2	2 ± 1	9 ± 3	0.47 ± 0.05 *	1.48 ± 0.08

Plants were cultivated from March to November in 30 L microplots filled with sand mixed with *Meloidogyne incognita* or *M. javanica* galled roots to achieve eight nematode density levels (*Pi*). Data are means ± standard errors of eight replicates. Values in the same row followed by *indicate differences

($p < 0.05$) between grafted and ungrafted plants according to the Wilcoxon nonparametric test. GI: galling index on a scale from 0 to 10, where 0 = complete and healthy root system and 10 = plants and roots dead (Zeck, 1971).

The disease severity was higher ($p < 0.05$) in ungrafted than in grafted tomato, irrespective of the *Pi*. The nematode reproduced less ($p < 0.05$) in grafted than ungrafted tomato at *Pi* densities between 5 and 100 eggs + J2s per 100 cm^3 soil but did not at higher *Pi*. Grafted tomato yielded more ($p < 0.05$) than ungrafted at *Pi* from 100 eggs + J2s per 100 cm3 soil (Table 1).

The relationship between *Pf/Pi* and *Pi* did not differ between *Meloidogyne* species in either ungrafted (intercept $p = 0.6104$; slope $p = 0.1213$) or grafted tomato (intercept $p = 0.7109$; slope $p = 0.3931$). Therefore, data were pooled together and a single relationship per each ungrafted or grafted tomato was estimated and compared, showing that they differed (intercept $p < 0.0001$; slope $p < 0.0001$; Figure 2). The maximum multiplication rate (a), the maximum nematode density (M) and the equilibrium density (E) of *Meloidogyne* on the susceptible cv. Durinta (ungrafted) were 1045.3, 5227 eggs + J2s per 100 cm^3 of soil, and 133 eggs + J2s per 100 cm^3 of soil, respectively. On grafted tomato, a, M and E values were 13.3, 153 eggs + J2s per 100 cm^3 of soil, and 64 eggs + J2s per 100 cm3 of soil, respectively.

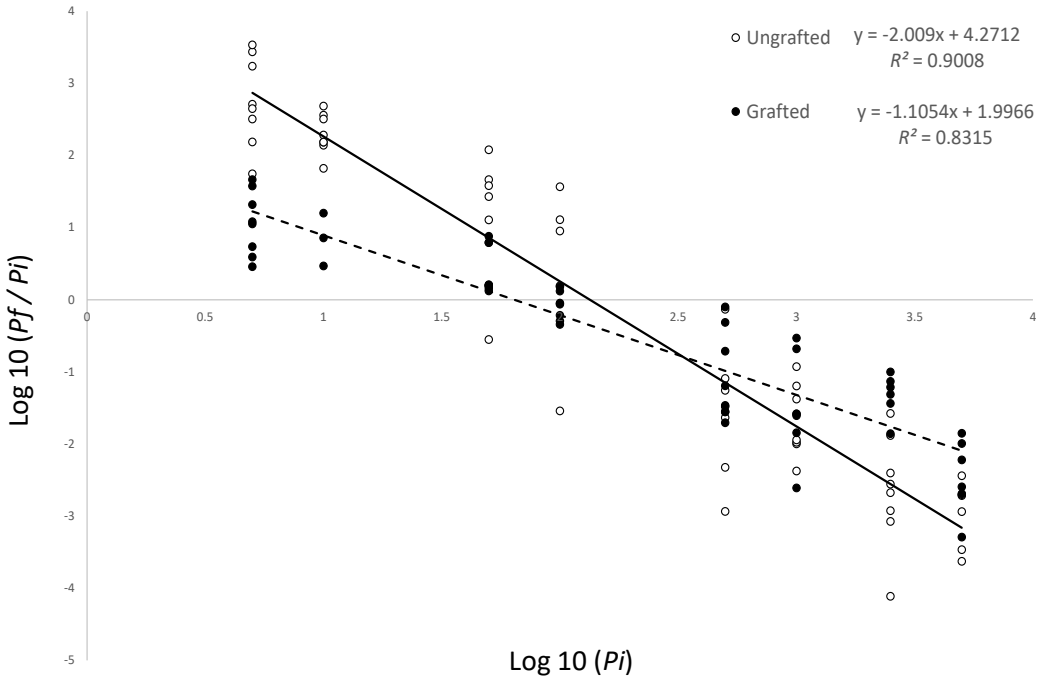


Figure 2. Relationship between reproduction rate (*Pf*/*Pi*) and the population densities at transplanting (*Pi*) of *Meloidogyne* species infecting the tomato cv. Durinta ungrafted or grafted onto *Solanum torvum* 'Brutus', cultivated from March to November in 30 L microplots. *Pf*, final nematode population density.

The relationship between *Pi* and the relative ungrafted tomato yield fitted to the Seinhorst damage function model irrespective of the *Meloidogyne* species ($y = 0.23 + (1–0.23) \times 0.95^{(Pi/1.6–}$

[1)]; *R2* = 0.949; Figure 3), but it did not for grafted tomato. The tolerance limit (*T*) was 1.6 eggs+ J2s ± 0.28) per 100 cm^3 of soil and the relative minimum yield (*m*) was 0.23 ± 0.045).

The values of the tomato fruit quality parameters are presented in Table 2. The *Pi* affected the quality of the tomato fruits produced by the ungrafted plants ($p < 0.05$). Indeed, the *Pi* of *M*. *incognita* was positively related to percentage of dry matter and Na content ($p = 0.0052$, $r = 0.8680$ and $p = 0.0153$, $r = 0.807$, respectively) and negatively to the content of Fe ($p = 0.0150$, $r = 0.809$). For *M*. *javanica*, the *Pi* was positively related to the content of dry matter, Na and ascorbic acid ($p = 0.0031$, $r = 0.857$; $p = 0.0002$, $r = 0.934$ and $p = 0.0041$, $r = 0.845$, respectively). Concerning the quality of the tomato fruits produced by the grafted plants, *Pi* of *M*. *incognita* was negatively related to the K content ($p = 0.025$, $r = -0.772$), while *Pi* of *M*. *javanica* was negatively related to the Fe content ($p = 0.0125$, $r = -0.784$) and positively related to lycopene content ($p = 0.049$, $r = 0.669$).

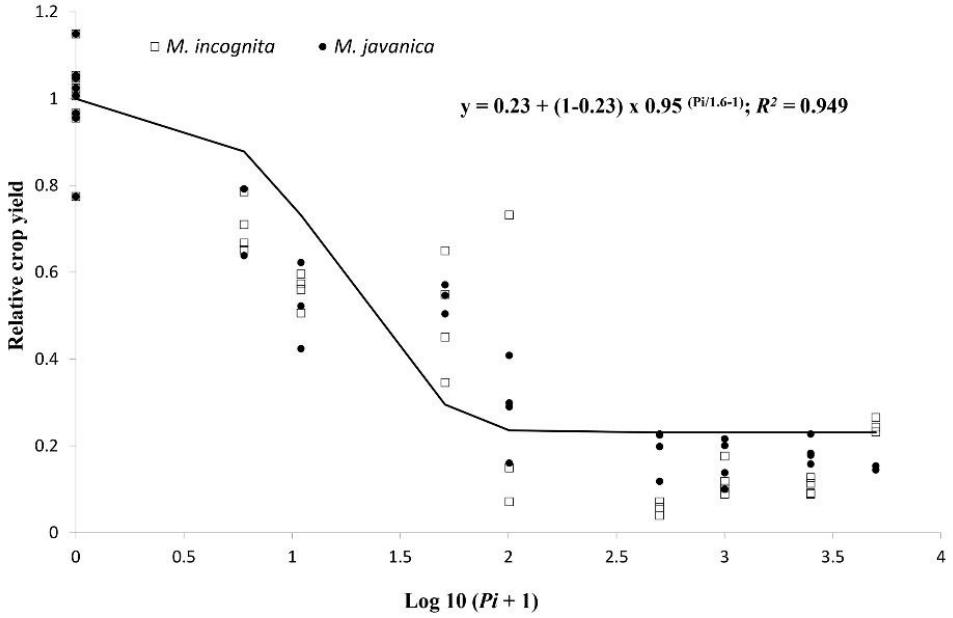


Figure 3. Seinhorst damage function model $y = m + (1 - m) \times 0.95^{(Pi/T-1)}$, where *y* is the relative crop yield, *m* is the minimum relative yield, *Pi* is the nematode population density at transplanting and *T* is the tolerance limit for ungrafted tomato cv. Durinta cultivated from March to November in 30 L microplots infested with *Meloidogyne incognita* or *M*. *javanica*.

No fitness cost for any of the *Meloidogyne* species was detected after developing on only one crop of tomato grafted onto *S*. *torvum* 'Brutus'. Nematode infection, reproduction and fertility did not differ ($p < 0.05$) between subpopulations of *M*. *incognita* or *M*. *javanica* developed on ungrafted or grafted tomato (Table 3).

Table 2. Values of the tomato fruit quality parameters (minimum and maximum) of the tomato cv. Durinta ungrafted and grafted onto the *Solanum torvum* 'Brutus' rootstock cultivated from March to November in 30 L microplots infested with nine *Pi* levels of *Meloidogyne incognita* (Mi) or *M. javanica* (Mj), from 0 to 5000 eggs + J2s/100 cm^3 of soil.

	Ungrafted		**Grafted**	
Parameter	***Mi***	***Mj***	***Mi***	***Mj***
SSC (°Brix)	4.6-5.8	4.2-4.8	4.2-4.7	3.9-4.7
pH	3.62-3.97	3.79-4.07	3.96-4.10	3.88-4.14
Dry matter (%)	6.39-8.33	6.55-7.89	5.65-6.86	6.14-6.61
Lycopene (mg/kg dw)	na	na	231.9-601.8	291.7-528.1
Ascorbic acid (g/kg dw)	0.59-2.17	0.54-1.45	0.4-1.73	0.52-2.73
mm (%)	6.4-8.5	6.7-7.5	6.0-8.5	7.0-7.8
Fe (mg/kg dw)	78.4-116.6	71.4-110.5	85.3-127.0	76.3-136.1
Ca (g/kg dw)	0.9-1.7	1.1-1.8	0.8-1.1	0.9-1.1
Mg (g/kg dw)	1.3-1.5	1.3-1.6	1.1-1.3	1.1-1.3
K (g/kg dw)	17.5-22.9	19.6-24.0	17.5-24.9	19.9-22.7
Na (g/kg dw)	3.2-7.1	3.2-7.0	3.2-3.7	3.4-4.4

Abbreviations: dw, dry weight; na, not assessed.

Table 3 Infection, reproduction and fertility of *Meloidogyne incognita* or *M. javanica* subpopulations developed on tomato ungrafted (T) or grafted onto *Solanum torvum* 'Brutus' (ST) on the susceptible eggplant cv. Cristal cultivated in 200 cm3 of soil during 45 days after inoculation with 200 J2s.

Subpopulation	**Infection (Egg mass per plant)**		**Reproduction (Eggs+J2 (x10^4) per plant)**		**Fertility (Eggs per egg mass)**	
	M.incognita	***M.javanica***	***M.incognita***	***M.javanica***	***M.incognita***	***M.javanica***
T	35 ± 5	47 ± 9	1.8 ± 0.6	2.9 ± 0.7	484 ± 125	588 ± 66
ST	45 ± 8	46 ± 9	2.2 ± 0.4	2.2 ± 0.4	500 ± 65	478 ± 33

Data are means ± standard errors of 10 replicates. Values in the same column did not differ ($p \geq 0.05$) between nematode subpopulations of the same species. Values in the same row did not differ ($p \geq 0.05$) between *Meloidogyne* species per nematode subpopulation according to the Student's *t* test or the non parametricl Wilcoxon test.

EFFECT OF TOMATO GRAFTED ONTO S. TORVUM 'BRUTUS' ON NEMATODE POPULATION DYNAMICS AND CROP YIELD IN PLASTIC GREENHOUSES

In the plastic greenhouse Agròpolis, the *Pi* ranged from 0 to 1830 J2s per 100 cm^3 of soil. The plots were grouped into four levels of nematode density (Table 4) that did not differ ($p \geq 0.05$) between plots in which ungrafted or grafted tomato were cultivated. Unfortunately, the scions of most of the grafted plants developed roots, so only the roots of *S. torvum* were used to assess the galling index and the nematode reproduction. Disease severity was lower ($p < 0.05$) in grafted than ungrafted tomato plants. Nematodes reproduced 56 and 154 times more ($p < 0.05$) on the ungrafted tomatoes cv. Durinta and cv. Candido than on the grafted ones, respectively. The tomato yield did not differ between grafted and ungrafted tomatoes at any *Pi* level.

Table 4. Galling index (GI) and nematode reproduction on ungrafted tomato cv. Durinta (T_D) and cv. Candido (T_C), and grafted onto *Solanum torvum* cv. Brutus (GT) cultivated in an experimental plastic greenhouse from March to November in soil infested with increasing densities of *Meloidogyne incognita* Agròpolis (*Pi*).

Pi	**Number of replicates**			**GI**			**Number of Eggs + J2 (x10^3) per plant**		
(J2 / 100 cm^3)	**T_D**	**T_C**	**GT**	**T_D**	**T_C**	**GT**	**T_D**	**T_C**	**GT**
<1	8	8	23	6 ± 0.6*	6 ± 0.6 *	1 ± 0.3	680 ± 364 *	804 ± 284 *	11 ± 4
1 - 60	11	10	40	7 ± 0.8*	8 ± 0.0 *	1 ± 0.2	899 ± 301 *	2467 ± 609 *	16 ± 4 *
61 - 120	5	10	9	8 ± 0.2*	8 ± 0.1 *	1 ± 0.6	554 ± 157 *	2156 ± 471 *	53 ± 30
>120	8	6	10	6 ± 0.6*	8 ± 0.2 *	2 ± 0.8	243 ± 92 *	550 ± 280 *	20 ± 4

Data are means ± standard errors of the corresponding replicates. Values in the same row followed by *indicate differences ($p < 0.05$) of paired comparisons between each ungrafted tomato cultivar and the grafted one according to the nonparametric Wilcoxon test. GI: galling index on a scale from 0 to 10, where 0 = complete and healthy root system and 10 = plants and roots dead (Zeck, 1971).

The *M. incognita* offspring from grafted tomato (ST) produced fewer ($p < 0.05$) egg masses on susceptible eggplant than that from ungrafted (T) but did not differ in either reproduction or fertility (Table 5). *S. torvum* 'Brutus' performed as resistant to both these *M. incognita* subpopulations (RI T = 5.5%; RI ST = 4.8%).

In the commercial plastic greenhouse Ametller the *Pi* ranged from 62 to 193 J2s per 100 cm3 of soil and did not differ ($p \geq 0.05$) between plots cultivated with tomato cv. Monterosa grafted onto the tomato rootstock Silex and those cultivated with tomato grafted onto *S. torvum*

'Brutus'. The galling index and the nematode reproduction in the tomato grafted onto Silex were 11.6-and 6.97-fold higher than in the tomato grafted onto *S. torvum* (Table 6).

Table 5. Root-knot nematode infection, reproduction and fertility on the resistant *Solanum torvum* and the susceptible eggplant cv. Cristal cultivated in 200 cm^3 pots after 45 days from inoculation with 200 J2s of *Meloidogyne incognita* developed on tomato ungrafted (T) or grafted onto *S. torvum* 'Brutus' (ST).

Sub-population	Infection (Egg mass per plant)		Reproduction (Eggs per plant)		Fertility (Eggs per egg mass)	
	Eggplant	*S. torvum*	Eggplant	*S. torvum*	Eggplant	*S. torvum*
T	58 ± 3.7 * †	0.4 ± 0.1	615 ± 106 †	34 ± 10	10 ± 1	13 ± 4
ST	33 ± 2.4 †	0.3 ± 0.1	565 ± 111 †	27 ± 10	14 ± 2	11 ± 8

Data are means ± standard errors of 14 replicates. Values in the same column followed by * indicate differences ($p < 0.05$) between subpopulations and the values in the same row followed by † indicate differences ($p < 0.05$) between susceptible or resistant germplasm according to Student's *t* test or the nonparametric Wilcoxon rank test.

Table 6. Nematode population density before transplanting (*Pi*) in soil naturally infested by *Meloidogyne incognita* and *M. javanica*, galling index (GI) and the number of eggs per plant at the end of the crop of the tomato cv. Monterosa grafted onto the tomato rootstock Silex (GT_S) or *Solanum torvum* 'Brutus' (GT_{ST}), cultivated in a commercial plastic greenhouse from July to December.

Rootstock	*Pi* (J2 per 100 cm^3)	GI	Number of eggs (x 10^3) per plant
GT_S	150 ± 25	5.8 ± 0.5 *	48.1 ± 9.8 *
GT_{ST}	106 ±22	0.5 ± 0.1	6.9 ± 1.8

Data are means ± standard errors of three replicates in the case of *Pi* and 12 in the rest of the data. Values in the same column followed by *indicate differences ($p < 0.05$) between grafted tomatoes onto Silex and Brutus rootstocks according to the nonparametric Wilcoxon test. GI: galling index on a scale from 0 to 10, where 0 = complete and healthy root system and 10 = plants and roots dead (Zeck, 1971).

The infective and reproductive capacity of the *Meloidogyne* offspring from the tomato rootstocks Silex (GT_S) or Brutus (GT_{ST}) was higher ($p < 0.05$) on the susceptible tomato cv. Durinta than on the resistant cv. Monika (Table 7). The tomato cv. Monika performed as resistant to both nematode subpopulations because the RI values were 2.7% and 7.4%.

Table 7. Infection, reproduction and fertility on the resistant tomato cv. Monika (R) and the susceptible cv. Durinta (S) cultivated in 200 cm^3 pots 45 days after inoculation with 200 J2s of *Meloidogyne* subpopulations developed on tomato grafted onto Silex (GT_S) or *Solanum torvum* 'Brutus' (GT_{ST}).

Sub-population	Infection (Egg mass per plant)		Reproduction (Eggs ($x10^2$) per plant)		Fertility (Eggs per egg mass)	
	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
GT_S	81 ± 8* †	4 ± 1	148 ± 22 * †	4 ± 1	183 ± 22	114 ± 28
GT_{ST}	2 ± 0.7	0.4 ± 0.1	4 ± 1	0.3 ± 0.2	180 ± 28	82 ± 20

Data are means ± standard errors of 14 replicates. Values in the same column followed by *indicate differences ($p < 0.05$) between nematode subpopulations. Values in the same row followed by † indicate differences ($p < 0.05$) between susceptible or resistant germplasm according to Student's *t* test or the nonparametric Wilcoxon test.

DURABILITY OF THE RESISTANCE AND FITNESS COST

All the nematode subpopulations developed on *S. torvum* rootstock showed less ($p < 0.05$) infective and reproductive capacity on the eggplant cv. Cristal than the nematode subpopulation developed on the ungrafted eggplant, but the female fertility was not affected irrespective of the number of times that they developed on *S. torvum* (Table 8). Afterward, the nematode subpopulations were inoculated onto *S. torvum* 'Brutus' to produce enough inoculum to determine the effect of increasing the number of *S. torvum* crops on the associated putative selection f or virulence and the fitness cost. At the end of the experiment, all the nematode subpopulations were assessed on susceptible eggplant, *S. torvum*, susceptible tomato cv. Durinta (ST) and resistant tomato cv. Monika (RT) (Table 9). In eggplant, smaller ($p < 0.05$) numbers of egg masses and eggs + J 2s p er p lant were observed in subpopulations developed on *S. torvum* (any number of times) than on eggplant; however, the female fertility was only reduced ($p < 0.05$) after being developed three or four times on *S. torvum*. In *S. torvum*, the number of egg masses, eggs + J2s per plant and female fertility did not differ ($p \geq 0.05$), irrespective of the number of times that the nematode subpopulation developed on it. In tomato, all the assessed nematode subpopulations showed less ($p < 0.05$) infection and reproduction on the resistant than on the susceptible tomato cultivar, but the female fertility did not differ ($p \geq 0.05$). The nematode infection and female fertility on susceptible or resistant tomato cultivars did not differ ($p \geq 0.05$) between nematode subpopulations. However, reproduction ($p < 0.05$) on the susceptible tomato was higher after growing three *(Subpop*2-ST) and four (*Subpop*3-ST) times on *S. torvum*. The reproduction index (RI) of the nematode

subpopulations in *S. torvum* ranged from 0.04% to 0.2%, indicating that *S. torvum* was highly resistant, whilst the resistant tomato was resistant (RI from 3.9% to 4.7%; Table 9).

Table 8. Infection, reproduction and fertility of *Meloidogyne* subpopulations, exposed to *Solanum torvum* one (*Subpop*1), two (*Subpop*2), three (*Subpop*3) times or not exposed (*Subpop*0), on the susceptible eggplant cv. Cristal cultivated in 200 cm^3 pots 55 days after nematode inoculation with 200 J2s.

Subpopulation	Infection (Egg mass per plant)	Reproduction (Eggs ($x10^2$) per plant)	Fertility (Eggs per egg mass)
*Subpop*0	180 ± 11	779 ± 60	433 ± 10
*Subpop*1	105 ± 21*	382 ± 154 *	321 ± 4
Subpop2	56 ± 26 *	195 ± 119 *	275 ± 5
*Subpop*3	91 ± 7*	550 ± 75*	592 ± 59

Data are means ± standard errors of 10 replicates. Values in the same column followed by *indicate differences ($p < 0.05$) between each nematode subpopulation and *Subpop*0 according to Student's *t* test or the nonparametricl Wilcoxon test.

HISTOPATHOLOGY

The roots of eggplant cv. Cristal exhibited less autofluorescence than those of *S. torvum* (Figure 4). Some J2s inside *S. torvum* roots were observed at 15 DANI (Figure 4A), while J2–J3 sedentary nematode stages were observed inside eggplant roots along with giant cells containing several nuclei (Figure 4B), irrespective of the plant germplasm in which the nematode subpopulation developed. At 70 DANI, the roots of *S. torvum* were less well clarified than those of eggplant. However, young females without egg masses of the *Subpop*1 subpopulation were observed (Figure 5A), whilst in eggplant, there were adult females and egg masses with plenty of eggs, most of them without hatching and some emerged J2 (Figure 5C). In *S. torvum* inoculated with nematode subpopulation *Subpop*0, adult females with a thin gelatinous matrix and some eggs were observed (Figure 5B). In contrast, females with egg masses mostly containing eggshells with few empty eggs and some emerged J2s were observed in eggplant (Figure 5D).

Discussion

This study investigated the potential use of *S. torvum* 'Brutus' as a tomato rootstock against RKNs, to be used in rotation with resistant tomato cultivars or tomato rootstocks to preserve the effectiveness of the *Mi1.2* resistance gene. Previous studies conducted to determine the compatibility of *S. torvum* as a rootstock of tomato have shown that tomato growth was delayed

Table 9. Infection, reproduction and fertility of *Meloidogyne incognita* subpopulations, developed one (*Subpop*0-ST), two (*Subpop*2-ST), three (*Subpop*2-ST) or four (*Subpop*3-ST) times on *Solanum torvum* in plastic greenhouse and/or climatic chamber, on the susceptible eggplant cv. Cristal, *S. torvum* 'Brutus', the susceptible tomato cv. Durinta (ST) and the resistant tomato cv. Monika (RT) cultivated in 200 cm^3 pots 55 days after inoculation with 200 J2s, and reproduction index (RI).

	Infection (Egg mass per plant)		**Reproduction (Eggs (x10^2) per plant)**		**Fertility (Eggs per egg mass)**		
RKN subpopulation	**Eggplant**	**S. torvum**	**Eggplant**	**S. torvum**	**Eggplant**	**S. torvum**	**RI (%)**
*Subpop*0-ST	148 ± 4 A	na	656 ± 97 A	na	438 ± 189 AB	na	na
*Subpop*1-ST	48 ± 5 C*	0.4 ± 0.2A	365 ± 41 AB*	0.1 ± 0.06 A	752 ± 34 A	10 ± 6 A	0.04
*Subpop*2-ST	57 ± 4 C*	0.5 ± 0.1A	92 ± 15 C*	0.2 ± 0.07 A	150 ± 19 C*	22 ± 7 A	0.2
*Subpop*3-ST	79 ± 6 B*	0.4 ± 0.1A	240 ± 33B*	0.2 ± 0.06 A	310 ± 38 B*	13 ± 6 A	0.06
	ST	**RT**	**ST**	**RT**	**ST**	**RT**	
*Subpop*0-ST	na	na	na	na	na	na	na
*Subpop*0-ST	74 ± 10 A	na	386 ± 59 B	na	537 ± 60 A	na	na
*Subpop*2-ST	106 ± 8 A*	6 ± 1 A	782 ± 70 A*	30 ± 8 A	751 ± 55 A	607± 100 A	3.9
*Subpop*3-ST	110 ± 14 A*	7 ± 1 A	704 ± 108A*	33 ± 10 A	633 ± 49 A	440 ± 42 A	4.7

Data are means ± standard errors of 10 replicates. Values in the same column followed by different letters indicate differences ($p < 0.05$) between subpopulations according to the nonparametric test of Kruskal–Walis. Values in the same row followed by *indicate differences ($p < 0.05$) between susceptible or resistant germplasm according to Student's *t* test or the nonparametric Wilcoxon test. na = not assessed due to insufficient nematode inoculum.

RI = (number of eggs per plant produced on resistant plants/number of eggs per plant produced on susceptible plants) × 100. The response was classified according to RI as highly resistant (RI <1%), resistant (1% ≤ RI < 10%), moderately resistant (10% ≤ RI < 25%), slightly resistant (25% ≤ RI < 50%), or susceptible (RI ≥50%).

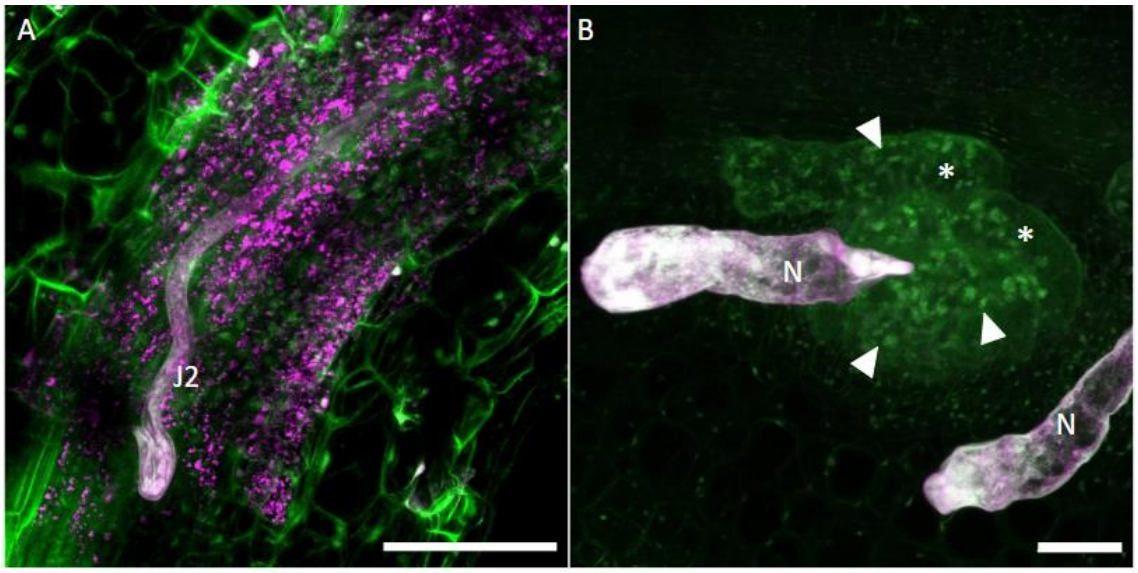


Figure 4. Laser-scanning confocal microscope images of *Meloidogyne incognita* 15 days after inoculation in the resistant rootstock *Solanum torvum* 'Brutus' (A), and the susceptible eggplant cv. Cristal (B). Second-stage juvenile (J2), nematode (N), cell nuclei (white arrowheads) and giant cells (asterisks) are indicated. Scale bar: 100 µm.

and yielded less compared to tomato grafted onto conventional tomato rootstocks (De Miguel et al., 2011). However, its use could be valuable for the management of *Mi1.2* against virulent RKN populations if tomato plants grafted onto *S*. *torvum* can reduce crop yield losses caused by these nematodes.

The results of our work have shown that the resistance of *S*. *torvum* 'Brutus' against *M*. *incognita* or *M*. *javanica* was not affected at increasing *Pi*, either in microplot or in field conditions. In fact, in microplot conditions, the maximum multiplication rate and the maximum density of RKNs on grafted tomato were 1.27% and 2.93% those achieved on ungrafted tomato, respectively. A similar performance was reported by García-Mendívil et al. (2019) who compared the same parameters between *S*. *torvum* and eggplant cv. Cristal. In addition, we found that grafted tomato did not experience significant crop yield losses whilst ungrafted did. The maximum crop yield losses of ungrafted tomato cv. Durinta were estimated at 77% according to the Seinhorst damage function model. Previous studies reported maximum crop yield losses of this tomato cultivar between 56% and 73% when cultivated in spring–summer in infested soil (Expósito et al., 2020; Giné and Sorribas, 2017). Our grafted tomato plants yielded between 2.9 and 7.5 times more than the ungrafted ones at *Pi* ≥ 100 eggs + J2s per 100 cm3 of soil in microplot conditions, although no differences were observed when plants were cultivated in a plastic greenhouse at *Pi* densities higher than the tolerance limit. However, in the plastic greenhouse experiments, a high number of the scions franked, thus changing the performance of the aboveground part of grafted plants.

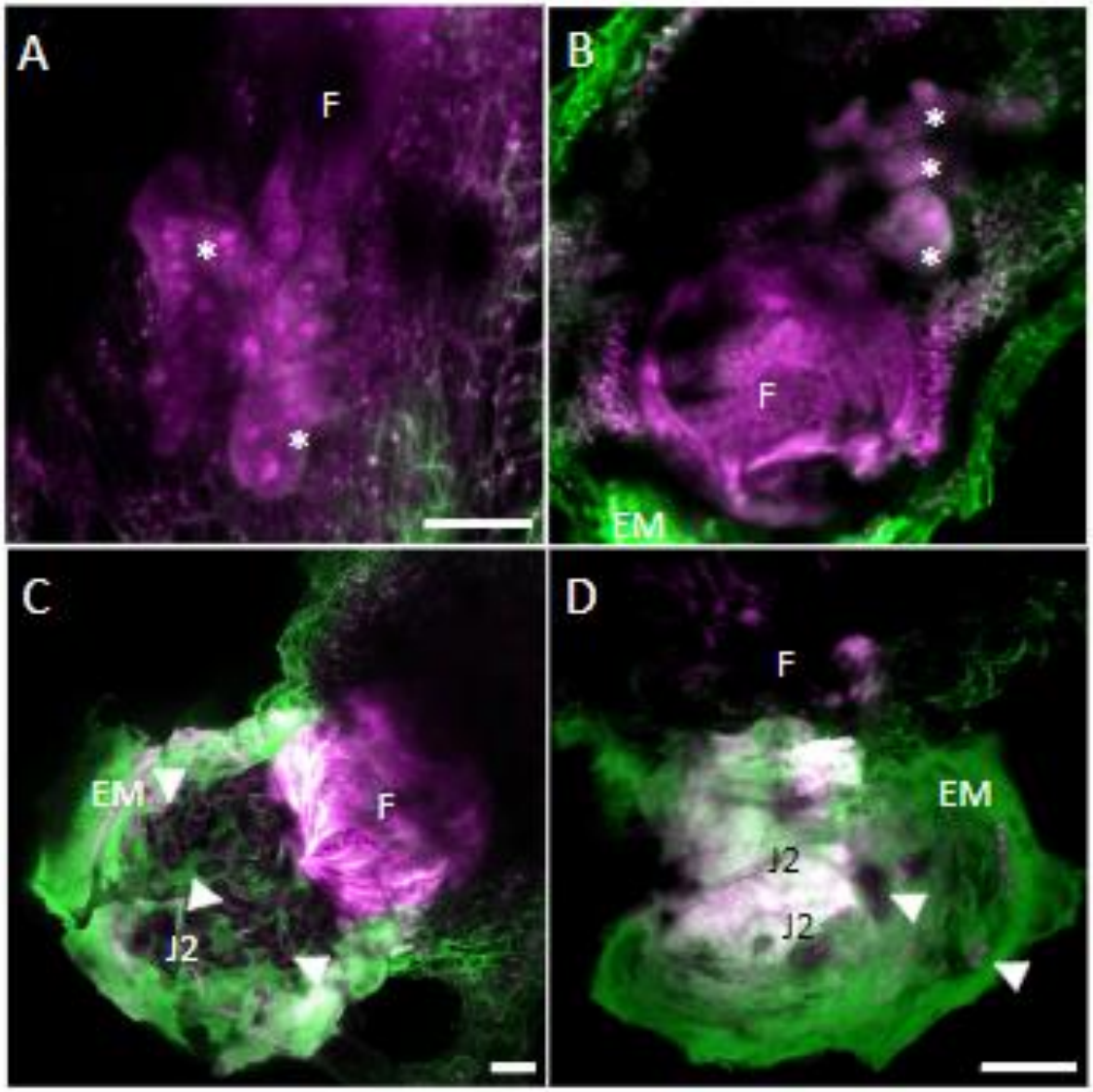


Figure 5. Laser-scanning confocal microscope images of roots of *Solanum torvum* 'Brutus' (A) or eggplant cv. Cristal (C) 70 days after inoculation of the *Meloidogyne incognita* subpopulation coming from *S*. *torvum* (Subpop1) and of the *M*. *incognita* subpopulation coming from eggplant cv. Cristal (*Subpop*0) on *S*. *torvum* (B) or eggplant cv. Cristal (D). Second-stage juvenile (J2), female (F), egg mass (EM), giant cells (asterisk), and some eggs and empty eggs (white arrowhead) are indicated. Scale bar: 100 µm.

Tomato fruit quality was not affected by grafting, according to the range of values obtained by chemical analyses, but it was dependent on the *Pi*. Similar results were obtained by Expósito et al. (2020), who compared tomato fruit quality produced in tomato cv. Durinta ungrafted or grafted onto the tomato rootstock cv. Aligator. Grafting rarely causes fruit quality changes (Grieneisen et al., 2018). In our study, tomato fruits produced by ungrafted tomato consistently had increasing percentage of dry matter and Na content with *Pi* but these parameters were not affected in grafted plants. It is known that both abiotic and biotic stresses can influence fruit quality parameters according to their intensity and duration at a given phenological stage of the plant (Nicoletto et al., 2019). In our study, fruits produced by different tomato sets were mixed and analysed. Thus, the putative changes in fruit quality that could occur in the different tomato sets were damped.

We investigated the durability of *S*. *torvum* resistance, showing that four continuous crops did not select for nematode virulence and the plants remained as highly resistant. Furthermore, the nematode subpopulations developed from one to four consecutive *S*. *torvum* crops showed less capacity to infect and to reproduce on the susceptible eggplant cv. Cristal after the second and the third *S*. *torvum* crop, respectively. However, the female fertility was not consistently affected from a given *S*. *torvum* crop. García-Mendívil and Sorribas (2019) found that the female fertility on the susceptible eggplant cv. Cristal was affected after two consecutive crops of eggplant grafted onto *S*. *torvum* that lasted 135 and 218 days respectively. In our experiments, each of subpopulations *Subpop*0-ST to *Subpop*3-ST was developed on *S*. *torvum* crops in pots for only 55 days; this ensured that there was only one nematode generation, in contrast to the experiment by García-Mendívil and Sorribas (2019), which enabled more than one generation to develop. Interestingly, we observed an increase in nematode reproduction in the susceptible tomato after two consecutive *S*. *torvum* crops, although not in the resistant tomato. A decrease in the fitness to parasitize a given susceptible plant germplasm of a given plant species (in this case eggplant) could change its ability to parasitize another one belonging to other plant species (e.g., tomato) to maintain its resilience, due to its adaptative potential (Castagnone-Sereno, 2006).

The histopathological study showed a delay in *M*. *incognita* infection and development on *S*. *torvum* in comparison to that on eggplant cv. Cristal, irrespective of the number of times that the nematode subpopulation developed in *S*. *torvum*. The resistance of *S*. *torvum* to RKNs has been attributed to sesquiterpenoids (including nematotoxic and nematicidal compounds) and chitinases (Bagnaresi et al., 2013), the expression of genes encoding class III peroxidases, fatty acid desaturases, defence hormone signalling and the biosynthesis of lignin, which is accumulated at the root tip (Sato et al., 2021), as well as the expression of nucleotide-binding and leucine-rich repeat genes and ABC transporters (Zhang et al., 2023). All these defence mechanisms can reduce the activity and viability of the infective J2s and affect nematode infection and development. Although female fertility was not severely affected, we found that nematode offspring produced in *S*. *torvum* did not show any increasing ability to parasitize this plant species. Thus, *S*. *torvum* showed higher and more stable resistance to *M*. *incognita* than other resistance genes against RKNs in fruiting solanaceous crops, such as tomato and pepper. In tomato, the level of resistance of tomato carrying the *Mi1.2* gene or of tomato grafted onto resistant tomato rootstocks progressively decreased with increasing number of successive crops cultivated in a plastic greenhouse, until a virulent nematode population was selected (Expósito et al., 2020; Giné and Sorribas, 2017; Verdejo-Lucas et al., 2009).

In summary, the results obtained in this and other studies indicate that *S*. *torvum* is a good candidate for inclusion as an eggplant rootstock in crop rotation sequences due to its resistance to the tropical RKN species, *M*. *enterolobii* and *M*. *luci*, and other soilborne plant pathogens. However, *S*. *torvum* is not recommended a s a tomato rootstock due to the scion–rootstock compatibility problems we observed. Indeed, the tomato scion franked frequently, mainly due to the narrower stem of *S*. *torvum* compared to that of tomato. In addition, there is an increased incidence of blossom-end rot, as has previously been reported (Lee and Oda, 2002) and a delay in tomato growth and yield. There have been attempts to improve the scion– rootstock compatibility, such as double grafting using eggplant as an intermediate rootstock between *S*. *torvum* and tomato, this did not improve tomato growth and yield quantity and precocity compared to tomato grafted onto a tomato rootstock (De Miguel et al., 2011).

Acknowledgements

The UPC authors acknowledge funding from the R + D + I project AGL2017-89785-R, financed by Ministerio de Ciencia e Innovación and by the European Regional Development Fund (FEDER) and the European Social Fund (FSE; PRE2018-084265, A.M.F.). The authors are also grateful to Laura Mateo from Semillas Fitó for providing seeds of *Solanum torvum* 'Brutus' and would like to extend their gratitude to Casa Amatllé for allowing us to conduct part of our experiments on their farms, as well as to Youssef Iboudaaten, for his technical assistance in the field. Furthermore, the authors also thank Dr Helio Adán García-Mendívil, Roger Nieto, Sheila Alcala, Maria Julià and Miquel Massip for their laboratory and field technical assistance. ICFO authors acknowledge funding from Fundació CELLEX; Ministerio de Economía y Competitividad— Severo Ochoa program for Centres of Excellence in R&D (CEX2019-000910-S); Research Centres of Catalonia (CERCA) program and Laserlab-Europe (871124); Ministerio de Ciencia e Innovación (MCIN/AEI/10.13039/501100011033); and Fondo Social Europeo (PRE2020-095721, MC).

Conflict of interest statement

The authors have no conflict of interest to declare.

Data availability statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

References

Abad, P., Castagnone-Sereno, P., Rosso, M. N., Engler, J. D. A., and Favery, B. (2009). "Invasion, feeding and development," in Root-knot Nematodes, Eds. R. N. Perry, M. Moens, and J. L. Starr (Wallingford: CABI international), 163–181.

AOAC. (2019). Official Methods of Analysis of AOAC INTERNATIONAL, Three-volume set, 21st Edition. AOAC INTERNATIONAL (Washington DC: AOAC international)

Bagnaresi, P., Sala, T., Irdani, T., Scotto, C., Lamontanara, A., Beretta, M., et al. (2013). *Solanum torvum* responses to the root-knot nematode *Meloidogyne incognita*. BMC genomics, 14, 1-21.

Castagnone-Sereno, P. (2012). *Meloidogyne enterolobii* (= *M. mayaguensis*): profile of an emerging, highly pathogenic, root-knot nematode species. Nematology, 4(2), 133–138.

Castagnone-Sereno, P. (2006). Genetic variability and adaptive evolution in parthenogenetic root-knot nematodes. Heredity, 96, 282–289.

De Miguel, A., Marsal, J. I., Goto, R., San Bautista, A., López-Galarza, S., Pascual, B., et al., (2011). Improving the affinity of tomato grafted on *Solanum torvum* using an intermediate rootstock. Acta Horticulturae, 898, 291-295.

Expósito, A., Pujolà., M., Achaerandio, I., Gińe, A., Escudero, N., Fullana, A. M., et al. (2020). Tomato and melon *Meloidogyne* resistant rootstocks improve crop yield but melon fruit quality is influenced by the cropping season. Frontiers in Plant Science*,* 11, 560024.

FAOstat. (2023). Food and Agriculture Organization of the United Nations. Agricultural production statistics. Available online at: https://openknowledge.fao.org/server/api/core/bitstreams/dc1b16d6-44af-4d50-9544-10fcb8a3779f/content [Accessed 23 July 2024].

Fullana, A. M., Expósito, A., Escudero, N., Cunquero, M., Loza-Alvarez, P., Giné, A., et al. (2023). Crop rotation with *Meloidogyne*-resistant germplasm is useful to manage and revert the (a)virulent populations of *Mi1.2* gene and reduce yield losses. Frontiers in Plant Science, 14,1133095.

García-Mendívil, H. A., Escudero, N., and Sorribas, F. J. (2019). Host suitability of *Solanum torvum* cultivars to *Meloidogyne incognita* and *M. javanica* and population dynamics. Plant pathology, 68(6), 1215-1224.

García-Mendívil, H. A. and Sorribas, F. J. (2019). Fitness cost but no selection for virulence in *Meloidogyne incognita* after two consecutive crops of eggplant grafted onto *Solanum torvum*. Plant Pathology, 68, 1602-1606.

Giné, A. and Sorribas, F.J. (2017). Quantitative approach for the early detection of selection for virulence of *Meloidogyne incognita* on resistant tomato in plastic greenhouses. Plant Pathology 2017, 66, 1338–1344.

Greco, N. and Di Vito, M. (2009). "Population dynamics and damage level" in Root-knot Nematodes, Eds. R. N. Perry, M. Moens, and J. L. Starr (Wallingford: CABI international), 246–274.

Grieneisen, M. L., Aegerter, B. J., Stoddard, C. S. and Zhang, M. (2018). Yield and fruit quality of grafted tomatoes, and their potential for soil fumigant use reduction. A meta-analysis. Agronomy for Sustainable Developmen*,* 38,29.

Hadisoeganda, W. W., and Sasser, J. N. (1982). Resistance of tomato, bean, southern pea, and garden pea cultivars to root-knot nematodes based on host suitability. Plant Dissease. 66 (2), 145–150.

Hussey, R. S., and Barker, K. R. (1973). Comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. Plant Dissease Report, 57, 1025–1028.

Jones, J. T., Haegeman, A. J., Danchin, E. G., Gaur, H. S., Helder, J. K., Helder G. J., et al. (2013). Top 10 plant parasitic nematodes in molecular plant pathology. Molecular Plant Pathology*,* 14, 946–961.

Lee, J. M., and Oda, M. (2002). Grafting of herbaceous vegetable and ornamental crops. HORTICULTURAL REVIEWS-WESTPORT THEN NEW YORK, 28, 61-124.

Nicoletto, C., Maucieri, C., Zanin, G., Vianello, F., and Sambo, P. (2019). "Vegetables Quality and Biotic Stress" in Plant Health Under Biotic Stress. Eds. Ansari, R. and Mahmood (Singapore: Springer), 107–128.

Öçal, S., Özalp, T. and Devran, Z. (2018). Reaction of wild eggplant *Solanum torvum* to different species of root-knot nematodes from Turkey. Journal of Plant Diseases and Protection*,* 125, 577–580.

Omwega, C. O., Thomason, I. J., and Roberts, P. A. (1988). A nondestructive technique for screening bean germ plasm for resistance to *Meloidogyne incognita.* Plant Disease*,* 72 (11), 970–972.

Pinheiro, J. B., da Silva, G. O., Biscaia, D., Magalhães, C. D. C., de Souza, L. R., Pereira, W. D. S., et al. (2022). Resistance sources to *Meloidogyne enterolobii* in wild *Solanum* species and interspecific hybrids. Horticultura Brasileira, 40, 275-280.

Ranil, R. H. G., Niran, H. L., Plazas, M., Fonseka, R. M., Fonseka, H. H., Vilanova, S., et al. (2015). Improving seed germination of the eggplant rootstock *Solanum torvum* by testing multiple factors using an orthogonal array design. Scientia Horticulturae, 193, 174–181.

Roberts, P. A., and Thomason, I. J. (1989). A review of variability in four *Meloidogyne* spp. measured by reproduction on several hosts including *Lycopersicon*. Agricultural Zoology Reviews*,* 3, 225-252.

Santos, D., Martins da Silva, P., Abrantes, I. and Maleita, C. (2020). Tomato Mi1.2 gene confers resistance to *Meloidogyne luci* and *M. ethiopica*. European Journal of Plant Pathology, 156(2), 571-580.

Sato, K., Uehara, T., Holbein, J., Sasaki-Sekimoto, Y., Gan, P., Bino, T., et al. (2021). Transcriptomic analysis of resistant and susceptible responses in a new model root-knot nematode infection system using *Solanum torvum* and *Meloidogyne arenaria*. Frontiers in Plant Science, 12, 680151.

Seinhorst, J. W. (1970). Dynamics of populations of plant parasitic nematodes. Annual review of Phytopathology, 8(1), 131-156.

Seinhorst, J. W. (1998). The common relation between population density and plant weight in pot and microplot experiments with various nematode plant combinations. Fundamental and Applied Nematology*,* 21, 459–468.

Sorribas, F. J., Ornat, C., Verdejo-Lucas, S., Galeano, M., and Valero, J. (2005). Effectiveness and profitability of the Mi-resistant tomatoes to control root-knot nematodes. *European Journal of Plant Pathology,* 111, 29–38.

Uehara, T., Tateishi, Y., Kadota, Y. and Iwahori, H. (2017). Differences in parasitism of *Meloidogyne incognita* and two genotypes of *M. arenaria* on *Solanum torvum* in Japan. Journal of Phytopathology, 165, 575–579.

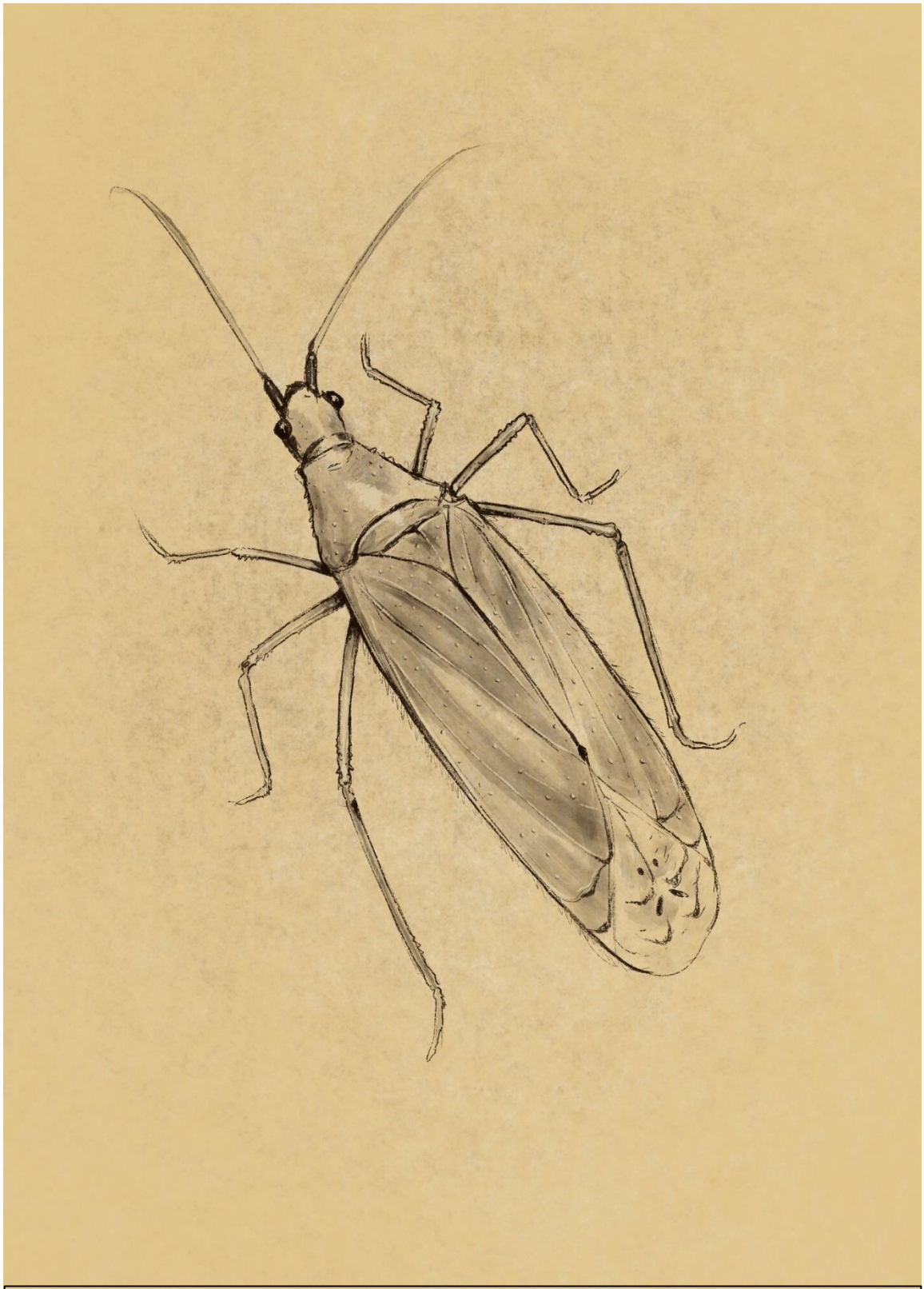
Vallverdú-Queralt, A., Martínez-Huélamo, M., Arranz-Martinez, S., Miralles, E., and Lamuela-Raventós, R. M. (2012). Differences in the carotenoid content of kétchups and gazpachos through HPLC/ESI(Li+)-MS/MS correlated with their antioxidant capacity. Journal of the Science of Food and Agriculture, 92(10), 2043–2049.

Verdejo-Lucas, S., Cortada, L., Sorribas, F. J., and Ornat, C. (2009). Selection of virulent populations of *Meloidogyne javanica* by repeated cultivation of Mi resistance gene tomato rootstocks under field conditions. Plant Pathology, 58(5), 990-998.

Zeck, W. M. (1971). Rating scheme for field evaluation of root-knot nematode infestations. *Pflanzenschutz Nachrichten.* Bayer*,* 24, 141–144.

Zhang, H., Chen, H., Tan, J., Huang, S., Chen, X., Dong, H., et al. (2023). The chromosome-scale reference genome and transcriptome analysis of *Solanum torvum* provides insights into resistance to root-knot nematodes. Frontiers in Plant Science, 14, 1210513.

Zijlistra, C., Donkers-Venne, C. and Fargette, M. (2000) Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterized amplified regions (SCAR) based PCR assays. Nematology, 2(8), 847-853.



Chapter 3

Nesiodiocoris tenuis, *Macrolophus pygmaeus* (Hemiptera: Miridae) and *(Z)*-3-hexenyl propanoate, induce systemic resistance in tomatoes against the root-knot nematode *Meloidogyne* spp.

Abstract

The management of *Meloidogyne* spp. in tomato crops presents significant challenges for sustainable agriculture. This study evaluates the potential of *Nesidiocoris tenuis*, *Macrolophus pygmaeus,* and *(Z)*-3-hexenyl propanoate - two zoophytophagous mirid species and one of the herbivory induced plant volatiles (HIPVs) they trigger - to induce systemic resistance in tomato plants (cv. Bodar) against *Meloidogyne incognita* and *M. javanica*. For this purpose, the expression of *PIN2* and *PR1* genes, related to the jasmonic acid (JA) and salicylic acid (SA) pathways, was assessed.

Exposure of tomato plants to 15 nymphs of either *N. tenuis* or *M. pygmaeus* for 24 or 48 hours, and to *(Z)*-3-hexenyl propanoate for 24 hours before inoculation with 200 second-stage juveniles of the nematodes significantly reduced nematode infectivity and reproduction. Notably, the *PIN2* gene expression in leaves was upregulated 9 and 14-fold increased by *N. tenuis* and *M. pygmaeus,* respectively, at 0 days after nematode inoculation (DANI) and was repressed by the nematode at 7 DANI with a 0.11-fold decreased, but not when plants were exposed to *M. pygmaeus* or *N. tenuis*, indicating a strong early defense response. However, the *PR1* expression levels did not show significant changes, suggesting a predominant role of the JA pathway over the SA pathway in the induced resistance.

Therefore, the induction of systemic resistance in tomato plants by *N. tenuis, M. pygmaeus,* and *(Z)*-3-hexenyl propanoate before nematode exposure is a promising strategy for nematode management, at least to suppress nematode infection and reproduction from the primary inoculum.

Keywords: Gene expression, Jasmonic acid, Nematode management, Root-knot nematode, Salicylic acid, *Solanum lycopersicum*

Introduction

Plant-parasitic nematodes (PPN) are key pathogens threatening global plant production and food security. It is estimated that PPN cause around 8.8-14.6% of crop yield losses (Singh et al., 2013). Among the PPN, root-knot nematodes (RKN), *Meloidogyne* spp., are the most limiting ones for vegetable production being *M. arenaria, M. incognita,* and *M. javanica* the most

widespread and damaging RKN species in tropical and subtropical agricultural areas (Hallmann and Meressa, 2018). Symptoms caused by RKN include the formation of galls on the roots, which interfere with the uptake of water and nutrients. These result in symptoms in the aboveground parts of the plant, such as yellowing, wilting, dwarfism, and, in severe cases, plant death. The extent of damage produced by RKN in a particular plant species depends on factors such as host status and plant tolerance, the nematode density at sowing or transplanting, and the environmental and soil conditions (Greco et al., 2009). In tomato, one of the most important cash crops in the Mediterranean basin, crop yield losses caused by RKN can reach between 62% and 72% (Giné et al., 2017; Expósito et al., 2020). During the last decades, RKN control has been mainly based on chemical nematicides (Djian-Caporalino, 2012; Talavera et al., 2012). However, the European Union's Directive on the Sustainable Use of Pesticides (2009/128/EC) and the Green Deal Program aim to achieve sustainable agriculture production to mitigate the effects of chemical pesticides on the environment, food products, farmers, and consumers. Consequently, environmentally friendly control methods must be implemented to reduce the nematode densities below the acceptable economic threshold. One of the approaches that has been extensively investigated is the induction of plant resistance (IR) by priming plant defenses through biotic and abiotic agents against pests and diseases. Primed plants activate defense responses faster and more robustly to biotic and abiotic stresses (Conrath, 2006 and 2009). Induced resistance in plants can be categorized into two main types: Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR). Biotrophic or hemibiotrophic pathogens typically activate SAR and lead to the expression of pathogenesis-related (PR) proteins. This process is dependent on salicylic acid (SA). On the other hand, ISR is triggered by root colonization by symbiotic bacteria or fungi or in response to herbivory. It is mediated by either the jasmonate or the ethylene pathways and does not involve PR protein expression (Conrath, 2009; Durrant and Dong, 2004; Molinari et al., 2011; Kloth and Dicke, 2022; Meena et al., 2022). Various abiotic and biotic elicitors have been reported as inducers of resistance against RKN. Notably, DL-b-amino-n-butyric acid (BABA) has increased tomato resistance to *M. javanica* (Oka et al., 1999). Similarly, SA and acibenzolar-S-methyl (ASM) have been reported to enhance resistance to *M. incognita* (Molinari and Baser, 2010). Furthermore, jasmonic acid (JA) and methyl jasmonate have effectively induced resistance against *M. javanica, M. incognita,* or *M. chitwoodi* (Cooper et al., 2005; Fujimoto et al., 2011; Vieira dos Santos et al., 2013). Regarding biotic inducers, various bacterial and fungal species have demonstrated their capability to induce resistance in tomatoes against RKN (Ayaz, 2021; Ghahremani et al., 2020; Martínez-Medina et al., 2017; Pocurull et al., 2020; Siddiqui et al., 2007; Vos et al., 2013; Xiang et al., 2018). However, there is limited knowledge about the ability of macro-organisms to induce this

resistance, particularly those that are zoophytophagous. These organisms are widely used in biological pest control, but they can also feed on plants when prey is scarce. It is precisely this phytophagy that the plant recognizes as an attack, thereby activating its natural defense mechanisms. This confers upon these natural enemies a positive attribute that goes beyond the mere predation they exert (Pérez-Hedo et al., 2022).

The zoophytophagous predators *Nesidiocoris tenuis* (Hemiptera: Miridae) and *Macrolophus pygmaeus* (Hemiptera: Miridae) are native to the Mediterranean region and colonize the tomato crops that are not intensively treated with chemical pesticides (Pérez-Hedo et al., 2021). Both mirid species are widely used to control different pests of tomato crops, such as whiteflies, lepidopterans, mites, thrips, and aphids (Arnó et al., 2010; Moreno-Ripoll et al., 2014; Pérez-Hedo et al., 2021; Pérez-Hedo and Urbaneja, 2016). However, due to their plant-feeding behavior, characterized by lacerating plant tissue with the stylet, injecting watery saliva into the surrounding cells, and ingesting the diluted cell content (Chinchilla-Ramírez et al., 2021), predatory mirids induce tomato plant defenses (Pérez-Hedo et al., 2022). *Nesidiocoris tenuis* and *M. pygmaeus* can activate metabolic pathways in tomato plants related to salicylic acid (SA) and jasmonic acid (JA), among others (Pérez-Hedo et al., 2015a and b). This activation enhances the plants' resistance to key pests such as the whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae), the South American tomato pinworm *Tuta absoluta* (Lepidoptera: Gelichiidae), the Western flower thrips *Frankliniella occidentalis* (Thysanoptera: Tripidae) and the two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae), significantly contributing to the protection and sustainability of tomato crops (Naselli et al., 2016; Pappas et al., 2015; Pérez-Hedo et al., 2018 and 2022; Silva et al., 2022; Zhang et al., 2018). When tomato plants are induced by mirid phytophagy, they release volatile compounds known as Herbivore-Induced Plant Volatiles (HIPVs) that attracts natural enemies, repel plant pests, and also act as alarm signals to neighboring plants, thus stimulating their defense mechanisms (Pérez-Hedo and Urbaneja, 2021; Pérez-Hedo et al., 2018 and 2021). One of those HIPVs is *(Z)*-3-hexenyl propanoate, which has been previously reported to attract the whitefly parasitoid, *E. formosa,* to repel *T. absoluta, B. tabaci,* and *F. occidelantallis,* and to induce plant defenses by upregulation of the gene expression of *PIN2* and *PR1* related to the jasmonic acid and salicylic acid pathways, respectively, and *Sl-PI-I*, a proteinase inhibitor 1 marker in exposed plants (Pérez-Hedo et al., 2021), enhancing the biological control of these pests.

Martinez-Medina et al., 2021 demonstrated that sustained leaf herbivory by the chewing insect *Manduca sexta* counteracts the ability of *M. incognita* to downregulate jasmonate-related root defenses. Based on this finding, we hypothesize that the phytophagous behavior of *N. tenuis*

and *M. pygmaeus* in the aboveground part of the plants as well as the HIPVs they trigger, may impact the infective and reproductive capacity of RKN in tomato roots. Therefore, this work conducted a series of pot experiments to assess the effect of both mirid species and the volatile *(Z)*-3-hexenyl propanoate on inducing plant resistance in tomatoes against *M. incognita* and *M. javanica*. Additionally, we evaluated the gene expression related to the JA and SA pathways in tomato leaves and roots.

Materials and methods

TOMATO POTTED-PLANT EXPERIMENTS

Plant material, nematode inoculum, insects, and volatile

Tomato seeds of the susceptible cv. Bodar (Seminis Seeds) were germinated in seedling trays using peat as a substrate. The seedlings were then transferred to a growth chamber (25 ± 2 ºC; 70 ± 10% relative humidity; 16:8 h L:D photoperiod). Once the plants had developed three true expanded leaves, they were transplanted into 200 cm^3 pots containing sterile river sand. Subsequently, they were allowed to root for one week within a growing chamber under the abovementioned conditions.

The RKN isolates used in the pot experiments were obtained from the nematode collection at the UPC Department of Agri-Food Engineering and Biotechnology. These isolates were maintained on the susceptible tomato cv. Durinta (Seminis Seeds). The experiments utilized *Meloidogyne javanica* (Mj05) for experiment 1 (Ornat et al., 2001) and *M. incognita* (Agròpolis) for experiment 2 (Giné and Sorribas, 2017). The nematode inoculum consisted of second-stage juveniles (J2), obtained by extracting eggs from tomato roots through blended maceration in a 5 % solution of commercial bleach (40 g/L NaOCl) for 10 min (Hussey and Barker, 1973). The egg suspension was filtered through a 74-µm aperture sieve to eliminate root debris and a 25-µm sieve screen to retain the eggs, which were then transferred into a pot. The egg suspension was placed in Baermann trays at 25 ± 2°C (Whitehead and Hemming, 1965). and the emerging second-stage juveniles (J2) were collected daily over 7 days using a 25-µm sieve and stored at 9 °C until their use.

The fourth-fifth instar nymphs of *N. tenuis* and *M. pygmaeus* used in the experiments were provided by Koppert Biological Systems.

The *(Z)*-3-hexenyl propanoate (Sigma-Aldrich. St. Louis, MO, USA) was first diluted in methanol at 1:100 (v/v) and then further diluted in water at 1:100 (v:v; volatile mix: water) so that the final

test concentration was $1:10^4$ (v/v). Then, volatile emitters were prepared from 2 x 2 cm filter paper and were impregnated, each with 10 µl of the diluted volatile (Pérez-Hedo et al. 2018).

Experimental design

Two consecutive pot experiments were conducted. In the first experiment, we assessed the ability of *N. tenuis* to induce resistance against *M. javanica* by infesting tomato plants with *N. tenuis* for either 24 h or 48 h before nematode inoculation or exposing tomato plants to the *(Z)*-3-hexenyl propanoate. Three isolated climatic chambers at the same conditions (25°C ± 2; 70% ± 5 relative humidity) were used for the experiments. In the first one, twelve tomato plants were placed inside each of two insect cages (47.5 x 47.5 x 93 cm; 680 µm mesh sieve), and the mirids were delivered at a ratio of 15 nymphs per plant. After 24 or 48 hours, the mirids were removed individually from each plant, and the plants were removed from the insect cages. Subsequently, each plant was inoculated with 200 J2 of *M. javanica* (Mj05). In the second chamber, twelve tomato plants were exposed to the volatile by holding two emitters close to the intact plants 24h before nematode inoculation with 200 J2 of *M. javanica* (Mj05). The emitters were maintained during the experiment. In the third chamber, twelve tomato plants only inoculated with the nematode were included as a control for comparison. Plants were watered as needed and fertilized with Hoagland's nutrient solution once a week. Soil temperature were recorded daily at 30-min intervals with a PT100 probe (Campbell Scientific Ltd) placed in the pots at a depth of 4 cm. Forty days after nematode inoculation (DANI), the tomato roots were carefully washed with tap water and submerged in a 15 mg/L erioglaucine solution (Acros Organics) to stain the egg masses (Omwega et al., 1988) before counting them to estimate the nematode infectivity. Afterward, the nematode eggs were extracted from tomato roots by blended maceration in a 10% commercial bleach solution (40 g/L NaOCl) for 10 min (Hussey and Barker, 1973), passed through a 74-µm sieve and collected in a 25-µm sieve to be counted under a microscope to estimate the nematode reproduction, and to calculate the nematode fertility as the number of eggs per egg mass.

The second experiment assessed the effect of *N. tenuis, M. pygmaeus,* and the volatile *(Z)*-3-hexenyl propanoate to induce resistance to the isolated Agròpolis of *M. incognita*, along with the dynamic expression of JA and SA-related genes. Tomato plants were exposed to *N. tenuis, M. pygmaeus,* or the volatile for 24 h previously to nematode inoculation and maintained during the experiment using the same procedure described above. The treatments consisted of plants inoculated with *M. incognita* (*Mi*), plants exposed to *N. tenuis* and inoculated with *M. incognita* (*N. tenuis* + *Mi*), plants exposed to *M. pygmaeus,* and inoculated with *M. incognita* (*M. pygmaeus* + *Mi*), and plants exposed to the volatile and inoculated with *M. incognita* (*(Z)*-3-HP

+ *Mi*). Each treatment was repeated 19 times. Fifteen replicates of each treatment inoculated with the nematode were used to assess the effect on nematode infectivity, reproduction, and fertility. The remaining 4 replicates were used to evaluate the expression of genes related to jasmonic acid (JA) and salicylic acid (SA) pathways (see below). Four additional treatments were included to assess the dynamics of gene expression: 8 plants non-nematode inoculated and non-exposed to the mirid species or the volatile (Control), 8 plants exposed to *M. pygmaeus* (*M. pygmaeus*), and 8 plants exposed to the plant volatile.

Forty days after nematode inoculation, the nematode infectivity and reproduction were determined, and the nematode fertility was calculated following the procedure described in Experiment 1.

Dynamic expression of JA and SA-related genes

The expression of genes *PR1* (precursor of pathogenesis-related protein) and *PIN2* (proteinase II inhibitor), which are marker genes for SA and JA pathways, respectively (Pérez-Hedo et al., 2015a and b), were determined from four individual tomato plants at two different time points after nematode inoculation: immediately after nematode inoculation (0 DANI) and at 7 DANI when the nematode infected the roots. At each assessment time, roots were washed using sterile distilled water and dried on sterile paper, and the aboveground part of the plant was cut from the root system. Both leaves and roots were quickly immersed in liquid nitrogen. The two parts of the plant were processed separately following the same procedure. Total RNA extraction, RNase-free DNase treatment, RT reaction, and SYBR PCR reaction were performed as described by Pérez-Hedo et al., 2021. Quantitative PCR was performed using the Smart Cycler II sequencing detector (Cepheid, Sunnyvale) with standard PCR conditions. EF1 expression was used for normalization as a standard control gene.

STATISTICAL ANALYSES

Statistical analyses were performed using JMP 16.2.0 (SAS Institute Inc.). Data was assessed for normality and homogeneity of the variances and transformed when needed. Data on the egg masses per plant, eggs per plant, and eggs per egg mass were submitted to one-way ANOVA. When it was significant ($P < 0.05$), the means were separated using the LSD test ($P < 0.05$). Data of the relative expression of the *PIN2* and *PR1* genes were relativized to the control treatment (fold change) and analyzed using the non-parametric Krustal-Wallis test because they did not fit a normal distribution. When the non-parametric analysis was significant ($P < 0.05$), groups were separated using the Dunn test ($P < 0.05$).

Results

TOMATO POTTED-PLANT EXPERIMENTS

In experiment 1, the exposition of tomato plants to *N. tenuis* for 24 or 48 h reduced nematode infectivity by 55.8% and 62.8%, respectively (F = 38.6; df = 3, *N. tenuis* 24h + *Mj*, $P < 0.0001$; *N. tenuis* 48h + *Mj*, $P < 0.0001$) and nematode reproduction by 68.4% and 72.1% (F = 44.5; df = 3, *N. tenuis* 24h + *Mj*, $P < 0.0001$; *N. tenuis* 48h + *Mj*, $P < 0.0001$), respectively. Regarding the exposure of tomato plants to *(Z)*-3-hexenyl propanoate, nematode infectivity was reduced by 51% (F = 38.6; df = 3, $P < 0.0001$) and nematode reproduction by 45% the (F = 44.5; df = 3, $P < 0.0001$). However, there were no significant differences in nematode fecundity between plants exposed to the mirids or the volatile and non-exposed plants (F = 6.38; df = 3, *N. tenuis* 24h + *Mj*, P = 0.059; *N. tenuis* 48h + *Mj*, P = 0.14; *(Z)*-3-hexenyl propanoate, P = 0.51) (Table 1).

In experiment 2, the exposure of tomato plants to *N. tenuis* or *M. pygmaeus* reduced nematode infectivity by 29.5% in both cases (F = 13.87; df = 3, *N. tenuis + Mi*, $P < 0.0001$; *M. pygmaeus + Mi*, P = 0.00013) and nematode reproduction by 37.2% and 23.6%, respectively (F = 9.72; df = 3, *N. tenuis + Mi*, $P < 0.0001$; *M. pygmaeus + Mi*, P = 0.021) compared to the control plants. However, no effects on nematode fecundity were observed (F = 5.71; df = 3, *N. tenuis + Mi*, P = 0.51; *M. pygmaeus + Mi*, P = 0.99) (Table 1). The exposure of the plants to *(Z)*-3-hexenyl propanoate did not reduce nematode infectivity (F = 13.87; df = 3, P = 0.30), but it did reduce nematode reproduction by 26.5% (F = 9.72; df = 3, P = 0.0012) and nematode fecundity 19.4% (F = 5.71; df = 3, P = 0.0027) compared to non-exposed plants.

DYNAMIC EXPRESSION OF JA AND SA-RELATED GENES

The expression of the *PIN2* gene in tomato plant leaves was upregulated 9.2 and 14-fold compared to the control after 24 h of exposure to *N. tenuis* or *M. pygmaeus* and just after nematode inoculation (0 DANI), respectively (*N. tenuis,* Z = 2.52, P = 0.011; *M. pygmaeus,* Z = 2.89, P = 0.003). No significant differences were observed in plants exposed to *(Z)*-3-hexenyl propanoate (Z = 1.70, P = 0.087). Regarding tomato roots, the expression of the *PIN2* gene did not differ (H = 5.319, P = 0.149) between treatments. Concerning the *PR1* gene expression in tomato leaves, no significant differences were detected between treatments (H = 7.698, P = 0.052). However, in tomato roots, the expression of PR1 was upregulated by 11.9 and 7.9-fold in plants exposed to *N. tenuis* and (Z)-3-hexenyl propanoate, respectively, compared to plants exposed to *M. pygmaeus* (*N. tenuis*, Z = 2.60, P = 0.009; *(Z)*-3-HP, Z = 2.26, P = 0.023) (Figure 1).

Table 1. Number of egg masses per plant, eggs per plant, and eggs per egg mass produced in the tomato cv. Bodar cultivated in pots in growth chambers 40 days after inoculation of 200 J2 of

Meloidogyne javanica (population Mj05) per plant 24 h or 48 h after being exposed to 15 nymphs per plant of *Nesiodiocoris tenuis* or 24 h after exposure to (Z)-3- hexenyl propanoate (Experiment 1), or inoculated with 200 J2 of *M. incognita* (population Agròpolis) 24 h after being exposed to 15 nymphs per plant of *N. tenuis* or *Macrolophus pygmaeus* or 24 h after being exposed to (Z)-3- hexenyl propanoate (Experiment 2).

Experiment	Treatment	Egg masses per plant	Eggs per plant (x100)	Eggs per egg mass
1	*Mj*	43 ± 2 a	210.0 ± 11.6 a	509 ± 34 ab
	N. tenuis 24h + *Mj*	19 ± 3 b	66.4 ± 10.4 c	365 ± 41 b
	N. tenuis 48h + *Mj*	16 ± 2 b	58.5 ± 8.1 c	388 ± 52 b
	(Z)-3- HP + *Mj*	21 ± 2 b	116 ± 8.4 b	572 ± 29 a
2	*Mi*	61 ± 2 a	432.9 ± 20.9 a	717 ± 29 a
	N. tenuis + Mi	41 ± 2 b	271.8 ± 16.1 b	662 ± 29 ab
	M. pygmaeus + Mi	44 ± 3 b	330.7 ± 37.2 b	736 ± 52 a
	(Z)-3- HP + *Mi*	55 ± 3 a	322.8 ± 19.0 b	583 ± 17 b

Data are means ± standard errors of 12 replicates for the experiment 1 and 15 replicates for the experiment 2. Data from the same experiment in the same column followed by different letter are significantly different according to the LSD test ($P < 0.05$)

At 7 DANI, the *PIN2* gene expression in tomato leaves was downregulated 0.11 and 0.09-fold in those plants inoculated with the nematode and those exposed to *(Z)*-3-hexenyl propanoate and inoculated with the nematode, respectively, compared to the control (*Mi*, $Z = 2.47$, $P = 0.013$; *(Z)*-3-HP + *Mi*, $Z = 2.53$, $P = 0.011$). No significant differences were found in the remaining treatments (*M. pygmaeus* + *Mi*, $Z = 0.6$, $P = 0.54$; *N. tenuis* + *Mi*, $Z = 1.06$, $P = 0.28$) (Figure 1). In tomato roots, PIN2 gene expression was downregulated in all treatments except those exposed to *N. tenuis*, regardless of nematode inoculation (*Mi*, 0.04-fold, $Z = 2.83$, $P = 0.004$; *M. pygmaeus*, 0.08-fold, $Z = 1.96$, $P = 0.049$; *M. pygmaeus* + *Mi,* 0.05-fold, $Z = 2.54$, $P = 0.011$; *N. tenuis*, $Z = 0.57$, $P = 0.56$; *N. tenuis* + *Mi*, $Z = 0.98$, $P = 0.16$; *(Z)*-3-HP, 0.05-fold, $Z = 2.57$, $P = 0.010$; *(Z)*-3-HP + *Mi*, 0.03-fold, $Z = 3.09$, $P = 0.002$) (Figure 1).

For the PR1 gene expression in tomato leaves at 7 DANI, it was downregulated by 0.02-fold in plants exposed to (Z)-3-hexenyl propanoate and inoculated with the nematode ($Z = 2.81$, $P = 0.004$). However, no significant differences were observed in roots (*Mi*, $Z = 1.90$, $P = 0.056$; *M.*

pygmaeus, *Z* = 1.03, *P* = 0.29; *M. pygmaeus* + *Mi, Z* = 0.57, *P* = 0.56; *N. tenuis*, *Z* = 0.28, *P* = 0.77; *N. tenuis* + *Mi*, *Z* = 1.90, *P* = 0.056; *(Z)*-3-HP, *Z* = 0.23, *P* = 0.81; *(Z)*-3-HP + *Mi*, *Z* = 1.09, *P* = 0.27) (Figure 1).

Discussion

The main objective of this study was to assess the capacity of *N. tenuis*, *M. pygmaeus* and the HIPV, *(Z)*-3-hexenyl propanoate to trigger systemic resistance against *Meloidogyne* incognita and *M. javanica*. Previous research has demonstrated that these zoophytophagous mirid species, as well as the exposition of volatiles can enhance tomato plant resistance against various pests, including spider mites, whiteflies, and thrips (Pérez-Hedo et al., 2021 and 2022). Moreover, the induction of resistance in tomato to the tomato spotted wilt virus has also been described (Bouagga et al., 2019). However, to the best of our knowledge, there is no information about its ability against RKN. The results of this study have shown that both mirid species and the exposure to (Z)-3-hexenyl propanoate volatile can induce systemic resistance in tomatoes against *M. incognita* and *M. javanica* in pot conditions.

The exposition of tomato plants to *N. tenuis* 24 h previous nematode inoculation was enough to reduce RKN infectivity and reproduction in experiments that allowed the completion of one nematode generation. Increasing the exposure time until 48 h did not improve the nematode suppressiveness. Both mirid species triggered physiological changes, mainly in the leaves, which interfered with the nematode's ability to infect the roots and to reproduce. The JA pathway was the most altered pathway in tomato plants after 24 h of exposure to the mirid species, which were forced to feed on the plants due to the absence of supplementary food. In the case of exposure to *(Z)*-3-hexenyl propanoate, although the nematode reproduction was reduced in both experiments, the infectivity was not affected in the second experiment. These results could be consistent with the expression levels of *PIN2* in leaves since it did not differ from the control. The JA pathway is known to mediate major anti-herbivore response (Kloth and Dicke, 2022; Schaller and Stintzi, 2008) and, in our case, seemed to play an important role in mediating induced resistance against *Meloidogyne*, countering its repression in the leaves and roots by the nematode during the infection, as has also been previously reported (Nahar et al., 2011; Song et al., 2021).

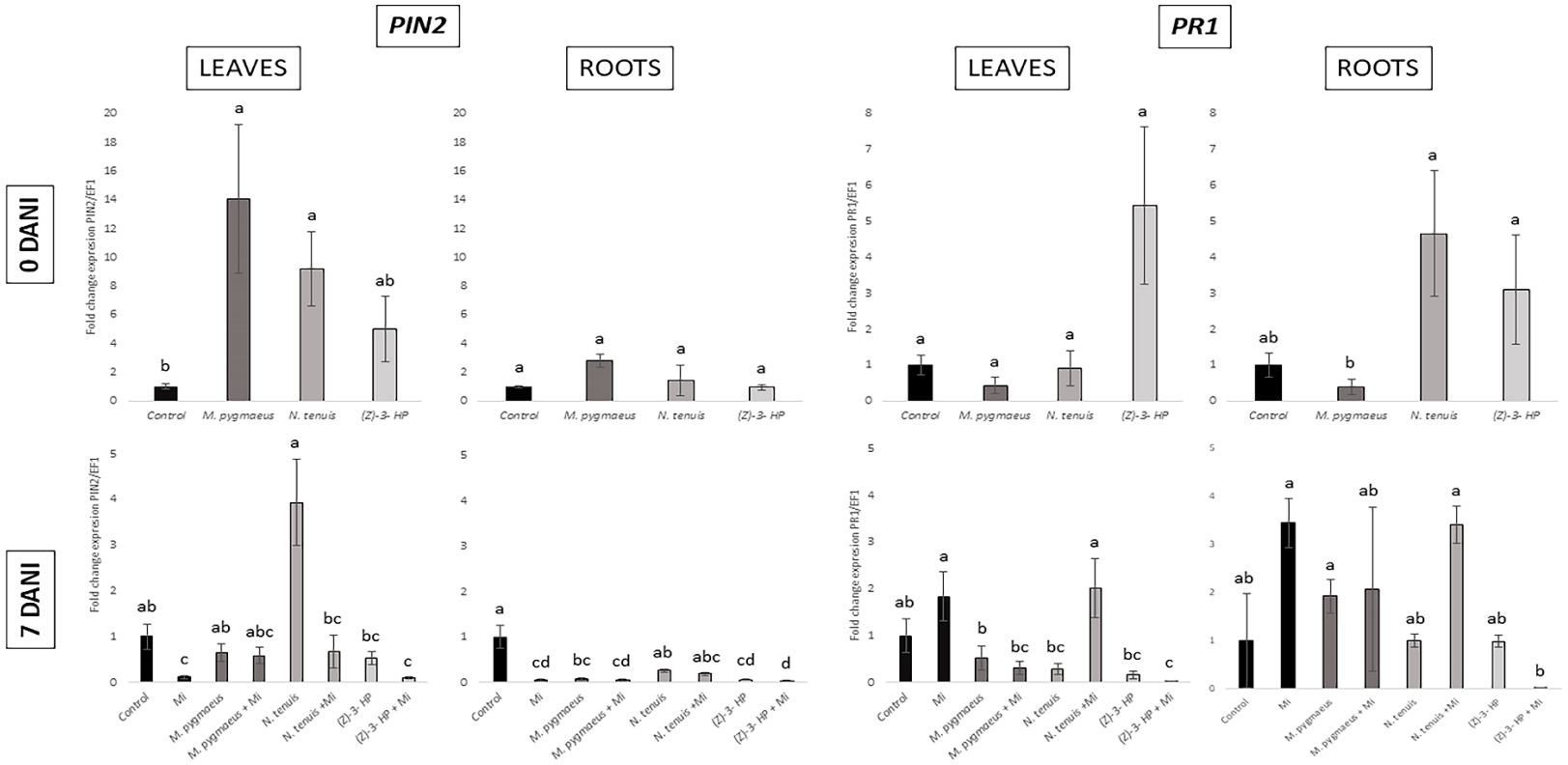


Figure 1. Expression analysis of the *PIN2* and *PR1* genes on the tomato cv. Bodar leaves and roots at 0 and 7 days after nematode inoculation (DANI) with 200 J2 per plant of *Meloidogyne incognita* (Agròpolis), 24 h after the exposure to 15 nymphs per plant of *Nesiodiocoris tenuis* or *Macrolophus pygmaeus* or 24 h after being exposed to (Z)-3-hexenyl propanoate. Plants non-exposed to mirid species and (Z)-3- hexenyl propanoate neither inoculated with *M. incognita* (Control), plants inoculated with *M. incognita* (*Mi*), plants exposed to *N. tenuis* (*N. tenuis*), plants exposed to *N. tenuis* and inoculated with *M. incognita* (*N. tenuis* + *Mi),* plants exposed to *M. pygmaeus* (*M. pygmaeus*), plants exposed to *M. pygmaeus* and inoculated with *M. incognita* (*M. pygmaeus* + *Mi*). plants exposed to (Z)-3- hexenyl propanoate ((Z)-3-HP), plants exposed to (Z)-3- hexenyl propanoate and inoculated with *M. incognita* ((Z)-3-HP + *Mi*). Transcript levels were normalized to the expression of *EF1* measured in the same sample. Each column and bar are the mean and standard error of 4 independent replicate analyses of transcript expression relative to a housekeeping gene and expressed as a ratio to the non-exposed to mirids neither inoculated with *M. incognita* plants (Control) (fold change). Different letters between treatments indicate differences according to Dunn's test ($P < 0.05$).

The overexpression of the *PIN2* gene in tomato leaves observed in this study was consistent with that reported by other authors. Indeed, Pérez-Hedo et al. 2015a and b, and 2018 also reported an increase in endogenous levels of JA-Ile in the apical part of the *N. tenuis*-exposed plants. Similarly, Zhang et al., 2018 reported an overexpression of genes related to the JA pathway and an increased concentration of 12-oxo-phytodienoic acid and jasmonic acid–isoleucine in leaves punctured by *M. pygmaeus.* In the case of the leaf chewer *Manduca sexta*, the phytophagy strongly activates the jasmonate biosynthesis in plant roots, affecting *M. incognita* reproduction (Martínez-Medina et al., 2021). Exogenous applications of JA or its derivatives, such as methyl jasmonate have also been demonstrated to reduce nematode infection, possibly by increasing toxic compounds to nematodes produced by roots, such as hytoectosteroids, flavonoids, and proteinase inhibitors (Cooper et al., 2005; Fujimoto et al., 2011; Gundlach et al., 1992; Soriano et al., 2004; Vieira dos Santos et al., 2013).

Concerning the SA hormone levels pathway, Pérez-Hedó et al., 2015b did not find significant changes between *N. tenuis*-exposed and non-exposed tomato plants. This is consistent with our results, where no changes in the relative expression of the *PR1* gene were observed in the leaves and roots after 24 h of plant exposure to the mirid species or the volatile. In addition, although no significant overexpression in roots was observed in tomato roots in plants inoculated with the nematode, regardless of the mirid species, previous research had shown the overexpression of SA-responsive genes in a susceptible tomato cultivar during the expansion of the feeding site induced by the nematode. Nevertheless, the response is posteriorly silenced by the nematode, leading to a compatible nematode-plant interaction (Shukla et al., 2018).

Both mirid species, *M. pygmaeus* and *N. tenuis,* are frequently used as biological control agents in integrated pest management programs in the Mediterranean basin. One of the strategies is to release the adults in nurseries, with food supplementation, for egg laying to achieve a uniform distribution in the field to anticipate the effect of early pest infestation (Calvo et al., 2012; Lenfant et al., 2000). Moreover, they can prime plants against pests and pathogens through their phytophagy activity and induce plant volatiles produced in response to herbivory (Pérez-Hedo et al., 2022).

The results obtained in our study have to be validated in field conditions to know the durability of the effect of the exposure to the mirid species or to the volatile since the nematode can complete more than one generation in a tomato crop. According to our results, it seems that this RKN management approach is effective in suppressing the capacity of the primary nematode inoculum, which remains in the soil or infected root debris and is responsible for the primary infection, to infect the tomato roots at transplanting and reproduce. However, the nematode

fecundity is not affected by any mirid species irrespective of the RKN species, but the volatile reduces *M. incognita* but not *M. javanica* fertility. This fact could affect the production of secondary nematode inoculum, extending its protection to the plant against this RKN species. In short, our study provides evidence that these novel tools— zoophytophagous mirid species and exposition to volatiles—could be integrated into RKN management strategies. Considering the limitations of existing control methods in terms of effectiveness, durability, and safety for the environment and human health, these new approaches may play a valuable role in comprehensive nematode management programs.

Conclusions

The induction of systemic resistance in tomato plants by *N. tenuis*, *M. pygmaeus* and exposition to *(Z)*-3-hexenyl propanoate prior to nematode exposure is a promising strategy for nematode management due to its efficacy in suppressing the primary nematode inoculum present in the soil. This study underscores the importance of integrating biological control agents into crop protection programs, providing a sustainable and environmentally friendly alternative to chemical nematicides. Further field validation is needed to confirm its long-term efficacy in real-world conditions.

Acknowledgements

The authors also are grateful to Koopert ® for providing the insects and to Mr. Roger Nieto, Ms. Sheila Alcala and Ms. Maria Julià for their technical support in the laboratory.

CRediT authorship contribution statement

Aïda Magdalena Fullana: Methodology, validation, formal analysis, investigation, data curation, writing – review & editing, visualization**. Ariadna Giné:** Validation, data curation, writing – review & editing, visualization, supervision**. Alberto Urbaneja:** Conceptualization, Validation, resources, writing – review & editing, supervision**. Meritxell Pérez-Hedo:** Conceptualization, Methodology, validation, formal analysis, investigation, resources, data curation, writing – review & editing, supervision, project administration and funding acquisition**. Francisco Javier Sorribas:** Conceptualization, methodology, validation, investigation, resouces, data curation, writing – review & editing, supervision, project administration and funding acquisition**. Alejando Expósito:** Conceptualization, methodology, validation, formal análisis, investigation, data curation, writing - original draft, visualization, supervision

Funding

This work was supported by project no. PID2020-113234RR-I00 and PID2021-129001OB-100 from the Spanish Ministry of Science and Innovation through the State Research Agency,

financed by MCIN and FEDER; Fondo Social Europeo (PRE2018-084265, Aïda Magdalena Fullana) and for the post-doctoral grant Funded by European Union-NextGenerationEU, Ministry of Universities and Recovery, Transformation and Resilience Plan, through a call from Universitat Politècnica de Catalunya (Grant Ref. 2022UPC-MSC-93765). Besides funding from "La red de excelencia (RED2018-102407-T) Aprendiendo de la naturaleza: interacciones multitróficas para la protección de cultivos y bosques" financied by MCIN/AEI/10.13039/501100011033

Data availability statement

Data is available on request from the corresponding author. The data supporting this study's findings are available from the corresponding author upon reasonable request.

References

Arnó, J., Castañé, C., Riudavets, J. and Gabarra, R. (2010). Risk of damage to tomato crops by the generalist zoophytophagous predator *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae). Bulletin of Entomological Research, 100, 105-115

Ayaz, M., Ali, Q., Farzand, A., Khan, A. R., Ling, H. and Gao, X. (2021). Nematicidal volatiles from *Bacillus atrophaeus* GBSC56 promote growth and stimulate induced systemic resistance in tomato against *Meloidogyne incognita*. International Journal of Molecular Sciences, 22,5049

Bouagga, S., Urbaneja, A., Depalo, L., Rubio, L. and Pérez-Hedo, M. (2019). Zoophytophagous predator-induced defences restrict accumulation of the tomato spotted wilt virus. Pest Managment Science, 76,561-567.

Calvo, F. J., Lorente, M. J., Stansly, P. A. and Belda, J. E. (2012). Preplant release of *Nesidiocoris tenuis* and supplementary tactics for control of *Tuta absoluta* and *Bemisa tabaci* in greenhouse tomato. Entomologia Experimentalis et Appicata, 143,111-119

Chinchilla-Ramírez, M., Garzo, E., Fereres, A., Gavara-Vidal, J., Ten Broeke, C. J., Van Loon, J. J., et al. (2021). Plant feeding by Nesidiocoris tenuis: Quantifying its behavioral and mechanical components. Biologycal Control, 152,104402

Conrath, U. (2006). Systemic acquired resistance. Plant Signaling Behavior, 1,:179-184

Conrath, U. (2009). Priming of induced plant defense responses. Advances in Botanical Research, 51,361-395.

Cooper, W. R., Jia, L. and Goggin, L. (2005). Effects of jasmonate-induced defenses on root-knot nematode infection of resistant and susceptible tomato cultivars. Journal of Chemical Ecology, 31, 1953-1967.

Djian-Caporalino, C. (2012). Root-knot nematodes (*Meloidogyne* spp.), a growing problem in French vegetable crops. EPPO Bull, 42,127-137.

Durrant, W. E. and Dong, X. (2004). Systemic acquired resistance. Annual Review of Phytopathology, 42,185-209.

Expósito, A., Pujolà, M., Achaerandio, I., Giné, A., Escudero, N., Fullana, A. M. et al. (2020). Tomato and melon *Meloidogyne* resistant rootstocks improve crop yield but melon fruit quality is influenced by the cropping season. *Frontiers in Plant Sci*ence, 11, 560024.

Fujimoto, T., Tomitaka, Y., Abe, H., Tsuda, S., Futai, K. and Mizukubo, T. (2011). Expression profile of jasmonic acid-induced genes and the induced resistance against the root-knot nematode (*Meloidogyne incognita*) in tomato plants (*Solanum lycopersicum*) after foliar treatment with methyl jasmonate. Journal of Plant Physiology, 168,1084-1097.

Ghahremani, Z., Escudero, N., Beltrán-Anadón, D., Saus, E., Cunquero, M., Andilla, J. et al., (2020). *Bacillus firmus* strain I-1582, a nematode antagonist by itself and through the plant. Frontiers in Plant Science, 11, 796.

Ghahremani, Z., Escudero, N., Saus, E., Gabaldón, T. and Sorribas, F. J. (2019). *Pochonia chlamydosporia* induces plant-dependent systemic resistance to *Meloidogyne incognita*. Frontiers in Plant Science, 10, 945

Giné, A. and Sorribas, F. J. (2017). Quantitative approach for the early detection of selection for virulence of *Meloidogyne incognita* on resistant tomato in plastic greenhouses. Plant Pathology, 66, 1338-1344.

Greco, N. and Di Vito, M. (2009). "Population dynamics and damage level", in Root-knot nematodes. Eds R.N. Perry, M. Moens, and J. L. Starr (Wallingford, CABI international), 246–274

Gundlach, H., Müller, M. J., Kutchan, T. M. and Zenk, M. H. (1992). Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. *Proceeding National Academy of Sciences*, 89, 2389-2393.

Hallmann, J. and Meressa, B. H. (2018). "Nematode parasites of vegetables", in Plant parasitic nematodes in subtropical and tropical agriculture. Eds R.A. Sikora, D. Coyne, J. Hallman and P. Timper, (Wallingford, CABI International), 346-410.

Hussey, R. S. and Barker, K. R. (1973). A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. Plant Diseases Reports, 57, 1025-1028.

Kloth, K. J. and Dicke, M. (2022). Rapid systemic responses to herbivory. *Current Opinion in Plant Biology*, 68, 102242.

Lenfant, C., Ridray, G. and Schoen, L. (2000). Eds. Biopropagation of *Macrolophus caliginosus* Wagner for a quicker establishment in Southern tomato greenhouses: IOBC/WPRS Working Group "Integrated Control in Protected Crops, Mediterranean Climate". Proceedings of the meeting; 2000 Apr 24-28; Antalya, Turkey, *Bulletin OILB/SROP,* 23,247-251.

Martínez-Medina, A., Fernandez, I., Lok, G. B., Pozo, M. J., Pieterse, C. M. and Van Wees, S. C. (2017). Shifting from priming of salicylic acid-to jasmonic acid-regulated defences by *Trichoderma* protects tomato against the root knot nematode *Meloidogyne incognita*. New Phytologist, 213, 1363-1377.

Martínez-Medina, A., Mbaluto, C. M., Maedicke, A., Weinhold, A., Vergara, F. and Van Dam, N. M. (2021). Leaf herbivory counteracts nematode-triggered repression of jasmonate-related defenses in tomato roots. Plant Physiology, 187, 1762-1778.

Meena, M., Yadav, G., Sonigra, P., Nagda, A., Mehta, T., Swapnil, P. et al., (2022). Role of elicitors to initiate the induction of systemic resistance in plants to biotic stress. Plant Stress, 5, 100103.

Molinari, S. and Baser, N., (2010). Induction of resistance to root-knot nematodes by SAR elicitors in tomato. Crop Protection, 29, 1354-1362.

Molinari, S. (2011). Natural genetic and induced plant resistance, as a control strategy to plant-parasitic nematodes alternative to pesticides. Plant Cell Reports, 30, 311-323.

Moreno-Ripoll, R., Gabarra, R., Symondson, W. O. C., King, R. A. and Agustí, N. (2014). Do the interactions among natural enemies compromise the biological control of the whitefly Bemisia tabaci?. Journal of Pest Science, 87, 133-141.

Nahar, K., Kyndt, T., De Vleesschauwer, D., Höfte, M. and Gheysen, G. (2011). The jasmonate pathway is a key player in systemically induced defense against root knot nematodes in rice. Plant Physiology, 157, 305-316.

Naselli, M., Urbaneja, A., Siscaro, G., Jaques, J. A., Zappalà, L., Flors, V. et al. (2016). Stage-related defense response induction in tomato plants by Nesidiocoris tenuis. International Journal of Molecular Science, 17, 1210.

Oka, Y., Cohen, Y. and Spiegel, Y. (1999). Local and systemic induced resistance to the root-knot nematode in tomato by DL-β-amino-n-butyric acid. Phytopathology, 89, 1138-1143.

Omwega, C. O., Thomason, I. J. and Roberts, P. A. (1988). A nondestructive technique for screening bean germ plasm for resistance to *Meloidogyne incognita*. Plant Disease, 72, 970-972.

Ornat, C., Verdejo-Lucas, S. and Sorribas, F. J. (2001). A population of Meloidogyne javanica in Spain virulent to the Mi resistance gene in tomato. Plant Disease, 85, 271-276.

Pappas, M. L., Steppuhn, A., Geuss, D., Topalidou, N., Zografou, A., Sabelis, M. W. et al., (2015). Beyond predation: the zoophytophagous predator *Macrolophus pygmaeus* induces tomato resistance against spider mites. PLoS One, 10, e0127251.

Pérez-Hedo, M. and Urbaneja, A. (2016). The zoophytophagous predator *Nesidiocoris tenuis*: a successful but controversial biocontrol agent in tomato crops. Advances in insect control and resistance management, 121-138.

Pérez-Hedo, M., Alonso-Valiente, M., Vacas, S., Gallego, C., Rambla, J. L., Navarro-Llopis, V. et al. (2021). Eliciting tomato plant defenses by exposure to herbivore induced plant volatiles. Entomologia Generalis, 41, 209-218.

Pérez-Hedo, M., Arias-Sanguino, A. M. and Urbaneja, A. (2018). Induced tomato plant resistance against *Tetranychus urticae* triggered by the phytophagy of *Nesidiocoris tenuis*. Frontiers in Plant Science, 9, 1419.

Pérez-Hedo, M., Bouagga, S., Jaques, J. A., Flors, V. and Urbaneja, A. (2015a). Tomato plant responses to feeding behavior of three zoophytophagous predators (Hemiptera: Miridae). Biological Control, 86, 46-51.

Pérez-Hedo, M., Bouagga, S., Zhang, N. X., Moerkens, R., Messelink, G., Jaques, J. A. et al., (2022). Induction of plant defenses: the added value of zoophytophagous predators. Journal of Pest Science, 95, 1501-1517.

Pérez-Hedo, M., Riahi, C. and Urbaneja, A. (2021). Use of zoophytophagous mirid bugs in horticultural crops: Current challenges and future perspectives. Pest Management Science, 77, 33-42.

Pérez-Hedo, M., Urbaneja-Bernat, P., Jaques, J. A., Flors, V. and Urbaneja, A. (2015b). Defensive plant responses induced by *Nesidiocoris tenuis* (Hemiptera: Miridae) on tomato plants. Journal of Pest Science, 88, 543-554.

Pocurull, M., Fullana, A. M., Ferro, M., Valero, P., Escudero, N., Saus, E. et al. (2020). Commercial formulates of *Trichoderma* induce systemic plant resistance to *Meloidogyne incognita* in tomato and the effect is additive to that of the Mi1.2 resistance gene. Frontiers in Microbiology, 10, 3042

Schaller, A. and Stintzi, A. (2008). "Jasmonate biosynthesis and signaling for induced plant defense against herbivory" in Induced plant resistance to herbivory. Eds A. Schaller (Dordrecht, Springer), 349-366.

Shukla, N., Yadav, R., Kaur, P., Rasmussen, S., Goel, S., Agarwal, M., et al., (2018). Transcriptome analysis of root-knot nematode (*Meloidogyne incognita*)-infected tomato (*Solanum lycopersicum*) roots reveals complex gene expression profiles and metabolic networks of both host and nematode during susceptible and resistance responses. Molecular Plant Pathology, 19, 615-633.

Siddiqui, Z. A., Baghel, G. and Akhtar, M. S. (2007). Biocontrol of *Meloidogyne javanica* by Rhizobium and plant growth-promoting rhizobacteria on lentil. World Journal of Microbiology Biotechnology, 23, 435-441.

Silva, D. B., Hanel, A., Franco, F. P., de Castro Silva-Filho, M. and Bento, J. M. S. (2022). Two in one: the neotropical mirid predator *Macrolophus basicornis* increases pest control by feeding on plants. *Pest Management Science*, 78, 3314-3323.

Singh, S. K., Hodda, M. and Ash, G. J. (2013). Plant-parasitic nematodes of potential phytosanitary importance, their main hosts and reported yield losses. EPPO bull, 43, 334-374.

Song, H., Lin, B., Huang, Q., Sun, T., Wang, W., Liao, J., et al. (2021). The *Meloidogyne javanica* effector Mj2G02 interferes with jasmonic acid signalling to suppress cell death and promote parasitism in Arabidopsis. Molecular Plant Pathology, 22, 1288-1301.

Soriano, I.R., Asenstorfer, R. E., Schmidt, O. and Riley, I. T. (2004). Inducible flavone in oats (*Avena sativa*) is a novel defense against plant-parasitic nematodes. Phytopathology, 94, 1207-1214.

Talavera, M., Sayadi, S., Chirosa-Rios, M., Salmeron, T., Flor-Peregrin, E. and Verdejo-Lucas, S. (2012). Perception of the impact of root-knot nematode-induced diseases in horticultural protected crops of south-eastern Spain. Nematology, 14, 517-527.

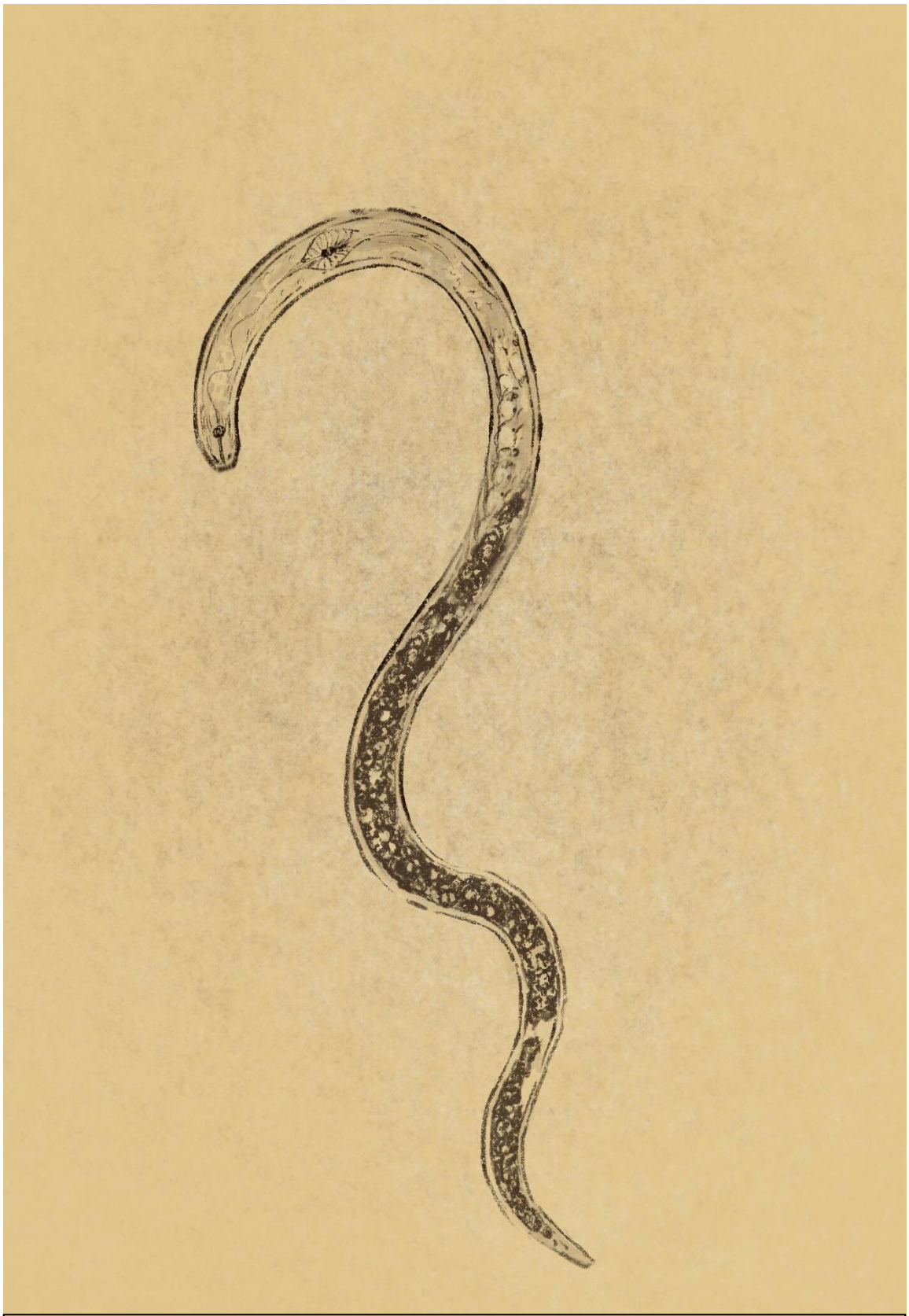
Vieira dos Santos, M. C., Curtis, R. H. C and Abrantes, I. (2013). Effect of plant elicitors on the reproduction of the root-knot nematode *Meloidogyne chitwoodi* on susceptible hosts. *European Journal of Plant Pathology*, 136, 193-202.

Vos, C., Schouteden, N., Van Tuinen, D., Chatagnier, O., Elsen, A., De Waele, D., et al. (2013). Mycorrhiza-induced resistance against the root–knot nematode *Meloidogyne incognita* involves priming of defense gene responses in tomato. Soil Biology and Biochemistry, 60, 45-54.

Whitehead, A. G. and Hemming, J. R. (1965). A comparison of some quantitative methods of extracting small vermiform nematodes from soil. Annals of Applied Biology, 55, 25-38.

Xiang, N., Lawrence, K. S. and Donald, P. A. (2018). Biological control potential of plant growth-promoting rhizobacteria suppression of *Meloidogyne incognita* on cotton and *Heterodera glycines* on soybean: A review. Journal of Phytopathology, 166, 449-458.

Zhang, N. X., Messelink, G. J., Alba, J. M., Schuurink, R. C., Kant, M. R. and Janssen, A. (2018). Phytophagy of omnivorous predator *Macrolophus pygmaeus* affects performance of herbivores through induced plant defences. *Oecologia*, 186, 101-113.



Chapter 4

Reactions of *Citrullus amarus* and *Cucumis metuliferus* to *Meloidogyne chitwoodi*, *Meloidogyne enterolobii* and *Meloidogyne luci*

Abstract

Meloidogyne chitwoodi, *M. enterolobii*, and *M. luci* are present in some EU countries, with restricted distributions, and plant resistance can be used to manage these nematodes. Two pot experiments were conducted under controlled conditions for 56 d to assess the host suitability of two potential rootstocks, *Cucumis metuliferus* BGV11135 and *Citrullus amarus* BGV5167, to one isolate of each nematode. The susceptible cucumber (*Cucumis sativus*) 'Dasher II', watermelon (*Citrullus lanatus*) 'Sugar Baby' and tomato (*Solanum lycopersicum*) 'Coração-de-Boi' were included for comparisons. A histopathological study using confocal-laser microscopy was also conducted 15 d after nematode inoculations. In the pot test, the rootstocks showed lower numbers of galls, egg masses, and eggs per plant than their susceptible ones. Reproduction indices of the rootstocks varied from immune to moderately resistant, depending on the isolate-rootstock combination. In the histopathological study, *M. enterolobii* and *M. luci* induced similar numbers of giant cells (GC) per feeding site in all germplasms. However, GC volumes and numbers of nuclei in rootstocks were lower than in the susceptible germplasms. GCs induced by *M. chitwoodi* were only detected in susceptible cucumber. These results emphasize the potential of *C. metuliferus* and *C. amarus* as effective, eco-friendly strategies for managing root-knot nematodes, and show the complex these host-pathogen interactions.

Keywords. Histopathology, plant resistance, root-knot nematodes, rootstocks.

Introduction

Plant-parasitic nematodes (PPN) have significant economic impacts on agriculture (Jones et al., 2013), leading to diminished crop yields quality (Elling, 2013). *Meloidogyne* spp., commonly known as root-knot nematodes (RKN), are obligate sedentary endoparasites of roots of many plant species, and are responsible for approx. half of crop yield losses attributed to PPN (Bent et al., 2008). In a compatible host, the RKN trigger formation of multinucleated giant cells (GC), from which the nematodes obtain the nutrients for development. RKN induce formation of host root galls, disrupting the uptake of water and nutrients and causing nonspecific symptoms in aerial plant parts, including stunting, nutrient deficiency, epinasty, and plant death, at high nematode population densities in soil. Disease severity depends on soil nematode population density at sowing or transplanting, and on host species and cultivar, cropping season, soil texture

and presence of potential nematode antagonists (Sorribas et al., 2020). Conversely, when compatibility between the host plant and the nematode is suboptimal, GCs often have inhibited growth, characterized by presence of multiple vacuoles, sparse nuclei, or cytoplasmic collapse. Another distinctive feature frequently observed is the absence of fluorescence in histopathological images, due to the probable accumulation of phenolic compounds surrounding the GCs, indicating hypersensitive responses to nematode infections (Phan et al., 2018; Expósito et al., 2020; Fullana et al., 2023). This defensive response results in suppression of nematode infection and reproduction, and, in some cases, increases in proportions of males in the populations (Ye et al., 2017).

Of the approx. 100 RKN species described to date, *Meloidogyne arenaria*, *M. incognita*, *M. javanica* (tropical species), and M. hapla (temperate species), are responsible for most yield crop losses attributed to *Meloidogyne* spp. (Jones et al., 2013). However, other RKN species, such as M. chitwoodi, M. enterolobii and M. luci, are gaining importance, because of their high pathogenicity in several economically important crops despite their limited global distributions (CastagnoneSereno, 2012; Elling, 2013; Maleita et al., 2022). *Meloidogyne chitwoodi* and *M. enterolobii* have been added to the EPPO A2 list of pests recommended for regulation as quarantine pests (EPPO, 2023a), and *M. luci* has been added to the EPPO Pest Alert List (EPPO, 2017). In Europe, populations of M. chitwoodi have been reported in Belgium, France, Germany, the Netherlands and Portugal in 2016 (EPPO, 2016). Currently, however, 17 other countries, including Spain, have been included (EPPO 2023b). The distribution of *M. enterolobii* is more limited than that of other *Meloidogyne* species, having been reported in Belgium, France, Italy, the Netherlands, Portugal and Switzerland (EPPO, 2023c). *Meloidogyne luci* is present in Greece, Italy, Portugal, Serbia, Slovenia and Turkey (EPPO, 2023d). Despite these restricted distributions, legislative measures have been implemented to eradicate these nematodes, and prevent their introduction into regions where they are absent. This emphasizes the need for increased surveillance and control measures against these emerging nematode species.

RKN control has traditionally relied on fumigant and non-fumigant nematicides. However, use of most of these have been prohibited or restricted, due to harmful environmental, human, and/or animal effects. In response, the European Union has adopted new policies that promote the use of integrated nematode management strategies, which prioritize environmentally friendly and safe approaches reflected in Directive 2009/128/CE and the European Green Deal. Plant resistance plays a key role in the available control strategies, because it suppresses nematode infection and/or reproduction (Roberts, 2002). Resistance is cost-effective, prevents nematode reproduction and crop yield losses (Sorribas et al., 2005), and its effect is extended

to following susceptible crops (Ornat et al., 1997; Hanna, 2000). Commercially available resistant vegetable cultivars or rootstocks for tropical RKN species are limited to the *Solanaceae* and *Cucurbitaceae* including tomato, pepper, eggplant and watermelon. However, some of the minor and temperate RKN species can reproduce on these plants, or their reproductive capacity is unknown.

Meloidogyne chitwoodi, *M. enterolobii* and *M. hapla* can reproduce on tomato carrying the *Mi1.2* resistance gene and pepper germplasm carrying the N resistance gene (Brown et al., 1997; Koutsovoulos et al., 2020). In addition, virulent isolates of *M. luci* able to overcome resistance conferred by the tomato *Mi1.2* gene have been reported (Aydinli et al., 2019). In cucurbits, the experimental melon rootstocks *Cucumis metuliferus* BGV11135 display resistance to *M. arenaria*, *M. incognita and M. javanica* (Expósito et al., 2018, and 2019), as well as *Citrullus amarus*, a commercial watermelon rootstock (García-Mendívil et al., 2019; Waldo et al., 2023). Additionally, three accessions of *C. metuliferus* 'Kino' exhibit resistance to *M. enterolobii*, *M. incognita* race 1, and *M. javanica* (Pinheiro et al., 2019). Waldo et al. (2023) also evaluated 108 different accessions of *C. amarus*, and some of these were resistant to *M. enterolobii*. Nevertheless, there is currently no available knowledge about the host suitability of *C. metuliferus* and *C. amarus* for the emerging RKN species *M. chitwoodi* and *M. luci*.

Histopathological studies conducted with laser scanning confocal microscopy have shown that GCs in resistant germplasms are less voluminous and have fewer nuclei than those in susceptible germplasm (Expósito et al., 2020; Fullana et al., 2023). The aim of the present study was to determine host suitability of *C. metuliferus* BGV11135 and *C. amarus* BGV5167 accessions for isolates of *M. chitwoodi*, *M. enterolobii*, and *M. luci*. Histopathological studies of each plant germplasm-RKN isolate combination were also carried out.

Materials and methods

NEMATODE INOCULA

Inocula consisted of second-stage juveniles (J2) of *M. chitwoodi* (PtCh), *M. enterolobii* (PtEn), and *M. luci* (PtL1) isolates selected from the RKN NEMATOlab collection (CFE, University of Coimbra) (Maleita et al., 2021). The isolates were maintained on the susceptible tomato (*Solanum lycopersicum*) cultivar 'Coraçãode-Boi' (Vilmorim-Mikado Ibérica, Alicante, Spain; Maleita et al., 2022), in a growth chamber maintained at 24 ± 2°C and 16 h light 8 h dark daily cycle. One week before nematode inoculations, nematode egg masses were hand-picked and placed in Baermann funnels to allow J2 emergence. After 24 h, the emerged J2 were discarded, and the remaining J2 were collected daily and kept at 4°C until the beginning of the experiment,

for a maximum of 5 d. Biochemical electrophoretic analyses of non-specific esterase enzymes were carried out to confirm the Meloidogyne species (Pais et al., 1986).

PLANT MATERIAL

Seeds of the *C. metuliferus* BGV11135 and *C. amarus* BGV5167 (COMAV-UPV, Valencia, Spain) were used in this study. The cucumber (*Cucumis sativus*) 'Dasher II' (Seminis Seeds) and the watermelon (*Citrullus lanatus*) 'Sugar Baby' (Batlle Seeds) were used as cultivars susceptible to tropical RKN species for comparisons (Giné et al., 2014; Lopez- Gomez et al., 2014). The susceptible tomato (*S. lycopersicum*) 'Coracao-de-Boi' was included as a control, to assess the viability of the nematode inocula. Seeds were germinated in Petri dishes with sterile filter paper soaked with sterile distilled water at 24 ± 1°C for 3 d in the dark. After germination, seedlings were transplanted (one per pot) into 50 cm3 pots containing a sterile mixture (1:1:2) of sandy loam soil, sand and a germination substrate (Siro Germinacao bioR). This substrate contains 2 kg $.m^{-3}$ of NPK 9-2-2. The seedlings were kept in a growth chamber for 3 weeks at 24 ± 2°C and a 16 h light 8 h dark daily cycle.

HOST SUITABILITY

Plants were transplanted into 200 cm3 capacity pots containing the soil mixture described above, and were each inoculated with 200 J2. The nematode inoculum was distributed in each pot into two 2 cm holes, located 1 cm away from the plant stem and 2 cm deep in the soil. Each plant germplasm-RKN isolate combination was repeated 10 times, and the experiment was conducted twice.

The plants were maintained in controlled climate chamber at 25 ± 2°C and 60% relative humidity with a 16 h light 8 h dark daily cycle for 56 days. The plants were watered at 2 d intervals, and were fertilized once each week with NUTREA 12-4-6 (Genyen, Crop Solutions), a liquid fertilizer containing 5% N, 8% P and 10% K. At the end of the experiment, plant roots were carefully washed free of soil with tap water, and were then immersed in a phloxine B (0.0015%) solution for 15 min to stain and visualize the nematode egg masses (Holbrook et al., 1983). The number of root galls and egg masses per plant were counted to estimate nematode penetration (galls) and infectivity (egg masses). Nematode eggs were extracted from each whole root system by blending maceration in a 1% NaOCl solution, using the procedure outlined by Hussey and Barker (1973), eggs were counted to estimate the final nematode population densities (*Pf*). Nematode fertility was calculated as the number of eggs per egg mass per plant, and reproduction index (RI), as the percentage of reproduction of a given *Meloidogyne* isolate in the resistant germplasm relative to that in the susceptible germplasm [RI = (*Pf* in resistant germplasm/*Pf* in

susceptible germplasm) × 100]. Levels of resistance were estimated according to the RI values, as immune (RI = 0), highly resistant (RI <1%), resistant (1% ≤ RI < 10%), moderately resistant (10% ≤ RI < 25%), slightly resistant (25% ≤ RI < 50%), or susceptible (RI ≥ 50%), based on the scale of Hadisoeganda and Sasser (1982).

HISTOPATHOLOGY

Fifteen plants of each plant germplasm (described above) were transplanted into 200 cm^3 capacity pots containing sterilized sand, and were maintained under the conditions described above. After 7 days, each susceptible plant germplasm-RKN isolate combination was inoculated with 200 J2, and each expected resistant plant germplasm-RKN isolate combination was inoculated with 600 J2, using the procedure described above. Each plant germplasm-RKN isolate combination was repeated five times. Fifteen days after nematode inoculation, five root systems of each RKN isolate-plant combination were washed free of subtrate, and were then fixed and rinsed following the procedure of Expósito et al. (2020). Images were acquired using a laser scanning confocal microscope (LSM 710 Axio Observer Z1 microscope with QUASAR detection unit; ZEN Black software) using a Plan-Neofluar 10×/0.3 objective, and Argon/2 (488 nm) and HeNe633 (633 nm) lasers, all of which are components from Carl Zeiss. Volumes were acquired with Z-stacks with a step size of 10 µm. The volumes and numbers of nuclei per GC, the numbers of GCs, and the volumes and numbers of nuclei per feeding site were determined using ImageJ and the TrakEM2 ImageJ plugin (ImageJ, version 1.50). This study was conducted once.

DATA ANALYSES

Statistical analyses were carried out using GraphPad Prism 7.00 (GraphPad Software). The normality of the data distributions and homogeneity of variances were determined with non-transformed or log10 (x+1) transformed data for parametric or non-parametric analyses. The nonparametric Mann-Whitney test was used to compare penetration (number of galls per plant), infectivity (number of egg masses per plant), reproduction (number of eggs per plant), and fecundity (number of eggs per egg mass) between the experimental repetitions. When significant differences (P ≤ 0.05) were observed, the values for each replicate were presented separately. Additionally, each parameter was compared between susceptible and the expected resistant germplasm of the same plant genus, or between paired comparisons of tomato plants and each of the susceptible cucurbit germplasms, by Student's t-test (P ≤ 0.05) when the data exhibited a normal distribution or Mann-Whitney test (P ≤ 0.05) if it did not. In addition, nonparametric Kruskal-Wallis analyses and Dunn's test (P ≤ 0.05) were used to compare each parameter between RKN isolate by plant germplasm combinations.

The numbers of nuclei per feeding site and GCs per feeding site, the volume of each GC, and the number of nuclei per GC from the histopathological study were compared ($P \leq 0.05$), between expected resistant and susceptible germplasms per plant genus, as well as the paired comparisons between tomato plants and each of the susceptible cucurbit germplasms. Data were compared using Student's t-test if the data fitted normal distributions; otherwise, the nonparametric Mann-Whitney test was used. In addition, nonparametric Kruskal-Wallis analysis and Dunn's test ($P \leq 0.05$) were used to compare each parameter among the RKN isolate by plant germplasm combinations.

Results

HOST SUITABILITY

Although general trends were observed, statistically significant differences ($P < 0.05$) were found between the experiments, results for each experiment are presented separately (Table 1). Second-stage juveniles of all RKN isolates penetrated the roots of each plant germplasm, leading to the formation of galls (Table 1). Among the susceptible germplasms, *M. chitwoodi* produced fewer ($P < 0.05$) galls on the cucurbit than on the tomato plants, while no differences ($P > 0.05$) were found between *M. enterolobii* and *M. luci*. Among the resistant germplasms, all the RKN isolates induced fewer ($P < 0.05$) galls than the susceptibles (Table 1). For nematode reproduction, all the RKN isolates developed until the adult female stage producing eggs, in all germplasms, except for *M. chitwoodi* in *C. metuliferus* (Table 1). Fewer ($P < 0.05$) egg masses per plant were produced in the resistant germplasms than in the susceptible germplasms of the same plant genus, except for *M. chitwoodi* in *Citrullus* spp. (Table 1). Concerning the levels of resistance of *C. amarus* to the RKN isolates, performed as resistant to *M. luci* (RI = 4.3 and 4.3%) in both experiments, and resistant or moderately resistant to *M. enterolobii* (RI = 6.7 and 12.2%) and *M. chitwoodi* (RI = 5.3 and 19.1%), depending on the experiment. Meanwhile, *C. metuliferus* was immune to *M. chitwoodi* (RI = 0), highly resistant to resistant to *M. enterolobii* (RI = 0.3 and 3.8%), and resistant *to M. luci* (RI = 1.6 and 1.8%).

Regarding the RKN isolates, *M. chitwoodi* produced fewer ($P < 0.05$) egg masses and eggs per plant on tomato plants than the other RKN isolates. *Meloidogyne luci* reproduced means of 5.5 and 11.3 more times in tomato than *M. chitwoodi* in experiment 1, and 2.6 and 1.7 more times than *M. enterolobii* in experiment 2 (Table 1).

Table 1. Number of galls, nematode egg masses and eggs per plant, and number of eggs per egg mass of *Meloidogyne chitwood*i, *M. enterolobii* or *M. luci*, in susceptible plants of *Solanum lycopersicum* 'Coração-de-Boi', *Cucumis sativus* 'Dasher II', and *Citrullus lanatus* 'Sugar Baby', or *Cucumis metuliferus* BGV11135 or *Citrullus amarus* BGV5167 rootstocks 56 d after inoculations with 200 second-stage juveniles per pot, in a climatic chamber in the two experiments.

	***Meloidogyne* species**	**Plant species**	**Galls**	**Egg masses per plant**	**Eggs per plant (10^2)**	**Egg per egg mass**	**Reproduction index (%)**	**Resistance level**
First experiment	***M. chitwoodi***	*S. lycopersicum*	>100 A	22 ± 4.0 C	74 ± 6.7 C	486 ± 111 A	nc	
		C. sativus	59 ± 6 B † *	1.8 ± 0.6 B †	0.9 ± 0.2 B †	23 ± 5 B †	0	
		C. metuliferus	7 ± 1 C	0 ± 0	0 ± 0	nc		I
		C. lanatus	28 ± 9 B † *	0.9 ± 0.4 B †	2.0 ± 0.4 C † *	120 ± 10 A †	5.3	
		C. amarus	8 ± 1 C	0.1 ± 0.1 B	0.01 ± 0.01 B	nc		R
	M. enterolobii	*S. lycopersicum*	>100 A	42 ± 2.3 B	144 ± 9.5 B	346 ± 22 A	nc	
		C. sativus	>100 A *	34 ± 2.3 A † *	44 ± 3.7 A † *	136 ± 16 A †	3.8	
		C. metuliferus	25 ± 2 B	0.8 ± 0.3 A	1.7 ± 1.4 A	179 ± 136 A		R
		C. lanatus	>100 A *	36 ± 3.0 A *	102 ± 9.0 A † *	295 ± 28 A	6.7	
		C. amarus	50 ± 5 B	4.2 ± 1.7 A	6.8 ± 2.5 A	191 ± 56 A		R
	M. luci	*S. lycopersicum*	>100 A	96 ± 4.5 A	382 ± 24.1 A	431 ± 25 A	nc	
		C. sativus	>100 A *	34 ± 2.3 A † *	36 ± 2.2 A † *	112 ± 10 A †	1.8	
		C. metuliferus	53 ± 3 A	0.3 ± 0.2 A	0.7 ± 0.5 A	218 ± 43 A		R
		C. lanatus	>100 A *	9 ± 2.0 B † *	24 ± 5.6 B † *	242 ± 50 A †	4.3	
		C. amarus	79 ± 5 A	0.8 ± 0.3 B	1.0 ± 0.6 AB	157 ± 106 A		R

Second experiment	***M. chitwoodi***	*S. lycopersicum*	>100 A	27 ± 3.0 B	57 ± 7.3 C	221 ± 30 C	nc	
		C. sativus	37 ± 2 † B *	5.6 ± 1.8 B †	5.6 ± 0.3 B †	90 ± 25 C †	0	I
		C. metuliferus	20 ± 1 B	0 ± 0	0 ± 0	nc		
		C. lanatus	33 ± 3 B † *	0.7 ± 0.4 B †	0.8 ± 0.5 C †	114 ± 39 B †	19.1	MR
		C. amarus	13 ± 2 C	0.2 ± 0.2 B	0.2 ± 0.2 B	nc		
	M. enterolobii	*S. lycopersicum*	>100 A	105 ± 9.0 A	380 ± 33.5 B	393 ± 55 B	nc	
		C. sativus	>100 A *	34 ± 5.0 A † *	224 ± 39.3 A † *	801 ± 172 A *	0.3	HR
		C. metuliferus	39 ± 5 A	0.3 ± 0.2 A	0.6 ± 0.4 A	200 ± 16 B		
		C. lanatus	>100 A *	79 ± A 7.0 † *	412 ± 26.9 A *	548 ± 50 A †	12.2	MR
		C. amarus	66 ± 6 A	8,3 ± 1.5 A	50 ± 10.4 A	603 ± 70 A		
	M. luci	*S. lycopersicum*	>100 A	121 ± 6.0 A	647 ± 22.5 A	544 ± 26 A	nc	
		C. sativus	>100 A *	36 ± 4.0 A † *	160 ± 23.0 A † *	442 ± 42 B	1.6	R
		C. metuliferus	25 ± 2 A	0.4 ± 0.2 A	2.6 ± 2.1 A	510 ± 162 A		
		C. lanatus	>100 A *	7 ± 2.0 B † *	27 ± 7.3 B † *	371 ± 65 AB †	4.3	R
		C. amarus	50 ± 5 B	0.6 ± 0.2 B	1.1 ± 0.5 B	222 ± 89 B		

Data are means ± standard errors of ten replicates. Data in each column followed by different letters are significantly different ($P < 0.05$) between root-knot nematode (RKN) isolates for a given plant germplasm, according to Dunn's test. Data for each column and each RKN isolate followed by * indicate significant differences ($P < 0.05$) between germplasms of the same genus, and by † indicate differences ($P < 0.05$) between *Solanum lycopersicum* and *Cucumis sativus* or *Citrullus lanatus*, as shown by Student's t tests or Mann-Whitney tests. nc = Not calculated. Reproduction index: percentage of the eggs produced in the resistant germplasm compared with those produced in the susceptible germplasm. Resistance level: I = immune (RI = 0), HR = highly resistant (RI < 1%), R = resistant (1% ≤ RI ≤ 10%), MR = moderately resistant (10% < RI ≤ 25%), SR = slightly resistant (25% < RI ≤ 50%) or S = susceptible (RI > 50%), as categorized by Hadisoeganda and Sasser (1982).

In *C. sativus, M. chitwoodi* produced fewer (P < 0.05) egg masses and eggs per plant than *M. enterolobii* and *M.luci*, which were not different for the numbers of eggs per egg mass in the second experiment. For *C. metuliferus*, *M. chitwoodi* induced fewer (P < 0.05) root galls than the other RKN isolates, but no reproduction was detected. In *Citrullus* spp., *M. enterolobii* produced more (P < 0.05) egg masses (4.0 to 112.9 times more in *C. lanatus*; 5.3 to 42.0 times more in *C. amarus*) and eggs per plant (4.3 to 515.0 times more in *C. lanatus*; 6.8 to 680.0 times more in *C. amarus*) than the other RKN isolates (Table 1).

HISTOPATHOLOGY

Fifteen days after nematode inoculations, only the *M.enterolobii* and *M. luci* isolates were able to infect the roots of all the assessed plant germplasms (Table 2; Figures 1 to 4). *Meloidogyne chitwoodi* only infected tomato and cucumber roots (Table 2; Figures 1 and 4). Despite *M. chitwoodi* J2 being observed inside the roots of *C. metuliferus* and *C. lanatus*, no GCs were induced (Figure 4, B and C); therefore, comparisons were only valid between tomato and cucumber. The number and volume of GCs per feeding site and the number of nuclei per GC and per feeding site did not differ (P > 0.05) between the tomato and cucumber plants (Table 2).

Meloidogyne enterolobii induced a similar (P > 0.05) number of GCs in *C. metuliferus* and cucumber. However, the volumes of the GCs in C. metuliferus were six times less (P < 0.05) than in cucumber, resulting in a 9.5-fold reduction (P < 0.05) in the total volume of GCs per feeding site. The number of nuclei per GC and per feeding site were 2.9 and 5.5 times greater (P < 0.05) in cucumber than in *C. metuliferus* (Table 2). Similar results were observed in watermelon. Although the nematodes induced similar (P > 0.05) numbers of GCs per feeding site in both *Citrullus* spp., the volumes per GC were 13.3 greater in *C. lanatus* and 8.5 times greater (P < 0.05) than in *C. amarus*. The numbers of nuclei per GC were 3.4 greater, and per feeding site were 2.8 greater (P < 0.05).

Meloidogyne luci induced a similar (P > 0.05) numbers of GCs in *C. metuliferus* and cucumber, but the GC volumes in *C. metuliferus* were 11 times less (P < 0.05) than in cucumber, resulting in a 12.1-fold reduction (P < 0.05) in total volume of GC per feeding site. However, the numbers of nuclei per GC and per feeding site did not differ (P > 0.05) (Table 2). In both *Citrullus* species, *M. luci* induced similar numbers (P > 0.05) of GCs, but GC volumes and numbers per feeding site in *C. amarus* were 2.5 and 3 times less (P < 0.05) than in in *C. lanatus*. In addition, 3.1 times fewer nuclei per GC (P < 0.05) and 3.2 times fewer feeding sites were observed in *C. amarus* than in *C. lanatus*.

Table 2. Number of giant cells per nematode feeding site (GC·fs-1), number of nuclei per giant cells (N·GC-1), number of nuclei per feeding site (N·fs-1), giant cell volume (GCV) and giant cell volume per feeding site (GCV·fs-1), in *Solanum lycopersicum* 'Coração-de-Boi', *Cucumis sativus* 'Dasher II' and *Citrullus lanatus* 'Sugar Baby' plants, and *Cucumis metuliferus* BGV11135 and *Citrullus amarus* BGV5167 rootstocks, 15 d after nematode inoculations with 200 or 600 second-stage juveniles per pot [a], in susceptible or rootstocks respectively.

Meloidogyne species	Plant species	GC/fs	N/GC	N/fs	GCV (μm^3 10^{-5})	GCV/fs (μm^3 10^{-5})
M. chitwoodi	*S. lycopersicum*	5 ± 1.0 A	14 ± 3.2 B	44 ± 8.8 B	8 ± 1.1 B	26 ± 3.0B
	C. sativus	4 ± 0.2 A	9 ± 1.7 B	29 ± 6.4 C	5 ± 0.9 C	22 ± 3.4 B
	C. metuliferus	na	na	na	na	na
	C. lanatus	na	na	na	na	na
	C. amarus	na	na	na	na	na
M. enterolobii	*S. lycopersicum*	5 ± 0.8 A	26 ± 3.1 A	131 ± 6.7 A	14 ± 1.9 AB	70 ± 7.8 A
	C. sativus	9 ± 0.9 A	20 ± 1.7 A*	181 ± 8.3 A*	12 ± 1.8 B*	114 ± 23.1 A*
	C. metuliferus	5 ± 0.8 A	7 ± 1.5 A	33 ± 5.6 B	2 ± 0.6 A	12 ± 2.0 A
	C. lanatus	5 ± 0.7 A	17 ± 2.8 A*	79 ± 10.7 A*†	40 ± 13.8 A*†	170 ± 32.8 A*†
	C. amarus	6 ± 1.7 A	5 ± 0.4 A	28 ± 3.2 A	3 ± 0.4 A	20 ± 3.0 A
M. luci	*S. lycopersicum*	4 ± 0.4 A	30 ± 5.2 A	138 ± 31.2 A	19 ± 4.7 A	87 ± 24.1 AB
	C. sativus	6 ± 0.4 A	16 ± 2.1 A†	89 ± 14.7 B	33 ± 5.5 A*†	181 ± 29.6 A*†
	C. metuliferus	9 ± 0.8 A	9± 1.4 A	59 ± 7.3 A	3 ± 0.8 A	15 ± 2.6 A
	C. lanatus	5 ± 0.5 A	22 ± 2.9 A*	112 ± 8.7 A*	12 ± 1.9 B*	65 ± 9.8 B*
	C. amarus	5 ± 0.7 A	7 ± 0.3 A	35 ± 3.3 A	5 ± 0.2 A	22 ± 1.8 A

Data are means ± standard errors for five replicates. Data in the same column followed by different letters are significantly different ($P < 0.05$) between root-knot nematode (RKN) isolates by a given plant germplasm, according to Dunn's test. Data in each column and for each RKN isolate followed by * are significantly different ($P < 0.05$) between germplasms of the same genus. † indicates differences ($P < 0.05$) between *Solanum lycopersicum* and *Cucumis sativus* or *Citrullus lanatus*, according to Student's t or Mann-Whitney tests. na = No available data because no infection was observed.

The majority of GCs induced by *M. enterolobii* and *M. luci* in *C. metuliferus* and *C. amarus* were almost empty, with few or no nuclei and with some necrotic areas compared to those in the respective susceptible plant germplasm (Figure 2 B and D, Figure 3 B and D).

Of the different RKN isolates, *M. enterolobii* induced formation of GCs that were 3.3 more voluminous ($P < 0.05$) than *M. luci* in *C. lanatus*, which resulted in a total mean GC volume per feeding site that was 2.6 times greater ($P < 0.05$). Nevertheless, no differences ($P > 0.05$) were observed in *C. amarus*. The numbers of nuclei per GC and per feeding site induced by *M. enterolobii* and *M. luci* in both *Citrullus* spp. did not differ ($P > 0.05$), but the numbers of nuclei per feeding site differed ($P < 0.05$) in *Cucumis* spp. (Table 2). Specifically, the number of nuclei per feeding site induced by *M. enterolobii* was 2 times greater in *C. sativus* and 0.56 times greater in *C.metuliferus*, compared to those induced by *M. luci* (Table 2). *Meloidogyne enterolobii* induced the formation of 1.8 times more GC volume ($P < 0.05$) in *S. lycopersicum* than *M. chitwoodi*, resulting in 2.7 more GC volume per feeding site ($P < 0.05$).

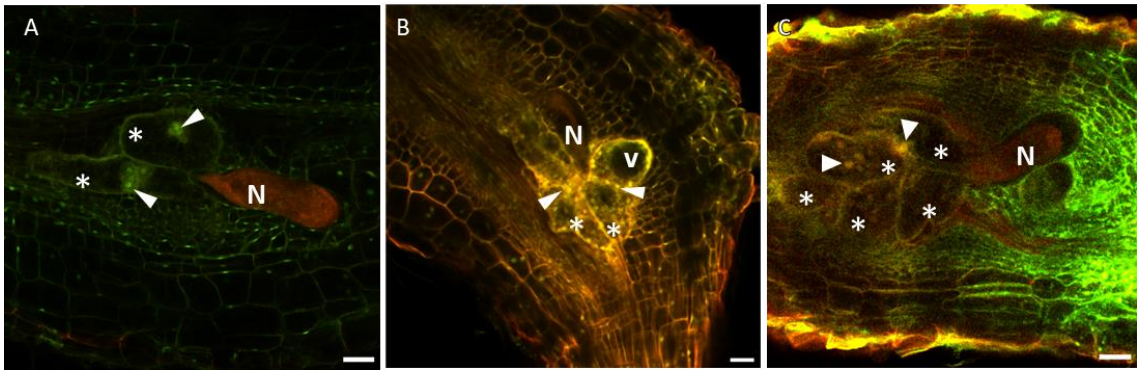


Figure 1. Laser scanning confocal microscope images of the infection sites of *Meloidogyne chitwoodi* (A), *Meloidogyne enterolobii* (B) and *Meloidogyne luci* (C), 15 d after inoculation, in *Solanum lycopersicum* 'Coração-de-Boi'. Nematode (N); vacuoles (v); giant cells (asterisks); and some nuclei (white arrowheads) are indicated. Scale bars = 50 µm.

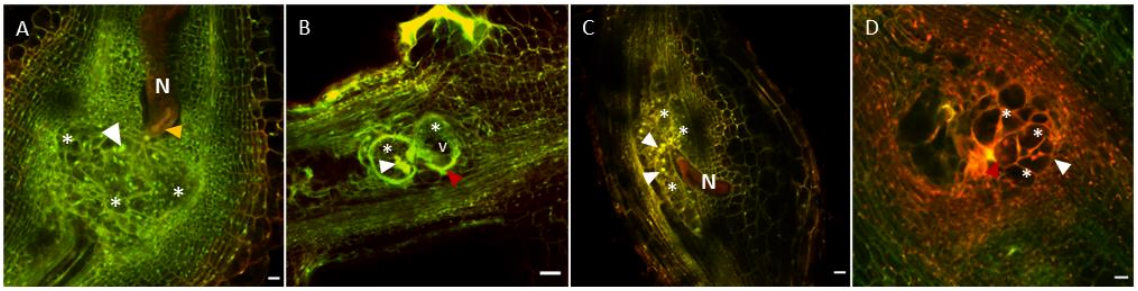


Figure 2. Laser scanning confocal microscope images of infection sites of *Meloidogyne enterolobii*, 15 d after inoculation, in *Cucumis sativus* 'Dacher II' (A), *Cucumis metuliferus* BGV11135 (B), *Citrullus lanatus* 'Sugar Baby' (C) or *Citrullus amarus* BGV5167 (D). Nematodes (N); vacuoles (v); giant cells (asterisks); some nuclei (white arrowheads); necrosed areas (red arrowheads); and a nematode oesophageal median bulb (yellow arrowhead) are indicated. Scale bars = 50 µm.

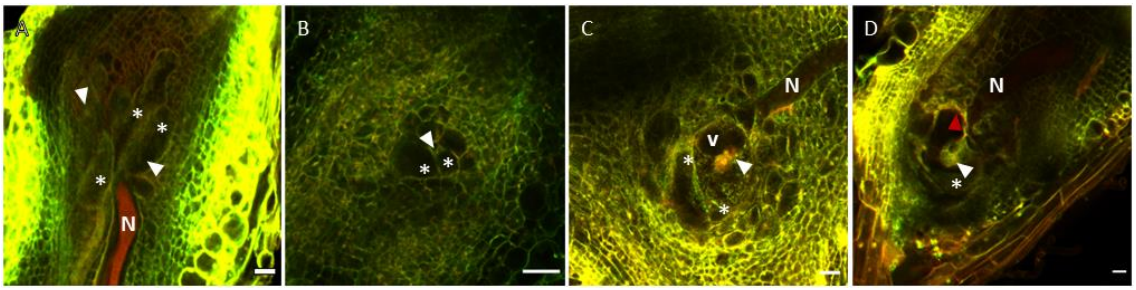


Figure 3. Laser scanning confocal microscope images of infection sites of *Meloidogyne luci* 15 d after inoculation in *Cucumis sativus* 'Dacher II' (A), *Cucumis metuliferus* BGV11135 (B), *Citrullus lanatus* 'Sugar Baby' (C), or *Citrullus amarus* BGV5167 (D). Nematodes (N); giant cells (asterisks); some nuclei (white arrowheads); and necrosed area (red arrowhead) are indicated. Scale bars = 50 µm.

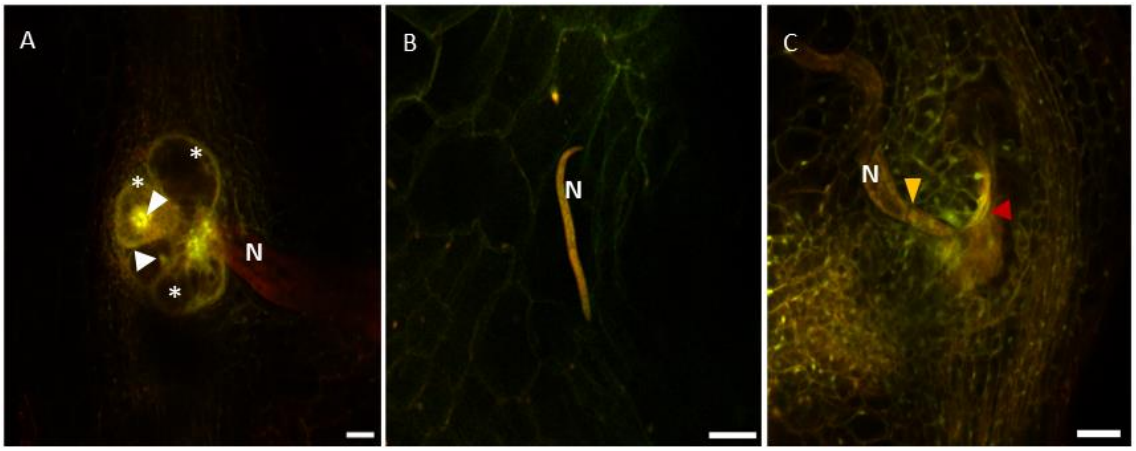


Figure 4. Laser scanning confocal microscope images of *Meloidogyne chitwoodi* infection sites, 15 d after inoculation in the cucumbers *Cucumis sativus* 'Dacher II' (A), *Cucumis metuliferus* BGV11135 (B), watermelon *Citrullus lanatus* 'Sugar Baby' (C), or *Citrullus amarus* BGV5167 (D). Nematodes (N); giant cells (asterisks); some nuclei (white arrowheads); necrosed area (red arrowhead), and an oesophageal median bulb (yellow arrowhead) are indicated. Scale bars = 50 µm.

Discussion

The main objective of this study was to determine host suitability of *C. metuliferus* BGV11135 and *C. amarus BGV5167* for the nematodes *M. chitwoodi, M. enterolobii* and *M. luci,* to provide insights into the potential use of these rootstocks for melon and watermelon crops, and to provide this information to assist management of RKN species. Previous studies have reported resistance of some *C. metuliferus* accessions to *M. incognita, M. arenaria, M. hapla, M. javanica* and *M. enterolobii* (Walters et al., 2006; Ye et al., 2017; Pinheiro et al., 2019), and that of *C. amarus* to *M. arenaria*, *M. enterolobii, M. incognita* and *M. javanica* (GarcíaMendívil et al., 2019; Waldo et al., 2023). The present paper is the first report on levels of resistance of *C. metuliferus* and *C. amarus* to *M. chitwoodi* and *M. luci*. In addition, cucumber may be included as a potential plant host of *M. chitwoodi*, because this nematode reproduced in this plant species, as in watermelon which is listed as a plant host (EPPO, 2023b).

The results of the present study have shown that the levels of resistance of *C. metuliferus* ranged from immune (RI = 0) to *M. chitwoodi*, highly resistant (RI < 1%) to resistant (1% ≤ RI < 10%) to *M. enterolobii*, and resistant to *M. luci*. *Citrullus amarus* ranged from resistant to moderately resistant (10% ≤ RI ≤ 25%) to *M. chitwoodi* and *M. enterolobii* and resistant to *M. luci*.

Several resistance mechanisms of *C. metuliferus* against RKN have been proposed, affecting root penetration, feeding site formation, nematode development, and sex differentiation (Fassuliotis, 1970; Walters et al., 2006). Xie et al. (2022) reported the emission of 18 volatiles by the roots of the CM3 accession of *C. metuliferus*, which had repellent effects on *M. incognita*. In the present study, substantial reductions of J2s root penetration of all the RKN isolates were observed, compared to that in cucumber, and only a low proportion of J2 achieved the adult female stage laying eggs (0% for *M. chitwoodi*, 2% for *M. enterolobii* and 1.1% for *M. luci*; averaged over two experiments). Some studies comparing the transcriptome of *C. metuliferus* and cucumber plants inoculated with *M. incognita* have proposed putative resistance mechanisms (Ling et al., 2017; Ye et al., 2017; Li et al., 2021). Ling et al. (2017) attributed resistance to differential expression in two host gene clusters related to cytoskeletons and RNA processing. Ye et al. (2017) attributed resistance to induction of phenylalanine ammonia-lyase and peroxidase activities after infection together with the expression of genes related to biosynthesis of phenylpropanoids and plant hormone signalling. Li et al. (2021) attributed resistance to upregulation of genes related to the Ca2+ signalling pathway at early stages of *M. incognita* infection, as well as the salicylic acid and jasmonate signalling pathways. In all these cases, nematode penetration and root infection were reduced, and nematode development was delayed. According to the present study results, the resistance mechanisms of *C. metuliferus* were highly effective against *M. chitwoodi*, because less J2 were able to penetrate, compared to *M. enterolobii* and *M. luci*, and no J2 reached the adult female stage. The histopathological analysis showed that *C. metuliferus* was not infected at 15 d after *M. chitwoodi* inoculation, and those that infected cucumber plants produced less voluminous GCs with a low numbers of nuclei per GC and per feeding site than did the other studied RKN species. For *M. enterolobii* and *M. luci*, reductions in nematode infection and reproduction were detected in *C. metuliferus* in comparison with cucumber, but J2, which were able to infect, to develop until the female stage and reproduce, produced a similar number of eggs per egg mass than in cucumber (except for *M. enterolobii* in the second experiment). However, a reduction in female fertility of *M. incognita* on *C. metuliferus* has been reported previously (Ye et al., 2017; Expósito et al., 2020). This result is important, because it could be an indicator of adaptation of a given percentage of individuals that could reproduce and increase populations after repeated cultivation. The

present histopathological study showed some differences from previous studies regarding the *C. metuliferus-M. incognita* relationship (Ye et al., 2017; Expósito et al., 2020), in which fewer nuclei per cell and per feeding sitewere reported.

Resistance of *C. amarus* to tropical *Meloidogyne* spp. has been attributed to its high root fibrosity in comparison with that of other cucurbits (Thies and Levi, 2007; Thies et al., 2015; García-Mendivil et al., 2019). Waldo et al. (2023) suggested that resistance to *M. enterolobii* is modulated by 11 single-nucleotide polymorphisms. Those in the locus QTL 3.1 influence root galling and egg mass formation, while those in QTL 4.1, 4.2, and 8.1 are associated with nematode egg production. In the present study, compared with those of watermelon, J2 root penetration of all the RKN isolates was reduced, and only a low proportion of J2 achieved the adult female stage laying eggs: 1.4% for *M. chitwoodi*, 10.5% for *M. enterolobii*, and 1.1% for *M. luci* (averaged for the two experiments). Watermelon is considered a poor host for the tropical *Meloidogyne* spp. due to their reduced reproduction rates (López-Gómez et al., 2014), but is a main host for *M. enterolobii* (EPPO, 2023b). This was observed in the present study, achieving levels of reproduction close to those in tomato. However, *M. enterolobii* reproduction in *C. amarus* reached 9.45% of that observed on watermelons, defining the *C. amarus* rootstock as an effective tool for managing this RKN.

Histopathological analyses revealed that neither *C. lanatus* nor *C. amarus* were infected by *M. chitwoodi* 15 days after inoculations. Reductions in the numbers of nuclei and GC volumes were observed in the combinations of remaining RKN-isolates in *C. amarus* compared with watermelon, which may affect nematode development and reproduction.

The results from the present study will provide valuable information for farmers to facilitate decision-making for implementing integrated RKN control strategies, including scenarios with a co-occurrence of RKN species and/or virulent nematode populations to specific host resistant genes. Resistance of these plant species to tropical RKN species in pot and field experiments (Ye et al., 2017; García-Mendívil et al., 2019), and the effectiveness for managing virulent RKN populations to the *Mi1.2* resistance gene in tomato (Expósito et al., 2018; Fullana et al., 2023) have been demonstrated. In addition, several accessions of *C. metuliferus* and *C. amarus* are resistant to other pathogens and diseases, such as *Fusarium oxysporum*, gummy stem blight, powdery mildew, and potyvirus (Gusmini et al., 2005; Guner et al., 2008; Tetteh et al., 2010; Keinath et al., 2019). These characteristics enhance agronomic value of these plant germoplasm. The strategic use of these rootstocks in rotations with other resistant plant germplasms can

alleviate the impacts of RKN on crop yield and contribute to reducing reliance on pesticides, as has been previously reported (Expósito et al., 2018; Fullana et al., 2023).

Funding

The UPC authors acknowledge funding from the R+D+i project AGL2017-89785-R, financed by MCIN/AEI/10.13039/501100011033 and by the FEDER and FSE (PRE2018-084265, Aïda Magdalena Fullana). In UC, this research was supported by FEDER funds through the Portugal 2020 (PT 2020), COMPETE 2020 and by the Portuguese Foundation for Science and Technology (FCT), under contracts UIDB/00102/2020, UIDP/00102/2020 (CERES), and UIDB/04004/2020 (DOI identifier 10.54499/ UIDB/04004/2020), UIDP/04004/2020 (DOI identifier 10.54499/UIDP/04004/2020) (CFE); and by 'Instituto do Ambiente, Tecnologia e Vida'. Duarte Santos is funded by FCT through the grant SFRH/BD/146196/2019.

Acknowledgments

The authors are grateful to the Institut de Conservació i Millora de l'Agrodiversitat Valenciana from Universitat Politècnica de Valencia (COMAV-UPV) for providing seeds of *Cucumis metuliferus* BGV11135 and *Citrullus amarus* BGV5167. Dr Teresa Rodrigues, iLAB– Microscopy and Bioimaging Lab, a facility of the Faculty of Medicine of University of Coimbra and a member of the national infrastructure PPBI-Portuguese Platform of BioImaging (POCI-01-0145-FEDER-022122) and supported by FSE CENTRO-04-3559-FSE-000142, gave technical support with the laser scanning confocal microscopy. The NEMATO-lab members are also acknowledged for their technical laboratory assistance.

References

Aydinli, G., Kurtar, E. S., Mennan, S. (2019). Screening of *Cucurbita maxima* and *Cucurbita moschata* genotypes for resistance against *Meloidogyne arenaria*, *M. incognita*, *M. javanica*, and *M. luci*. Journal of Nema tology 51, 2019–2057.

Bent, E., Loffredo, A., McKenry, M. V., Becker, J. O., Borneman, J. (2008). Detection and investigation of soil biological activity against *Meloidogyne incognita*. Journal of Nematology 40(2), 109–118

Brown, C. R., Mojtahedi, H., Santo, G.S., Williamson, V.M. (1997). Effect of the *Mi* gene in tomato on reproductive factors of *Meloidogyne chitwoodi* and *M. hapla*. Journal of Nematology 29(3), 416-419

Castagnone-Sereno, P. (2012). *Meloidogyne enterolobii* (= *M. mayaguensis*): profile of an emerging, highly pathogenic, root-knot nematode species. Nematology 14(2), 133–138.

Elling, A. A. (2013). Major emerging problems with minor *Meloidogyne species*. Phytopathology 103, 1092–1102.

EPPO (2016). PM 7/41 (3) *Meloidogyne chitwoodi* and *Meloidogyne fallax*. Bulletin OEPP/EPPO 46(2), 171– 189

EPPO (2017). EPPO Alert List: Addition of *Meloidogyne luci* together with *M. ethiopica*. EPPO Reporting Service, 2017/2018. Available at: https://gd.eppo.int/reporting/article-6186. Accessed August 05, 2023

EPPO (2023) a. EPPO Alert List 2013-10. Available at: https://eppo.int/ACTIVITIES/plant_quarantine/alert_list. Accessed November 03, 2023

EPPO (2023) b. *Meloidogyne chitwoodi.* EPPO global database. Available at: https://gd.eppo.int/taxon/MELGCH. Accessed November 03, 2023

EPPO (2023) c. *Meloidogyne enterolobii*. EPPO global database. Available at: https://gd.eppo.int/taxon/MELGMY. Accessed August 05, 2023

EPPO (2023) d. *Meloidogyne luci*. EPPO global database. Available at: https://gd.eppo.int/taxon/MELGLC. Accessed August 05, 2023

Expósito, A., Munera, M., Giné, A., López-Gómez, M., Cáceres, A., Picó, B. et al. (2018). *Cucumis metuliferus* is resistant to root-knot nematode *Mi1.2* gene (a)virulent isolates and a promising melon rootstock. Plant Pathology 67, 1161–1167.

Expósito, A., García, S., Giné, A., Escudero, N., Sorribas, F. J. (2019). *Cucumis metuliferus* reduces *Meloidogyne incognita* virulence against the *Mi1.2* resistance gene in a tomato-melon rotation sequence. Pest Management Science 75, 1902–1910.

Expósito, A., Pujolá, M., Achaerandio, I., Giné, A., Escudero, N., Fullana, M. A., et al. (2020). Tomato and melon *Meloidogyne* resistant rootstocks improve crop yield but melon fruit quality is influenced by the cropping season. Frontiers Plant Science 11, 560024-14.

Fassuliotis, G. (1970). Resistance of *Cucumis* spp. to the root-knot nematode, *Meloidogyne incognita acrita.* Journal of Nematology 2, 174–178

Fullana, A. M., Expósito, A., Escudero, N., Cunquero, M., Loza-Alvarez, P., Giné, A., et al. (2023). Crop rotation with *Meloidogyne*-resistant germplasm is useful to manage and revert the (a)virulent populations of *Mi1. 2* gene and reduce yield losses. Frontiers Plant Science 14, 1133095-13.

García-Mendívil, H. A., Munera, M., Giné, A., Escudero, N., Picó, M. B., Gisbert, C., et al. (2019). Response of two *Citrullus amarus* accessions to isolates of three species of *Meloidogyne* and their graft compatibility with watermelon. Crop Protection 119, 208–213.

Giné, A., López-Gómez, M., Vela, M. D., Ornat, C., Talavera, M., Verdejo-Lucas, S., et al. (2014). Thermal requirements and population dynamics of root-knot nematodes on cucumber and yield losses under protected cultivation. Plant Pathology 63(6), 1446–1453.

Guner, N., Wehner, T. C., Pitrat, M. (2008). Overview of potyvirus resistance in watermelon. In: *Proceedings of the IXth EUCARPIA Meeting on Genetics and Breeding of* Cucurbitaceae. (M Pitrat ed.), Institut National de la Recherche Agronomique, Avignon, France, 445–451.

Gusmini G., Song R., Wehner T.C., (2005). New sources of resistance to gummy stem blight in watermelon. *Crop Science* 45(2), 582–588.

Hadisoeganda W.W., Sasser J.N., (1982). Resistance of tomato, bean, southern pea, and garden pea cultivars to root-knot nematodes based on host suitability. *Plant Disease* 66, 145–150.

Hanna, H. Y. (2000). Double-cropping muskmelons with nematode-resistant tomatoes increases yield, but mulch color has no effect. HortScience 35, 1213– 1214. doi: org/10.21273/HORTSCI.35.7.1213

Holbrook, C. C., Knauf, D. A., Dickson, D. W. (1983). A technique for screening peanut for resistance to *Meloidogyne incognita*. Plant Disease 57, 957–958

Hussey, R. S., Barker, K. R. (1973). A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. Plant Disease Report 57, 1025– 1038

Jones, J. T., Haegeman, A. J., Danchin, E. G., Gaur, H. S., Helder, J. K., Jones, M., G., K., et al., (2013). Top 10 plantparasitic nematodes in molecular plant pathology. Molecular Plant Pathology 14, 946–961.

Keinath, A. P., Wechter, W. P., Rutter, W. B., Agudelo, P. A. (2019). Cucurbit rootstocks resistant to *Fusarium oxysporum f.* sp. niveum remain resistant when coinfected by *Meloidogyne incognita* in the field. Plant Disease 103, 1383–1390.

Koutsovoulos, G. D., Poullet, M., Elashry, A., Kozlowski, D. K., Sallet E., Da Rocha, M., et al. (2020). Genome assembly and annotation of *Meloidogyne enterolobii*, an emerging parthenogenetic root-knot nematode. Scientific Data 7(1), 324.

Li, X., Sun, Y., Yang, Y., Yang, X., Xue, W., Wu, M., et al. (2021). Transcriptomic and histological analysis of the response of susceptible and resistant cucumber to *Meloidogyne incognita* infection revealing complex resistance via multiple signalling pathways. Frontiers Plant Science 12, 675429-675441. Ling, J., Mao, Z., Zhai, M., Zeng, F., Yang, Y., Xie, B. (2017). Transcriptome profiling of *Cucumis metuliferus* infected by *Meloidogyne incognita* provides new insights into putative defense regulatory network in Cucurbitaceae. Scientific Reports 7, 3544–3559.

López-Gómez, M., Giné, A., Vela, M. D., Ornat, C., Sorribas F. J., Talavera, M., et al., (2014). Damage functions and thermal requirements of *Meloidogyne javanica* and *Meloidogyne incognita* on watermelon. Annals of Applied Biology 165(3), 466–473.

Maleita, C., Cardoso, J. M., Rusinque, L., Esteves, I., Abrantes, I. (2021). Species-specific molecular detection of the root knot nematode *Meloidogyne luci*. Biology 10, 775–788.

Maleita, C., Correia, A., Abrantes, I., Esteves, I. (2022). Susceptibility of crop plants to the root-knot nematode *Meloidogyne luci*, a threat to agricultural productivity. Phytopathologia Mediterranea 61, 169–179.

Ornat, C., Verdejo-Lucas, S., Sorribas, F. J. (1997). Effect of the previous crop on population densities of *Meloidogyne javanica* and yield of cucumber. Nematropica 27, 85–90.

Pais, C. S., Abrantes, I., Fernandes, M. F. M., Santos, M. S. N. A. (1986). Tecnica de electroforese aplicada ao estudo das enzimas dos nematodes-das-galhasradiculares, *Meloidogyne* spp. Ciência Biológica Ecology and Systematics 6, 19–34.

Phan, N. T., Waele, D., Lorieux, M., Xiong, L., Bellafiore, S. (2018). A hypersensitivity-like response to *Meloidogyne graminicola* in rice (*Oryza sativa*). Phytopathology 108(4), 521–528.

Pinheiro, J. B., Silva, G. O. D., Oliveira, V. R., Amaro, G. B., Morais A. A. D. (2019). 'Prospection of genetic resistance resources to root-knot nematodes in cucurbit genotypes. Horticultura Brasiliera 37, 343–347.

Roberts, P. A. (2002). 'Concepts and consequences of resistance' in: Plant Resistance to Parasitic Nematodes. Eds. J. L. Starr, R. Cook, J. (Bridge Wallingford, UK: CAB International), 25–41

Sorribas, F. J., Ornat, C., Verdejo-Lucas, S., Galeano, M., Valero, J. (2005). Effectiveness and profitability of the Mi-resistant tomatoes to control root-knot nematodes. European Journal of Plant Pathology 111: 29–38.

Sorribas, F. J., Djian-Caporalino, C., Mateille, T. (2020). 'Nematodes' in: Integrated pest and disease management in greenhouse crops. Eds. M. L. Gullino, R. Albajes, P. C. Nicot (Springer Cham, Switzerland), 147– 174

Tetteh, A. Y., Wehner, T. C., Davis, A. R. (2010). Identifying resistance to powdery mildew race 2W in the USDA-ARS watermelon germplasm collection. Crop Science 50(3), 933-939.

Thies, J. A., Levi, A. (2007). Characterization of watermelon (*Citrullus lanatus* var. citroides) germplasm for resistance to root-knot nematodes. HortScience 42(7), 1530–1533.

Thies, J. A., Ariss, J. J., Hassell, R. L., Buckner, S., Levi, A. (2015). Accessions of *Citrullus lanatus* var. citroides are valuable rootstocks for grafted watermelon in fields infested with root-knot nematodes. HortScience 50, 4–8

Waldo, B. D., Branham, S. E., Levi, A., Wechter, W. P., Rutter, W. B. (2023). Distinct Genomic Loci Underlie Quantitative Resistance to *Meloidogyne enterolobii* Galling and Reproduction in *Citrullus amarus*. Plant Disease 107(7), 2126–2132.

Walters, S. A., Wehner, T. C., Daykin, M. E., Barker, K. R. (2006). Penetration rates of root-knot nematodes into *Cucumis sativus* and *C. metuliferus* roots and subsequent histological changes. Nematropica 36, 231–242

Xie, X., Ling, J., Mao, Z., Li, Y., Zhao, J., Yuhong, Y., et al. (2022). Negative regulation of root-knot nematode parasitic behavior by root-derived volatiles of wild relatives of *Cucumis metuliferus* CM3. Horticulture Research 9, uhac051.

Ye, D. Y., Qi, Y. H., Cao, S. F., Wei, B. Q., Zhang, H. S. (2017). Histopathology combined with transcriptome analyses reveals the mechanism of resistance to *Meloidogyne incognita* in *Cucumis metuliferus*. Journal of Plant Physiology 212, 115–124.

General discussion

General discussion

Plant resistance is the most environmentally, friendly and, economically viable practice for suppressing population densities of the most frequent RKN species in the Mediterranean basin and reducing vegetable crop yield losses (Sorribas et al., 2005). Moreover, the benefit of using plant resistance is also evident in the following crop in a rotation sequence because of the reduction in the primary inoculum (Giné and Sorribas, 2017b; Ornat et al., 1997). Unfortunately, vegetable-resistant cultivars or rootstocks of the RKN species are only commercially available for tomato (*Mi1.2* gene), pepper (*N, Me1, Me3/Me7* genes), eggplant (*Solanum torvum*), and watermelon (*Citrullus amarus*), and repeated use of plant germplasm carrying the same resistance gene leads to the selection of resistance-breaking populations. In fact, virulent *Meloidogyne* populations to the *Mi1.2* resistance gene of tomato, *Me3/Me7*, and N resistance genes of pepper, as well as *S. torvum* rootstock have been described (Castagnone-Serono et al. 2001; Ornat et al., 2001; Ros-Ibáñez et al., 2014; Thies, 2011; Uehara et al., 2017; Verdejo-Lucas et al., 2012;). The use of more than one resistance gene either by pyramiding them in a single plant genotype, alternating, in mixture, or in sequences with susceptible ones, has been proposed to avoid selection for virulence (Djian-Caporalino et al., 2014). However, this PhD thesis proposal has certain limitations. First, these authors pyramided the *Me1* and *Me3* resistance genes in an experimental pepper line, which is the most efficient method for suppressing RKN without selecting for virulence. Gene pyramiding could also be used in other important commercial crops, such as tomato, because several other RKN-R genes in addition with *Mi1.2* have been described, but nowadays, there are no commercial cultivars carrying different RKN resistance genes. In relation to alternating resistant RKN crops, Expósito et al. (2019) showed that alternating two different sources of resistance, grafted tomato onto the resistant rootstock 'Aligator' and melon grafted onto *C. metuliferus,* in rotation sequences did not prevent the selection of virulence to the *Mi1.2* resistance gene, although the level of virulence was attenuated; but, if several resistance sources were used, it would be possible to avoid virulence selection to specific R genes, but to our knowledge, there is no information on this for vegetable crops, and consequently it was assessed in Chapter 1. With regard to mixing RKN-resistant germplasms carrying different R genes, this approach can only be carried out in pepper crops for which several R genes are commercially available, but the main constraint is that the pepper cultivars would only differ in the R gene having the same physicochemical and organoleptic characteristics; otherwise, it entails additional work for the farmer, who would harvest by cultivar in order not to mix them for commercial reasons, which would mean an

increase in production costs. Another possibility is to use vegetable-resistant rootstocks that are compatible with the cash crop without affecting crop yield quantity and quality, but differ in the resistance source. In this thesis, tomato grafted onto the eggplant rootstock *S. torvum* was assessed to determine its effect on RKN population dynamics, tomato crop yield quantity and quality, and the putative selection for virulence (Chapter 2). In addition to plant resistance mediated by R genes, plant resistance can also be induced by biotic and abiotic agents (Conrath, 2006), and could be used as new sources of resistance in order to be used in rotation or mixing plant resistant germplasm to increase the number and/or the expression of plant defence mechanisms being more effective in suppressing RKN and, perhaps, decreasing the possibility of selecting for virulence. Characterization of these inducer agents can provide new possibilities for the management of RKN in vegetable crops based on plant resistance. This issue was studied in Chapter 3.

According to the arguments presented above, the main aim of this thesis was to obtain basic and applied knowledge to answer questions that can help to design management strategies for *Meloidogyne*-resistant plant germplasm to avoid the selection of virulent nematode populations. Specifically, we investigated how crop rotation with different sources of resistance affects the development of nematode populations and virulence selection to specific R genes (Chapter 1). In tomato monoculture cultivation, is it possible to use *S. torvum* as tomato rootstock without affecting crop yield quantity and quality in order to be used in rotation with the resistance gene *Mi1.2* to favour its durability? (Chapter 2); would it be possible to use plant resistance inducers on susceptible tomato with the intention of extend the period of time between two crops carrying the *Mi1.2* resistance gene or to be used as mixing resistance sources? (Chapter 3); and what is the level of resistance of cucurbit rootstocks to quarantine and regulated RKN species (Chapter 4) in order to have valuable information as an additional control method to be used in addition to the eradication ones to reduce the possibility of survive?

The results of this PhD thesis have shown that crop rotation with different sources of resistance significantly affects the development of nematode populations and virulence selection for specific R genes. Indeed, at the end of a rotation sequence including four different resistant sources, the RKN densities of an avirulent nematode population to all the resistance sources used were 6% of that at the beginning without selection for virulence to any of the resistance sources. Interestingly, when the rotation sequence was conducted in a soil infested by a *Mi1.2* virulent population, the nematode density in the soil at the end of the rotation was below the detection level, and the virulence status of the RKN population was reversed. These results

highlight differences in the mode of action of the plant defense mechanisms of the plant germplasm used in this PhD thesis because cross-virulence was not detected. Additionally, rotation sequences with resistant plant germplasm yielded 1.8 and 2.8 more times than rotation sequences with susceptible germplasm cultivated in soil infested by an avirulent or virulent RKN population, respectively.

Although crop rotation is a mandatory or recommended agronomical practice in organic or integrated production standards, monoculture is practiced on some farms owing to market demands and economic viability. Tomato is one of the main vegetable crops cultivated in monoculture, for which only one RKN resistance gene is currently present in commercial resistant cultivars or rootstocks, favoring virulence selection. In this scenario, the use of alternative compatible tomato rootstocks to be used in rotation or mixture with the *Mi1.2* resistance gene is needed to preserve RKN resistance. In this PhD thesis, the eggplant rootstock *S. torvum* was selected as it has been reported to be resistant against virulent RKN populations to the N and *Mi1.2* resistance genes in pepper and tomato, respectively (García-Mendívil, et al., 2019a; Öçal et al., 2018; Pinheiro et al., 2022; Uehara et al., 2017). Tomato plants grafted onto *S. torvum* avoided the build-up of RKN populations and produced between 2.9 and 7.5 more times than the ungrafted ones in microplot conditions at *Pi* ≥ 100 eggs+J2 per 100 cm^3 of soil, but no differences were observed in plastic greenhouse where a high number of the scions franked. Furthermore, the incidence of blossom-end rot increased and a delay in tomato growth and yield was observed, as previously reported (de Miguel et al., 2011; Lee and Oda, 2003). However, *S. torvum* did not selected for nematode virulence after four repeated crops in pot conditions, and the ability to infect and reproduce in susceptible eggplant after the second and third crop was observed. In fact, a delay in *M. incognita* infection and development in *S. torvum* compared with that in eggplant cv. Cristal, irrespective of the number of times that the nematode subpopulation developed in *S. torvum*, was observed in the histopathological study. Another interesting finding was an increase in nematode reproduction in the susceptible tomato after two consecutive *S. torvum* crops, but not in the resistant tomato. Alternating resistant and susceptible plant germplasms have been proposed by Talavera et al. (2009) could only be useful when alternating resistant and susceptible plant germplasms of the same plant species; otherwise, the remaining nematode inoculum at the end of the resistant one could increase at damaging levels for the following crop depending on its host status.

Besides to RKN-resistance genes, plant resistance can also be induced by priming plant defenses against pests and diseases through biotic and abiotic agents. Various abiotic and biotic elicitors have been reported to stimulate the DL-β-amino-n-butyric acid (BABA), salicylic acid (SA),

jasmonic acid (JA), and methyl jasmonate (MJ) pathways, which induce resistance to *Meloidogyne* species (Cooper et al., 2005; Fujimoto et al., 2011; Martínez-Medina et al., 2017; Oka et al., 1999). Some root symbiont microorganisms, such as *Trichoderma* spp., *Bacillus firmus*, and *Pochonia chlamydosporia,* have been reported to induce defense against *Meloidogyne* (Ghahremani et al., 2019 and 2020; Pocurull et al., 2020). Other biotic agents that can induce plant defenses against *Meloidogyne* are insect pests through herbivory (Martínez-Medina et al., 2021). In recent years, several studies have demonstrated the ability of zoophytophagous insects such as *Nesidiocoris tenuis* and *Macrolophus pygmaeus* to induce plant resistance against pests such as red spider mites, whiteflies, thrips, and the TSWV virus (Bouagga et al., 2019; Pérez-Hedo et al., 2022), but there is no information about its effect on RKN, which was studied in the current PhD thesis. These zoophytophagous species are either present or released in commercial tomato crops and nurseries. Our studies have confirmed that 24 h after exposure of tomato plants to *N. tenuis* and *M. pygmaeus*, overexpression of the *PIN2* gene, associated with the JA metabolic pathway, is triggered in the aerial parts of the plant. At the end of the experiments, nematode reproduction was suppressed by up to 68% and 27%, respectively, compared to the non-exposed ones. However, in the absence of insect pests, *N. tenui*s can cause plant damage. Interestingly, Pérez-Hedo et al. (2018) observed that plants on which *N. tenuis* feeds produce volatile compounds that can induce resistance of the neighboring plants against pests. The VOC responsible for this priming was identified as (Z)-3-hexenyl propanoate and diffusors were designed to be released in the field. They demonstrated that this VOC was effective in inducing defenses against *Spodoptera exigua*, *Frankliniella occidentalis*, and *Trialeurodes vaporariorum* in peppers cultivated in greenhouses (Riahi et al., 2023) and was also effective in citrus orchards (Perez-Hedo et al., 2024). In our pot experiments, the induction of tomato plants exposed to (Z)-3-hexenyl propanoate 24 h before nematode infection and nematode reproduction was reduced by up to 47%. However, it is necessary to verify whether these results are valid for multiple generations of nematodes and to determine whether alterations in plant defenses remain effective under field conditions, where plants are subjected to various stress factors.

In this PhD thesis, we mainly focused on two *Meloidogyne* species, *M. incognita* and *M. javanica*, as they are two of the four most widely distributed species worldwide (Jones et al., 2013). In Spain, the three most common species are *M. arenaria*, *M. incognita*, and *M. javanica*; however, other emerging species may be concerning. The proliferation of *M. hapla* has been documented in Spain (Robertson et al., 2006; Talavera et al., 2019), because it can break the *Mi1.2* resistance gene in tomatoes (Brown et al., 1997). Other species, such as *M. chitwoodi*, *M. enterolobii*, and

M. luci, are gaining importance owing to their high pathogenicity in various economically significant crops despite their limited global distribution (Castagnone-Sereno, 2012; Elling, 2013; Maleita et al., 2022). *M. chitwoodi* and *M. enterolobii* are also able to parasitize tomato germplasm carrying the *Mi1.2* gene and peppers with the N gene (Brown et al., 1997; Koutsovoulos et al., 2020), and some *M. luci* isolates have been reported to be virulent to the *Mi1.2* resistance gene (Aydinli et al., 2019). The fact that we have now categorized the melon rootstock *Cucumis metuliferus* BGV11135 and the watermelon rootstock *Citrullus amarus* BGV5167 as resistant, with different levels of expression, to those quarantine or regulated RKN species make them valuable tools to be used as complementary tools to those conducted for its eradication in order to decrease its ability to stabilize in any growing area in the current context of globalization.

According to the answers to the questions posed at the beginning of this section, the management strategies of *Meloidogyne*-resistant plant germplasm to avoid the selection of virulent nematode populations validated in field conditions is only one: crop rotation with more than two different sources of resistance. Another possible strategy that has to be validated in field conditions in monoculture scenarios is the alternation of at least two different sources of resistance mediated by R-genes, along with the use of plant resistance inducers to be used in susceptible germplasm or to enhance the plant defense mechanisms of resistant plant germplasm. A key point when rootstocks are used as resistant plant germplasm of a given cash crop is their compatibility without significant effects on crop yield quantity and quality in order to be adopted by farmers.

Conclusions

Conclusions

i. Crop rotation with solanaceous and cucurbit crops grafted onto four different sources of resistance to *Meloidogyne* has been proven to be effective for managing both avirulent and partially *Mi1.2* virulent population of *M. incognita* and to reduce crop yield losses. Regarding the avirulent population, the rotation sequence does not select for virulence to any specific resistance source. Remarkably, the partially *Mi1.2* virulent population was efficiently suppressed by the rotation with resistant germplasm and the level of virulence to the *Mi1.2* gene decreased at the end of the rotation with susceptible germplasm.

ii. *Solanum torvum* has been proven to be resistant to *M. incognita* and *M. javanica* irrespective of the initial population densities without experiencing significant tomato yield losses and without selecting for nematode virulence after four consecutive crops, in field and pot experiments. However, *S. torvum* is not recommended as a tomato rootstock due to observed scion–rootstock compatibility issues. Indeed, the tomato scion franked frequently, mainly due to the narrower stem of *S. torvum* compared to tomato. In addition, there was a high incidence of blossom-end rot and a delay in tomato growth and yield. Additionally, an increased in nematode reproduction in tomato after three consecutive *S. torvum* crops was observed. Therefore, it is essential to use properly *S.torvum* to avoid undesirable problems caused by the nematode.

iii. *Nesidiocoris tenuis*, *M. pygmaeus*, and the compound *(Z)*-3-hexenyl propanoate, a volatile produced by tomato plants on which *N. tenuis* feed, are useful for suppressing the capacity of the primary nematode inoculum. The infectivity and reproduction of *Meloidogyne* were reduced by both mirid species, while *(Z)*-3-hexenyl propanoate was reduced only the nematode reproduction. Then, susceptible tomato cultivars exposed to the three agents can be used in rotation sequences with *Mi1.2* resistant germplasm to improve the durability of the resistance gene in monocrop scenarios. The effectiveness of such rotation should be studied.

iv. *Cucumis metuliferus* BGV11135 and *Citrullus amarus* BGV5167 are valuable tools for the management of the regulated root-knot nematode species: *M. chitwoodi, M. enterolobii* and *M. luci,* along with other control methods, in order to be eradicated in case they are detected.

General introduction and discussion references

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Abad, P., Castagnone-Sereno, P., Rosso, M.N., Engler, J.D.A. and Favery, B. (2009). “Invasion, feeding and development” in: Root-knot nematodes. Eds. R.N. Perry, M. Moens and J. L. Starr (Wallingford, CABI international), pp. 163–181

Abd-Elgawad, M. M. (2022). Exploiting plant–phytonematode interactions to upgrade safe and effective nematode control. Life 12 (11), 1916.

Argento, S., Melilli, M. G., and Branca, F. (2019). Enhancing greenhouse tomato-crop productivity by using Brassica macrocarpa guss. Leaves for controlling root-knot nematodes. Agronomy, 9(12), 820.

Aydinli, G., Kurtar, E. S., Mennan, S., (2019). Screening of *Cucurbita maxima* and *Cucurbita moschata* genotypes for resistance against *Meloidogyne arenaria, M. incognita, M. javanica*, and *M. luci.* Journal of Nematology, 51, 2019–2057.

Beckers, G. J. M., and Spoel, S. H. (2006). Fine-tuning plant defence signalling: salicylate versus jasmonate. Plant biology, 8(01), 1-10.

Bent, E., Loffredo, A., McKenry, M. V., Becker, J. O., Borneman, J., (2008). Detection and investigation of soil biological activity against *Meloidogyne incognita*. Journal of Nematology 40(2), 109–118.

Bouagga, S., Urbaneja, A., Depalo, L., Rubio, L., and Pérez-Hedo, M. (2019). Zoophytophagous predator-induced defences restrict accumulation of the tomato spotted wilt virus. Pest Managment Science, 76, 561-567.

Brown, C. R., Mojtahedi, H., Santo, G. S., and Williamson, V. M. (1997). Effect of the Mi gene in tomato on reproductive factors of *Meloidogyne chitwoodi* and *M. hapla*. Journal of Nematology, 29(3), 416.

Castagnone-Sereno P. (2012). *Meloidogyne enterolobii* (= *M. mayaguensis*): profile of an emerging, highly pathogenic, root-knot nematode species. Nematology, 14(2): 133–138.

Castagnone-Sereno, P. (2002). Genetic variability of nematodes: a threat to the durability of plant resistance genes?. Euphytica, 124(2), 193-199.

Castagnone-Sereno, P., and Danchin, E. G. J. (2014). Parasitic success without sex–the nematode experience. Journal of Evolutionary Biology, *27*(7), 1323-1333.

Castagnone-Sereno, P., Bongiovanni, M., and Dalmasso, A. (1993). Stable virulence against the tomato resistance Mi gene in the parthenogenetic root-knot nematode *Meloidogyne incognita*. Phytopathology, 83(8), 803-812.

Castagnone-Sereno, P., Bongiovanni, M., and Djian-Caporalino, C. (2001). New data on the specificity of the root-knot nematode resistance genes Me1 and Me3 in pepper. Plant Breeding, 120(5), 429-433.

Chitwood, D. J., and Perry, R. N. (2009). “Reproduction, physiology and biochemistry” in Root-knot nematodes (Wallingford UK, CABI), 182-200.

Collange, B., Navarrete, M., Peyre, G., Mateille, T., Tchamitchian, M. (2011) Root-knot nematode (*Meloidogyne*) management in vegetable crop production: The challenge of an agronomic system analysis. Crop Protection, 30, 1251–1262

Conrath, U. (2006). Systemic acquired resistance. Plant signaling and behavior, 1(4), 179-184.

Cook, R. J. (1983). The nature and practice of biological control of plant pathogens. American Phytopathological Society, *539*.

Cooper, W.R., Jia, L. and Goggin, L. (2005). Effects of jasmonate-induced defenses on root-knot nematode infection of resistant and susceptible tomato cultivars. Journal of Chemical Ecology, 31, 1953-1967.

Cortada, L., Sorribas, F. J., Ornat, C., Kaloshian, I., and Verdejo-Lucas, S. (2008). Variability in infection and reproduction of *Meloidogyne javanica* on tomato rootstocks with the Mi resistance gene. Plant Pathology, 57(6), 1125-1135.

Curto, G., Dongiovanni, C., Sasanelli, N., Santori, A., and Myrta, A. (2014). Efficacy of dimethyl disulfide (DMDS) in the control of the root-knot nematode *Meloidogyne incognita* and the cyst nematode *Heterodera carotae* on carrot in field condition in Italy. In VIII international symposium on chemical and non-chemical soil and substrate disinfestation, *1044*, 405-410.

Daunay, M. C., and Dalmasso, A. (1985). Multiplication de *Meloidogyne javanica*, *M. incognita* et *M. arenaria* sur divers Solarium. Revue Nématol, 8(1), 31-34.

de Medeiros H. A., de Araújo Filho J. V., de Freitas L. G., Castillo P., Rubio M. B., Hermosa R., et al. (2017). Tomato progeny inherit resistance to the nematode *Meloidogyne javanica* linked to plant growth induced by the biocontrol fungus *Trichoderma atroviride*. Science Reports, 7,40216.

de Miguel, A., Marsal, J. I., Goto, R., San Bautista, A., López-Galarza, S., Pascual, B., et al., (2011) Improving the affinity of tomato grafted on *Solanum torvum* using an intermediate rootstock. Acta Horticulturae, 898, 291-295.

Dhivya, R., Sadasakthi, A., and Sivakumar, M. (2014). Response of wild solanum rootstocks to root-knot nematode (*Meloidogyne incognita* Kofoid and White). International Journal of Plant Science, 9, 117-122

Djian-Caporalino, C., Fazjari, A., Arguel, M. J., Vernie, T., Casteele, C. V. D., Faure, I., et al. (2007). Mapping of the dominant Me genes cluster conferring resistance to root-knot nematodes in pepper (Capsicum annuum L.), 71-85.

Djian-Caporalino, C., Mateille, T., Bailly-Bechet, M., Marteu, N., Fazari, A., Bautheac, P., et al. (2019). Evaluating sorghums as green manure against root-knot nematodes. Crop Protection, 122, 142-150.

Djian-Caporalino, C., Molinari, S., Palloix, A., Ciancio, A., Fazari, A., Marteu, N., Ris, N., and Castagnone-Sereno, P. (2011). The reproductive potential of the root-knot nematode *Meloidogyne incognita* is affected by selection for virulence against major resistance genes from tomato and pepper. European Journal of Plant Pathology. 131, 431.

Djian-Caporalino, C., Palloix, A., Fazari, A., Marteu, N., Barbary, A., Abad, P., et al. (2014). Pyramiding, alternating or mixing: comparative performances of deployment strategies of nematode resistance genes to promote plant resistance efficiency and durability. BMC plant biology, *14*, 1-13.

Djian-Caporalino, C., Pijarowski, L., Januel, A., Lefebvre, V., Daubeze, A., Palloix, A., et al. (1999). Spectrum of resistance to root-knot nematodes and inheritance of heat-stable resistance in in pepper (*Capsicum annuum L*.). Theoretical and Applied Genetics, *99*, 496-502.

Elling A. A. (2013). Major emerging problems with minor *Meloidogyne* species. Phytopathology, 103: 1092–1102.

EPPO, 2017. EPPO Alert List: Addition of *Meloidogyne luci* together with *M. ethiopica*. EPPO Reporting Service, 2017/2018. Available at: https://gd.eppo.int/reporting/article-6186. Accessed August 05, 2023.

EPPO, 2023a. EPPO Alert List 2013-10. Available at: https:// www.eppo.int/ACTIVITIES/plant_quarantine/alert_list. Accessed November 03, 2023.

Expósito, A., García, S., Gińe, A., Escudero, N., and Sorribas, F. J. (2019). *Cucumis metuliferus* reduces *Meloidogyne incognita* virulence against the *Mi1.2* resistance gene in a tomato–melon rotation sequence. Pest Managment Science. 75 (1), 902–1910.

Expósito, A., Munera, M., Giné, A., López-Gómez, M., Cáceres, A., Picó, B., et al. (2018). *Cucumis metuliferus* is resistant to root-knot nematode Mi1. 2 gene (a) virulent isolates and a promising melon rootstock. Plant Pathology, *67*(5), 1161-1167.

Expósito, A., Pujolà., M., Achaerandio, I., Gińe, A., Escudero, N., Fullana, A. M., et al. (2020). Tomato and melon *Meloidogyne* resistant rootstocks improve crop yield but melon fruit quality is influenced by the cropping season. Frontiers in Plant Science. 11.

Fazari, A., Palloix, A., Wang, L., Yan Hua, M., Sage-Palloix, A. M., Zhang, B. X., and Djian-Caporalino, C. (2012). The root-knot nematode resistance N-gene co-localizes in the Me-genes cluster on the pepper (*Capsicum annuum L.)* P9 chromosome. Plant Breeding, 131(5), 665-673.

Fujimoto, T., Tomitaka, Y., Abe, H., Tsuda, S., Futai, K. and Mizukubo, T. (2011). Expression profile of jasmonic acid-induced genes and the induced resistance against the root-knot nematode (*Meloidogyne incognita*) in tomato plants (*Solanum lycopersicum*) after foliar treatment with methyl jasmonate. Journal of Plant Physiology, 168, 1084-1097

García-Mendívil, H. A., Escudero, N., and Sorribas, F. J. (2019a) Host suitability of *Solanum torvum* cultivars to *Meloidogyne incognita* and *M. javanica* and population dynamics. Plant pathology, 68(6), 1215-1224.

García-Mendívil, H. A., Munera, M., Giné, A., Escudero, N., Picó, M. B., Gisbert, C., et al. (2019b). Response of two *Citrullus amarus* accessions to isolates of three species of *Meloidogyne* and their graft compatibility with watermelon. Crop Protection 119, 208–213.

Ghahremani, Z., Escudero, N., Beltrán-Anadón, D., Saus, E., Cunquero, M., Andilla, J., et al. (2020). *Bacillus firmus* strain I-1582, a nematode antagonist by itself and through the plant. Frontiers in plant Science, 11, 796.

Ghahremani, Z., Escudero, N., Saus, E., Gabaldón, T., and Sorribas, F. J. (2019). *Pochonia chlamydosporia* induces plant-dependent systemic resistance to *Meloidogyne incognita*. Frontiers in plant science, 10, 945.

Giné, A., and Sorribas, F. J. (2017b). Quantitative approach for the early detection of selection for virulence of *Meloidogyne incognita* on resistant tomato in plastic greenhouses. Pant Pathology 66(8), 1338-1344.

Giné, A., Bonmatí, M., Sarro, A., Stchiegel, A., Valero, J., Ornat, C., et al. (2013). Natural occurrence of fungal egg parasites of root-knot nematodes, M*eloidogyne* spp. in organic and integrated vegetable production systems in Spain. BioControl, 58(3), 407-416.

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

Giné, A., González, C., Serrano, L. and Sorribas, F. J. (2017a) Population dynamics of *Meloidogyne incognita* on cucumber grafted onto the Cucurbita hybrid RS841 or ungrafted and yield losses under protected cultivation. European Journal of Plant Pathology, 148, 795–805.

Gisbert, C., Gammoudi, N., Munera, M., Giné, A., Pocurull, M., Sorribas, F. J., and Picó, M. B. (2015). Evaluation of two potential Cucumis spp. resources for grafting melons. In V International Symposium on Cucurbits, 1151, 157-162.

Grant, M. R., and Jones, J. D. (2009). Hormone (dis) harmony moulds plant health and disease. Science, 324(5928), 750-752.

Guerrero-Díaz, M. D. M., Lacasa-Martínez, C. M., Hernández-Piñera, A., Martínez-Alarcón, V., and Lacasa-Plasencia, A. (2013). Evaluation of repeated biodisinfestation using Brassica carinata pellets to control *Meloidogyne incognita* in protected pepper crops. Spanish Journal of Agricultural Research, 11(2), 485-493.

Hallman, J., and Meressa, B. H. (2018). "Nematode parasites of vegetables," in Plant parasitic nematodes in subtropical and tropical agriculture. Eds. R. A. Sikora, D. Coyne, J. Hallman and P. Timper (Wallingford: CABI International), 346–410.

Hare, W. W. (1956). Resistance in pepper to *Meloidogyne incognita* acrita Phytopathology. 46,98-104.

Hendy, H., Dalmasso, A. and Cardin, C. (1985): Differences in resistant *Capsicum annuum* attacked by different *Meloidogyne* species. Nematologica, 31, 72-78.

Hu, Y., You, J., Li, C., Hua, C., and Wang, C. (2017). Exogenous application of methyl jasmonate induces defence against *Meloidogyne hapla* in soybean. Nematology, 19(3), 293-304.

Hugo, H. J., and Malan, A. P. (2010). Occurrence and control of plant-parasitic nematodes in irrigation water: a review. South African Journal of Enology and Viticulture, 31 (2), 169-180

Jones, J. T., Haegeman, A. J., Danchin, E. G., Gaur, H. S., Helder, J. K., et al. (2013). Top 10 plant-parasitic nematodes in molecular plant pathology. Molecular Plant Pathology, 14, 946–961.

Katan, J. (1981). Solar heating (solarization) of soil for control of soilborne pests. Annual Review of Phytopathology, 19,211–236.

Khan, M. T. A., Mukhtar, T., and Saeed, M. (2019). Resistance or susceptibility of eight aubergine cultivars to *Meloidogyne javanica*. Pakistan Journal of Zoology, *51*(6), 2187-2192.

Kidwell, M. G., and Lisch, D. R. (2000). Transposable elements and host genome evolution. Trends in ecology & evolution, 15(3), 95-99.

Koutsovoulos G.D., Poullet M., Elashry A., Kozlowski D.K., Sallet E. et al. (2020). Genome assembly and annotation of *Meloidogyne enterolobii*, an emerging parthenogenetic root-knot nematode. Scientific Data, 7(1), 324.

Lee, J. M., and Oda, M. (2003) Grafting of herbaceous vegetable and ornamental crops. HORTICULTURAL REVIEWS-WESTPORT THEN NEW YORK, 28, 61-124.

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

López-Gómez, M., Talavera, M. and Verdejo-Lucas, S. (2016), Differential reproduction of *Meloidogyne incognita* and *M. javanica* in watermelon cultivars and cucurbit rootstocks. Plant Pathology, 65, 145-153.

Lopez-Perez, J. A., Le Strange, M., Kaloshian, I., and Ploeg, A. T. (2006). Differential response of Mi gene-resistant tomato rootstocks to root-knot nematodes (*Meloidogyne incognita*). Crop Protection, 25(4), 382-388.

Maleita, C., Correia, A., Abrantes, I., Esteves, I. (2022). Susceptibility of crop plants to the root-knot nematode *Meloidogyne luci*, a threat to agricultural productivity. Phytopathologia Mediterranea, 61, 169–179.

Martínez-Medina, A., Fernandez, I., Lok, G. B., Pozo, M. J., Pieterse, C. M., and Van Wees, S. C. (2017). Shifting from priming of salicylic acid-to jasmonic acid-regulated defences by *Trichoderma* protects tomato against the root knot nematode *Meloidogyne incognit*a. New phytologist, 213(3), 1363-1377.

Martínez-Medina, A., Mbaluto, C. M., Maedicke, A., Weinhold, A., Vergara, F., and van Dam, N. M. (2021). Leaf herbivory counteracts nematode-triggered repression of jasmonate-related defenses in tomato roots. Plant Physiology, 187(3), 1762-1778.

Murata, G., and Uesugi, K. (2021). Parasitism of *Solanum torvum* by *Meloidogyne hapla* populations from Japan. Journal of Phytopathology, 169(2), 122-128.

Nilusmas, S., Mercat, M., Perrot, T. et al. (2016) A multi-seasonal model of plant-nematode interactions and its use to identify durable plant resistance deployment strategies. V International Symposium on Models for Plant Growth, Environment Control and Farming Management in Protected Cultivation, Avignon 19–22

Núñez-Zofío, M., Larregla, S., Garbisu, C., Guerrero, M. M., Lacasa, C. M., and Lacasa, A. (2013). Application of sugar beet vinasse followed by solarization reduces the incidence of *Meloidogyne incognita* in pepper crops while improving soil quality. Phytoparasitica, 41, 181-191.

Nyczepir, A. P., and Thomas, S. H. (2009). "Current and future management strategies in intensive crop production systems" in Root-knot nematodes (Wallingford UK, CABI), 412-443.

Öçal, S., Özalp, T. and Devran, Z., (2018) Reaction of wild eggplant *Solanum torvum* to different species of root-knot nematodes from Turkey. Journal of Plant Diseases and Protection, 125, 577–580.

Oka, Y., Cohen, Y. and Spiegel, Y., (1999). Local and systemic induced resistance to the root-knot nematode in tomato by DL-β-amino-n-butyric acid. Phytopathol, 89:1138-1143.

Orion, D., Kritzman, G., Erbe, E. F., and Chitwood, D. J. (2001). A role of the gelatinous matrix in the resistance of root-knot nematode (Meloidogyne spp.) eggs to microorganisms. Journal of Nematology, *33*(4), 203.

Ornat, C., and Sorribas, F. J. (2008). Integrated management of root-knot nematodes in Mediterranean horticultural crops. Integrated management and biocontrol of vegetable and grain crops nematodes, *2*, 295-319.

Ornat, C., Verdejo-Lucas, S., and Sorribas, F. J. (1997). Effect of the previous crop on population densities of *Meloidogyne javanica* and yield of cucumber. Nematropica 27, 85–90.

Ornat, C., Verdejo-Lucas, S., and Sorribas, F. J. (2001). A population of *Meloidogyne javanica* in Spain virulent to the *Mi* resistance gene in tomato. Plant Disease. 85(3), 271-276.

Pérez-Hedo, M., Arias-Sanguino, A. M. and Urbaneja, A. (2018). Induced tomato plant resistance against *Tetranychus urticae* triggered by the phytophagy of *Nesidiocoris tenuis*. Frontiers in Plant Science, 9, 1419.

Pérez-Hedo, M., Bouagga, S., Jaques, J. A., Flors, V. and Urbaneja, A. (2015a). Tomato plant responses to feeding behavior of three zoophytophagous predators (Hemiptera: Miridae). Biological Control, 86, 46-51

Pérez-Hedo, M., Bouagga, S., Zhang, N. X., Moerkens, R., Messelink, G., Jaques, J. A., et al., (2022). Induction of plant defenses: the added value of zoophytophagous predators. Journal of Pest Science, 95, 1501-1517.

Pérez-Hedo, M., Gallego-Giraldo, C., Forner-Giner, M. Á., Ortells-Fabra, R., and Urbaneja, A. (2024). Plant volatile-triggered defense in citrus against biotic stressors. Frontiers in Plant Science, 15, 1425364.

Pérez-Hedo, M., Urbaneja-Bernat, P., Jaques, J. A., Flors, V. and Urbaneja, A. (2015b). Defensive plant responses induced by *Nesidiocoris tenuis* (Hemiptera: Miridae) on tomato plants. Journal of Pest Science, 88, 543-554

Petrillo, M. D., and Roberts, P. A. (2005). Fitness of virulent *Meloidogyne incognita* isolates on susceptible and resistant cowpea. Journal of nematology, *37*(4), 457.

Phan, N. T., Waele, D., Lorieux, M., Xiong, L., Bellafiore, S. (2018). A hypersensitivity-like response to *Meloidogyne graminicola* in rice (*Oryza sativa*). Phytopathology, 108(4): 521–528.

Pieterse, C. M., Leon-Reyes, A., Van der Ent, S., and Van Wees, S. C. (2009). Networking by small-molecule hormones in plant immunity. Nature chemical biology, 5(5), 308-316.

Pinheiro, J. B., da Silva, G. O., Biscaia, D., Magalhães, C. D. C., de Souza, L. R., Pereira, W. D. S., et al. (2022) Resistance sources to *Meloidogyne enterolobii* in wild *Solanum species* and interspecific hybrids. Horticultura Brasileira, 40, 275-280.

Pinheiro, J. B., Silva, G. O. D., Oliveira, V. R., Amaro, G. B., Morais, A. A. D. (2019). Prospection of genetic resistance resources to root-knot nematodes in cucurbit genotypes. Horticultura Brasiliera 37: 343–347.

Pinkerton, J. N., Ivors, K. L., Miller, M. L., and Moore, L. W. (2000). Effect of soil solarization and cover crops on populations of selected soilborne plant pathogens in western Oregon. Plant Disease, *84*(9), 952-960.

Pocurull, M., Fullana, A. M., Ferro, M., Valero, P., Escudero, N., Saus, E., et al. (2020). Commercial formulates of T*richoderma* induce systemic plant resistance *to Meloidogyne incognita* in tomato and the effect is additive to that of the *Mi-1.2* resistance gene. Frontiers in Microbiology, 10, 3042.

Riahi, C., Urbaneja, A., Gallego, C., and Pérez-Hedo, M. (2023). Exploring the potential of plant volatiles to enhance pest management in sweet pepper plants. In Integrated Control in Protected Crops Temperate and Mediterranean Climate, 154-157.

Roberts, P. A., and Thomason, I. J. (1989). A review of variability in four *Meloidogyne* spp. measured by reproduction on several hosts including L*ycopersicon*. English, Journal article, 3, Agricultural Zoology Reviews. 225–252.

Robertson, L., López-Pérez, J. A., Bello, A., Díez-Rojo, M. A., Escuer, M., Piedra-Buena, A., et al. (2006). Characterization of *Meloidogyne incognita, M. arenaria* and *M. hapla* populations from Spain and Uruguay parasitizing pepper (*Capsicum annuum* L.). Crop Protection, 25(5), 440-445.

Ros, C., Sánchez, F., Martínez, V., Lacasa, C. M., Hernández, A., Torres, et al. (2016). El cultivo de brásicas para biosolarización reduce las poblaciones de *Meloidogyne incognita* en los invernaderos de pimiento del Sudeste de España. Información Técnica Económica Agraria, 112(2), 109-126.

Ros-Ibáñez, C., Robertson, L., Martínez-Lluch, M., Cano-García, A., and Lacasa Plasencia, A. (2014). Development of virulence to *Meloidogyne incognita* on resistant pepper rootstocks. Spanish Journal of Agricultural Research. 12, 225–232.

Santos, D., Martins da Silva, P., Abrantes, I. and Maleita, C. (2019) Tomato *Mi-1.2* gene confers resistance to *Meloidogyne luci* and *M. ethiopica*. European Journal of Plant Pathology, 156, 571–580.

Sasser, J. N., Eisenback, J. D., Carter, C. C., and Triantaphyllou, A. C. (1983). The international *Meloidogyne* project-its goals and accomplishments. Annual Review of phytopathology, 21(1), 271-288.

Schomaker, C. H. and Been T. H. (2006). "Plant growth and population dynamics" in Plant Nematology. Eds: R. Perry, M. Moens, J. L. Dtarr (Wallingford, CAB International), 275–295.

Seinhorst, J.W. (1998) The common relation between population density and plant weight in pot and microplot experiments with various nematode plant combinations. Fundamental and Applied Nematology, 21, 459–468.

Shukla, N., Yadav, R., Kaur, P., Rasmussen, S., Goel, S., Agarwal, M., et al. (2018). Transcriptome analysis of root-knot nematode (*Meloidogyne incognita*) -infected tomato (*Solanum lycopersicum*) roots reveals complex gene expression profiles and metabolic networks of both host and nematode during susceptible and resistance responses. Molecular Plant Pathology, 19 (3), 615–633.

Shukla, P. S., Mantin, E. G., Adil, M., Bajpai, S., Critchley, A. T., and Prithiviraj, B. (2019). Ascophyllum nodosum-based biostimulants: Sustainable applications in agriculture for the stimulation of plant growth, stress tolerance, and disease management. Frontiers in plant science, 10, 462648.

Sigüenza, C., Schochow, M., Turini, T., and Ploeg, A. (2005). Use of *Cucumis metuliferus* as a rootstock for melon to manage Meloidogyne incognita. *Journal of Nematology*, 37(3), 276.

Sikora, R. A. (1992). Management of the antagonistic potential in agricultural ecosystems for the biological control of plant parasitic nematodes. Annual review of phytopathology, 30(1), 245-270.

Snapp, S. S., Swinton, S. M., Labarta, R., Mutch, D., Black, J. R., Leep, R., et al. (2005). Evaluating cover crops for benefits, costs and performance within cropping system niches. Agronomy journal, 97(1), 322-332.

Snyder, D. W. (2004). Analysis of Genes Expressed by *Meloidogyne incognita* Males: Generation of ESTs and Comparative Genomics.

Sorribas, F. J., Djian-Caporalino, C., and Mateille, T. (2020). "Nematodes" in Integrated pest and disease management in greenhouse crops, vol. Vol 9. Eds. M. L. Gullino, R. Albajes and P. C. Nicot (Cham, Switzerland: Springer), 147–174.

Sorribas, F. J., Ornat, C., Verdejo-Lucas, S., and Galeano, M., Valero, J. (2005). Effectiveness and profitability of the *Mi*-resistant tomatoes to control root-knot nematodes. European Journal of Plant Pathology 111: 29–38.

Sorribas, J., and Ornat, C. (2011). Estrategias de control integrado de nematodos fitoparásitos. Enfermedades causadas por nematodos fitopárasitos en España. Phytoma-*SEF.* Valencia, 115-127.

Stirling, G.R. (1991). Biological control of nematodes: Progress, problems and prospects. CAB International, Wallingford, 282

Talavera, M., Miranda, L., Gómez-Mora, J. A., Vela, M. D., and Verdejo-Lucas, S. (2019). Nematode management in the strawberry fields of southern Spain. Agronomy, 9(5), 252.

Talavera, M., Sayadi, S., Chirosa-Ríos, M., Salmerón, T., Flor-Peregrín, E. and Verdejo-Lucas, S. (2012). Perception of the impact of root-knot nematodeinduced diseases in horticultural protected crops of south-eastern Spain. Nematology, 14, 517-27.

Talavera, M., Verdejo-Lucas, S., Ornat, C. et al. (2009) Crop rotations with Mi gene resistant and susceptible tomato cultivars for management of root-knot nematodes in plastic houses. Crop Protection, 28:662–667.

Talavera-Rubia, M., Vela-Delgado, M. D., and Verdejo-Lucas, S. (2022). A costbenefit analysis of soil disinfestation methods against root-knot nematodes in Mediterranean intensive horticulture. Plants, 11 (20), 2774.

Thies, J. A., Ariss, J. J., Hassell, R. L., Olson, S., Kousik, C. S., and Levi, A. (2010). Grafting for management of southern root-knot nematode, *Meloidogyne incognita*, in watermelon. Plant Disease, 94(10), 1195-1199.

Thies, J. A., Fery, R. L., Mueller, J. D., Miller, G., and Varne, J. (2003). Response of bell pepper cultivars near-isogenic for the N gene to *Meloidogyne incognita* in field trials. HortScience, *38*(7), 1394-1396.

Thies, Judy A. (2011). Virulence of *Meloidogyne incognita* to expression of N gene in pepper. Journal of nematology, 43(2), 90-4.

Triantaphyllou, A. C. (1985). Cytogenetics, cytotaxonomy and phylogeny of root-knot nematodes. 113-126

Uehara, T., Tateishi, Y., Kadota, Y., and Iwahori, H. (2017). Differences in parasitism of *Meloidogyne incognita* and two genotypes of *M. arenaria* on *Solanum torvum* in Japan. Journal of Phytopathology, 165(9), 575-579.

van Loon, L. C., Rep, M., and Pieterse, C. M. (2006). Significance of inducible defense-related proteins in infected plants. Annual Review of Phytopathology., 44(1), 135-162.

Vela, M. D., Giné , A., Ló pez-Gómez, M., Sorribas, F. J., Ornat, C., Verdejo-Lucas, S., et al. (2014). Thermal time requirements of root-knot nematodes on zucchini-squash and population dynamics with associated yield losses on spring and autumn cropping cycles. European Journal of Plant Pathology, 140, 481–490.

Verdejo-Lucas, S., Cortada, L., Sorribas, F. J., and Ornat, C. (2009). Selection of virulent populations of *Meloidogyne javanica* by repeated cultivation of *Mi* resistance gene tomato rootstocks under field conditions. Plant Pathology, 58(5), 990-998.

Verdejo-Lucas, S., Ornat, C., Sorribas, F. J., and Stchiegel, A. (2002). Species of root-knot nematodes and fungal egg parasites recovered from vegetables in Almería and Barcelona, Spain. Journal of Nematology, 34(4), 405.

Verdejo-Lucas, S., Talavera, M., and Andrés, M. F. (2012). Virulence response to the Mi. 1 gene of *Meloidogyne* populations from tomato in greenhouses. Crop Protection, 39, 97-105.

Widmer, T. L., and Abawi, G. S. (2000). Mechanism of suppression of *Meloidogyne hapla* and its damage by a green manure of Sudan grass. Plant disease, 84(5), 562-568.

Williamson, V. M., and Roberts, P. A. (2009). "Mechanisms and genetics of resistance" in Root-knot nematodes (Wallingford UK, CABI), 301-325.

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Monika, and from roots of the last tomato crop (PTMPWT) in the melon rootstock *C. metuliferus* and pepper rootstock 'Oscos' too. The columns represent the mean and the bars represent the standard error of 15 replicates.

CHAPTER 2

Figure 1. (A) Experiment to investigate putative selection of nematode virulence to *Solanum torvum* and the cost to nematode fitness of using the *S. torvum* 'Brutus' rootstock for successive crops. Diagram shows successive crops used for development of different nematode subpopulations. Black squares refer to crops cultivated in the plastic greenhouse at Agròpolis, while grey squares refer to plant growth in climate chambers. (B) Fitness cost experiment where subpopulations were tested on susceptible eggplant cv. Cristal. (C) Experiment of durability of the resistance of *S. torvum* after repeated cultivation and the effect on nematode growth and reproduction on following susceptible or resistant crop. Nematode subpopulations *Subpop*0 to *Subpop*3 were multiplied on another crop of *S. torvum* and then tested on reisistant tomato, susceptible tomato, *S. torvum* and eggplant.

Figure 2. Relationship between reproduction rate (*Pf*/*Pi*) and the population densities at transplanting (*Pi*) of *Meloidogyne* species infecting the tomato cv. Durinta ungrafted or grafted onto *Solanum torvum* 'Brutus', cultivated from March to November in 30 L microplots. *Pf*, final nematode population density.

Figure 3. Seinhorst damage function model $y = m + (1 - m) \times 0.95^{(Pi/T - 1)}$, where *y* is the relative crop yield, *m* is the minimum relative yield, *Pi* is the nematode population density at transplanting and *T* is the tolerance limit for ungrafted tomato cv. Durinta cultivated from March to November in 30 L microplots infested with *Meloidogyne incognita* or *M*. *javanica*.

Figure 4. Laser-scanning confocal microscope images of *Meloidogyne incognita* 15 days after inoculation in the resistant rootstock *Solanum torvum* 'Brutus' (A), and the susceptible eggplant cv. Cristal (B). Second-stage juvenile (J2), nematode (N), cell nuclei (white arrowheads) and giant cells (asterisks) are indicated. Scale bar: 100 µm.

Figure 5. Laser-scanning confocal microscope images of roots of *Solanum torvum* 'Brutus' (A) or eggplant cv. Cristal (C) 70 days after inoculation of the *Meloidogyne incognita* subpopulation coming from *S*. *torvum* (*Subpop*1) and of the *M*. *incognita* subpopulation coming from eggplant cv. Cristal (*Subpop*0) on *S*. *torvum* (B) or eggplant cv. Cristal (D). Second-stage juvenile (J2), female (F), egg mass (EM), giant cells (asterisk), and some eggs and empty eggs (white arrowhead) are indicated. Scale bar: 100 µm.

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Table 9. Infection, reproduction and fertility of *Meloidogyne incognita* subpopulations, developed one (*Subpop*0-ST), two (*Subpop*2-ST), three (*Subpop*2-ST) or four (*Subpop*3-ST) times on *Solanum torvum* in plastic greenhouse and/or climatic chamber, on the susceptible eggplant cv. Cristal, *S. torvum* 'Brutus', the susceptible tomato cv. Durinta (ST) and the resistant tomato cv. Monika (RT) cultivated in 200 cm^3 pots 55 days after inoculation with 200 J2s, and reproduction index (RI).

CHAPTER 3

Table 1. Expression analysis of the *PIN2* and *PR1* genes on the tomato cv. Bodar leaves and roots at 0 and 7 days after nematode inoculation (DANI) with 200 J2 per plant of *Meloidogyne incognita* (Agròpolis), 24 h after the exposure to 15 nymphs per plant of *Nesiodiocoris tenuis* or *Macrolophus pygmaeus* or 24 h after being exposed to (Z)-3- hexenyl propanoate. Plants non-exposed to mirid species and (Z)-3- hexenyl propanoate neither inoculated with *M. incognita* (Control), plants inoculated with *M. incognita* (*Mi*), plants exposed to *N. tenuis* (*N. tenuis*), plants exposed to *N. tenuis* and inoculated with *M. incognita* (*N. tenuis* + *Mi),* plants exposed to *M. pygmaeus* (*M. pygmaeus*), plants exposed to *M. pygmaeus* and inoculated with *M. incognita* (*M. pygmaeus* + *Mi*). plants exposed to (Z)-3- hexenyl propanoate ((Z)-3-HP), plants exposed to (Z)-3- hexenyl propanoate and inoculated with *M. incognita* ((Z)-3-HP + *Mi*). Transcript levels were normalized to the expression of *EF1* measured in the same sample. Each column and bar are the mean and standard error of 4 independent replicate analyses of transcript expression relative to a housekeeping gene and expressed as a ratio to the non-exposed to mirids neither inoculated with *M. incognita* plants (Control) (fold change). Different letters between treatments indicate differences according to Dunn's test ($P < 0.05$). Expression analysis of the *PIN2* and *PR1* genes on the tomato cv. Bodar leaves and roots at 0 and 7 days after nematode inoculation (DANI) with 200 J2 per plant of *Meloidogyne incognita* (Agròpolis), 24 h after the exposure to 15 nymphs per plant of *Nesiodiocoris tenuis* or *Macrolophus pygmaeus* or 24 h after being exposed to (Z)-3- hexenyl propanoate. Plants non-exposed to mirid species and (Z)-3- hexenyl propanoate neither inoculated with *M. incognita* (Control), plants inoculated with *M. incognita* (*Mi*), plants exposed to *N. tenuis* (*N. tenuis*), plants exposed to *N. tenuis* and inoculated with *M. incognita* (*N. tenuis* + *Mi),* plants exposed to *M. pygmaeus* (*M. pygmaeus*), plants exposed to *M. pygmaeus* and inoculated with *M. incognita* (*M. pygmaeus* + *Mi*). plants exposed to (Z)-3-hexenyl propanoate ((Z)-3-HP), plants exposed to (Z)-3- hexenyl propanoate and inoculated with *M. incognita* ((Z)-3-HP + *Mi*). Transcript levels were normalized to the expression of *EF1* measured in the same sample. Each column and bar are the mean and standard error of 4 independent replicate analyses of transcript expression relative to a housekeeping gene and expressed as a ratio to the non-exposed to mirids neither inoculated with *M. incognita* plants (Control) (fold change). Different letters between treatments indicate differences according to Dunn's test ($P < 0.05$).

CHAPTER 4