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Characterization of resistant Cucumis germplasm to manage root-knot nematodes Meloidogyne spp.

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Characterization of resistant *Cucumis* germplasm to manage root-knot nematodes *Meloidogyne* spp.

PhD Dissertation
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“Gentle soul, never be afraid to face the goal.
Don't you know the light you see is your own soul.
In the end we come full circle again”

-Nick Barber

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Resumen

La resistencia vegetal mediante el injerto de plantas susceptibles sobre patrones resistentes es una medida eficaz para controlar las poblaciones del nematodo agallador (RKN) y reducir las pérdidas de producción de los cultivos. Sin embargo, el uso reiterativo de genes R específicos podría seleccionar poblaciones virulentas para esos genes. En España, la rotación de cultivos de solanáceas y cucurbitáceas es común y actualmente se encuentran disponibles varios cultivares y portainjertos comerciales resistentes para cultivos de solanáceas. Sin embargo, en el caso de los cultivos de cucurbitáceas, solo unos pocos están disponibles y ninguno para pepino o melón. Algunas especies de cucurbitáceas silvestres se han caracterizado por ser resistentes a *Meloidogyne*, como algunas especies del género *Cucumis*. La información sobre la respuesta del huésped a poblaciones de áreas específicas de producción, el efecto sobre la dinámica poblacional, la compatibilidad patrón-variedad y el efecto del nematodo sobre la cantidad y calidad de la producción es esencial para caracterizar el nuevo germoplasma a introducir en los sistemas productivos. *Cucumis metuliferus* es un patrón prometedor para melón y pepino, pero hay poca información sobre los parámetros mencionados anteriormente. Se espera que la rotación de genes R en cultivares o portainjertos de solanáceas con los de cucurbitáceas podría reducir la tasa de crecimiento de la población del nematodo, así como la probabilidad de seleccionar para virulencia de genes R específicos mejorando la durabilidad de la resistencia. Los resultados obtenidos en este doctorado serán útiles para proporcionar germoplasma resistente capaz de ser utilizado como patrón de melón y pepino, para proponer alternativas del uso de resistencia vegetal y para mejorar su durabilidad reduciendo las pérdidas de rendimiento del cultivo y también el uso de métodos de control químico con el fin de mejorar la sostenibilidad en los sistemas de producción hortícolas. En consecuencia, el objetivo principal de esta tesis doctoral fue evaluar la respuesta de la resistencia de *Cucumis metuliferus* a *Meloidogyne* spp. y su compatibilidad con melón, la durabilidad de la resistencia en rotación con tomate resistente *Mi*1.2 y el efecto del portainjerto y las densidades de nematodos sobre el rendimiento y la calidad del fruto tanto de tomate como de melón. Los objetivos específicos fueron i) Evaluar la respuesta como huésped de diferentes líneas de *Cucumis metuliferus* frente a aislados (a)virulentos *Mi*1.2 de *Meloidogyne* spp. y su compatibilidad con melón y ii) Determinar el efecto de una rotación tomate-melón de tres años sobre la dinámica poblacional de *M. incognita*, el rendimiento del cultivo (cantidad y calidad) y la durabilidad de la resistencia tanto del gen *Mi*1.2 de tomate como de *C. metuliferus*.

***Cucumis metuliferus* es resistente a aislados (a)virulentos del gen *Mi*1.2 del nematodo agallador de la raíz y un patrón de melón prometedor**: Se llevaron a cabo experimentos en macetas para caracterizar la respuesta de dos líneas de *Cucumis metuliferus* (BGV11135 y BGV10762) del Instituto de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV-UPV), contra aislados (a)virulentos del gen *Mi*1.2 de *Meloidogyne arenaria*, *M. incognita* y *M. javanica* de España, su respuesta histopatológica y la compatibilidad y el efecto sobre las propiedades fisicoquímicas del melón. Una semana después del trasplante en macetas de 200 cm^3, las plantas se inocularon con 1 J2 cm^{-3} de arena esterilizada y se mantuvieron en una cámara de crecimiento a 25°C durante 40 días. El pepino susceptible cv. Dasher II o el melón cv. Paloma se incluyeron como controles susceptibles para su contraste. Se evaluó el número de masas de huevos y el número de huevos por planta, y se calculó el índice de reproducción (IR) como el porcentaje de huevos producidos en las líneas de *C. metuliferus* en comparación con los producidos en los cultivares susceptibles. Los estudios histopatológicos se realizaron utilizando muestras de raíz infectadas de pepino de 2 µm de sección embebidas en resina epoxi obtenidas con un Ultramicrotomo y observadas al microscopio óptico. La compatibilidad y la calidad del fruto se evaluó injertando tres variedades, dos de tipo Charentais y uno de tipo Piel de Sapo, y se cultivaron en condiciones hidropónicas en un invernadero comercial. El nivel de resistencia de ambas líneas de *C. metuliferus* varió de muy resistente (RI <1%) a resistente (1% ≤ RI ≤ 10%) independientemente de los aislados de *Meloidogyne*. Las células gigantes inducidas por *Meloidogyne* spp. en *C. metuliferus* fueron en general poco desarrolladas con múltiples vacuolas en comparación con las del pepino. Además, se observaron células gigantes sin citoplasma y áreas necróticas que rodeaban al nematodo. Las plantas de melón injertadas en la línea BGV11135 de *C. metuliferus* crecieron como plantas autoinjertadas sin afectar negativamente los parámetros de calidad del fruto.

***Cucumis metuliferus* reduce la virulencia de *Meloidogyne incognita* contra el gen de resistencia *Mi*1.2 en una secuencia de rotación tomate-melón y mejora el rendimiento del cultivo, pero la calidad del fruto del melón está influenciada por la época de cultivo**: El tomate susceptible cv. Durinta, no injertado o injertado sobre el patrón resistente “Aligator”, ambos seguidos por el melón susceptible cv. Paloma, no injertado o injertado sobre *Cucumis metuliferus* BGV11135, y en orden inverso, se cultivaron de 2015 a 2017 en las mismas parcelas en un invernadero de plástico, infestadas o no, con *Meloidogyne incognita*. Para cada cultivo, se determinaron las densidades de nematodos del suelo, el índice de agallas, el número de huevos por planta y el rendimiento del cultivo (cantidad y calidad). Se evaluó la relación entre las densidades de *M. incognita* en el suelo al trasplante (Pi) de cada cultivo y el rendimiento del cultivo y se estimó la tolerancia (T) y el rendimiento relativo mínimo del cultivo (m) mediante el modelo de pérdidas de producción de Seinhorst al final de cada cosecha. Al final de cada cultivo, se evaluó la selección de virulencia en experimentos en macetas. Además, se contrastó el volumen y el número de núcleos de células gigantes individuales y el número de células gigantes, su volumen y número de núcleos por sitio de alimentación en tomate y melón susceptibles con los del tomate resistente y *C. metuliferus* 15 días después de la inoculación de nematodos en maceta. En la rotación tomate-melón, las densidades de nematodos aumentaron progresivamente para el tomate injertado, siendo mayores que para las plantas no injertadas al final del estudio;

pero no así en la rotación melón-tomate. Los cultivos injertados rindieron más que los no injertados en las parcelas infestadas. La T estimada para el tomate no injertado fue levemente mayor, pero m fue menor (34%) que para el tomate injertado (67%). La concentración de sodio en los frutos de tomate no injertado, pero no en del tomate injertado, aumentó con las densidades de nematodos en la primavera de 2015 y 2016. La T estimada de melón no injertado no difirió de la del melón injertado cultivado en primavera, pero sí cuando se cultivó en verano. El rendimiento relativo del cultivo de melón sin injertar fue menor (2%) que el del cultivo injertado en primavera (62%) o verano (20%). La concentración de sodio en frutos de melón de plantas no injertadas aumentó con la densidad de nematodos. No se encontraron variaciones en la calidad del fruto del melón injertado cultivado en primavera, aunque se registró menos contenido de materia seca y sólidos solubles totales en las densidades más altas de nematodos cuando se cultivó en verano. Se detectó virulencia contra el gen *Mi*1.2, pero no contra *C. metuliferus*. La reproducción de *M. incognita* en el tomate resistente fue de alrededor del 120% que en el cultivar susceptible después del primer cultivo de tomate injertado, pero disminuyó al 25% al final del experimento. Se observó un menor número de células gigantes por sitio de alimentación tanto en el tomate como en el melón susceptible en comparación con los germoplasmas resistentes, pero fueron más voluminosas y tuvieron un mayor número de núcleos por célula gigante y por sitio de alimentación.

Las principales conclusiones obtenidas de este trabajo fueron que *C. metuliferus* es resistente a las tres principales especies comunes de *Meloidogyne*, incluidos los aislados virulentos del gen de resistencia *Mi*1.2. Los estudios histopatológicos mostraron células gigantes poco desarrolladas inducidas por *Meloidogyne javanica* en *C. metuliferus* y áreas necróticas que rodeaban al nematodo. En tomate resistente cv. Monika y *C. metuliferus*, *M. incognita* indujo la formación de más células gigantes, pero poco desarrolladas y con menor número de núcleos por célula gigante que en tomate y melón susceptibles. *C. metuliferus* BGV11135 es un patrón compatible con melones tipo cantaloupe y piel de sapo sin afectar la calidad del fruto. El melón y tomate injertado en "*C. metuliferus*" y "Aligator" respectivamente, no aumentó el rendimiento del cultivo en suelos no infestados de nematodos. La calidad de los frutos producidos en plantas injertadas estuvo dentro de los estándares. La secuencia de rotación primavera-verano melón-tomate proporcionó más rendimiento de peso de fruto que la de tomate-melón en nuestras condiciones agroambientales. En suelos infestados de *Meloidoyne incognita*, el melón injertado rindió significativamente más que el no injertado independientemente de la temporada de cultivo. Sin embargo, el melón injertado fué más tolerante y experimentó menos pérdidas máximas de rendimiento cuando se cultivó en primavera-verano en comparación con la cosecha de verano-otoño. Además, algunos parámetros de calidad del fruto del melón se vieron afectados por el nematodo en la cosecha de verano-otoño, pero no en la primavera-verano. La tasa de reproducción del nematodo se vio afectada por la temporada de cultivo, el material vegetal, la densidad de población inicial y la virulencia de genes R específicos. En melón, la tasa de reproducción del nematodo en plantas no injertadas fue mayor en el cultivo de primavera en comparación con las plantas resistentes. Sin embargo, cuando se cultivó en verano la tasa de reproducción fue menor debido a la alta mortalidad producida por las condiciones de estrés. En tomate, la tasa de reproducción en plantas injertadas aumentó progresivamente en cada cultivo, siendo superior al tomate no injertado al final del tercer cultivo de tomate de la secuencia de rotación tomate-melón debido a la selección de

virulencia. Se observó virulencia al gen *Mi*1.2 en el patrón “Aligator” después del primer cultivo de tomate, pero no en *C. metuliferus* BGV11135. Consecuentemente, la alternancia de estas dos especies resistentes no fue suficiente para evitar la selección de virulencia al gen *Mi*1.2, aunque su nivel se redujo después de utilizar *C. metuliferus* en rotación. El coste biológico de la subpoblación virulenta al gen *Mi*1.2 en el tomate susceptible se demostró por una menor capacidad de infectar y reproducirse, así como la reducción de la fertilidad de las hembras con respecto a la subpoblación avirulenta. En melón, la subpoblación virulenta al gen *Mi*1.2 mostró una menor capacidad de reproducción y una menor fertilidad de las hembras con respecto a la subpoblación avirulenta. El coste biológico de la subpoblación virulenta al gen *Mi*1.2 se detectó solo después del tercer cultivo de tomate injertado. En consecuencia, se necesita un número mínimo de cultivos para fijar el carácter en la población, en nuestras condiciones experimentales, tres cultivos de tomate injertados alternados en "Aligator". *Cucumis metuliferus* es un excelente portainjerto para ser incluido en las estrategias de manejo integrado de *Meloidogyne* en sistemas de producción hortícola, debido a su resistencia y tolerancia al nematodo, su efecto en la reducción del nivel de virulencia al gen *Mi*1.2 y su compatibilidad con melón sin afectar la calidad del fruto.

Characterization of resistant *Cucumis* germplasm to manage root-knot nematodes *Meloidogyne* spp.

Alejandro Expósito Creo

Abstract

Plant resistance through grafting susceptible scions onto resistant rootstocks is an effective measure to supress root-knot nematode (RKN) populations and to reduce crop yield losses. However, the reiterative use of specific *R* genes could select virulent populations for those genes. In Spain, crop rotation including solanaceous and cucurbitaceous crops is common and currently several commercial resistant cultivars and rootstocks are available for solanaceous crops. However, in the case of cucurbit crops, only few are available and not one for cucumber or melon. Some wild cucurbit species have been characterized as resistant to RKN, as some *Cucumis* species. The information regarding the host suitability to nematode populations from specific production areas, the effect on the population dynamics, the rootstock-scion compatibility and the effect of the nematode on the crop yield quantity and quality is essential to characterize new putative germplasm to be included in the agronomic systems. *Cucumis metuliferus* is one promising rootstock for melon and cucumber, but there is little information regarding the above mentioned parameters. It is expected that rotating *R* genes in solanaceous cultivars or rootstocks with those in cucurbits might reduce the nematode population's growth rate, as well as, the probability to select for virulence to specific *R* genes improving the resistance durability. The results obtained in this PhD will be useful to provide resistant germplasm able to be used as rootstock for melon and cucumber, to propose alternatives of the use of plant resistance to improve its durability reducing the crop yield losses and also the use of chemical control methods in order to enhance the sustainability in horticulture production systems. Accordingly, the main objective of this PhD thesis was to evaluate the resistance response of *Cucumis metuliferus* to *Meloidogyne* spp. and its compatibility with melon, its resistance durability in crop rotation with *Mi*1.2 resistant tomato and the effect of the rootstocks and nematode population densities on both tomato and melon yield and fruit quality. The specific objectives were i) To evaluate the host suitability of different accessions of *Cucumis metuliferus* against (a)virulent *Mi*1.2 isolates of *Meloidogyne* spp. and its compatibility with melon, and ii) To determine the effect of a three years tomato-melon rotation on the population dynamics of *M. incognita*, the crop yield (quantity and quality), and the durability of the resistance of both tomato *Mi*1.2 gene and *C. metuliferus* R genes.

***Cucumis metuliferus* is resistant to root-knot nematode *Mi*1.2 gene (a)virulent isolates and a promising melon rootstock**: Pot experiments were carried out to characterize the response of two *Cucumis metuliferus* accessions (BGV11135 and BGV10762) from the Institute for Conservation and Improvement of Valencian Agrodiversity (COMAV-UPV), against *Mi*1.2 gene (a)virulent *Meloidogyne arenaria, M. incognita* and *M. javanica* isolates from Spain, it's histopathological response and the compatibility and the effect on physicochemical properties of fruit melons. One week after transplanting into 200 cm^3-pots, plants were inoculated with 1 J2 cm^{-3} of sterilized sand and maintained in a growth chamber at 25ºC for 40 days. The susceptible cucumber cv. Dasher II or melon cv. Paloma were included as susceptible control for comparison. The number of egg masses and the number of eggs per plant were assessed, and the reproduction index (RI) was calculated as the percentage of eggs produced on the *C. metuliferus* accessions compared to those produced on the susceptible cultivars. Histopathological studies were conducted using infected cucumber root samples of 2 µm section embedded in epoxy resin obtained using an Ultramicrotome and observed in light microscope. The compatibility and the fruit quality were assessed by grafting three scions, two of Charentais type and one of type Piel de Sapo, and cultivated under hydroponic conditions in a commercial greenhouse. The resistance level of both *C. metuliferus* accessions ranged from highly resistant (RI < 1%) to resistant (1% ≤ RI ≤ 10%) irrespective of *Meloidogyne* isolates. Giant cells induced by *Meloidogyne* spp. on *C. metuliferus* were in general poorly developed with multiple vacuoles compared to those on cucumber. Furthermore, giant cells without cytoplasm and necrotic areas surrounding the nematode were observed. Melon plants grafted onto *C. metuliferus* accession BGV11135 grew as selfgrafted plants without negatively impacting fruit quality traits.

***Cucumis metuliferus* reduces *Meloidogyne incognita* virulence against the *Mi*1.2 resistance gene in a tomato-melon rotation sequence and improve crop yield but melon fruit quality is influenced by the cropping season**: The susceptible tomato cv. Durinta, ungrafted or grafted onto cv. Aligator resistant rootstock, both followed by the susceptible melon cv. Paloma, ungrafted or grafted onto *Cucumis metuliferus* BGV11135, and in reverse order, were cultivated from 2015 to 2017 in the same plots in a plastic greenhouse, infested or not with *Meloidogyne incognita*. For each crop, the soil nematode densities, galling index, number of eggs per plant and crop yield (quantity and quality) were determined. The relationship between *M. incognita* densities in soil at transplanting (*Pi*) of each crop and the crop yield was assessed and the tolerance (*T*) and the minimum relative crop yield (*m*) were estimated by the Seinhorst's damage model at the end of each crop. At the end of each crop, the virulence selection was evaluated in pot experiments. In addition, the volume and the number of nuclei of single giant cells and the number of giant cells, its volume and number of nuclei per feeding site in susceptible tomato and melon were compared to those in the resistant tomato and *C. metuliferus* 15 days after nematode inoculation in pot test. In the tomato-melon rotation, the nematode densities increased progressively for the grafted tomato, being higher than for the ungrafted plants at the end of the study; but not so in the melon-tomato rotation. The grafted crops yielded more than the ungrafted ones in the infested plots. The estimated *T* for ungrafted tomato was slight higher but *m* was lower (34%) than for grafted tomato (67%). Sodium concentration in fruits from ungrafted but not from grafted tomato increased with nematode densities in spring 2015 and 2016. The

estimated ungrafted melon *T* did not differ from that of grafted melon cultivated in spring, but it did when it was cultivated in summer. The relative crop yield of ungrafted melon was lower (2%) than the grafted cultivated in spring (62%) or summer (20%). Sodium concentration in melon fruits from ungrafted plants increased with the nematode density. No variations in fruit quality from grafted melon cultivated in spring were found, although less dry matter and total soluble solids content at highest nematode densities were registered when it was cultivated in summer. Virulence against the *Mi*1.2 gene was detected, but not against *C. metuliferus*. Reproduction of *M. incognita* on the resistant tomato was around 120% that on the susceptible cultivar after the first grafted tomato crop, but it decreased to 25% at the end of the experiment. Lower number of giant cells per feeding site was observed in both susceptible tomato and melon compared to the resistant germplasms but they were more voluminous holding higher number of nuclei per giant cell and per feeding site.

The main conclusions obtained from this work was that *C. metuliferus* is resistant to the main three common *Meloidogyne* species including virulent nematode isolates to the *Mi*1.2 resistant gene. The histopathological studies have shown poorly developed giant cells induced by *Meloidogyne javanica* in *C. metuliferus* and necrotic areas surrounding the nematode. In resistant tomato cv. Monika and *C. metuliferus, M. incognita* induced the formation of more giant cells but poorly developed and with less number of nuclei per giant cell than in susceptible tomato and melon. *C. metuliferus* BGV11135 was a compatible rootstock with cantaloupe and piel de sapo type melons without affecting the melon fruit quality. Grafting melon and tomato onto "*C. metuliferus"* and "Aligator" rootstocks respectively did not increase the crop yield in non-nematode infested soil. The quality of the fruits produced in grafted plants was within the standards. The spring-summer rotation sequence melon-tomato provided more fruit weight yield than the tomato-melon one in our agroenvironmental conditions. In *Meloidoyne incognita* infested soil, grafted melon yielded significantly more than the ungrafted irrespective of the cropping season. However, grafted melon was more tolerant and experienced less maximum yield losses when cultivated in spring-summer compared to the summer-autumn crop. In addition, some melon fruit quality parameters were affected by the nematode in the summer-autumn crop but not in the spring-summer. The reproduction rate of the nematode was affected by the cropping season, the plant material, the initial population density and the virulence to specific R genes. In melon, the reproduction rate of the nematode in ungrafted plants was higher in the spring crop compared to the resistant plants. However, when it was cultivated in summer the reproduction rate was lower due to the high mortality produced by the stressful conditions. In tomato, the reproduction rate in grafted plants increased progressively in each crop, being higher than the ungrafted tomato at the end of the third tomato crop of tomato-melon rotation sequence due to virulence selection. Virulence to the *Mi*1.2 was observed in the "Aligator" rootstock after the first tomato crop, but not in *C. metuliferus* BGV11135. Thus, alternating these two different resistant species was not enough to prevent virulence selection to the *Mi*1.2 gene, although its level was reduced after using *C. metuliferus* in rotation. The fitness cost of the virulent *Mi*1.2 subpopulation in the susceptible tomato were shown by a reduced ability to infect and to reproduce, as well as the reduced fertility of the females respect to the avirulent subpopulation. In melon, the virulent *Mi*1.2 subpopulation showed a reduced ability to reproduce and a reduced fertility of the females respect to the avirulent subpopulation. The fitness cost of the virulent *Mi*1.2

subpopulation was detected only after the third grafted tomato crop. Then, a minimum number of crops are needed to fix the character in the population, three alternating grafted tomato crops onto 'Aligator' in our experimental conditions. *Cucumis metuliferus* is as excellent rootstock to be included in integrated management strategies for RKN management in horticulture production systems, due to its resistance and tolerance to the nematode, its effect on reducing the level of nematode virulence to the *Mi*1.2 gene, and its compatibility with melon without affecting its fruit quality.

GENERAL INTRODUCTION

Second-stage juvenile (J2) of *Meloidogyne* spp. Picture: Ariadna Giné Blasco

Characterization of resistant *Cucumis* germplasm to manage root-knot nematodes *Meloidogyne* spp.

Alejandro Expósito Creo

PhD Dissertation

Meloidogyne spp.

Root-knot nematodes (RKN) *Meloidogyne* spp, are the most harmful plant-parasitic nematodes for vegetable production worldwide (Hallmann and Meressa, 2018). They are highly adapted obligate root parasites of thousands of cultivated and adventitious plants with a worldwide distribution, especially in warm temperate and tropical regions and consequently, it is foreseeable due the global warming to be an increasing limiting factor in the coming years for agriculture production. The genus *Meloidogyne* comprises around 100 species, but three of them: *M. arenaria*, *M. incognita* and *M. javanica* cause the most important yield losses worldwide (Jones et al., 2013). This is because their wide range of host plants and their parthenogenetic mode of reproduction. In addition, their rapid rate of development leads to complete several generations per cropping season increasing the damage to the crops. The main symptom caused by RKN in the belowground part of the plant is the root-knot or galls induced by the nematode, which block the water and the nutrient uptake by the plant and produce symptoms such as dwarfing, wilting (Figure 1) and in severe attacks, the death of the plant.

In addition, plant physiology disruption induced by the nematode leads to a reduction in the quality of the different plant products (Greco and Di Vito, 2009). Furthermore, secondary infections by other pathogens often occur in nematode-infected tissues (Moens et al., 2009). To complete its live cycle, RKN needs a suitable living host. The life cycle (Figure 2) comprises the egg, four juvenile stages and the adult stage. Juveniles of first (J1) stage along with the preinfective second (J2) stage and the males are vermiform, whilst third (J3) and fourth (J4) juvenile stage and the female increase width. First-stage juvenile moults into J2 inside the egg. Afterwards, the J2 hatches and moves into the soil attracted by root stimulants (Dutta et al., 2012) until reach the plant host and then penetrates into the root using the stylet, inyecting enzymes secreted by the esophagus that allows the cell-wall degradation. Then, the J2 moves through the intercellular space until the vascular cylinder, in which the nematode induces the formation of hypertrophied cells, called giant cells (Abad et al., 2009). Giant cells are multinucleated and very active metabolically with a dense cytoplasm becoming a permanent nematode feeding site. Thus, the nematode development and reproduction depend on the induction of giant cells (Nyczepir and Thomas, 2009).

Figure 1. Symptoms of root-knot in roots and wilting in tomato plants caused by *Meloidogyne incognita*. Pictures: Alejandro Expósito Creo.

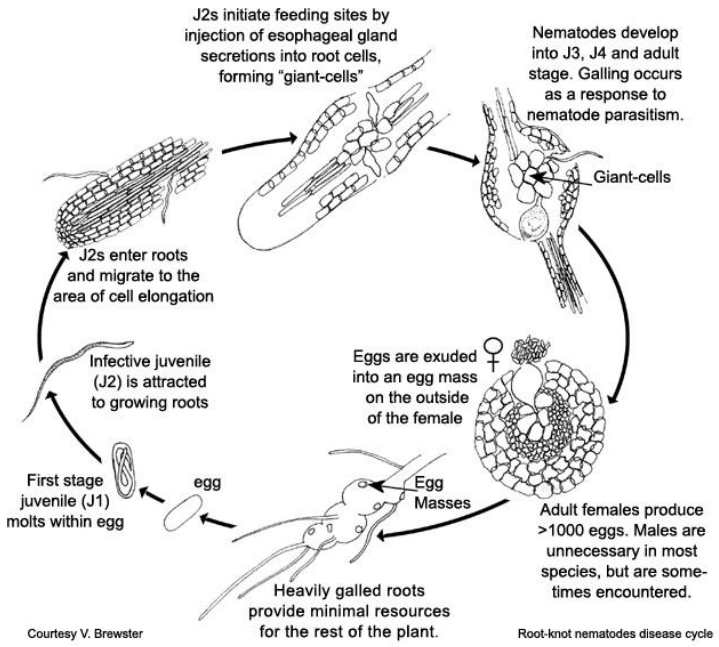

Figure 2. Life cycle of *Meloidogyne* spp. (Mitkowski and Abawi, 2003).

Once the J2 has been infected, it will moult to J3, J4 and adult. Sex differentiation is hormonally regulated which is affected by environmental factors (Taylor and Sasser, 1978; Papadopaoulu and Triantaphyllou, 1982). Under favourable conditions, the juveniles will develop to females that will lay a large amount of eggs into a gelatinous matrix, protecting them from desiccation until a suitable host is available. Males are rare and their frequency increases under unfavorable environmental conditions, such as, scarcity of food or stressed plants, being a mechanism to regulate the nematode population density (Fassuliotis, 1970; Walters et al*.,* 2006).

Meloidogyne are poikilothermic animals (Tyler, 1933). That is, the rate of nematode development from one stage to the other and for life cycle completion depends on soil temperatures and the rate at which it is accumulated by the nematode. The nematode development begins above a basal temperature (Tb), achieving the maximum development rate at the optimum temperature. Over it, the development rate decreases until the maximum temperature from which no development occurs. The accumulated temperature above Tb in a day is a degree day (DD), and a given species needs to accumulated a certain DD, known as thermal constant (S) (Table 1). Thermal requirements of some RKN species in vegetable crops have been obtained (summarized in Sorribas et al., 2020), being useful to predict the development stage of the nematode providing information on the number of nematode generations in a given crop and its influence on crop yield losses and the population growth rate. In addition, the information provided will be useful to apply specific control methods for each vulnerable stage of development.

Table 1. Thermal requirements for life cycle completion of *Meloidogyne* spp. on tomato (Summarized in Sorribas et al., 2020)

Meloidogyne spp.	Base Temperature (Tb; ºC)	Thermal constant (S)[a]
M. incognita	10.1	400
M. javanica	13.0	345
M. hapla	8.3	553
M. hispanica	10.4	526

[a]Accumulated degree days (ºC) over Tb

Population dynamics and yield losses

The population size depends on the life cycle of the nematode, the plant host status and the environmental conditions (Schomaker and Been, 2006; Greco and Di Vito, 2009). The host plant status refers to the ability of the nematode to feeds and reproduces on a given plant species, and the tolerance of the plant to support nematode densities without suffering yield losses. The knowledge of these parameters is essential to design management control strategies to reduce nematode populations and maintain the densities under the economic yield thresholds. In this scenario, two different phases can be differenciate. The first one is when a living plant host is available and the nematode can develop inside the plant. Then, the relation between the population density at sowing or transplanting (*Pi*) and at the end of the crop (*Pf*) is well represented by a logistic function (Figure 3) with three differentiated areas. The first one, is represented by a linear relationship, where *Pf* increases proportionally to *Pi*. That is, *Pf = aPi,* where *a* is the maximum multiplication rate due to the absence of limiting factors for the nematode development.

The maximum multiplication rate occurs at low *Pi*. In the second area, *Pf* does not increase proportionally to *Pi* because intraspecific competition begins and a proportion of nematodes do not infect roots or the fecundity rate decreases. In this area, the maximum density (*M*) is achieved. The third area is characterized by a reduction of *Pf* at increasing *Pi* because there are not enough food resources to maintain the nematode density at sowing or transplanting. In this area, the equilibrium density (*E*) of the population, defined as the maximum nematode density at sowing or transplant that the plant can support at the end of the crop (*Pf*=*Pi*; *Pf*/*Pi*=1), is achieved (Greco and Di Vito, 2009), and it can be calculated according to the expression $E = M\,(a\text{-}1)\,/\,a$ (Schomaker and Been, 2006). *a* and *E* are used to categorize the plant host status. Good hosts have high values of *a* and *E* whilst low values are indicators of poor and resistant hosts. Different examples of the relationship between *Pi* and *Pf* for different plant host status are shown in the Figure 3.

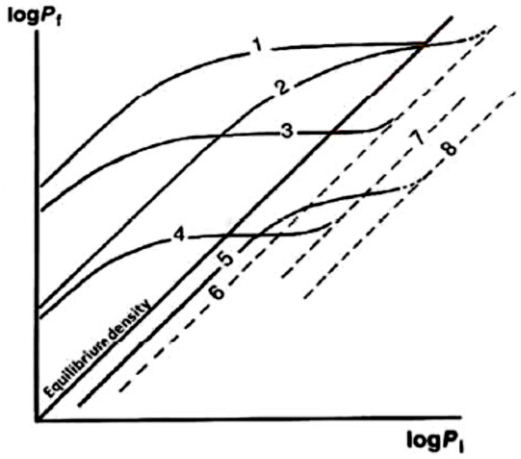

Figure 3. Relationship between *Pi* and *Pf* for good (lines 1 and 2), intermediate (line 3), poor (lines 4 and 5), and non-hosts (lines 6, 7 and 8) plants (Seinhorst, 1965).

These parameters could also be calculated by modelling the relationship between the reproduction rate (*Pf*/*Pi*), which is the number of times that the population density at sowing or transplanting (*Pi*) increases at the end of the crop (*Pf*). (Figure 4)

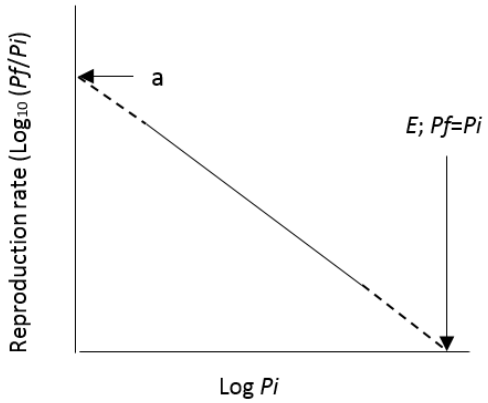

Figure 4. Relation between *Pi* and the reproduction rate (*Pf*/*Pi*). (Adapted from Ferris, 1985).

The second one is when the nematode is outside the plant when no host is available. In this case, RKN can survive as eggs in the egg masses depending in soil temperature, aeration and moisture. The gelatinous matrix protects every development stage inside from desiccation, and when dehydrated, the egg hatching is inhibited. This is important because the percentage of J2 hatching decreases proportionally to moisture level. In addition, in moist soils the J2 emerged will consume their own reserves at a rate related to the soil temperature (Goodell and Ferris, 1989). In addition, high temperatures can be letal for the nematode. Wang and McSorley (2008) founded that 100% of J2 died when exposed to 39, 40, 41 and 42ºC for 48, 46, 17 and 14 h respectively.

The crop yield is related to the *Pi*, the plant tolerance and the number of generations that the nematode can complete during a cropping period (Sorribas et al., 2020). Seinhorst (1965), described a model relating *Pi* with the relative crop yield (*y*), to estimate the plant tolerance (*T*), that is, the maximum *Pi* which crop yield losses begins; and the minimum relative crop yield (*m*) that occurs at highest *Pi*, being $y = 1$ at $Pi \leq T$, and $y = m + (1-m)\ 0.95^{(Pi/T-1)}$ at $Pi \geq T$ (Figure 5). *Meloidogyne* spp. can severely reduce the crop yield. The maximum yield losses caused by the nematode in tomato, cucumber, zucchini and watermelon cultivated in plastic greenhouse in Spain, have been reported in 66%, 88%, 52% and 37%, respectively (Giné et al., 2017a, 2017b; López-Gómez et al., 2014; Vela et al., 2014).

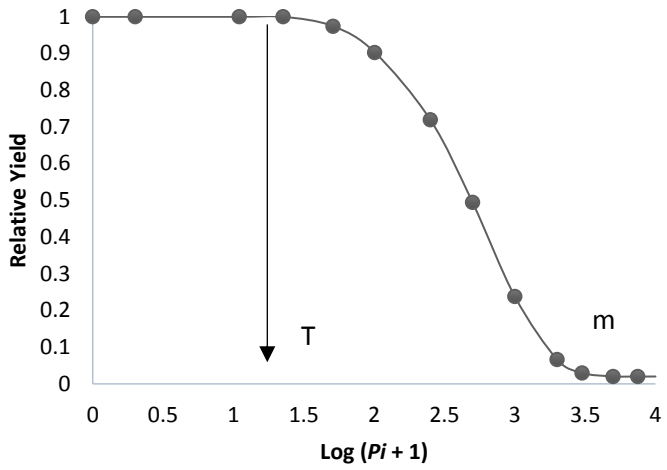

Figure 5. Seinhorst damage function model $y = m + (1-m)\ 0.95^{(Pi/T-1)}$, where y = relative yield, m = minimum yield, Pi = initial population, T = tolerance limit (adapted from Seinhorst, 1965)

Control management strategies

Nowadays, chemical nematicides are commonly used against *Meloidogyne* (Talavera et al., 2012, Sorribas et al., 2020). However, due the concern for human health and the environment contamination, their restriction or prohibition in integrated and organic production systems, as well as, their limit use due to the European Directive 2009/128/EC, are deep reasons to search for non-chemical alternatives to their manage. Nematode management should be preventive and permanent using durable and sustainable control methods. Different methods for controlling RKN have been widely research (Nyczepir and Thomas, 2009). For instance, preventive strategies to maintain a free-disease field from avoiding the entry of the nematodes in the production systems through sanitation of the tools between infested and non-infested areas, the use of free-disease seedlings from certificate nurseries and the control of weeds as alternative hosts should be implemented as important preventive strategies. Once the nematode is stablished in the field, the best management strategy for a given production area should be designed and used considering the nematode densities in soil. When RKN densities are high, it is imperative to reduce them to manageable levels before stablish any crop. For example, soil solarisation or soil biofumigation using plants from Brassicaceae and Alliaceae families, wich release toxic compounds as isothiocyanates and hydrogen cyanide (HCN), has been effectively used to control RKN during the warmest season in the southern and eastern European countries (Summarized in Sorribas et al., 2020). Afterwards, when RKN drop to densities under the economic threshold, different control methods can be used to inhibit nematode population buildup. Among them, the use of organic amendments has proven to stimulate the natural soil microbiota that plays and important role against RKN.

For example, soils conducted under organic farming, which have more content of organic matter, finer textured particles, FDA, β-glucosaminidase, and urease

activity compared to soils conducted under integrated farming, showed higher levels of egg parasitism to *Meloidogyne* (Giné et al., 2013). This tactic could be complemented with the use of biological based formulates including nematode antagonistic, some of which, as for example *Bacillus firmus* or *Trichoderma* spp. have been shown to induce system resistance to RKN in tomato, which seems to be additive in resistant plants with the *R*-gene *Mi*1.2 (Ghahremani et al., 2020; Pocurull et al., 2020). Another important management tool is plant resistance, which will be extensively explained in the next section.

Plant resistance to RKN in cucurbitacae and solanaceae families

Plant resistance to nematodes is the ability of the plant to supress the infection, development and/or reproduction of the nematode (Roberts, 2002). Resistance to pathogens could be quantitative or qualitative. The quantitative response is mediated by different genes, where each of them contributes partially to the resistance (Kou and Wang, 2010). In contrast, the qualitative resistance is triggered by a gen-by-gen interaction between the resistant gene of the plant (*R*-gene) and the avirulent gene of the pathogen (*Avr* gene), and both needs to be present for the resistance expression (Jones and Dangl, 2006). If some of them are absent or inactive, the interaction results in infection. The effect of the resistance on the population dynamics is the reduction of the reproduction rate and the equilibrium density of the nematode population (Talavera et al., 2009; Giné and Sorribas, 2017). Resistant plants to RKN are usually tolerant to them, reducing significantly the yield losses in the actual crop (Giné and Sorribas, 2017) and in the following crop in the rotation sequence (Ornat et al., 1997; Thies et al., 2004).

Among solanaceous, tomato was the first crop with commercial resistant cultivars to *M. arenaria, M. incognita* and *M. javanica*. The resistance is conferred by the *Mi*1.2-resistance-gene and was introgressed in *S. lycopersicum* from *Solanum peruvianum* by embryo culture by Smith in the 1940's. This *R*-gene is the best characterized and serves as a basis for comparison with other genes (Williamson and Roberts, 2009). The *Mi*1.2 gene encodes a large plant defence protein in an inactive conformation in absence of *Meloidogyne*, but the conformation is activated by elicitors from the nematode leading to a hypersensitive response at the site of infection (Williamson and Roberts, 2009). Despite the effectiveness of the *Mi*1.2 gene to manage RKN, its expression can be affected by constant soil temperatures higher than 28 ^{o}C (Dropkin, 1969). Fluctuant soil temperatures, higher than 28 ^{o}C, are not enough to reduce the resistance significantly (Verdejo-Lucas et al., 2013). Moreover, the homozygosis or heterozygosis of the genes in the plant seems to play and important role in the resistance response to the nematode, as for example, in the *Mi*1.2 resistant tomato gene. In addition, nematodes have developed the ability to silence the resistance mechanisms of the plant leading to a compatible interaction (López-Pérez et al., 2006; Bhattarai et al., 2007; Cortada et al., 2008 and 2009).

Furthermore, several single dominant *R*-genes (from *Mi1* to *Mi9*), and some of them resistant against *Mi*1.2-virulent RKN populations and stable at high soil temperatures (32 ^{o}C) have been identified and mapped in different chromosomes of

tomato (Rashid et al., 2017). In pepper, three *R*-genes can be found introduced in commercial cultivars and rootstocks (*Me1, Me3* and *N*) (Williamson and Roberts, 2009; Barbary et al., 2015). Additionally, resistance to RKN can be found in several wild accessions of the Solanaceae family; for example, in *Solanum arcanum, S. sisymbrifolium, S. sparsipilum,* and *S. torvum* (Kouassi et al., 2005; Jablonska et al., 2007; Dias et al., 2012; Bagnaresi et al., 2013; García-Mendívil et al., 2019).

In the case of cucurbits, those are usually grafted onto *Cucurbita maxima* x *C. moschata* hybrids due to their vigour along with their resistance or tolerance to fusarium wilt and *Monosporascus*. Unfortunately, those hybrids are susceptible to *Meloidogyne* (Thies et al., 2010; Lopez-Gómez et al., 2015; Giné et al., 2017). However, some wild cucurbit species have been described as resistant to RKN. For example, the new resistant *Citrullus amarus* cv. Strongback rootstock for watermelon has been released recently by the USDA (Kemble et al., 2019). Recently, Kantor et al. (2018) pointed out that some metabolic profile of the roots of different lines of *Citrullus amarus* that could have nematicidal activity were higher compared to the watermelon cv. Charleston grey and cv. Crimsom sweet. Regarding to the *Cucumis* genera, no commercial resistant rootstocks are available, though resistance to *Meloidogyne* has been found in wild species, such as *C. africanus, C. anguria C. ficifolius, C. metuliferus, C. postulatus, C. subsericeus* and *C. zeyheri* since the 1960's (Fassuliotis, 1967; Thies et al., 2014; Liu et al., 2015). *Cucumis metuliferus* or "kiwano" is a vegetable crop used in Africa for its fruit characteristics. It has been proven its therapeutical effects, including hypoglycemic, antimicrobial and antiviral properties (Summarized in Usman et al., 2015). The wild bitter forms are rich in cucurbitacins, wich are toxic for consumption. *C. metuliferus* has been reported as resistant to RKN and used as a melon rootstock in previous works (Sigüenza et al., 2005; Guan et al., 2014). Resistance in *C. metuliferus,* has been associated with poor developed of giant cells and high male production rate (Fassuliotis, 1970). In addition, gene expression related to plant defence mechanisms against RKN was modified compared to cucumber (Ye et al., 2017). There have been different programs to introgress resistance to *Meloidogyne* in commercial cultivars, but, unfortunately, intraspecific hybridation between nematode resistant *Cucumis* has been unsuccessful (Fassuliotis and Nelson, 1988; Walters and Wehner, 2002). Grafting is a widely spread technique, which has been increasing in the last years, where the tissue of a scion plant is joined to the root of another compatible plant to prevent abiotic and biotic stresses. The use of grafted plants can affect the quality, the storability, and the nutritive values of the fruits. For this reason, is necessary the knowledge of particular scion-rootstock compatibility to be used by growers (Kyriacou et al., 2017). *C. metuliferus* seems a good candidate rootstock to introduce in infested RKN areas, however, the host suitability to RKN populations from the vegetable production areas, the effect on the population dynamics, the rootstock-scion compatibility and the effect of the nematode on the crop yield quantity and quality is unknown.

Thus, increasing the availability of a diversity of RKN resistance germplasm for economical important crops used in the rotation sequences will theoretically help to reduce the nematode population growth rate, crop yield losses, and preserve the durability of the resistance.

Virulence selection to RKN, fitness cost and management

Virulence can be defined as the ability of the nematode to overcome plant resistance. Virulence selection is subject to different factors and can be progressive (Verdejo-Lucas et al., 2009; Giné and Sorribas, 2017), or occur suddenly (Ornat et al., 2001; Cortada et al., 2008; Barbary et al., 2016). The repeated cultivation of the same *R*-gene leads to a selection of virulent populations. This phenomenon has been widely reported for the *Mi*1.2 resistant gene in tomato cultivars or rootstocks (Noling, 2000; Verdejo-Lucas et al., 2009; Giné and Sorribas, 2017), and to the *Me*3, *Me*7 and *N* in pepper (Djian-Caporalino et al., 2011; Thies et al., 2011). Acquired virulence is a genetically inherited and stable character (Castagnone-Sereno et al., 1993), but it probably needs a minimum amount of continuous exposure to the resistant germplasm to become fixed in the population.

The loss of resistance is an important problem, as shown by the increasing frequency of virulent RKN populations in commercial areas in the recent years (Tzortzakakis et al., 2005; Devran and Söğüt, 2010; Verdejo-Lucas et al., 2012) and due to the time needed to find sources of resistance to be introgressed via breeding to commercial cultivars or rootstocks for grafting. In order to avoid virulence selection, different strategies should be considered. When it is available, the use of resistance plants with more than one resistant gene (Pyramided *R*-genes), for example, pepper carrying the *Me1* and *Me3* showed to be more effective than alternating those genes separated and than the use of a single *R*-gene. (Djian-Caporalino et al., 2014). Similar results were reported for potato germplasm containing the $GpaIV_{adg}$ and *Gpa5* genes pyramided, where fewer *Globodera pallida* cysts were developed compared to genotypes carrying each single gene separated (Dalton et al., 2013). It is accepted that the acquisition of virulent status brings changes in the fitness of the nematode population in the susceptible plant hosts, compared to avirulent nematodes (Petrillo and Roberts, 2005; Djian-Caporalino et al., 2011; García-Mendívil and Sorribas, 2019). In fact, rotation sequences including resistant and susceptible crops have been proposed as a strategy to reduce the level of virulence and to reduce crop yield losses (Talavera et al., 2009; Nilusmas et al., 2016 and 2020).

Alternating different *R*-genes is foreseeable to reduce the problem, because acquired virulence for one gene does not compromise other *R* genes preserving all genes involved (Dijan-Caporalino et al., 2011). However, the number of resistant crops, the *R*-genes involved and the order in a rotation sequence need to be assessed.

In this PhD, the impact of *C. metuliferus* as a melon rootstock as well as its contribution to the durability of the resistance in crop rotation with the *Mi*1.2 resistant gene was evaluated.

In addition, population dynamics and the effect of the nematode on fruit quantity and quality was assessed in spring or summer in order to stablish the best rotation scheme.

OBJECTIVES

The main objective of this PhD was to evaluate the resistance response of *Cucumis metuliferus* to *Meloidogyne* spp. and its compatibility with melon, its resistance durability in crop rotation with *Mi*1.2 resistant tomato and the effect of the rootstocks and nematode population in both tomato and melon yield and fruit quality. This general objective was divided in two specific objectives:

1- To evaluate the host suitability of different accessions of *Cucumis metuliferus* against (a)virulent *Mi*1.2 isolates of *Meloidogyne* spp. and its compatibility with melon. (Chapter 1 and 3).

2- To determine the effect of a three years tomato-melon rotation on the population dynamics of *M. incognita*, the crop yield (quantity and quality), and the durability of the resistance of both tomato *Mi*1.2 gene and *C. metuliferus* R genes. (Chapter 2 and 3).

CHAPTER 1

Cucumis metuliferus is resistant to root-knot nematode *Mi*1.2 gene (a)virulent isolates and a promising melon rootstock.

Fruit of melon cv. Paloma. Picture: Alejandro Expósito Creo

Cucumis metuliferus is resistant to root-knot nematode *Mi*1.2 gene (a)virulent isolates and a promising melon rootstock.

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Abstract

Pot experiments were carried out to characterize the response of two *Cucumis metuliferus* accessions (BGV11135 and BGV10762) against *Mi*1.2 gene (a)virulent *Meloidogyne arenaria, M. incognita* and *M. javanica* isolates and to determine the compatibility and the effect on physicochemical properties of fruit melons. In addition, histopathological studies were conducted. One week after transplanting, plants were inoculated with 1 J2 cm^{-3} (200 cm^3-pots) of sterilized sand and maintained in a growth chamber at 25ºC for 40 days. The susceptible cucumber cv. Dasher II or melon cv. Paloma were included for comparison. The number of egg masses and number of eggs per plant were assessed, and the reproduction index (RI) was calculated as the percentage of eggs produced on the *C. metuliferus* accessions compared to those produced on the susceptible cultivars. The compatibility and fruit quality were assessed by grafting three scions, two of Charentais type and one of type Piel de Sapo, under commercial greenhouse conditions. The resistance level of both *C. metuliferus* accessions ranged from highly resistant (RI < 1%) to resistant (1% ≤ RI ≤ 10%) irrespective of *Meloidogyne* isolates. Melon plants grafted onto *C. metuliferus* accession BGV11135 grew as selfgrafted plants without negatively impacting fruit quality traits. Giant cells induced by *Meloidogyne* spp. on *C. metuliferus* were in general poorly developed compared to those on cucumber. Furthermore, necrotic areas surrounding the nematode were observed. *C. metuliferus* accession BGV11135 could be a promising melon rootstock to manage *Meloidogyne* spp., irrespective of their *Mi*1.2 (a)virulence without melon fruit quality reduction.

Key words: *Cucumis melo*, grafting, histopathology, horned cucumber, *Meloidogyne* spp., plant resistance.

Introduction

Root-knot nematodes (RKN), *Meloidogyne* spp., are the most damaging plant parasitic nematodes for vegetable production worldwide (Sikora and Fernández, 2005). Nonetheless, the ability of RKN species to develop in a given plant species, to reproduce on it, and to affect its productivity differs according to the plant's host status. Regarding cucurbit crops, one of the most widely cultivated groups around the world, zucchini-squash and watermelon are a susceptible and a poor-host, respectively, but both are tolerant (López-Gómez et al*.,* 2014 and 2015). Melon and cucumber, on the other hand, are susceptible and get severely damaged by RKN (Di Vito et al*.,* 1983; Giné et al*.,* 2014 and 2017). In Spain, crop rotation schemes including solanaceous and cucurbit crops are very common (Ornat et al*.*, 1997; Talavera et al., 2012; Giné and Sorribas, 2016), but resistant cucurbit cultivars or rootstocks are not commercially available. According to the European directive 2009/128/CE grafting onto resistant-tolerant rootstocks is a promising non-chemichal way to suppress RKN populations and to reduce yield losses of the most susceptible-intolerant cucurbit crops. Plant resistance is an effective and profitable control method (Sorribas et al*.,* 2005) to reduce the RKN reproduction rate and the equilibrium density (Talavera et al., 2009; Giné and Sorribas, 2017). This prevents subsequent yield losses on the following crop (Ornat et al*.,* 1997) which are directly related to nematode population densities in the soil at planting stage (Seinhorst, 1965). Grafting is also an effective tool for controlling other soil borne pathogens (Lee and Oda, 2010).

In this sense, cucurbit crops are usually grafted onto *Cucurbita* hybrids, which are resistant to fusarium wilt but susceptible to *Meloidogyne* spp*.* (Thies et al., 2010; López-Gómez et al., 2016; Giné et al., 2017). However, resistance to RKN has been found in wild *Cucumis* spp., including accessions of *C. africanus, C. anguria*, *C. ficifolius, C. metuliferus, C. myriocarpus, C. postulatus, C. subsericeus*, and *C. zeyheri* (Fassuliotis, 1967; Sigüenza et al., 2005; Kokalis-Burelle and Rosskopf, 2011; Pofu and Mashela, 2011; Guan et al., 2014; Liu et al., 2015). Moreover, some of these *Cucumis* species are resistant to pathogenic fungi, such as *Fusarium oxysporum* f. sp. *melonis* (Liu et al*.,* 2015) and *Monosporascus cannonballus* (Dias et al., 2001). The inclusion of RKN resistant cucurbit rootstocks in the solanaceous-cucurbitaceous rotation sequence could be helpful to manage RKN, including the isolates that are virulent to the *Mi*1.2 resistance gene of tomato. Such isolates have increased in the last years due the reiterative use of resistant germplasm. (Tzortzakakis et al., 2005; Devran and Sögüt, 2010; Verdejo-Lucas et al., 2012). Nonetheless, as far we know, there is no information about the host suitability of *C. metuliferus* accessions to *Mi*1.2 virulent RKN isolates.

C. metuliferus is a compatible rootstock for melon but can affect fruit quality traits, such as the total soluble solids content (º Brix) and the flesh firmness, depending on melon type and agronomic conditions (Guan et al*.,* 2014). When testing for putative rootstocks, the evaluation on their impact on the scion's qualitative traits should be considered. The objectives of this study were to assess the host suitability of *C. metuliferus* against several RKN (a)virulent isolates, its compatibility as a rootstock to melon and the effects on fruit quality. Complementary, histopathological studies were conducted

to identify resistance mechanisms of *C. metuliferus* against *M. javanica*.

Materials and methods

Nematode inoculum

RKN isolates belonging to *M. arenaria, M. incognita* and *M. javanica* were used in the experiments. The information on RKN species, code, origin and the (a) virulent status against tomato cultivars carrying the *Mi*1.2 gene is presented in Table 1.1. The RKN isolates were maintained on the susceptible tomato cv. Durinta (Seminis Seeds, USA and Canada). Second stage juveniles (J2) were used as inoculum. Eggs were extracted from tomato roots by blender maceration in a 5% commercial bleach (40 g L^{-1} NaOCl) solution for 5 min (Hussey and Barker, 1973). The egg suspension was then passed through a 74 µm aperture sieve to remove root debris, and eggs were collected on a 25 µm sieve and placed on Baermann trays (Whitehead and Hemming, 1965) at 25°C. Nematodes were collected daily using a 25 µm sieve during 7 days and stored at 9ºC until inoculation. *Meloidogyne* species identification were confirmed according to the morphology of the perineal pattern of the females, and by SCAR-PCR markers (Zijlstra et al., 2000).

Table 1.1. *Meloidogyne* isolates from Spain, geographic origin, (a)virulent status against tomato cultivars carrying the *Mi 1.2*, and reference.

Species	Isolate	Geographic origin	(a)virulent status	Reference
M. arenaria	MA68	Barcelona	Avirulent	NP*
	MAAl06	Almería	Virulent	Verdejo-Lucas et al*.,* 2012
M. incognita	MIAl15	Almería	Partial virulent	Verdejo-Lucas et al*.,* 2012
	Agropolis	Barcelona	Avirulent	Giné and Sorribas, 2017
	Garriga	Barcelona	Avirulent	NP
M. javanica	Bay	Murcia	Avirulent	NP
	MJ05	Barcelona	Avirulent	Ornat et al*.,* 2001
	Tugues	Barcelona	Avirulent	NP
	MJ27	Barcelona	Virulent	Ornat et al*.,* 2001
	MJLg	Almería	Virulent	NP

*NP: Not published

Response of C. metuliferus *accessions to RKN isolates*

Three experiments were carried out to evaluate the response of *C. metuliferus* against (a)virulent RKN isolates. In the first experiment, accessions BGV11135 and BGV10762 of *C. metuliferus* from the Institute for conservation and improvement of Valentian agrodiversity (COMAV-UPV) collection (Valencia, Spain) and cucumber cv. Dasher II (Seminis Seeds, USA and Canada), used as susceptible control, were assessed against the Agropolis (*M. incognita*) and MJ05 (*M. javanica*) avirulent isolates. Each plant-RKN isolate combination was replicated 10 times. The experiment was carried out once. In the second experiment, the response of only the *C. metuliferus* accession BGV11135 against avirulent isolates of *M. arenaria* (MA68), *M. incognita* (Agropolis and Garriga) and *M. javanica* (Bay, MJ05 and Tugues) was assessed because this accession showed the most consistent resistance response against the RKN isolates in the previous experiment. The susceptible standard cucumber cv. Dasher II was included for comparison. The experiment was repeated once. Each plant-RKN isolate combination was replicated 7 and 8 times in the first and second experiment repetition, respectively. In the third experiment, the response of the *C. metuliferus* accession BGV11135 and the susceptible melon cv. Paloma (Fitó, Spain) was assessed against four *Mi*1.2 virulent RKN isolates belonging to *M. arenaria* (MAAl06), *M. incognita* (MIAl15) and *M. javanica* (MJ27 and MJLg). The avirulent *M. javanica* isolate MJ05 was included as standard for comparison. The experiment was repeated once.

Each plant-RKN isolate combination was replicated 8 times.

All experiments were conducted following the same procedure. Seeds of *C. metuliferus* were surface disinfested using a 20% bleach commercial solution (40g L^{-1} NaOCl) during 2 min and washed two times in sterilized distilled water. Seed germination was done on a cotton matrix saturated with sterilized distilled water in Petri dishes and the seeds were incubated two days at 37ºC. Afterwards, germinated seeds were sown in sterile vermiculite and maintained in a growth chamber at 25±2ºC with a 16:8 h (light:darkness) photoperiod programme for a week. Then, seedlings were individually transplanted into 200 cm^3 pots containing sterile river sand and inoculated with 1 J2 cm^{-3} of soil a week after transplanting. Inoculated plants were maintained in a growth chamber during 40 days. Plants were watered as needed along the experiment and fertilized with a slow release fertilizer (15% N, 9% P_2O_5, 12% K_2O, 2% MgO_2, microelements; Osmocote Plus). Soil temperatures were recorded daily at 30-min intervals with a PT100 probe (Campbell Scientific Ltd) placed into the pots at 4 cm depth. At the end of the experiment, roots were carefully washed, weighed and immersed in a 0.01% solution of erioglaucine to assess the number of egg masses (Omwega et al*.,* 1988). RKN eggs were extracted from roots by maceration in a 10% commercial bleach solution (40g L^{-1} NaOCl) (Hussey and Barker, 1973) and counted. The reproduction index (RI) was calculated as the percentage of the number of eggs per plant in the experimental accessions compared to that on the susceptible cucumber cv. Dasher II or melon cv. Paloma. The response of the accessions was categorized according to the 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).

Histopathology

Seeds of *C. metuliferus* BGV11135 and cucumber cv. Dasher II were germinated and transferred to growth pouches as reported by Atamian et al*.* (2012). Plantlets were placed in a growth chamber at 25±2ºC with a 16:8 h (light:darkness) photoperiod programme, and inoculated at two true leaf expanded stage with 2500 J2 of the *M. javanica* MJ05 isolate. After 12 days, roots were carefully washed and cut in pieces of 10 mm. Then, roots containing galls were selected and fixed in 2.5% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) overnight at 4°C and washed three times with the same buffer. Afterwards, root pieces were post-fixed in 1% (w/v) osmium tetroxide in 0.1 M sodium phosphate buffer (pH 7.2) for 1 h and washed three times with the same buffer and dehydrated in an acetonitrile series (30–100%) before embedding in epoxy resin (Embed 812, Aname®) and polymerizing at 60ºC for 48h. Semithin (2 µm) sections of samples were obtained in a Reichert-Jung Ultracut E Ultra Microtome Leica EM UC6 (Leica Microsysteme GmbH Wien, Austria) and left to dry on a slide previous to be stained with Richardson's blue (Azure II in dH_20:Methylene blue in 1% sodium borate, 1:1; v/v). The sections were mounted in a DPX mountant for histology and observed under a Leica DM4000 B microscope (Leica Microsystems, Mannheim, Germany). Sections were photographed using a Leica DFC300 FX 1.4-megapixel digital colour camera equipped with the Leica software application suite LAS V3.8 (Leica Microsystems).

Compatibility and fruit quality assessment

The performance of *C. metuliferus* BGV11135 as a potential rootstock was evaluated using the cv. Vedrantais (COMAV-UPV, Spain) and Paloma (Fitó Seeds, Spain) of Charentais melon (*Cucumis melo* L. var*. cantalupensis* Naudin) and cv. Finura (Rijk Zwaan, Netherlands) of Piel de Sapo melon (*Cucumis melo* L. var*. inodorus* Naudin) as scions. Plants were selfgrafted (used as control treatment) and grafted onto *C. metuliferus* BGV11135 using the cleft procedure (Lee and Oda, 2010). Plants were grown under hydroponic conditions in a commercial greenhouse at Fundación Cajamar (Paiporta, València, Spain) during the spring-summer of 2017. Plant vigor was evaluated at 30 and 60 days after transplanting in a visual scale of 0 (low) to 4 (high). The flowering time was recorded as the number of days after transplanting at which the first female flower appeared. In order to evaluate the impact of grafting on fruit quality, each fruit (8 per treatment) was characterized for the following fruit traits: weight (g), length and width (cm), rind (mm), flesh thickness (cm) and firmness (kg cm^{-2}) (Penetrometer (8 mm) FHT-803®, Melrose, MA), pH (pH-indicator paper pH1-14 Merck, Darmstadt, Germany), total soluble solids (Digital rephractometer Atago®, Tokyo, Japan), and flesh color (Colorimeter Minolta CR-400®, New Jersey, USA) using the color parameters Hunter *L*, *a* and *b*, where the *L* value indicates lightness (from 0 to 100), the *a* value redness (+) or greenness (-), and the *b* value yellowness (+) or blueness (-).

Statistical analysis

Statistical analysis was performed using SAS system V9 (SAS Institute, Inc., Cary, NC, USA). Data on number of eggs masses and eggs per plant were submitted to non-parametric analysis by the npar1way procedure to compare between replications of the same experiment, and considered as the same experiment if no differences were found ($P \geq 0.05$) by the Kruskal-Wallis test. Comparisons were made between the number of eggs masses and eggs per plant produced on each *C. metuliferus* accession and those on the susceptible cucumber or melon cultivars, as well as between *C. metulifeurs* accessions in the first experiment. Moreover, a comparison was made between RKN isolates per each plant material. Paired comparisons of fruit quality traits between all grafted and selgrafted cultivars were performed by Student t-test because data were normally distributed.

Results

Response of C. metuliferus *accessions against* Meloidogyne *spp. isolates*

The number of egg masses and eggs per plant on both *C. metuliferus* accessions were significantly lower ($P < 0.05$) than on the susceptible cucumber cv. Dasher II, irrespective of the *Meloidogyne* isolates (Table 1.2). Both *C. metuliferus* accessions (BGV11135 and BGV10762) responded as highly resistant (RI < 1%) or resistant (1% ≤ RI ≤ 10%) to RKN depending on the nematode isolate. The MJ05 isolate produced more ($P < 0.05$) egg masses and eggs per plant on BGV10762 than BGV11135 accessions. The infective and reproductive ability of the *Meloidogyne* isolates differed ($P < 0.05$) on both *C. metuliferus* BGV11135 and the cucumber cv. Dasher II. The nematode isolates Agropolis and Garriga of *M. incognita*, and MJ05 of *M. javanica* produced the highest number of egg masses and eggs per plant ($P < 0.05$) compared to the remaining RKN isolates on *C. metuliferus*. *M. arenaria* isolate MA68 produced the highest amount of egg masses on cucumber, although reproduction was higher in the Agropolis and Garriga isolates of *M. incognita* ($P < 0.05$). The accession BGV11135 of *C. metuliferus* was classified as resistant against most RKN isolates assessed. Regarding the *Mi*1.2 gene virulent isolates, the BGV11135 accession responded as highly resistant (RI < 1%), resistant (1% ≤ RI ≤ 10%) or moderately resistant (10% ≤ RI < 25%) (Table 1.3).

Histopathology

M. javanica isolate MJ05 induced giant cells in both *Cucumis* species (Figure 1.1), but those produced in *C. metuliferus* were in general poorly developed with multiple vacuoles compared to those on cucumber. Furthermore, giant cells without cytoplasm and necrotic areas surrounding the nematode were observed.

Table 1.2. Number of egg masses plant^{-1}, eggs plant^{-1}, and reproduction index (RI) of *M. arenaria M. incognita* and *M. javanica* isolates on the *C. metuliferus* accessions BGV11135 and BGV10762 in the experiment 1 and BGV11135 in the experiment 2, and on the cucumber cv. Dasher II.

Experiment	Species	Isolate	Eggs masses plant^{-1}			Eggs plant^{-1} (x 100)			RI (%)	
			C. metuliferus		Cucumber	*C. metuliferus*		Cucumber		
			BGV10762	BGV11135	Dasher II	BGV10762	BGV11135	Dasher II	BGV10762	BGV11135
Experiment 1	*M. incognita*	Agropolis	1 ±0.2 b*	2±0.5 a*	78±9.7 a	2.1±0.9 b*	3.7±1.1 a*	526±72 a	0.4±0.2	1±2
	M. javanica	MJ05	4 ±0.6 a*	1±0.3 a*	44±13.6 b	16±4.1 a*	4.3±1.3 a*	407±118 a	4±1	1±3
Experiment 2	*M. arenaria*	MA68	-	1 ± 0.3 b*	58 ± 3.2 a	-	0.3 ± 0.1 b*	3.9 ± 1.3 d	-	8 ± 4
	M. incognita	Agropolis	-	2 ± 0.3 a*	35 ± 4.9 b	-	4.7 ± 1.1 a*	178 ± 31 a	-	1 ± 1
		Garriga	-	4 ± 0.7 a*	32 ± 0.3 b	-	8.6 ± 2.2 a*	157 ± 18 a	-	4 ± 2
	M. javanica	Bay	-	0,4 ± 0.2 b*	11 ± 1.2 d	-	0.08 ± 0.05 b*	32 ± 6.8 c	-	<1
		MJ05	-	3 ± 0.6 a*	33 ± 1.23 b	-	3.6 ± 0.9 a*	51 ± 14 bc	-	7 ± 2
		Tugues	-	0,3 ± 0.2 b*	19 ± 2.4 c	-	0.64 ± 34 b*	68 ± 972 b	-	3±2

Data are mean ± standard error of 10 and 15 replicates in experiment 1 and 2 respectively. Data within the same column and experiment followed by the same letter did not differ ($P < 0.05$) according to the Kruskal-Wallis test. Data of egg masses plant^{-1} or eggs plant^{-1} within the same raw followed by * indicate differences ($P < 0.05$) between each *C. metuliferus* accessions and cucumber according to the Kruskal-Wallis test.

RI (Reproduction index): (number of eggs on the *C. metuliferus* accession / number of eggs on the cucumber cv. Dasher II) *100.

Table 1.3. Number of eggs plant^{-1} of avirulent (MJ05), partially virulent (MIAl15) and virulent (MAAl06, MJLg and MJ27) isolates to the *Mi 1.2* gene on *C. metuliferus* accession BGV11135 and melon cantaloupe cv. Paloma and reproduction index (RI) in experiment 3.

Species	Isolate	Eggs plant^{-1} (x 100)		RI (%)
		BGV11135	Paloma	
M. arenaria	MAAl06	0.6 ± 0.2 b*	4.4 ± 2.4 b	13.4 ± 4.7
M. incognita	MIAl15	10 ± 3.8 *	133 ± 25 a	7.5 ± 2.8
M. javanica	MJLg	11 ± 5 a*	88 ± 35 a	13 ± 6
	MJ27	0	6.1 ± 2.1 b	0
	MJ05	3.9 ± 2.2 ab*	159 ± 17 a	2.4 ± 1.3

Data are mean ± standard error of 16 replicates. Data within the same column followed by the same letter did not differ ($P < 0.05$) according to the Kruskal-Wallis test. Data of eggs plant^{-1} followed by * indicate differences ($P < 0.05$) between the *C. metuliferus* accession and melon cv. Paloma according to the Kruskal-Wallis test.

RI (Reproduction index): (number of eggs on the *C. metuliferus* accession / number of eggs on the melon cv. Paloma) *100.

Compatibility and fruit quality assessment

C. metuliferus used as rootstock did not affect the plant growth of Charentais and Piel de Sapo melons. Grafted plants of each cultivar showed similar vine vigour and flowering time than the corresponding selfgrafted plants. There were no significant effects of the rootstock on fruit's external and internal quality in the two Charentais melons cultivars, except from a slight increase of the flesh's thickness for cv. Paloma (Table 1.4).

Each grafted Charentais melon cultivar maintained its fruit size, rind and flesh firmness, and flesh quality (º Brix, pH and colour). Grafting the Piel de Sapo melon cv. Finura onto *C. metuliferus* increased both fruit weight and length, although they were softer, sweeter and the flesh presented a lighter colour compared to the selfgrafted plants (Table 1.4).

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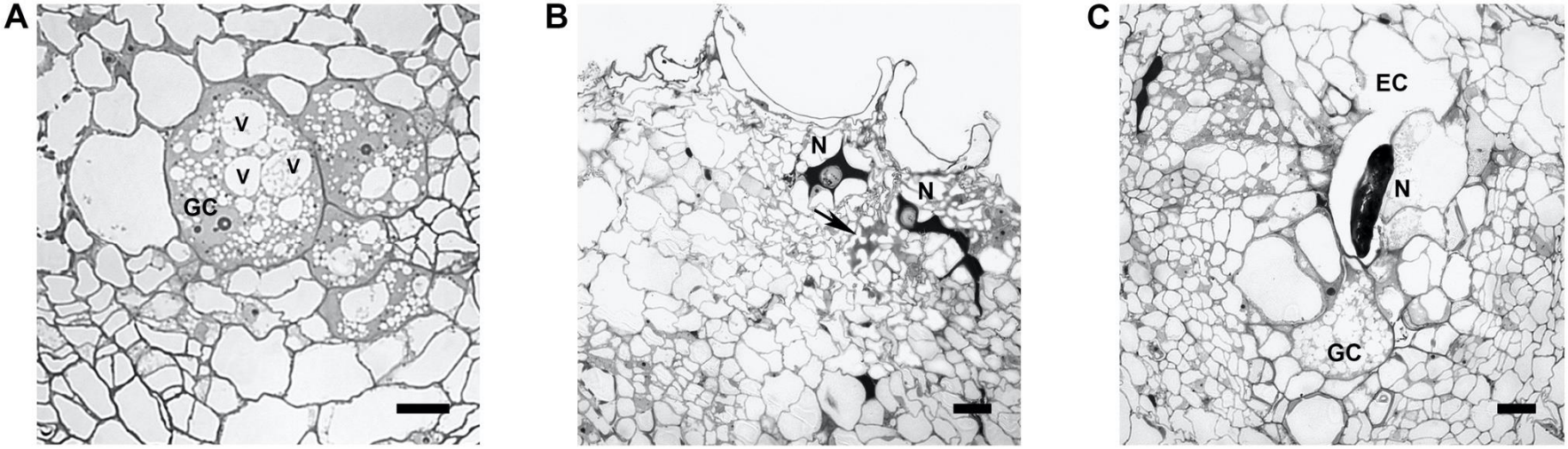

Figure 1.1. Light microscope images of 2 lm tranversal sections of cucumber cv. Dasher II (A) and *Cucumis metuliferus* accession BGV11135 (B, C) infected roots by *Meloidogyne javanica* (MJ05) 12 days after inoculation. GC, giant cells; V, vacuole; N, nematode; EC, empty cell; arrows indicates the nematode-necrosed area around the nematode. Bars = 20 µm

Table 1.4. Quality parameters of fruit of the Charentais melon cv. Vedrantais (VED) and cv. Paloma (PAL) and the Piel de sapo melon cv. Finura (FIN) from plants selfgrafted and grafted onto *C. metuliferus* BGV11135.

Genotype	Fruit weight (g)	Fruit length FL (cm)	Fruit width FW (cm)	Rind thickness (mm)	Flesh thickness (cm)	Cavity thickness (cm)	Rind firmness ($kg.cm^{-2}$)	Flesh firmness ($kg.cm^{-2}$)	*ºBrix*[1]	*pH*	*L*[2]	*a*[2]	*b*[2]
VED-VED	723.4 ± 26.5	10.6 ± 0.2	11.3 ± 0.1	3.3 ± 0.3	26.4 ± 1.3	47.4 ± 1.2	11.7 ± 0.7	1.0 ± 0.3	14.4 ± 0.5	6.2 ± 0.1	53.4 ± 1.1	11.1 ± 0.2*	23.9 ± 1.2
C.metuliferus-VED	758.1 ± 49.8	10.6 ± 0.2	11.7 ± 0.3	3.5 ± 0.2	27.1 ± 0.7	46.6 ± 1.8	11.4 ± 0.8	1.3 ± 0.2	14.2 ± 0.4	6.1 ± 0.1	55.5 ± 0.4	13.1 ± 0.6	24.9 ± 0.5
PAL-PAL	811.9 ± 48.5	11.8 ± 0.2	11.6 ± 0.3	3.0 ± 0.1	24.5 ± 0.6*	54.5 ± 1.5	13.0 ± 0.0	3.6 ± 0.1	15.9 ± 0.2	6.0 ± 0.0	62.3 ± 0.8	14.1 ± 0.4*	27.8 ± 0.1
C.metuliferus-PAL	907.1 ± 24.4	12.3 ± 0.1	11.9 ± 0.1	2.9 ± 0.2	27.7 ± 0.9	53.4 ± 1.9	12.8 ± 0.2	3.2 ± 0.2	15.3 ± 0.3	6.1 ± 0.1	62.0 ± 1.2	12.9 ± 0.3	27.5 ± 0.4
FIN-FIN	1340.5 ± 48*	16.4 ± 0.2*	12.8 ± 0.2	4.1 ± 0.1*	34.9 ±0.2	53.6 ± 0.7	13.0 ± 0.0	2.3 ± 0.1*	14.5 ± 0.2*	6.0 ± 0.0	58.3 ± 0.3*	-2.5 ± 0.1	8.4 ± 0.2
C.metuliferus-FIN	1552.6 ± 85.9	17.4 ± 0.2	13.3 ± 0.2	3.2 ± 0.1	33.4 ± 1.0	56.9 ± 1.3	13.0 ± 0.0	1.8 ± 0.1	15.5 ± 0.2	6.0 ± 0.0	64.3 ± 0.9	-2.6 ± 0.1	8.9 ± 0.1

Data are mean ± standard error of 8 replicates. Values of each parameter in the same cultivar followed by * are significantly different according to Student-t test ($P < 0.05$).

[1]Brix: soluble solid content measured in fruit flesh as Brix degrees.
[2]Hunter *L*, *a*, *b* colour parameters measured in fruit flesh: *L* value indicates lightness (from 0 to 100), the *a* value redness (+) or greenness (-), and the *b* value yellowness (+) or blueness (-).

Discussion

The *C. metuliferus* accessions assessed in this study were highly resistant (RI < 1%) or resistant (1% ≤ RI ≤ 10%) to most RKN isolates tested. This is in agreement with previous reports by other authors (Fassuliotis, 1967 and 1970; Sigüenza et al*.,* 2005; Walters et al*.,* 2006; Guan et al*.,* 2014; Ye et al., 2017). The host suitability of *C. metuliferus* was not affected by the *Mi*1.2 (a)virulence of the nematode isolate. The frequency of detection of virulent *Mi*1.2 populations of *Meloidogyne* in commercial growing areas is increasing since the last century (Tzortzakakis et al., 2005; Devran and Sögüt, 2010; Verdejo-Lucas et al., 2012), wich is a serious problem that needs to be solved. Verdejo-Lucas et al. (2012) reported for example that 48% of the RKN populations from 29 fields sampled in Almeria (Spain), the most important tomato growing area under protected cultivation in Europe, were virulent. Selection of virulence to the *Mi*1.2 gene in field conditions can be progressive (Verdejo-Lucas et al*.,* 2009; Giné and Sorribas, 2017) or can occur suddenly (Ornat et al*.,* 2001) depending on the genetic background of the plant and/or the nematode population (Ornat et al., 2001; Cortada et al., 2008). Different strategies for managing the selection for virulence on solanaceous crops have been assessed. Such strategies were mainly based on the rotation of tomato germplasm carrying the *Mi*1.2 resistance gene with susceptible cultivars (Talavera et al., 2009; Giné and Sorribas, 2017) or on pyramiding multiple R-genes in pepper (Djian-Capporalino et al., 2014). Until now, no virulent RKN populations to *C. metuliferus* have been reported. Including new sources of resistance to RKN, as such on *C. metuliferus,* could thus be a useful tool for managing RKN, irrespective of their (a)virulence. Moreover, it could be difficult to select for virulence to resistant genes on solanaceous crops in rotation schemes with susceptible cucurbits grafted onto resistant rootstocks. In addition, the RKN population able to reproduce on both resistant solanaceous crops and *C. metuliferus* could be an indicator of the durability of the resistance due the high specificity of resistance genes. This hypothesis should be verified in long-term experiments.

Fassulotis (1967 and 1970) reported the resistance response of *C. metuliferus* accession C-701 to *M. incognita*. They conducted histopathological studies, and observed small giant cells affecting nematode development and increasing the proportion of males. However, no hypersensitive response was observed. Similar results were found by Walters et al*.,* (2006) in the accession PI482454 inoculated with *M. arenaria, M. hapla, M. incognita* or *M. javanica.* Recent studies (Ye et al*.,* 2017) have reported a reduction of the number of *M. incognita* J2 in roots of the *C. metuliferus* accession PI482443 at 7 compared to at 4 days after inoculation (dpi), indicating death or emigration from roots and a delayed development of the nematodes remaining in the roots. Empty or poorly developed giant cells with multiple vacuoles were observed at 7 and 14 dpi, with giant cells appearing to be collapsed or without cytoplasm. In addition, several genes related to plant defence mechanisms were significantly modified and, in contrast with previous reports (Fassuliotis, 1970; Walters et al., 2006), hypersensitive necrosis was observed (Ye et al., 2017). The results of this study are consistent with those previously reported, in which giant cells were multivacuolated or appeared collapsed without cytoplasm. Furthermore, necrotic areas were observed. These results indicate that the *C. metuliferus* genetic background could

play an important role in the interaction with *Meloidogyne* spp.

Grafting can affect fruit quality depending on the rootstock-scion interactions, climatic and agronomic conditions (Leonardi et al., 2017). For instance, fruit melons of cultivars Supermarket or Proteo grafted onto *C. metuliferus* contained less º Brix than the ungrafted plants in one out two cropping seasons (Trionfetti-Nisini et al., 2002). Guan et al. (2014) reported less º Brix content and flesh firmness in galia but not in honeydew melons grafted onto *C. metuliferus* conducted in a conventional manner. However, no differences were found when plants were conducted under organic farming. In this study, no differences were found on growth or fruit quality between selfgrafted cantaloupe melon cv. Vedrantais and cv. Paloma and those grafted onto *C. metuliferus*. These results are in agreement with those reported by Gisbert et al. (2017) who did not find differences among fruit quality from ungrafted, selfgrafted or grafted cv. Vedrantais onto *C. metuliferus*. Conversely, grafted melon Piel de Sapo cv. Finura onto *C. metuliferus* affected fruit weight and length. Nonetheless, these changes do not reduce the commercial value of the fruits as the market of Piel de Sapo melons accepts a wide range of fruit sizes and variability in shapes. The changes in parameters associated with flesh quality (higher º Brix, lower flesh firmness and lighter flesh color) might be associated to a more advanced ripening state of the melons grafted onto *C. metuliferus*. Effects on fruit quality in grafted plants due to growing cycle alterations have been reported previously (Davis et al., 2008; Soteriou et al., 2014). Therefore, these effects could be reduced adapting the harvesting period for each rootstock-scion combination.

In conclusion, the *C. metuliferus* accession BGV11135 could be a promising melon rootstock to manage *Meloidogyne* spp. irrespective of their *Mi*1.2 (a)virulence, without reducing melon fruit quality. In addition, the *C. metuliferus* accessions assessed in this study are highly resistant to fusarium wilt (Gisbert et al., 2014), and tolerant to *Monosporascus cannonballus* in field conditions (Perpiñà et al., com pers).

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CHAPTER 2

Cucumis metuliferus reduces *Meloidogyne incognita* virulence against the *Mi*1.2 resistance gene in a tomato-melon rotation sequence

Plantlets of tomato cv. Durinta. Picture: Alejandro Expósito Creo.

Cucumis metuliferus reduces *Meloidogyne incognita* virulence against the *Mi*1.2 resistance gene in a tomato-melon rotation sequence

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Abstract

The susceptible tomato cv. Durinta, ungrafted or grafted onto cv. Aligator resistant rootstock, both followed by the susceptible melon cv. Paloma, ungrafted or grafted onto *Cucumis metuliferus* BGV11135, and in reverse order, were cultivated from 2015 to 2017 in the same plots in a plastic greenhouse, infested or not with *Meloidogyne incognita*. For each crop, the soil nematode densities, galling index, number of eggs per plant and crop yield were determined. Moreover, virulence selection was evaluated in pot experiments. In the tomato-melon rotation, the nematode densities increased progressively for the grafted tomato, being higher than for the ungrafted plants at the end of the study; but not so in the melon-tomato rotation. The grafted crops yielded more than the ungrafted ones in the infested plots. Virulence against the *Mi*1.2 gene was detected, but not against *C. metuliferus*. Reproduction of *M. incognita* on the resistant tomato was around 120% that on the susceptible cultivar after the first grafted tomato crop, but this decreased to just 25% at the end of the experiment. Alternating different resistant plant species suppresses nematode population growth rate and yield losses. However, although do not prevent the selection of virulence, the level was reduced.

Key words: *Cucumis melo*, grafting, resistance durability, root-knot nematode, *Solanum lycopersicum*.

Introduction

Root-knot nematodes (RKN), *Meloidogyne* spp., are the most harmful parasitic nematodes for vegetable crops worldwide (Sikora and Fernández, 2005). Vegetable yield losses caused by RKN under protected cultivation have been estimated to reach maximum values of 88% in cucumber, 62% in tomato, 39% in zucchini-squash, and 37% in watermelon (Giné et al., 2014; López-Gómez et al., 2014; Vela et al., 2014; Giné and Sorribas, 2017). Currently, chemical control, either alone or combined with other methods, is frequently used to manage RKN densities (Djian-Caporalino, 2012; Talavera et al., 2012). Nonetheless, the use of pesticides must be reduced in accordance with European directive 2009/128/CE via increased application of natural pest control mechanisms, in line with integrated pest management. Among such natural mechanisms, plant resistance is the most effective, economical and environmentally friendly control method; and it is easy for farmers to use (Sorribas et al., 2005; Giné and Sorribas, 2017). However, the effectiveness of plant resistance decreases or is lost entirely after repeated cultivation of the same resistance gene or *R*-gene (Verdejo-Lucas et al., 2009; Giné and Sorribas, 2017).

Alternating the *R*-genes in crops via rotation sequences could prevent the selection of RKN populations that are virulent against each gene, and thus improve resistance durability. Unfortunately, there is little diversity among *R*-genes in commercial vegetable cultivars or rootstocks to the most widespread RKN species: *M. arenaria, M. incognita* and *M. javanica.* Within solanaceous and cucurbit crops, the most economically important cultivated vegetables worldwide, three *R*-genes can be found in commercial pepper (*Me1, Me3* and *N*), and only one in tomato (*Mi*1.2) (Williamson and Roberts, 2009; Barbary et al., 2015). Additionally, resistance to RKN in the Solanaceae family has been found in several wild accessions; for example, in *Solanum arcanum, S. sisymbrifolium, S. sparsipilum,* and *S. torvum* (Kouassi et al., 2005; Jablonska et al., 2007; Dias et al., 2012; Bagnaresi et al., 2013). For cucurbit crops, no cultivars resistant to RKN are commercially available, and they are mostly grafted onto hybrid *Cucurbita* rootstocks that are resistant to fusarium wilt but susceptible to RKN (Thies et al., 2010; López-Gómez et al., 2016; Giné and Sorribas, 2017). Nonetheless, resistance has also been found in wild accessions of different cucurbit genera: *Cucumis,* including *C. africanus, C. anguria, C. ficifolius, C. metuliferus* and *C. myriocarpus* (Liu et al., 2015); and *Citrullus*, including *Citrullus lanatus* var. *citroides* (Thies et al., 2015). All these species represent putative germplasm that could be used as commercial rootstocks or in breeding programmes to obtain commercial resistant cultivars.

In the case of *C. metuliferus*, the resistance response to RKN is associated with hindrance of larval development, delayed development from second-stage juveniles (J2) to adults, increased maleness of J2 (Fassuliotis, 1970; Walters et al., 2006), migration of J2 from the root, differential expression of several genes related to plant defence mechanisms (Ye et al., 2017), and the appearance of necrotic areas surrounding the nematode (Expósito et al., 2018). Rotation sequences including solanaceous and cucurbits species in protected cultivation are very common, because these crops represent the main source of income for many growers (Ornat et al., 1997; Thies et al., 2004; Djian-Caporalino, 2012; Talavera et al., 2012; Giné and Sorribas,

2017). So, alternating resistant solanaceous cultivars with resistant cucurbitaceous ones could be an efficient way to manage RKN densities by preventing the selection of virulent populations and consequently reducing crop yield losses. *C. metuliferus* is resistant to RKN populations that are (a)virulent against the *Mi*1.2 gene, and it is compatible melon rootstock (Expósito et al., 2018).

To the best of our knowledge, however, there is no information available on the effect of rotating *C. metuliferus* with RKN-resistant crops on the potential selection of RKN populations that are virulent against both the *Mi*1.2 tomato gene and *C. metuliferus*. Selection of RKN for their virulence can be detected by an increase in the final RKN population density on the resistant germplasm, compared to that on the susceptible germplasm, at the end of the crop (*Pf*), for a given initial RKN density at transplanting (*Pi*). That is, the RKN population growth rate (the relationship between the rate of multiplication (*Pf*/*Pi*) and *Pi*) on resistant germplasm tends to be similar to that of the susceptible one (Giné and Sorribas, 2017). In addition, virulence is tested for by comparing RKN reproduction on resistant versus susceptible germplasm in pot experiments at constant soil temperatures above 28ºC, using the field nematode population as an inoculum (Sorribas et al., 2005; Verdejo-Lucas et al., 2009). Moreover, the reproduction index (RI), that is, the proportion of RKN reproduction on the resistant germplasm compared to that on the susceptible germplasm, allows to estimate the level of plant resistance (Hadisoeganda and Sasser, 1982) as well as nematode virulence to a given *R*-gene(s) (Sorribas et al., 2005; Verdejo-Lucas et al., 2009).

The efficacy of alternating resistant germplasm could be affected by soil temperatures. In the case of the *Mi*1.2 gene, its expression may be reduced at soil temperatures over 32ºC (Dropkin, 1969), depending on the time spent under these conditions (Verdejo-Lucas et al., 2013; de Carvalho et al., 2015). So, the sequence of the crops in rotation must be considered to select the most suitable for achieving the highest level of nematode suppression and therefore to maximize crop yield without compromising the durability of any resistance gene(s). Thus, the objective of this study was to determine the effect of three-year rotation sequences including tomato and melon, ungrafted or grafted onto RKN-resistant germplasm, on nematode suppression, disease severity, crop yield and putative virulence selection; as well as the optimal sequence of crops in the rotation scheme.

Materials and methods

Plastic greenhouse experiments

The experiment was carried out in a 700 m^2 experimental plastic greenhouse located in Viladecans (Barcelona, Spain) over three growing seasons (2015, 2016 and 2017). The soil 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^{-1} electrical conductivity. The plastic greenhouse was solarized from July to September in 2014. Afterwards, 75% of the soil was infested with the avirulent *Mi*1.2 gene isolate Agropolis from *M. incognita* by planting infected tomato cv. Durinta (Seminis Seeds) in October 2014 and harvesting them in February 2015. The tomato plants were obtained from a commercial nursery and were inoculated

with 100 eggs and 100 J2 per polystyrene tray cell 7 days before transplanting. The *M. incognita* isolate was obtained in 2010 from roots of the susceptible tomato cv. Durinta, grown in a plot previously cultivated with susceptible tomato or cucumber, or maintained in black fallow since 2007. The nematode isolate was maintained in susceptible tomato cultivated in pots and identified by the morphology of the perineal pattern and by sequence-characterized amplified region (SCAR) markers (Zijlstra et al., 2000). The *Mi*1.2 gene and *C. metuliferus* avirulence status of the isolate were determined previously (Giné and Sorribas, 2017; Expósito et al., 2018). The remaining 25% of the soil was planted with non-inoculated tomato cv. Durinta, which did not show nematode infection and reproduction at the end of the crop cycle. The experiment consisted of four treatments: i) susceptible tomato cv. Durinta grafted onto the resistant rootstock Aligator (previously PG76) (Gautier seeds) (GT) followed with susceptible melon cv. Paloma (Fitó Seeds) grafted onto the resistant *C. metuliferus* accession BGV11135 from the Institute for Conservation and Improvement of Valencian Agrodiversity (COMAV-UPV) collection (Valencia, Spain) (GM); ii) ungrafted tomato cv. Durinta (T) followed by ungrafted melon cv. Paloma (M); iii) GM-GT; and iv) M-T. Each treatment was cultivated in both *M. incognita* infested and non-infested plots. Crops were grown from March to July and July to November each year in two rotation schemes, tomato-melon (GT-GM, T-M) and melon-tomato (GM-GT, M-T); except in 2017, when only the spring crop of each rotation (March to September) was grown (Figure 2.1). Each treatment was replicated 10 times. Individual plots of 3.75 m^2 consisted of 2.5 m long, containing 4 plants with 0.55 m between each. Plots within a row were spaced 0.9 m, with 1.5 m between rows. Grafted or ungrafted plants were cultivated in the same plot each year to determine the effect of alternating resistant plant species on *M. incognita* densities, disease severity, crop yield and the durability of the resistance of both the *Mi*1.2 tomato gene and *C. metuliferus*. The soil in each plot was prepared separately to avoid cross contamination. Plants were irrigated as needed via a drip irrigation system and fertilized with a solution of NPK (15-5-30) at 31 kg ha^{-1}, and iron chelate and micronutrients at 0.9 kg ha^{-1}. Weeds were removed manually before and during the growing season. Soil temperature and water content were recorded at 1 h intervals with 5TM digital soil probes (Decagon Devices, Inc.) placed at a depth of 15 cm. Tomato and melon fruits were harvested and weighed when they reached commercial standards, and values were expressed as kg $plant^{-1}$. Initial nematode population densities were determined at transplanting (*Pi*) and finally at the end (*Pf*) of each crop. Soil samples consisted of eight cores taken from the top 30 cm of soil with a 2.5 cm diameter auger, which were mixed and passed through a 4 mm-pore sieve to remove stones and roots. For each experimental plot, J2 were extracted from 500 cm^3 of soil using Baermann trays (Whitehead and Hemming, 1965) and incubated at 27°C±2°C for 1 week. Afterwards, the J2 were collected using a 25 µm aperture screen, counted, and expressed as J2 250 cm^{-3} of soil. At the end of each crop cycle, roots were carefully removed from the soil, washed and weighed, and then the galling index was evaluated on a scale from 0 to 10: 0 = complete and healthy root system, and 10 = plants and roots dead (Zeck, 1971).

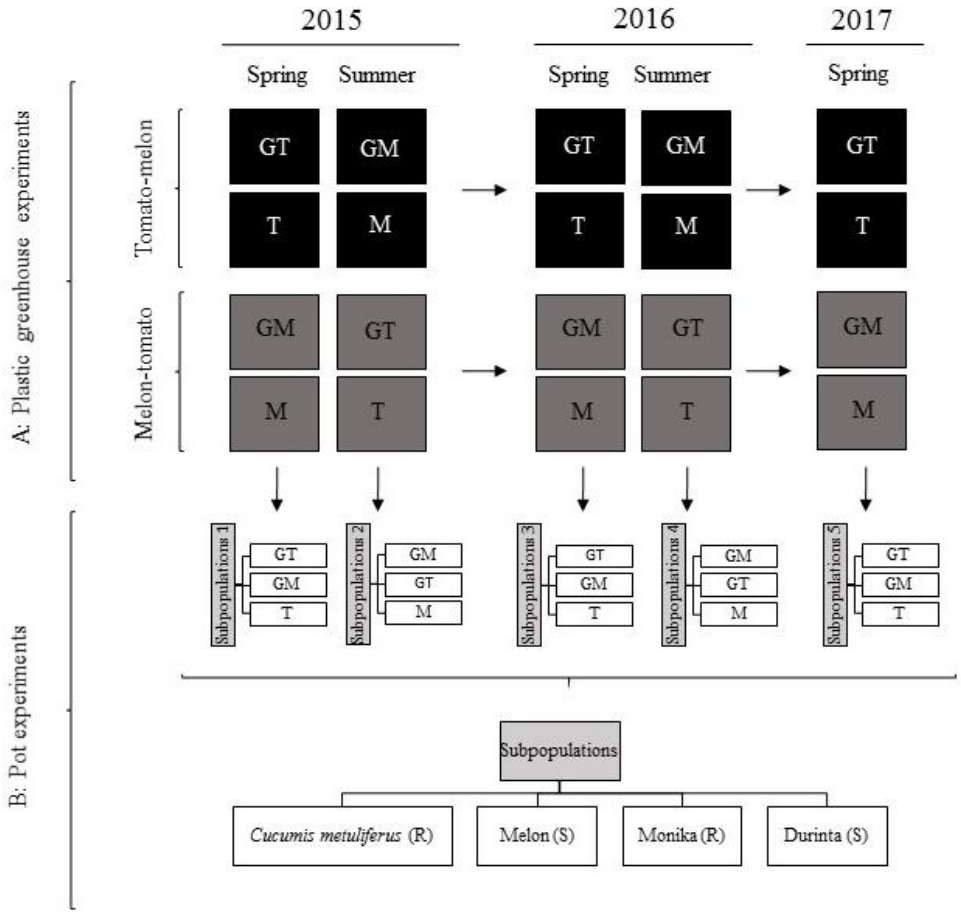


Figure 2.1. A: Rotation schemes for 2015, 2016 and 2017 for the tomato-melon (GT-GM;T-M) or melon-tomato (GM-GT;M-T) including susceptible tomato (T) and susceptible melon (M) ungrafted or grafted onto the resistant tomato rootstock cv. Aligator (GT) or resistant *Cucumis metuliferus* (GM) accession BGV11135 respectively in a plastic greenhouse infested with *Meloidogyne incognita* to determine the nematode suppression, disease severity and crop yield. B: Pot experiments conducted with the subpopulations extracted after each crop of the rotation scheme to determine the putative selection of virulence.

After that, roots of the plants from the same plot were chopped, homogenized, and two 20 g samples of roots were used to determine the number of eggs. The eggs were extracted from roots by maceration in a 10% solution of commercial bleach (40 g L^{-1} 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, counted and expressed as eggs $plant^{-1}$. The remaining root samples were used to obtain nematode inoculum to assess putative virulence selection. The nematode multiplication rate was calculated as *Pf* (J2 250 cm^{-3} soil + eggs $plant^{-1}$) / *Pi* (J2 250 cm^{-3} soil), and the relationship between *Pf*/*Pi* and *Pi* was established for each crop and year, in order to determine the putative virulence selection, according to Giné and Sorribas. (2017).

Virulence selection

The experiments were conducted at the end of each crop cycle. The nematode inoculum consisted of J2 obtained from eggs produced on each plant material: tomato cv. Durinta ungrafted or grafted onto the cv. Aligator rootstock, and melon cv. Paloma ungrafted or grafted onto C. metuliferus (Figure 2.1). The eggs were extracted from roots by blender maceration in a 5% solution of commercial bleach (40 g L^{-1} NaOCl) for 5 min (Hussey and Barker, 1973), as previously described. The egg suspension was placed on Baermann trays at 27°C±2°C. Nematodes were collected daily for 7 days using a 25 µm sieve, and stored at 9ºC until inoculation. The resistant tomato cv. Monika (Syngenta, Switzerland), the susceptible cv. Durinta, the resistant C. metuliferus BGV11135 and the susceptible melon cv. Paloma were used in the experiments. Seeds of C. metuliferus were germinated as reported in Expósito et al. (2018). Tomato seeds were sowed in sterile vermiculite at 25ºC±2ºC. Seedlings were maintained in a growth chamber at 25ºC±2ºC with a 16:8 h (light:dark) photoperiod, for a week. Afterwards, the plants were individually transplanted into 200 cm^3 pots containing sterile river sand and maintained under the same conditions as before. Plants with three true leaves were singly inoculated with 1 J2 cm^{-3} of soil. Each plant-subpopulation combination was replicated 10 times. After the first experiment, the avirulent population from the tomato-melon rotation was selected, because no differences were observed between subpopulations from the ungrafted tomato or melon. The plants were maintained in the growth chamber under the same conditions as described previously for 40 days. They were watered as needed and fertilized with a slow release fertilizer (15% N, 9% P_2O_5, 12% K_2O, 2% MgO_2, microelements; Osmocote Plus). Soil temperatures were recorded at 30 min intervals with a PT100 probe (Campbell Scientific Ltd.) inserted into the pots at a depth of 4 cm. At the end of the experiments, roots were carefully washed and weighed. The nematode eggs were extracted from the roots, as previously described. The RI for each subpopulation was calculated as the percentage of the number of eggs per plant in the resistant *C. metuliferus* or tomato cv. Monika, in relation to that in the susceptible melon cv. Paloma or tomato cv. Durinta, respectively. The response of the tomato cv. Monika and *C. metuliferus* was categorized according to the RI as highly resistant (RI < 1%), resistant (1% ≤ RI < 10%), moderately resistant (10% ≤ RI < 25%), slightly resistant (25% ≤ RI < 50%) or susceptible (RI ≥ 50%). In addition, two experiments were conducted to assess the infectivity, the fecundity and the level of virulence of the subpopulations of the J2 extracted from the soil at the end of the summer crop in 2016, and from those extracted from eggs collected at the end of the spring crop in 2017. The experiments were carried out following the same procedures described previously. The infectivity was considered to be the number of J2 capable of infecting and developing into females laying eggs; and it was expressed as the number of egg masses per plant. The number of egg masses was counted after dying by submerging the whole root system in a 0.01% solution of erioglaucine for 30 min (Omwega et al., 1988). The fecundity was evaluated as the number of eggs laid by each female and expressed as the number of eggs egg $mass^{-1}$.

Statistical analysis

Statistical analyses were performed using IBM SPSS statistics v.23 (IBM Corp.). Data for *Pi* and *Pf*/*Pi* were transformed to $\log_{10}$ (x) to linearize them, and subjected to regression analysis for each crop and year, in order to determine the population growth rate. Linear regressions were compared between years for each crop. When no differences were found (intercept and slope $P > 0.05$), the data were pooled to construct a single general model. Regression lines of the grafted and ungrafted crops for each rotation scheme were compared between years, or between general models if no differences were found between years. The galling index and crop yield data were compared between grafted and ungrafted plants for each crop and year; and the crop yield was also compared between infested and non-infested plots. The optimal rotation sequence was determined by comparing the rotation sequences, considering the overall yield of grafted crops in 2015 and 2016, cultivated in infested plots. Comparisons were carried out by means of the non-parametric Wilcoxon signed rank test, as the data did not fit a normal distribution. Data on number of egg masses, eggs plant^{-1}, and eggs egg mass^{-1} from the virulence selection experiments were compared between resistant and susceptible germplasm, or between nematode subpopulations. All the data were subjected to the non-parametrical Wilcoxon signed rank test or the Kruskal-Wallis test ($P \leq 0.05$), due to the non-normal distribution of the data.

Results

Plastic greenhouse experiment

The dates of cultivation of each crop, the minimum, maximum and average soil temperatures during cultivation and the range of nematode densities at transplanting each crop are presented in Table 2.1.

In the tomato-melon rotation scheme, the relationship between *Pi* and *Pf*/*Pi* for ungrafted tomato (T) did not differ between 2015 and 2017 (intercept $P = 0.1122$; slope $P = 0.2992$); however, both these differed from the relationship in 2016 (intercept $P = 0.0002$; slope $P = 0.0127$). For grafted tomato, the relationship between *Pi* and *Pf*/*Pi* differed between 2016 and 2017 (intercept $P < 0.0001$; slope $P = 0.7059$). The population growth rate on ungrafted tomato was higher than on grafted tomato (intercept $P = 0.0008$; slope $P = 0.7156$) in 2016, but it was lower in 2017 (intercept $P < 0.0001$; slope $P = 0.1379$) (Figure 2.2A). The grafted tomato showed a lower ($P < 0.05$) galling index than the ungrafted tomato in 2015 and 2016, but a high index ($P < 0.05$) in 2017 (Table 2.2). The grafted tomato cultivated in infested plots yielded between 64% and 88%, with respect to that in non-infested plots; and between 1.45 and 1.8 times more than the ungrafted tomato in infested plots (Table 2.2).

Table 2.1. Rotation sequence, cultivation dates, soil temperatures and nematode density ranges at transplanting (*Pi*) the ungrafted susceptible tomato cv. Durinta (T) or grafted onto the resistant tomato rootstock cv. Aligator (GT), and the ungrafted susceptible melon cv. Paloma (M) or grafted onto the resistant *Cucumis metuliferus* accession BGV11135 (GM) cultivated in a plastic greenhouse infested with *M. incognita* in 2015, 2016 and 2017.

Rotation sequence	Year	Crop	Dates		Soil T (ºC)			*Pi* range (J2 250cm^{-3})
			Start	End	Min	Max	Av	
Tomato-melon	2015	GT/T	24/3	16/7	17.6	31.9	24.1	0-1611
		GM/M	22/7	26/10	18.3	30.5	24.2	0-4438
	2016	GT/T	15/3	21/7	13.1	29.4	22.1	0-1496
		GM/M	22/7	26/10	18.4	30.5	25.2	0-4657
	2017	GT/T	19/4	12/9	13.8	29.8	24	0-5222
Melon-tomato	2015	GM/M	24/3	16/7	17.6	31.9	24.1	0-1134
		GT/T	22/7	29/10	18.1	30.5	24.1	0-3970
	2016	GM/M	20/4	26/7	14	30	22.5	0-3312
		GT/T	27/7	7/11	17.1	30.6	25.1	0-1395
	2017	GM/M	5/4	28/8	13.1	29.8	24.6	0-6680

Regarding the summer melon crop, no differences were found in the population growth rate of the grafted melon between 2015 and 2016 (intercept P = 0.12; slope P = 0.8466). In fact, in melon, only in 2015 were significant regressions found, and the population growth rate differed from that of the grafted melon (intercept $P < 0.0000$; slope $P = 0.2959$) due to the high mortality. A total of 98% of melon plants showed galling index values of 10 at the end of the crop, and this was 40% in 2016 (data not shown). A lower galling index was recorded on grafted than ungrafted melon each year ($P < 0.05$). The grafted melon cultivated in infested plots yielded between 11% and 35% less than that in non-infested plots; but between 8 and 13 times more than the ungrafted melon in infested plots (Table 2.2).

In the melon-tomato rotation scheme, the relationship between *Pi* and *Pf*/*Pi* for ungrafted and grafted melon did not differ between years (ungrafted melon, 2015 vs 2016: intercept $P = 0.1153$, slope $P = 0.8537$; 2015 vs 2017: intercept $P = 0.4832$, slope $P = 0.7631$; 2016 vs 2017: intercept $P = 0.4589$, slope $P = 0.7818$; grafted melon, 2015 vs 2016: intercept $P = 0.0852$, slope $P = 0.4593$; 2015 vs 2017: intercept $P = 0.3058$, slope $P = 0.9019$; 2016 vs 2017; intercept $P = 0.9856$, slope $P = 0.4894$).

Table 2.2. Galling index (GI) and yield (kg plant^{-1}) in the rotation sequence tomato-melon (GT-GM;T-M) and melon-tomato (GM-GT;M-T) of susceptible tomato cv. Durinta, ungrafted (T) or grafted onto the resistant tomato rootstock cv. Aligator (GT) and susceptible melon cv. Paloma, ungrafted (M) or grafted onto the resistant *Cucumis metuliferus* BGV11135 (GM) cultivated in *Meloidogyne incognita* infested or non-infested plots in a plastic greenhouse for three years.

Rotation sequence	Year	Season	Crop	GI†	Yield (kg plant^{-1})	
					Infested	Non-infested
Tomato-melon	2015	Spring	GT	2 ± 0.2*	3.6 ± 0.2 *b	4.1 ± 0.1 a
			T	8.2 ± 0.1	2 ± 0.2 b	4.4 ± 0.2 a
		Summer	GM	4.3 ± 0.4*	1.3 ± 0.1 *a	2 ± 0.4 a
			M	9.9 ± 0.1	0.1 ± 0.1 b	2.1 ± 0.4 a
	2016	Spring	GT	3.9 ± 0.1*	2.7 ± 0.2 *b	3.7 ± 0.2 *a
			T	6 ± 0.2	1.7 ± 0.2 b	2.7 ± 0.2 a
		Summer	GM	4.6 ± 0.8*	0.8 ± 0.2 *a	0.9 ± 0.1 a
			M	8.2 ± 0.4	0.1 ± 0.1	NA‡
	2017	Spring	GT	7.1 ± 0.3*	2.9 ± 0.2 *b	4.5 ± 0.2 a
			T	6.5 ± 0.1	2 ± 0.2	NA‡
Melon-tomato	2015	Spring	GM	4.1 ± 0.2*	3.2 ± 0.3 *a	2.5 ± 0.2 b
			M	8.7 ± 0.2	0.8 ± 0.1 b	2.5 ± 0.2 a
		Summer	GT	1.9 ± 0.2*	2 ± 0.2 *a	2.4 ± 0.2 a
			T	7.2 ± 0.2	0.8 ± 0.1 b	2.1 ± 0.2 a
	2016	Spring	GM	3.3 ± 0.2*	2 ± 0.2 *a	1.7 ± 0.2 a
			M	5.6 ± 0.3	0.2 ± 0.1 b	1.4 ± 0.1 a
		Summer	GT	5 ± 0.3*	1.6 ± 0.1 b	2 ± 0.2 a
			T	5.9 ± 0.2	1.6 ± 0.1	NA‡
	2017	Spring	GM	5.1 ± 0.3*	3.1 ± 0.3 *a	3.4 ± 0.3 a
			M	6.1 ± 0.2	0.3 ± 0.1	NA‡

Data are mean of 40 plants ± standard error. Values followed by * are different between grafted and ungrafted plants according to the Wilcoxon signed rank test (P<0.05). Values of yield in the same row followed by the same letter are not different according to the Wilcoxon signed rank test (P<0.05).

†GI: Galling index (Zeck, 1971)
‡NA: Not available, due to cross contamination.

The general linear model of the population growth rate for ungrafted melon was higher than for grafted melon (intercept P< 0.0001; slope P = 0.1506) (Figure 2.2C). The grafted melon showed a lower (P < 0.05) galling index than the ungrafted melon each year (Table 2.2). Regarding melon yield, the grafted melon produced 1.3 times more (P < 0.05) in infested than non-infested plots in 2015; but did not differ the other years. However, the ungrafted melon cultivated in infested plots produced between 68% and 86% less than in non-infested plots. The grafted melon yielded between 4 and 10.3 times the ungrafted in infested plots (Table 2.2). In the following tomato crops, the population growth rate for ungrafted tomato did not differ between years (intercept P < 0.9828; slope P = 0.9592), but it did for grafted tomato (2015 vs 2016: intercept P < 0.0001; slope P = 0.8600) being higher in 2016 than in 2015, but lower than for grafted tomato (Figure 2.2D). A lower galling index was recorded for grafted than for ungrafted tomato each year. The grafted tomato cultivated in infested plots yielded 20% less than that in

non-infested plots in 2016, and did not differ from that of the ungrafted tomato in infested plots (Table 2.2).The comparison between rotation sequences considering the overall yield of grafted crops cultivated in infested plots in 2015 and 2016 were 15% higher in the melon-tomato rotation sequence than the tomato-melon sequence ($P < 0.05$).

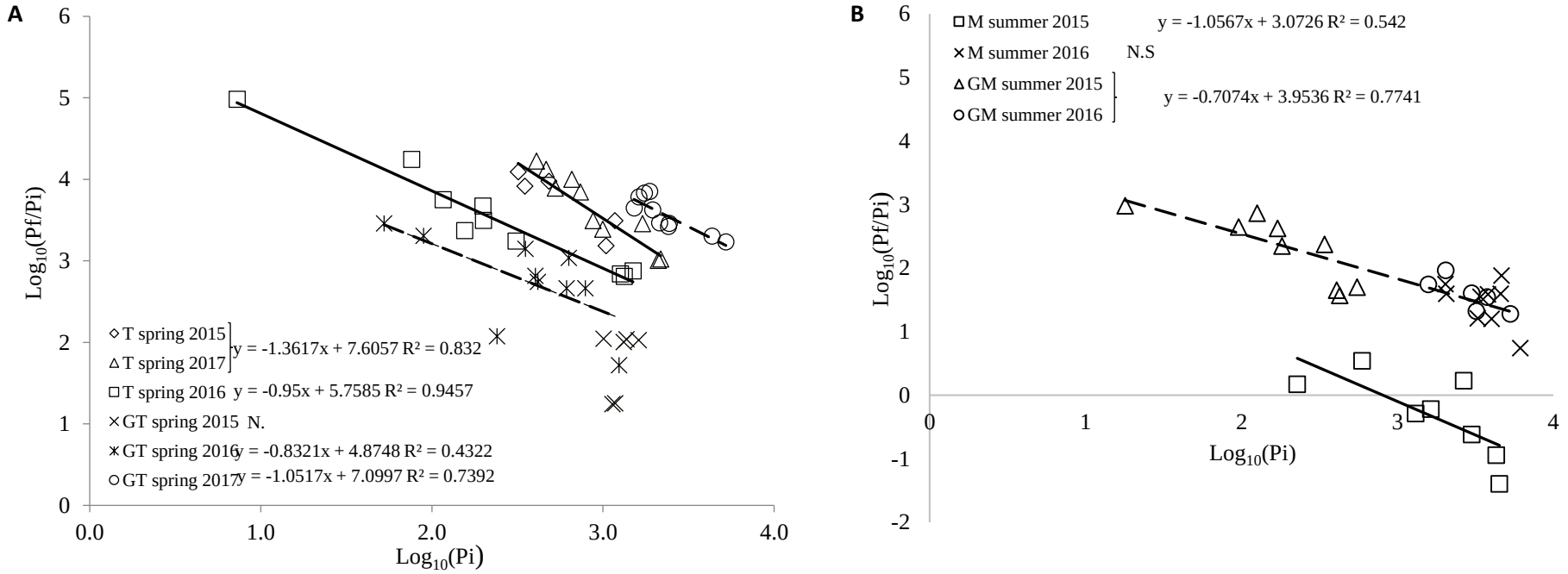
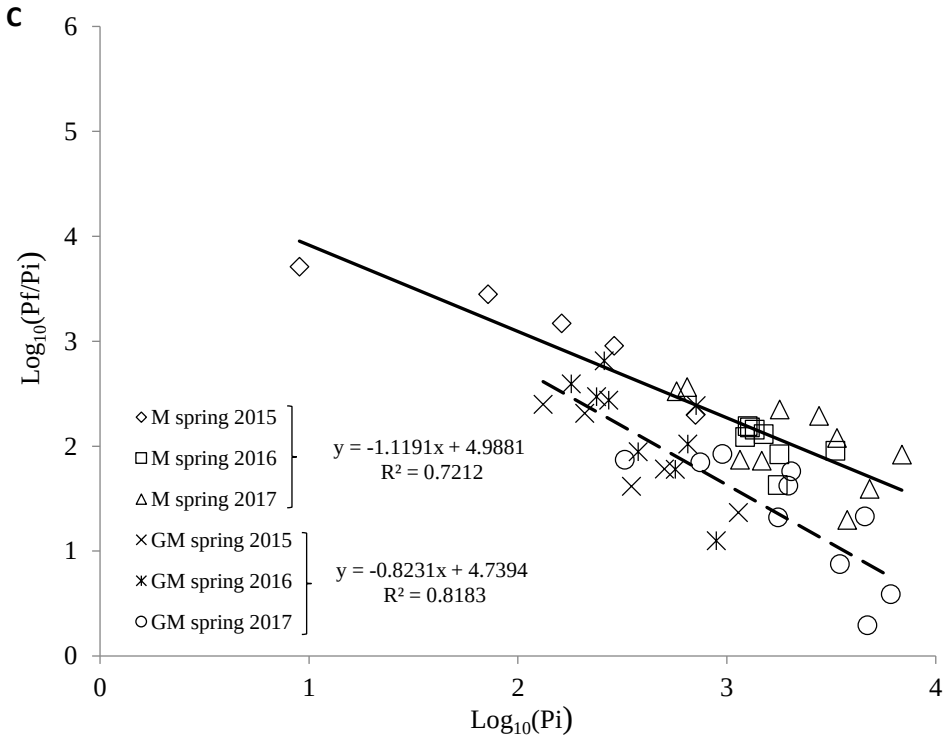
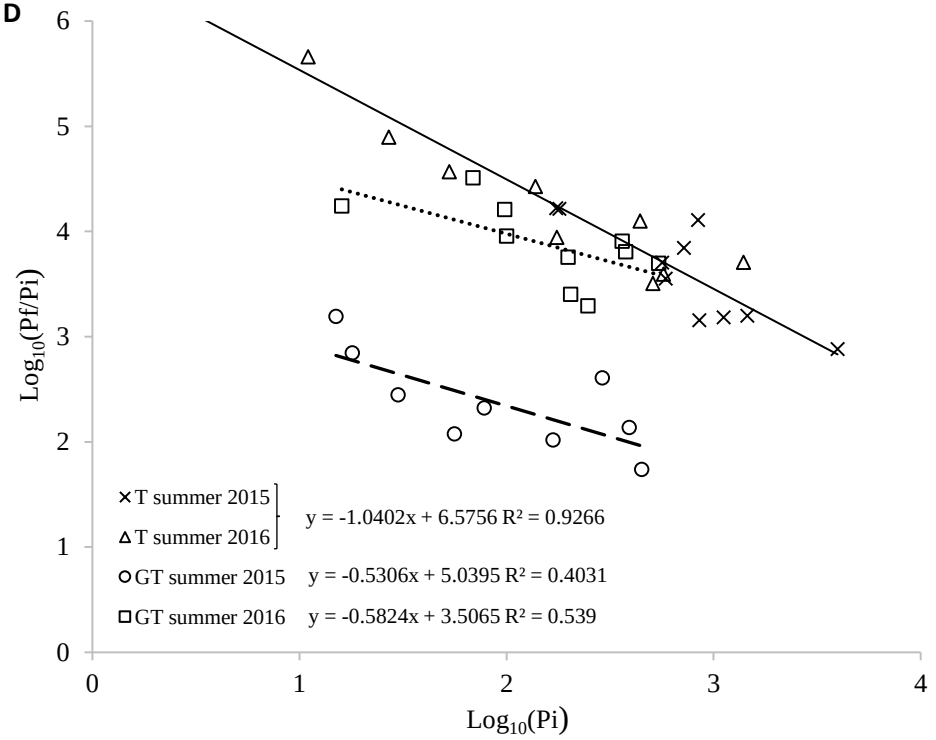


Figure 2.2. Relationship between the *Meloidogyne incognita* nematode reproduction rate (*Pf/Pi)* and the population densities at transplanting (*Pi*) for the susceptible tomato cv. Durinta, ungrafted (T) or grafted onto the resistant tomato rootstock cv. Aligator (GT), and for the susceptible melon cv. Paloma ungrafted (M) or grafted onto the resistant *Cucumis metuliferus* accession BGV11135 (GM) cultivated in a plastic greenhouse during 2015, 2016 and 2017 in a tomato-melon (A and B) or melon-tomato (C and D) rotation scheme. N.S: Not significant.


C
Log10(Pf/Pi)
Log10(Pi)
M spring 2015
M spring 2016
M spring 2017
y = -1.1191x + 4.9881
R² = 0.7212
GM spring 2015
GM spring 2016
GM spring 2017
y = -0.8231x + 4.7394
R² = 0.8183


D
Log10(Pf/Pi)
Log10(Pi)
T summer 2015
T summer 2016
y = -1.0402x + 6.5756 R² = 0.9266
GT summer 2015
y = -0.5306x + 5.0395 R² = 0.4031
GT summer 2016
y = -0.5824x + 3.5065 R² = 0.539

Virulence selection bioassays

The RI for the resistant tomato cv. Monika of the subpopulations from the ungrafted tomato or melon throughout the study ranged from <1% to 5%, corroborating that the tomato cv. Monika was resistant and thus, the nematode subpopulations were avirulent against the *Mi*1.2 gene. However, the subpopulations from roots of the first grafted tomato cultivated in both spring-summer and summer-autumn in the plastic greenhouse were fully virulent against the *Mi*1.2 gene, according to their RI for cv. Monika: RI =120% and 118%, respectively. Nonetheless, after cropping the following grafted melon, the RI decreased to 39% when cultivated in summer-autumn 2015, and to 14% when cultivated in spring-summer 2016. After that, the RI ranged from 13% to 31% (Figure 2.3).

The RI for *C. metuliferus* ranged from <1% to 13%, irrespective of the plant germplasm in which the subpopulation was developed. So, no virulence selection was observed in this plant germplasm, as it mainly reacted as resistant (1% ≤ RI < 10%) over the three years (Figure 2.3).

The infectivity and reproduction of the subpopulations obtained from soil after cropping grafted melon or grafted tomato in 2016 were higher ($P < 0.05$) than those of the subpopulation obtained after cropping ungrafted tomato. Nonetheless, the fecundity of the subpopulation obtained after cropping ungrafted tomato was higher than after cropping grafted melon on the resistant tomato cv. Monika. For the susceptible tomato cv. Durinta, the reproduction of the subpopulation after cropping grafted melon was lower than after cropping ungrafted tomato (Table 2.3). The infectivity, reproduction and fecundity of the nematode subpopulation obtained from grafted tomato roots at the end of the crop in 2017 were lower than the ungrafted tomato subpopulation on the susceptible cv. Durinta ($P < 0.05$). Moreover, the reproduction and fecundity of the subpopulation from grafted tomato were also lower ($P < 0.05$) than those of the subpopulation from ungrafted tomato, on melon cv. Paloma (Table 2.4).

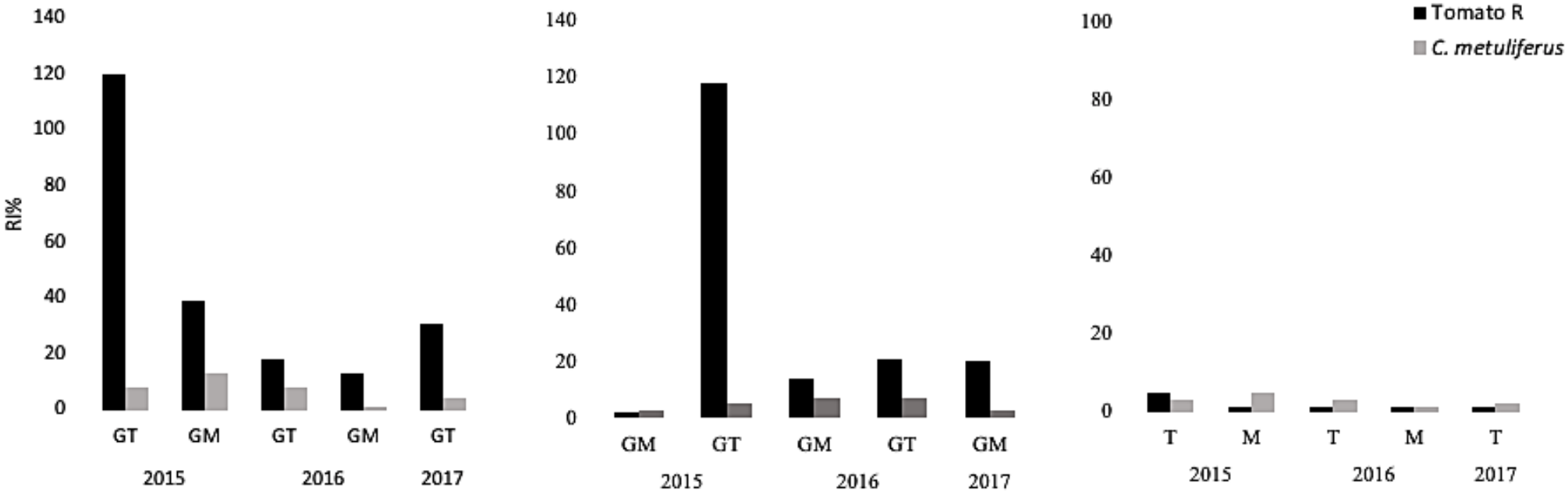


Figure 2.3. Reproduction index (RI: percentage of the eggs plant^{-1} produced in the resistant germplasm respect those produced in the susceptible germplasm), of the *Meloidogyne incognita* subpopulations obtained from roots of the susceptible tomato cv. Durinta, ungrafted (T) or grafted onto the resistant tomato rootstock cv. Aligator (GT) and susceptible melon cv. Paloma, ungrafted (M) or grafted onto the resistant *Cucumis metuliferus* accession BGV11135 (GM) cultivated in a plastic greenhouse in 2015, 2016 and 2017 in a tomato-melon (GT-GM;T-M) or melon-tomato (GM-GT; M-T) rotation sequence.

Table 2.3. Number of egg masses plant^{-1}, eggs plant^{-1} and eggs egg mass^{-1} produced on the resistant tomato cv. Monika (R) and the susceptible cv. Durinta (S) in 200cm^3 pot experiments inoculated with 1J2 cm^{-3} of the *Meloidogyne incognita* subpopulations obtained from soil after cropping grafted tomato (GT), grafted melon (GM) or tomato (T) in 2016.

	Egg masses plant^{-1}			Eggs plant^{-1} (x100)			Eggs Egg mass^{-1}		
Cultivar	GT	GM	T	GT	GM†	T	GT	GM	T
Monika (R)	29 ±3 a	32 ± 3 a	9 ± 1 b	195 ± 19 a	161 ±21 a	85 ± 15 b	693 ± 46 ab	531 ± 63 b	904 ± 58 a
Durinta (S)	102 ± 8 a*	76 ± 6 b*	96 ± 6 ab*	1124 ± 104 a*	433 ± 46 b*	743 ± 70 a*	1240 ± 205 a*	560 ± 38 b	790 ± 77 ab

Data are mean ± standard error of 16 replicates. Values of the same parameter in the same row followed by different letters are significantly different according to the Kruskal-Wallis test ($P < 0.05$). Values of the same column followed by * are different according to the Wilcoxon signed rank test ($P < 0.05$).
†GT: subpopulation from the melon-tomato rotation scheme, GM-GT-GM-GT; T: subpopulation from the melon-tomato rotation scheme, M-T-M-T; GM: subpopulation from the tomato-melon rotation scheme, GT-GM-GT-GM;

Table 2.4. Number of egg masses plant^{-1}, eggs plant^{-1} and eggs egg mass^{-1} produced on the resistant tomato cv. Monika (R), the susceptible cv. Durinta (S), the resistant *Cucumis metuliferus* BGV11135 (R), and the susceptible melon cv. Paloma (S) in 200cm^3 pot experiments inoculated with 1J2 cm^{-3} of the *Meloidogyne incognita* subpopulations obtained from roots after cropping grafted tomato (GT), grafted melon (GM) or tomato (T) in 2017.

	Egg masses plant^{-1}			Eggs plant^{-1} (x100)			Eggs Egg mass^{-1}		
Plant	GT	GM	T	GT†	GM	T	GT	GM	T
Monika (R)	14 ± 1 a	16 ± 2 a	1 ± 0 b	65 ± 9 a	126 ± 21 a	4 ± 1 b	454 ± 41 b	748 ± 63 a	288 ± 49 b
Durinta (S)	40 ± 4 b*	74 ± 7 a*	77 ± 7 a*	212 ± 26 b*	619 ± 58 a*	873 ± 71 a*	545 ± 52 b	839 ± 55 ab	1211 ± 93 a*
C. metuliferus (R)	4 ± 1 b	6 ± 1 a	6 ± 1 a	20 ± 4 a	20 ± 4 a	17 ± 3 a	418 ± 84 a	355 ± 47 a	334 ± 49 a
Melon (S)	52 ± 3 b*	72 ± 6 a*	67 ± 6 ab*	439 ± 27 b*	721 ± 57 a*	1003 ± 53 a*	851 ± 57 b*	1040 ± 84 b*	1617 ± 151 a*

Data are mean ± standard error of 16 replicates. Values of the same parameter in the same row followed by different letters are significantly different according to the Kruskal-Wallis test ($P < 0.05$). Values of the same column and crop followed by * are different according to the Wilcoxon signed rank test ($P < 0.05$).
†GT: subpopulation from tomato-melon rotation GT-GM-GT-GM-GT; GM: subpopulation from the melon-tomato rotation scheme, GM-GT-GM-GT-GM; T: subpopulation from the tomato-melon rotation scheme, T-M-T-M-T.

Discussion

The management of RKN is a challenge in intensive horticulture in which crop yield losses can be very important for farm economies. The use of plant resistance is an easy environmentally friendly way to suppress the nematode population growth and has a high benefit-to-cost ratio. Nonetheless, this strategy must be used correctly to avoid the selection of virulent nematode populations. The selection of *Mi*1.2 virulent populations due the reiterative use of resistant germplasm has been reported previously (Eddaoudi et al., 1997; Noling, 2000; Cortada et al., 2009; Giné and Sorribas, 2017), and it has become an important problem, as shown by the increasing frequency of virulent RKN populations in commercial areas in recent years (Tzortzakakis et al., 2005; Devran and Söğüt, 2010; Verdejo-Lucas et al., 2012). Thus, it is very important to include different *R*-genes, because the overlapping of signalling and the recognition of the resistance pathways may result in cross-selection (Petrillo et al., 2006). Along these lines, our working hypothesis was that alternating crops of two different resistant plant species can prevent the selection of virulence against each *R*-gene(s) thereby improving their durability. However, the results of this study have shown that this strategy is not enough to prevent the selection of virulence against one of them; but it does contribute to reducing disease severity and to improving crop yields.

The resistant cv. Aligator rootstock selected an *M. incognita* population with virulence against the *Mi*1.2 gene after the first tomato crop, irrespective of the crop season. This tomato rootstock was previously reported to be highly resistant in pot experiments and also after being cultivated for one season (March to July) in a plastic greenhouse (Cortada et al., 2008 and 2009). Nonetheless, the Aligator rootstock selected a virulent *M. javanica* population in plastic greenhouse experiments after being repeatedly cultivated for three seasons in the same plots (Verdejo-Lucas et al., 2009). This virulence selection was corroborated in pot experiments that show a progressive increase in the level of virulence, year by year, resulting in the resistance being overcome before the third tomato crop (*RI* = 90). Virulence selection is subject to different factors and can be progressive (Verdejo-Lucas et al., 2009; Giné and Sorribas, 2017), or occur suddenly (Ornat et al., 2001; Cortada et al., 2008; Barbary et al., 2016). Acquired virulence is a genetically inherited and stable character (Castagnone-Sereno et al., 1993), but it probably needs a minimum amount of continuous exposure to the resistant germplasm to become fixed in the population. Otherwise, if the population is not continuously exposed, the level of virulence of the population may decrease to a certain intermediate level, as observed with the inclusion of *C. metuliferus* in the rotation scheme. It is accepted that the acquisition of virulent status brings about changes in the fitness of the nematode population with respect to other susceptible plant hosts, compared to avirulent nematodes (Petrillo and Roberts 2005; Djian-Caporalino et al., 2011). The infectivity, reproduction and fecundity fitness of the subpopulation selected with *Mi*1.2 virulence against the susceptible tomato and melon were reduced with respect to the avirulent subpopulation after the third grafted tomato crop, but not after the second. This indicates that a minimum of three resistant tomato crops are needed to affect the fitness of the intermediate virulent population selected. So, in a nematode population in which (a)virulent individuals coexists, virulence could be

counter-selected in susceptible germplasm (Djian-Caporalino et al., 2011). Thus, including some more resistant plant species in the rotation scheme alone, or alternating with susceptible ones in order to increase the time elapsed between two crops with the same *R*-gene, could prevent virulence selection. However, even if it does not, virulence could not be fixed in the nematode population and the frequency of virulent individuals would decrease over time. In fact, rotation sequences including resistant and susceptible crops have been proposed as a strategy to reduce the level of virulence and to reduce crop yield losses (Talavera et al., 2009; Nilusmas et al., 2016). Other strategies to manage the emergence of virulent populations have been reported, such as pyramiding *R*-genes. For example, pepper germplasm containing both *Me1* and *Me3* resistance genes pyramided, totally suppressed the emergence of virulent isolates under both laboratory and field conditions (Djian-Caporalino et al., 2014). Similar results were reported with potato germplasm containing the $GpaIV_{adg}$ and *Gpa5* genes pyramided, in which fewer *Globodera pallida* cysts developed than in genotypes carrying each single gene (Dalton et al., 2013). Regarding tomato, several single dominant *R*-genes that are also resistant against *Mi*1.2-virulent RKN populations and stable at high soil temperatures (32^{o}C) have been identified and mapped in different chromosomes (Rashid et al., 2017). Such genes could be pyramided in order to obtain stronger and durable resistance in tomato. Similarly, transplanting plants primed by microorganisms which express faster and stronger resistance against RKN (Martinez-Medina et al., 2016) could reduce virulence selection. In addition, the inclusion of other practices in the rotation sequence, before the selection of virulent populations, such as the use of resistant plants or other plant species as a trap cover crop (Navarrete et al., 2016), soil solarization or biofumigation (Guerrero et al., 2006), could also avoid the virulence selection due to the reduced level of nematode infestation of the soil.

In this study, intermittent soil temperatures over 28^{o}C were registered at the end of the spring crop and at the beginning of the summer crop; but the possibility that this triggered the breaking of the resistance is ruled out in accordance with previous work (Verdejo-Lucas et al., 2013). High soil temperatures could help the nematode to breakdown the *Mi*1.2 gene, but this is not plausible as the nematode subpopulations obtained from roots after the first susceptible crop or *C. metuliferus,* which were similarly affected by these high soil temperatures, did not show an increase of *RI* in pot experiments at soil temperatures below 28^{o}C. In addition, the lack of resistance induced by exposure to high soil temperature is reversed over time, regardless of additional exposure and nematode infection (de Carvalho et al., 2015).

Conclusions

Alternating crops of different resistant plant species suppress nematode population growth rate and crop yield losses. Moreover, although this strategy does not prevent virulence selection, the resultant level of virulence is reduced.

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CHAPTER 3

Tomato and melon *Meloidogyne* resistant rootstocks improve crop yield but melon fruit quality is influenced by the cropping season.

Grafted and ungrafted tomato cv. Durinta (left) and melon cv. Paloma (right) onto Aligator and *C. metuliferus* BGV11135 respectively. Picture: Alejandro Expósito Creo

Tomato and melon *Meloidogyne* resistant rootstocks improve crop yield but melon fruit quality is influenced by the cropping season.

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Abstract

Four rotation sequences consisting of ungrafted tomato cv. Durinta – melon cv. Paloma or tomato grafted onto the resistant rootstock 'Aligator'– melon grafted onto the resistant *Cucumis metuliferus* accession BGV11135, and in reverse order, were conducted from 2015 to 2017 in a plastic greenhouse infested or not with *Meloidogyne incognita* to determine the plant tolerance (*T*), the minimum relative crop yield (*m*) and fruit quality. The relationship between *M. incognita* densities in soil at transplanting (*Pi*) of each crop and the crop yield was assessed and *T* and *m* were estimated by the Seinhorst's damage model. In addition, the volume and the number of nuclei of single giant cells and the number of giant cells, its volume and the number of nuclei per feeding site in susceptible tomato and melon were compared to those in the resistant tomato and *C. metuliferus* 15 days after nematode inoculation in pot test. The relationship between the *Pi* and the relative crop yield fitted the Seinhorst's damage model in both ungrafted and grafted tomato and melon, but not for all years and cropping seasons. The estimated *T* for ungrafted and grafted tomato did not differ but *m* was lower in the former (34%) than the latter (67%). Sodium concentration in fruits from ungrafted but not from grafted tomato increased with nematode densities in spring 2015 and 2016. The estimated ungrafted melon *T* did not differ from the grafted melon cultivated in spring, but it did when it was cultivated in summer. The relative crop yield of ungrafted melon was lower (2%) than the grafted cultivated in spring (62%) and summer (20%). Sodium concentration in melon fruits from ungrafted plants increased with nematode densities. No variations in fruit quality from grafted melon cultivated in spring were found, although less dry matter and soluble solid content at highest nematode densities were registered when it was cultivated in summer. Lower number of giant cells per feeding site was observed in both susceptible tomato germplasms compared to the resistant ones but they were more voluminous and held higher number of nuclei per giant cell and per feeding site.

Key words: Crop yield losses, *Cucumis melo, C. metuliferus,* Plant tolerance, Root-knot nematodes, *Solanum lycopersicum.*

Introduction

Tomato (*Solanum lycopersicum*) and melon (*Cucumis melo*) are two of the major horticultural crops worldwide with annual productions of 5.163.466 and 655.677 tonnes in 2017, respectively (FAOSTAT, 2017). Root-knot nematodes (RKN), *Meloidogyne* spp., are one of the most important limiting soil borne pathogens for vegetable production (Hallmann and Meressa, 2018). Among the more than 100 RKN species described, *M. arenaria, M. incognita, M. javanica* and *M. hapla* are the most damaging species, which are worldwide distributed, have a wide range of host plants and reproduce by parthenogenesis (Jones et al., 2013), allowing an exponential increase of nematode densities at the end of the crop from low densities at planting (Greco and Di Vito, 2009).

RKN are obligate sedentary endoparasitic nematodes. The infective second-stage juvenile (J2) moves between the soil particles and penetrates the host plant roots near to the elongation zone. The J2 moves intercellularly to the root tip, turns after the casparian strips, enter into the vascular cylinder to establish a feeding site and becomes sedentary. A feeding site is composed by five to seven multinucleate and hypertrophied cells, called giant cells, which supply nutrients to the nematode for the rest of its life cycle (Abad et al., 2009). After that, the parasitic J2 undergoes three moults to reach the adult female that lays the eggs in a gelatinous matrix, the egg mass, located outside or into the root. The embryogenesis leads to the J1 that moults inside the egg and becomes J2 until hatching occurs. The hypertrophy and hyperplasia of root parenchyma cells lead to the formation of galls that reduce the water and nutrients uptake in the infected plants, which can show aboveground symptoms, such as, dwarfism, wilting and nutrient deficiency. The severity of the symptoms can range from asymptomatic to plant death depending on nematode densities in soil and the plant tolerance. Crop yield losses due to RKN under different environmental conditions have been summarized by Greco and Di Vito, (2009). Regarding fruiting vegetables cultivated under protected or open fields, maximum yield losses of 88% and 75% have been reported for ungrafted and grafted cucumber onto *Cucurbita* hybrid rootstock, respectively; 65% and 57% for ungrafted and grafted melon onto *Cucurbita* hybrid rootstock; 56% for tomato; 39% for zucchini; and 37% for watermelon (Kim and Ferris, 2002; Ploeg and Phillips, 2001; Giné et al., 2014 and 2017; López-Gómez et al., 2014; Vela et al., 2014). In addition, RKN could affect fruit quality reducing its nutritive value. For instance, Vinay (2018) reported a reduction of the lycopene content in tomato fruits up to 37% and an increase of titratable acidity, total soluble solids and vitamin C up to 20%, being 75% and 21% respectively, when plants were cultivated in soil inoculated at a rate of 6 J2 g^{-1} of soil compared to the non-inoculated.

RKN control has been mainly conducted by non-fumigant and fumigant nematicides (Nyczepir and Thomas, 2009). However, the current legal regulations, such as the European directive 2009/128/CE, promote the use of alternative control methods in order to reduce their harmful effects to the environment and human health. Plant resistance has been proven to be an effective, economic, environmental and human health friendly control method against RKN (Sorribas et al., 2005; Starr and Mercer, 2009; Williamson and Roberts, 2009) able to be used in

integrated nematode management strategies. Plants bearing resistance genes lead to an incompatible plant-RKN interaction by the activation of several plant genes that suppress giant cell formation and/or induction of cell apoptosis affecting nematode development and/or reproduction (Shukla et al., 2018). Plant resistance genes to some RKN species have been reported in several crops (reviewed in Williamson and Roberts, 2009), but only a few of them have been introgressed into commercial fruiting vegetable cultivars including tomato and pepper. Nonetheless, several sources of plant resistance against RKN that are able to be used in plant breeding programs or as rootstocks have been reported (Lee et al., 2010). Commercial RKN resistant rootstocks are currently available for aubergine, pepper, and tomato. Regarding cucurbit crops, the watermelon rootstock *Citrullus amarus* 'Strongback', released by the USDA-ARS (Kemble et al., 2019), will be commercially available soon. But currently, there is none available for melon or cucumber although RKN resistant wild *Cucumis* species that could be used in breeding programs or as rootstocks have been described, such as *C. africanus, C. anguria, C. dipsaceus, C. ficifolius, C. hystrix, C. metuliferus, C. myriocarpus, C. proferatum, C. pustualtus, C. subsericeus, C. zambianus* and *C. zeyheri* (Liu et al., 2015; Expósito et al., 2018; Cheng et al., 2019). Despite the effectiveness of plant resistance against RKN, virulent nematode populations able to circumvent plant defence mechanisms can be selected after repeated cultivation of resistant plants bearing the same *R*-gene (Verdejo-Lucas et al., 2009; Thies, 2011; Ros-Ibáñez et al., 2014; Expósito et al., 2019). Consequently, plant resistance will be effective and durable only if it is adequately used, as for example in rotation sequences with different resistance genes. In a previous study, cropping melon grafted onto *C. metuliferus* followed by tomato grafted onto the resistant rootstock 'Aligator' or viceversa, reduced the reproduction rate of the nematode and yielded more compared to ungrafted crops; and also reduced the level of virulence to the *Mi*1.2 gene after cropping grafted melon onto *C. metuliferus* (Expósito et al., 2019).

Grafting vegetables onto resistant rootstocks is an effective management method against biotic and abiotic stresses that also provide yield stability (Rouphael et al., 2018). However, physicochemical fruit quality, storability, and nutritive value can be affected by grafting, being necessary the knowledge of particular scion-rootstock compatibility to be used by growers (Kyriacou et al., 2017). In order to know the tolerance of grafted plants to RKN, two parameters have to be considered: the tolerance limit (*T*), that is, the maximum nematode population that do not cause crop yield losses, and the minimum relative yield (*m*) at high nematode densities (Seinhorst, 1998).

Thus, the main objective of this study was to determine the plant tolerance, the minimum relative crop yield and fruit quality of ungrafted and grafted tomato cv. Durinta onto the resistant rootstock 'Aligator', and ungrafted and grafted melon cv. Paloma onto the resistant *C. metuliferus* accession BGV11135, cultivated in a rotation sequence of ungrafted tomato-ungrafted melon, grafted tomato-grafted melon and viceversa, conducted from 2015 to 2017 in plots infested or not with *M. incognita* in a plastic greenhouse. In addition, histopathology analyses were conducted to determine the number and the volume of giant cells per feeding site and the number of nuclei per giant cell and per feeding site in susceptible tomato and melon and being compared to those in the

resistant germplasm 15 days after nematode inoculation in pot test.

Materials and methods

Plant material

The susceptible tomato cv. Durinta (Seminis Seeds, USA and Canada) (T), the resistant tomato rootstock 'Aligator' (previously PG76) (Gautier seeds, France) (GT), the susceptible melon cv. Paloma (Fitó Seeds, Spain) (M), and the resistant *C. metuliferus* accession BGV11135 (GM) (Institute for Conservation and Improvement of Valencian Agrodiversity collection, COMAV-UPV, Valencia, Spain) were used in the plastic greenhouse experiment conducted to determine the damage function models, and the effect of grafting and nematode densities in fruit quality parameters. Plantlets were produced by the commercial nursery HishtilGS (Malgrat de Mar, Spain). Rootstocks seeds of tomato and melon were germinated in 104-cell polystyrene trays, and those of tomato and melon cultivars in 216-cell polystyrene trays during 2 days in a growth chamber at 25 °C ± 1 ºC and 90% relative humidity in the darkness. After that, plantlets were transferred to a greenhouse bench. Plantlets were watered and weekly fertilized with a 5-3-7 NPK liquid fertilizer. After 15 days, melon plants were grafted using the one cotyledon grafting method (Davis et al., 2008). Tomato plants were grafted after 25 days using the tube grafting method (Lee et al, 2010). Grafted plants were placed in a healing room at 25 ºC ± 1 ºC and 90% relative humidity for 5 days. After that, plants were acclimated in the shadow for one day and then, were transferred to a greenhouse bench for 10 days before transplanting.

The optical histopathology study was conducted with the majority of plant material used in the plastic greenhouse experiment, but the resistant tomato rootstock 'Aligator' was replaced by the resistant tomato cv. Monika (Syngenta Crop Protection AG, Basel, Switzerland), because it was no longer commercially available in Spain at the time of the study was conducted. Seeds were sown into vermiculite and incubated at 25 ^{o}C ± 2 ^{o}C and 16:8 h light:dark photoperiod in a growth chamber. Three-leaf stage plants were transferred to 200 cm^3 pots filled with sterilized sand at 121 ^{o}C for 1 h and repeated after 1 day. Afterwards, plants were fertilized with a slow release fertilizer (15% N, 9% P_2O_5, 12% K_2O, 2% MgO2, microelements: Osmocote Plus), watered as needed and maintained in a growth chamber at the same growing conditions described previously until nematode inoculation.

Damage functions models

The experiment was conducted over three growing seasons (2015, 2016 and 2017) in a 700 m^2 experimental plastic greenhouse located in Viladecans (Barcelona, Spain). The plastic greenhouse management history, the characteristics of the experiment and its design are described in Expósito et al. (2019). In brief, the experiment consisted of eight treatments replicated 10 times: grafted tomato (GT), grafted melon (GM), tomato (T) and melon (M) cultivated in both *M. incognita* infested and non-infested plots. Four individual rotation schemes were conducted in the same plots in 2015 and 2016: GT-GM, T-M, GM-

GT and M-T from March to July (spring crop) and July to November (summer crop). In 2017 only the spring crop was carried out. Grafted and ungrafted melon and tomato were cultivated from April to August and from April to September, respectively. Individual plots consisted in a row of 2.5 m long and 1.5 m wide containing 4 plants spaced 0.55 m between them. Plots were spaced 0.9 m within a row and 1.5 m between rows. The soil of each plot was prepared separately to avoid cross contamination. The soil was loamy sand textured, with 1.8 organic matter (w/w) and 0.5 dS m^{-1} electric conductivity. Plants were irrigated and fertilized by a drip irrigation system with a solution of NPK (15-5-30) at 31 kg ha^{-1}, and iron chelate and micronutrients at 0.9 kg ha^{-1}. Weeds were removed manually during the growing seasons. Soil temperature and water content were recorded with four sensors (5TM digital soil probes, Decagon Devices, Inc.) at 1 h intervals placed at a depth of 15 cm randomly in the plots. Tomato and melon fruits were collected and weighed when they reached the commercial standards, and the relative crop yield was calculated as the crop yield in a RKN infested plot in relation to the mean crop yield in non-infested plots. The nematode population densities were determined at transplanting (*Pi*) and consisted of eight cores taken from the upper 30 cm of the soil with a 2.5 cm diameter auger, mixed and sieved through a 4 mm-pore sieve to remove stones and roots. For each experimental plot, J2 were extracted from 500 cm^3 of soil using Baermann trays (Whitehead and Hemming, 1965) and incubated at 27 °C ± 2 °C for 1 week. Then, the J2 were collected with a 25 µm aperture screen sieve, counted, and expressed as J2 250 cm^{-3} of soil. The relationship between *Pi* and the relative crop yield (kg $plant^{-1}$) was estimated per each crop to determine its compliance with the Seinhorst damage function model ($y = m+(1-m)\ 0.95^{(Pi/T-1)}$) (Seinhorst, 1998).

Fruit quality assessment

The third tomato cluster at the red ripening stage and one melon fruit when fully slip per each plant, when they were available, were used for fruit quality analyses. Fruits were conserved at 10 ºC ± 1 ºC until processed. All the parameters were analysed twice. When it was available, the official methods of analysis (AOAC) were used (George and Latimer, 2019). Tomato and melon colour was determined by using a Minolta colorimeter CR-400 model (Minolta Camera, Osaka, Japan) in the CIElab colour space. Lightness (L^*), a^* and b^* values were recorded, and hue angle (H) and chroma (C^*) parameters were calculated as: $H = \tan^{-1}(b^*/a^*)$ and chroma: $C^* = (a^{*2} + b^{*2})^{1/2}$. Fruit flesh firmness was measured using a Texture Analyser TA.TXPlus (Stable Microsystems, Ltd., UK) interfaced to a personal computer. Firmness was evaluated as the maximum force (N) needed to depress 4 mm into the fruit with a 4 mm diameter stainless steel flat end probe (P/4). Six measurements were conducted by sample for colour and firmness. Chemical analyses were conducted from melon and tomato flesh obtained by crushing melon flesh from each single melon or all tomato fruits from each cluster. The soluble solid content (*SSC*) was measured with a digital refractometer (model PR-101, Atago, Co., Tokyo, Japan) at 20 °C and the results were expressed as °Brix. The pH and titratable acidity (*TA*) were determined according to AOAC 981.12 and AOAC 942.15, respectively, and expressed as g citric acid·kg^{-1} dry weight (*dw*). The dry matter content was obtained following the gravimetric method (AOAC 931.04) and

was expressed as percentage of the fruit dry weight in relation to the fresh fruit weight. After that, dried samples were kept in a muffle furnace and incinerated at 475 °C until white ashes were obtained (AOAC 940.26). Then, mineral content was assessed. Sodium and potassium content were determined by flame atomic emission spectrometry Corning 410 C (England). Iron, calcium and magnesium were determined by atomic absorption spectrometry Varian SpectrAA-110 (Australia). The results were expressed as g kg^{-1} *dw*, except for iron (mg kg^{-1} *dw*). Ascorbic acid content was measured using a titration method (AOAC 967.21) and oxalic acid as an extracting solution (Teixeira et al., 2012) and the results were expressed in g of ascorbic acid·kg^{-1} *dw*. The total phenolic content (*TPC*) of oxalic-aqueous extract was assessed according to the Folin-Ciocalteu assay (Singleton et al., 1999) and the results were expressed as g of gallic acid equivalent (*GAE*) kg^{-1} *dw*. The antioxidant activity of the oxalic-aqueous extracts of fruit samples was performed using the oxygen radical absorbance capacity (*ORAC*) assay (Gorjanovic et al., 2013). The results were expressed as mmol of Trolox equivalents (*TE*) kg^{-1} *dw*. Carotenoid extracts were obtained as proposed by Rodriguez-Amaya et al. (2004). Total carotenoid content was analyzed by UV-Vis Spectrophotometry following the method stated by Scott (2001). Melon extracts were measured at λ = 450 nm (β-carotene, maximum absorbance) and tomato extracts at λ= 470 nm (lycopene, maximum absorbance) in a Nicolet Evolution 300 Spectrophotometer (Thermo electron Corporation, Basingstoke, UK). Results were expressed in mg of carotenoid kg^{-1} *dw* (β-carotene for melon; lycopene for tomato).

Optical histopathology

A histopathology study with laser-scanning confocal microscopy of cleared galled-roots was performed. Three-leaf stage plants of the susceptible tomato cv. Durinta and melon cv. Paloma and the resistant tomato cv. Monika and *C. metuliferus* BGV11135 were transplanted in 200 cm^3 pots filled with sterilized sand. Five days later, 1 or 3 *M. incognita* J2 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. In order to obtain the nematode inoculum, eggs were extracted from tomato roots by blender maceration in a 5% bleach solution (40 g L^{-1} NaOCl) for 5 min (Hussey and Barker, 1973). Then, the suspension was filtered through a 74 µm sieve screen to remove root debris, and eggs were collected on a 25 µm sieve screen and placed on Baermann trays (Whitehead and Hemming, 1965) maintained at room temperature. J2 emerged during the first 24 h were discarded. After that, J2 were collected on a 25 µm sieve screen every two days for 6 days and kept at 9 ºC until inoculation. 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 in Cabrera et al. (2018) with some modifications. In brief, galled-root pieces were handpicked and introduced in a vial containing 1 mL of sodium phosphate buffer (10 mM, pH = 7). The pieces were fixed in sodium phosphate buffer (10 mM, pH = 7) with glutaraldehyde 4% under soft vacuum for 15 min, and maintained at 4 ˚C overnight. Afterwards, pieces were rinsed for 10 min with sodium phosphate buffer and 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 two weeks. Afterwards, 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 BABB solution. Fluorescence images were acquired with an inverted Leica TCS 5 STED CW microscope (Leica Microsystem) equipped with a 10x 0.40NA HCX PI 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 µm. Each volume was optically sectioned to produce a collection of Z-stack images (step size of 2 - 3 µm). For the GC volume measurements, images were segmented using TrakEM2 ImageJ plugin (ImageJ, version 1.50i).

Statistical analyses

Statistical analyses were performed using the SAS system V9 (SAS Institute, Inc., Cary, NC, USA). The nonlinear procedure proc nlin was used to determine the compliance of the relationship between the initial population densities (*Pi*) and the relative crop yield (*y*) with the Seinhorst damage-function model $y = m + (1-m)\ 0.95^{(Pi/T-1)}$ when $Pi \geq T$, and $y = 1$ when $Pi < T$, where *m* is the minimum relative yield, and *T* is the tolerance limit (Seinhorst, 1998). The relative crop yield was calculated as the crop yield for a given *Pi* / mean crop yield at *Pi* = 0. Twenty data per treatment and cropping season were used. Seinhorst's damage function models obtained per each crop were contrasted considering confidence intervals at 95% of *m* and *T,* and a general model was constructed with pooled data when no differences were found.

Pi were grouped in classes represented in both treatments in order to determine the effect of grafting ($Pi < T$) and nematode densities ($Pi > T$) on fruit quality. Data were submitted to non-parametrical analysis by the npar1way procedure to compare between grafted and ungrafted plants for a given *Pi* classes by the Wilcoxon test and by the Kruskal-Wallis test to determine the effect of nematode densities per treatment per each cropping season.

The number of nuclei and GC per feeding site, the volume of each GC and the volume of GC per feeding site from the histopathology study were compared between resistant and susceptible germplasm per each crop using the JMP v.15 (SAS Institute, Inc.) software. Data were submitted to non-parametric Wilcoxon test or Student's *t*-test ($P < 0.05$).

Results

Damage function models

The relationship between *Pi* and the relative crop yield fitted the Seinhorst's damage model for both ungrafted and grafted tomato and melon crops in 2016 and 2017 and some cropping seasons (Figure 3.1A and B). Minimum and maximum average soil temperatures at 15 cm depth during spring crops were 13.1 and 31.9 ºC, respectively, and 17.1 and 30.6 ºC during the summer crops. Grafted and ungrafted tomato cultivated in spring in non-infested plots yielded 4.1 and 3.9 kg plant^{-1} on average, respectively, and 2.2 and 2.0 kg plant^{-1} when cultivated in summer. At the end of the spring tomato crop cultivated in 2016, 4 out of 5 plots cultivated with ungrafted plants in non-infested soil were reinfested by the same nematode population. *Pi* densities at the beginning of the following melon crop ranged from 0 to 3494 J2 250 cm^{-3} of soil. In spring 2016, the minimum relative crop yield (*m*) and the tolerance (*T*) of grafted tomato cultivated in a *Pi* range from 0 to 1237 J2 250 cm^{-3} were 0.67 ± 0.03 and 5 ± 2 J2 250 cm^{-3} of soil, respectively (R^2 = 0.99, P < 0.05). For ungrafted tomato cultivated in a *Pi* range from 0 to 1496 J2 250 cm^{-3} of soil, the *T* value (10 ± 7 J2 250 cm^{-3} of soil) did not differ from that estimated for the grafted one, but the *m* value did (0.41 ± 0.19). In spring 2017, *m* and *T* values for ungrafted tomato cultivated in a *Pi* range from 0 to 2174 J2 250 cm^{-3} of soil were 0.27 ± 0.26 and 32 ± 25 J2 250 cm^{-3} of soil, respectively, (Figure 3.1A) and did not differ from those estimated in spring 2016. Then, a single model was constructed with the pooled data for ungrafted tomato, which provided estimated values of *m* and *T* of 0.34 ± 0.12 and 15 ± 7 J2 250 cm^{-3} of soil, respectively (R^2 = 0.96, P < 0.0001). The relationship between *Pi* and the relative tomato crop yield cultivated in summer did not fit the Seinhorst damage function model, irrespective of grafting.

Regarding melon, grafted and ungrafted melon cultivated in non-infested plots in spring yielded on average 2.5 and 2.4 kg plant^{-1}, respectively, and 1.5 and 1.6 kg plant^{-1} when cultivated in summer. At the end of the spring melon crop cultivated in 2016, 4 out of 5 plots cultivated with ungrafted plants in non-infested soil were reinfested by the same nematode population. *Pi* in the following tomato crop ranged from 0 to 241 J2 250 cm^3 of soil. Values of *m* and *T* for ungrafted crop cultivated in a *Pi* range from 0 to 7306 J2 250 cm^{-3} of soil in summer 2016 were 0.06 ± 0.06 and 32 ± 11, respectively (R^2= 0.94; P < 0.0001) (Figure 3.1B). Concerning grafted melon cultivated in a *Pi* range from 0 to 12258 J2 250 cm^{-3} of soil in summer 2016, the estimated *m* and *T* values were 0.2 ± 0.08 and 3 ± 3 J2 250 cm^{-3} of soil, respectively (R^2 = 0.97; P < 0.0001). In spring 2017, *m* and *T* values for grafted melon were 0.62 ± 0.1 and 56 ± 32 J2 250 cm^{-3} of soil, respectively, when cultivated in a *Pi* range from 0 to 6086 J2 250 cm^{-3} of soil (R^2 = 0.99; P < 0.0001), and 0.07 ± 0.05 and 27 ± 6 J2 250 cm^{-3} of soil, respectively, for ungrafted melon cultivated in a *Pi* range from 0 to 6680 J2 250 cm^{-3} of soil (R^2= 0.99; P<0.0001). The estimated Seinhorst damage function models for ungrafted melon cropped in summer 2016 and in spring 2017 did not differ according to the confidence interval values of *m* and *T*. Consequently, a single model was constructed with the pooled data for ungrafted melon. The estimated *m* and *T* values were 0.02 ± 0.02 and 33 ± 7 J2 250 cm^{-3} of soil, respectively (R^2 = 0.97; P < 0.0001).

Fruit quality

The range (minimum and maximum values) of the fruit quality parameters of tomato and melon fruits produced on ungrafted and grafted plants cultivated in spring or summer in infested and non-infested soil are presented in Tables 3.1 and 3.2, respectively. Tomato fruit quality parameters produced on plants cultivated in spring and summer 2015 and in spring 2017 in non-infested plots did not differ ($P > 0.05$) irrespective of grafting. However in 2016, lycopene, Na and *TPC* were higher ($P < 0.05$) in fruits produced in ungrafted than in grafted plants (1057 ± 71 *vs.* 663 ± 45 mg lycopene kg^{-1} *dw*; 2.7 ± 0.1 *vs.* 2 ± 0.1 g of Na kg^{-1} *dw*; and 4.5 ± 0.2 *vs.* 3 ± 0.5 g *GAE* kg^{-1} *dw*) (Figure 3.2A, B. C). Increasing nematode densities did not affect ($P > 0.05$) any of the tomato fruit quality parameters from grafted plants, but it did from ungrafted ones. The Na concentration in tomato fruits produced on ungrafted plants cultivated in infested plots was higher than those cultivated in non-infested plots in spring 2015 and 2016 (Figure 3.3A). Moreover, lower ($P < 0.05$) *TPC* was found in fruits from ungrafted tomato plants cultivated in a *Pi* range from 135 to 572 J2 250 cm^{-3} of soil (3.6 ± 0.1 g *GAE* kg^{-1} *dw*) than those cultivated in a *Pi* range from 0 to 27 J2 250cm^{-3} of soil (6.5 ± 0.3 g *GAE* kg^{-1} *dw*) in summer 2016 (Figure 3.3B).

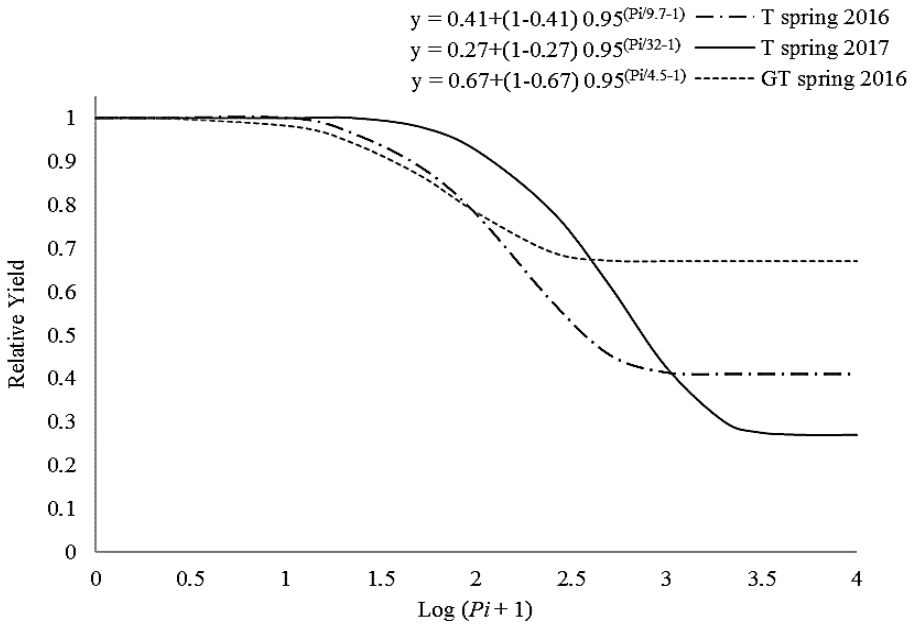
Concerning melon, higher ($P < 0.05$) Na content was found in fruits from ungrafted plants respect to the grafted ones cultivated in non-RKN infested plots irrespective of the cropping season. Dry matter and *SSC* also differed ($P < 0.05$) between melon fruits produced on ungrafted and grafted plants cultivated in summer 2015 and spring 2016 (Table 3.3). However, higher ($P < 0.05$) Na and dry matter content were found in fruits produced on ungrafted melon cultivated in infested soil in spring 2015, as well as of Na and *SSC* when cultivated in spring 2017. About fruits produced on grafted plants, the majority of the quality parameters were not affected by RKN densities, except dry matter and *SSC* that were lower ($P < 0.05$) at high nematode densities when cultivated in summer but not in spring (Table 3.3).

Optical histopathology

Fifteen days after *M. incognita* inoculation, the nematode induced 1.8 more ($P < 0.05$) giant cells (GCs) in *C. 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 *C. metuliferus* (Table 3.4).

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 (Figure 3.4 A and B).

A



B

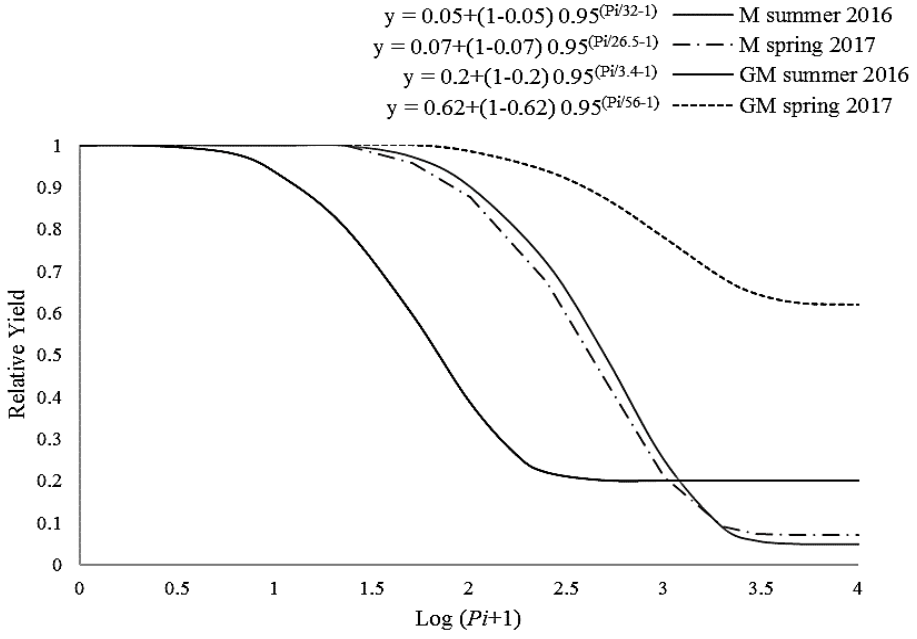


Figure 3.1. Seinhorst damage function model $y = m + (1 - m)\ 0.95^{(Pi/T-1)}$, where *y* is the relative crop yield, *m* is the minimum relative yield, *Pi* is the nematode population density at transplanting and *T* is the tolerance limit for A) ungrafted tomato cv. Durinta (T) or grafted onto the resistant rootstock 'Aligator' (GT); and for B) ungrafted melon cv. Paloma (M) or grafted onto the resistant rootstock *C. metuliferus* (GM) cultivated in *M. incognita* infested soil in a plastic greenhouse.

Table 3.1. Values of fruit quality parameters (minimum and maximum) of the tomato cv. Durinta ungrafted (T) and grafted (GT) onto cv. Aligator, cultivated in infested and non-infested *M. incognita* plots in plastic greenhouse in spring or summer during three years (2015-2017), and those reported by the department of Agriculture of United States of America (USDA), and by Coyago-Cruz et al. (2017) for the cluster tomato cv. Tigerella, Palamós and Byelsa, and the cherry tomato cv. Lazarino and Summerbrix.

Parameter	GT				T				USDA*	Coyago-Cruz et al., 2017
	Spring		Summer		Spring		Summer			
	Infested	Non-infested	Infested	Non-infested	Infested	Non-infested	Infested	Non-infested		
L*	38.2 – 42.6	38 – 44.7	40.3 – 44.6	43 – 46.3	37.5 – 40.5	37.8 – 41.2	38 – 47.1	40.4 – 41.1	n.a	33.4 – 43.9
AE	31 – 41.7	30.2 – 41.8	39.4 – 44.4	41.1 – 47	30 – 43.9	32.1 – 42.7	39.9 – 47.8	41.3 – 43.7	n.a	n.a
Chroma	26.6 – 38.2	19.3 – 41.5	36 – 41.5	35.8 – 39.3	21.8 – 39.6	21.5 - 41	34.6 – 44.6	38 – 40.3	n.a	31.6 – 46.7
Hue	37.4 – 52.8	37.7 – 63.3	37.5 – 45.6	38.8 – 49	38.2 – 48.1	37.2 – 51.8	37.3 – 49.5	38.1 – 39	n.a	40 – 63.7
TSS (ºBrix)	3.5 – 5.8	3.5 – 5.3	4.3 – 5.4	4.6 – 5.4	4 – 6.6	3.9 – 5.8	4.1 – 5.1	4.5 – 4.7	n.a	4.7 – 7.9
dm (%)	5.9 – 7.3	6 – 8.5	5.7 – 6.7	6 - 7	5.8 – 8.5	6.4 – 7.1	5.6 – 6.8	5.9 – 6	5.5	n.a
Lycopene (mg lycopene kg dw^{-1})	251 - 885	37 - 1275	332 - 457	371 - 485	90 - 976	113 - 1185	261 - 704	261 - 390	396	252 - 1510
T.A (g citric acid kg dw^{-1})	6.6 - 7	4.7 - 7	6.1 – 8.9	7 – 7.5	0.2 – 7.4	0.2 – 6.9	0.4 – 0.9	0.4 – 0.8	n.a	n.a
TPC (g GAE kg dw^{-1})	4.1 – 7	1.8 – 5.3	4.2 - 6	4.3 - 7	3.6 – 6.1	3.8 – 4.8	3.1 – 6.2	3.5 – 7.4	n.a	2.2 – 4.3
Vitamin C (g ascorbic acid kg dw^{-1})	1.5 – 4.1	1.5 – 6.7	2.3 – 2.7	2.4 – 3.6	1.9 – 3.6	1.5 – 3.1	1.8 – 3.4	3 – 3.6	2.1	n.a
Antioxidant activity (mmol Trolox kg dw^{-1})	8.2 - 57	8.4 – 63.8	28 – 81.2	26.8 – 66.3	8.7 – 79.6	10.7 – 74.2	20.3 – 70.4	41.4 – 57.5	n.a	n.a
pH	4 – 4.5	3.9 – 4.5	3.9 – 4.4	4 – 4.4	3.9 – 4.5	4 – 4.5	3.9 – 4.4	4.2 – 4.3	n.a	n.a
mm (%)	7.9 – 12.2	6.8 – 9	8 – 8.9	7.8 – 9.4	7.7 – 9.8	7.6 – 9.2	7 – 9	8.3 – 9.1	n.a	n.a
Fe (mg kg dw^{-1})	43.6 – 75.3	34.7 – 73.2	46.5 - 66	50.1 - 68	11 – 99.2	31.4 – 85.3	48.6 – 66.1	53.3 – 69.7	41.5	n.a
Ca (g kg dw^{-1})	1.2 – 2.8	1.7 – 3.5	0.9 – 2.8	1.6 – 3.3	0.8 – 2.3	1.4 – 2.4	0.9 – 3.1	1.6 – 2.5	1.5	n.a
Mg (g kg dw^{-1})	1.1 – 1.6	1 – 1.7	1.2 – 1.5	1.3 – 1.5	1.1 – 1.7	1.2 – 1.7	1 – 1.6	1.4 – 1.6	1.7	n.a
K (g kg dw^{-1})	15.3 – 26.5	13.2 – 27	22.1 - 29	21.6 – 28.5	11.2 – 26.5	12.3 – 29.1	19.9 – 29.2	23.5 – 27.8	36.5	n.a
Na (g kg dw^{-1})	1.7 – 5	1.8 – 3.9	2 - 5	1.9 – 2.9	2 – 6.8	1.6 – 4	2.3 – 3.7	2.3 – 3.1	0.77	n.a

The original values reported by USDA, and Coyago-Cruz et al. (2017) were adapted to the units used in this study. n.a: Data not available.

Table 3.2. Values of fruit quality parameters (minimum and maximum) of the cantaloupe melon cv. Paloma ungrafted (M) and grafted (GM) onto *C. metuliferus* BGV11135 cultivated in infested and non-infested *M. incognita* plots in plastic greenhouse in spring or summer during three years (2015-2017), and those reported by the department of Agriculture of United States of America (USDA), and by Colla et al. (2006) for the melon cantaloupe cv. Cyrano, grafted or ungrafted onto *C. maxima* x *C. moschata,* and by Lester et al. (2008) for the honeydew melon cv. Orange Dew.

Parameter	GM				M				USDA*	Colla et al., 2006	Lester et al., 2008
	Spring		Summer		Spring		Summer				
	Infested	Non-infested	Infested	Non-infested	Infested	Non-infested	Infested	Non-infested			
L*	50.5 – 84.7	45.8 – 80	49.5 – 72.4	54.5 – 66.5	39.9 – 71.3	49.3 – 69.6	57.5 – 72.5	54.9 – 66.5	n.a	53.1 – 58.3	n.a
AE	51.6 – 68.9	50.6 – 59.7	49.2 – 58.4	50.5 – 54.6	53 – 59.7	50.6 – 55.3	50.5 – 56.2	58.4 – 61.4	n.a	n.a	n.a
Chroma	18.2 – 47.2	21.2 – 45.2	19.5 – 41.2	33 – 47.3	19.6 – 45.2	28.4 – 42.3	22.9 – 41.6	38.1 – 45.2	n.a	n.a	n.a
Hue	39.4 – 79.6	44.6 – 84.1	66.6 – 81.8	64.8 – 75.1	79.6 – 83.9	80.4 – 85.9	60.4 – 81.3	67.7 – 74.1	n.a	n.a	n.a
TSS (ºBrix)	10.4 – 15.7	8.5 – 14.8	10 - 17	12.2 - 16	6.4 – 14.7	10.1 – 14.8	9 – 14.9	11.1 – 17.2	n.a	10.1 – 12.6	8.6 – 13.3
dm (%)	12.8 – 24.3	9.4 – 15.4	9.6 – 15.6	12.8 – 15.4	8.5 – 14.7	8.2 – 14	6.9 – 15.4	11.5 – 16.8	9.85	10.4 – 13.2	9 – 12.1
B-carotene (mg β-carotene kg dw^{-1})	18-157	12-136	20 - 102	38.3 – 51.7	14 - 151	13 - 73	19 – 54.3	31 – 40	206	n.a	214 - 215
T.A (g citric acid kg dw^{-1})	5.2 – 20.9	6.8 – 15.9	6.7 – 24.9	5.1 – 22.1	5.1 – 17.6	9.6 – 38.2	7.2 – 22.4	15 – 18	n.a	n.a	n.a
TPC (g GAE kg dw^{-1})	1 – 3.8	2 – 3.5	1.8 – 4.1	2.6 – 3.3	0.9 – 5.9	1.6 - 6	1 - 4	1.5 – 3.2	n.a	n.a	n.a
Vitamin C (g ascorbic acid kg dw^{-1})	0.8 – 2.4	0.8 – 1.9	1.4 – 2.2	1.4 – 1.9	1.1 – 2.3	0.4 – 1.6	1.1 – 2.5	1.3 – 1.7	3.7	n.a	1.3 – 1.4
Antioxidant activity (mmol Trolox kg dw^{-1})	20.3 – 42.3	21 – 43.1	3.3 – 30.2	7.3 – 30	10.7 – 38.7	8.7 – 35.2	7 – 28.5	17.4 – 22.6	n.a	n.a	n.a
pH	4.9 – 6.9	5.6 - 7	5.1 – 6.7	6.1 – 6.5	5.6 – 7.6	6 – 6.9	5.1 – 6.2	5.7 – 6.5	n.a	6 – 6.7	n.a
mm (%)	6 – 14.2	7.8 – 12.3	7.8 – 12.2	6.6 – 11.6	6.4 – 11.6	7 – 14	6.2 – 8.6	6.5 – 8.9	n.a	n.a	n.a
Fe (mg kg dw^{-1})	27 - 70	41.9 - 70	49 – 60.6	54.7 - 61	32 - 85	43.5 – 81.7	45.2 – 63	43.1 – 53.7	21.4	n.a	20
Ca (g kg dw^{-1})	1.4 – 2.5	1.3 – 2	0.8 – 3.2	0.9 – 2.6	1.6 – 2.2	1.7 – 2.4	1.4 – 3.4	1.6 – 2.6	0.9	n.a	0.1
Mg (g kg dw^{-1})	0.7 – 1.3	0.9 – 1.2	0.8 – 2.1	0.9 – 2.1	0.9 – 1.7	1 – 1.7	0.9 – 2.3	1 – 1.9	1.2	n.a	0.6 – 0.9
K (g kg dw^{-1})	17.4 – 24.1	19.5 – 25.9	15.3 – 23.9	17.3 – 21.1	23.6 – 24.8	21.9 - 27	19.4 – 21.9	18.1 – 20	27.2	31.3 – 34.9	21.6 – 23.2
Na (g kg dw^{-1})	2 – 3.4	2.1 – 3.3	2.3 – 4.6	2.3 – 4.6	2.7 – 13.4	3 – 4.2	3 – 6.2	3.9 – 4.7	1.6	0.9 - 14	1.6 – 2.6

The original values reported by USDA, Colla et al. (2006) and Lester et al. (2008) were adapted to the units used in this study; n.a: Data not available.

Table 3.3. Soluble solid content (*SSC*), dry matter (*Dm*) and sodium content in ungrafted melon cv. Paloma (M) or grafted onto the resistant rootstock *C. metuliferus* BGV11135 (GM) cultivated in soil infested with increasing *Meloidogyne incognita* densities at transplanting (*Pi*) in a plastic greenhouse during three years (2015-2017).

Year	Season	*Pi* range (J2 250cm^{-3})	*SSC* (ºBrix)		*Dm* (%)		Na (g kg^{-1} *dw*)	
			GM	M	GM	M	GM	M
2015	Spring	0	12.2 ± 0.3 a	12.2 ± 0.2 a	12.3 ± 0.2 a	12.5 ± 0.1 a	3.7 ± 0.2 a	4.9 ± 0.1 b*
		72-349	12.3 ± 0.3 a	10.0 ± 0.3 b*	12.4 ± 0.1 a	9.9 ± 0.2 b*	4.4 ± 0.2 a	8.5 ± 0.6 a*
		502-709	12.2 ± 0.4 a	10.5 ± 0.7 ab	12.3 ± 0.4 a	10.0 ± 0.3 b*	4.1 ± 1 a	8.3 ± 0.8 a*
	Summer	0	15.2 ± 0.2 a	16.4 ± 0.4*	14 ± 0.4 a	15.6 ± 0.4*	4.5 ± 0.4 a	6.1 ± 0.3*
		96-427	13.4 ± 0.5 b	n.a	12.3 ± 0.3 b	n.a	6.5 ± 0.8 a	n.a
2016	Spring	0	13.8 ± 0.4 a	12.6 ± 0.3 a*	14.0 ± 0.4 a	12.3 ± 0.3 a*	2.5 ± 0.1 a	3.6 ± 0.2 a*
		15-48	12.9 ± 0.1 a	12.8 ± 0.1 a	12.6 ± 0.4 a	12.4 ± 0.2 a	2.5 ± 0.1 a	3.9 ± 0.2 a*
	Summer	0†	13.3 ± 0.5 a	12.6 ± 0.7	14.1 ± 0.5 a	13.4 ± 0.8	3.1 ± 0.1 b	4.3 ± 0.4*
		1581-3772	10.8 ± 0.3 b	n.a	10.5 ± 0.9 b	n.a	3.8 ± 0.2 a	n.a
2017	Spring	0†	14.0 ± 0.2 a	14.7 ± 0.2 a	13.6 ± 0.7 a	13.7 ± 0.6 a	2.7 ± 0.2 a	4.1 ± 0.4 ab*
		203-951	13.1 ± 0.6 a	14.2 ± 0.2 a	12.5 ± 1.3 a	13.6 ± 0.5 a	1.8 ± 0.3 a	3.8 ± 0.2 b*
		1156-3476	11.9 ± 0.9 a	11.7 ± 0.5 b	12.8 ± 0.3 a	12.3 ± 0.5 a	2.3 ± 0.4 a	5.1 ± 0.3 a*

Data are mean ± standard error of 5 replicates. Data in the same column and cropping season followed by the same letter did not differ ($P < 0.05$) according to the non-parametric Mann-Whitney or Kruskal-Wallis test. Data within the same row per quality parameter followed by * indicate differences between germplasm according to the non-parametric Mann-Whitney test ($P<0.05$).

n.a: Range of *Pi* not represented in the treatment; †: *Pi* = 0 included nematode densities below the plant tolerance according to the Seinhorts damage function model.

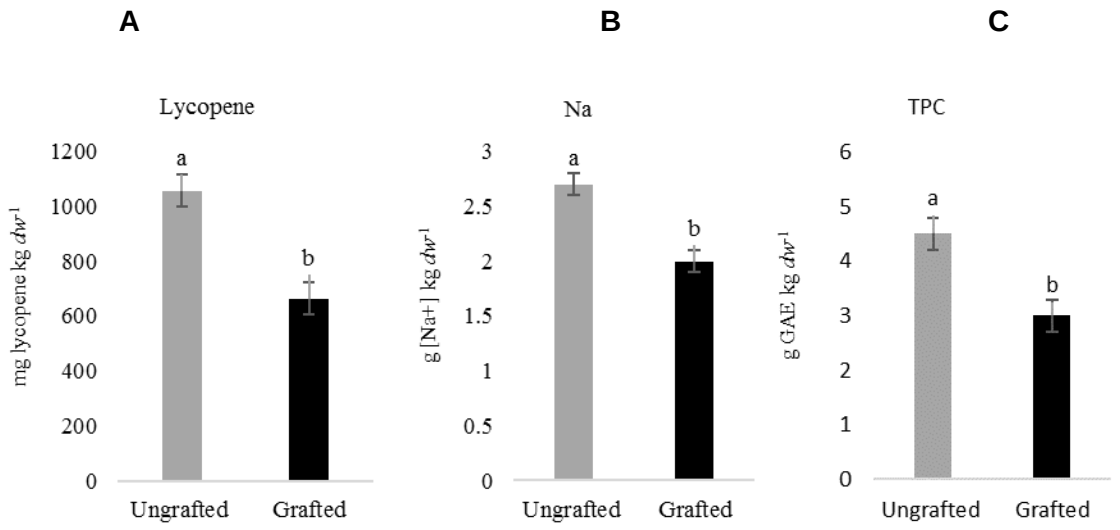


Figure 3.2. Effect of grafting on lycopene (A), sodium concentration [Na^+] (B) and total phenolic compound (TPC) (C) in tomato cv. Durinta fruits produced in spring 2016. Data are mean ± standard error (n = 5). Column with the same letter did not differ ($P < 0.05$) according to the non-parametric Wilcoxon test.

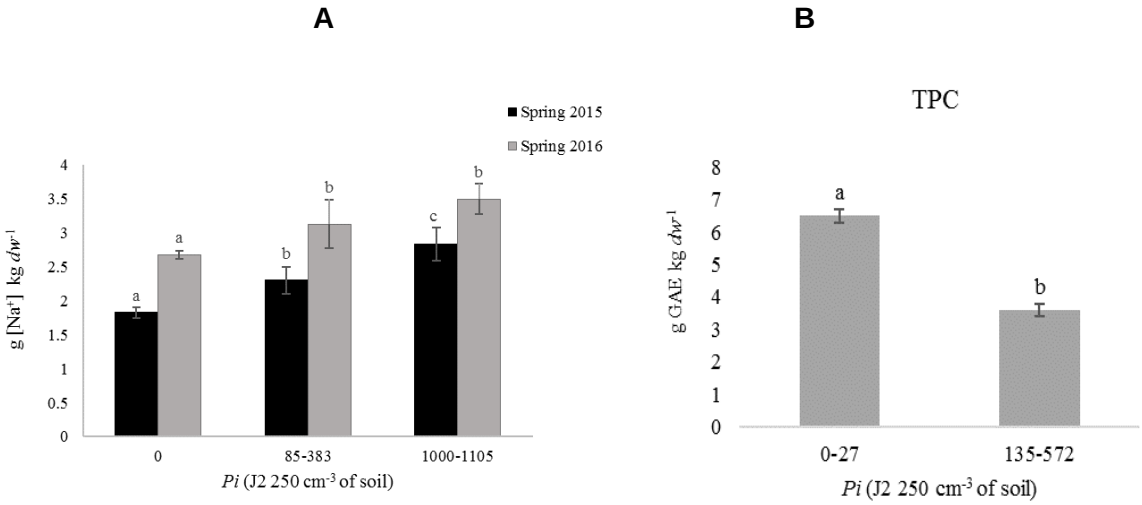


Figure 3.3. Effect of nematode density at transplanting (*Pi*) on (A) sodium concentration [Na^+] in tomato fruits produced on ungrafted tomato cv. Durinta (T) cultivated in spring 2015 and 2016, and on (B) phenolic compounds in summer 2016. Data are mean ± standard error (n = 5). Column of the same year with the same letter did not differ ($P < 0.05$) according to the non-parametric Kruskal-Wallis test (A) and Wilcoxon test (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 3.4). However, GCs volume per feeding site did not differ between tomato cultivars, but the number of nuclei per feeding site did, being higher ($P < 0.05$) in susceptible than in resistant tomato (Table 3.4). In resistant tomato, several GCs did not emit fluorescence and no nuclei were observed compared to the voluminous and multinucleated GCs observed in the susceptible tomato (Figure 3.4 C and D).

Discussion

The results of this study provide novel information on the effect of nematode densities and the cropping season on grafted tomato and melon tolerance to *M. incognita*, crop yield losses, and fruit quality.

Expósito et al. (2019) found that tomato yield did not differ between ungrafted and grafted tomato onto the tomato rootstock 'Aligator' cultivated in non-nematode infested soil, but it did in infested. The results of the present study have shown that the tolerance of ungrafted and grafted tomato cv. Durinta onto 'Aligator' to *M. incognita* cultivated in the same season and year did not differ but the later suffered a 36% less relative yield losses (59% *vs.* 23%). Di Vito et al. (1991) found that the tolerance to *M. incognita* of the susceptible cv. Ventura and the resistant cv. Disa N did not differ (0.55 J2 cm^{-3} of soil) but yield losses were lower in the resistant than in the susceptible tomato (30% *v*s 100%) in microplot conditions. In our study, the tolerance to *M. incognita* of the susceptible tomato cv. Durinta cultivated in spring was similar to that previously reported by Giné and Sorribas (2017).

Grafting did not influence the majority of fruit quality parameters of tomato cultivated in non-infested soil, except lycopene, Na and *TPC* that were lower in fruits from grafted than ungrafted plants but only in one out of three years. It is known that grafting can affect tomato fruit quality depending on the scion-rootstock combination and environmental conditions, including abiotic and biotic factors (Fernández-García et al., 2004; Turhan et al., 2011; Vrcek et al., 2011; Di Gioia et al., 2013; Erba et al., 2013). Nonetheless, Grieneisen et al., (2018) conducted an extensive review of data from 159 publications to point light on the effect of grafting on tomato yield and fruit quality. They concluded that grafting rarely causes fruit quality changes and that self-grafted plants yielded similarly than ungrafted plants.

However, the occurrence of abiotic and /or biotic stresses and its intensity during a given phenological stage of the plant can lead to changes in fruits and vegetables quality such as an increase of bioactive compounds (Nicoletto et al., 2019; Toscano et al., 2019).

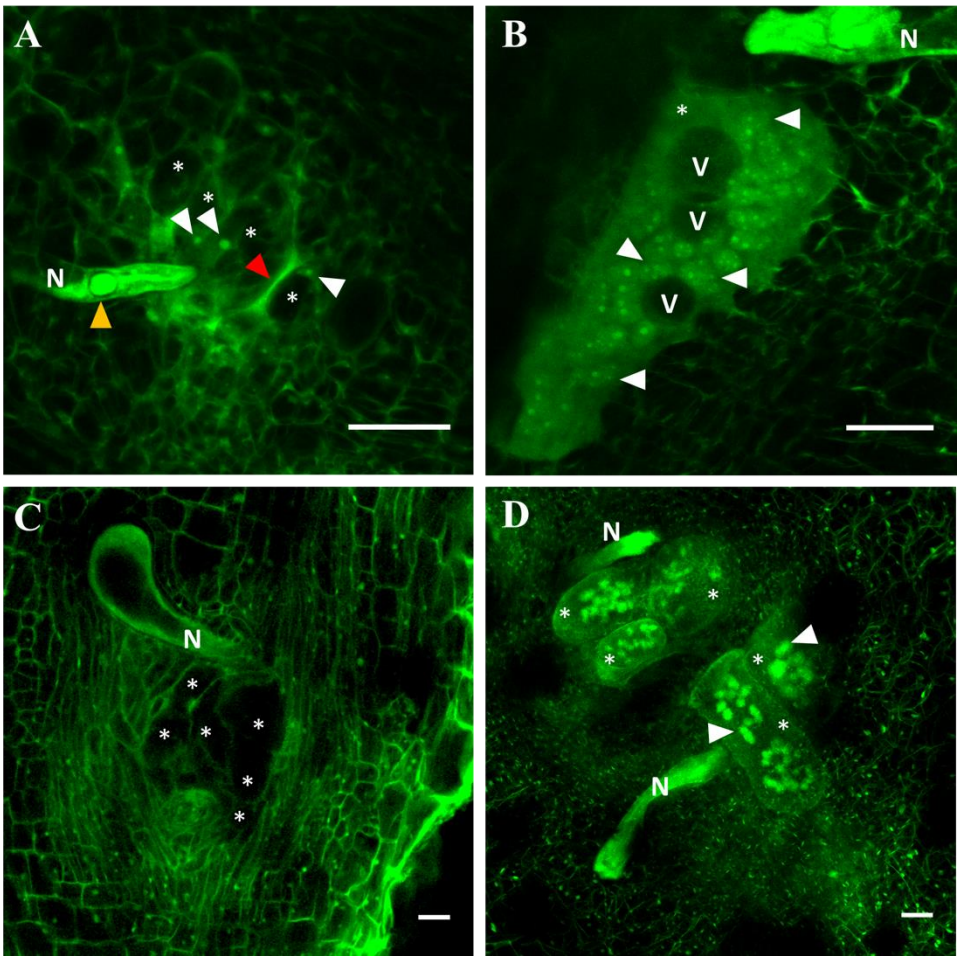


Figure 3.4. Laser scanning confocal microscope images of giant cells induced by *Meloidogyne* 15 days after inoculation in the resistant *Cucumis metuliferus* BGV11135 (A), the susceptible melon cv. Paloma (B), the resistant tomato cv. Monika (C) and the susceptible cv. Durinta (D). Nematode (N), vacuoles (V), giant cells (asterisk), some nuclei (white arrowhead), esophageal median bulb (yellow arrowhead) and necrosed area (red arrowhead) are indicated. Scale bar: 50 µm

Table 3.4. Giant cell volume (GCV), GC volume per feeding site (GCV fs^{-1}), number of nuclei per GC (N GC^{-1}), number of nuclei per feeding site (N fs^{-1}), and number of cells per feeding site (NC fs^{-1}) in the resistant (R) C. metuliferus BGV11135 and tomato cv. Monika and the susceptible (S) 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.

Host plant (host status)	GCV (μm^3 10^5)	GCV fs^{-1} (μm^3 10^5)	N GC^{-1}	N fs^{-1}	NC fs^{-1}
C. metuliferus (R)	0.45 ± 0.1 *	3.41 ± 0.8*	1.2 ± 0.7*	9.2 ± 5.5*	8.0 ± 1.1*
Melon cv. Paloma (S)	7.97 ± 1.5	33.19 ± 9.9	17.1 ± 1.8	72.0 ± 7.8	4.5 ± 1.0
Tomato cv. Monika (R)	3.14 ± 0.4*	26.84 ± 3.7	0.9 ± 0.4*	7.0 ± 3.0*	8.7 ± 1.2*
Tomato cv. Durinta (S)	11.42 ± 1.9	45.94 ± 7.3	13.7 ± 1.0	56.2 ± 7.3	4.1 ± 0.4

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

Interestingly, there is a crossing-talk between signalling pathways allowing plant plasticity to be adapted to environmental situations (Martinez-Medina et al., 2017; Ghahremani et al., 2020). Atkinson et al. (2011) studied the effect of water stress and *M. incognita* (10 eggs g^{-1} soil) alone and in combination on the nutritional fruit quality of tomato cv. Shirley cultivated in pots in a growth chamber. They found that the second cluster produced by nematode inoculated plants had less dry matter content than that produced by non-inoculated, contrarily to the results obtained from the fifth cluster that in addition had more content of phenolic compounds. When both kinds of stresses were combined, the percentage of fruit dry matter of the second cluster was similar to that the water stressed plants alone. It seems that the initial nematode densities at transplanting was not enough to affect the quality of the second cluster fruits but increasing nematode density after completion of the first generation affected the fifth cluster. In our study, that was conducted in non-controlled conditions, in which the third cluster fruit was used for assessing fruit quality parameters when they reached the commercial standards, increasing nematode densities at transplanting did not affect the quality of fruits produced by grafted plants. However, the *TPC* in fruits from ungrafted tomato decreased at nematode densities between 135 and 572 J2 250 cm^{-3} of soil in summer 2016, and Na concentration increased in spring 2015 and 2016. The range of Na content in tomato fruits were between 2.1 and 8.8 times higher than that reported by USDA (2020a) (Table 3.1).

The tomato cultivar and crop management can affect the concentration of nutritional compounds as it has been reported by Erba et al. (2013) who found values of Na content in three tomato cultivars between 4.8 and 17.6 higher than that reported by USDA (2020a) depending on the tomato cultivar, N fertilization, and fungicide application.

In relation to melon, Expósito et al. (2019) found that the yield of ungrafted and grafted melon onto *C. metiluferus* cultivated in non-nematode infested soil did not differ irrespective of the cropping season. In the present study, the estimated tolerance to *M. incognita* of ungrafted and grafted melon cultivated in spring did not differ but maximum yield losses did, being 98% for ungrafted and 38% for grafted melon. Reports about grafted melon tolerance to RKN and yield losses are scarce. Kim and Ferris (2002) estimated the tolerance to *M. arenaria* and yield losses of melon cv. Geumssaragi-euncheon grafted onto the *Cucurbita* hybrid rootstock 'Shintoza' cultivated at nematode densities between 0 and 2980 J2 per 100 cm^{-3} of soil, being 0 J2 100 cm^{-3} of soil and 57%, respectively. According to these results, *C. metuliferus* is more tolerant to RKN and experience less yield losses than the *Cucurbita maxima* x *C. moschata* rootstock.

In fact, plant tolerance and crop yield losses of grafted cucumber onto the *Cucurbita* hybrid rootstock 'RS841' did not differ from ungrafted but the nematode population growth rate did, being higher in grafted than ungrafted cucumber, indicating that it was not resistant to the nematode (Giné et al., 2017). Plant species supporting high nematode population growth rates leave high nematode densities at the end of the crop causing more yield losses to the following one. *C. metuliferus* has been proven to suppress nematode population growth rate compared to melon, being an indicator of its resistance against the nematode (Expósito et al., 2018). Under

an agronomic point of view, rootstocks bearing resistance and tolerance genes to RKN are needed to manage them and to avoid crop yield losses.

Regarding melon fruit quality, it has been reported that the *C. metuliferus* accession BGV11135 did not affect physical fruit traits, *SSC* and pH when cultivated in hydroponic system (Expósito et al., 2018). But fruit quality can be affected according to the scion-rootstock combination and the cultivation system. For example, Guan et al. (2014) did not find differences on flesh firmness and *SSC* between ungrafted melon cv. Honey Yellow and grafted onto *C. metuliferus* cultivated under both conventional and organic standards, but did in fruits from grafted melon cv. Arava cultivated under both cropping systems as well as less *SSC* was found when cultivated under conventional system. In our study, lower Na content was measured in fruits from grafted than ungrafted plants cultivated in non-infested soil.

Interestingly, increasing nematode densities increased Na content in fruits from ungrafted but not from grafted plants. Nonetheless, the levels of Na reached in melon fruits from both grafted and ungrafted plants (1.8 to 8.5 g Na kg^{-1} *dw*) were in the range of that reported by Colla et al. (2006) but slight higher in ungrafted melon than that reported by Lester (2008) and USDA (2020b) (Table 3.2). Furthermore, increasing nematode densities reduced the *SSC* and the dry matter content in fruits produced in ungrafted plants in spring and in those produced in grafted plants cultivated in summer. Ploeg and Phillips (2001), found an increase in the percentage of dry matter of the aereal plant part of melon cv. Durango after 8 weeks of cultivation in pots non-inoculated and inoculated with an increasing nematode density from 0.06 to 15 J2 100 g^{-1} of soil. In field conditions, significant yield reduction was observed due to a reduction in the number of fruits at increasing nematode densities over *T.* It seems that the metabolic activity of the nematode would compete with fruit development which could be inhibited.

In this line, the effect of suboptimal growing conditions, as for example high temperatures and radiation levels which are achieved in the Mediterranean areas at transplanting during the summer season can affect plant metabolism. Heat stress can affect plant photosynthesis and the phenylpropanoid pathway. Moreover, ROS can be accumulated in the tissues and the plant will activate antioxidants mechanisms to protect cell structures from oxidation. In addition, light excess can induce severe damage to the photosystem II (Toscano et al., 2019). These stresses will lead to a reduction in the potential yield of the crop and potential changes in the fruit quality. Thus, the selection of the best season for cropping is also necessary to maximize its efficiency as it was previously described for cucumber-*M. incognita* and for zucchini-*M. incognita* (Giné et al., 2014 and 2017; Vela et al., 2014). These studies found that cucumber and zucchini were more tolerant and suffered lower yield losses when cultivated in spring than in summer or autumn. Similar results were observed in our study for grafted melon, which was more tolerant and experienced less yield losses when cultivated in spring instead of summer. So, it is expected that the damage of the nematode infection increase and the tolerance were reduced under those stressful conditions due the required energy to overcome RKN infection and the abiotic stress together. Grafting onto tolerant rootstocks has been used widely to overcome the damage to different abiotic stresses, including high temperatures (Tao et al. 2020). Consequently, screening for resistant-RKN and tolerance to abiotic stress will

increase the availability of scion-rootstock combinations for agriculture production to overcome RKN and sub-optimal growing conditions.

The histopathological study provided interesting information related to the number and volume of giant cells and the number of nuclei into them. Giant cells formation is a key factor for a successful plant-nematode interaction after the nematode arrive into 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 as 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 *M. javanica* in *Arabidopsis* roots, and to compare GCs formed in the *Arabidopisis* 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.

The results of our study have shown that both resistant *C. metuliferus* and tomato cv. Monika had more number of giant cells per feeding site than melon and susceptible tomato 15 days after *M. incognita* inoculation, but they were smaller, less voluminous, with fewer nuclei and some of them were empty of cytoplasm. Previous histopathological studies reported some of the observations pointed out in this study. Fassuliotis (1970) observed small GCs in *C. metuliferus* accession C-701 compared with those induced by *M. incognita* in melon; the nematode developed slow and a 20% of juveniles' differentiated to males. Walters et al. (2006) observed elongated GCs conforming abnormal in shape feeding sites in *C. metuliferus* accession 482454 compared with melon. More recently, Ye et al. (2017) observed that the most of the GC were empty of cytoplasm in the *C. metuliferus* accession PI 482443-*M. incognita* interaction 14 days after nematode inoculation along with a slow nematode development compared with cucumber. 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 *C. metuliferus* accession BGV11135–*M. javanica* interaction compared to cucumber. Interestingly, the major number of GCs found in both resistant *C. metuliferus* and tomato could be due to an attempt of the nematode to achieve enough nutrients for its life cycle completion. 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 *Mi*1.2 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).

Our research pointed out the importance to use grafted fruiting vegetables onto resistant rootstocks to decrease yield losses caused by RKN without conferring significant non-desirable quality traits. According to our data, the use of grafted plants could not be necessary to increase crop yield in absence of RKN because crop yield did not differ in our scenario. Nonetheless, rootstocks also bear other sources of resistance against soil-borne plant pathogens increasing its interest to be included in integrated disease management strategies. For example, *C.*

metuliferus is also resistant to Monosporascus root rot and Fusarium wilt as well as to vine decline (Castro et al., 2020). Some other putative hybrid *Cucumis* rootostcks, such as *C. ficifolius* x *C. anguria* and *C. ficifolius* x *C. myriocarpus,* which are tolerant to *Monosporascus cannonballus,* and resistant to *Fusarium oxysporum* f.sp *melonis* and to RKN and did not affect the quality of melon fruit compared to non-grafted or self-grafted (Cáceres et al., 2017), will increase the number of possible rootstocks that could be available for growers in the near future.

Special attention should be pay to the selection of the optimal cropping season in order to maximize the performance of grafted plants as it was observed in this study. The main effect of RKN on tomato and melon yield was on quantity but not in quality since the most fruit quality parameters assessed were in the range of values previously reported for these crops.

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GENERAL DISCUSSION

Root-knot nematodes have become an increasing problem for agriculture production since the restriction of soil fumigants, due the concern for human health and the environment, and the intensification of agriculture production systems. In consequence, it is necessary to find environmental-safe alternatives wich can reduce RKN damage under the acceptable economical levels. This PhD thesis provides novel information regarding the characterization of *Cucumis metuliferus* germplasm to manage RKN. This germplasm was used as a melon rootstock in crop rotation with resistant tomato to control RKN in the agroenvironmetal conditions in Spain. *C. metuliferus* or horned cucumber is a wild African relative to cucumber and melon and has been performed as highly resistant and tolerant to RKN, and has shown its compatibility with melon as rootstock for grafting. In addition, this rootstock is highly resistant to *Monosporascus* root rot and to *Fusarium oxysporum* f. sp *melonis* (Castro et al., 2020), which are two major diseases in melon production.

The *C. metuliferus* acessions BGV11135 and BGV10762 assessed in this thesis were highly resistant (RI < 1%) or resistant (1% ≤ RI ≤ 10%) to most of the RKN isolates tested, including the *Mi*1.2 virulent ones. In this germplasm, *Meloidogyne* induced empty or poorly developed giant cells with multiple vacuoles, similarly as have been reported by other authors (Fassuliotis, 1970; Walters, et al., 2006; Ye et al., 2017). In addition, the number of giant cells induced by *Meloidogyne* in both *C. metuliferus* and the resistant tomato cv. Monika were higher but less voluminous and with fewer nuclei compared to the susceptible melon and tomato respectively. The reduction in the number of nuclei observed in the giant cells (GC) of the resistant plants compared to the susceptibles, as well as, the higher number of giant cells, could be an indicator that the nematode is not acquiring enough nutrients for each GC and need to induce more to complete its life cycle. The understanding of the changes in the giant cells' structures induced by the nematodes through the resistance mechanisms is critical to comprehend the physiological response of the plant-nematode interaction. This, combined with transcriptomic tools to understand the genetic nature of the resistance could be the basis for future introgressions to susceptible cultivars or rootstocks via natural breeding or transgenic lines. In addition, the optical histopathology used in this study could be used as a diagnostic tool for screening resistance wich can be complemented with the classical plant bioassays methods.

The use of *C. metuliferus* as a melon rootstock in field conditions and its resistance to RKN has been previously described (Sigüenza et al., 2005; Guan et al., 2014). However, the effect in crop rotation with a solanaceous crop, wich is a common rotation sequence in Spain, the effect of the growing period in the performace of the rootstocks and on the fruit quantity and quality and the response to the potential virulent populations need to be assesed before commercial use by farmers. Our study point out, that these two resistant crops in rotation supress the

nematode population growth rate, reduce the severity of the disease and the crop yield losses compared to the ungrafted plants, but the performace is dependent on the number of times the crop is repeteated and the growing season. In fact, when the grafted melon was cultivated in spring suffered less yield losses and was more tolerant compared to summer. Simmilar results have been previously described for cucumber and zucchini infected with *M. incognita* (Giné et al., 2014 and 2017; Vela et al., 2014) Therefore, the effect of the growig period in the performance of the crops is essential to increase the tolerance to RKN. Also, the effect of the growing season in combination with nematodes could affect the quality of the fruits. The dry matter content of melons was reduced when increasing nematode densities in both ungrafted and grafted plants, as well as, the SSC in grafted plants produced in summer, probably due the combination of RKN infection and suboptimal growing conditions. In tomato, the fruit quality from grafted plants was not affected by increasing nematode densities. However, the total phenolic content in fruits from ungrafted tomato decreased at high nematode densities only in the summer of 2016 and an increase of the sodium concentration was detected in spring 2015 and 2016. The information regarding the effect of the increasing nematode densities in the fruit quality is scarce, but other variables seems to play an important role in how nematode is affecting the fruit quality (Erba et al., 2013). Grafting can also affect fruit quality (Davis et al., 2008), however, in our study the majority of the quality traits were not affected compared to ungrafted plants. These results are in agreement with those reported by Gisbert et al. (2017) who did not find differences among fruit quality of the ungrafted melon cv. Vedrantais or, selfgrafted or grafted onto *C. metuliferus*. Conversely, they found that melon Piel de Sapo cv. Finura grafted onto *C. metuliferus* affected fruit weight and length. However, these changes do not reduce the commercial value of the fruits as the market accepts a wide range of fruit sizes and shapes variability. In tomato, grafting did not influence the majority of fruit quality parameters, except lycopene, sodium and total phenolic content in 2016 that were lower in grafted fruits than ungrafted plants. Nonetheless, the marketable quality was not affected by grafting onto the “Aligator” rootstock.

After three years of experiments, no virulence selection of nematodes was detected in *C. metuliferus*. Ye et al, (2017) pointed out that fifteen unigenes with coexpression affecting the phenylpropanoid biosynthesis, plant hormone signal transduction and plant-pathogen interaction might be involved in the resistance of *C. metuliferus* accession PI482443 to *M. incognita*. Consequently, the quantitative nature of the resistance seems to decrease the risk of selection for virulence in the same period compared to the qualitative resistance of germplasms carrying single *R*-genes as our results pointed out. The rotation of these two resistant germplasm was not enough to supress virulence selection for both of them and in contrast to *C. metuliferus,* after the first tomato crop on the resistant “Aligator” rootstock, a *Mi*1.2 virulent *M. incognita* population was selected, irrespective of the crop season. The selection of virulent populations to this rootstock was previously reported (Verdejo-Lucas et al., 2009), though, the resistance was not completely overcome until the third tomato crop.

Nevertheless, after cropping melon grafted onto *C. metuliferus,* the virulence to the *Mi*1.2 was drastically reduced. In addition, the infectivity, reproduction and

fecundity fitness of the subpopulation selected with *Mi*1.2 virulence against the susceptible tomato and melon were reduced with respect to the avirulent subpopulation after the third grafted tomato crop, but not after the second. This indicates that a minimum of three resistant tomato crops were needed to affect the fitness of the intermediate virulent population. Nonetheless, introducing different germplasm in a rotation sequence could alter the fitness status of the nematode populations as recent studies pointed out that using another resistant rootstock did not reduce the infectivity and fecundity in the susceptible germplasm compared to the avirulent population, contrary as using the rootstock "Aligator" during three years (data not published). Consequently, it seems that as high is the selection pressure for virulence, the fitness cost associated in the susceptible plants is higher. Hence, the fitness acquired could be variable depending on several factors, including the plant background (Nilusmas et al., 2020).

Therefore, identify the resistant germplasm against RKN to maximize the durability of the resistance and induce high fitness cost on the susceptible plants is important in the case to use strategies alternating resistant and susceptible plants. For example, Nilusmas et al, (2020), proposed a mathematical model in which the relative gain of cropping one resistant tomato crop followed by two susceptible can increase the gain in 40% compared to only use resistant cultivars. Similarly, Talavera et al, (2009) proposed that the best crop sequence was two resistant tomato crops followed by one susceptible to reduce crop yield losses. Nonetheless, when available, the best option to avoid the selection for virulence is using different *R*-genes with different mechanisms of resistance and, if is possible, pyramided in the same variety, as for example, pepper germplasm containing both *Me1* and *Me3* resistance genes, which totally suppressed the emergence of virulent isolates under laboratory and field conditions (Djian-Caporalino et al., 2014). In potato, germplasm with the $GpaIV_{adg}$ and *Gpa5* genes pyramided, showed similar results in which fewer *Globodera pallida* cysts were developed compared to the genotypes carrying each single gene separated (Dalton et al., 2013). However, pyramiding resistant genes into elite cultivars is a very difficult process using conventional breeding. In the last years, the use of molecular tools has been facilitating the introgression of those gens into the plants (Suh et al., 2013). Meanwhile, alternating *R*-genes separately are the best option compared to spatial mixing or successive cropping of the same *R*-gene (Caporalino et al., 2014).

The characterization of other resistant germplasms that selected virulence has been not reported, can be good candidates to be introduced in the crop rotations as for example *Citrullus amarus* and *Solanum torvum,* which are RKN resistant rootstocks for watermelon and eggplant respectively (García-Mendívil et al., 2019; 2020). Consequently, the use of resistant crops in the rotation in which no virulent populations are selected is a key point to reduce the nematode densities, including virulent populations for other *R*-genes and to reduce the overall yield losses in the crop rotation. Consequently, including more sources of resistance in the rotation schemes and the time elapsed between two crops with the same *R*-gene might reduce even more the virulence until its suppression. Nevertheless, the number of resistant species, as well as, its order in the sequence, must be evaluated. New research in multi-resistance crop sequence to avoid the selection of

virulence is ongoing and the results will provide valuable information on the use of those resistant germplasm to avoid RKN virulence and therefore reduce more the nematode densities and the crop yield losses.

In addition to plant resistance provided by *R*-genes, different soil microbial components can induce systemic resistance to the plants, such as *Bacillus firmus*, *Pochonia chlamydosporia,* or *Trichoderma* spp. (Ghahremani et al., 2019, 2020; Pocurull et al., 2020), and could play an important role on *R*-genes durability.

In fact, Pocurull et al. (2020) pointed out that the use of *Trichoderma asperellum* (T22) or *Trichoderma harzianum* (T34) induced systemic resistance in tomato, but not in cucumber, and this resistance was additive to the Mi1.2 gene.

The combined use of *R*-genes with plant systemic resistance microbial inducers could inhibit the selection of virulent nematode populations. These hypotheses should be assessed and could provide valuable information to design solid integrated nematode management strategies. In addition, good plant resistance manual along with or inside good agronomic practises could be elaborated in order to preserve the durability of the resistance and to promote the use of alternatives to chemical control, reducing plant-parasitic nematode densities and crop yield losses in the line of sustainable agriculture development.

CONCLUSIONS

1. *Cucumis metuliferus* accessions BGV11135 and BGV10762 are resistant to *M. arenaria M. incognita* and *M. javanica* including virulent *Mi1*.2 isolates. The histopathological studies have shown poorly developed giant cells induced by *Meloidogyne javanica* in *C. metuliferus* and necrotic areas surrounding the nematode. In resistant tomato cv. Monika and *C. metuliferus, M. incognita* induced the formation of more giant cells but poorly developed and with less number of nuclei per giant cell than in susceptible tomato and melon.

2. *C. metuliferus* BGV11135 is a compatible rootstock with cantaloupe and piel de sapo type melons without affecting the melon fruit quality, and affected only some shape characteristics in the piel de sapo type that is not important at commercial level.

3. Grafting melon and tomato onto "*C. metuliferus"* and "Aligator" rootstocks respectively did not increase the crop yield in non-nematode infested soil. The quality of the fruits produced in grafted plants was within the standards.

4. The spring-summer rotation sequence melon-tomato provided more fruit weight yield than the tomato-melon one in our agroenvironmental conditions. In *Meloidoyne incognita* infested soil, grafted melon yielded significantly more than the ungrafted irrespective of the cropping season. However, grafted melon was more tolerant and experienced less maximum yield losses when cultivated in spring-summer compared to the summer-autumn crop. In addition, some melon fruit quality parameters were affected by the nematode in the summer-autumn crop but not in the spring-summer.

5. The reproduction rate of the nematode was affected by the cropping season, the plant material, the initial population density and the virulence to specific R genes. In melon, the reproduction rate of the nematode in ungrafted plants was higher in the spring crop compared to the resistant plants. However, when it was cultivated in summer the reproduction rate was lower due to the high mortality produced by the stressful conditions. In tomato, the reproduction rate in grafted plants increased progressively in each crop, being higher than the ungrafted tomato at the end of the third tomato crop of tomato-melon rotation sequence due to virulence selection. Virulence to the *Mi*1.2 was observed in the "Aligator" rootstock after the first tomato crop, but not in *C. metuliferus* BGV11135. Thus, alternating these two different resistant species was not enough to prevent virulence selection to the *Mi*1.2 gene, although its level was reduced after using *C. metuliferus* in rotation.

6. The fitness cost of the virulent *Mi*1.2 subpopulation in the susceptible tomato were shown by a reduced ability to infect and to reproduce, as well as the reduced fertility of the females respect to the avirulent subpopulation. In melon, the virulent *Mi*1.2 subpopulation showed a reduced ability to reproduce and a reduced fertility of the females respect to the avirulent subpopulation. The fitness cost of the virulent *Mi*1.2 subpopulation was detected only after the third grafted tomato crop. Then, a minimum number of crops are needed to fix the character in the population, three alternating grafted tomato crops onto 'Aligator' in our experimental conditions.

7. *Cucumis metuliferus* is as excellent rootstock to be included in integrated management strategies for RKN management in horticulture production systems, due to its resistance and tolerance to the nematode, its effect on reducing the level of nematode virulence to the *Mi*1.2 gene, and its compatibility with melon without affecting its fruit quality.

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