Tomato rootstocks for the control of *Meloidogyne* spp.

Characterization and evaluation of the resistance response conferred by the Mi-1 gene in tomato rootstocks.

Laura Cortada González

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Front cover: Plantlet of tomato rootstocks Maxifort obtained at the commercial nursery Cristalplant S.A in Almería (Spain). Picture by Laura Cortada.

All photographs by Laura Cortada, except: Introduction (Picture "Almería-Spain" obtained from the Web site of the United Nations Environment Program DEWA/GIRD-Europe*"* from the publication *"One planet, many people: Atlas of our changing environment* Web site: http://www.grid.unep.ch/activities/global_change/atlas/atlas_examples.php (Visited 16th November, 2009)*;* Fig. 5 (a, b), Fig. 7 (a, b) and Fig. 8 (a, b), by Oscar Simon Bueno; Fig. 7 (c), by Dr. Miguel Talavera.; Text Box 4 (a), by Dr. Francesc Xavier Sorribas; Text Box 4 (c) from Wikipedia Commons; *"Wooden Letters"*, by http://eltallerliterario.com.ar/tag/literatura. Pictures are reproduced with the authorization from the owner of the copyright or have been obtained under the terms of the GNU Free Documentation License, Version 1.3 or any later version published by the Free Software Foundation; with no Invariant Sections, no Front-Cover Texts, and no Back-Cover Texts.

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IRTA

Tomato rootstocks for the control of *Meloidogyne* spp.

Characterization and evaluation of the resistance response conferred by the *Mi-1* gene in tomato rootstocks

PhD Dissertation

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A la memoria de mis abuelos, Juan Antonio y Marina.
Porque pienso en vosotros cada día.

A mis padres por su apoyo incondicional y su cariño.
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"Our challenges may be new. But those values upon which our success depends (hard work and honesty, courage and fair play, tolerance and curiosity...) these things are old. These things are true... "

B.H. Obama

“La amistad duplica las alegrías y divide las angustias por la mitad”

Sir. F. Bacon

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Abstract

Tomato rootstocks are becoming an important non-chemical method to control diseases worldwide. Grafting improves plant growth and vigor and allows the cultivation of appreciated tomato varieties in pathogen infested soils. Due to these advantages, the use of rootstocks has increased in detriment of tomato cultivars. Despite the adaptability of tomato rootstocks to different conditions and their high tolerance to stress, little information is available about the ability of tomato rootstocks carrying the *Mi-1* resistant gene to control root-knot nematodes of the genus *Meloidogyne* under different agronomic conditions. The objective of this thesis was to determine the suitability of the *Mi-1* tomato rootstocks to manage tomato infections by *M. arenaria*, *M. incognita* and *M. javanica* and their effectiveness was compared with that of resistant tomato cultivars.

The response of 10 *Mi-1* tomato rootstocks to a *Mi*-avirulent population of *M. javanica* was determined in pot tests conducted in a greenhouse in spring (March to July) when temperatures remained below the *Mi-1* functionality resistance threshold (28 °C), and in summer (July to September) when daily average temperatures exceeded the temperature threshold for *Mi-1* expression. These same rootstocks were additionally evaluated in field conditions by exposing them to high population densities of the nematode (March to July). Results on infectivity and reproduction below 28 °C for pot tests and field trials indicated a wide variability in the resistance response of the rootstocks ranging from highly or intermediate resistance (PG-76, Gladiator, MKT-410; Brigeor, 42851, 43965, Big Power and He-man) to fully susceptible (Beaufort and Maxifort). At high temperature conditions, only two rootstocks (PG-76 and He-man) were able to inhibit the reproduction of *M. javanica*. Rootstocks PG-76, Brigeor, Beaufort and Maxifort were challenged to different populations of *M. arenaria*, *M. incognita* and *M. javanica*. Rootstock PG-76 was highly resistant to all the populations tested, whereas the

response of Brigeor ranged from highly to moderate resistance; rootstocks Beaufort and Maxifort showed reduced resistance or complete inability to suppress nematode reproduction. In addition, the response of the latter rootstocks varied according to the population tested. Thus, Beaufort and Maxifort were susceptible to the two tested populations of *M. javanica* and Maxifort also was to one of *M. incognita.*

Molecular characterization of the resistance phenotype was performed for all the tomato hybrid rootstocks and cultivars previously tested. The markers PM3, PMi, Mi23, designed for the characterization of the *Mi*-locus of hybrid tomato rootstocks (*S. lycopersicum* × *S. habrochaites* and *S. lycopersicum* × *S. chilense*) were used for PCR reactions. In silico analyses were done with specific markers for the *Mi-1.2* gene (Mint-up/do, C1/2, C2S4, IMO-F1/R1, and VIGS). Results indicated that markers PMi and Mi23 were polymorphic for the *Mi-1* locus in wild *Solanum* species (*S. chilense*, *S. habrochaites* and *S. peruvianum*) and for *S. lycopersicum* (marker Mi23). Marker PM3 was able to detect the *Mi-1.2* gene in *S. lycopersicum* × *S. habrochaites* hybrid rootstocks (Beaufort and Maxifort), but not in the *S. chilense* hybrids (Tyrmes). As marker PM3 is located outside the coding sequence (CDS) of the *Mi-1.2* gene, expression of this homolog could not be determined in Beaufort and Maxifort. In silico results indicated that none of the currently available specific markers for the *Mi-1.2* gene could distinguish this homolog from the other *Mi*-homologs present in both *S. lypcopersicum* and *S. peruvianum* species. A new marker Pau-Do, in combination with C2S4, was designed to amplify a 1,494 bp fragment in the CDS of the *Mi-1.2* gene. Amplification with Pau-Do and C2S4 primers of cDNA from roots and leaves of Beaufort and Maxifort indicated that the *Mi-1.2* gene was expressed in both rootstocks, despite their susceptible phenotypic response to some *Meloidogyne* populations.

The durability of the *Mi-1* gene after repeated cultivation of resistant tomato rootstocks was determined through field trials performed along three consecutive years using rootstocks PG-76 and Brigeor. Results indicate that rootstock PG-76 responded as highly resistant after the first cropping cycle, although it showed intermediated resistance and became fully susceptible after the second and the third cropping cycles, respectively. Rootstock Brigeor and the resistant tomato cultivar Monika used as control, retained intermediate resistance levels at the end of the third year, although bioassays confirmed that selection of virulence occurred more rapidly in plots with rootstock PG-76 followed by Brigeor, and the resistant tomato cultivar Monika in the third place. Data shown that virulent nematode isolates were rapidly selected from an original avirulent *M. javanica* population after repeated cultivation of resistant tomato rootstocks in the field.

Determination of the virulent phenotype of the selected *M. javanica* populations in the field experiments was performed at the molecular level using the MVC marker. This marker was designed to distinguish selected from naturally virulent populations of root-knot nematodes. The populations analyzed included two Japanese selected virulent populations of *M. incognita* and *M. javanica*, the three Spanish virulent populations selected in the field trials, and one naturally virulent population and one avirulent population, both of the *M. javanica* species. DNA samples were obtained from individual juveniles (J2) or adult females from all the selected virulent populations. Experiments included water samples free of nematodes (5-μm filtered), obtained from the draining-water of a plant infected by a Japanese selected virulent population. Amplification of DNA only occurred in samples of filtered water, but not in those containing only nematode genetic material. Sequencing and BLAST of the DNA fragments amplified by the MVC molecular marker, established a strong correlation of the amplified bands with several proteins from the betaproteobacteria species *Acidovorax avenae* spp. *citrulli*, *Verminephrobacter eiseniae* and the genus *Diaphorobacter* spp.

Overall, these results showed that the MVC marker is not related to a nematode virulence gene (*avr*) but to betaproteobacteria.

Finally, a search for new root-knot nematode resistant *Mi*-homologs in accessions of the wild *Solanum* species *S. chilense*, *S. habrochaites*, *S. peruvianum* and *S. huaylasense* was done. From the nine accessions analyzed, only the *S. huaylasense* accession LA-1358 was able to inhibit reproduction of a population of *M. arenaria* to similar levels than the resistant tomato cultivar Anairis. Nevertheless, the resistance response of *S. huaylasense* accession LA-1358 was nematode-species specific, as it was resistant to *M. arenaria*, but susceptible to *M. javanica*. Reproduction of *M. incognita* was highly variable and did not differ from reproduction on resistant or susceptible tomato cultivars.

Resumen

Los patrones de tomate se han convertido en un importante método no químico para el control de diversas enfermedades que afectan a este cultivo a nivel mundial. El injerto mejora el crecimiento de las plantas y aumenta su vigor, permitiendo el cultivo de variedades de tomate apreciadas por el consumidor en suelos infestados por diversos patógenos. Gracias a estas ventajas, el uso de los patrones ha incrementado a lo largo de las últimas décadas en detrimento del de los cultivares de tomate tradicionales. A pesar de la elevada capacidad de adaptación de estos patrones a distintas condiciones ambientales y de su alta tolerancia al estrés, existe poca información sobre la capacidad de los patrones resistentes de tomate portadores del gen *Mi-1* para controlar nematodos fitoparásitos del género *Meloidogyne* en distintas condiciones agronómicas. Por lo tanto, el objetivo de esta tesis fue determinar la idoneidad del uso los patrones de tomate resistentes como herramienta para el manejo de las infecciones causadas por *M. arenaria*, *M. incognita* y *M. javanica*, y la de comparar su efectividad respecto a la de los cultivares de tomate resistentes.

Se determinó la respuesta de resistencia de 10 patrones de tomate a una población avirulenta de *Meloidogyne javanica* en ensayos en maceta. Éstos se llevaron a cabo en primavera (marzo-julio), cuando las temperaturas permitían la expresión fenotípica de la resistencia proporcionada por el gen *Mi-1* (28 ºC) y en verano (julio a septiembre), cuando las temperaturas diarias promedio superan dicho umbral. Los mismos patrones resistentes también se evaluaron en campo durante los meses de marzo a julio mediante su exposición a altas densidades poblacionales del nematodo. Los resultados sobre inefectividad y reproducción en los ensayos en maceta y campo efectuados a temperaturas inferiores a los 28 ºC, revelaron una gran variabilidad en la respuesta de resistencia de los patrones que osciló entre altamente resistente o moderadamente resistente (PG-76, Gladiator, MKT-410; Brigeor, 42851, 43965, Big Power y He-man),

hasta completamente susceptible (Beaufort y Maxifort). Cuando las temperaturas excedieron los 28 °C, únicamente dos patrones (PG-76 y He-man) fueron capaces de inhibir la reproducción de *M. javanica*. La respuesta de resistencia de los patrones PG-76, Brigeor, Beaufort y Maxifort también se evaluó frente a distintas poblaciones de *M. arenaria*, *M. incognita* y *M. javanica*. El patrón PG-76, fue altamente resistente a todas las poblaciones analizadas, mientras que la respuesta de Brigeor osciló entre altamente resistente y moderadamente resistente; los patrones Beaufort y Maxifort mostraron menor resistencia que los anteriores o fueron totalmente incapaces de inhibir la reproducción del nematodo. Además, la respuesta de los dos últimos patrones varió en función de la población analizada; así, Beaufort y Maxifort fueron susceptibles a la infección causada por las dos poblaciones de *M. javanica* estudiadas y Maxifort también lo fue a una población de *M. incognita*.

Se llevó a cabo una caracterización a nivel molecular de resistencia proporcionada por el gen *Mi-1* en todos los patrones híbridos y cultivares de tomate previamente estudiados. Para, ello, se emplearon los marcadores moleculares PM3, PMi y Mi23, diseñados específicamente para la caracterización del locus *Mi* en patrones híbridos de tomate (*S. lycopersicum* × *S. habrochaites*, *S. lycopersicum* × *S. chilense*), mediante técnicas de PCR. También se realizaron análisis bioinformáticos empleando marcadores específicos (Mint-up/do, C172, C2S4, IMO-F1/R1, y VIGS) para determinar la presencia del gen *Mi-1.2* en los patrones estudiados. Los resultados mostraron que los marcadores de PMi y Mi23 son capaces de amplificar numerosos homólogos del gen *Mi-1* en diversas especies de tomate salvaje (*S. chilense*, *S. habrochaites* y *S. peruvianum*) y también en *S. lycopersicum* (marcador Mi23). El marcador PM3 amplificó el gen *Mi-1.2* en los patrones Beaufort y Maxifort (híbridos de *S. lycopersicum* × *S. habrochaites*) pero no resultó efectivo para los híbridos de *S. chilense*. Debido a que el marcador molecular PM3 se halla ubicado fuera de la secuencia codificadora (CDS) del gen *Mi-1.2*, no se pudo determinar la

expresión de este homólogo en Beaufort y Maxifort. Análisis bioinformáticos indicaron que ninguno de los marcadores específicos diseñados para amplificar el gen *Mi-1.2* disponibles en la actualidad, es capaz de distinguir este homólogo de otros homólogos presentes en las especies *S. lypcopersicum* y *S. peruvianum*. El nuevo marcador molecular Pau-Do fue diseñado para amplificar de forma específica, en combinación con el primer C2S4, un fragmento de 1.494 pb en la secuencia codificadora del gen *Mi-1.2*. El empleo de ambos marcadores permitió la amplificación del gen *Mi-1.2* en el cDNA procedente de raíces y hojas de Beaufort y Maxifort. Este hecho indicó que el gen de resistencia *Mi-1.2* se expresa en ambos patrones, a pesar del fenotipo de susceptibilidad que Beaufort y Maxifort presentan frente a algunas poblaciones de *Meloidogyne*.

La durabilidad de la resistencia proporcionada por el gen *Mi-1* después del cultivo reiterado de patrones de tomate se determinó mediante experimentos de campo realizados a lo largo de tres años consecutivos en los que se emplearon los patrones resistentes PG-76 y Brigeor. El patrón PG-76 fue altamente resistente al nematodo después del primer ciclo de cultivo, mostró una respuesta de resistencia intermedia y resultó susceptible al finalizar el segundo y el tercer año de cultivo, respectivamente. El patrón Brigeor y el cultivar de tomate resistente Monika empleado como control, mantuvieron un nivel de resistencia intermedio al final del tercer ciclo de cultivo, a pesar de que ensayos posteriores realizados en maceta confirmaron la aparición de dicha virulencia, la cual se produjo más rápidamente en las parcelas cultivadas con el patrón PG-76, seguidas por aquellas sembradas con el patrón Brigeor y con el cultivar resistente Monika, en tercer lugar. Estos resultados mostraron que el cultivo reiterado de los patrones de tomate resistentes en el campo dio lugar a una selección rápida de aislados virulentos a partir de una población avirulenta de *M. javanica*.

El fenotipo virulento de las poblaciones del *M. javanica* seleccionadas en los experimentos de campo se analizó a nivel

molecular utilizando el marcador de virulencia MVC. Este marcador se diseñó para distinguir poblaciones virulentas de *Meloidogyne* seleccionadas tras el uso reiterado de variedades resistentes de tomate de los aislados naturalmente virulentos. Se analizaron dos poblaciones japonesas de *M. incognita* y *M. javanica* cuya virulencia había sido seleccionada mediante el uso repetido de cultivares resistentes de tomate, las tres poblaciones españolas virulentas seleccionadas en los ensayos de campo y una población naturalmente virulenta y una avirulenta, ambas de la especie *M. javanica*. Las muestras de ADN se obtuvieron de individuos juveniles (J2) o de hembras adultas. En los experimentos se incluyeron muestras de agua sin nematodos (previamente filtradas por un filtro de 5-μm), procedentes del drenaje recogido después del riego de una maceta que contenía una planta infectada por una población virulenta japonesa. La amplificación de ADN mediante el marcador MVC sólo se produjo en las muestras de agua filtrada y sin embargo, no se produjo en aquellas que contenían únicamente el material genético de los nematodos. Las secuencias de ADN obtenidas, fruto de la amplificación con el marcador MVC, se compararon con las secuencias disponibles en las bases de datos públicas mediante el programa informático BLAST. Los resultados mostraron una estrecha correlación de las secuencias MVC con las de diversas proteínas de las especies de betaproteobacterias *Acidovorax avenae* spp. *citrulli*, *Verminephrobacter eiseniae* y del género *Diaphorobacter* spp. Estos experimentos revelaron que el marcador de MVC no está relacionado con un gen de virulencia del nematodo (*avr*) sino con betaproteobacterias.

Finalmente, se estudió la existencia de homólogos del gen *Mi* de resistencia a nematodos en las especies de tomate silvestre *Solanum chilense*, *S. habrochaites*, *S. peruvianum* y *S. huaylasense*. De las nueve variedades analizadas, sólo la variedad LA-1358 de la especie *S. huaylasense* fue capaz de inhibir la reproducción de una población de *M. arenaria* a niveles similares a los del cultivar de tomate resistente Anairis, empleado como control. La respuesta de

resistencia de la variedad LA-1358 de *S. huaylasense* varió en función de la especie del nematodo estudiada ya que ésta se comportó como resistente frente a *M. arenaria*, pero fue susceptible frente a *M. javanica*. En cambio, la reproducción de *M. incognita* fue muy variable y no difirió de la reproducción alcanzada en ambos cultivares de tomate empleados como control.

Evolution of the area under protected cultivation in southeastern Spain: Aerial image of the plastic houses of Campo de Dalias County from 1974 to 2004. In the last 20 years the area covered by plastics has increased in 300%

S P A I N
El Ejido
Roquetas de Mar
La Mojonera
Campo de Dalias
Balerma
Almerimar
Punta Entinas-Sabinar
Alboran Sea
0 3 6
24 Jan 1974

Dalias
Almeria
El Ejido
Roquetas de Mar
La Mojonera
Campo de Dalias
Balerma
Almerimar
Punta Entinas-Sabinar
Alboran Sea
0 3 6
18 Jul 2004

Introduction

Tomato is one of the most important horticultural crops worldwide. It represents an important source of iron (2%) and vitamin A (5%) in the world's basic diet (FAO, 2001). Deficiencies of vitamin A and iron are of greatest public health significance in the world today (Chadha and Oluoch, 2003).

According to the Food and Agricultural Organization (FAOSTAT, web site), China was the first tomato world producer in 2007 closely followed by India, Iran and Turkey. Far behind these Asiatic countries, appear the productions of America, Europe, Africa and Oceania. In the European Union (EU), tomato is the first vegetable crop in terms of quantities (around 15.3 million t in 2007) (EUROSTAT, 2008a). The tomato production has remained quite stable from 2001 to 2006, although a slight decrease occurred between 2006 and 2007. Within the EU, tomato is of special relevance in the Mediterranean region where more than 60% of the production comes from Italy and Spain (EUROSTAT, 2008a). Although Spain (3,664 million t) is the second producer after Italy (6,026 million t), it is the first European exporter to countries like United States of America, Russia, Norway, Greece or Germany (Figure 1a). The most outstanding importations to Spain are those proceeding from Morocco (13 thousand t). The production of this country is concentrated and exported to Europe at the same time that the Spanish tomato production, turning Morocco into the most direct competitor for the Spanish producers (Text Box 1). The remaining importations represent a minority and mainly come from other European countries (Figure 1b). The final EU net trades for tomato in 2007 were positive, and exportations (710 thousand t) widely exceeded the foreign importations (246 thousand t) (EUROSTAT, 2008a).

Provisional data provided by the Ministry of Environment, Rural and Marine Affairs of Spain in 2007 (MARM, 2007) indicates that Spain dedicated 56, 7 thousand hectares to tomato production, which was the smallest surface area for this crop since 1990. Nevertheless, the reduction of the cultivated area was compensated by the highest average yield increases (6.77 t/ha) in the last decade.

The investment on mechanization of the farms would be the main reason for the increased productivity. From the total surface area (ha) cultivated in Spain, 99% is occupied by irrigated areas and the production of tomato is mainly performed outdoor (64.2%) and the rest, under plastic houses (35.8%).

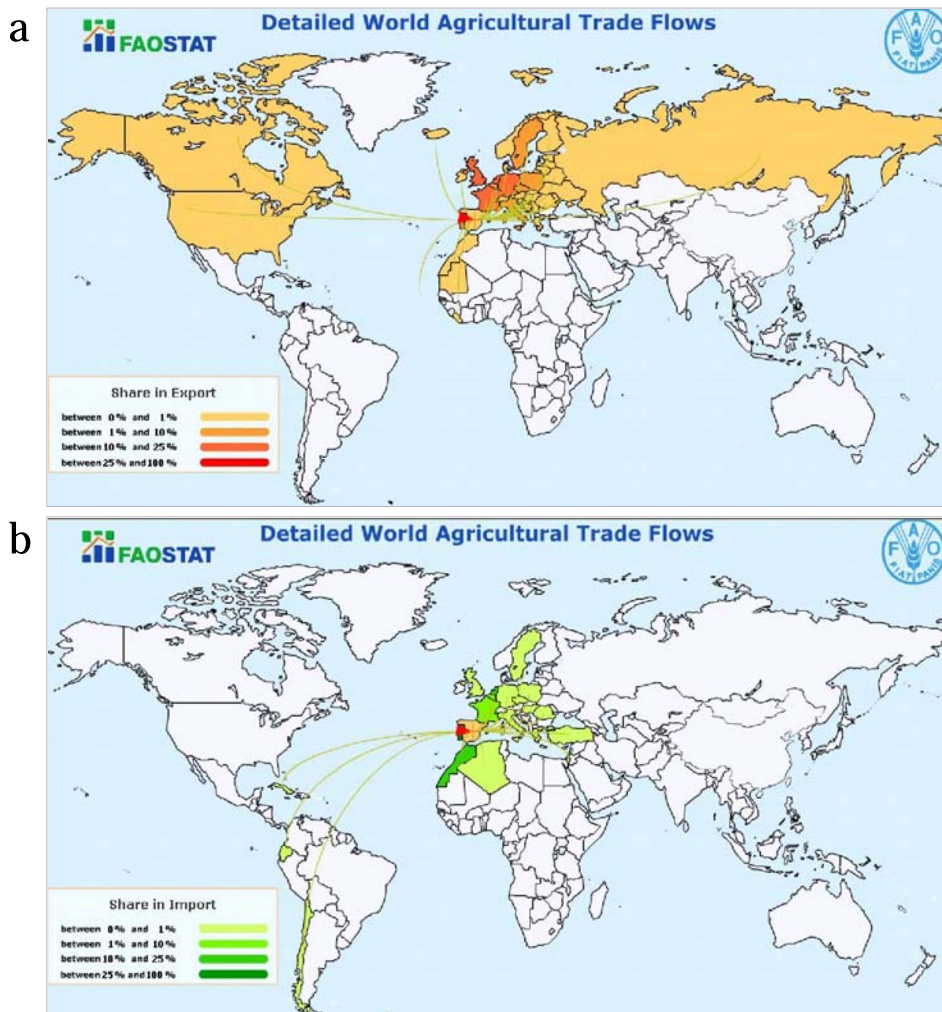


Figure 1. World map of the commercial relationships of Spain with other countries related to tomato during 2005. **(a)** Exportations. **(b)** Importations (FAOSTAT).

According to the Statistics of the Marine and Rural Area Yearbook (MARM, 2008), production of tomato in Spain is specially concentrated from June to September with a total production of more than 2,000 thousand t for this period. Productions obtained from January to May (984 thousand t) and those from October to December (705 thousand t) are less significant.

The main tomato producers among the Spanish regions are: Andalusia, Extremadura, Murcia, Canary Islands, Castile–La Mancha, Aragon, Navarre and Catalonia (Fig. 2). Almeria and Murcia are the major provinces for production of fresh tomato in greenhouses, and both are mainly focused on exportation. Production in Almeria is characterized by small-scale family farms and the use of simple plastic structures called “parral”. Cultivation is mostly in “enarenado”, which consists on a 30-cm layer of soil placed on top of the natural soil, 2-3 cm of organic compost and a 10-cm top-layer of sand. In this province, the area under protected cultivation (plastic multi-tunnels) has largely increased but the glasshouse area is relatively small. In Murcia, the production systems are characterized by large-scale fields owned by companies with modern greenhouses (Costa and Heuvelink, 2007).

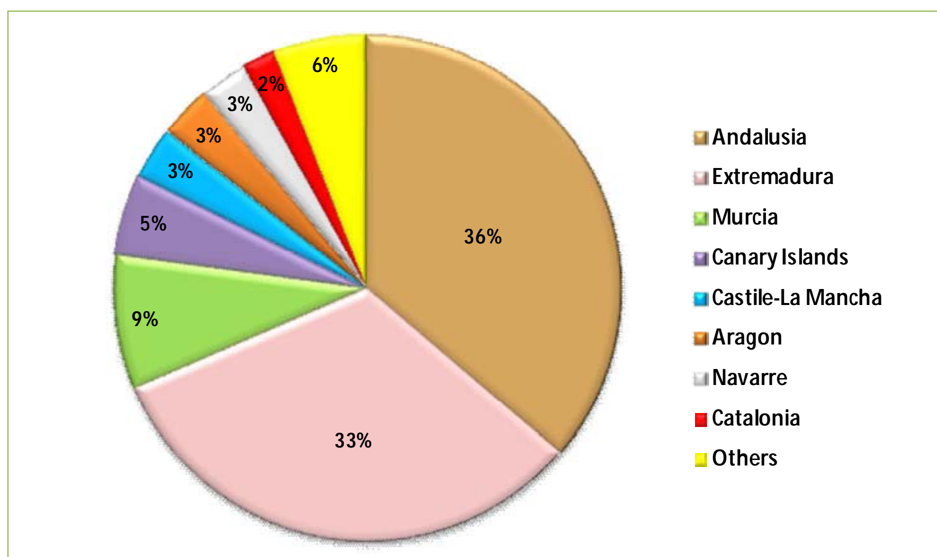


Figure 2. Percentual distribution of the total tomato production in the Spanish regions.

Text Box 1: The controversy with the Moroccan tomato

Tomato production in Morocco represents a threat for the Spanish producers of cherry tomato, especially those from Almeria and Murcia. This fact has been attributed to the high increases of the African exportations and the low prices of the tomato in Morocco which is exported to the UE coinciding with the Spanish tomato production (Nantes, 2009; http:www.abc.es). An agreement reached in 2001 between the EU and the Kingdom of Morocco established a maximum quota for the Moroccan exportations, although Morocco has been systematically breaking the UE trading agreements (Anónimo, 2009; http:www.elpais.com). The European Group of Tomato Producers (EGTP), which includes France, The Netherlands, United Kingdom, Poland and Spain, has publicly denounced the irregularities committed by Morocco over the last years concerning to these exportation quotes. In addition, the price of the Moroccan tomato is far below (0.36 €/kg) the preferential duty fees established by the UE to this country (0.46 €/kg) (COAG, 2009; http://www.agroinformación.com). Nevertheless, African producers regret the critics received arguing that most producers working right now in Morocco are from EU countries, especially from Spain and France (Fernandez, 2009). Displacement of tomato production to developing countries is mainly attributed to lower salaries, cheaper farm lands and relaxed policies concerning the use of pesticides. The European Anti-Fraud Office (EAFO) has confirmed the irregularities related to tomato exportations from Morocco, and has demanded an increased control of these exportations to "verify the observance of the EU legislation about hygiene, food security, traceability, and those related with environmental issues, requested for the European producers".

The genus *Meloidogyne* Goeldi, 1892

The phylum *Nematoda* comprises a large number of species adapted to several habitats, ranging from free living nematodes to obligate parasitic species. Within this phylum, sedentary endoparasitic nematodes from the *Heteroderide* family are responsible for a reduction of 12% of the annual worldwide production, estimated in more than 1 billion dollar per year (Sasser and Freckman, 1987).

Two major groups can be distinguished within the *Heteroderidae* family, the cyst-nematodes and the root-knot nematodes. The cyst-nematodes comprises those species where the adult endoparasitic female that contains the fully embrionated eggs inside their hardened cuticle-body, is visible at the

external surface of the root. When infection occurs, the response of the host cells to nematode feeding is the formation of a *syncytium* that consists of the union of the infected cells to their adjacent through cell wall degradation and protoplast fusion. The most damaging species within this group belong to the genus *Globodera* and *Heterodera*. The potato cyst-nematodes, *G. pallida* and *G. rostochiensis* are becoming increasingly dominant in intensive potato production areas; *H. avenae* affects wheat producing millionaire economic losses in USA, Australia, Europe or India, and *H. glycines* is considered a major threat for production of soybean worldwide (Manzanilla-López et al., 2004).

In the root-knot nematodes group, the mature sedentary females remain inside the root tissue and lay their eggs in a gelatinous egg mass. When the second-stage juvenile (J2) infects the plant and feeds on a cell, it produces an abnormal cell growth followed by multiple acytokinetic mitosis that lead to the formation of a large multinucleate *giant cell* (Williamson and Hussey, 1996). The most damaging species of *Meloidogyne* are *M. arenaria* (Neal) Chitwood, *M. incognita* (Kafoid and White) Chitwood and *M. javanica* (Treub) Chitwood, as they can infect more than 2,000 different hosts including horticultural and extensive crops, ornamental plants and fruit trees (Hussey, 1985). They have a broad geographic distribution in temperate and tropical regions in a latitudinal range comprised between 40 ˚N and 33 ˚S. The species *M. arenaria* infects a wide range of crops and is specially relevant to peanut crops of the African continent; *M. incognita* is highly pathogenic to basic crops for human consumption as rice, maize, potato, soybean, banana or yam, and also for added-value crops like tobacco, coffee, sugar cane, sugar beet or cotton (Lamberti, 1997); *M. javanica* is extremely polyphagous and mainly affects vegetables, cotton and tobacco. Among these three species, *M. javanica* is the most abundant in the Mediterranean basin, followed by *M. incognita* and *M. arenaria* (Ibrahim, 1985; Ornat and Sorribas, 2008).

In Spain, other species of *Meloidogyne* have been recorded in addition to those already cited (Andrés et al., 1998). Species such as *M. hapla, M. artiella*, *M. hispanica* and *M. baetica* are important pests for several horticultural crops and fruit trees. Although *M. hapla* is present in the Mediterranean area it is more commonly found in northern regions of Europe infecting winter crops. Nevertheless, *M. hapla* has been identified in Spain infecting strawberry (*Fragaria vesca*) and kiwifruit (*Actinidia deliciosa*) (Abelleira and Mansilla, 1993). *M. artiella* is mainly distributed in the Mediterranean area infecting leguminous crops like chickpea (*Cicer*

arietinum) or durum wheat (*Triticum durum*), cereals like barley (*Hordeum vulgare*) or sorghum (*Sorghum vulgare*) and crucifer crops such as cabbage (*Brassica oleracea*) or radish (*Raphanus sativus*) among others (Greco et al., 1992; Hernández et al., 2005); *M. hispanica* was first described infecting peach rootstocks (*Prunus persica silvestris*) although it can also infect tomato, pepper and watermelon (Hirschmann, 1986); finally, *M. baetica* was described in southern Spain infecting wild olive trees (*Olea europaea* spp. *sylvestris*) (Castillo et al., 2003). Additional *Meloidogyne* species have been found in the Iberian Peninsula infecting herbaceous and woody dicotyledonous plants: *M. silvestris* n. sp. was reported parasitizing the European holly (*Ilex aquifolium*) (Castillo et al., 2009); *M. dunensis* is a parasitic specie of the European sea rocket (*Cakile maritima*) (Palomares Rius et al., 2007),; and *M. lusitanica* has been described infecting the olive tree (*Olea europaea*) in Portugal (Abrantes et al., 1991). The geographic distribution of these *Meloidogyne* species in the Iberian Peninsula can be observed in Figure 3. Other *Meloidogyne* species that affect crops of local economic interest are described in Table 1.

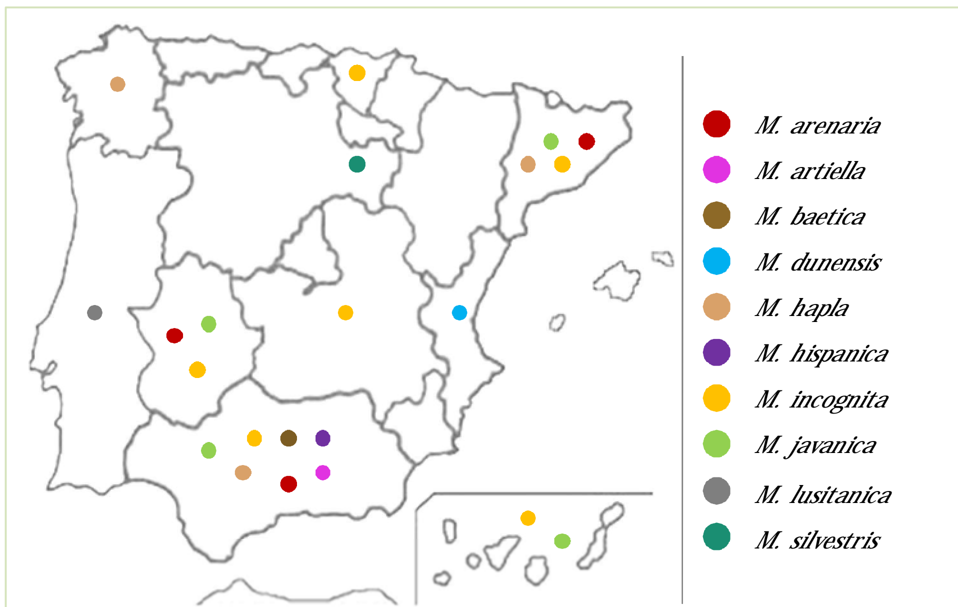


Figure 3. Geographic localization of the ten *Meloidogyne* species described in the Iberian Peninsula.

Table 1[1]. Other *Meloidogyne* species recognized as important pest for crops of economic interest.

Species	Plant hosts	Geographic distribution
M. acronea	Cotton, sorghum	Southern Africa
M. africana	Coffee	Africa
M. arabica	Coffee	Costa Rica
M. brevicultata	Tea	India
M. chitwoodi	Cereals, potatoes, sugar beet	Australia, Europe, North and South America
M. coffeicola	Coffee	South America
M. decalineata	Coffee	Africa
M. exigua	Coffee	South America
M. graminicola	Rice	North America, South America, South-east Asia
M. fallax	Alfalfa, carrot, potatoes, sugar beet	Australia, Europe, South Africa
M. konaensis	Coffee	Hawaii
M. mayaguensis	Coffee, guava, *Solanaceae*	Cuba, Puerto Rico, Senegal, South Africa
M. naasi	Grasses	Europe, New Zealand, North America
M. oryzae	Wheat, potatoes, rice, tomatoes	Surinam
M. salasi	Rice	Costa Rica, Panama
M. mali	Cherry trees, apple trees	Japan

[1]Adapted from Manzanilla-López et al. (2004).

Identification of root-knot nematodes at the species level is essential, particularly when more than one crop is grown and *Meloidogyne* control is planned using rotation of susceptible and resistant crops. Nevertheless, this may not be sufficient as some of these species like *M. arenaria* or *M. incognita* appear as races with a specific range of hosts (Hartman and Sasser, 1985) (Table 2). Although *Meloidogyne* races have been established, recent diferencial host tests, including a wider range of hosts, indicate that there can be an enormous variability on reproduction of populations of different geographic origin, beyond what is described in Table 2 (Noe, 1992; Robertson et al., 2006).

Table 2. Differential host range test for four species of the *Meloidogyne.*

Nematode specie	Cotton	Tobacco	Pepper	Water-melon	Peanut	Tomato
Meloidogyne incognita						
Race 1	-	-	+	+	-	+
Race 2	-	+	+	+	-	+
Race 3	+	-	+	+	-	+
Race 4	+	+	+	+	-	+
Meloidogyne arenaria						
Race 1	-	+	+	+	+	+
Race 2	-	+	-	+	-	+
Meloidogyne javanica	+	+	-	+	-	+
Meloidogyne hapla	-	+	+	-	+	+

Symbol (+) indicates that the host supports nematode reproduction and (-) indicates absence of reproduction. The plant species used to perform this test are: cotton (*Gossipum hirsutum*) cv. Deltapine 61, tobacco (*Nicotiana tabacum*) cv. NC 95, pepper (*Capsicum annuum*.) cv. Early California Wonder, peanut (*Arachis hypogea*) cv. Florunner; watermelon (*Citrullus vulgaris*) cv. Charleston Grey, and tomato (*Solanum lycopersicum*) cv. Rutgers (Hartman and Sasser, 1985).

Life cycle, Symptoms and Pathogenesis

The *Meloidogyne* spp. life cycle consists of four developmental stages. Nematodes emerge from the eggs as second-stage juveniles (J2) (Fig. 5g). After hatching, the infective J2 migrate into the root and penetrate through the elongation area, the lateral roots or the adjacent areas of the pre-infected tissues. Inside the roots, J2 migrate through the intercellular space to the vascular cylinder to establish a feeding site. Once established, J2 initiate the growing process shedding to the 3rd and 4th molt and becoming adult females or males (Fig. 4 b to h). Males migrate outside of the root while sedentary females remain feeding on it. Adult females are pear-shaped and lay a gelatinous matrix which remains attached to the posterior end of the female body (Fig. 5f). Eggs are deposited within an egg mass of about 500 to 1,500 eggs (Ornat and Sorribas, 2008) which sometimes can be visible at the external surface of the root (Fig. 5a-5d). In normal conditions, the presence of males is not necessary

for reproduction as *M. arenaria*, *M. incognita,* and *M. javanica* reproduce by obligatory parthenogenesis (Tyler, 1944). The life cycle occurs between 10 ˚C (basal temperature) and 32 ˚C. *Meloidogyne* needs about 600 to 700 accumulated degree-days (DD˚) of soil temperature to complete one generation (Ferris et al., 1985). Nematode survival in the absence of a host is also conditioned by temperature: the optimum temperatures for survival of eggs and juveniles ranges from 5 ˚C to 10 ˚C (Ornat and Sorribas, 2008).

Nematode secretions (Text Box 2) injected into a plant cell, interact with the citoplasmatic receptors to initiate a series of proteomic modifications in the infected cell (Hussey et al., 2002). Few hours after nematode infection, the cell undergoes several morphological, physiological and molecular changes to become a *giant cell* (Fig. 6). Transformed cells are metabolically very active and have a dense cytoplasm with abundant organelles, ribosomes and mitochondria. The numerous invaginations that appear in the cell wall contribute to increase the contact area between the cell and the rest of the vascular tissues, enhancing the nutrient flux to the giant cell (Abad et al., 2003).

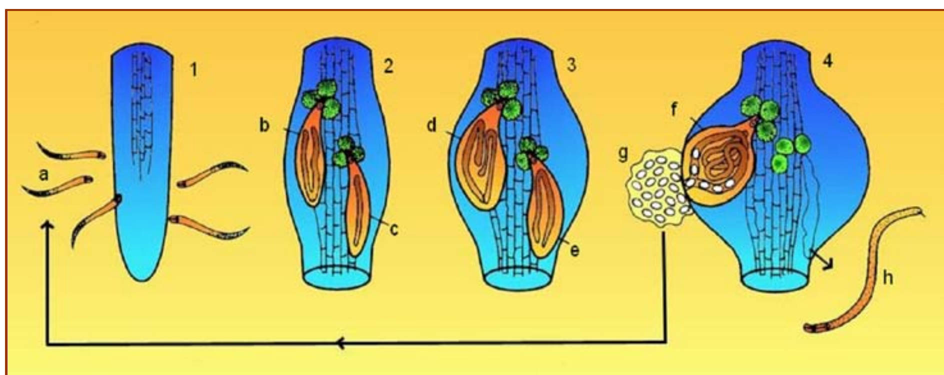


Figure 4. Diagram of the life cycle of *Meloidogyne*. **(1)** Penetration of second-stage juveniles (J2) in the plant **(a)** through the apical root tip. **(2)** Development of juveniles of third-stage (J3) to females **(b)** or males **(c)** after the establishment of the feeding site, and the formation of the giant cells (green), inducing the formation of the root gall. **(3)** Development of the fourth-stage juveniles (J4) to females **(d)** and males **(e)**. **(4)** Final maturation of the female **(f)** and formation of the gelatinous egg mass at the external surface of the root **(g)**; migration of adult males **(h)** outside of the gall (Figure adapted by V. Vermaerke and K. Spruyt from Niebel et al., (1994). and reprinted with permission from Elsevier).

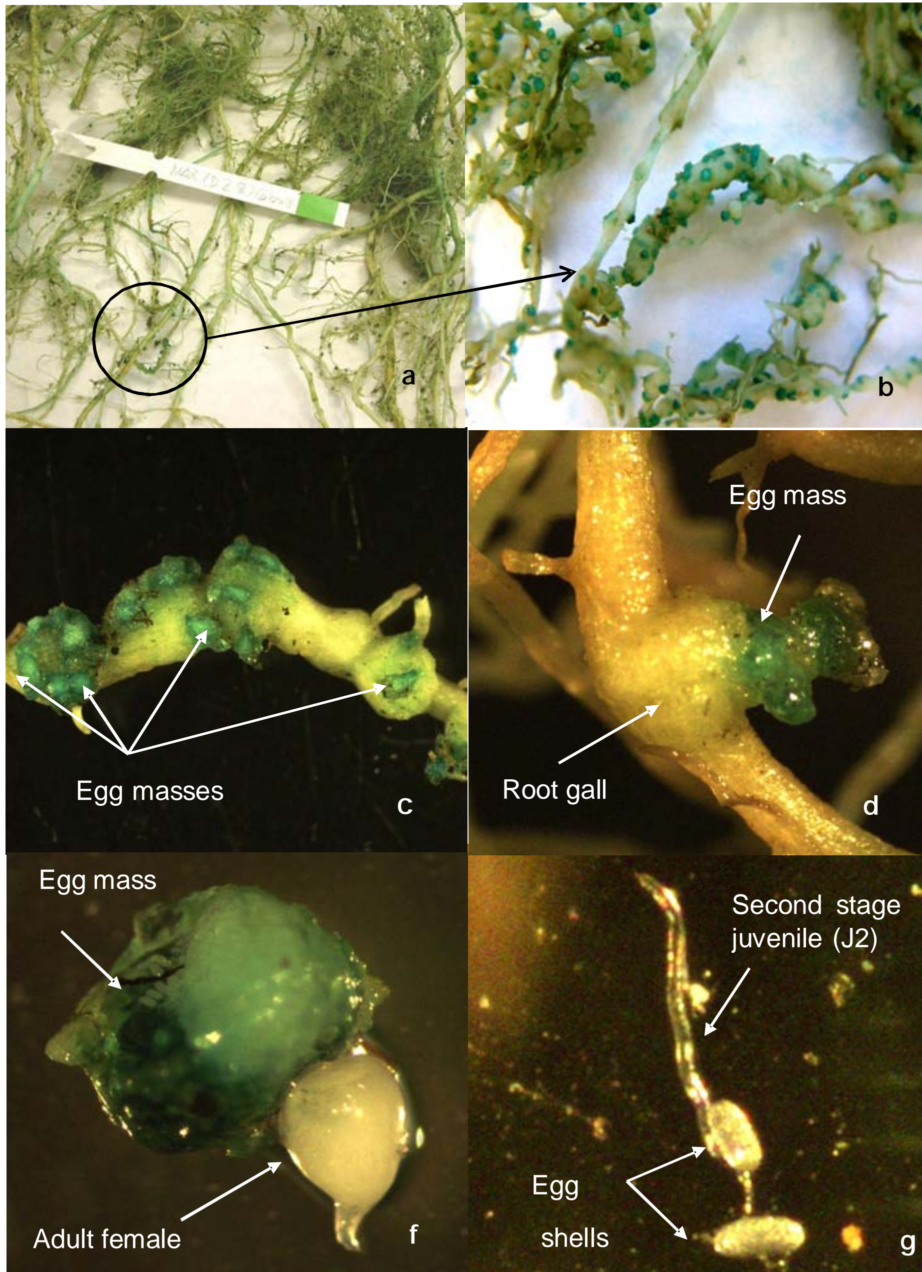


Figure 5. Roots of a susceptible tomato infected by *Meloidogyne javanica*. **(a)** General view of the infected root system of a tomato. **(b)** Closer view of the circled area and observation of blue egg masses on the external surface of the root. Egg masses were stained in blue with erioglaucine for easy counting. **(c)** Detail of the distortion of the root tissue (galls) caused by nematode infection. **(d)** Close-up view of a gall that contains an adult sedentary female with an egg mass attached to the posterior end of the body. **(f)** Pear-shaped female with an attached egg mass. **(g)** Second-stage juvenile emerged from an egg.

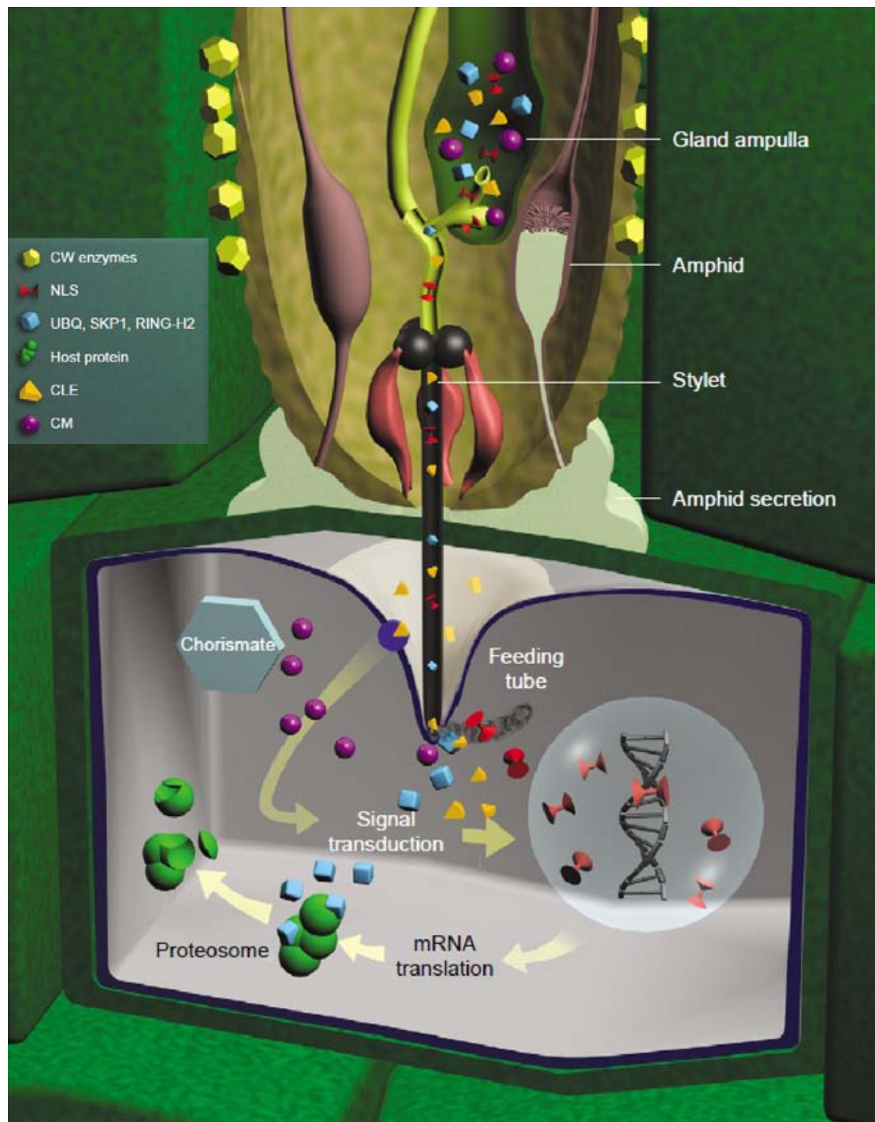


Figure 6. Interaction model of *Meloidogyne* spp. to the infected cell. This figure shows the molecular mechanism used by the nematode to alter the metabolism of the feeding cell through the interaction of the nematode secretions with the citoplasmatic receptors. Cell wall (CW)-modifying proteins (endoglucanases, pectolytic enzymes, xylanases and expansins) may be secreted to aid the migration of infective juveniles through host plant tissues. Other nematode gland cell secretions might have multiple roles in the formation of specialized feeding cells by the nematode, including: effects on host cell metabolism by secreted chorismate mutase (CM); signaling by secreted nematode peptides such as homologs to plant CLAVATA3/ESR-related (CLE) peptides; selective degradation of host proteins through the ubiquitin (UBQ)-proteasome pathway by UBQ, S-phase kinase-associated protein 1 (Skp-1) and RING-H2 secreted from the nematode; and potential effects of secreted nematode proteins that contain nuclear localization signals (NLS) within the host cell nucleus (Figure reprinted from Davis et al. (2004) with permission from Elsevier).

Text Box 2: Nematode secretions

Sedentary endoparasitic nematodes have evolved a feeding strategy that allows them to feed from a single cell or a group of cells for **prolonged periods of time.** The formation of the *syncytia* (cyst nematodes) and the *giant-cells* (root-knot nematodes) implies a dramatic modification of the cell gene expression. The evolutionary adaptations for sedentary parasitism imply the acquisition of a **stylet** and the development of a complex **oesophageal glandular system**. The secretory gland cells from the nematode oesophagus are the main source of secretions involved in plant parasitism and it has been suggested that some genes that encode oesophageal gland secretions of plant-parasitic nematodes were acquired via horizontal gene transfer from prokaryotic microbes (i. e. -1,4-endoglucanases) (Davis et al., 2000). In the oesophagic glands, secretion proteins are stored as secretory granules that **vary in size, morphology and content** among nematode species and **between the different glands** of a nematode (one dorsal and two subventrals) **within a specific life stage**: subventral glands are more active in second-stage juveniles when nematodes have to penetrate and establish their feeding site, while the activity of the dorsal gland predominates during the sendentary parasitic stages (i.e. adult females). Variation in the composition of the secreted proteins of the glands also occurs. Both glands remain active during infection and removal of a nematode during the parasitic interaction results in the degradation of the feeding cells (Hussey et al., 2002).

Individual root-knot nematodes are able to induce the formation of five to seven giant cells that can contain about 100 nuclei each. Due to the hyperplasia of the cortical cells, the root tissue becomes distorted and forms the root galls which are a characteristic symptom of *Meloidogyne* spp. infection (Figs. 5c, d and 7). Nematode damage can be assessed according to a gall rating scheme. For instance, the Zeck index (Zeck, 1971) classifies damage to the root system in a scale from 0 to 10, where 0 represents a complete and healthy root system (no galls observed) and 10 indicates plants and roots death.

The presence of root-knot nematodes in a crop may not be apparent as the aerial symptoms of *Meloidogyne* infection in a plant are unspecific and very similar to those caused by nutrient deficiencies or to other diseases caused by pathogens that attack the root system (e.g. *Fusarium* spp.). Root galling constricts the absorption of nutrient and water uptake by the plant, producing leaf chlorosis (Fig. 8a), stunting and wilting especially at the hottest hours of the day. Infection by root-knot nematodes increases plant susceptibility to other pathogens and reduces growth and yield (Fig. 8b and c). In severe attacks, plants may finally die. In addition, water stress caused by nematodes alters the quality of the tomato fruits (Roberts and May, 1986).

Figure 7. Root symptoms of infection caused *Meloidogyne javanica* in tomato. **(a)** General view of the galls in an infected root system. **(b)** Detail of galls and egg masses present in the lateral roots. **(c)** Severly infected roots of the tomato plant indicate a heavy soil infestations.

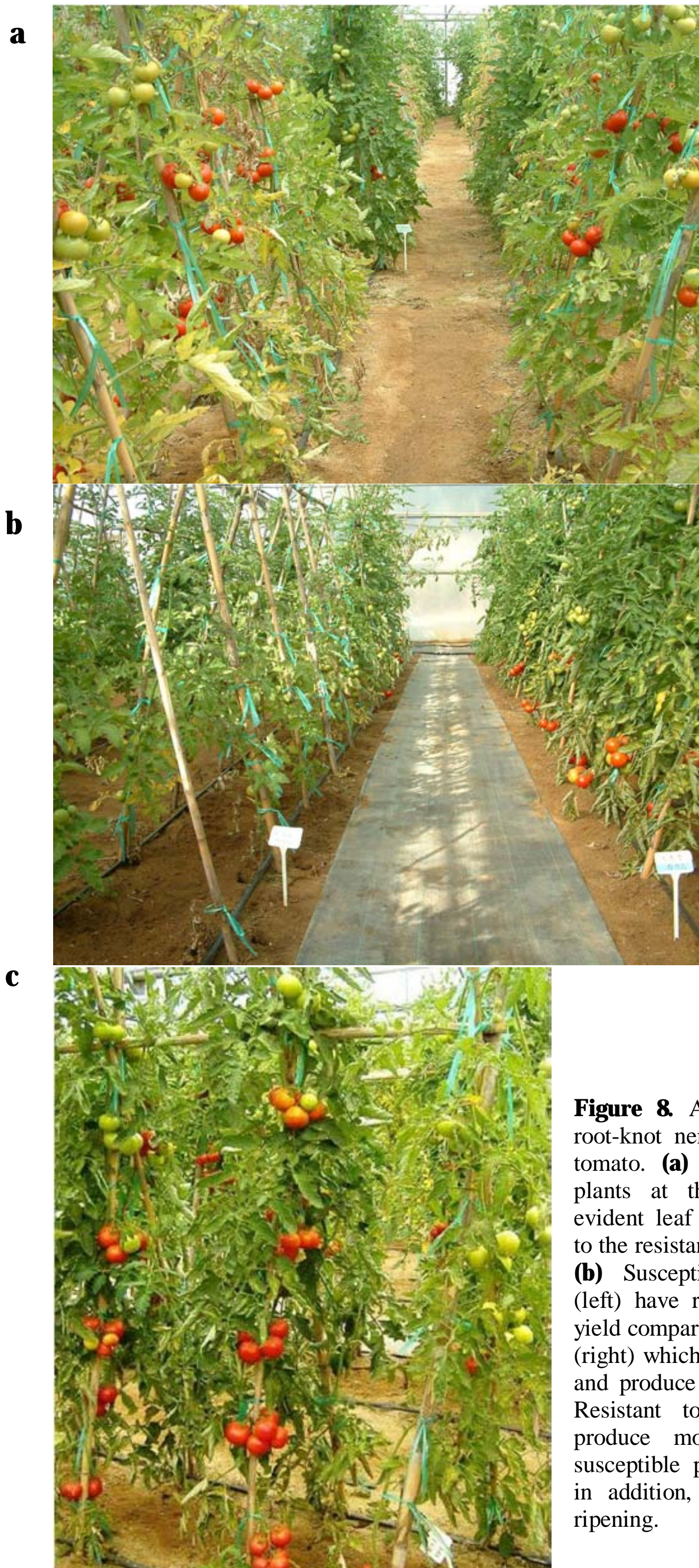


Figure 8. Aerial symptoms of root-knot nematode infection in tomato. **(a)** Susceptible tomato plants at the front show an evident leaf chlorosis compared to the resistant plants at the back. **(b)** Susceptible tomato plants (left) have reduced growth and yield compared to resistant plants (right) which have more biomass and produce more tomatoes. **(c)** Resistant tomato plants (left) produce more tomatoes than susceptible plants (right) which in addition, present a retarded ripening.

Control methods

The constant advance of the Mediterranean agriculture in the last forty years entailed a large number of initiatives to increase agricultural yields. Along the past decades, the use of chemical compounds became essential to maintain the productivity of agriculture around the world. The use of chemicals to control nematodes was first reported in 1881 when carbon disulfide was used against *H. schachtii* in sugar beet (Rich et al., 2004). The following chemical compound used was chloropicrin in the early 1920s. This gas was an efficient broad spectrum biocide employed regularly for more than eighty years. The next step forward in the use of chemicals for nematode control occurred during the 1940s, when the great majority of synthetic molecules were discovered. By that time, three of the most used products were synthesized: the D-D mixture (1,3-dichloropropane and 1,2-dichloropropene), ethylene dibromide (1,2-dibromoethane) and methyl bromide (bromomethane) (Rich et al., 2004). Since the 19th century more than sixteen different chemical products had been used as nematicides. A list of the main substances and their effects upon human health are presented in Table 3.

Methyl bromide (MeBr) has been the most effective and widely used soil fumigant worldwide. This volatile gas has a wide biocide activity, rapid action and can be used to control fungi, bacteria, insects, nematodes and weeds. The harmful effects of MeBr on the environment were reported in 1992 when this gas was identified as an ozone depleting agent in the Montreal Protocol (Watson et al., 1992). The bromines contained in MeBr are fifty times more destructive to the ozone layer than the chlorine found in chlorofluorocarbons (CFCs) and the United Nations Scientific Panel estimated that MeBr was responsible for 5 to 10% of worldwide ozone depletion (UNEP, 2001). Damage caused by MeBr to human health includes alterations of the nervous central system, respiratory depression, and eye and skin irritation. In cases of acute toxicity it can lead to death.

Developed countries committed themselves to reduce progressively the consumption of MeBr from the baseline of 1991 until its complete phase out in 2005, except for critical use exemptions. Developing countries signing the Montreal Protocol agreed to freeze consumption in 2002 at the 1995-98 average level and to reduce consumption from the baseline by 20% in 2005 and 100% in 2015**.**

Table 3[1]**.** List of synthetic chemical compounds used as nematicides along history and their toxic effects for human health.

Compound name and classification[2]	Effects on human health[3]
Inorganic	
Carbon Disulfide	Mainly affects the central nervous system and causes respiratory disorders. In laboratory experiments with animals its harmful effects on fetal development have been demonstrated.
Organohalogenated	
Chloropicrin	Irritant and corrosive for respiratory system and skin. Exposition to higher levels produces eye and skin burns, hyperventilation and bronchial collapse, blue coloring of the skin, accumulation of liquid in lungs and breath pain.
Halogenated organic	
Methyl Bromide; D-D Mixture; Ethylene dibromide; DBCP	Headache and weakness. Prolonged exposures cause accumulation of liquid in lungs, muscular shivering and renal failure. In contact with the skin it causes irritation and blisters. 1, 2-dicloropropane can cause sickness, headache and nauseas, lesions in liver and kidneys or anaemia. Acute exposures to methyl bromide and D-D mixture can cause comma or even lead to death. Men exposed to DBCP can have more female babies that males, produce less sperm and can become sterile.
Ditiocarbamat	
Metham Sodium	Irritant causes allergic dermatitis by skin contact. Irritation of the respiratory system by inhalation. Asthma. Neurotoxic. In some animals a carcinogenic effect has been observed, although it has not been demonstrated for humans.
Organophosphate	
Thionazin; Fensulfothion; Fenamiphos; Terbufos; Cadusafos; Fosthiazate	Excess of perspiration and salivation. Muscular spasms, weakness, muscular shivering and lack of coordination. Sickness, headache, nauseas, abdominal pain, diarrhoea. Respiratory depression and liquid accumulation in lungs. Blurred vision. Neurotoxic. In acute exposures it can produce strokes and loss of consciousness.
Carbamate	
Aldicarb; Ethoprophos Oxamyl; Carbofuran	Excess of perspiration and salivation. Muscular spasms, weakness, muscular shivering and lack of coordination. Sickness, headache, nauseas, abdominal pain, diarrhoea. Neurotoxic. In acute exposures it can produce depression, hypertension, and cardio-respiratory depression, diphneas and pulmonary edemas.

[1] Table adapted from Rich et al., (2004); [2] Classification of the chemical category according to the Pesticide Action Network Database (PAN, 2008); [3]Agency of Toxic Substances and Disease Registry (TSDR, 2008) and PAN (PAN, 2008).

Countries that completely eliminated the use of MeBr in tomato before 2005 were Australia, Japan, New Zeland, Portugal, Spain and United Kingdom. Nevertheless, in other developed countries like Italy, France or USA the use of MeBr for critical uses was very important until the end of 2005 (UNEP, 2006). Before 1998, Spain was the fourth MeBr consumer in the world, after USA, Japan and Italy (UNEP, 2006). At present, only four chemical non-volatile nematicides have been maintained in the Annex 1 of the Directive 91/414/CEE of the new European Directive on Plant Protection Substances for 2010 (Text Box 3).

Text Box 3: The approval of plant protection products in the European Union

As laid down in Directive 91/414/EEC, in 1993 the European Commission (2009) launched a work programme to perform a wide review for all active substances used in plant protection products within the European Union. In this review process, each substance had to be evaluated as to whether it could be used safely with respect to human health (i.e. consumers, farmers, local residents) and the environment. There were about **1,000 active substances** (or products containing them) on the market at the time the Directive was adopted. Decisions taken in 2001 enabled the review programme to be finalised in March 2009. The review of existing pesticides has led to the removal from the market of those pesticides which cannot be used safely. Of the 1,000 active substances on the market in at least one Member State before 1993, **26 %** (equivalent to 250 substances) **have passed** the EU safety assessment. Nevertheless, **67%** of the substances **have been eliminated** because the technical dossiers of these products were either not submitted, incomplete or withdrawn by industry. In addition, about **70 substances failed the review** and have been removed from the market, because the evaluation did not show safe use with respect to human health and the environment. This review on toxic substances provides assurances that the substances **currently available on the market are acceptable** for human health and for the environment.

The aproved substances belong to two main groups of nematicides: the oximine-carbamates (Oxamyl) and the organophosphates (Fenamiphos, Etoprophos and Fosthiazate). The emulsifiable formulation of these nematicides makes them specially indicated for application through drip irrigation systems. These nematicides are directly lethal to nematodes and insects and their primary action is a result of direct contact. Once these nematicides reach more than 8 cm deep into soil, their action is mostly due to sublethal effects, including modification of the nematode behavior. Non-fumigant nematicides have been successfully used to decrease densities of root-knot nematodes in many regions. Nevertheless, repeated applications are needed to maintain reduced nematode densities and consistent yield increases. These nematicides brake down in the soil or plants by hydrolysis or oxidation, but their properties relative to movement and soil persistence vary according to their solubility in water or to other ambiental factors like soil moisture, texture, type of irrigation or the amount of organic matter in the soil (Verdejo-Lucas and McKenry, 2004).

The increasing awareness of producers and consumers about the risks that chemical pesticides represent for human health and the environment has stimulated the search of new harmless methods to control pests, weeds and pathogens. The non-chemical alternatives that can be used to control root-knot nematodes are:

I. **Solarization** uses the sun energy to control soil pathogens. A transparent polyethylene film is used to cover the soil and increase the temperature of the first 30 cm up to 45 °C. This system is considered very effective in arid, humid and temperate regions. Nevertheless, this method is more efficient to eliminate sedentary stages of parasitic nematodes than mobile stages (Halbrendt and LaMondia, 2004) as they migrate deep into the soil escaping from high temperatures and afterwards, most of them return to the upper soil layers with plough labors (Bello et al., 2001).

II. **Biofumigation** is based on the use of the resulting volatile substances from biodegradation of organic matter to control plant pathogens. Biofumigants were included as a non-chemical alternative to MeBr by the Methyl Bromide Technical Options Committee (MBTOC) in 1997. When biofumigation is used in combination with other techniques (e.g. solarization) it is a very useful and efficient method to control root-knot

nematodes in integrated pest management programs (Medina-Míguez, 2002). In addition, biofumigation improves soil characteristics. Any organic remain can act as a biofumigant, although the efficiency of this method depends on the biofumigant composition, the dosage and application system (Bello et al., 2002). The biofumigants most commonly used in Spain are goat, sheep and cow manure or crop remains from rice, mushroom, olive, brassicas and ornamental gardens.

III. **Steaming** treatments are of special interests for sterilization of greenhouse structures or containers. Pathogens and pests are efficiently eliminated when steam rises to lethal temperatures or when physical damages are incurred to their resting structures (e.g. cysts), even in cases of heavy soil infestations. It has also been reported the beneficial effect of steaming in the production and growing of subsequent crops (Albajes et al., 1999). In the field, over the chemical treatments, it has the advantage that allows fast planting after soil cooling. Although it may be a useful technique, it requires exhaustive weed removing and soil preparation for a good steam penetration and the efficiency may depend on the soil texture. It can also have undesirable effects over soil biodiversity (UNEP, 2001). It is also an expensive method that is practical only for small areas.

IV. **Soil-less artificial substrates** were widely used during the decade of the 1980s to avoid soil disinfestations. The use of artificial substrates is specially recommended to prevent diseases caused by pathogens that need the soil or any kind of inert substrate to complete their life cycle. The most used substrates are vermiculite, pine bark, coconut bark or mineral wools. Although they provide a clean substrate at planting, the re-circularized water can be a source for spreading diseases that are introduced into the soil-less cultures system (Ploeg and Edwards, 2008). If plantlets are previously infected by root-knot nematodes, the soil-less cultures cannot prevent the spreading of the disease (Tzortzakakis, 2004; Hallman et al., 2005).

V. **Biological control** is based on the use of natural enemies to control or reduce population densities of pathogens and pests. The organism that has adverse effects on nematode populations are commonly called nematode antagonists. They include fungi, bacteria, insects and other invertebrates. Parasitism and predation are the main mechanisms of action of these antagonists. Among these, the hyperparasite bacterium

Pasteruria spp. and the egg parasitic fungi have been extensively studied and are considered the most important antagonists regulating nematode populations in the soil (Chen and Dickson, 2004).

VI. **Sanitation:** This prophylactic measure prevents nematode introduction in new productive areas. Sanitation includes the inspection and certification of nematode-free planting material, the cleaning of equipment and quarantine measures to minimize inoculum dispersion (Halbrendt and LaMondia, 2004).

VII. **Cultural practices** have been successfully used to control soil-borne pathogens and to minimize undesirable effects of continuous monoculture. They include:

a. **Planting Date**: Modification of planting dates can be a useful tool for nematode management. Crop displacing when soil temperatures are too high or too low for nematode infection and development has been used to reduce nematode damage. High temperatures can reduce resistance to root-knot nematodes, and therefore planting in hot seasons should be carefully considered. This breaking-resistance phenomenon has been reported in tomato (*Solanum lycopersicum*) (Dropkin, 1969), alfalfa (*Medicago sativa*) (Griffin, 1969), cotton (*Gossipum hirsutum*) (Carter, 1982), sweet potato (*Ipomoea batatas*) (Jatala and Russell, 1972) and common bean (*Phaseolus vulgaris*) (Mullin et al., 1991). Additionally, planting with low temperatures allows plant establishment and a delay in root invasion.

b. **Trap Crops**: Some short cycle crops like lettuce (*Lactuca sativa*), radish (*Raphanus sativus*), Chinese-cabbage (*Brassica rata* subsp. *pekinensis*) and Chinese salt-word (*Brassica rata* subps. *chinensis*) can be used as trap crops to reduce nematode inoculum in soil (Cuadra et al., 2000). In north-eastern Spain, temperatures between the months of September and October are high enough to allow lettuce infection by root-knot nematodes, although nematodes cannot complete their life cycle due to subsequent decrease of soil temperatures in November. In January, the crop is harvested reducing the nematode inoculum in the soil (Ornat et al., 2001).

c. **Root destruction**: Nematode populations can remain active in soil during warm and humid years in the presence of a plant host. Removing

plant roots from the precedent crop interrupt nematode life cycle which in turn, reduces nematodes survival and prevents population increases of *Meloidogyne* spp. (Ornat et al., 1999).

d. **Fallowing:** Reduces nematode population densities in soil by starvation. It is more effective when combined with root destruction rather than fallowing alone. It has been observed that survival rates of nematodes in the field are positively correlated to the length of the fallowing periods (Ornat et al., 1999). Nevertheless, it may not be a profitable option and sometimes it has detrimental effects on the soil such as increased risk of erosion or loss of soil organic matter and beneficial flora (e.g. mycorrhizae) (Halbrendt and LaMondia, 2004).

e. **Weed control**: Is of special importance as hundreds of different species are hosts of the nematode, which contribute to preserve *Meloidogyne* populations in the fields during fallow periods or winter conditions (Ornat and Sorribas, 2008).

f. **Crop Rotation:** Non-host crops can be used to remove the food source of nematodes from an infested soil. This method is difficult to implement in perennial crops. In general terms, the magnitude of the benefits is generally positively correlated with the number of cropping seasons between planting of susceptible crops. It is effective for those nematode species that have a narrow range of hosts (e.g. cyst nematodes) but not for those with a wide spectrum of parasitism (e.g. root-knot nematodes). Crop rotation can be very useful, although it has some disadvantages like the need for accurate identification of the nematode species, the host range of the nematode, the ability of the nematode to survive in the absence of a host, the presence of alternative hosts (e.g. weeds) or the economics of crop rotation (Halbrendt and LaMondia, 2004).

VIII. **Plant resistance** is based on the use of varieties that present resistance genes to certain pathogens. Resistant plant suppresses pathogen reproduction and is an effective tactic to improve crop yield when nematode population densities exceed the damage threshold (Starr et al., 2002). Tolerant crops cannot be included within this category, as they do not suppress nematode reproduction although they may provide sustained yields. A specific use of plant resistance is **grafting.** This technique consists of the union of the aerial part of a susceptible plant

(scion) with a root system (rootstock) resistant to fungi, bacteria, insects and/or nematodes. This method allows the cultivation of susceptible plants in soils severely infested with pathogens. In addition, grafted plants provide higher yields and improved tolerance towards stress conditions (e.g. salinity, high boron concentrations or low temperatures) due to their increased vigor (Edelstein, 2004).

Plant resistance in tomato

Plant resistance to parasitic nematodes is defined as the ability of a plant that has one or more resistance genes to inhibit or suppress nematode reproduction (Roberts, 2002). In nematology, resistance can be classified as: complete, intermediate (partial) or non-resistant (susceptible) (Barker, 1993).

Resistant tomato cultivars inhibit pathogen reproduction when compared to susceptible cultivars and increase crop productivity (Philis and Vakis, 1977; Rich and Olson, 1999; Roberts, 2002). They do not represent an additional cost for farmers and are useful in rotation systems preceded by susceptible crops (Hanna et al., 1993; Ornat et al., 1997; Talavera et al., 2009). Moreover, plant resistance is compatible with integrated pest management strategies and organic farming. According to FAO *"resistant cultivars are without doubt the easiest and most convenient choice for farmers*" (Lamberti, 1997). Resistant tomatoes are an economically feasible alternative to MeBr to control populations of root-knot nematodes in infected soils (Besri et al., 2003; Sorribas et al., 2005). In tomato, resistance is associated to the *Mi-1* gene. This gene inhibits reproduction of three root-knot nematode species, *M. arenaria, M. incognita* and *M. javanica*, but not of *M. hapla* (Brown et al., 1997). The most important limitation of the *Mi-1* gene is that resistance is only phenotipically expressed when soil temperature is below 28 ˚C. When temperatures exceed this maximum threshold, nematodes reproduce similarly on resistant and susceptible cultivars (Dropkin, 1969).

The *Mi-1* gene considerably reduces nematode reproduction, although differences in the efficiency of the resistant cultivars to control nematode populations have been observed (Netscher, 1976; Roberts and Thomason, 1989; Tzortzakakis et al., 1998; Sorribas and Verdejo-Lucas, 1999). Such differences

could be attributed to a different dosage of the *Mi-1* gene, which means that homozygous cultivars would suppress nematode reproduction more efficiently than the heterozygous ones. Nevertheless, scientific evidence seems to indicate that this hypothesis is not a general trend (Tzortzakakis et al., 1998; Jacquet et al., 2005). Therefore, differences among resistant cultivars have been usually attributed to the high variability within nematode species (Roberts and Thomason, 1989; Sorribas and Verdejo-Lucas, 1994; Tzortzakakis and Gowen, 1996; Eddaoudi et al., 1997; Tzortzakakis et al., 1998) or to the genetic background of the resistant cultivars, which are both key factors in the plant-nematode interactions. A study performed with *M. incognita* corroborated that nematode infection of a plant cell involves the differential expression of more than 3,000 genes compared to a non-infected cell, pointing out the great variety of cellular mechanisms associated to the *Mi*-mediated resistant response of a plant (Jammes et al., 2005).

The *Mi-1* gene

The *Mi-1* gene was first discovered in *Solanum peruvianum* accession PI-128657 and later introduced through embryo rescue in the commercial *S. lycopersicum* (Smith, 1944), and all resistant tomato cultivars commercialized nowadays originate from that single resistant hybrid (Ammati et al., 1986). The name of the gene refers to *M. incognita* as pathogenicity assays were initially performed with this nematode species. Resistance is conferred by this single dominant gene located at the telomeric proximal end in the short arm of chromosome 6 (Messeguer et al., 1991) which is transmitted to the progeny through Mendelian inheritance segregation (Gilbert and McGuire, 1955). The length of the introgressed area that spans the *Mi-1* gene varies among resistant cultivars and it has been greatly reduced in some lines respect to the original hybrid (Messeguer et al., 1991; Ho et al., 1992).

Several *Mi*-homolog genes (MiGHs) have been mapped in the vicinity of the *Mi-1* in *S. lycopersicum* and *S. peruvianum* species (Milligan et al., 1998; Seah et al., 2007). Clusters that belong to the *S. peruvianum* introgression are described as cluster 1p and 2p and those from *S. lycopersicum* are named 1e and 2e (Fig. 9) (Seah et al., 2007a). The *Mi*-homologs from *S. peruvianum* are designated with a number (e.g. *Mi-1.1* or *Mi-1.4*), to distinguish them from the

homologs from *S. lycopersicum* that are described with a letter (e.g. *Mi-1A* or *Mi-1B*). Among all the MiGHs present in both *Solanum* species, only the *Mi-1.2* gene is able to confer resistance to root-knot nematodes. In addition, this gene also inhibits reproduction of the potato aphid *Macrosiphum euphorbiae* (Rossi et al., 1998) and the biotypes B and Q of *Bemisia tabaci* (Nombela et al., 2003) (Text Box 4).

Text Box 4: Spectrum of resistance of the *Mi-1.2* gene

So far the ***Mi-1.2*** gene is the **only** known gene that provides resistance to three organisms that are phylogenetically very distant: **nematodes** (a), **aphids** (b), and **white flies** (c). As opposed to what happens when a nematode infects the root system of a resistant tomato cultivar, no hypersensitive response can be observed on leaf tissues when tomato plants are attacked by either insect species. Introgression of the *Mi-1.2* resistance gene in eggplant revealed that the gene confers resistance to nematodes but not to aphids, although introgression of the *Mi-1.2* gene in susceptible tomato cultivars gave resistance to nematodes and aphids (Goggin et al., 2006). The metabolic pathway that leads to such a differential resistant response of the *Mi-1.2* gene in these **species still remains unsolved**, although it is known that the resistance response in all these species is established by a **gene-for-gene** interaction (Rossi et al., 1998; Nombela et al., 2003). Several studies have been conducted to determine the resistance response of the *Mi-1.2* gene to different aphid isolates of several geographic origins, and results showed that this resistance gene presents both species specificity and isolate specificity resistance response (Atkinson et al., 2003).

Four of these fourteen known MiGHs are considered pseudogenes: two in *S. peruvianum* (*Mi-1.3* and *Mi-1.5*) and two in *S. lycopersicum* (*Mi-1A* and *Mi-1D*). These pseudogenes present large insertions and deletions that result in a lack of a complete open reading frame (ORF) compared to *Mi-1.2*. The homologs *Mi-1.6* and *Mi-1C* have single nucleotide mutation which also results in a truncated ORF. All the MiGHs are transcribed, except for the pseudogenes. Excluding these, the identity of the sequences for all the MiGHs is extremely high ranging from 92.9% to 96.7%. The MiGHs belong to the family of the plant resistance genes know as *R* genes that interact with pathogens through a *gene-for-gene* model described by Flor (1971) which postulates that *"for each*

gene able to establish in a plant an efficient resistance mechanism there is a homolog gene that conditions avirulence in the pathogen'. In addition to the *Mi-1* gene, several genes have been mapped at the telomeric end of the chromosome 6 in tomato in a large cluster of *R* genes (Grube et al., 2000), that includes *Ol-1*, that confers resistance to powdery mildew (*Oidium lycopersicum*) (van der Beek et al., 1994); *Am* gene, that confers resistance to most strains of alfalfa mosaic virus (AMV) (Parrella et al., 2004); alleles *Cf-2* and *Cf-5* for resistance to *Cladosporium fulvum* (Dixon et al., 1998); the *Ty-1* and *Ty-3* alleles that confers tolerance to tomato yellow leaf curl virus (TYLCV) (Ji et al., 2007); the *Bw-5* allele for resistance to *Ralstonia solaracearum* (Thoquet et al., 1996), and the *Cm6* allele for resistance to *Clavibacter michiganensis* (Sandbrink et al., 1995). These *R* genes confer qualitative or quantitative resistance to other pathogens, and all were identified in different *Solanum* species like *S. chilense*, *S. habrochaites*, *S. peruvianum* or *S. pimpinellifolium* (Grube et al., 2000).

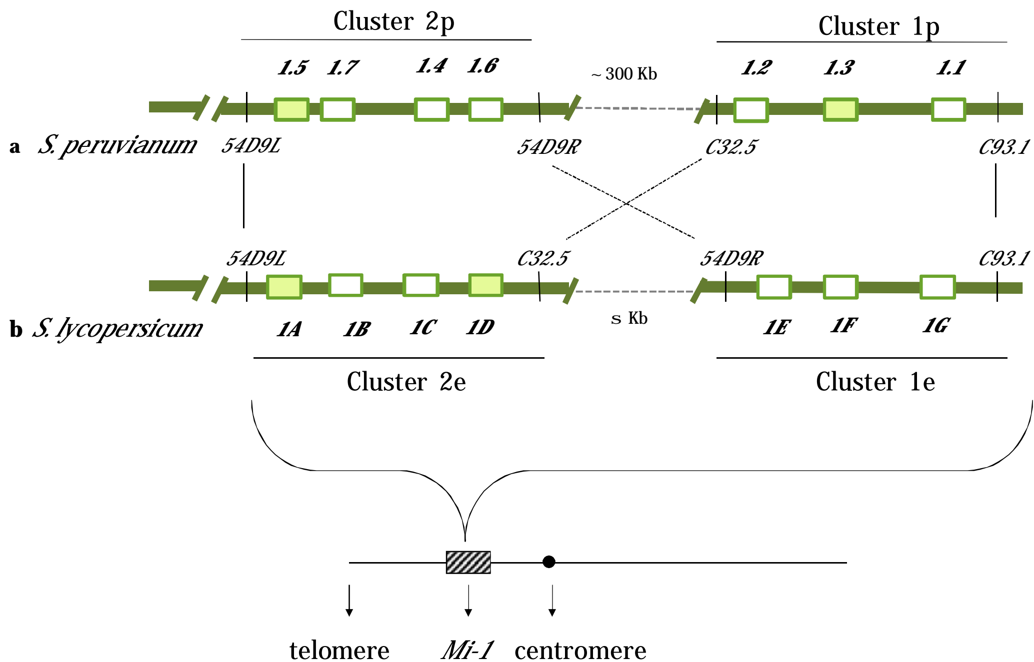


Figure 9. Comparison of the *Mi-1* locus located at the telomeric region in the short arm of chromosome 6. **(a)** Introgression of *Solanum peruvianum* in the genetic background of *S. lycopersicum*. **(b)** Short arm of chromosome 6 in the susceptible species *S. lycopersicum*. The MiGHs that present a truncated open reading frame (ORF) appear as colored boxes (Adapted from Seah et al., 2007).

The *R* genes are characterized by the presence of a nucleotide binding site (NBS) domain that is responsible for the union of the protein to the ATPs and interferes in the process of cellular apoptosis, and a leucine-rich repeat (LRR) region responsible for the protein interaction and pathogen recognition (Williamson, 1999) (Fig. 10). *R* genes can be classified according to the final destiny of their codified proteins inside the cell. The *Mi-1* gene belongs to the group of proteins that remain into the cytoplasm of the cell. Other *R* genes like *Cf-2* and *Cf-5* have been cloned, and the sequence of the LRR domain indicates that both *R* proteins are located at the membrane of the cell (Dixon et al., 1998). The similarity between the *Mi-1* gene and other nematode resistance genes (*Nem-R*) has been proved. This is the case of *Hero* gene that confers resistance to the cyst nematode species *G. rostochiensis* and partial resistance to *G. pallida*, *Gpa2* for resistance to *G. pallida*, and *Gro1-4* that confers resistance to a narrow range of pathotypes of *G. rostochiensis* (Williamson and Kumar, 2006).

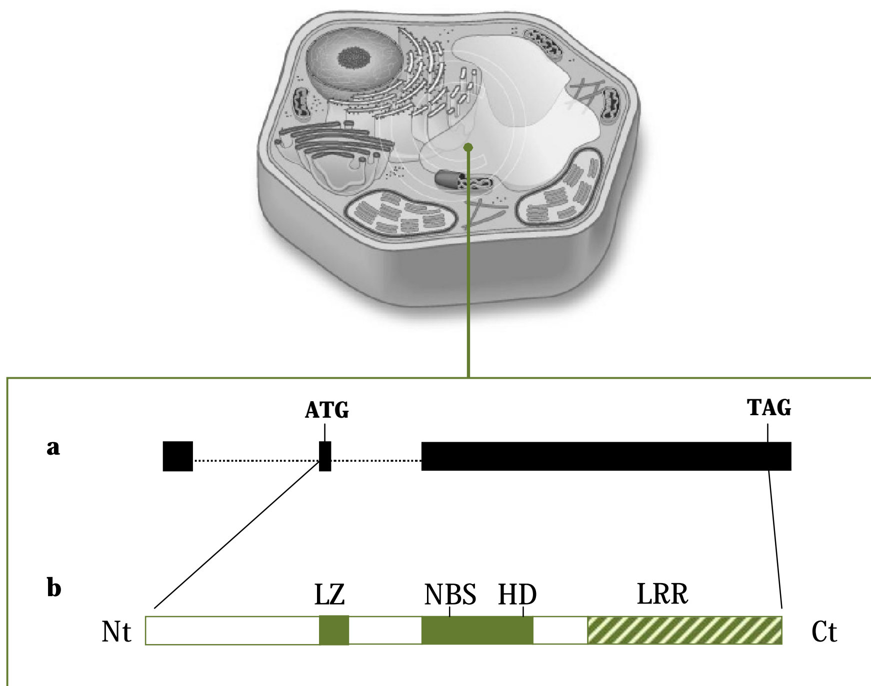


Figure 10. Diagram of the structure of the protein codified by the *Mi-1.2* gene located in the cytoplasm of the cell. **(a)** Transcript of the *Mi-1.2* gene with introns as solid black bars and exons as dotted lines; **(ATG)** Transcription start and **(TAG)** transcription stop codons. **(b)** Protein structure of *Mi-1.2*; **(Nt)** Protein amino terminus; **(Ct)** carboxyl terminus; **(LZ)** Indicates the position of a leucine zipper; **(NBS)** Nucleotide Binding Site; **(HD)** Hydrophobic domain; **(LRR)** Leucine-rich repeat region (adapted from Williamson, 1998).

The LRR domain in the carboxyl extreme (Ct) of the protein indicates that plant-pathogen recognition is located inside the cell once the nematode has penetrated to establish the feeding site (Hwang, 2000). In an infected cell, the initial recognition of an avirulence gene (*Avr*) from the pathogen by a *R* gene from the plant, activates a series of metabolic pathways to initiate a hypersensitive response (HR) that finally leads to cell death. Cellular necrosis occurs 12 h after the infection of the plant roots by the J2 (Williamson and Hussey, 1996). This mechanism interrupts giant cell development and prevents the establishment of the feeding site in the roots (Fig. 11a).

Cell death is preceeded by several biochemical and molecular responses (Melillo et al., 2006; Bleve-Zacheo et al., 2007). An example is the increased activity of the anionic peroxidases as a part of the so-called *oxidative burst* that helps to protect the plant from the invasion of nematodes or other pathogens (Gheysen and Fenoll, 2002). When no recognition occurs between the *Avr-R* genes (compatible interaction) the absence of the hypersensitive response allows the establishment of the feeding site by the J2, leading to plant infection (Fig. 11b).

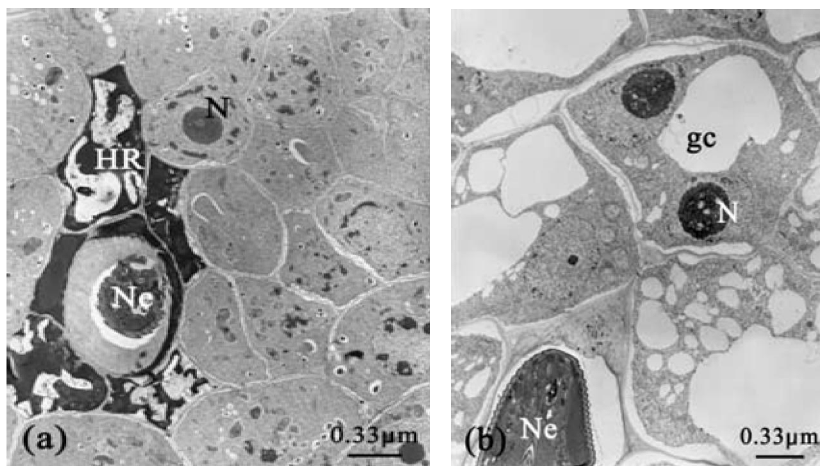


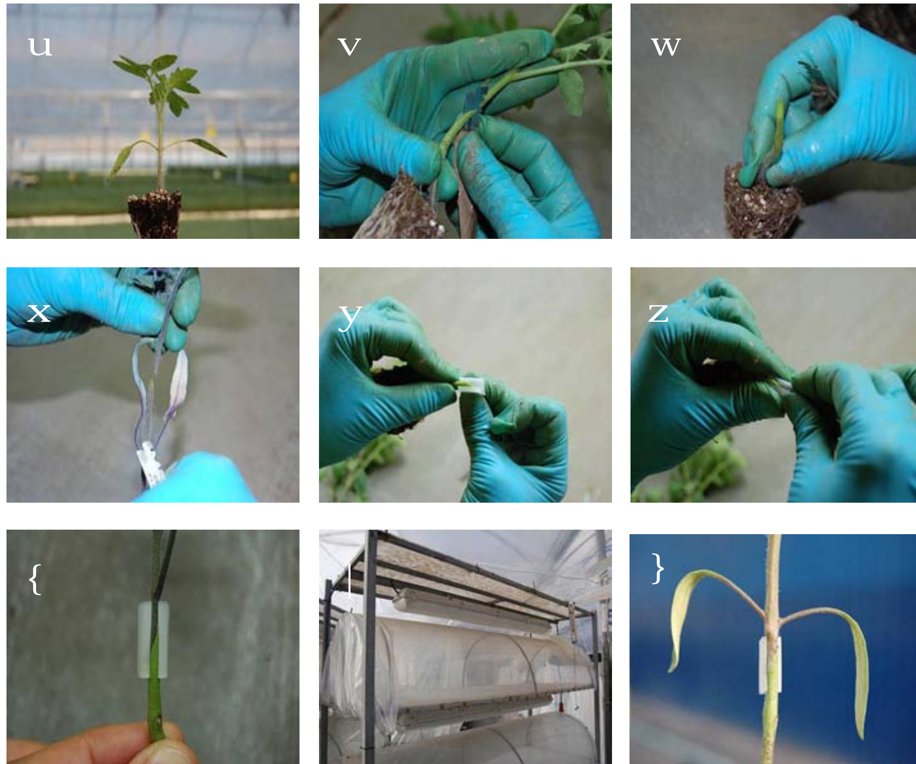
Figure 11. Histological section of a tomato root showing the feeding site established by a second-stage juvenile (J2) of *Meloidogyne incognita.* **(a)** Hypersensitive reaction (HR) of a cell infected by an avirulent isolate. **(b)** Absence of HR in a cell infected by a virulent isolate. **(HR)** Hypersensitive response; **(Ne)** nematode; **(N)** cell nucleus; **(gc)** giant cell (Figure reprinted from Melillo et al., (2006) with permission from Willey-Blackwell).

Tomato rootstocks

Although plant resistance is an economical, environmentally safe and a harmless control method for human health, fruit characteristics of resistant tomato cultivars are not always accepted by consumers. Therefore, grafting is a good alternative for producing commercially appreciated tomato varieties in pathogen infested soils. In agronomy grafting is defined as the union of two related plants using a resistant root system (rootstock) and the aerial part of a cultivar (scion), which allows cultivation of certain species susceptible to pathogens in infested soils (de la Torre, 2005). In horticulture, grafting is frequently used in intensive cropping systems. First reports on grafted vegetable crops are from the seventeenth century in Korea although grafting was first used commercially in the 20th century in Asia (Edelstein, 2004) when wild *Solanum* species were used to introduce new agronomical traits into hybrid cultivars (Osborn et al, 2007). Efforts have been mainly focused on the acquisition of new resistance genes to fungi, virus and nematodes.

As the introgression of new resistance genes from wild *Solanum* species into commercial varieties had several difficulties, the use of tomato rootstocks became essential to fight against some pathogens (Santos et al., 2004). The first pathogen that was controlled using tomato rootstocks was the corky root disease caused by the fungus *Pyrenochaeta lycopersici* (Hogenboom, 1970), followed by root-knot nematode resistance. The *Solanaceous* species most frequently grafted are: tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), pepper (*Capsicum annuum*), cucumber (*Cucumis sativus*), melon (*Cucumis melo*) and water-melon (*Citrullus lanatus*). Grafting of eggplants started in the 1950s, followed by cucumber and tomato around the 1960s and 1970s, respectively. In Spain, tomato is the second horticultural crop in the application of the grafting technique. More than 45 million of tomato plants are grafted each year (de la Torre, 2005), and the trend is towards an increased use in future years. Although commercial rootstocks still need to overcome several obstacles. Nowadays the percentage of grafting abortions is still too high: the incompatibility between the rootstock and the scion results in the formation of a callus in the grafting union that interrupts sap transportation from the roots and leads to plant death (Text Box 5). In addition, this technique requires especial machinery and trained personnel to perform the grafting, that finally increases between 2 to 3 times the prices of the grafted plants.

Text Box 5: Grafting techniques



Grafting involves splicing the fruit-producing **shoot** (called **'scion'**) of a desirable cultivar onto the disease resistant rootstock from another cultivar. The simplest grafting technique consists of the cut with a razor of rootstock and scion plants at the same angle (images 1 to 4), followed by the matching of the scion with the rootstock. A silicon band helps to tight the union between the rootstock and the scion to increase the contact of the vascular tissues of the grafted plant (images 5 and 6) and promotes cicatrization (image 7). Proper handling and acclimatization are important for grafted plants to survive, as plants should be protected from desiccation. Optimum conditions are: temperatures from 24 ˚C to 27 ˚C; 95% to 100% of relative humidity and darkness for 3-5 days while the cut ends heal together (in picture 8, special grafting carts where plants remain under strictly controlled conditions). Finally the union cicatrizes (image 9) and plants can be moved to the greenhouse.

The two rootstocks most widely used for greenhouse tomato production in United States are **'Maxifort' and 'Beaufort'** due to their **increased rate of grafting success** and the **high compatibility** that both rootstocks offer to scions (UCIM, 2009).

Tomato rootstocks are generally hybrid plants between a traditional tomato cultivar (*S. lycopersicum*) and a wild *Solanum* species, usually *S. habrochaites* or *S. chilense*. Tomato rootstocks are useful because they increase crop yield due to their high vigorous root system (Pogonyi et al., 2005) that enhances nutrient absorption of the grafted plants (Leonardi and Giuffrida, 2006). In addition, rootstocks improve tomato yield under termic-stress conditions (Rivero et al., 2003) or in soils with high salinity concentrations (Fernández-García et al., 2002; Estañ et al., 2005), and improves fruit quality compared to traditional tomato cultivars (Fernández-García et al., 2004). The ability of the *Mi*-tomato rootstocks to suppress *Meloidogyne* spp. reproduction is variable and the response to the nematode has been reported as highly resistant (Ioannou, 2001), moderately resistant (Verdejo-Lucas and Sorribas, 2008) or susceptible (Graf et al., 2001; López-Pérez et al., 2006; Tzortzakakis et al., 2006). Such variability in the phenotypic resistant response of tomato rootstocks to root-knot nematodes could be attributed to differences in the genetic background of the hybrid rootstocks, in the aggressiveness of the nematode populations or to differences in inoculum pressure, although no clear conclusions can be drown from the literature.

Durability of *Mi-1* resistance gene

In tomato, the *Mi-1* gene confers resistance to root-knot nematodes but it does not confer immunity to the plant, that is, there is always a small proportion of the nematode population which is able to reproduce successfully on the roots of a resistant plant (Roberts and Thomason, 1989). Certain populations of *Meloidogyne* can increase their reproduction rate on resistant tomatoes when they are repeatedly maintained under the selection pressure of the *Mi-1* gene (Jarquin-Barberena et al., 1991; Sorribas et al., 2005; Castagnone-Sereno et al., 2006). *Meloidogyne* populations are considered virulent when their reproduction on a resistant plant does not differ from reproduction on a susceptible cultivar (Roberts, 1995). The increase of the reproduction rate of a population can be considered as an indicator of its aggressiveness. Little is known about how avirulent parthenogenetic nematodes become virulent after selection pressure of the *Mi-1* gene. A hypothesis proposes that mutational events frequently occur during parthenogenetic mitosis leading to essential changes in the genome of the progeny (Castagnone-Sereno et al., 2006). In this

way, breaking resistant populations appear when virulent individuals within a population are selected displacing the avirulent ones that are unable to reproduce in the presence of the *Mi-1* gene (Roberts, 1995). On the other hand, some populations never become virulent despite they undergo through the same selection pressure (Jarquin-Barberena et al., 1991). Natural virulent populations also occur without previous exposure to the *Mi-1* gene (Ornat et al., 2001; Xu et al., 2001).

The appearance of virulent populations of *Meloidogyne* spp. has been described in commercial fields with a background of cultivation of resistant tomato cultivars, although in any case the possibility of an original virulent population present in the field could be dismissed (Kaloshian et al., 1996; Tzortzakakis and Gowen, 1996; Eddaoudi et al., 1997). Virulent populations of *Meloidogyne* spp. have been selected through continuous cultivation of resistant plants in pot tests, although this phenomenon has been detected in field conditions to a lesser extent. In a study by Sorribas et al. (2005), a resistant cultivar was cropped for three consecutive years in the same experimental plots to determine if repeated cultivation selected for virulent populations; at the end of the study a significant increase in nematode aggressiveness (eggs/ g of root) was observed on the resistant cultivar, although the population was not considered as virulent. Although increased aggressiveness may occur in the field, some theories suggest that virulence rarely occurs because it implies a cost in nematode fitness in the absence of the selection pressure exerted by a resistant host (Roberts, 1995; Block et al., 1997; Castagnone-Sereno et al., 1992, 2006), although no conclusive evidences have been obtained about this subject yet (Gleason et al., 2008). Nevertheless, in addition to genetic resistance, several environmental factors may have an incidence in the selection for virulence, which could explain why the *Mi-1* gene has remained durable along time in commercial fields. The variability in biological systems is expressed through differences in the rates of development and response to environmental conditions. Since these differences occur in the field, the status of the environmental biotic and abiotic factors select in favor of increased reproduction, survival, or fitness of an individual of a particular genetic complement (Ferris and Wilson, 1987). Salinity, temperature, or soil texture, are abiotic factors that influence *Meloidogyne* reproduction and alter its population dynamics in field conditions (Bird and Wallace, 1965; Edongali et al., 1982). Whether the virulence is intrinsic to the population or artificially selected through the continuous use or resistant cultivars, once a population becomes virulent this character remains genetically stable and will be

transmitted to the progeny even in the absence of a resistance host (Bost and Triantaphyllou, 1982; Roberts, 1995).

Virulent populations of *M. incognita* able to overcome the *Mi-1* gene in tomato did not reproduce on resistant pepper and *vice versa*, which suggests a strong gene-for-gene interaction between the crop and nematode population (Castagnone-Sereno et al., 1996). As resistance genes to *Meloidogyne* in pepper (*Me1, Me3*) and tomato (*Mi-1*) belong to the *R* gene family, there is a chance that selection for virulence may be caused by common genetic mechanisms in pepper and tomato rootstocks although this hypothesis has not been tested. Although the (a)virulence spectrum within nematode isolates is very complex, the resistance conferred by the *Mi-1* gene is not too unstable genetically to invalidate its use to control root-knot nematodes (Roberts, 1995). Until the present date, there are no reports on selection for virulence in *Meloidogyne* spp. populations by nematode resistant rootstocks although this phenomenon has been reported for resistant pepper rootstocks (*C. annum*) after cultivation during two consecutive cropping seasons (Lacasa et al., 2002; Ros et al., 2004, 2006).

To determine the suitability of tomato rootstocks as a non-chemical method to control root-knot nematode populations it will be necessary to fully characterize their resistance response to different *Meloidogyne* spp. and establish their effect upon selection for virulence. Preventing the appearance of virulent populations of nematodes is essential to preserve the durability of the *Mi-1* gene and to maintain plant resistance as a feasible alternative to soil fumigation through time.

Additional genes for root-knot nematode resistance and their application in tomato

The original *Solanum* species where *Mi-1.2* was first identified is *S. peruvianum*. Later studies performed in the 1990s demonstrated that there are other *Mi* genes related to root-knot nematode resistance in the genomic profile of this species (Table 4) (Ammati et al., 1986, Yaghoobi et al., 1995; Veremis and Roberts, 1996a, 1996b, 2000; Eddaoudi et al., 1997). These genes segregate independently form *Mi-1* (Cap et al., 1993; Veremis and Roberts, 1996a,

1996b) and are mainly located in chromosomes 6 and 12 of tomato, although few of them have been mapped. All these MiGHs are monogenic-dominant and it has been proved that some induce a similar HR than the *Mi-1* gene when plants are infected by *Meloidogyne* spp. Nevertheless, some MiGHs present interesting characteristics which makes of them of special interest for their ability to inhibit reproduction of virulent nematode isolates or to maintain a phenotypic resistance response when soil temperatures are above 28 ˚C (e.g. *Mi-3* and *Mi-5*). The most recently discovered MiGH is *Mi-9* from *S. arcanum*, which is located in the short arm of chromosome 6 very close to *Mi-1* (Ammiraju et al., 2003; Jablonska et al., 2007).

Table 4. MiGH(s) present in wild *Solanum* species for resistance to root-knot nematodes.

Gene	*Meloidogyne* species	Chromosome	Resistance to high temperatures	Resistance to (a)virulent isolates	Reference
Mi-2	*M. incognita*	Not mapped	Active at 32 ˚C	avirulent	Cap et al., 1993
Mi-3	*M. incognita*	12	Inactive >28 ˚C	virulent	Yaghoobi et al, 1995
Mi-4	*M. arenaria*	Not mapped	Active at 32 ˚C	avirulent	Veremis and Roberts, 1996b
Mi-5	*M. incognita*	12	Active at 32 ˚C	avirulent	Veremis and Roberts, 1996a
Mi-6	*M. incognita*	6	Active at 32 ˚C	avirulent	Veremis and Roberts, 1996b
Mi-7	*M. incognita*	6	Inactive >28 ˚C	virulent	Veremis and Roberts, 1996b
Mi-8	*M. incognita*	6	Inactive >28 ˚C	virulent	Veremis and Roberts, 1996b
Mi-9	*M. arenaria* *M. incognita* *M. javanica*	6	Active at 32 ˚C	avirulent	Ammiraju et al., 2003; Jablosnka et al., 2007

Stacking of MiGHs in tomato could be a useful strategy to prevent nematode reproduction and maintain the durability of the *Mi-1* gene. Nevertheless, the incompatibility between the germplasm of the wild tomato *S. peruvianum* and edible tomato *S. lycopersicum* is still the main obstacle for

the introgression of these genes to obtain new resistant hybrids (Ammiraju et al., 2003; Veremis and Roberts; 2000). A transgene of *Mi-1.2* was successfully expressed in a susceptible tomato cultivar resulting in the acquisition of resistance to root-knot nematodes and potato aphids (Rossi et al., 1998). In order to introduce resistance to root-knot nematodes in susceptible crops like tobacco or eggplant, some interesting work has been performed using "bridge lines", embryo rescue and genetically engineering (Williamson, 1998; Williamson and Hussey, 1996). Pursuit of this goal seems constrained by the fact that some *R* genes do not function properly in other plants different from tomato. Most likely this phenomenon occurs because there is no direct recognition between the *Avr-R* genes, and a pathogen-independent *R* protein is required for the activation in the *Mi*-mediated resistance pathway (Atkinson et al., 2003; Bent and Mackey, 2007) (Text Box 6). Therefore, as *Mi*-stacking in other cultivars different from tomato is not feasible at the moment the only way to preserve the durability of tomato resistance and prevent the appearance of virulent isolates of *Meloidogyne* spp. is an accurate management of the *Mi-1* gene.

Text Box 6: Genetically modified organisms for root-knot nematode control

The **inclusion of the *Mi-1.2* gene** in other crops different from tomato through **transgenesis** has been contemplated by several authors and works have been performed in tobacco and eggplant (Goggin et al., 2006). In tobacco, the introduction of the *Mi-1.2* gene in the genome did not result in any resistance response to *Meloidogyne* spp infection. In eggplant, results were more promising although resistance in this species was less efficient than in tomato. Although tobacco and eggplant are both *Solanaceous* species, **introgression of the *Mi-1.2* gene has not been successfully** achieved. Similar unsuccessful results were obtained in experiments performed with *Arabidopsis thaliana* (Williamson and Kumar, 2006).

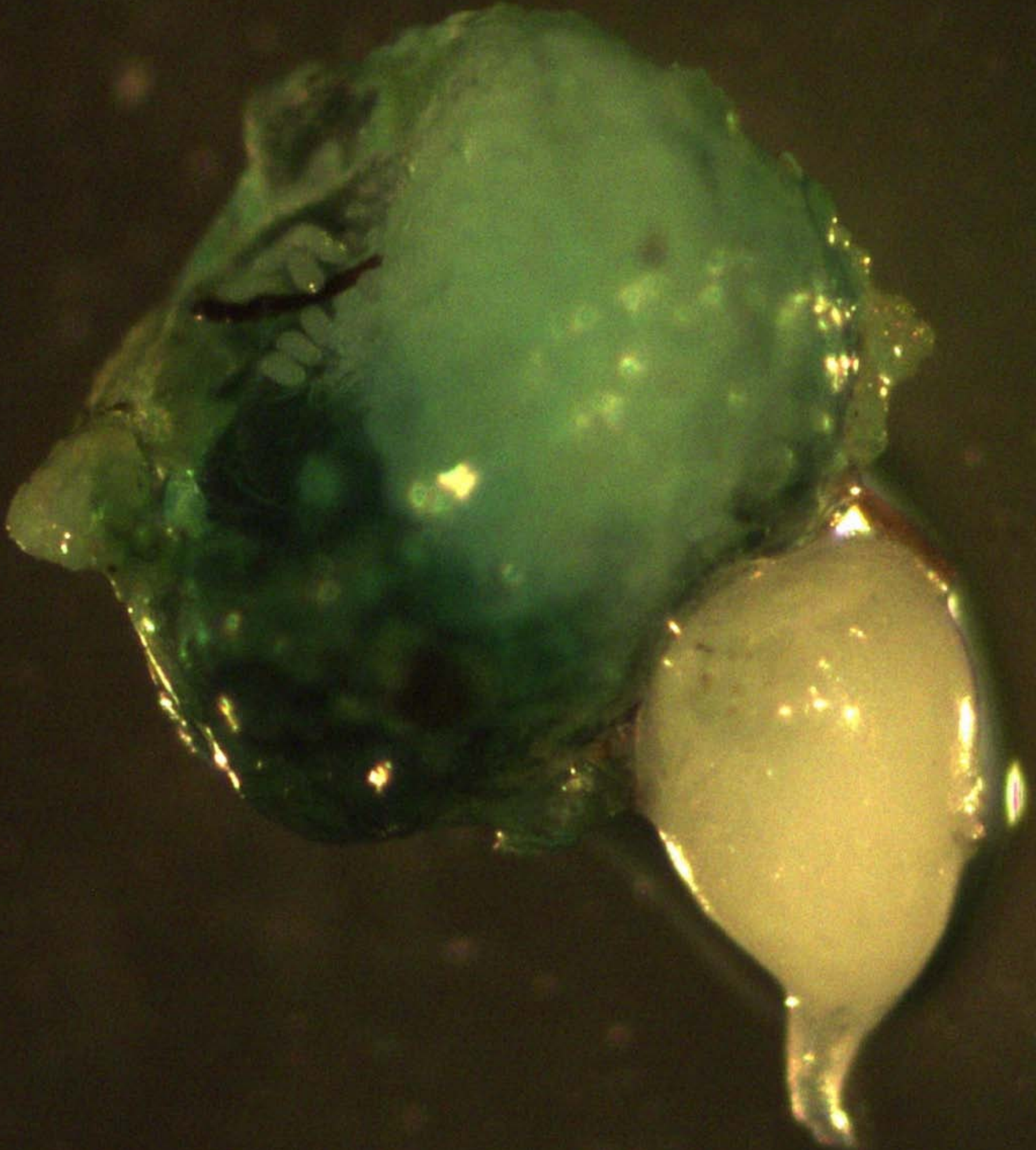
This failure has been mainly attributed to the **absence of auxiliary *Mi*-genes in** the plants that received the transgenic vector, which would be fundamental to obtain the HR of the infected cell. Therefore, the introduction of the *Mi-1.2* gene in other horticultural crops through transgenic techniques has not been successful for the moment. At present, only China is commercializing genetically modified tomatoes with resistance to virus (EUROSTAT, 2008b).

Resistance to nematodes has been achieved with the transgenic insertion of proteinase inhibitors (PIs), which are proteins that play an important

role in the natural plant defense strategies. The use of engineering techniques to control phytopathogenic nematodes is widely reviewed by Atkinson et al. (2003). Pot experiments showed that the transgenic insertion of the same PI in the genome of *Arabidopsis thaliana* has different effectors upon pathogen reproduction depending on the nematode species. Thus, while the PI insertion reduced the number of females that reached to adult maturation for *H. schachtii*, the fecundity of females was also reduced for *M. incognita*. The effectiveness of the PI insertion has been proved in tomato to control *G. pallida* (Urwin et al., 2001), in potato for *M. incognita* and *Nacobbus aberrans;* in rice for *M. incognita*, and in banana for *Rotylenchus reniformis*.

Nowadays, the genetically modified (GM) tomato cultivars are resistant to pests, pathogens and include new characteristics for consumers (e.g. enhanced carotenoids contents). Detrimental effects have been neither observed for GM tomatoes expressing the Crystal Protein Cry1Ab from *Bathillus thuringiensis* nor for GM tomatoes expressing the Coat Protein (CP) from the cucumber mosaic virus (CMV) in mice (Domingo, 2007). Nevertheless, the effects that GM foods have upon the environment and the human health (e.g. toxicity, allergenicity) are still largely unknown. Some reviews on the toxicologic effects related to the consumption of GMO by different animal species, indicates that some of them have a submicroscopic effect in vivo (e.g. acceleration of cellular metabolic rates and transmembrane traffic, increase of lactic dehydrogenase 1 synthesis), that remains unnoticed when only macroscopic variables are observed (Domingo, 2007; Magaña-Gómez and Calderón de la Barca, 2009). So far while GMO still need to overcome multiple obstacles to become accepted tool to control pests and pathogens, plant resistance remains the best strategy to inhibit reproduction of root-knot nematodes of the genus *Meloidogyne* as it is an effective, safe and environmentally friendly control method.

Adult female of *Meloidogyne javanica* obtained from an infected root of a susceptible tomato cultivar. Note the gelatinous egg mass attached at the posterior end of the body. The blue color of the egg mass was obtained after root staining with an erioglaucine solution



Objectives

The general objective of the thesis was to evaluate and characterize the *Mi*-mediated resistance response in tomato rootstocks as a management strategy to control nematodes of the genus *Meloidogyne* spp. The effectiveness of tomato rootstocks to suppress nematode reproduction was compared with traditional tomato cultivars. Secondly, the resistance provided by the *Mi*-gene in tomato rootstocks was assessed to determine if rootstocks are a durable strategy to control root-knot nematodes in field conditions. For this purpose, the pathogenic and agronomic factors that influence the *Mi-1* phenotypic response were examined. Characterization and the evaluation of the resistance response conferred by the *Mi-1* in tomato rootstocks have been tackled to determine:

1.- The resistance response of tomato rootstocks and the influence of the cropping season on the phenotypic expression of the *Mi-1* mediated resistance.

To determine the ability of *Mi-1* tomato rootstocks to control root-knot nematode populations, the phenotypic resistant response of a pool of resistant rootstocks was determined under different experimental conditions. In a first phase, bioassays were performed in a glasshouse to characterize the response of ten *Mi-1* tomato rootstocks to one avirulent population of *M. javanica* when soil temperatures were below 28 ˚C (spring crops), and when temperatures frequently exceed the phenotypic expression threshold of the *Mi-1* gene (summer crops) (Chapter 1). Experiments were performed in a plastic house to validate the resistance response of the rootstocks in real agronomic conditions by subjecting them to a continuous inoculum pressure exerted by an avirulent *M. javanica* population infesting the soil of the plastic house (Chapter 1).

2.- The variability of the *Mi-1* mediated resistance related to the infective root-knot nematode population.

The variability of the resistance response of *Mi-1* tomato cultivars related to the root-knot nematode population had been reported, although no information was available for *Mi-1* tomato rootstocks. Therefore, in a second phase, the specific

plant-nematode interaction between the *Mi-1* resistant tomato rootstocks and seven populations of either *M. arenaria*, *M. incognita* or *M. javanica* species was characterized in bioassays conducted in a glasshouse (Chapter 2).

3.-The capacity of the existing molecular markers for the characterization of *Mi-1* locus and detection of the *Mi-1.2* gene in tomato hybrid rootstocks.

Molecular markers are an important tool for selection of resistant hybrids in breeding programs. The REX-1 marker was specifically designed to detect the *Mi-1* locus in *S. lycopersicum* × *S. peruvianum* hybrids and has been commonly used to trace *Mi-1* resistance in tomato cultivars. The introduction of new *Solanum* species in the genetic background of tomato hybrid rootstocks has prevented the utility of the REX-1 marker anymore. To determine the effects of the allelic condition of the *Mi-1* locus in the resistance response of tomato rootstocks, a molecular characterization of this locus was done using markers for interspecific tomato hybrids; detection of the *Mi-1.2* gene in resistant tomato rootstocks was done using molecular markers existing in literature and developing new markers (Chapter 3).

4.-The suitability of tomato rootstocks as a long term strategy to manage root-knot nematodes of the genus *Meloidogyne* spp.

Resistant tomato cultivars have proved to be a useful alternative to control root-knot nematodes, although their repeated cultivation in nematode-infested soil has lead to the appearance of virulent populations of root-knot nematodes, both in experimental and field conditions. This situation had not been reported for *Mi-1* tomato rootstocks. Thus, the durability conferred by the *Mi-1* resistance gene in tomato rootstocks was studied in the long-term under field conditions to determine whether repeated cultivation of resistance of tomato rootstocks selects virulent nematode populations or not. Experiments were performed in a plastic house naturally infected with an avirulent population of *M. javanica* along three consecutive cropping cycles (Chapter 4).

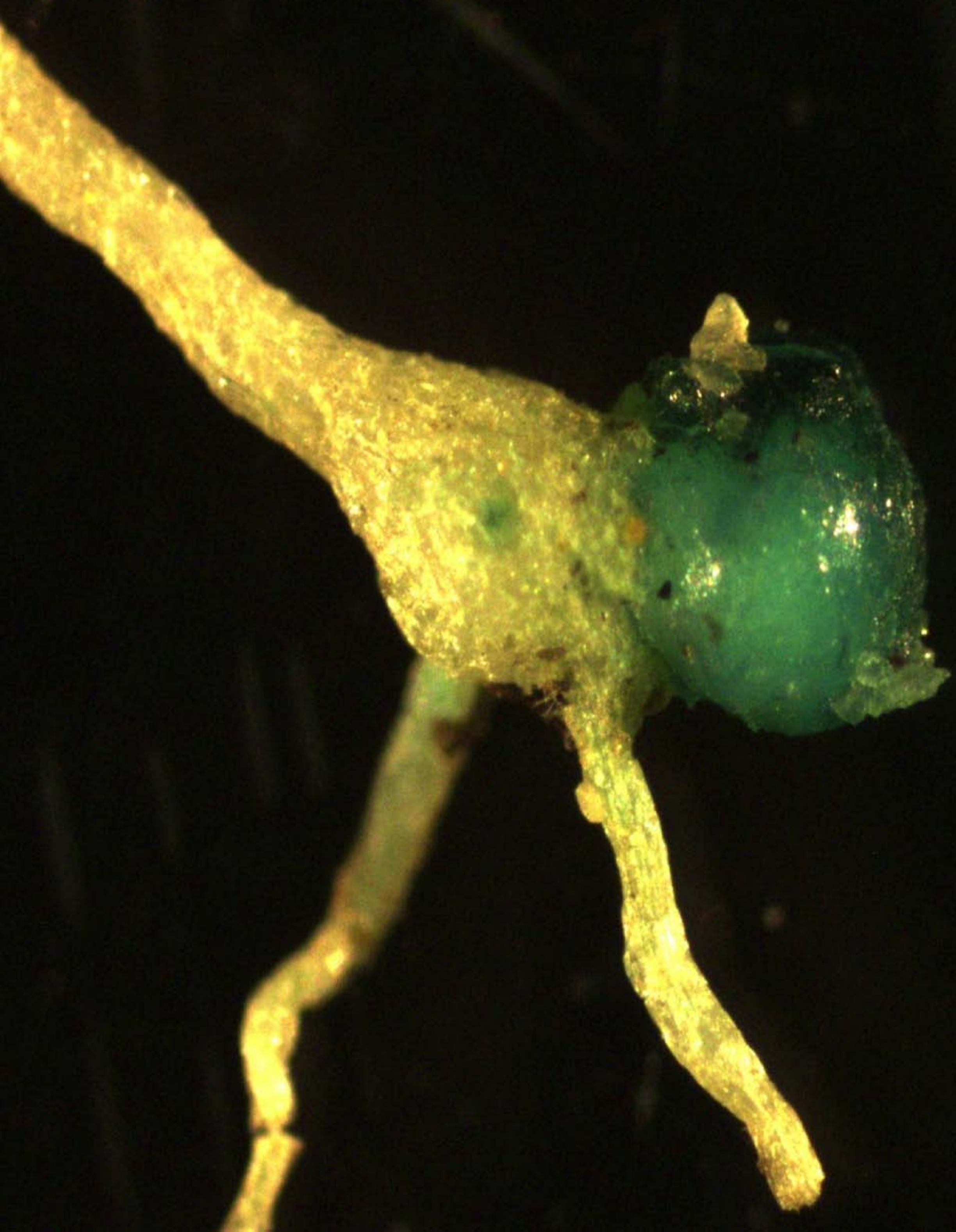
5.-The molecular characterization of selected virulent populations of *M. javanica* after repeated cultivation of *Mi-1* tomato rootstocks

Root-knot nematode virulence has not been precisely characterized at the molecular level. The selection of virulent populations of *Meloidogyne* spp. after repeated cultivation of *Mi-1* tomato rootstocks was confirmed by pathogenicity assays. The MVC marker was used to characterize at the genetic level the

virulent populations of *M. javanica* in selected the previous study and to establish a correlation between the virulence observed in field and this genetic marker (Chapter 5).

6.- The presence of new sources of resistance to root-knot nematodes in wild *Solanum* species.

The use of new *Solanum* species as a source for novel resistance genes to root-knot nematodes is becoming a rising trend. Therefore a screening for new resistant *Mi*-homologs in the genome of wild *Solanum* species was initiated. One accession of a the recently described species *Solanum huaylasense* was challenged to three avirulent populations of either *M. arenaria*, *M. incognita* or *M. javanica* species and to one naturally virulent populations of *M. javanica* to determine its resistance response to the nematode (Chapter 6).



A gelatinous egg mass observed at the external surface of a galled root. The adult female remains protected inside the gall of the root.

1 Variability in infection and reproduction of *Meloidogyne javanica* on tomato rootstocks with the *Mi* resistance gene

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The response of 10 commercial or experimental tomato rootstocks with the *Mi* resistance gene to an initial inoculum of a *Mi*-avirulent population of *Meloidogyne javanica* was determined in pot tests conducted in spring and summer. In a field test, the rootstocks were subjected to continuous exposure to high initial population densities (2,050 ± 900 second-stage juveniles (J2) per 250 cm^3 soil of the nematode. The presence of the *Mi* locus in the resistant rootstocks and cultivars was confirmed using the PCR co-dominant markers REX-1 and Mi23. Nematode infectivity (egg masses) and reproduction (eggs g^{-1} root) were highly variable in the spring tests. Rootstocks PG-76, Gladiator and MKT-410 consistently responded as highly resistant, with nematode multiplication rate (Pf/Pi) < 1 and reproduction index (RI) < 10%, and they were as efficient as standard resistant tomato cultivars at nematode suppression. The relative resistance levels of rootstocks Brigeor, 42851, 43965, Big Power and He-Man varied depending on the susceptible standard used for reference or the duration of the test. Rootstocks Beaufort and Maxifort were susceptible to *M. javanica* (Pf/Pi > 50 and RI > 50%). Rootstocks PG-76 and He-Man, and the resistant tomato cv. Caramba showed high levels of resistance in the test conducted in summer, whereas MKT-410 and 42851 and the resistant tomato cv. Monika were moderately resistant. In the field, seven rootstocks showed high levels of resistance and one (He-Man) showed an intermediate level, whereas Beaufort and Maxifort were susceptible.

Key words: host-plant resistance, Mi23 marker, REX-1 marker, root-knot nematodes, susceptibility, tomato rootstock

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Introduction

The tomato (*Solanum lycopersicum*) *Mi* gene confers resistance to three major root-knot nematode species, *Meloidogyne incognita*, *M. javanica*, and *M. arenaria* (Roberts and Thomason, 1989). In 1940's, *Mi* was identified in the wild relative of tomato *Solanum peruvianum* and later was introgressed into cultivated tomato (Smith 1944). The resistance mediated by *Mi* is heat sensitive. At soil temperatures above 28°C *Mi* tomatoes display susceptible or reduced resistance phenotypes (Dropkin, 1969). The *Mi* gene was mapped to the short arm of tomato chromosome 6 and several molecular markers in this region were developed (Kaloshian et al., 1998; Seah et al., 2007a; Seah et al., 2007b). In the *Mi* locus, three genes, *Mi-1.1*, *Mi-1.2* and *Mi-1.3*, with nucleotide-binding site and leucine rich-repeat motifs were identified. One of these genes, *Mi-1.2*, conferred resistance to root-knot nematodes (Milligan et al., 1998). We refer to this gene as *Mi*.

Tomatoes carrying the *Mi* gene are effective for controlling *Meloidogyne* spp. as they suppress nematode development and/or reproduction and can be cultivated in nematode-infested soils without significant yield reduction (Philis and Vakis, 1977; Rich and Olson, 1999; Sorribas et al., 2005). In addition, the *Mi* resistance gene can have a carry over effect on the subsequent crop (Hanna et al., 1993; Ornat et al., 1997). Despite these characteristics, the use of resistant tomatoes is not widespread as a management strategy. Reasons for this include undesirable horticultural traits and the ever increasing demand of growers for high yielding and /or specific fruit qualities.

One way to overcome losses caused by plant-parasitic nematodes in desirable susceptible genotypes would be grafting these varieties onto rootstocks with the *Mi* resistance gene. At present, cultivation of grafted vegetables is expanding in Europe, and has been adopted as a non-chemical alternative to methyl bromide in several countries (MBTOC report, 2006). In Spain, grafting tomatoes started over a decade ago to reduce incidence of the corky root disease (*Pyrenochaeta lycopersici*), a limiting factor for growing tomatoes at the time due to the lack of varieties with effective resistance to the fungus (Santos et al., 2004). Grafting offers several advantages as growth promotion, yield increases or low temperature tolerance when compared to non-grafted plants (Ioannou, 2001; Miguel, 2002; Lee, 2003). Tomato rootstocks are also suitable for cultivation in pathogen-infested soils as they incorporate different resistant genes including the *Mi* gene (Marín Rodríguez, 2005). Nevertheless, information on their suppressive effect on *Meloidogyne* spp. is limited to few reports that indicate great differences in terms of nematode infectivity and/or

reproduction among the rootstocks. For instance, the resistant rootstock cv. Brigeor reduced root galling (Miguel, 2002) and nematode reproduction (Graf et al., 2001) but its effectiveness depended on temperature (Ioannou, 2001). The resistant rootstock cv. SC 6301 reduced root galling (Miguel, 2002) but showed moderate levels of nematode reproduction (Verdejo-Lucas and Sorribas, 2008). In contrast, the resistant rootstock cv. Beaufort supported high population densities of *M. incognita* in glasshouse tests (López-Pérez et al., 2006). These findings suggest variability in the response of the rootstocks to the nematode species could be caused by the genetic background of the rootstocks or to changes in the resistant response caused by environmental factors.

This paper reports the response of 10 tomato rootstocks to an initial inoculum density of a population of *M. javanica* in glasshouse tests conducted in spring and summer. The response of these rootstocks exposed to high and continuous population densities of the nematode in a plastic house field infested with *M. javanica* is also reported.

Materials and Methods

Rootstock response to an initial *M. javanica* inoculum in spring

Ten tomato rootstocks with the *Mi* resistance gene were exposed to an initial inoculum density of a *Mi* avirulent population of *M. javanica* (code Mj-05) (Ornat et al., 2001) in a glasshouse. Two experiments were conducted. Experiment one was run from 6 March to 13 July 2006, allowing the nematode to complete two generations (130 days post-inoculation). Each rootstock was replicated eight times and plants were arranged at random in a complete block design on a glasshouse bench. The main characteristics of the rootstocks are described in Table 1. The commercial tomato cultivars cvs. Monika and Caramba (resistant) and Durinta and Tyrmes (susceptible) were included as standards for reference. Seedlings were transplanted singly into 1.5-L pots containing steam-sterilized river sand and were allowed to grow for one week before they were inoculated with the nematode. Nematode inoculum was obtained from infected tomato (cv. Roma) roots collected from pot cultures maintained in a glasshouse. Roots were macerated in a 0.5% NaOCl solution in a food blender at ca 1,000 rpm for 5 min (Hussey and Barker, 1973). The eggs suspension was passed through a 74-μm-aperture sieve to remove root debris

and the dispersed eggs collected on a 25-μm-sieve were used as inoculum. Plants were inoculated with approximately 6000 eggs per plant by adding aliquots (4-5 mL) of the egg suspension into two holes made in the soil 3 cm apart from the base of the plant. Plants were watered as needed and fertilized with a slow-release fertilizer (15% N +10% P_2O_5 +12% K_2O + 2% MgO_2 + microelements). At harvest, tops were cut at ground level and the root systems washed free of soil and weighed. Egg masses were stained for ease for counting by immersion of the entire root system into a 0.1-g L^{-1} erioglaucine solution (Aldrich Chemical Company) (Omwega et al., 1988) for 2h. The number of eggs per plant was determined by extracting the eggs from two 10-g root subsamples in a 0.5% NaCOl solution for 10 min. Eggs were expressed per gramme fresh root. The multiplication rate of the nematode (Pf/Pi) was calculated as the eggs per plant (Pf) divided by the initial inoculum density (Pi). The index of infectivity (II) and reproduction (RI) were used to assess the level of resistance of the rootstocks. The II was calculated as the number of egg masses on the resistant rootstock or cultivar divided by egg masses on susceptible cultivar × 100, and the RI as the Pf on the resistant rootstock or cultivar divided by the Pf on susceptible cultivar × 100. A rootstock was considered as resistant when the II and the RI differed statistically from the susceptible standards. Once a rootstock differed from a susceptible standard and was considered resistant it was classified as highly resistant or intermediate resistant according to their RI (Hadisoeganda and Sasser, 1982).

Experiment two was run from 9 March to 18 July 2006 (132 days post-inoculation). The same rootstocks were exposed to an initial inoculum density of 6,000 eggs of *M. javanica* per plant. Each rootstock was replicated 16 times, and eight plants were harvested after completion of one nematode generation (63 days post-inoculation), and the other eight plants after two nematode generations (132 days post-inoculation). Plants were arranged at random according to a complete randomized block design. The remaining experimental conditions, plant maintenance and assessments of nematode infectivity and reproduction were the same as for experiment one.

Rootstock response to an initial *M. javanica* inoculum in summer

To determine if the rootstocks retained their relative resistance levels when cultivated in the hottest season of the year, a pot test was conducted from 19 July to 5 September 2006 in a glasshouse.

Table 1. Main characteristics and resistances of the tomato rootstocks and cultivars used to determine variability in infection and reproduction of a Mi avirulent population of Meloidogyne javanica.

Variety	REX-1 profile	Mi23 profile	Parental species	Availability	Seed company	Resistances
Rootstock						
PG76	Mi/Mi [a]	Mi/Mi[a]	S. lycopersicum × Solanum sp[b]	Experimental	Gautier Seeds	HR: TMV/ Fol:2 /For / Va/ Vd/ Pl/ Ma/ Mi/ Mj [c]
Gladiator	Mi/Mi	Mi/Mi	S. lycopersicum × Solanum sp	Commercial	Rijk Zwaan	HR: ToMV/ Fol 0,1/ For/ PI/ Va IR: Mi
MKT-410	Mi/mi	Mi/mi	S. lycopersicum × Solanum sp	Experimental	Agriset Seeds	Not available
Brigeor	Mi/Mi	Mi/Mi	S. lycopersicum × S. habrochaites	Commercial	Gautier Seeds	HR: TMV/ Fol:2/ For/ V/ Ma/ Mi/ Mj
42851	Mi/Mi	Mi/mi	S. lycopersicum ×S. habrochaites	Experimental	Syngenta Seeds	Not available
43965	Mi/Mi	Mi/Mi	S. lycopersicum × S. habrochaites	Experimental	Syngenta Seeds	Not available
BigPower	Mi/Mi	Mi/Mi	S. lycopersicum × S. habrochaites	Commercial	Rijk Zwaan	HR: ToMV /Fol 0-1, For/ Pl; IR: Mi
He-man	Mi/Mi	Mi/Mi	S. lycopersicum × S. habrochaites	Commercial	Syngenta Seeds	HR: ToMV/ Va/ Vd/ Fol: 1, 2/ For/ Ff/ Sbl; IR: Ma/ Mi/ Mj
Beaufort	Mi/Mi	Mi/Mi	S. lycopersicum × S. habrochaites	Commercial	De Ruiter Seeds	HR: ToMV/ Fol:0,1/ For/ Pl/ Va/ Vd/ Ma/Mi/ Mj
Maxifort	Mi/Mi	Mi/Mi	S. lycopersicum × S. habrochaites	Commercial	De Ruiter Seeds	HR:ToMV/ Fol:0,1/ For/ Pl/ Va/ Vd/ Ma/ Mi/ Mj
Cultivar						
Caramba	Mi/mi	Mi/mi	S. lycopersicum × S. peruvianum	Commercial	De Ruiter Seeds	HR: ToMV/ Ff:1-5/Fol:0,1/ Va/ Vd/ Ma/ Mi/ Mj
Monika	Mi/mi	Mi/mi	S. lycopersicum × S. peruvianum	Commercial	Syngenta Seeds	HR: ToMV: 0-2/ Fol:1/ Va/ VdIR: Mi/ Ma/ Mj
Tyrmes	mi/mi	mi/mi	S. lycopersicum × Solanum sp	Commercial	Syngenta Seeds	HR: ToMV: 0-2/ Fol:1,2/ Va/ Vd; T: TYLCV
Durinta	mi/mi	mi/mi	S. lycopersicum × S. lycopersicum	Commercial	Western Seeds	HR: ToMV/ Fol:1-2/ Va/ Vd

[a] Mi/Mi (homozygous resistant); Mi/mi (heterozygous resistant); mi/mi (homozygous susceptible).[b] S. lycopersicum × Solanum sp: unknown parental species. [c] Information from the seed companies' descriptions. **HR**: High resistance; **IR**: Intermediate resistance; **T**: Tolerance. **TMV**: Tobacco Mosaic Virus; **ToMV**: Tomato Mosaic Virus; **TYLCV**: Tomato yellow leaf curl virus; **Ff: 1-5**: Fulvia fulva races 1, 2, 3, 4, 5; **Fol: 0-2**: Fusarium oxysporum f. sp. lycopersici races 0, 1, and 2; **For**: Fusarium oxysporum f. sp. radicis-lycopersici; **Pl**: Pyrenochaeta lycopersici; **Sbl**: Stemphylium botryosum f. sp. lycopersici, **Va**: Verticillium albo-atrum; **Vd**: Verticullium dahliae; **Ss**: Stemphylium solana; **Si:** Silvering; **Cmm**: Clavibacter pv. michiganensis; **Pst**: Pseudomonas syringae pv tomato; **Mi, Ma, Mj**: Meloidogyne incognita, M. arenaria, M. javanica.

The rootstocks were inoculated with 6,000 eggs of *M. javanica* per plant, and they were harvested after completion of one nematode generation (48 days post-inoculation).Each rootstock was replicated eight times according to a complete randomized block design. Experimental conditions, assessments of nematode infectivity and reproduction were similar to those described for the spring experiments.

Soil temperatures were registered daily at 30 min intervals by placing soil probes into the potted soil. The mean daily soil temperature was calculated as the maximum plus the minimum temperatures divided by two. The number of degree-days accumulated by *M. javanica* was calculated using a base temperature of 13 ˚C and 343 ˚C as the minimum thermal time requirement for one generation (Trudgill, 1995; Tzortzakakis and Trudgill, 1996).

Rootstock response to continuous exposure to high population densities of *M. javanica* in the field

The study was conducted in an unheated plastic house with a history of root-knot nematode problems caused by *M. javanica* (Sorribas et al., 2005). The plastic house was located at Cabrils, Barcelona, Spain, and the soil was a sandy loam with 85.8% sand, 8.1% silt and 6.1% clay, pH 8.1, 0.9% organic matter w/w and 0.40 dS m^{-1} electric conductivity. To determine nematode-infestation levels, composite soil samples were collected from each plot before starting the study. Individual samples consisted of five soil cores taken to 30 cm deep with a sampling tube (2.5 cm diameter). Samples were mixed thoroughly and nematodes extracted from a 500-cm^3 soil subsample using Baermann trays. Second-stage juveniles (J2) that migrated to the water were collected one week later, concentrated on a 25-μm-pore sieve, counted and expressed as J2 per 250 cm^{-3} of soil. The average infestation levels were 2,050 ± 900 J2 per 250 cm^{-3} of soil.

One-month-old seedlings of each tomato rootstocks (Table 1) were transplanted in the field on 15 March and allowed to grow until 26 July 2007. Resistant tomato cvs Monika and Caramba and susceptible tomato cv. Durinta were included as standards for reference. Plots of 12.30 m^2 consisting of four rows with six plants per row placed 50 cm and 55 cm within and between rows. A total of 24 plants per plot were transplanted; of these, 12 plants corresponded to the tested materials and the remaining ones to cv. Durinta. The resistant

tomato cultivars and rootstocks were arranged at random within each plot. The resistant and susceptible plants were transplanted alternating in a way that each resistant tomato was next to two susceptible ones placed either in the same or opposite row. The infested soil of the plastic house served as the initial source of high nematode inoculum. Continuous inoculum pressure exerted on the resistant plants was provided by the susceptible Durinta neighbour plant (Esmenjaud et al., 1992, 1996) once the nematode had completed one generation on this cultivar (approximately, 6 weeks after transplanting). To determine rootstock tolerance to nematode damage, the root gall index of tomato plants was assessed at the end of the experiment. Plants were dug from the soil, examined and rated on a scale of 0 to 10, where 0 = a complete and healthy root system (no galls observed) and 10 = plants and roots dead (Zeck, 1971). To determine nematode reproduction, roots from each resistant plant were chopped individually in 1-cm-long segments and two 10-g sub-samples used to extract eggs by blender maceration in a 0.05% NaOCl solution for 10 min. Roots from 12 susceptible Durinta plants per plot were combined, chopped, and two 10-g sub-samples processed. The RI of the nematode was calculated as eggs g^{-1} root on the resistant plant /eggs g^{-1} root on the susceptible cv. Durinta × 100.

Detection of the *Mi* resistance gene presence

The presence of the *Mi* resistance gene in the rootstocks was determined using the PCR-based co-dominant markers REX-1, commonly used to detect the presence of the *Mi* gene, and the SCAR Mi23 specifically designed to detect root-knot nematode resistance in tomato breeding programs (Seah et al., 2007b). Both genetic markers are located on the short arm of chromosome 6 in the vicinity of the *Mi* resistance gene (Williamson et al., 1994; Seah et al, 2007a). REX-1 flanks the *Mi* gene at the telomeric proximal end of the short arm of chromosome 6. The chosen marker Mi23 flanks *Mi* at the centromeric proximal end of the short arm of chromosome 6. This co-dominant SCAR marker allows the amplification of a 380 bp genomic fragment from the *Mi* region in *S. peruvianum* (*Sp*-Mi23-locus), and a fragment of 480 bp from the *mi* region in tomato (*Sl*-Mi23-locus). The resistant and susceptible cultivars were used as controls. DNA was extracted from two leaflets in a plastic bag with 1 mL extraction buffer (0.35 M Sorbitol, 0.1 M Tris pH 7.5, 0.005 M EDTA and 0.02 M $NaHSO_3$ added just before use). After sealing the bag, tissue was macerated by rolling over a 25-mL-pipette until the leaflets were cleared. The

liquid containing the plant cells was spun at 13,800 g at 4 ˚C and the pellet was resuspended in 100 μL extraction buffer, 100 μl lysis buffer (0.2 M Tris pH 7.5, 0.05 M EDTA, 2.0M NaCl, 2 % v/v CTAB) and 40 μL 5% Sarkosyl. The lysed cells were extracted with chloroform and precipitated with equal volume of isopropanol. The pellet was washed with 70% ethanol and DNA resuspended in 30 μL TE (10 mM Tris pH 7.5, 1 mM EDTA).

REX-1 primers were: REX-F1 (5'-TCGGAGCCTTGGTCTGAATT-3') and REX-R2 (5'-GCCAGAGATGATTCGTGAGA-3'). The Mi23 primers were: Mi23F (5'-TGG AAA AAT GTT GAA TTT CTTTTG-3') and Mi23R (5'-GCA TAC TAT ATG GCT TGT TTA CCC-3'). PCR reaction was carried out in 25-μL volumes using 50-100 ng DNA template. A Similar PCR mix [buffer (10 mM Tris pH 9.0, 50 mM KCl, 0.1% Triton X-100); 0.4-1.0 μM of each primer; 0.25-0.4 mM of each dNTP and 0.5 unit Taq polymerase] was used for both sets of primers except 2.5 mM $MgCl_2$ was used for REX-1 and 1.75 mM $MgCl_2$ for Mi23. The amplification conditions were: 94 ˚C for 3 min followed by 35 cycles of 30 s at 94 ˚C, 1 min at 57 ˚C and 1 min at 72 ˚C, followed by 10 min at 72 ˚C. REX-1 amplified products were digested with *Taqa* I (Biolab) restriction enzyme according to Williamson et al. (1994). For both REX-1 and Mi23 markers, 20 μL of the PCR products were resolved on 1.5% agarose gel in 1× TAE buffer.

Statistical analyses

The General Linear Model procedure of SAS version 8 (SAS Institute Inc., Cary, NC) was used for statistical analyses. Data on egg masses per plant, eggs g^{-1} root, and Pf/Pi were transformed to $\log_{10}(x+1)$ before analysis. To establish the host status of the rootstocks, a Tukey's studentized range test ($P < 0.05$) was used to separate means when the statistical analysis was significant ($P < 0.05$). A multiple comparison procedure that compares all treatments with a single control was used to identify rootstocks expressing resistance to *M. javanica*. Host suitability of individual rootstocks or cultivars was compared with that of the susceptible standard cv. Durinta by Dunnett the *t*-test (Dunnett, 1955). Data on infectivity and reproduction indexes of *M. javanica* in the pot tests were transformed to $\log_{10}(x+0.001)$ before analysis. Because results after one or two nematode generations did not differ, data were pooled and the new set of data analyzed. Data on the RI of the nematode in the field was transformed to

$\log_{10}(x)$ before analysis. A correlation analysis was done to determine the relationship between the index of infectivity and reproduction.

Results

Rootstock response to an initial *M. javanica* inoculum in spring

Mean daily soil temperatures in the pots from 3 March to 18 July 2006 were below 28 ˚C. The minimum soil temperature was 13.5 ˚C and the maximum 26 ˚C in experiment one (x = 20.2 ˚C), and 13.7 ˚C and 26.4 ˚C, respectively, in experiment two (x = 20.7 ˚C).

As expected, number of egg masses and eggs of *M. javanica* on the resistant cultivars were lower (P <0.05) than on the susceptible ones after one (Tables 2) or two nematode generations (Table 3). The Pf/Pi values were < 1 on both resistant cultivars after one generation (Table 2) but slightly > 1 after two generations (Table 3). More (P <0.05) egg masses were produced on susceptible cv. Durinta than Tyrmes (Tables 2 and 3). Egg production was similar between these two cultivars, although approximately three times more eggs were recorded on Durinta than Tyrmes, irrespective of the duration of the tests (Tables 2 and 3). The response of the rootstocks to nematode infection and reproduction was highly variable after one or two generations. As a result, variable numbers of plants became infected after one generation but only one out of eight inoculated plants did of rootstocks PG-76, Gladiator and MKT-410 (Table 2). Eggs g^{-1} root on rootstocks cvs. PG-76, Gladiator, MKT-410, and Brigeor did not differ from both resistant standards. On rootstocks 42851, 43965, and Big Power, the number of eggs masses and eggs g^{-1} root did not differ from the resistant cultivars. Eggs g^{-1} root on rootstocks He-man, Beaufort, and Maxifort were similar to both susceptible standards (Table 2). The Pf/Pi values ranged from 0.02 to 3.8 times the Pi on the rootstocks and population increases (Pf/Pi >1) were only recorded on Beaufort and Maxifort.

After two generations, six plants of rootstock PG-76, nine of Gladiator and six of MKT-410 out of 16 inoculated plants each became infected (Table 3). The number of egg masses on these rootstocks was lower (P <0.05) than on the resistant cultivars. A similar number of egg masses was recorded on rootstocks 42851, 43965, and Brigeor than on the resistant cultivars. Eggs masses on rootstocks Big Power and He-man did not differ from those on susceptible

Tyrmes. In contrast, numbers of egg masses on Beaufort and Maxifort and susceptible Durinta were similar (Table 3). Regarding nematode reproduction, eggs g^{-1} root on rootstocks PG-76 and MKT-410 were lower ($P < 0.05$) than on both resistant cultivars (Table 3) whereas eggs g^{-1} root on rootstocks Brigeor, 42851, 43965 and Big Power were similar to both resistant cultivars. Rootstock He-man and susceptible Tyrmes showed similar eggs g^{-1} root. Eggs g^{-1} root on rootstocks Beaufort and Maxifort did not differ from both susceptible cultivars. Pf/Pi values ranged from 0.1 to 70 times the Pi on the rootstocks and population increases (Pf/Pi > 1) were recorded on six out of 10 rootstocks; the highest values on Beaufort and Maxifort (Table 3).

Table 2. Numbers of eggs masses per plant, eggs g^{-1} of root, multiplication rate (Pf/Pi), and infectivity and reproduction indexes of a *Mi* avirulent population of *Meloidogyne javanica* on tomato rootstocks with the *Mi* resistance gene after one generation of the nematode in a pot test conducted in a glasshouse in spring.

Plant Material	Infected plants	Egg masses /plant	Eggs g^{-1} root	Pf / Pi[a]	Infectivity index (%)[b]		Reproduction index (%)[c]	
					Durinta	Tyrmes	Durinta	Tyrmes
Rootstock								
PG-76	0	0 ± 0 g*	3 ± 5 f*	0.02 ± 0.03 c*	0 ± 0 e	0 ± 0 e	0.7 ± 1 e	2 ± 4 ef
Gladiator	1	4 ± 1 fg*	12 ± 11 ef*	0.05 ± 0.05 c*	0.2 ± 1 e	1 ± 3 e	2 ± 2 e	7 ± 7 def
MKT-410	1	0.2 ± 0.7 fg*	5 ± 10 f*	0.02 ± 0.04 c*	0.1 ± 0.4 e	1 ± 2 e	1 ± 1 e	2 ± 5 f
Brigeor	6	5 ± 5 def*	16 ± 20 ef*	0.1 ± 0.1 c*	3 ± 3 bcd	16 ± 15 bcd	3 ± 4 de	10 ± 13 def
42851	7	3 ± 2 defg*	22 ± 16 de*	0.1 ± 0.1 c*	2 ± 1 bcd	10 ± 7 bcd	4 ± 3 de	13 ± 10 de
43965	5	3 ± 3 efg*	32 ± 22 cde*	0.1 ± 0.1 c*	1 ± 2 cde	8 ± 9 cde	6 ± 4 d	19 ± 13 cd
Big Power	3	2 ± 4 efg*	40 ± 49 de*	0.3 ± 0.4 c*	1 ± 2 de	7 ± 11 de	12 ± 16 cd	38 ± 50 cd
He-man	7	21 ± 22 cd*	117 ± 81 bcd	0.7 ± 0.5 bc*	11 ± 12 abc	65 ± 70 abc	29 ± 22 bc	93 ± 69 bc
Beaufort	8	92 ± 76 ab	674 ± 445 ab	3.5 ± 2.6 a	49 ± 40 a	285 ± 236 ab	141 ± 106 a	445 ± 335 ab
Maxifort	8	90 ± 65 ab	671 ± 311 a	3.8 ± 2.4 a	48 ± 35 a	280 ± 203 ab	154 ± 97 a	485 ± 306 a
Cultivar								
Caramba	7	5 ± 3 de*	13 ± 11 ef*	0.1 ± 0.1 c*	3 ± 2 abcd	16 ± 10 abcd	3 ± 3 de	10 ± 9 def
Monika	3	1 ± 2 efg*	10 ± 7 ef*	0.1 ± 0.04 c*	1 ± 1 de	4 ± 5 de	2 ± 2 de	7 ± 5 def
Tyrmes	8	32 ± 23 bc*	159 ± 66 abc	0.8 ± 0.3 c*	17 ± 12 ab		32 ± 13 b	
Durinta	7	188 ± 81 a	523 ± 385 ab	2.5 ± 1.5 ab		584 ± 251 a		316 ± 196 ab

Values were transformed $\log_{10}(x+1)$ or $\log_{10}(x+0.001)$ before analysis. Values are back-transformed mean ± standard deviation of eight replicated plants except for Durinta that only had seven plants. Values in the same column followed by different lower-case letters are significantly different according to Tukey's studentized range test ($P < 0.05$). Values in the same column with * indicate differences between a tomato rootstock or cultivar and the susceptible standard cv. Durinta according the Dunnett's *t*-test ($P < 0.05$).

[a] Eggs per plant /egg inoculum.

[b] Egg masses per plant on the resistant rootstock or cultivar/ egg masses per plant on susceptible standard cv. Durinta or Tyrmes × 100.

Correlation analyses showed a significant correlation (P <0.05) between the index of infectivity and reproduction irrespective of the susceptible cultivar used for reference or the duration of the tests (Fig. 1). Rootstocks showed variable levels of relative resistance when nematode infectivity or reproduction was referred to a single susceptible cultivar; in general, Durinta provided lower indexes (Table 2). Variability in the resistance levels was even greater after two nematode generations (Table 3). Rootstocks Beaufort and Maxifort consistently showed lower (P <0.05) resistance levels than the remaining ones or no resistance at all irrespective of the susceptible cultivar used for reference or the duration of the tests.

Table 3. Number of eggs masses per plant, eggs g^{-1} of root, multiplication rate (Pf/Pi), and infectivity and reproduction indexes of a *Mi* avirulent population of *Meloidogyne javanica* on tomato rootstocks with the *Mi* resistance gene after two generations of the nematode in pot tests conducted in a glasshouse in spring.

Plant Material	Infected plants	Egg masses /plant	Eggs g^{-1} root	Pf / Pi[a]	Infectivity index (%)[b]		Reproduction index (%)[c]	
					Durinta	Tyrmes	Durinta	Tyrmes
Rootstock								
PG-76	6	2 ± 3 g*	22 ± 18 gh*	0.1 ± 0.1 b*	0.2 ± 0.3 e	2 ± 3 f	0.2 ± 0.2 g	0.9 ± 1 f
Gladiator	9	2 ± 2 g*	76 ± 63 fgh*	0.3 ± 0.3 b*	0.2 ± 0.2 e	2 ± 2 ef	0.4± 1 fg	2 ± 2 ef
MKT-410	6	5 ± 12 g*	133 ± 295 h*	0.5 ± 1.2 b*	0.6 ± 1 e	2 ± 4 f	0.7 ± 2 fg	3 ± 6 ef
Brigeor	15	18 ± 27 f*	170 ± 169 fg*	0.8 ± 0.9 b*	2 ± 3 d	9 ± 9 d	1 ± 1 efg	5 ± 6 def
42851	14	15 ± 16 f*	271 ± 501ef*	1.2 ± 2.1 b*	2 ± 2 d	15 ± 20 cd	2 ± 3 efg	10 ± 21 de
43965	16	40 ± 48 ef*	582 ± 514 de*	3 ± 3 b*	5 ± 6 cd	17 ± 13 cd	4 ± 4 d	21 ± 19 cd
Big Power	9	239 ± 225 cd*	1051 ± 1136 cd*	8 ± 9 b*	28 ± 26 ab	72 ± 67 abc	11 ± 13 cd	42 ± 49 c
He-man	16	63 ± 40 de*	2225 ± 1402 bc*	13 ± 10 b*	7 ± 5 bc	59 ± 63 de	18 ± 13 bc	95 ± 80 b
Beaufort	16	350 ± 441 bc*	7863 ± 7662 ab	55 ± 86 a*	41 ± 53 ab	367 ± 676 ab	76 ± 120 a	491 ± 879 a
Maxifort	16	1411 ± 1394 a	9676 ± 6845 ab	70 ± 64 a*	165 ± 160 a	940 ± 723 a	94 ± 84 a	455 ± 325 a
Cultivar								
Caramba	15	13 ± 7 f*	245 ± 154 def*	1.1 ± 0.7 b*	2 ± 1 cd	13 ± 12 cd	2 ± 1 efg	9 ± 8 d
Monika	13	16 ± 15 f*	309 ± 322 def*	2.0 ± 1.5 b*	2 ± 2 d	10 ± 10 de	2 ± 2 ef	9 ± 7 d
Tyrmes	16	192 ± 171 cd*	3228 ± 1816 abc	15 ± 9 b*	22 ± 20 b		22 ± 12 b	
Durinta	16	850 ± 446 ab	12144 ± 4878 a	73 ± 31 a		740 ± 563 a		550 ± 294 a

Values were transformed $\log_{10}(x+1)$ or $\log_{10}(x+0.001)$ before analysis. Values are back-transformed mean ± standard deviation of 16 replicated plants except for Big Power that only had nine plants. Values in the same column followed by different lower-case letters are significantly different according to Tukey's studentized range test (P <0·05). Values in the same column with * indicate differences between a tomato rootstock or cultivar and the susceptible standard cv. Durinta according the Dunnett's *t*-test (P <0·05).

[a] Eggs per plant /egg inoculum.

[b] Egg masses per plant on the resistant rootstock or cultivar / egg masses per plant on susceptible standard cv. Durinta or Tyrmes × 100.

[c] Eggs per plant on the resistant rootstock or cultivar /eggs per plant on susceptible standard cv. Durinta or Tyrmes × 100.

Rootstock response to an initial *M. javanica* inoculum in summer

Mean daily soil temperatures in the pots soil from 19 July to 18 September 2006 ranged from 20.8 ˚C to 35.1 ˚C (x = 24 ˚C). Mean soil temperatures above 28 ˚C were registered during the first week post-nematode inoculation. High infectivity and reproduction values were recorded on the resistant rootstocks in summer. Number of egg masses and egg production on resistant cultivar Caramba but not on Monika were significantly lower (P <0.05) than on both susceptible cultivars (Table 4). The nematode produced similar number of egg masses and eggs g^{-1} root on both susceptible cultivars in this test. The number of egg masses and eggs g^{-1} root on rootstocks PG-76, MKT-410 and He-man were lower (P <0.05) than those on the susceptible cultivars.

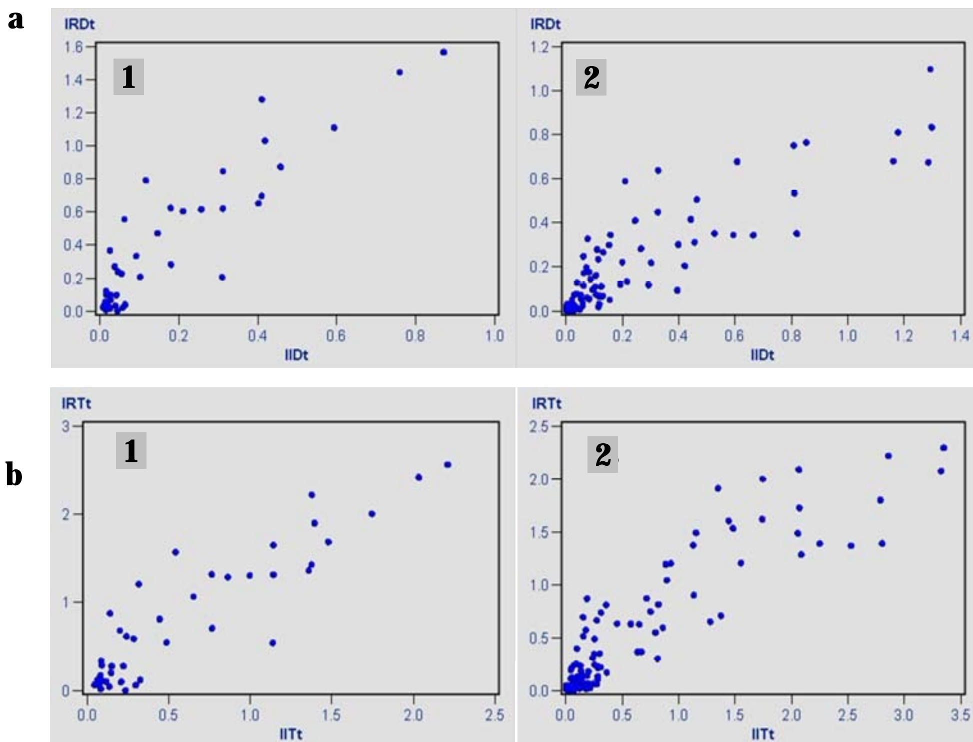

Figure 1[1]. Pearson Correlation of the Infectivity Index (II) and the Resistance index (RI) referred to the susceptible cultivars Durinta **(a)** and Tyrmes **(b)**, after one (1) and two genetarions (2).

1 This figure does not appear in the published paper of Cortada et al., 2008.

The remaining rootstocks did not differ from the susceptible cultivars in the number of egg masses or egg production. Pf/Pi values ranged from 3 to 25 times the Pi on the rootstocks after one generation, and population increases (Pf/Pi >1) occurred in all resistant plant materials except PG-76 and He-man which maintained the initial inoculum levels.

Table 4[2]. Number of eggs masses per plant, eggs g^{-1} of root, multiplication rate (Pf/Pi), and infectivity and reproduction indexes of a *Mi* avirulent population of *Meloidogyne javanica* on tomato rootstocks with the *Mi* resistance gene after one generation of the nematode in a pot test conducted in a glasshouse in summer.

Plant Material	Infected plants	Egg masses /plant	Eggs g^{-1} root	Pf / Pi[a]	Infectivity index (%)[b]		Reproduction index (%)[c]	
					Durinta	Tyrmes	Durinta	Tyrmes
Rootstock								
PG-76	2	4 ± 3 f*	136 ± 109 g*	0.6 ± 0.5 f*	1 ± 1 e	3 ± 2 e	3 ± 3 e	4 ± 4 e
Gladiator	8	58 ± 46 bcd*	4334 ± 5582 abcde	7 ± 8 abcde*	20 ± 16 abc	37 ± 29 abcd	40 ± 42 abcd	52 ± 55 abcd
MKT-410	6	25 ± 34 cdef*	867 ± 1280 efg*	3 ± 4 def*	9 ± 12 bcde	16 ± 22 cde	15 ± 21 cde	20 ± 28 de
Brigeor	8	117 ± 142 abc	4927 ± 2863 abc	16 ± 23 abc	40 ± 49 abc	74 ± 90 abc	89 ± 126 ab	117 ± 165 abc
42851	7	24 ± 30 cdef*	1285 ± 1577 def*	5 ± 7 cdef*	8 ± 10 cde	15 ± 19 cde	27 ± 37 bcd	36 ± 49 cd
43965	8	125 ± 109 ab	6362 ± 5694 ab	14 ± 7 abc	43 ± 38 ab	79 ± 69 ab	79 ± 38 ab	104 ± 49 abc
Big Power	8	194 ± 92 ab	5922 ± 1206 a	21 ± 8 a	67 ± 32 a	123 ± 58 a	116 ± 42 a	153 ± 55 a
He-man	3	5 ± 6 ef*	414 ± 300 fg*	1 ± 0.4 ef*	2 ± 2 de	4 ± 3 e	6 ± 2 de	7 ± 3 de
Beaufort	8	224 ± 193 ab	5174 ± 3065 abc	22 ± 17 ab	78 ± 67 a	142 ± 122 ab	123 ± 91 a	162 ± 120 ab
Maxifort	8	201 ± 107 ab	8277 ± 6081 a	25 ± 18 ab	69 ± 37 a	127 ± 68 a	135 ± 97 a	178 ± 128 ab
Cultivar								
Caramba	4	10 ± 5 def*	963 ± 601 cdef*	1.6 ± 0.8 ef*	4 ± 2 cde	6 ± 3 de	9 ± 5 de	11 ± 6 de
Monika	2	59 ± 60 bcde*	1313 ± 983 bcdef*	5 ± 4 bcdef*	21 ± 21 abcd	32 ± 39 bcde	29 ± 22 abcd	39 ± 29 bcd
Tyrmes	8	158 ± 123 ab	4272 ± 3513 abcd	14 ± 11 abcd	55 ± 43 a		76 ± 62 abc	
Durinta	8	289 ± 124 a	6053 ± 2499 a	20 ± 10 ab		183 ± 78 a		145 ± 69 ab

Values were transformed $\log_{10}(x+1)$ or $\log_{10}(x+0.001)$ before analysis. Values are back-transformed mean ± standard deviation of eight replicated plants except for Caramba and PG-76 that only had five plants. Values in the same column followed by different lower-case letters are significantly different according to Tukey's studentized range test (P< 0·05). Values in the same column with * indicate differences between a tomato rootstock or cultivar and the susceptible standard cv. Durinta according the Dunnett's t test (P< 0·05).

[a]Eggs per plant /egg inoculum.

[b]Egg masses per plant on the resistant rootstock or cultivar / egg masses per plant on susceptible standard cv. Durinta or Tyrmes ×100.

[c]Eggs per plant on the resistant rootstock or cultivar /eggs per plant on susceptible standard cv. Durinta or Tyrmes × 100.

[2] This table does not appear in the published paper of Cortada et al., 2008.

Rootstocks PG-76 and He-man and resistant Caramba showed high resistance levels whereas moderate resistance levels were found in rootstock MKT-410 and 42851, and resistant cultivar cv. Monika.

Rootstock response to continuous exposure to high population densities of *M. javanica* in the field

Mean daily soil temperatures in the plastic house from 15 March to 19 July 2007 ranged from 15.1 ˚C to 28.5 ˚C (x =23.6 ˚C). Disease incidence on the rootstocks, measured as number of infected plants, ranged from one infected plant of MKT-410 to all plants infected of Brigeor, Beaufort, and Maxifort with two to five out of eight replicated plants for the remaining rootstocks (Table 5). Gall ratings on rootstocks and resistant cultivars were lower ($P < 0.05$) than on susceptible cv. Durinta. Rootstocks Beaufort and Maxifort showed moderate gall ratings. Egg g^{-1} root was lower ($P < 0.05$) on the resistant than on the susceptible plants with the exception of rootstocks He-man, Beaufort, and Maxifort that did not differ from susceptible Durinta (Table 5). Remarkable differences in the RI of the rootstocks were found, and these ranged from 1% to 68% (Table 4).

Detection of the *Mi* resistance gene

The REX-1 amplified product digested with *Taq*I from all rootstocks except MKT-410 resulted in two bands of approximate 570 bp and 160 bp in size indicating that the *Mi* locus is homozygous resistant in these rootstocks (Fig. 2a). Rootstock MKT-410 displayed three bands of approximate 750 bp, 570 bp and 160 bp in size indicating that the *Mi* locus is in heterozygous form (Fig. 2a). Molecular data were repeated at least twice for all plant material obtaining consistent results (data not shown). In addition to the rootstock MKT-410, the two root-knot nematode resistant tomato Monika and Caramba were also heterozygous for the *Mi* locus and displayed the three REX-1 bands (Fig. 2a). The susceptibles cvs. Tyrmes and Durinta displayed a single band of 720 bp indicating that the *Mi* locus is in homozygous susceptible form (Fig. 2a). The REX-1 genotyping data for rootstocks Beaufort and Maxifort disagreed with the nematode resistance phenotypic results from both glasshouse and plastic house field tests. The REX-1 indicated that these rootstocks have a region similar to

the introgressed *Mi* gene, but the biological assays provided susceptibility responses to the nematode.

Table 5. Root galling, eggs g^{-1} of root and reproduction index of *Meloidogyne javanica* on tomato rootstocks with *Mi* resistance gene subjected to continuous exposure to high population densities of the nematode in a field plastic house.

Plant material	Transplants	Infected plants	Gall rating[a]	Eggs g^{-1} root	Reproduction index (%)[b]
Rootstock					
PG-76	8	2	0.1 ± 0.3 (0-1) d*	358 ± 353 d*	0.6 ± 0.5 d
Gladiator	6	2	0.2 ± 0.4 (0-1) d*	767 ± 730 cd*	1.4 ± 1.4 cd
MKT-410	7	1	0.5 ± 1.2 (0-3) d*	1469 ± 2362 cd*	2 ± 4 cd
Brigeor	7	7	1.9 ± 0.7 (1-3) cd*	6250 ± 7803 bc*	10 ± 12 bc
42851	8	2	0.5 ± 1.1 (1-3) d*	1245 ± 1442 cd*	2 ± 2 cd
43965	8	5	1.6 ± 1.3 (1-3) cd*	6209 ± 9375 bc*	10 ± 14 bcd
Big Power	7	4	1.1 ± 1.2 (1-3) d*	3350 ± 3750 bcd*	6 ± 6 bcd
He-man	8	3	2.0 ± 1.7 (3-4) bcd*	18800 ± 21496	31 ± 32 ab
Beaufort	8	7	4.0 ± 0.9 (3-5) b*	37295 ± 25557 a	68 ± 54 a
Maxifort	7	7	3.6 ± 1.3 (2-5) bc*	34954 ± 19205 a	59 ± 29 a
Cultivar					
Caramba	8	5	0.9 ± 0.8 (1-2) d*	446 ± 286 cd*	0.8 ± 0.5 cd
Monika	7	3	1.0 ± 1.0 (1-2) d*	3540 ± 5563 bcd*	6 ± 8 bcd
Durinta	8	7	6.4 ± 1.7 (3-8) a	56870 ± 25283 a	

Values were transformed $\log_{10}(x+1)$ or $\log_{10}(x)$ before analysis. Values are back-transformed mean ± standard deviation of eight replicated plants except for Durinta that is mean of composite root samples of 12 plants. Values in the same column followed by different lower-case letter are significantly different according Tukey's studentized range test (P<0·05). Values in the same column with * indicate differences between a tomato rootstock or cultivar and the susceptible standard cv. Durinta according the Dunnett's t test (P< 0·05).

[a] Based on a scale from 0 (none) to 10 (dead plants). In parentheses, range of gall rating.

[b] Eggs g^{-1} root on resistant tomato /eggs g^{-1} root on susceptible tomato × 100.

Therefore, the Mi23 marker was selected to confirm the genotypes of these rootstocks, and the results indicated that except for rootstock MKT-410 and 42851, all rootstocks were homozygous for the *Sp*-Mi23 locus as only a single band of 380 bp was amplified (Fig. 2b). Rootstocks MKT-410 and 42851 and the resistant standards displayed two bands of 380 bp (*Sp*-Mi23-locus) and 430 bp (*Sl*-Mi23-locus) indicating that they were heterozygous for this region. The susceptible standards amplified a 480 bp fragment indicating that they were homozygous susceptible for this locus. It should be noted that two different genotypes were obtained for rootstock 42851 depending on the co-dominant

markers used; the REX-1 region in this rootstock was homozygous whereas the Mi23 region was heterozygous (*Sp*-Mi23-locus and *Sl*-Mi23-locus were amplified) indicating the presence of a recombination event between these two markers.

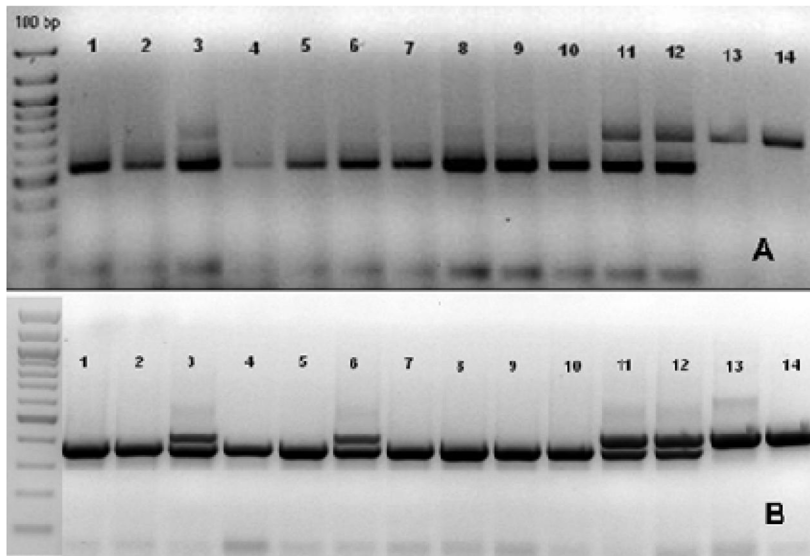

Figure 2. Detection of the *Mi-1.2* gene in tomato rootstocks and cultivars by molecular analyses. **(a)** REX-1 marker profile in tomato rootstocks (lanes 1-10) and control tomato cultivars (lanes 11-14). PCR amplification products with primers REXF1/REXR2 were digested with *Taq*αI enzyme and resolved on 1·5 % agarose gel. **(b)** Mi23 marker profile in tomato rootstocks (lanes 1-10) and control tomato cultivars (lanes 11-14). PCR with primers Mi23F/R amplifies the non-coding region between *Mi-1.2* and *Mi-1.3* in *S. peruvianum* introgressed region (380 bp) and the non-coding region between *Mi-1A* and *Mi-1B* in *S. lycopersicum* background (430 bp). Lanes 1, PG-76 (resistant); lanes 2, Gladiator (resistant); lanes 3, MKT-410 (resistant); lanes 4, Brigeor (resistant); lanes 5, 42851 (resistant); lanes 6, 43965 (resistant); lanes 7, Big Power (resistant); lanes 8, He-man (resistant); lanes 9 Beaufort (susceptible); lanes 10 Maxifort (susceptible); lanes 11, Caramba (resistant), lanes 12, Monika (resistant), lanes 13, Tyrmes (susceptible), lanes 14, Durinta (susceptible).

Discussion

Although molecular analysis confirmed the presence of the *Mi* resistance gene in the studied tomato rootstocks, remarkable differences were shown in nematode infectivity and reproduction when they were subjected to an initial

inoculum of an avirulent population of *M. javanica*. Thus, three rootstocks PG-76, Gladiator, and MKT-410, were consistently highly resistant (Pf/Pi <1 and RI <10%) and were as efficient as the resistant cultivars in nematode suppression. The relative resistance levels of five rootstocks, Brigeor, 42851, 43965, Big Power, and He-man, varied depending on the susceptible cultivar used for reference or the duration of the test. Nevertheless, Brigeor and 42851 showed good levels of resistance, whereas Big Power and He-man showed much lower resistance levels. Previously, the intermediate resistance of Big Power had been described only to *M. incognita* (Marín Rodríguez, 2005). Two rootstocks cvs. Beaufort and Maxifort were susceptible to *M. javanica* (Pf/Pi >50 and RI >50%), and Beaufort was susceptible to *M. arenaria* (Graft et al., 2001) and *M. incognita* (López-Pérez et al., 2006).

The susceptible response of Maxifort does not appear to have been reported before. Beaufort and Maxifort (*S. lycopersicum* × *S. habrochaites*) (Table 1), described as highly resistant to *M. arenaria*, *M. incognita* and *M. javanica* (Marín Rodríguez, 2005), should be nematode resistant, according to the REX-1 marker. The detection of false positives for root-knot nematode resistance in these rootstocks is most likely due to the genetic background of Beaufort and Maxifort (Table 1). The molecular marker REX-1 was developed to detect the presence of the introgressed *Mi* region in hybrids of *S. lycopersicum* × *S. peruvianum* (Williamson et al., 1994), but it might not be equally useful for hybrids between *S. lycopersicum* and other *Solanum* species. This fact was reported by El Mehrach et al. (2005) in tomato hybrid lines with introgressions of *S. habrochaites* and *S. chilense* on chromosome 6. Therefore, the newly developed co-dominant marker Mi23, (Seah et al., 2007b) was used. This marker was also been tested with other wild species of *Solanum* with similar results (Seah et al., 2007b). Since only a few accessions of wild tomatoes were tested with this marker, it is unclear whether *Sp*-Mi23 allele is universally associated with root-knot nematode resistant genotypes in *Solanum* species. According to Mi23 marker, all resistant rootstocks and cultivars carried the *Mi* resistance gene either in homozygous or heterozygous forms. However, the root-knot resistance phenotypic data indicates that Mi23 like REX-1 is unable to distinguish between resistant and susceptible genotypes in hybrids of *S. lycopersicum* × *S. habrochaites* and *S. lycopersicum* × *Solanum* spp.

Since nematode resistance in these rootstocks was from *Solanum* species other than *S. peruvianum*, it was uncertain whether the resistance is conferred by the *Mi* gene or a homologue of *Mi*. It is also difficult to speculate on the genetic distances between the nematode resistance gene and the markers used in

this study. Assuming that the genetic distance between the nematode resistance gene and SCAR Mi23 in these *Solanum* species is similar to that between *Mi* and Mi23 in *S. peruvianum* (Seah et al., 2007a, 2007b), then the nematode resistance gene was most likely present in these rootstocks. The nematode susceptibility of rootstocks Beaufort and Maxifort could be explained by spontaneous mutation in the resistance gene. A single nucleotide change can eliminate the function of a gene. In fact, two *Mi* homologues *Mi-1.6* and *Mi-1G* have only single nucleotide mutations which resulted in nonfunctional truncated proteins (Seah et al*.,* 2007a). Absence of resistance could also be caused by resistance gene silencing by the methylation process (Liharska, 1998). The dosage effect of the *Mi* gene in resistant tomato cultivars was associated with variability in root-knot nematode responses, suggesting that homogozygous cultivars were more resistant than heterogozygous ones, but with some exceptions (Tzortzakakis et al., 1998, Jacquet et al., 2005). The response of some rootstocks did not conform to the dosage effect model proposed for tomato cultivars with introgressions from *S. peruvianum.* Several possible explanations for this behaviour have been offered in this paragraph. Nevertheless, more research is needed to better understand the nature of lack of resistance in some rootstocks.

The efficiency of the nematode resistance was greatly reduced in most resistant materials in the hottest time of the year. Yet, some rootstocks, e.g. PG-76, MKT-410 and 42851, still showed high or moderate resistance levels despite soil temperatures raised above 28 ˚C in the first week post-nematode inoculation, which suggests that the resistance in these rootstocks may be conferred by a heat stable gene other than *Mi.* The response of cv. He-man needs further investigation because low numbers of plants were infected in this test. Resistant Caramba retained a high relative resistance level, whereas Monika only a moderate level suggesting, that the genetic background might play a role in the stability of *Mi* resistance at high temperatures.

The results of the field study conducted under continuous exposure to high population densities of *M. javanica* confirmed the differential response of the rootstocks carrying the *Mi* resistance gene previously identified in the glasshouse tests. Seven out of 10 rootstocks showed high levels of field resistance (RI <10%). Once more, Beaufort and Maxifort were susceptible (RI >50%) but were more tolerant to nematode infection (root galling) than susceptible Durinta as they suffered less nematode damage. The variability in nematode infection and reproduction on the tomato rootstocks can be largely attributed to their genetic background as they were challenged with a single

isolate of *M. javanica*. The field population came from the same *M. javanica* population (MJ-05) as the site had been artificially infested with this isolate in 2004 by pre-inoculating tomato seedlings before transplanting. Although such variability in infectivity and nematode reproduction had been documented on tomato cultivars (Roberts and Thomason, 1989, Ornat et al., 2001, Jacquet et al., 2005) as well as rootstocks (López-Pérez et al., 2006, Graf et al., 2001) such large differences were unexpected.

The category of nematode resistance in a plant phenotype can change depending on the experimental conditions set for screening since nematode resistance is relative to reproduction on a susceptible plant host. Thus, the choice of the cultivar used as standard for susceptibility was of great relevance and seemed more crucial than the duration of the test (one over two generations). Nematode genotype will also affect the categorization of the rootstock resistance response, although this factor was not addressed in this study. Nematode reproduction on resistant tomato genotypes was explained by the interaction between the plant genotype and nematode isolate but not by either factor alone (Jaquect et al., 2005). The evaluation of the rootstocks after two nematode generations allowed detection of population increases and changes in the relative levels of resistance in some rootstocks that were otherwise gone unnoticed in tests involving only one generation. Susceptible Tyrmes was selected because its agronomic characteristics are similar to those of the resistant cultivar Caramba whereas those of susceptible Durinta matched resistant Monika. Tyrmes is tolerant to *Tomato yellow leaf curl virus* (TYLCV), and reduced root-knot nematode infectivity on geminivirus resistant plants has been reported (Mahajan and Chhabra, 1977). There was a positive correlation between the infectivity and the reproduction index, although the RI offered a better level of agreement between the seed companies' descriptions and the present results, as the infectivity index can be sometimes misleading (Roberts and Thomason, 1989). However, this index would be especially useful in the initial steps of the evaluation process; to reduce the duration of the test and save labour.

In summary, *M. javanica* infected and reached high reproduction levels on some resistant tomato rootstocks just after two nematode generations in spring and a single generation in summer. Cultivation of these rootstocks in nematode-infested soil confirmed the differential response of the rootstocks. These results are relevant for breeding programs as well as nematode management. They emphasize the need for marker-assisted selection to be complemented with biological characterization of the resistant response.

Different resistant and susceptible cultivars and nematode isolates should be included as reference standards to diminish variation due to genetic background and inter- and intra-specific variability in the genus *Meloidogyne*. These results raise concerns over the durability of the resistance in tomato rootstocks. The appearance of resistance breaking populations may be preceded by increased nematode infectivity and reproduction (Sorribas et al*.,* 2005). Therefore, attention should be paid to the possible emergence of virulent nematode genotypes in areas infested with the nematode and cultivated with resistant tomato rootstocks, as selection for virulence has been reported on resistant pepper rootstocks only after two cropping cycles (Ros et al., 2004, 2006).

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Comparison of the vigor of roots of Maxifort (left) and MKT-410 (right) rootstocks. Despite the number of secondary roots, the length and the thickness of the roots, reproduction on Maxifort was considerably higher that on resistant roostock MKT-410. Maxifort presented a susceptible phenotypic response when plants were inoculated with *Meloidogyne javanica* and *M. incognita* populations.

2 Response of tomato rootstocks carrying the *Mi*-resistance gene to populations of *Meloidogyne arenaria*, *M. incognita* and *M. javanica*

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The response of four *Mi*-resistance gene tomato rootstocks to seven populations of *Meloidogyne* was determined in pot tests conducted in a glasshouse. Rootstocks PG-76 (*Solanum lycopersicum* × *Solanum sp*) and Brigeor (*S. lycopersicum* × *S. habrochaites*) and resistant cultivar Monika (*S. lycopersicum*) were assessed against one population of *M. arenaria*, three of *M. incognita*, and three of *M. javanica*. Rootstocks Beaufort and Maxifort were assessed against one population of *M. arenaria*, two of *M. incognita* and two of *M. javanica*. Rootstock PG-76 was highly resistant (reproduction index < 10%) to all the populations, whereas rootstock Brigeor and cultivar Monika were highly to moderate resistant. Rootstocks Beaufort and Maxifort showed reduced resistance or inability to suppress nematode reproduction and their responses varied according to the population tested. Beaufort and Maxifort were susceptible to the two populations of *M. javanica* as Maxifort was to one of *M. incognita*. The reproduction index of the nematode was higher ($P < 0.05$) on Maxifort than Beaufort for all root-knot nematode populations.

Key words: genetic variability, resistance, root-knot nematodes, SCAR-PCR, Solanum habrochaites, Solanum lycopersicum.

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Introduction

Meloidogyne is the most important plant parasitic nematode genus that causes serious yield losses in tomato (*Solanum lycopersicum*, formerly *Lycopersicon esculentum*) worldwide. *Meloidogyne javanica* and *M. incognita* are most common root-knot nematode found in vegetable production areas in Spain whereas *M. arenaria* is found but to a lesser extent (Sorribas and Verdejo-Lucas, 1994; Verdejo-Lucas et al., 2002).

The use of new control methods against soil pathogens has increased in the last two decades to circumvent the toxicity and environmental impact of traditional pesticides used in agriculture (e.g. methyl bromide). Plant resistance is an economically, sustainable and environmentally friendly alternative to conventional and organic agriculture (Roberts and Thomason, 1996; Besri, 2003; Sorribas et al., 2005). In tomato, the *Mi*-resistance gene, introgressed from *S. peruvianum* (Smith, 1944), strongly reduces development and reproduction of *M. arenaria*, *M. javanica* and, *M. incognita* at soil temperatures below 28˚C (Dropkin, 1969). Tomato cultivars carrying the *Mi*-resistance gene are not immune to root-knot nematodes and support some level of reproduction (Roberts and Thomason, 1989). Reproduction on resistant tomatoes has been explained by the interaction between plant genotype and nematode isolate, but not by either factor alone (Jacquet et al., 2005). The inter- and intra-specific genetic variability in the genus *Meloidogyne* contributes to variation in the response of *Mi*-resistance gene tomatoes with introgressions from *S. peruvianum* which can result in reduced levels of nematode suppression (Roberts and Thomason, 1996; Ornat et al., 2001; Castagnone-Sereno, 2002).

At present, grafting vegetables is expanding in Europe, and it is primarily used to increase their vigour and yield. In tomato, most commercially available rootstocks are interspecific hybrids of *S. lycopersicum* and S*. habrochaites* (formerly, *L. hirsutum*) or other wild *Solanum* species. They incorporate the *Mi*-resistance gene in addition to other resistance genes to manage diseases caused by bacteria, fungi, and viruses. The response of *Mi*-resistance gene tomato rootstocks against root-knot nematodes varied greatly depending on plant genotype and ranged from highly resistant to fully susceptible (Graf et al., 2001; López-Pérez et al., 2006; Cortada et al., 2008; Verdejo-Lucas and Sorribas, 2008). However, little is known on the contribution of the nematode-genotype observed variation in levels of nematode suppression. The objective of this study was to determine variation in the resistance response of four tomato

rootstocks against different populations of *M. arenaria*, *M. incognita* and *M. javanica*.

Materials and Methods

The root-knot nematode populations were held on susceptible cv Roma, and had never been exposed to the *Mi*-resistance gene. They included one population of *M. arenaria* (code MA-68), three of *M. incognita* (codes MI-ALM, MI-CROS and MI-26), and three of *M. javanica* (codes MJ-IBIZA, MJ-05 and MJ-Q21). The identity of these populations was confirmed before the start of the study by molecular SCAR-PCR markers according to Zijlstra et al. (2000). The tomato rootstocks were PG-76, Brigeor, Beaufort, and Maxifort and the resistant cultivar Monika. All had been described as highly resistant to *M. arenaria*, *M. incognita* and *M. javanica* (Marín Rodríguez, 2005). The susceptible cultivar Durinta was included as a reference standard for comparison. The main characteristics and resistances of the tomatoes are described in Table 1.

Pot tests were conducted to determine nematode reproduction on rootstocks PG-76, and Brigeor, and cultivar Monika. Seedlings were transplanted singly into 1.5 l pots containing steam-sterilized river sand, and were allowed to grow for one week before inoculation. Nematode inoculum was obtained from infected tomato cv. Roma by macerating the roots in a 0.5% NaOCl solution in a food blender at 1,000 rpm for 5 min. (Hussey and Barker, 1973). Therefore macerated roots were passed through a 74-μm-aperture sieve to remove root debris, and the dispersed eggs were collected on a 25-μm-sieve. Plants were inoculated with approximately 3,000 eggs of *M. arenaria* MA-68, *M. incognita* MI-ALM, MI-CROS, and MI-26, *M. javanica* MJ-IBIZA, MJ-05, and MJ-Q21. Each tomato-population combination was replicated eight times. Plants were maintained in a glasshouse for 8 weeks. They were watered as needed and fertilized with a slow-release fertilizer (15% N +10% P_2O_5 +12% K_2O + 2% MgO_2 + microelements). At the end of each test, the number of eggs g^{-1} of fresh root was determined by macerating two 10-g root sub-samples in a 0.5% NaCOl solution for 10 min, as described previously.

Table 1. Main characteristics and resistances of the *Mi*-resistance gene tomatoes used to determine variation in the response to different populations of *Meloidogyne arenaria*, *M. incognita* and *M. javanica*.

Variety	Mi23 profile[a]	Parental Species[b]	Seed Company	Resistances[c]
Rootstock				
PG-76	Mi/Mi	S. lycopersicum × Solanum sp	Gautier Seeds	HR: TMV/ Fol:2 /For / Va/ Vd/ Pl/ Ma/ Mi/ Mj
Brigeor	Mi/Mi	S. lycopersicum × S. habrochaites	Gautier Seeds	HR: TMV/ Fol:2/ For/ V/ Ma/ Mi/ Mj
Beaufort	Mi/Mi	S. lycopersicum × S. habrochaites	De Ruiter Seeds	HR: ToMV/ Fol:0,1/ For/ Pl/ Va/ Vd/ Ma/Mi/ Mj
Maxifort	Mi/Mi	S. lycopersicum × S. habrochaites	De Ruiter Seeds	HR: ToMV/ Fol:0,1/ For/ Pl/ Va/ Vd/ Ma/ Mi/ Mj
Cultivar				
Monika	Mi/mi	S. lycopersicum × S. peruvianum	Syngenta Seeds	HR: ToMV: 0-2/ Fol:1/ Va/ Vd IR: Mi/ Ma/ Mj
Durinta	mi/mi	S. lycopersicum × S. lycopersicum	Western Seeds	HR: ToMV/ Fol:1-2/ Va/ Vd

[a] *Mi/Mi* (homozygous resistant); *Mi/mi* (heterozygous resistant); *mi/mi* (homozygous susceptible).
[b] *S. lycopersicum* × *Solanum* sp: unknown parental species.
[c] Information from the seed companies' descriptions. HR: High resistance; TMV: *Tobacco Mosaic Virus*; ToMV: *Tomato Mosaic Virus*; TYLCV: *Tomato yellow leaf curl virus*; Ff: 1-5: *Fulvia fulva races* 1, 2, 3, 4, 5; Fol: 0-2: *Fusarium oxysporum* f. spp. *lycopersici* races 0, 1, and 2; For: *Fusarium oxysporum* f. spp. *radicis-lycopersici*; Pl: *Pyrenochaeta lycopersici*; Sbl: *Stemphylium botryosum* f. spp. *lycopersici*, Va: *Verticillium albo-atrum*; Vd: *Verticullium dahliae*; Ss: *Stemphylium solana*; Si: Silvering; Cmm: *Clavibacter* pv. *michiganensis*; Pst: *Pseudomonas syringae* pv tomato; Mi, Ma, Mj: *Meloidogyne incognita*, *M. arenaria*, *M. javanica*.

The response of the tomato rootstocks was categorized according to the reproduction index (RI) as, highly resistant (RI <10%), moderately resistant (10 RI < 50%) or susceptible (RI 50%) (Hadisoeganda and Sasser, 1982). The RI was calculated as number of eggs per plant on resistant rootstock or cultivar divided by the number of eggs per plant on the susceptible cv. Durinta × 100. Rootstocks Beaufort and Maxifort were inoculated with populations *M. arenaria* MA-68, *M. incognita* MI-ALM and MI-CROS, and *M. javanica* MJ-IBIZA and MJ-05. It was not possible to test *M. incognita* MI-26 and *M. javanica* MJ-Q21 populations with Beaufort and Maxifort due to insufficient inoculum. Preparation of nematode inoculum and experimental conditions were similar to those described previously except for the combinations Beaufort and Maxifort with *M. incognita* MI-CROS and *M. javanica* MJ-05 that were maintained in the glasshouse for 12 instead of 8 weeks.

The general linear model procedure of the SAS software version 8 (SAS institute Inc., Cary, NC) was used for statistical analysis. The number of eggs g^{-1} of root and eggs per plant were transformed to x to achieve normality of data, and then subjected to analysis of variance. The Tukey's studentized range test was used to compare means when the ANOVA analysis was significant ($P < 0.05$). Soil temperatures were registered daily at 30 min intervals by placing temperatures probes into the potted soil. Temperatures were below 28 ˚C for the duration of the tests and ranged from 11.2 to 24.6 ˚C (x = 19.3 ˚C).

Results and Discussion

Resistant rootstocks PG-76 and Brigeor supported lower number of eggs g^{-1} of root ($P < 0.05$) than susceptible Durinta (Table 2). Both rootstocks showed similar ability to inhibit nematode reproduction irrespective of the populations tested. Egg production was similar on rootstock Brigeor and cultivar Monika, but differences between rootstock PG-76 and cultivar Monika were observed with populations *M. incognita* MI-CROS, and *M. javanica* MJ-05 and MJ-Q21. Nematode reproduction (eggs g^{-1} root) on resistant Monika was lower ($P < 0.05$) than on susceptible control Durinta for all combinations. Rootstock PG-76 was highly resistant to the seven populations of *Meloidogyne* as RI values ranged from 0.02% (MI-CROS) to 3.3% (MJ-Q21) (Fig. 1). Rootstock Brigeor was highly resistant to four nematode populations but moderately resistant to

M. incognita MI-26, and two *M. javanica* populations. The RI values for cv. Monika ranged from 4.4% (MI-ALM) to 27.3% (MI-26) (Fig. 1).

The number of eggs g^{-1} of root on rootstock Beaufort was lower (P <0.05) than on susceptible Durinta inoculated with *M. arenaria* MA-68, and *M. incognita* MI-ALM and MI-CROS, but was not different from the susceptible control when inoculated with *M. javanica* MJ-IBIZA and MJ-05 (Table 3). Egg production on rootstock Maxifort was lower (P <0.05) than on susceptible Durinta inoculated with *M. arenaria* MA-68, *M. incognita* MI-CROS and *M. javanica* MJ-05, but the number of eggs g^{-1} of root did not differ from the susceptible tomato with the remaining populations (Table 3).

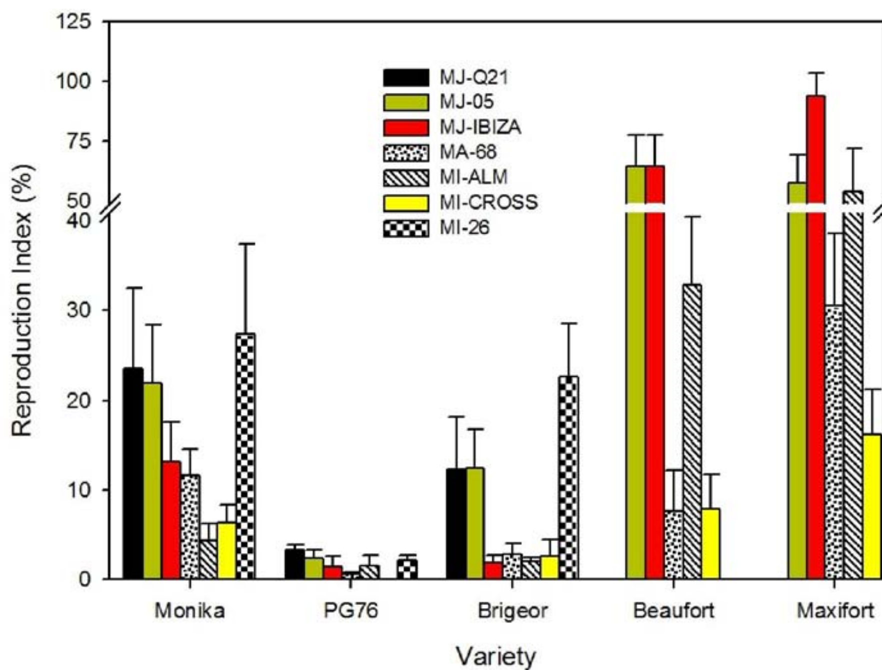

Figure 1. Reproduction index (RI) of one population of *Meloidogyne arenaria* (MA-68), three populations of *M. incognita* (MI-ALM, MI-CROS, MI-26), and three populations of *M. javanica* (MI-IBIZA, MJ-O5, MJ-Q21) on *Mi*-resistance gene tomato cultivar Monika, and rootstocks PG-76, Brigeor, Beaufort, and Maxifort. RI: eggs per plant on a resistant tomato divided by eggs per plant on susceptible control × 100.

Table 2. Numbers of eggs g^{-1} of root of three populations of Meloidogyne javanica (MJ-IBIZA, MJ-05, MJ-Q21), one of M. arenaria (MA-68), and three of M. incognita (MI-ALM, MI-CROS, MI-26) on Mi-resistance gene tomato rootstocks PG-76 and Brigeor, and resistant cultivar Monika and susceptible cultivar Durinta eight weeks after the inoculation of 3,000 eggs per plant.

	M. javanica			M. arenaria	M. incognita		
Tomato	MJ-Q21	MJ-05	MJ-IBIZA	MA-68	MI-ALM	MI-CROS	MI-26
PG-76	290 ±165 c	168 ± 185 c	354 ± 844 b	16 ± 19 b	74 ± 146 b	3 ± 6 c	351 ± 275 b
Brigeor	1182 ± 1291 bc	875 ±1042 bc	611 ± 794 b	107 ± 100 b	73 ± 67 b	382 ± 672 bc	3216 ± 1837 b
Monika	3189 ± 3670 b	2118 ± 2223 b	1564 ± 1423 b	564 ± 401 b	159 ± 160 b	1110 ± 1140 b	4294 ± 3974 b
Durinta	10488 ±5115 a	13696 ± 7104 a	12511 ± 3310 a	6452 ± 3910 a	3570 ± 1602 a	12315 ± 3576 a	14921 ± 8708 a

Values are back-transformed mean ± standard deviation of seven replicated plants.
Values within the same column sharing the same letter are not significantly different according to Tukey's studentized range test ($P<0.05$).

Rootstock Beaufort was highly resistant (RI <10%) to *M. arenaria* MA-68 and *M. incognita* MI-CROS, moderately resistant to *M. incognita* MI-ALM (RI = 33%), and fully susceptible to both *M. javanica* populations. Rootstock Maxifort responded as moderately resistant to *M. arenaria* MA-68 and *M. incognita* MI-CROS, and as susceptible (RI >50%) to *M. incognita* MI-ALM, and the two *M. javanica* populations (Fig. 1). Rootstocks Beaufort and Maxifort inoculated with *M. javanica* MJ-IBIZA and MJ-05 resulted in very high RI values that were not significantly different from the susceptible control (P > 0.05).

Table 3. Numbers of eggs g^{-1} root of two populations of *Meloidogyne javanica* (MJ-IBIZA and MJ-05), one of *M. arenaria* (MA-68), and two of *M. incognita* (MI-ALM and MI-CROS), on *Mi*-resistance gene tomato rootstocks Beaufort and Maxifort and susceptible cultivar Durinta eight or twelve weeks after the inoculation of 3,000 eggs per plant.

	Duration of the tests				
	8 weeks			12 weeks	
	M. javanica	*M. arenaria*	*M. incognita*	*M. javanica*	*M. incognita*
Tomato	MJ-IBIZA	MA-68	MI-ALM	MJ-05	MI-CROS
Beaufort	9859 ± 2204 a	508 ± 635 c	1197 ± 694 b	6908 ± 3998 ab	2846 ± 4024 b
Maxifort	15669 ± 6865 a	1627 ± 1079 b	1811 ± 1359 ab	6403 ± 3778 b	4658 ± 3671 b
Durinta	12511 ± 3310 a	6436 ± 1034 a	3570 ± 1602 a	12239 ± 4881 a	27697 ± 7847 a

Values are back-transformed mean ± standard deviation of seven replicated plants. Values in the same column sharing the same letter are not significantly different according to Tukey's studentized range test (P<0.05).

There was strong effect of the tomato genotype on their response to nematode population. A total of 31 nematode population-tomato genotype combinations were tested in this study. Of these, 15 combinations resulted in a highly resistant response, 11 moderately resistant, and 5 were susceptible responses: three involved rootstock Maxifort, and two, rootstock Beaufort. Molecular analysis using co-dominant marker REX-1 (Williamson et al., 1994) and the PCR-based co-dominant SCAR marker Mi23 (Seah et al., 2007b) had been performed. Both indicated that all tomato rootstocks were homozygous resistant for *Mi-1* locus and that resistant cultivar Monika was heterozygous

(Cortada et al., 2008). The marker Mi23 was specifically designed for interespecific tomato hybrids lines with *S. habrochaites* as were rootstocks Brigeor, Beaufort and Maxifort. However, the pathogenicity tests showed variable results. Tzortzakakis et al. (1998) and Jacquet et al. (2005) have suggested that *Mi-1* homozygous locus might protect better against the nematode compared to the *Mi-1* heterozygous locus, but no consistent effect was found in this study. For instance, rootstocks Beaufort and Maxifort were susceptible to both populations of *M. javanica* whereas cv. Monika was resistant. The molecular markers were unable to distinguish variation in the resistant response which emphasizes the need to use different nematode populations to characterize plant resistance. High soil temperatures as a cause for resistance breaking were discarded because soil temperatures remained below 28 ˚C during the tests.

Remarkable changes were revealed in some rootstocks depending on the population, and they were best illustrated for rootstock Beaufort which was highly resistant to *M. arenaria* MA-68 and *M. incognita* MI-CROS, moderately resistant to *M. incognita* MI-ALM, and susceptible to both populations of *M. javanica*. Conversely, highly resistant responses were consistently obtained on PG-76 challenged to seven populations.

Several hypotheses could explain the susceptibility of rootstocks Beaufort and Maxifort against the two populations of *M. javanica*. The lack of resistance could be attributed to gene silencing by a methylation process (Liharshka, 1998) or a spontaneous mutation in the sequence of *Mi-1.2* gene that could inhibit gene expression (Seah et al., 2007a). The absence or the mutation of genes necessary in the signaling pathway of *Mi-1.2* gene like *Rme1* (Martínez de Ilarduya et al., 2004), *Hsp90* or *Sgt1* (Bhattarai et al., 2007) could also explain the susceptible phenotype of Beaufort and Maxifort. Nevertheless, the differential responses of both rootstocks were related to the nematode population which reinforces the concept that each plant-nematode combination has a specific interaction pattern. Changes from resistant to susceptible responses have been reported in tomato cultivars with introgressions from wild *Solanum* species when a single plant genotype was challenged to different *Meloidogyne* isolates (Sorribas and Verdejo-Lucas, 1999; Tzortzakakis et al., 2006). On the other hand, little is known about root-knot nematode avirulence effectors (*Avr*) (Fuller et al., 2008) and the reason why some isolates can reproduce on resistant plants whereas others never overcome the resistance of the *Mi-1.2* gene (Jarquin- Barberena et al., 1991). Virulence could be due to lack of or modification of those nematode gene products that activates plant

defense genes against nematodes (Nem-*R* genes) (Williamson and Kumar, 2006). Nowadays, the specific interaction of the nematode and the *Mi-1.2* resistance signaling pathway remains to be solved. As all nematode-rootstock combinations were not tested simultaneously, main effects could not be statistically analyzed but differences in the phenotypic expression of the *Mi-* resistance gene were apparent as tomato rootstocks and cultivar were not equally effective in suppressing nematode reproduction. As a general trend, rootstock PG-76 was the most effective, followed by Brigeor, cv. Monika, Beaufort, and Maxifort. These results are in agreement with those of Cortada et al. (2008) using a single population of *M. javanica* (MJ-05) regarding to the differential response of tomato rootstocks and the ranking in the resistance levels.

The differences found in the resistant responses of tomato rootstocks have implications in root-knot nematode management. The success of growing resistant tomato rootstocks in nematode-infested soils could vary according to locally-occurring populations of *Meloidogyne*, and this could limit their usefulness as an alternative to chemical control. The susceptibility of Beaufort to populations of *M. incognita* and *M. arenaria* has already been reported (Graf et al., 2001; López-Pérez et al., 2006). The extremely vigourous root system of the rootstocks and the presence of additional resistance genes in their genome may help to counteract other soil-borne diseases, and in turn, contribute to increased tomato yields, but they may not be effectively enough to control root-knot nematodes.

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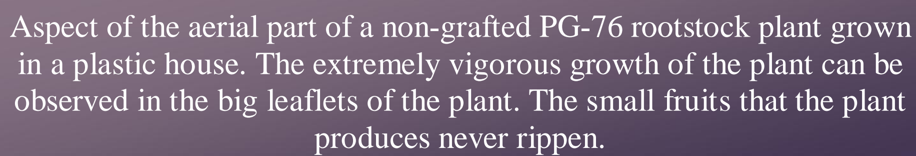
Comparison of the vigor of the aerial part of the resistant rootsock Brigeor (upper plant) and the susceptible cultivar Durinta (lower plant) four weeks after planting in a plastic house. The soil was naturally infested by an avirulent *Meloidogyne javanica* population.



Aspect of the aerial part of a non-grafted PG-76 rootstock plant grown in a plastic house. The extremely vigorous growth of the plant can be observed in the big leaflets of the plant. The small fruits that the plant produces never rippen.

3 Molecular markers for detection of *Mi-1.2* resistance gene in tomato hybrid rootstocks

L. Cortada[1], *S. Mantellin*[2], *S. Verdejo-Lucas*[1], and *I. Kaloshian*[2]

In crop breeding programs, the use of molecular markers saves time, money and effort. In tomato, the co-dominant REX-1 marker is used to detect the *Mi-1.2* gene in *S. lycopersicum* × *S. peruvianum* hybrids. Nevertheless, this marker is not suitable for use with hybrid tomato rootstocks (*S. lycopersicum* × *S. habrochaites* or *S. lycopersicum* × *S. chilense*) since false positives were reported in screenings for root-knot nematode resistance. In this paper, we evaluate the reliability of 4 available PCR-based molecular markers defined by primer pairs: Mi23, PM3, PMi, and intron 1, for detection of resistance mediated by *Mi-1.2* gene and characterization of the *Mi*-locus in tomato hybrid rootstocks. Primers annealing to the *Mi-1.2* gene, Mint-up/do, C1/2, C2S4, IMO-F1/R1 or VIGS-F were assessed in silico for their ability to anneal to known *Mi*-homologs (MiGHs) in *S. lycopersicum* and *S. peruvianum* genomes. Markers PM3-Fb/Rb and Pau-Do, could distinguish the *Mi-1.2* gene among the *S. peruvianum* and *S. lycopersicum* MiGHs, although PM3-Fb/Rb could detect the *Mi-1.2* resistance gene in *S. lycopersicum* × *S. habrochaites* hybrid rootstocks but not in *S. lycopersicum* × *S. chilense* hybrids. Pau-Do, in combination with C2S4, was successfully used to determine *Mi-1.2* gene expression in *S. lycopersicum* × *S. habrochaites* hybrids. The markers specifically designed for tomato rootstocks (PMi-F3/R3 and Mi23) could not determine the allelic condition of the *Mi-1* locus as both amplified *Mi-1.2-like* bands in susceptible tomato cultivars (*S. lycopersicum*).

Key words: Beaufort, Maxifort, Meloidogyne spp., root-knot nematodes, Solanum spp.

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Introduction

The introduction of new agronomic traits into tomato (*Solanum lycopersicum; Sl*) cultivars from wild *Solanum* species started early in the 20th century (Osborn et al., 2007). Breeding efforts have mainly focused on the acquisition of genes that confer resistance to various pathogens and pests including fungi, viruses and nematodes. In this process, several disease resistance genes have been introduced to tomato and mapped to the short arm of chromosome 6. *Ol-1* and the *Am* genes that confer resistance to powdery mildew (*Oidium lycopersicum*) (van der Beek et al., 1994) and most strains of *alfalfa mosaic virus* (AMV) (Parrella et al., 2004), respectively, were introduced from *S. habrochaites*, *Cf-2* and *Cf-5* for resistance to *Cladosporium fulvum* was obtained from *S. pimpinellifolium* and *S. lycoperscium var. cerasiforme*, respectively (Dixon et al., 1998); *Ty-1* and *Ty-3* introgressed from *S. chilense* conferring resistance to *tomato yellow leaf curl virus* (TYLCV) (Ji et al., 2007)*;* and the *Mi-1.2* gene, from *S. peruvianum* (*Sp*) conferring resistance to root-knot nematodes (*Meloidogyne arenaria, M. incognita*, and *M. javanica*) (Roberts and Thomason, 1986) from *S. peruvianum* (Smith, 1944).

The introduction of the *Mi-1.2* gene had a high impact on this crop which is currently the only commercially available source of resistance to root-knot nematodes in tomato. Plant resistance against root-knot nematodes is an economical alternative to soil fumigation (e.g. methyl bromide) that allows cultivation of resistant tomato in nematode-infested fields without significant yield losses (Sorribas et al., 2005). The resistance gene (*R* gene) *Mi-1.2* is a member of the plant *R* gene family encoding proteins with nucleotide-binding site and leucine-rich repeat motifs (Milligan et al., 1998). In resistant tomato, seven *Mi-1.2* gene homologs (MiGHs), all located on the short arm of chromosome 6, are from the *S. peruvianum* introgression and are organized into two different clusters: cluster 1p (*Mi-1.1*, *Mi-1.2* and *Mi-1.3)* and cluster 2p (*Mi-1.4* to *Mi-1.7)* (Seah et al., 2007a). In susceptible tomato, the seven MiGHs are also organized in two clusters: 1e (*Mi-1E*, *Mi-1F* and *Mi-1G)* and 2e (*Mi-1A*, *Mi-1B*, *Mi-1C* and *Mi-1D)* (Seah et al., 2007a). Despite the high homology among these 14 MiGHs*,* only *Mi-1.2* has been shown to confer resistance to root-knot nematodes (Milligan et al., 1998). Additional root-knot nematode resistance genes have been identified in a number of accessions of wild *Solanum* species such as *Mi-2, Mi-3, Mi-4*, *Mi*-5, *Mi-6*, *Mi-7* and *Mi-8* from

S. peruvianum (Yaghoobi et al., 1995; Veremis and Roberts, 1996a, 1996b; Ammiraju et al., 2003) and *Mi-9* from *S. arcanum* (Jablonska et al., 2007).

As pathogenicity assays are time consuming and require specialized skills, molecular marker analysis evolved rapidly as a reliable tool in tomato breeding programs to monitor the transfer of genes from wild *Solanum* accessions into commercial tomato cultivars. In tomato, root-knot nematode resistance has been traditionally traced either with the isozyme acid phosphatase (*Aps-1*) (Rick and Forbes, 1974) or the co-dominant PCR marker REX-1 (Williamson et al., 1994). REX-1 is located on the short arm of chromosome 6 and is tightly linked to the *Mi-1* locus (Kaloshian et al., 1998) and the reliable results obtained with this marker made it widely used in most tomato breeding programs (Osborn et al., 2007). Because this marker was designed to detect the introgression of the region of the short arm of chromosome 6 containing the *Mi-1* locus from a specific accession of *S. peruvianum*, its use to detect the presence of root-knot nematode resistance originating from other *Solanum* species is questionable. El Mehrach et al. (2005) reported false positives when REX-1 was used in screens for root-knot nematode resistance in begomovirus resistant tomatoes with introgressions from *S. habrochaites* and *S. chilense*. In a bioassay performed to determine root-knot nematode resistance in commercial and experimental tomato rootstocks, the hybrids Beaufort and Maxifort (both hybrids: *S. lycopersicum* × *S. peruvianum* × *S. habrochaites*), responded as susceptible to two a *Mi-1.2* avirulent population of *M. javanica*, in disagreement with the REX-1 genotyping data (Cortada et al., 2008, 2009). As this result was initially interpreted as a possible false positive, the co-dominant SCAR-PCR marker Mi23 was used to confirm the genotypes of these two rootstocks. Mi23 was designed for tomato hybrid rootstocks (Seah et al., 2007b) and despite being successfully tested with several wild *Solanum* species (Seah et al., 2007b) it was also unable to detect the susceptible phenotypes of Beaufort and Maxifort (Cortada et al., 2009). The proximity of the Mi23 marker to the *Mi-1.2* resistance gene, and the low recombination rate in the *Mi* locus, suggests that the *Mi-1.2* gene is present in Beaufort and Maxifort. Since the genetic background of these rootstocks included a *Solanum* species other than *S. peruvianum*, it is uncertain whether the intended resistance source was the *Mi-1.2* gene or a distinct MiGH.

The purpose of this work was to investigate the presence of the *Mi-1.2* resistance gene in tomato hybrid rootstocks Beaufort and Maxifort. We evaluated available PCR markers that amplify the *Mi-1.2* gene or are tightly linked to it and have been specifically designed to detect root-knot nematode

resistance in hybrid tomato rootstocks, to determine their utility for marker-assisted selection in tomato breeding programs. Bioinformatics and PCR analyses were performed with tomato cultivars, rootstocks and wild *Solanum* species and accessions. Nematode pathogenicity assays were also performed in order to determine the resistance phenotype of the wild *Solanum* species analyzed.

Materials and methods

Plant material and growth conditions

Plant materials used for DNA analyses are described in Table 1. Seeds were germinated in seedling trays filled with an organic planting mix (Sun Gro Horticulture) and maintained in a greenhouse at 22 ˚C to 26 ˚C. After germination, seedlings were supplemented with a slow release fertilizer (NPK: 17-7-10; Osmocote® Pro, Sierra Chemical).

PCR-based markers

PCR-based markers were employed for characterization of the *Mi* locus in tomato varieties and wild tomato species (Table 2). These were REX-1, Mi23, PMi and PM3 (Fig. 1a) (Williamson et al., 1994; El Mehrach et al., 2005; Seah et al., 2007a). Intron-1 was also used to fingerprint MiGHs (Fig. 1b) (Jablonska et al., 2007).

Bioinformatics analysis

Bioinformatics analyses were performed to identify the locations of markers linked to the *Mi* locus. The AnnHyb software (http://www.bioinformatics.org/annhyb/) was used for in silico analyses considering that the salts and the primers concentrations of the virtual PCR reaction ranged from 25 to 50 mM and from 250 to 1000 nM, respectively. In addition, analysis was performed with primers C1/2, C2S4 (Milligan et al., 1998), C1/2Do (Martínez de Ilarduya and Kaloshian, 2001), IMOF1, IMOR1 (Bendezu, 2004), and VIGS-F (Li at al., 2006) designed to amplify *Mi-1.2* (Fig.

1b). Anaylsis tested whether these primers could also detect in silico MiGHs from resistant tomato cultivar Motelle (introgression from the *S. peruvianum*, *Sp*) and from susceptible tomato cultivar Heinz-1706 (*S. lycopersicum*, *Sl*) (Seah et al., 2007a).

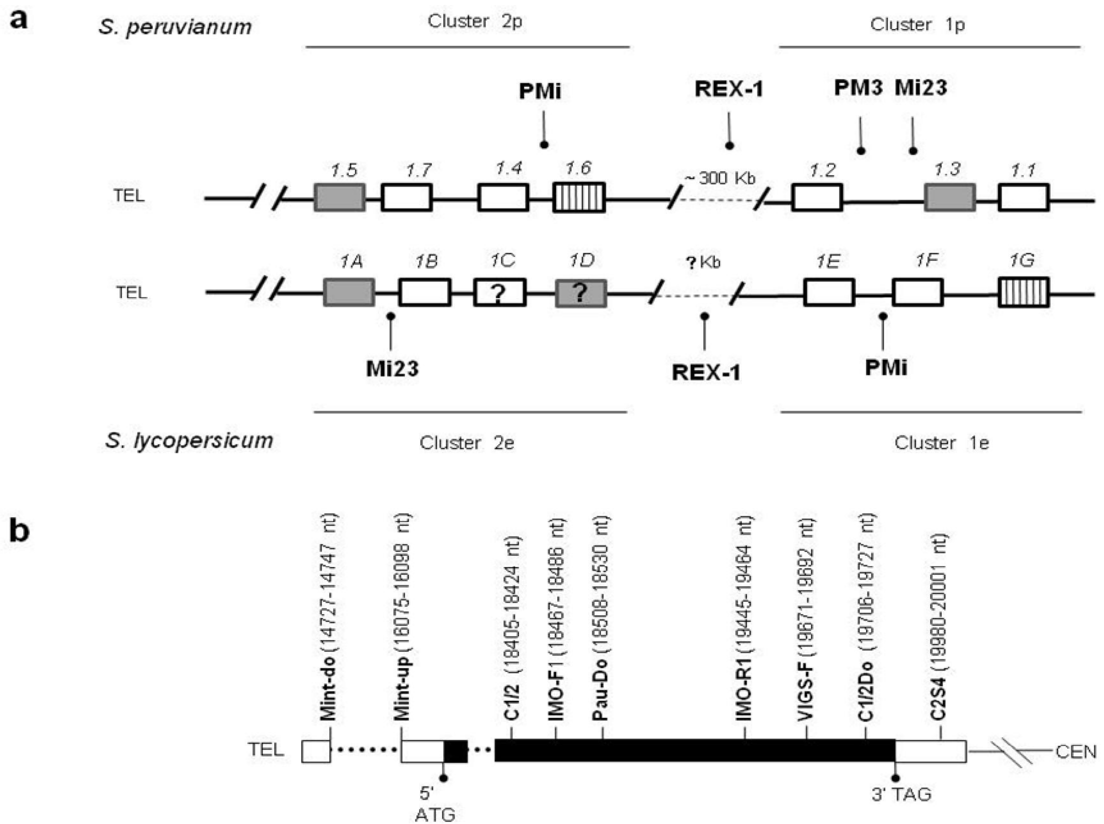

Figure 1. (a) Location of PCR markers on the short arm of chromosome 6 of *Solanum peruvianum* and *S. lycopersicum*. MiGHs are shown as white boxes, pseudogenes as grey boxes and truncated genes in hatched boxes. In cluster 2e, the order between 1C and 1D is not resolved. Figure adapted from Seah et al. 2007a. **(b)** Location of primers in the *Mi-1.2* resistant gene. In parenthesis, the nucleotide position of each primer is shown, according to the *Mi-1.2* genomic sequence obtained from Genbank accession U81378. Solid bars show exons, dotted line show introns, and the white bar represents the 3' and 5' untranslated regions of the gene.

Table 1 Characteristics of the tomato cultivars and hybrid rootstocks used for DNA analyses.

Solanum species	Root-knot nematode resistance genotype[a]	TYLCV tolerance[b]	References of resistance phenotype to root-knot nematodes
S. lycopersicum			
Heinz 1706, Moneymaker, Durinta, UC82B, Pearson[c], Pixie[c], Castlemart[c], Ailsa Craig[c], Chatham[c]	mi/mi	ty/ty	Seah et al., 2007a; Williamson et al., 1994; Cortada et al., 2008, 2009
S. lycopersicum × S. peruvianum			
VFN, Motelle	Mi/Mi	ty/ty	Williamson et al., 1994, Cortada et al. 2008, 2009
Caramba, Monika	Mi/mi	ty/ty	
S. lycopersicum × S. peruvianum × S. habrochaites			
Brigeor, Heman, Beaufort, Maxifort	Mi/Mi or Mi/mi	ty/ty	Cortada et al. 2008, 2009
S. lycopersicum × S. peruvianum × Solanum sp[d]			
Gladiator, Big Power	Mi/Mi or Mi/mi	ty/ty	Cortada et al. 2008, 2009
PG-76[e], MKT-410[e], 42851[e], 43965[e]	Mi/Mi or Mi/mi	unknown	
S. lycopersicum × S. chilense			
Tyrmes	mi/mi	Ty/Ty or Ty/ty	Cortada et al., 2008
S. chilense			
LA-1932, LA-1959, LA-2746	unknown	unknown	This paper
LA-1969	unknown	Ty/Ty or Ty/ty[f]	
S. habrochaites			
LA-3864	unknown	unknown	This paper
LA-0386, LA-1777	unknown	Ty/Ty or Ty/ty[g]	
S. peruvianum			
LA-1336	unknown	unknown	This paper
PI 270435-2R2, PI270435-3MH	Mi/M[h]	unknown	Veremis and Roberts 1996a, 1996b

[a] Mi/Mi: homozygous resistant; Mi/mi: heterozygous resistant; mi/mi: homozygous susceptible. [b] Information on tolerance to Tomato yellow leaf curl virus (TYLCV) obtained from seed companies; TY/TY: homozygous tolerant, Ty/ty: heterozygous tolerant; ty/ty: homozygous susceptible. [c] Susceptible phenotype to root-knot nematodes is Kaloshian I, unpublished data. [d] Tomato hybrid rootstocks with unknown parental species (Solanum sp.). [e] Experimental rootstocks. Information about resistance to root-knot nematodes has been obtained from seeds companies. [f] Information obtained from the Tomato Genetics Resource Center (TGRC), University of California, Davis (http://tgrc.ucdavis.edu/). [g] Vidavsky and Czosnek, 1998. [h] Clones PI 270435 2R2 present the root-knot nematode resistant homologs Mi-1, Mi-2 and Mi-8 and clone 3MH Mi-1, Mi-6 and Mi-7 (Veremis and Roberts, 1996a, 1996b).

Table 2 Primers used and products amplified in PCR or predicted using bioinformatics analysis in resistant tomato with *Solanum peruvianum* introgression or susceptible tomato *S. lycopesicum*.

Marker	Primer	Sequence (5'- 3')	Marker type	Amplified product (bp)		Reference
				Resistant	Susceptible	
REX1	REX-F1 REX-R2	TCGGAGCCTTGGTCTGAATT GCCAGAGATGATTCGTGAGA	co-dominant	570, 160[a]	730[a]	Williamson et al., 1994
Mi23	Mi23F Mi23R	TGGAAAAATGTTGAATTTCTTTTG GCATACTATATGGCTTGTTTACCC	co-dominant	380	430	Seah at el., 2007b
PMi	PMiF3 PMiR3	GGTATGAGCATGCTTAATCAGAGCTCTC CCTACAAGAAATTATTGTGCGTGTGAATG	co-dominant	550	350	El Mehrach et al., 2005
PM3	PM3-Fb PM3-Rb	CACACATGAGGTATGTTCGTATTATGG TCACAGCCTAGCTTTTGAATCAGTACC		500	no product	El Mehrach et al., 2005
Intron-1	Mint-do Mint-up	TTCTCTAGCTAAACTTCAGCC TTTTCGTTTTTCCATGATTCTAC	co-dominant	622 (Mi-1.1)[b] 1372 (Mi-1.2) 1186 (Mi-1.4)	1353 (Mi-1.C)[b] 981 (Mi-1.F)	Jablonska et al., 2007

[a] After restriction with *Taq*I enzyme.
[b] Based on bioinformatics analysis, DNA size equivalent to the respective MiGHs according to NCBI sequence information of accessions no. U81378, DQ863286, DQ863287, DQ863288, DQ863289, DQ863290, DQ863291, DQ863292, DQ863293 (Seah et al., 2007a).

DNA isolation and PCR

DNA extraction was carried out according to Cortada et al. (2008). The amplification conditions for intron-1 is described by Jablonska et al. (2007); REX-1 by Williamson et al. (1994); PM3 and REX-1 multiplex-PCR and PMi by El Mehrach et al. (2005), and Mi23 by Seah et al. (2007b). The *ubiquitin-3* (*Ubi-3*) PCR conditions were: 95 ˚C for 3 min followed by 35 cycles of 30 s at 95 ˚C, 1 min at 58 ˚C and 30 s at 72 ˚C, followed by 5 min at 72 ˚C. For all PCR reactions, 20 *μ*L of the PCR products were resolved on 1.5% agarose gel in 1×TAE buffer, except for the intron-1 reactions where 45 *μ*L were resolved in the same conditions.

RNA isolation and RT-PCR

RNA from leaf tissues was extracted using a hot phenol protocol (Bhattarai et al., 2007) and extractions from roots were performed according to Lambert et al. (1999). RNA samples were treated with *RQ1* RNase-free DNase (Promega) and followed by phenol/chloroform extraction. For cDNA synthesis, 3.5 to 5 *μ*g of DNase treated RNA was used with oligo$(dT)_{20}$ primer and ThermoScript™ reverse transcriptase (Invitrogen) according to manufacturer's recommendation. To amplify *Mi-1.2*, PCR was carried out in 25 *μ*L volume using 1 *μ*L of cDNA template, except for Beaufort leaves where 4 *μ*L was used. Primers Pau-Do (5'-CCTTTGACAATCTATTTG TTGAC-3') and C2S4 (5'- CTAAGAGGAATCTCATCACAGG-3') (Milligan et al. 1998) were used (Fig. 1b).

Amplification conditions were 95 ˚C for 5 min followed by 30 cycles of 1 min at 94 ˚C, 1 min at 61 ˚C and 72 ˚C for 2 min, followed by 8 min at 72 ˚C. Tomato *Ubi3* gene was used as internal control for PCR. The amplified products were resolved on 1.5% agarose gel in 1×TAE buffer.

Nematode screens

Cuttings from tomato accessions (Table 1) and cultivars Monika (resistant) and Durinta (susceptible) were treated with Rizhopon AA growing hormone (3- indolbutiric acid at 1%; Rizhopon) and rooted in seedling trays in vermiculite. Each accession was replicated eight times and rooted cuttings of

the commercial tomato cultivars Monika (resistant) and Durinta (susceptible) were included as controls. Cuttings were maintained in a growth chamber at 25 ˚C until new leaves were produced. Four-week-old rooted cuttings were transplanted singly into 500 cm^3 pots containing a mixture of steam-sterilized river sand and peat (v/v) and used for nematode assays a week later. *Mi*-avirulent nematode inoculum was obtained from infected tomato (cv. Roma) roots collected from pot cultures maintained in a glasshouse. The identity of the *M. javanica* population was confirmed by SCAR-PCR using primers Fjav (5'-GGTGCGCGATTGAACTGAGC-3') and Rjav (5'-CAGGCCCTTCAG TGGAACTATAC-3') (Ziljstra et al., 2000). Roots were macerated in a 0.5% NaOCl solution and eggs collected (Hussey and Barker, 1973). Infective second-stage-juveniles (J2) were obtained from hatched eggs as described by Martínez de Ilarduya et al. (2001). Five hundred J2, collected after 72 h, were used as inoculum per plant.

Plants were maintained in a growth chamber at 20 ˚C- 22 ˚C temperature until the first nematode generation was completed determined by the informatics application GENERA (Ornat et al., 2008; https://deab.upc.edu/genera). Plants were fertilized with a slow-release fertilizer (Osmocote®, Sierra Chemical). At harvest, root systems were washed free of soil and weighed. The number of eggs per plant was determined by extracting the entire root system in NaCOl as described for the nematode inoculum. Eggs were expressed per gram of fresh root. An accession was considered resistant when the number of eggs per gram of root did not differ statistically from the resistant cultivar Monika.

Statistical analyses

The general linear model procedure of the Statistica (StatSoft, Inc. 2004) software was used for statistical analysis. The variable "number of eggs per g of root" was transformed into cubic root to comply with test assumptions, and then subjected to analysis of variance. The Dunnett studentized range test was used to compare means of each *Solanum* accessions to resistant control cv. Monika when the One-way ANOVA analysis was significant ($P <0.05$).

Results

Bioinformatics analysis

Since the development of the markers used in this study, substantial progress has been made in sequencing the chromosomal regions that contain MiGHs from both resistant (with *Sp* introgression) and susceptible tomato (Seah et al. 2007a). We have used in silico bioinformatics analysis to determine potential amplification of a product using the primer pairs for markers (Mi23, PMi, PM3) on the short arm of chromosome 6 where the MiGHs reside.The locations of the amplified products are indicated in relation to MiGHs in figure 1b for both the *Sp* introgressed region and the corresponding *Sl* region. Our bioinformatics analysis indicates that Mi23 primers could anneal to a region in clusters 2e and 1p in *Sl* and *Sp*, respectively (Fig. 1a). Similarly, PMi primers could anneal to a region in clusters 1e and 2p in *Sl* and *Sp*, respectively. The PM3 primers could anneal only to a region in cluster 1p in *Sp* (Fig. 1a).

Nematode bioassays

Root-knot nematode resistance phenotypes of all rootstocks and tomato cultivars used in this study was previously determined (Table 1). The resistance phenotype was only known for two of the wild *Solanum* species and the rest were assayed for nematode susceptibility. Nematode reproduction on wild *Solanum* accessions was significantly different ($P < 0.05$) from that on the resistant cultivar Monika (Fig. 2) and therefore they were considered susceptible.

PCR marker analysis

Previously, REX-1 marker (Fig. 1a) analysis of the rootstocks used in this study showed that all were *Sp* homozygous, except for MKT-410, which was heterozygous, both profiles indicating resistant genotypes even though a subset of these rootstocks were root-knot nematode susceptible (Cortada et al., 2008). Similarly, it was shown that the Mi23 marker could not distinguish

between nematode resistant and susceptible rootstocks. Although REX-1 has been tested in numerous tomato cultivars and shown clear polymorphism between *Sl* and *Sp* alleles and nematode resistance introgression, Mi23 has not been extensively tested with tomato cultivars.

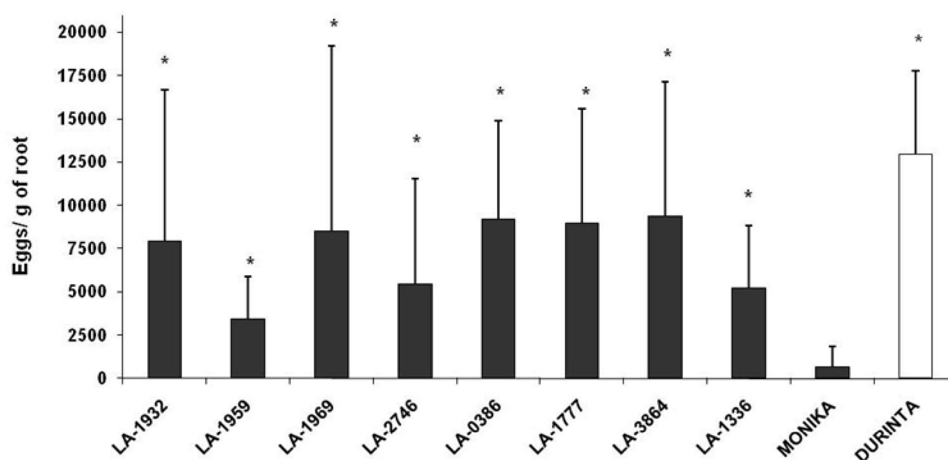

Figure 2. Reproduction of a *Mi-1* avirulent population of *Meloidogyne javanica* on *Solanum species*. *Solanum chilense* (LA-1932, LA-1959, LA-1969, LA-2746), *S. habrochaites* (LA-0386, LA-1777, LA-3864), *S. peruvianum* (LA-1336), tomato resistant cultivar Monika, and susceptible cultivar Durinta. Displayed values show back-transformed mean ± standard deviation of eight replicated plants except for *S. chilense* accessions LA-1959, LA-1969, and LA-2746 that had 6, 4 and 7 plants, respectively. Stars indicate significant differences between a *Solanum* accession and the resistant control Monika, according the Dunnett's t test ($P < 0{\cdot}05$).

Using Mi23 primers for PCR on tomato cultivars amplified a 380 bp fragment from nematode resistant tomato and a 430 bp fragment from most susceptible tomato cultivars (Fig. 3a). However, both fragments were amplified from two nematode susceptible tomato cultivars (Fig. 3a, lanes 11-12) indicating that this marker also cannot distinguish between resistant and susceptible tomato cultivars. The inability of this marker to discern between root-knot nematode resistance and susceptibility was further evident when Mi23 was tested with *S. chilense*, *S. habrochaites* and *S. peruvianum*, species that constitute one or more parent of the tomato rootstocks (Table 1). Although this marker could distinguish between root-knot nematode resistant and susceptible *S. peruvianum* accessions (Fig. 3b, lanes 9-10), no consistent

profile linked to nematode resistance was identified among the other *Solanum* species (Fig. 3b).

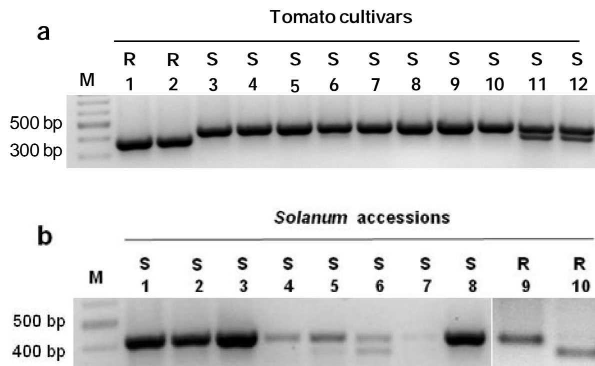

Figure 3. Mi23 marker profile in tomato cultivars and *Solanum* species. **a** Lanes: (1) VFN; (2) Motelle; (3) Heinz-1706; (4) Moneymaker; (5) Durinta; (6) UC82; (7) Castlemart;(8) Pearson; (9) Pixie; (10) Castlemart; (11) Ailsa Craig; (12) Chatham. **b** Lanes: (1) LA-1932; (2) LA-1959; (3) LA-1969; (4) LA-2746; (5) LA-0386; (6) LA-1777; (7) LA-3864; (8) LA-1336; (9) PI 270435 clone 2R2; (10) PI 270435 clone 3MH. R indicates resistance and S susceptibility to root-knot nematodes according to Ammati et al. (1986), Kaloshian unpublished, or this study. Lane M is 100 bp DNA ladder.

We also tested PMi marker with tomato cultivars, rootstocks and wild tomato accessions. The primers PMiR3/F3 amplified about 530 bp fragment from the *Sp* resistant allele (Fig. 4a, lane 15) and 350 bp fragment from the *Sl* susceptible allele (Fig. 4a, lane 14). Both DNA fragments were amplified from the heterozygous cultivars (Fig. 4a, lanes 11-12). The susceptible hybrid cultivar Tyrmes (*S. lycopersicum* × *S. chilense*, Fig. 4a, lane 13) gave a distinct banding pattern, which included the *Sl* susceptible allele in addition to two (450 bp and 600 bp) distinct fragments. All the fragments amplified from the hybrid rootstocks were similar to those found in the commercial cultivars. Two of the susceptible rootstocks amplified a fragment similar to the *Sp* resistance allele (Fig. 4a, lanes 9–10), indicating that PMi cannot distinguish between resistant and susceptible hybrid rootstocks. Furthermore, screening the accessions from several *Solanum* species indicated that PMi is highly polymorphic (Fig. 4b) and cannot distinguish between resistant and susceptible accessions (Fig. 4b, lanes 8-9).

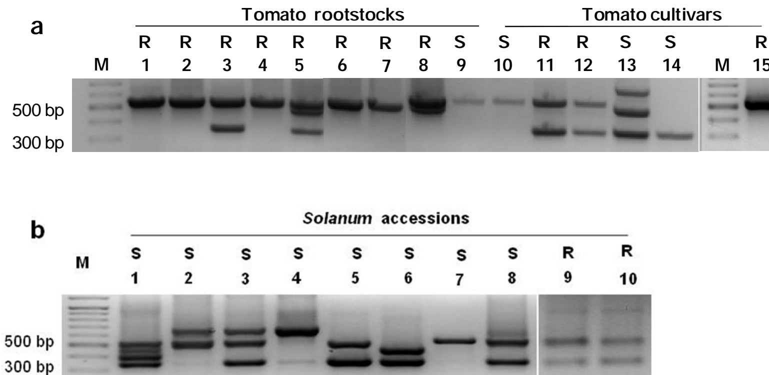

Figure 4. PMi marker profile in tomato hybrid rootstocks, cultivars and in *Solanum* species. **a** Lanes: (1) PG-76; (2) Gladiator; (3) MKT-410; (4) Brigeor; (5) 42851; (6) 43965; (7) Big Power; (8) Heman; (9) Beaufort; (10) Maxifort; (11) Caramba; (12) Monika; (13) Tyrmes; (14) Durinta; (15) VFN. **b** Lanes: (1) LA-1932; (2) LA-1959; (3) LA-1969; (4) LA-2746; (5) LA-0386; (6) LA-1777; (7) LA-3864; (8) LA-1336; (9) PI 270435 clone 2R2; (10) PI 270435 clone 3MH. R indicates resistance and S susceptibility to root-knot nematodes according to Ammati et al. (1986), Cortada et al. (2008), commercial sources or this study. Lane M is 100 bp DNA ladder.

Primers PM3-Fb/Rb amplified a 500 bp fragment from the *S. peruvianum* introgression (*Sp-PM3*-locus) located in close proximity 3'of *Mi-1.2* (Fig. 1). Since this primer pair does not amplify a fragment in tomato cultivars without wild species introgressions, it is designed for use in multiplex reactions with REX-1 primers (El Mehrach et al., 2005). A multiplex-PCR reaction was performed to amplify both PM3 and the REX-1 markers. The multiplex-PCR amplified a 720 bp fragment representing REX-1 in all the tomato rootstocks and cultivars tested (Fig. 5a). Similarly, a 500 bp fragment was amplified representing PM3 in all rootstock and cultivars except in the susceptible cultivar Durinta (Fig. 5a, lane 14). Surprisingly, a 500 bp fragment was also amplified from the susceptible cultivar Tyrmes (Fig. 5a, lane 13). In addition, analysis of susceptible accessions from *S. chilense* with PM3 primers amplified the 500 bp fragment in three out of four accessions tested (Fig. 5b, lanes 2-4). There was no amplification of this marker in *S. habrochaites* as expected from susceptible phenotypes (Fig. 5b, lanes 5-7) however it was also not amplified from two resistant accessions of *Sp* (Fig. 5b, lanes 9-10). The REX-1 marker did not amplify from the *S. habrochaites* accessions (Fig. 5b, lanes 5-7), although Ubi3 amplified

successfully from the same DNA source (Fig. 5c), indicating that REX-1 is not present in these accessions.

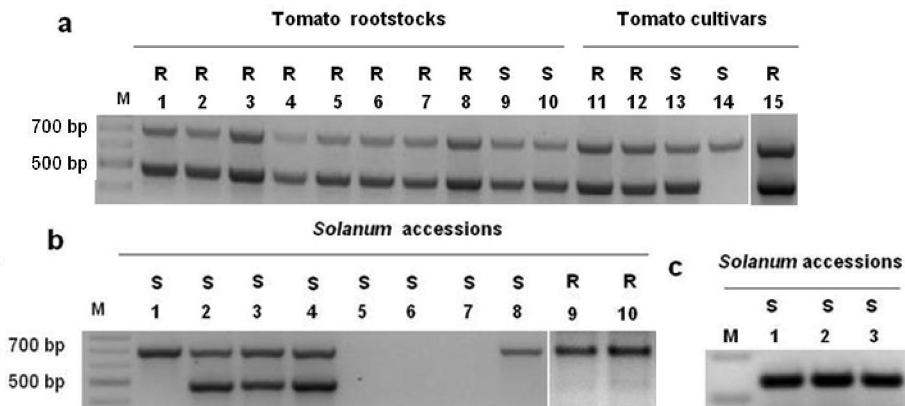

Figure 5. Multiplex PCR of PM3 and REX-1 markers of tomato hybrid rootstocks, cultivars and *Solanum* accessions. **a** Lanes: (1) PG-76; (2) Gladiator; (3) MKT-410; (4) Brigeor; (5) 42851; (6) 43965; (7) Big Power; (8) Heman; (9) Beaufort; (10) Maxifort; (11) Caramba; (12) Monika; (13) Tyrmes; (14) Durinta; (15) VFN. **b** Lanes: (1) LA-1932; (2) LA-1959; (3) LA-1969; (4) LA-2746; (5) LA-0386; (6) LA-1777; (7) LA-3864; (8) LA-1336; (9) PI 270435 clone 2R2; (10) PI 270435 clone 3MH. **c** Amplification of tomato *Ubi3* gene in *S. habrochaites* accessions. Lanes: (1) LA-0386; (2) LA-1777; (3) LA-3864. R indicates resistance and S susceptibility to root-knot nematodes according to Ammati et al. (1986), Cortada et al. (2008), commercial sources or this study. Lane M is 100 bp DNA ladder.

Fingerpriting of MiGHs

To identify MiGHs in the tomato rootstocks and cultivars, PCR was used to amplify the intron-1 region (Fig. 1b). Amplification of intron-1 from tomato rootstocks and cultivars produced a highly diverse range of fragments (Fig. 6) indicating the presence of different MiGHs. Resistant tomato rootstocks and cultivars have at least a single large fragment (1,400 bp) in common which is not seen in the susceptible cultivars (Fig. 6a and 6b). However, this large fragment is present in the susceptible rootstocks Beaufort and Maxifort (Fig. 6a, lanes 9-10). It is interesting to note that susceptible tomato cultivars, with no nematode resistance introgression, have also variable MiGHs.

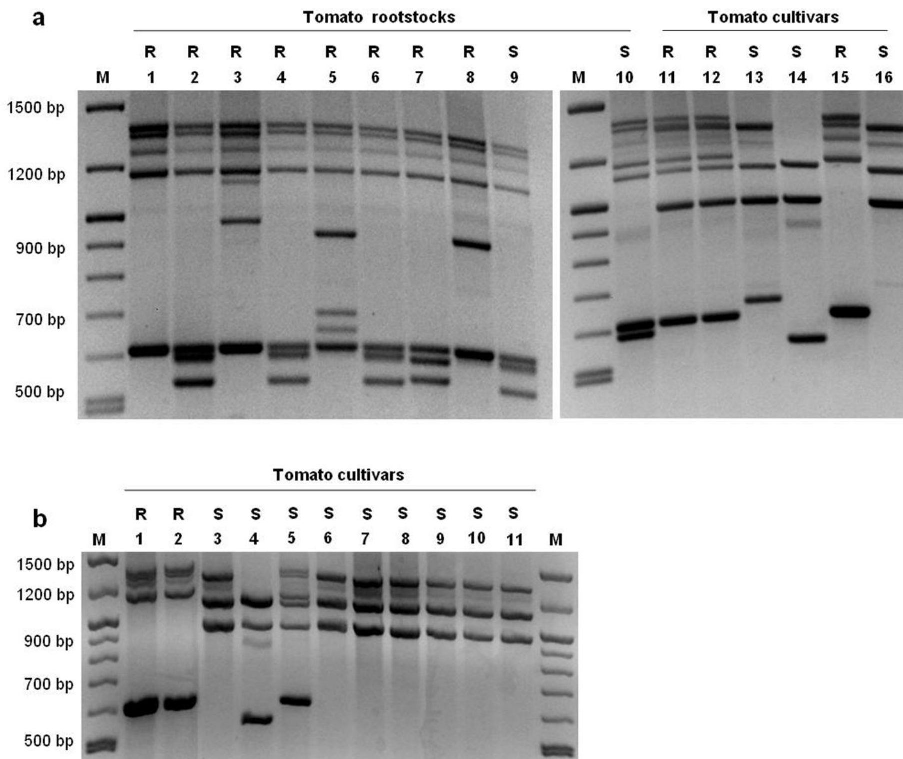

Figure 6. *Mi* intron 1 profile of tomato hybrid rootstocks and cultivars. Primers Mint-up and Mint-do were used in PCR to amplify intron 1. **a** Lanes: (1) PG-76; (2) Gladiator; (3) MKT-410; (4) Brigeor; (5) 42851; (6) 43965; (7) Big Power; (8) Heman; (9) Beaufort; (10) Maxifort; (11) Caramba; (12) Monika; (13) Tyrmes; (14) Durinta; (15) Motelle; (16) Moneymaker. **b** Lanes: (1) VFN; (2) Motelle; (3) Moneymaker; (4) Durinta; (5) UC82; (6) Castlemart; (7) Pearson; (8) Pixie; (9) Castlemart; (10) Ailsa Craig; (12) Chatham. R indicates resistance and S susceptibility to root-knot nematodes according to Cortada et al. (2008), Kaloshian I, unpublished, or commercial sources. Lane M is 100 bp DNA ladder.

Expression of *Mi-1.2* in rootstocks Beaufort and Maxifort

To assess the expression of *Mi-1.2* in rootstocks Beaufort and Maxifort, existing *Mi-1.2* primers, C1/2, C1/2Do, C2S4, IMO-F, IMO-R and VIGS-F, were used in all possible combinations in bioinformatics analysis to determine their specificity. None of these primer combinations were specific to *Mi-1.2* (Table 3). Therefore, a new primer, Pau-Do (Fig. 1b), was designed based on existing sequence information that could amplify only *Mi-1.2* when used in combination with primer C2S4 (Fig. 1b). This predicted *Mi-1.2*

cDNA amplification product was 1,494 bp. PCR was performed with Beaufort and Maxifort root and leaf cDNAs and Pau-Do and C2S4 primers. Resistant tomato cultivar Motelle was used as control for *Mi-1.2* and the expected size fragment was amplified (Fig. 7). A similar size product was amplified from both leaves and roots of Beaufort and Maxifort indicating that *Mi-1.2* is expressed in these rootstocks (Fig. 7).

Table 3. In silico bioinformatics analysis performed with primers C1/2, C1/2Do, C2S4; VIGS-F, IMO-F and IMO-R and the known MiGHs from *Solanum peruvianum* and *S. lycopersicum*. The length of the DNA fragments amplified from the MiGHs by the primer pair combination is indicated.

MiGHs	C1/2 C2S4	C1/2 IMO-R	C1/2D0 C2S4	VIGS-F C2S4	IMO-F IMO-R	IMO-F C2S4
Solanum peruvianum						
Mi-1.1	nm[a]	nm	nm	nm	nm	nm
Mi-1.2	1,579 bp	1,060 bp	296 bp	331 bp	998 bp	1.535 bp
Mi-1.3[b]	nm	nm	nm	nm	nm	nm
Mi-1.4	nm	1,048 bp	nm	nm	nm	nm
Mi-1.5[b]	nm	nm	nm	nm	nm	nm
Mi-1.6	1,579 bp	1,054 bp	284 bp	nm	992 bp	1,517 bp
Mi-1.7	1,581 bp	1,060 bp	280 bp	315 bp	nm	nm
Solanum lycopersicum						
Mi-1.A[b]	nm	nm	nm	nm	nm	nm
Mi-1.B	nm	1,069 bp	nm	nm	nm	nm
Mi-1.C	1,597 bp	1,060 bp	265 bp	331 bp	nm	nm
Mi-1.D[b]	nm	nm	nm	nm	nm	nm
Mi-1.E	nm	1,051 bp	nm	nm	nm	nm
Mi-1.F	nm	nm	nm	nm	989 bp	nm
Mi-1.G	nm	1,060 bp	nm	nm	998 bp	nm

[a] *nm* indicates that there is no match of the primers with the genomic sequence of the MiGHs.
[b] Pseudogenes with a truncated ORF (Seah et al., 2007b).

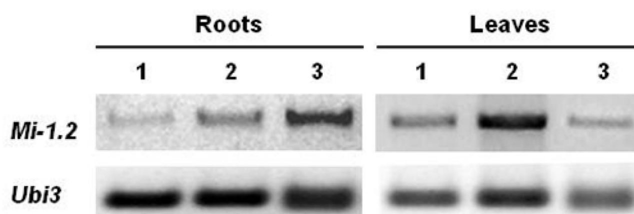

Figure 7. *Mi-1.2* gene expression in tomato cultivar Motelle (lane 1) and rootstocks Beaufort (lane 2) and Maxifort (lane 3). cDNA from leaves and roots were amplified using Pau-Do and C2S4 primers. Tomato *Ubi3* gene was used as an internal control for cDNA. RT-PCR products were resolved in 1 - 1.5% agarose gels and stained with ethidium bromide.

Discussion

The parental species of the resistant tomato hybrid rootstocks Beaufort and Maxifort were not available, as seed breeders refused to share this information. This fact prevented the characterization of the *Mi* locus through marker screening of an F_2 population and the derived F_3 lines. As none of the markers available could determine the presence of the *Mi-1.2* gene in the genome of Beaufort and Maxifort, a new molecular marker was developed. Pau-Do, in combination with C2S4 marker, was used to determine expression of the *Mi-1.2* gene in rootstocks Beaufort and Maxifort. RT-PCR results indicated that the *Mi-1.2* gene is expressed in leaves and roots of both rootstocks, although no quantitative comparative conclusions can be drown from our results as tissues were not coming from the same plants and the amount of DNase-treated total RNA used for RT-PCR varied among samples.

The Mi-1.2 gene confers a broad resistance-spectrum against *Mi*-avirulent isolates of the *M. arenaria*, *M. incognita* and *M. javanica* species, despite some variability has been reported among resistant tomato cultivars (Williamson and Kumar, 2006). This resistant gene has been successfully used to inhibit reproduction of root-knot nematodes populations worldwide since it was introgressed into cultivated tomato (*S. lycopersicum)* (Roberts, 1995). Neither the methylation of the *Mi-1.2* gene (Liharshka, 1998) nor a

mutation of the *R* genes located upstream in the *Mi-1* signal transduction pathway (e.g. *Rme1, Hspo90* or *Sgt1)* (Martínez de Ilarduya et al., 2001; Bhattarai et al., 2007b) were considered as feasible hypothesis; both types of mutation would have caused a complete loss of the functionality of the *Mi-1.2* gene preventing the expression of a resistant response to any root-knot nematode population tested. Thus, the nematode-isolate specific phenotype observed in Beaufort and Maxifort (Cortada et al., 2009) did not correspond to the expected phenotype of a resistant tomato plant that hosts a non-mutated *Mi-1.2* gene in its genome.

One of the hypotheses considered is that the introgression of new genes from *S. habrochaites* might have, somehow, affected the post-transcriptional expression of the *Mi-1.2* gene in Beaufort and Maxifort. The most straightforward prediction of the gene-for-gene model (Flor, 1971) is that NBS-LRR plant *R* proteins recognize a single pathogen *Avr* effector and that recognition involves a direct binding between these two proteins. However, more recent findings are consistent with the so-called *guard model* for *R-Avr* gene interactions. This model predicts that the *R* proteins detect modifications of host proteins (e.g. *Rme1*) targeted by several unrelated effectors rather than the effectors themselves (van der Voosen et al., 2005; Jones and Dangl, 2006). Based on this model, it could be feasible that the hybrid genetic background of Beaufort and Maxifort interacts to the *Avr* effectors of the *Mi*-avirulent nematode isolates tested in a different manner than resistant tomato cultivars to elicit the resistance response.

The second hypothesis considered was that the *Mi-1.2* gene was present in the genome of Beaufort and Maxifort, although it was not functional. A mutation in the sequence of the gene impeded the induction of a conformational change in the NBS-LRR protein required to initiate signaling or resulted in a truncated ORF that prevented resistance expression, as it has been reported for other MiGHs (Hwang and Williamson, 2003; Seah et al., 2007a). Under this statement, it can be speculated that in the absence of a major resistance response conferred by the *Mi-1.2* gene, the underlying resistant phenotype of other MiGH(s) present in the genetic background of both rootstocks was revealed. A nematode-specific resistance response has been reported in several MiGHs (e.g. *Mi-3*, *Mi-7* or *Mi-8*) from *S. arcanum* and *S. peruvianum* (Ammati et al., 1985, 1986; Roberts et al., 1990; Veremis and Roberts; 1996a, 1996b). In other Solanaceous crops like pepper (*Capsicum annuum*), some *Me*-homologs present a resistance response that varies in time and intensity (e.g. *Me1* and *Me3*) or that is nematode isolate-

dependent (e.g. *Me2*, *Me4*, *Me5*) (Bleve-Zacheo et al., 1997; Castagnone-Sereno et al., 2001). The presence of additional homologs to those of the *S. peruvianum* and *S. lycopersicum* species in the genetic background of Beaufort and Maxifort is supported by the genetic profile of intron-1. Individual silencing of the MiGH(s) would be useful to determine the role that each homolog plays in the resistant response of tomatoes to root-knot nematodes (Seah et al., 2007a). Whether the nematode-isolate specific resistance response of Beaufort and Maxifort is linked to the *Mi-1.2* gene or to a MiGH(s) from the *S. habrochaites* genetic background remains unknown. Cloning of the whole gene sequence would be needed for a complete identification of the gene.

The great polymorphism of the MiGHs present in the wild *Solanum* species has been corroborated by the molecular markers tested (Mi23, PMi, and PM3) as they amplified gDNA in all the *Solanum* species analyzed (*S. peruvianum*, *S. lycopersicum*, *S. habrochaites*, and *S. chilense*). Only, the PM3 marker (El Mehrach et al., 2005), specifically designed to trace the *Mi-1.2* gene in tomato hybrid lines, amplified a *Sp-PM3*-like band in the *S. habrochaites* hybrid rootstocks. Analyses with the Mi23 marker indicated that the regions comprised between the *Mi-1.2* and *Mi-1.3* genes in cluster 1p (*S. peruvianum*) and between *Mi-1A* and *Mi-1B* genes in cluster 2e (*S. lycopersicum*) are highly conserved among these two species. Polymorphism was additionally observed in susceptible cultivar Tyrmes (*S. lycopersicum* × *S. chilense*) which showed a PM3-profile different from that described for tomato lines with *S. chilense* introgressions (El Mehrach et al., 2005). The *Sp-PM3*-like fragment amplified in Tyrmes, however, could not be clearly attributed to the introgression of the *Ty-1* locus of *S. chilense* (Vidavsky and Czosnek 1998; Zamir et al., 1994), as similar bands were amplified in accessions LA-1959 and LA-2746 although no tolerance to TYLCV has been reported for these two accessions (TGRC; http://tgrc.ucdavis.edu/). In silico analyses with specific markers for amplification of the *Mi-1.2* gene (C1/2, C1/2Do, C2S4, VIGS-F and IMO-F/R) indicated that they all amplify *Mi-1.2*-like bands in the MiGHs of the *S. lycopersicum* and *S. peruvianum* species. These molecular markers should be hence discarded for molecular analyses, as they lead to detection of false positives in tomato hybrid rootstocks and in resistant tomato cultivars (El Mehrach et al., 2005).

Mint-PCR marker could not determine the presence of *Mi-1.2* gene in Beaufort and Maxifort, although it was able to indicate different parental origin between tomato rootstocks obtained by different parental species (e.g.

Tyrmes *vs.* Brigeor), and differences within species, between different parental accessions (e.g. Beaufort *vs.* Maxifort *vs.* Heman). The distinct Mint-band intron-1 patterns observed in susceptible tomato cultivars revealed a recent and rapid divergence process in *S. lycopersicum*. Although the MiGHs have been classified as a fast evolving type-I of *R* genes (Seah et al., 2007a), the variability observed in the fingerprint of MiGHs in the *S. lycopersicum* cultivars analyzed was unexpected, due to the recently speciation of the *Solanum* genus (Bretó et al., 1993), These new phylogenetic differences were not observed when other PCR markers were used for the characterization of the *Mi*-locus in *S. lycopersicum*.

Despite the positive results obtained with the molecular markers Pau-Do and C2S4 to detect expression of the *Mi-1.2* gene in *S. habrochaites* hybrid lines, a wider pool of accessions from different *Solanum* species should be analyzed. Unfortunately, the idea that we amplified a homolog different from *Mi-1.2* gene from the *S. habrochaites* parental accessions of Beaufort and Maxifort cannot be completely abandoned. It has to be mentioned that the design of the Pau-Do molecular marker was based on the sequences presently available from *Sp* and *Sl* MiGHs. The sequences of all MiGHs from *S. peruvianum* and *S. lycopersicum*, their organization in clusters and their positioning in the short arm of chromosome 6 are recent discoveries (Seah et al., 2007a) that clearly contrast with the limited knowledge about MiGHs from other wild *Solanum* species. Several approaches have been done to map the *R* genes of wild tomatoes, although the high diversity among races and between accessions challenges the characterization of each *Solanum* species (Grube et al., 2000). The unknown sequences of the MiGHs of the wild *Solanum* species and the high inter- and intra-genetic variability of the accessions used in crop breeding programs, also limits the availability of molecular markers to specifically trace a single *R* gene within a wide family of *R* homologs.

If molecular screenings in tomato hybrid rootstocks had not been performed side by side with pathogenicity tests, the *Mi-1.2* gene could be missing in some commercial tomato rootstocks although they would host conserved homologue copies of the *Mi-1* gene from other wild *Solanum* species, which would be susceptible or partially resistant to *Meloidogyne* spp. More exhaustive assays related to the tomato rootstock genome should be performed to clarify this hypothesis. Due to the variability observed among different *Solanum* species, the high polymorphisms of the MiGHs present in the genome of these wild species, and the lack of reliable

molecular markers for the characterization of the *Mi-1* locus, pathogenicity assays cannot yet be substituted by marker-assisted selection in tomato breeding programs.

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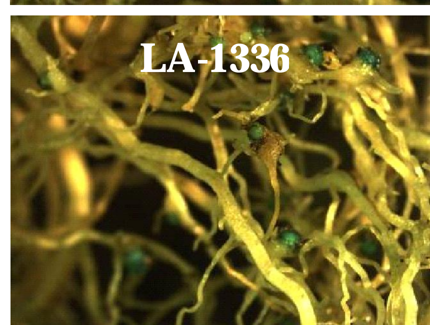
Stained blue egg masses of an avirulent population of *M. javanica* attached at the external surface of the roots of wild *Solanum* accessions of the species *S. chilense* (LA-1932, LA-1959, LA-2746, LA-1969), *S. habrochaites* (LA-3864, LA-0386, LA-1777) and *S. peruvianum* (LA-1336), and to the resistant tomato cultivar (*S. lycopersicum* × *S. peruvianum*) Monika and the susceptible cultivar (*S. lycopersicum*) Durinta.



General view of a plastic house in which field trials for selection of virulent nematode populations where performed during three consecutive years. The soil was artificially infected with an avirulent population of *Meloidogyne javanica.*

4 Selection of virulent populations of *Meloidogyne javanica* by repeated cultivation of *Mi* resistance gene tomato rootstocks under field conditions

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Field trials were conducted in a plastic house artificially infested with an avirulent population of *Meloidogyne javanica* to determine the durability of the resistance mediated by the *Mi* gene in tomato rootstocks after repeated cultivation for three consecutive years. Treatments included an experimental rootstock cv. PG-76 (*Solanum lycopersicum* × *Solanum* sp), a commercial rootstock cv. Brigeor (*S. lycopersicum* × *S. habrochaites*), a resistant tomato cv. Monika (*S. lycopersicum* × *S. peruvianum*); and a susceptible cv. Durinta (*S. lycopersicum*). Based on the reproduction index (RI: number of eggs per g root on the resistant cultivar divided by number of eggs per g root on the susceptible cultivar × 100), rootstock cv. PG-76 responded as highly resistant (RI = 7%) after the first cropping cycle (3.4 nematode generations), showed intermediated resistance (RI = 33%) after the second cropping cycle (3.3 generations), and was fully susceptible (RI = 94%) after the third one (3.3 generations). In contrast, rootstock cv. Brigeor and resistant cv. Monika retained intermediate resistance levels (RI = 41% and 25%, respectively) after the third cropping cycle. Virulent nematode populations were rapidly selected from an avirulent one after repeated cultivation of resistant tomatoes under field conditions. Bioassays conducted under controlled conditions confirmed that selection for virulence occurred more rapidly in plots with cv. PG-76 followed by Brigeor and Monika. The nematode population in the field not exposed to *Mi* resistance remained avirulent to *Mi* genotypes. The genetic background of the resistant rootstocks and the frequency of cropping were critical factors for the appearance of virulent nematode populations. Irrespective of nematode infection, all resistant tomatoes yielded more than the susceptible cultivar.

Key words: durability, root-knot nematodes, Solanum habrochaites, virulence

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Introduction

Root-knot nematodes, *Meloidogyne* spp., are major pests of vegetable crops of intensive agriculture in several areas of the Mediterranean basin including Spain (Ornat et al., 2001, Verdejo-Lucas and Sorribas, 2008). Grafting vegetables is expanding in Europe and has been adopted alone or in combination with other control measures as a non-chemical alternative to soil fumigation, especially in the Mediterranean region (MBTOC, 2006). Within the Solanaceae, tomato (*Solanum lycopersicum*, formerly, *Lycopersicon esculentum*), pepper and eggplant are the most successfully grafted crops. Most tomato rootstocks are interspecific hybrids of *Solanum lycopersicum* × *S. habrochaites* and they incorporate resistances to viruses, fungi and root-knot nematodes. The wild type *S. habrochaites* is primarily used to confer increased vigour to the root system of the grafted plants. These rootstocks improve nutrient absorption (Leonardi et al., 2006), yield and fruit quality under various stress conditions (Fernández-García et al., 2002; Rivero et al., 2003; Estañ et al., 2005).

Disease resistance in plants must be durable, that is, it should provide an efficient protection against the target organism during prolonged and widespread use in environments conducive to disease development (Johnson, 1981). In tomato, resistance to *Meloidogyne incognita*, *M. javanica* and *M. arenaria* is conferred by the *Mi* resistance gene (Roberts and Thomason, 1989) that was identified in the wild relative of tomato *S. peruvianum* and later introgressed into cultivated tomato *S. lycopersicum* (Smith, 1944). This gene has been the source of resistance to root-knot nematodes for more than 40 years in all resistant tomato cultivars worldwide, and may be considered as a very stable resistance gene in terms of durability (Kaloshian et al., 1996; Castagnone-Sereno, 2002). However, virulence, defined as the ability of the nematode to reproduce on a host plant that possesses one or more resistance genes, occurs naturally in *Meloidogyne* populations, apparently without previous exposure to the *Mi* resistance gene (Kaloshian et al., 1996; Ornat et al., 2001), and their frequency of occurrence is increasing in certain regions (Tzorztzakakis et al., 2005).

Virulence in root-knot nematodes can also be selected after repeated exposure to *Mi* resistant plants (Bost and Triantaphyllou, 1982; Jarquin-Barberena et al., 1991, Xu et al., 2001). The selection of virulent nematode populations depends on the nematode genetic composition (Castagnone-Sereno et al; 1994) and the frequency of virulent individuals present in a field

population (Roberts, 2002). The response of resistant tomato rootstocks to root-knot nematodes ranges from highly resistant to fully susceptible (López-Pérez et al., 2006, Cortada et al., 2009). It is assumed that the durability of the *Mi* resistance gene of the rootstocks in the field is as durable as the F_1 hybrid cultivars. However, no information currently exits about the durability of tomato rootstocks in the field. Therefore, it is important to determine if root-knot nematode resistance in tomato rootstocks is durable under field conditions to establish their utility for nematode management.

This paper reports the results of field trials conducted in a plastic house infested with an avirulent *Mi* population of *M. javanica*, to determine the durability of the *Mi* resistance gene in tomato rootstocks after repeated cultivation over three consecutive years. Bioassays were conducted to determine the (a)virulence status of the nematode field population after exposure to the *Mi* resistance gene for one, two or three cropping cycles.

Materials and methods

The study was conducted in an unheated plastic house of 800 m^2 located at Cabrils, Barcelona, Spain. The soil was a sandy loam with 85.8% sand, 8.1% silt and 6.1% clay, pH 8.1, 0.9% organic matter w/w, and 0.40 dS m^{-1} electric conductivity. The soil had been artificially infested with *M. javanica* (code MJ-05) (Ornat et al., 2001) originally isolated from infested soil collected in a plastic house located in Cabrera (Barcelona) in 1994. Single egg-mass cultures were initially established in monoxenic transformed tomato root cultures.

In 1998, the nematode was transferred from monoxenic cultures to potted tomato plants in a glasshouse, to obtain inoculum to infest the field site. Pre-inoculated tomato seedlings were transplanted to the plastic house and allowed to grow for 4 months. Lettuce and susceptible tomato were grown in rotation from 1999 to 2003 in the plots used for this study. In September 2003, the soil of the plastic house was fumigated with methyl bromide, and in spring 2004, the soil was reinfested with the same nematode isolate as before. The identity of *M. javanica* was confirmed by SCAR-PCR (Zijlstra et al., 2000) before the start of this study and at the end of each cropping cycle. The avirulence status of the field *M. javanica* population was confirmed using second-stage juveniles (J2) recovered from the plastic house soil and infecting selected resistant tomatoes.

Twenty plots of 9.6 m^2 (3.5 m long × 2.75 m wide) each were marked in the plastic house. They consisted of four rows with six plants per row spaced 50 cm within the row and 55 cm between rows. Four plant treatments were investigated: (i) the experimental resistant tomato rootstock cv. PG-76 (*S. lycopersicum* × *Solanum* sp., *Mi/Mi*); (ii) the resistant tomato rootstock cv. Brigeor (*S. lycopersicum* × *S. habrochaites*, *Mi/Mi*); (iii) the resistant tomato cv. Monika (*S. lycopersicum* × *S. peruvianum*, *Mi/mi*); and (iv) the susceptible tomato cv. Durinta (*S. lycopersicum*, *mi/mi*). Both rootstocks were selected because they consistently responded as highly resistant and resistant, respectively, to *M. javanica* under glasshouse and field conditions (Cortada et al., 2008). The commercial tomato cultivars cv. Monika (resistant) and cv. Durinta (susceptible) were included as controls. Each treatment was replicated five times in a stratified randomized block design. Tomatoes were cultivated for about 4 months and each plot received the same treatment in 2005 (22 February to 7 July), 2006 (3 March to 10 July) and 2007 (22 March to 7 July). Plots were maintained free of weeds between crops. Rootstocks were grafted with susceptible cv. Durinta in 2006 and 2007, but were left ungrafted in 2005. Grafting was performed by a commercial nursery.

Crop management

Soil preparation was carried out by hand hoeing plots individually to prevent cross contamination from treatments. Plants received water as needed through a drip irrigation system and were fertilized weekly with a solution consisting of NPK (15-5-30) at 31 kg ha^{-1}, together with iron chelate with micronutrients at a rate of 0.9 kg ha^{-1}, respectively. The ingredients were mixed in a tank and delivered through the irrigation system. Tomato plants were vertically trained using canes, and were pollinated by a colony of *Bombus* bees placed into the plastic house at first blossom. After the final tomato harvest, plants were cut at ground level and removed from the plastic house. Weeds were removed manually during and between crops. Soil temperatures were recorded daily at 30 min intervals with temperature probes placed at 15 cm deep. Mean daily soil temperatures are provided in Fig. 1. The number of degree-days accumulated by *M. javanica* was calculated using a base temperature of 13 ˚C and 343 ˚C as the minimum thermal time requirement for one generation (Tzortzakakis and Trudgill, 1996).

Densities of *M. javanica* and evaluation of nematode damage

Composite soil samples were collected at the beginning and at the end of each cropping cycle to estimate initial (Pi) and final (Pf) population densities of the nematode. Individual samples consisted of eight soil cores taken to 30 cm deep with a sampling tube (2.5 cm diameter). Samples were mixed thoroughly and nematodes were extracted from 500 cm^3 soil sub-samples using Baermann trays. Second-stage juveniles were collected 1 week later, concentrated on a 25-μm-aperture sieve, counted and expressed as J2 per 250 cm^3 of soil. Disease incidence was measured as the percentage of plants with galled roots. Disease severity was assessed using the root gall index of tomato plants.

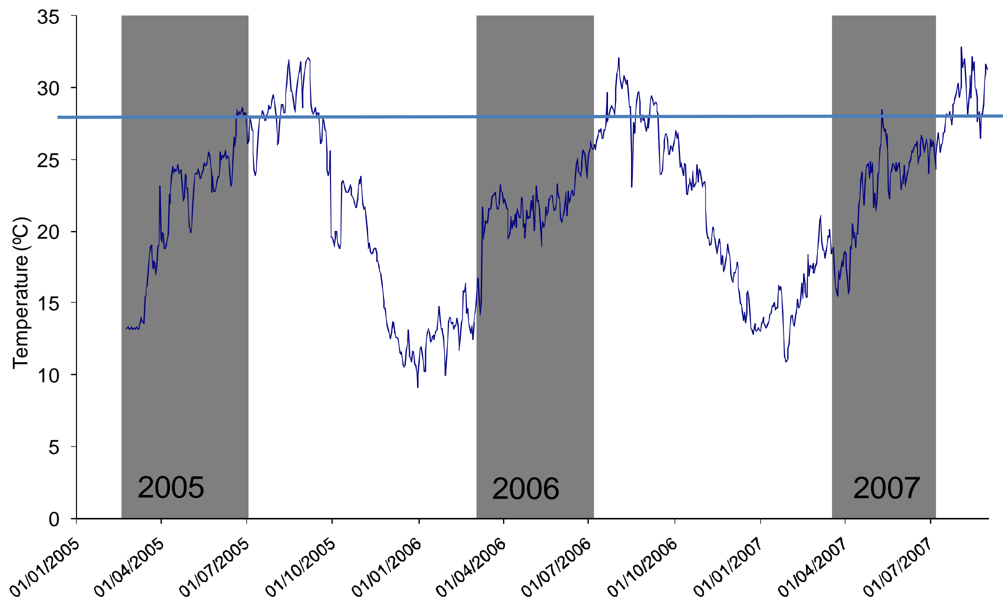

Figure 1. Mean daily soil temperatures at 15 cm depth from 22 February 2005 to 7 July 2007 in a plastic house infested with a *Mi* avirulent population of *Meloidogyne javanica* cultivated with resistant tomatoes containing the *Mi* resistance gene for three consecutive years in a plastic house. Shaded areas indicate the periodof cropping.

Eight plants per plot from the two central rows were dug from the soil, examined, and immediately rated on a scale of 0 to 10, where 0 = a complete and healthy root system and 10 = plants and roots were dead (Zeck, 1971). To determine egg production, roots from the eight plants used for gall rating were bulked, cut into 1 cm-long segments and two 30 g subsamples used to extract eggs by blender maceration in a 0.5% NaOCl solution for 10 min. (Hussey and Barker, 1973). The number of eggs was expressed as eggs g^{-1} of fresh root weight. The reproduction index (RI) of the nematode was calculated as the number of eggs g^{-1} root on the resistant material divided by eggs g^{-1} root on the susceptible material $\times 100$ (Triantaphyllou, 1975). Based on the RI, nematode response was classified as highly resistant (RI 10%), intermediate resistant (10% <RI 50%), or susceptible (RI >50%) (Hadisoeganda and Sasser, 1982).

Testing for virulence

Bioassays were conducted in pots using as inoculum the J2 that had survived in the soil from the preceding crop as inoculum. Each year, three sub-populations of the nematode were generated; one per each resistant material, and called sub-populations P1 (2005), P2 (2006) and P3 (2007) (Fig. 2, Table 1).

Nematode juveniles from plots with susceptible cv Durinta were included as control since they had never been exposed to the *Mi* resistance gene. Tests were conducted as the nematode sub-populations were generated. Each sub-population was inoculated onto a resistant (cv. Monika) or susceptible (cv. Durinta) tomato growing in 1.5 L pots containing steam sterilized sand. To obtain J2 inocula, composite soil samples were collected from the five replicated plots of each plant treatment in March 2006 (J2 P1), 2007 (J2 P2), and 2008 (J2 P3). The soil was bulked, mixed, and J2 recovered from soil using Baermann trays. Tomatoes were inoculated with 350 J2 per plant of the P1 sub-populations, 200 J2 per plant of the P2 sub-populations or 600 J2 per plant of the P3 sub-populations. There were six (P1) or 10 times (P2 and P3) plants per treatment combination. Pots were arranged at random on a bench in an air conditioned glasshouse set at 25˚C. Plants were watered as needed and fertilized with a slow-release fertilizer (15 N + 10 P + 12 K + 2MgO + microelements). The number of eggs g^{-1} root was determined 10 weeks after nematode infestation. Eggs were extracted from roots as described previously. The RI of the nematode was calculated in the same way as the field study.

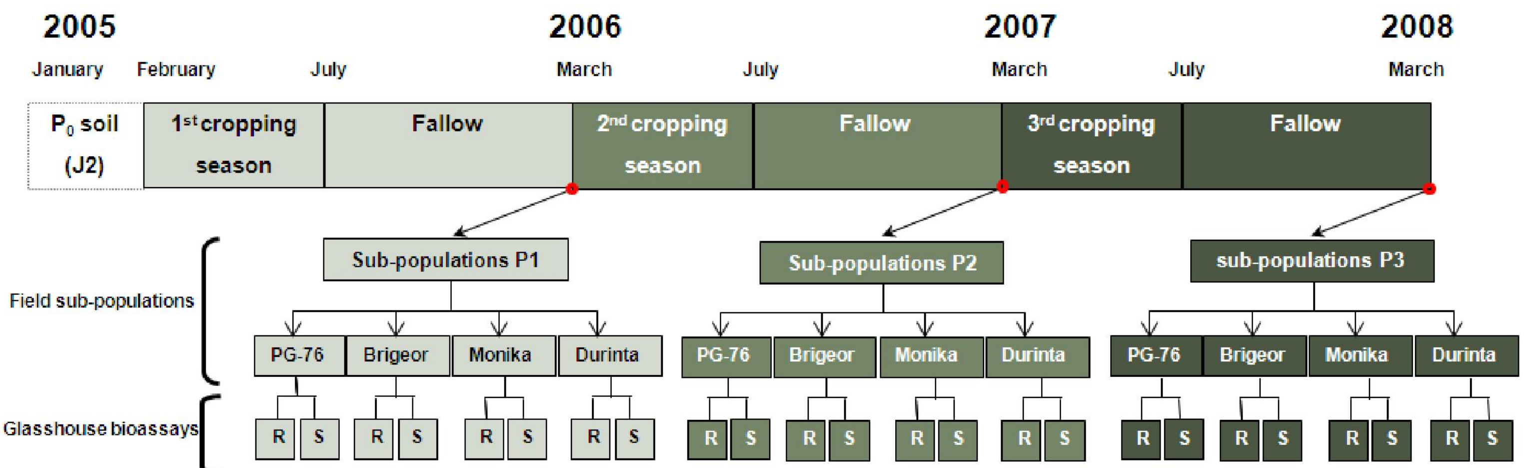

Figure 2. Schematic diagram of the field trials conducted in a plastic house infested with an Mi avirulent population of Meloidogye javanica to determine the durability of Mi resistance gene in tomato rootstocks after repeated cultivation for three consecutive years, and bioassays to test for virulence of the field population after exposure to the Mi resistance gene for one, two or three cropping cycles. R: Mi resistant plants, S: **mi** susceptible plants

Crop yield

To determine tomato yield, fruits produced from eight plants (the same ones used for gall rating and egg extraction) from each plot were harvested once per week from the first six fruit sets. Cumulative yield was expressed as kg m^{-2}.

Statistical analysis

Statistical analyses were performed using the general linear model procedure (Proc GLM) of the SAS software version 8 (SAS institute Inc.). The number of nematodes in soil and eggs g^{-1} root were transformed to $\log_{10}(x + 1)$, and along with data on gall rating and yield of tomato were subjected to analysis of variance. When the overall *F*-test was significant (P <0·05), means were separated by the Least Significant Difference (LSD) method. Data on the RI were transformed to $\log_{10}(x)$ and means were separated by the LSD method.

Results

Meloidogyne javanica completed 3.4, 3.3, and 3.3 generations per cropping cycle in 2005, 2006 and 2007, respectively. In 2005, maximum values exceeded 28 ˚C, and ranged from 28.6 ˚C to 31.3 ˚C (x = 29.9 ˚C) for an average of 12 h per day (range from 9 to 14h) from 21 to 30 June. They were below 28 ˚C in 2006, and maximum values of 28.9 ˚C were recorded for 6h only on 10 May in 2007.

Densities of *M. javancia* and evaluation of nematode damage

Repeated cultivation of the resistant tomatoes did not affect Pi values but there was a progressive increase in Pf values and number of eggs g^{-1} root from one cropping cycle (Fig. 1) to the next (Table 2). On susceptible cv. Durinta, very high levels of the nematodes were observed in soil and roots and did not differ between cropping cycles. With regards to the effect of plant treatment on the

disease parameters, Pf values and eggs g^{-1} root were lower ($P < 0 \cdot 05$) on the resistant tomatoes compared to susceptible cv. Durinta after the first cropping cycle (2005) (Table 2).

Table 1. Number of eggs $g^{-1}$of root on *Mi* resistance gene (R) and susceptible (S) tomato cultivars produced by three sets of sub-populations of *Meloidogyne javanica* generated after exposure to tomatoes containing the *Mi* resistance gene for one (P1), two (P2) or three (P3) cropping cycles to test for virulence in a glasshouse. Tests were conducted as sub-populationswere generated.

Sub-populations and source of J2[a] inoculum	Inoculum (J2 per plant)	Preceding crop	Inoculated cultivar	Eggs g^{-1} root[b]	RI (%)[c]
P1 = Pi 2006	350	PG-76 (R)	Monika (R)	82 ± 89 b	12 ± 13
			Durinta (S)	702 ± 890 a	
		Brigeor (R)	Monika	240 ± 173 a	24 ±17
			Durinta	1020 ± 854 a	
		Monika (R)	Monika	110 ± 111 b	13 ± 13
			Durinta	868 ± 685 a	
		Durinta (S)	Monika	456 ± 564 b	8 ± 10
			Durinta	5832 ± 4172 a	
P2 = Pi 2007	200	PG-76 (R)	Monika	387 ± 258 a	90 ± 38
			Durinta	490 ± 313 a	
		Brigeor (R)	Monika	580 ± 213 b	40 ± 14
			Durinta	1477 ± 527 a	
		Monika (R)	Monika	313 ± 72 b	33 ± 7
			Durinta	976 ± 304 a	
		Durinta (S)	Monika	72 ± 57 b	9 ± 7
			Durinta	816 ± 462 a	
P3 = Pi 2008	650	PG-76 (R)	Monika	2620 ± 1790 a	107 ± 25
			Durinta	2459 ± 886 a	
		Brigeor (R)	Monika	1359 ± 675 a	51 ± 73
			Durinta	2650 ± 1857 a	
		Monika (R)	Monika	1852 ± 1240 a	180 ± 120
			Durinta	1028 ± 557 a	
		Durinta (S)	Monika	288 ± 263 b	11 ± 10
			Durinta	2523 ± 1544 a	

[a] J2= secondstage juveniles of *M. javanica*
[b] Values were transformed to $\log_{10}(x+1)$ before analysis. Values are untransformed means ± standard deviations of 6 (sub-populations P1) or 10 (sub-populations P2 and P3) plants per treatment combination. Means separation within resistant and susceptible tomatoes assessed by the LSD test ($P < 0 \cdot 05$). Different lower case letters indicate significant differences.
[c] Eggs g^{-1} root on resistant tomato / eggs g^{-1} root on susceptible tomato × 100.

However, in the second cropping cycle (2006), these parameters were higher (P <0·05) on rootstock cv. PG-76 compared to resistant cv. Monika, and in the third cycle (2007) they were similar on rootstock cv. PG-76 and susceptible cv. Durinta. According to the RI, rootstock cv. PG-76 responded as highly resistant (RI = 7%) after the first cropping cycle, resistant (RI = 33%) after the second cycle, and fully susceptible (RI = 94%) after the third cycle (Table 2). Rootstock cv. Brigeor and tomato cv. Monika responded as highly resistant after the first (RI = 4%) and second cropping cycles (RI = 9%) and resistant after the third cycle (RI = 41% Brigeor and 25% Monika). Rootstock cvs PG-76 and Brigeor and tomato cv. Monika showed similar levels of relative resistance after the first cropping cycle (2005) but rootstock cv. PG-76 showed lower (P <0·05) resistance level compared to cvs. Brigeor and Monika after the third cycle (2007).

Table 2. Initial (Pi) and final (Pf) population densities, eggs g^{-1} of root and reproduction index (RI %) of *Meloidogyne javanica* on selected rootstocks and cultivars of tomato containing the *Mi* resistance gene for three consecutive seasons in a plastic-house infested with the nematode.

		Juveniles 250 cm^{-3} soil			
Treatment	Year	Pi	Pf	Eggs g^{-1} root	RI (%)[a]
Rootstock					
PG-76 (R)[b]	2005	172 ± 224 a A[c]	205 ± 112 b B	2284 ± 2946 b B	7 ± 9 b A
	2006	210 ± 193 a B	2892 ± 2392 a B	12870 ± 12450 a B	33 ± 32 b A
	2007	290 ± 473 a A	4606 ± 2090 a AB	29860 ± 11083 a A	94 ± 35 a A
Brigeor (R)	2005	206 ± 237 a A	266 ± 216 b B	1143 ± 966 c B	4 ± 3 b A
	2006	132 ± 128 a B	717 ± 498 b BC	3418 ± 1496 b BC	9 ± 4 b A
	2007	196 ± 143 a A	8917 ± 8917 a A	12934± 6885 a B	41 ± 22 a B
Cultivar					
Monika (R)	2005	178 ± 210 a A	216 ± 283 b B	1359 ± 334 b B	4 ± 1 b A
	2006	357 ± 354 a B	662 ± 443 ab C	3570 ± 3717 ab C	9 ± 10 b A
	2007	180 ± 127 a A	2545 ± 3050 a B	8015 ± 6370 a B	25 ± 20 a B
Durinta (S)	2005	236 ± 123 c A	13476 ± 10044 a A	31924 ± 2496 a A	
	2006	2063 ± 1222 a A	14927 ± 6700 a A	39064 ± 11437 a A	
	2007	485 ± 186 b A	12658 ± 10090 a A	31834 ± 13973 a A	

[a] Eggs g^{-1} root on resistant tomato / eggs g^{-1} root on susceptible tomato × 100.
[b](R): *Mi* resistant plants, (S): *mi* susceptible plants.
[c] Values are means ± standard deviations of five replicated plots per treatment. Values within tomato rootstock or cultivar in the same column followed by different lower-case letters are significantly different according to LSD Test (P <0·05). Values within year in the same column followed by different capital letters are significantly different according to LSD Test (P <0·05).

Gall ratings were lower (P <0·05) on the resistant cultivars compared to the susceptible cv. Durinta throughout the study, although disease incidence (number of plants with galled roots) and severity (gall rating) increased progressively as the resistant cultivars were repeatedly cultivated in the same *M. javanica* infested plots (Fig. 3). Plants with gall rating index 5 (25% of the root system severely damaged and not functioning) were considered as nematode susceptible. The gall rating on rootstock cv. PG-76 was higher (P <0·05) in the second and third cropping cycles compared to the first cycle. On rootstock cv. Brigeor, the gall rating was higher (P <0·05) only in the third cycle compared to the two previous cycles. On tomato cv. Monika, gall ratings did not differ between cropping cycles.

Testing for virulence

The sub-populations of P1 and P2 from plots with resistant tomatoes produced lower (P <0·05) number of eggs g^{-1} root on the resistant than susceptible cultivars (Table 1) with the exceptions of sub-population P1 from plots with rootstock Brigeor and sub-population P2 from plots with rootstock cv. PG-76. All sub-populations of P3 from resistant tomatoes produced similar eggs g^{-1} root on the resistant and susceptible tomato cultivars. Sub-populations of P1, P2, and P3 from plots with susceptible Durinta produced lower (P <0·05) number of eggs g^{-1} root on the resistant than susceptible cultivars (Table 1), indicating that the *Mi* resistance gene mediated resistance was functional in the resistant tomato cultivar used in the virulence testing. The resistant cultivar used in these virulence tests, expressed a high level of resistance to the four P1-sub-populations of the nematode after exposure to the *Mi* resistance gene for one cropping cycle. However, after two cropping cycles, the resistant cultivar showed no resistance to the P2 sub-population from plots with rootstock PG-76, and only intermediate resistance to those from plots with rootstock cv. Brigeor or tomato cv. Monika (Table 1). After the third cropping cycle, the resistant tomato cultivar responded as susceptible to all P3 sub-populations from plots with resistant rootstocks and tomato cultivars. In contrast, the sub-populations from plots with susceptible cv. Durinta not exposed to the *Mi* resistance gene remained avirulent irrespective of the frequency of cropping (Table 1).

Crop yield

Cumulative yield was higher (P <0·05) in plots with resistant compared to susceptible tomatoes in each cropping cycle (Table 3).

Table 3. Cumulative tomato yield (kg m^{-2}) of rootstocks containning *Mi* resistance gene grafted with susceptible cv. Durinta, grown in *Meloidogyne javanica*-infested plots for three consecutive years in a plastic house.

Treatment	Year	kg m^{-2}
Rootstocks		
PG 76 (R)[a]	2005	na[b]
	2006	17.65 ± 1.43 a[c] A
	2007	7.88 ± 0.97 b B
Brigeor (R)	2005	na
	2006	19.09 ± 1.55 a A
	2007	10.36 ± 1.53 b A
Cultivars		
Monika (R)	2005	13.47 ± 1.79 a A
	2006	14.51 ± 1.37 a B
	2007	8.38 ± 1.83 b B
Durinta (S)	2005	7.58 ± 1.97 a B
	2006	3.86 ± 2.18 b C
	2007	5.17 ± 1.17 ab C

[a] (R): *Mi* resistant plants, (S): *mi* susceptible plants.
[b] Not available.
[c] Yield values are means ± standard deviations of 40 plants (eight plants per plot × five plots per treatment). Means separation within plant treatment done by the LSD test (P <0·05). Different lower-case letters in the same column within tomato rootstock or cultivar indicate significant differences. Different capital letters within year indicate significant differences.

The resistant plants yielded more (P <0·05) fruits in the second (2006) compared to the third cropping cycle (2007). No yield data was evaluated for the rootstocks in the first cycle since they were not grafted (2005). Cumulative yield was higher (P <0·05) in tomatoes grafted on rootstock cv. Brigeor than on rootstock cv. PG-76 in 2007. Resistant cv. Monika consistently had higher fruit yield (P <0·05) than susceptible cv. Durinta. Grafting cv. Durinta onto either cv. PG-76 or Brigeor rootstock produced significantly higher yield compared to the ungrafted Durinta plants.

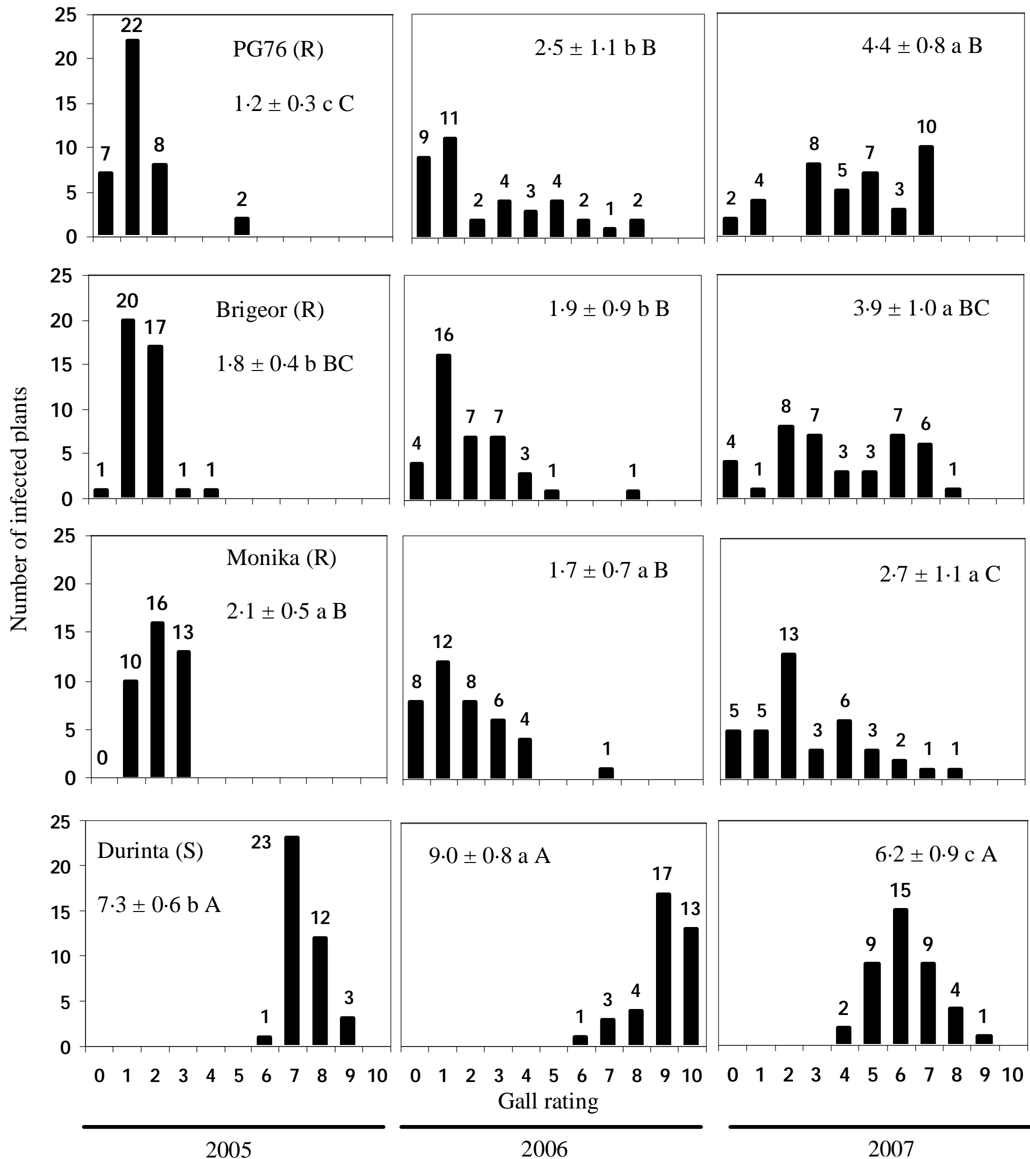

Figure 3. Change over the years in *Meloidogyne javanica* disease incidence (percentage of infected plants) and severity (gall rating) for tomato rootstocks containing the *Mi* resistance gene cvs. PG-76 and Brigeor, and tomato resistant cv. Monika and susceptible cv Durinta. Plants were grown in a plastic house infested with the nematode. Gall rating based on a scale from 0 (none, healthy plant) to 10 (dead plants). Bars represent the number of plants with a given gall rating. Values in each square are means ± standard deviations of 40 plants per treatment. Significant differences according to LSD Test ($P < 0.05$) are represented in lower case letters within tomato rootstock or cultivar and with capital letters within year. (R): *Mi* resistant plants, (S): *mi* susceptible plants. A few plants did not survive until the end of the cropping cycle; 1 plants of cv. PG-76 in 2005 and 2007, 2 plants in 2006; 1 plant of cv. Brigeor in 2006, 1 plant of cv. Monika in 2005, 2006 and 2007, 1 plant of cv. Durinta in 2005, and 2 plants in 2006.

Discussion

Virulent populations of *M. javanica* were rapidly selected from a *Mi* avirulent population after repeated cultivation of resistant tomatoes for only two or three cropping cycles under field conditions. However, the change in the relative resistance levels of the resistant materials was not abrupt but gradual. The resistance level decreased as the frequency of cropping increased, particularly with rootstock cv. PG-76 that became fully susceptible after the third cropping cycle. The resistance in rootstock cv. Brigeor and cv. Monika was not completely overcome after the third cycle. Although repeated cultivation gradually increased the mean gall ratings on the rootstocks, it did not increase the mean gall rating on cv. Monika in which selection for virulence occurred at lower speed. The faster selection of virulence on rootstock cv. PG-76 and Brigeor compared to cv. Monika suggest different mechanisms of nematode resistance in these tomato cultivars. The genetic background of the tomatoes was a critical factor for the selection of virulence. The frequency of cropping was also crucial as virulent nematode populations only appeared if resistant tomatoes were repeatedly cultivated in the same plots. The increased reproduction of *M. javanica* on the resistant plants cannot be attributed to *Mi* resistance breakdown due to high temperatures. Temperature records were above 28˚C for 10 days in 2005 (two weeks before the end of the cropping cycle) but during this cropping cycle the three resistant tomatoes showed high resistance levels, and resulted in suppression of nematode reproduction by 93% (PG-76) or 96% (Brigeor and Monika). In a previous experiment, rootstock PG-76 retained a high resistance level despite soil temperatures being above 28˚C in the first week after nematode inoculation (Cortada et al., 2008).

Selection for virulence was somehow unexpected because these resistant rootstocks had provided consistently high degree of resistance in previous experiments under both glasshouse controlled conditions (one nematode generation) and in the field after one cropping cycle (3.3 nematode generations) (Cortada et al., 2008). Also, rootstock cv. PG-76 has shown high resistance levels to several species and populations of *Meloidogyne* under glasshouse controlled conditions whereas rootstock cv. Brigeor showed more variable results (Cortada et al., 2009). Cultivar Monika provided similar resistance responses to those reported in previous studies (Sorribas et al., 2005; Verdejo-Lucas and Sorribas, 2008). The genetic differences in rootstock cvs. PG-76 and Brigeor, could contribute differentially to the durability of the nematode

resistance. In addition, the genetic composition of the nematode can also contribute to the reduction in the durability of the resistance (Castagnone-Sereno et al., 1994; Jacquet et al., 2005).

The results of the virulence bioassays confirmed the field data regarding the selection for virulence after repeated cultivation of resistant tomato genotypes. The nematode population in the field remained *Mi* avirulent after one cropping cycle irrespective of the planted tomato genotype. In the second cropping cycle, the nematode population became virulent in plots with rootstock cv. PG-76, and after the third cycle, virulence appeared in plots with rootstock cv. Brigeor and tomato cv. Monika. Selection of virulent nematode populations on resistant tomato cultivars over generations have been previously reported but under controlled conditions (Bost and Triantaphyllow, 1982; Jarquin-Barberena et al., 1991, Xu et al., 2001). However, selection of virulent nematode populations has not been previously reported on tomato rootstocks under field conditions. The acquired virulence by the nematode will probably remain stable because virulence is genetically inherited and stable once acquired (Castagnone-Sereno et al., 1993). Although the mechanisms involved in the selection for virulence are largely unknown, one possibility is that two copies of the *Mi* resistance gene in homozygous tomato cultivars might protect better against the nematode compared to one copy in hybrid tomatoes (Tzortzakakis et al., 1998; Jacquet et al., 2005). However, this *Mi* dosage effect was not observed here as the *Mi* locus in rootstock cv. PG-76 and Brigeor is homozygous whereas in tomato cv. Monika is heterozygous (Cortada et al., 2008).

Recently, several wild *Solanum* species have been used as novel breeding materials to combine pathogen resistances and improved agronomical traits under a wide range of conditions. For example, *S. habrochaites* accession LA1777 confers resistance to both *Tomato yellow leaf curl virus* (TYLCV) (Vidavsky and Czoskez, 1998) and *Cucumber mosaic virus* (CMV) (Cillo et al., 2007), as well as for increase productivity in suboptimal temperatures (Hanson et al., 2007). Since the genetic background of the material used to develop hybrid PG-76 and Brigeor rootstocks are not well described, it is possible that instead of the *Mi* resistance gene, other *Mi*-homologues have been selected for in these hybrid rootstocks. Several *Mi*-homologues are present in *Solanum* spp. and these homologues may not be equally effective or stable as the *Mi* resistance gene. Alternatively, these *Mi*-homologues might play a role in selecting for virulent nematode populations by a yet unknown mechanism.

Further investigations are necessary using additional nematode-tomato genotype combinations to determine if tomato rootstocks rapidly select virulent populations in different environmental conditions. From an agronomic perspective, the *Mi* resistance gene provided resistance and tolerance to *M. javanica* in cv. Monika, but only tolerance in tomato rootstocks. The tolerance in both rootstocks could be attributed to the deep and massive root systems. Although the use of tomato rootstocks can be profitable and are considered an ecological alternative to chemical control, nematode management should include rotation of resistant rootstocks with susceptible genotypes to preserve the durability of the resistance. The frequency of the rotations, however, needs to be determined.

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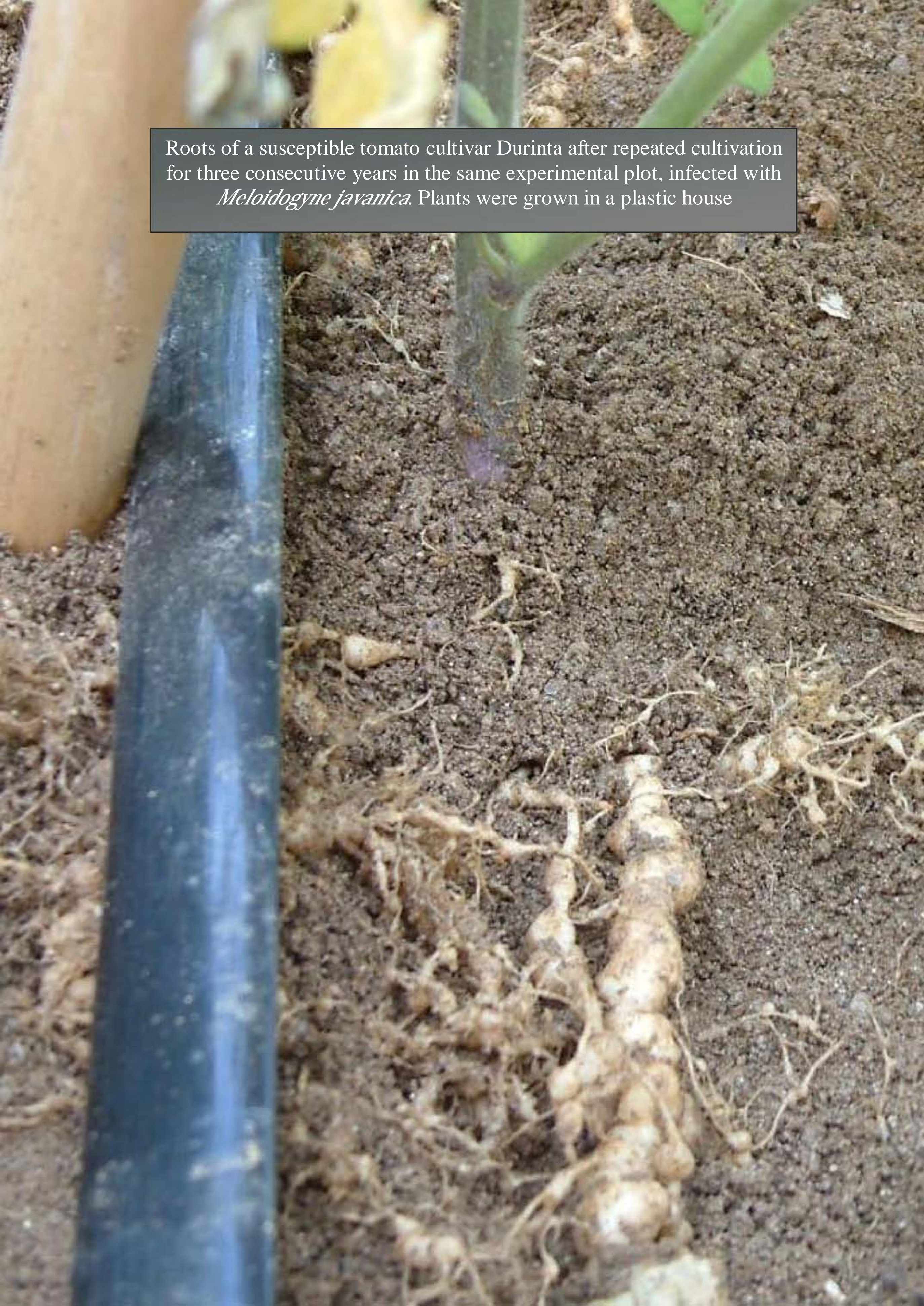

Roots of a susceptible tomato cultivar Durinta after repeated cultivation for three consecutive years in the same experimental plot, infected with *Meloidogyne javanica*. Plants were grown in a plastic house

5 Molecular marker MVC for characterization of selected virulent populations of root-knot nematodes is correlated with three genera of betaproteobacteria

L. Cortada[1], H. Sakai[2], S Verdejo-Lucas[1], T. Mizukubo[2]

In tomato, resistance to root-knot nematodes is mediated by the *Mi* resistance gene. This gene confers resistance to the three most important species of *Meloidogyne*. *M. arenaria*, *M. incognita* and *M. javanica*. Nevertheless, the *Mi*-gene is unable to inhibit reproduction of selected and naturally virulent populations of root-knot nematodes. As pathogenicity assays are difficult to perform and time consuming, molecular markers have been developed for the easy identification of virulent populations of *Meloidogyne*. The SCAR MVC molecular marker, differentiated selected virulent from naturally virulent populations. This marker was used to compare acquired virulence in populations of *M. javanica* form Spain. The original populations used to develop de MVC marker were included as control for relevance. Results indicated that the MVC marker was not able to amplify genomic DNA extracted from single juveniles and females of any of the populations studied, either from Spain or Japan. In silico analyses performed with the recently published complete genomic of *M. incognita*, and of several betaproteobacterial species, indicated that the MVC marker is related to bacterial enzymes from the species *Verminephrobacter eiseniae* EF01-2, *Acidovorax avenae* subsp. *citrulli* AAC00-1 and the genus *Diaphorobacter* spp., and not to a *Meloidogyne* virulence locus (MVC).

Key words: Acidovorax spp., Diaphorobacter spp., egg mass, Meloidogyne spp., Verminephorobacter eiseniae

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Introduction

In tomato, plant resistance to *Meloidogyne arenaria*, *M. incognita* and *M. javanica* is conferred by a single dominant gene named *Mi* (Willamson et al., 1994). Plant resistance conferred by the *Mi* gene has been widely used to successfully control root-knot nematode since the 1940s (Ornat and Sorribas, 2008). In nematology, virulence is defined as the ability of a nematode to infect and reproduce on a resistant plant, evolving into a fertile female (Roberts, 1995). Virulent populations can be classified into two groups: as selected virulent and naturally virulent. Virulent selected populations appear after repeated exposure to the selection pressure of the *Mi* gene (Bost and Thriantaphyllou, 1982; Jarquin-Barberena et al., 1991; Eddaoudi et al., 1997; Williamson, 1998; Gleason, 2003; Verdejo-Lucas et al., 2009), whereas naturally virulent populations are able to reproduce successfully on resistant tomato cultivars despite never having been exposed to the *Mi* gene (Netcher, 1976; Prot, 1984; Kaloshian et al., 1996; Ornat et al., 2001; Xu et al., 2001). Virulence against the *Mi* gene might appear under polygenic control and is stably inherited and maintained over generations (Jarquin-Barberena et al., 1991; Castagnone-Sereno et al., 2003).

The gene-for-gene model postulated by Flor (1971) indicates that for every avirulence gene (*Avr*) present in the pathogen there is a resistant gen (*R*) in the host that prevents infection and the establishment of the pathogen in the plant. So far, *Avr* genes have been cloned from bacteria, viruses and oomycetes but not from nematodes (Williamson and Gleason, 2003) although there is genetic evidence that avirulence in some plant-parasitic nematode species is transmitted to the progeny as a single dominant trait (Gleason et al., 2008). This *Avr-R* gene model has already been proved for *Globodera rostochiensis* and the *R* gene *H1* (Bakker, 2002), although experiments to confirm this model in the root-knot nematode-resistant plant pathosystem are scarce (McK Bird et al., 2009). In 2003, Chen and Roberts were able to demonstrate that a gene-for-gene interaction occurs between an *Avr* dominant gene present in *M. hapla* (with sexual reproduction) and a dominant *R* gene from the bean cultivar NemaSnap. Unfortunately, the strictly parthenogenetic mode of reproduction of *M. arenaria*, *M. incognita* and *M. javanica* prevents from performing experiments to determine progeny segregation of *avr* alleles in these species.

Many efforts have been done along the past years to characterize virulent populations through a molecular approach. The protein *MAP-1,* the first

candidate coding for a nematode *Avr* gene (Semblat et al., 2001), was found in those avirulent nematode populations of *Meloidogyne* subjected to the *Mi* selection pressure (*M. arenaria*, *M. incognita* and *M. javanica*), but not in *M. hapla* or *M. fallax* (Semblat et al., 2001). However, a general functional evidence correlating this candidate gene to virulence or avirulence has not been established (Gleason et al., 2008). Recently, the new candidate *Cg-1* gene has been related with avirulence of *M. javanica* (Gleason et al., 2008). Although no similarities have been found between *Cg-1* and other sequences on the Genebank, silencing experiments performed with dsRNA indicated that *Cg-1* is required in the nematode for the expression of the *Mi*-mediated resistance response in the plant. In 2001, Xu et al. developed a SCAR-PCR molecular marker to differentiate selected from naturally virulent populations of *Meloidogyne* genus. These authors suggested that virulent populations of *Meloidogyne* spp. do not share a common origin but are originated by similar mutational events in the MVC (*Meloidogyne* virulence related) locus. In the opposite way, other molecular studies have shown that virulence has different molecular origins between virulent populations rather than being the result of a mutational event on a single locus (Abad et al., 2003; Gleason et al., 2008; Semblat et al., 2000; Tzortzakakis et al., 1999). So far, characterization of virulent root-knot nematode populations using the MVC molecular marker has only been feasible for Asiatic virulent isolates (Bleve-Zacheo et al., 2007), suggesting that this marker may be only useful for eastern Asiatic nematode isolates. Therefore, none of the candidates coding for a nematode *Avr* gene screened to date can be used to characterize virulent nematode populations from all around the world at the molecular level.

The recent publication of the complete genome of *M. incognita* and *M. hapla* (McK Bird et al., 2009) opened an opportunity to establish a correlation between the sequences of the MVC locus and the corresponding gene(s) in the genome of *M. incognita*. In silico analyses resulted in no relation of the MVC locus with any nematode gene(s), although there was a strong similarity of the MVC sequences to three different genera of anaerobic protobacteria. Experiments presented in this paper show that the MVC locus is strongly related to the genome of three different genera of anaerobic protobacteria that have been recently sequenced, but not to a root-knot nematode virulence locus.

Materials and Methods

Blast analyses of the MVC-allelic sequences

Bioinformatic analyses were performed with the MVC-allelic sequences (GenBank accession no. AB047761 to AB047767) described by Xu et al. (2001) corresponding to three selected virulent populations, one naturally virulent population and to three avirulent *M. javanica* populations. The nucleotide BLAST search revealed a close relationship between the sequences of the MVC locus and those of three different genera of betaproteobacteria. The closest homology for the MVC-allelic sequences analyzed was consistently obtained with the betaproteobacteria genera *Acidovorax* spp., *Diaphorobacter* spp., and the species *Verminephrobacter eiseniae* (EF01-2). Pairwise sequence similarities are presented in Table 1. No correlation of the sequences of the MVC locus were found to any nematode species, including *M. incognita*, *M. hapla* and *Caenorhabditis elegans*, nor to any other eukaryotic organism.

Nematode samples

Two Japanese laboratory-selected virulent populations of *M. javanica* (MJON-VI) and *M. incognita* (MIYN-VI), three *M. javanica* selected virulent populations (PG-76-P3, Brigeor-P3, Monika-P3), one avirulent population (Durinta-P3), and one naturally virulent (MJ-27) from north-eastern Spain were used for molecular analyses. Characteristics of the root-knot nematode populations are described in Table 1.

Draining-water samples were obtained from a potted resistant tomato plant (cv. Momotaro) infected by the MIYN-VI population. The plant was watered with distilled water and draining water was collected on a beaker and stored at 4°C. This draining-water, so called "non-filtered water" (NFW), was then passed through a 5 μm-pore-sieve of nylon to retain J2 and nematode eggs (5 μm-FW) and finally, an aliquot of this 5 μm-FW water was filtered through a 0.2-μm cellulose acetate filter to retain bacterial and fungal spores (0.2 μm-FW).

Table 1. Proportion of nucelotide sequence similarities between seven MVC-allelic sequences (Xu et al., 2001) from selected virulent populations (VI), naturally virulent populations (NVI), and avirulent (AV) populations of Meloidogyne arenaria, M. incognita and M. javanica from Japan, and the betaproteobacterial species Verminephrobacter eiseniae EF01-2, and the genera Acidovorax spp. and Diaphorobacter spp. of betaproteobacteria.

	MVC allelic sequences						
	Virulent (VI)				Avirulent (AV)		
Proteobacteria Species	MAYG-VI	MINN-VI	MJON-VI	MIGD-NVI	MAMG-AV	MIIK-AV	MJON-AV
V. eiseniae (EF01-2)	79%	78%	78%	79%	79%	79%	80%
Acidovorax spp.	77%	77%	77%	77%	77%	77%	77%
Diaphorobacter spp.	77%	76%	77%	78%	77%	76%	77%

Table 2. Populations of Meloidogyne spp. used for molecular analyses.

Species	*Population*	*Origin*	*Mi-virulence*	*Selecting host*	*References*
M. javanica	MJ-27	Barcelona, Spain	VI-natural	naturally virulent	Ornat et al., 2001
	PG-76-P3	Barcelona, Spain	VI-selected	*Mi-1* tomato rootstock	Verdejo-Lucas et al., 2009
	Brigeor-P3	Barcelona, Spain	VI-selected	*Mi-1* tomato rootstock	Verdejo-Lucas et al., 2009
	Monika-P3	Barcelona, Spain	VI-selected	*Mi-1* tomato rootstock	Verdejo-Lucas et al., 2009
	MJON-VI	Okinawa, Japan	VI-selected	*Mi-1* tomato cultivar	Xu et al., 2001
	Durinta-P3	Barcelona, Spain	AV	*mi*-tomato cultivar	Verdejo-Lucas et al., 2009
M. incognita	MIYN-VI	Yamanashi, Japan	VI-selected	*Mi-1* tomato cultivar	Xu et al., 2001

Ten females and their corresponding egg masses of the population MJON-VI, and three females of the population MIYN-VI, were dissected from the roots of infected resistant tomato plants (cv. Momotaro) with the help of a forceps under a stereo microscope. Egg masses of the population MJON-VI were placed into screw-cap vials filled with 2 mL-sterile distilled water. Vials were left at room temperature after hatching of second stage juveniles (J2) for 15 days. Forty females each of the populations PG-76-P3, Brigeor-P3, Monika-P3, MJ-27, and Durinta-P3 were hand-picked from roots of infected resistant tomato plants (cv. Monika) as described. Females were kept at -80 ˚C until DNA extraction.

DNA extraction

From water: one milliliter of the NFW, 5 µm-FW and 0.2 µm-FW samples were pipetted separately into 1.5 mL eppendorf tubes and spun at 13,000 rpm for 20 min at 4 ˚C. Supernatant was removed and 20 µL of 0.1% SDS lysis buffer (Sakai et al., 2008) were carefully added by repeated pipetting on the walls of the eppendorf tubes, to clean and drug the pellet to the bottom of the tube. Incubation was held at 50 ˚C in a thermal cycler for 2 h followed by 95 ˚C for 10 min. Finally, 980 µL of sterilized water were added and the tubes were vortexed. Vials each containing single egg mass of the MJON-VI population were vigorously shaken and hatched J2 were left to settle at the bottom of the vials for three hours. One mL of each the supernatant phase and the sediment containing J2 and the remains of the gelatinous matrix (GM) of the egg mass was pipetted separately into 1.5 mL eppendorf tubes, spun and processed as described for water samples.

From nematodes: DNA extraction of individual J2 of the MJON-VI population was performed in twenty J2 from two egg masses (ten J2 from each EM_1 and EM_2), adapting the protocol of Sakai et al. (2008). A suspension of J2 was added to a sterilized Petri dish, and individual J2 were picked with the aid of a sterilized needle and placed on a 5 µL-drop of sterilized Milli-Q water on a slide glass. Single J2 were crushed with a sterilized filter paper chip (1mm × 1mm). Paper chips were then introduced individually in a 0.5 mL eppendorf tube containing 4 µL of the DNA 0.1% SDS lysis buffer; DNA extraction of single females of the MIYN-VI population was performed following the same protocol. Samples were incubated at 55 ˚C for 1h and 30 min; the lysate was extracted twice with 1 volume of chloroform, and samples were left at -70 ˚C

for 15 min after addition of the 3M Sodium Acetate for DNA precipitation. DNA extraction of females from the Spanish populations was performed following a protocol adapted from Cenis (1993).

PCR analyses

PCR analyses were performed with the co-dominant marker MVC-F3/R2 and the dominant marker MVC-VF/R2 (Xu et al., 2001). PCR conditions were those described by Xu et al. (2001), except that PCR reactions were held at a final volume of 40 μL with 10× PCR Buffer and 0.5 units of Ex Taq DNA-polymerase (Takara Bio). Specific amplification conditions for all samples were those described by Xu et al. (2001), although amplification was performed with 40 cycles and the denaturation was held at 95 ˚C. Nematode DNA extraction was tested by PCR amplification of the ITS region (Ferris et al.; 1993). The MVC-F3/R2 amplified products were digested overnight with the Nde I restriction enzyme (Takara Bio) following the manufacturer protocol. For all PCR reactions, 5 μL of the PCR products were resolved on 1.5% agarose gel in 0.5 × TBE buffer.

DNA sequencing

PCR products obtained with the dominant marker MVC-VF/R1 were cut from the gel and purified with a MinElute Gel Extraction Kit (Qiagen). DNA labeling was performed using BigDye® Terminator v1.1 (Applied Biosystems) followed by purification with Dye Ex® 2.0 Spin Kit (Qiagen). Sequencing was performed in a 370xl DNA analyzer (Applied Biosystems). DNA sequences were manually corrected using Bioedit v 7.0.9 program.

Results

Amplification with the MCV-molecular markers in absence of nematodes

PCR reactions carried on water samples showed that amplification of MVC-F3/R2 and MVC-VF/R2 molecular markers occurred in water samples from

NFW, in 5 μm-FW but not in 0.2 μm-FW. This fact suggested that DNA amplification occurred in samples without nematode J2 (Fig. 1a and b), but that contained organisms that were retained by a 0.2 μm cellulose filter.

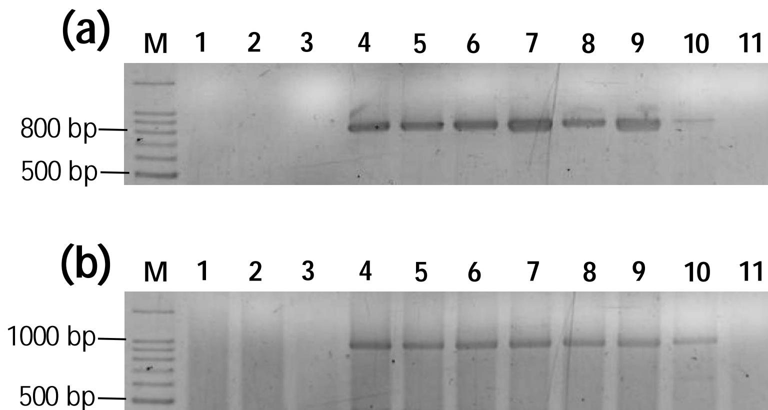

Figure 1. MVC marker profile of draining-water samples collected from a potted plant infected by a *Meloidogyne incognita* (MIYN-VI) population. **(a)** Co-dominant molecular marker MVC-F3/R2. **(b)**. Dominant molecular marker MVC-VF/R1. Lanes: (1 to 3) 0.2 μm-filtered water, (4 to 6) 5 μm-filtered water, (7 to 9) non-filtered water, (10) Supernatant of egg mass no. 3 (EM_3), (11) MiliQ sterilized water. Lane M indicates 100 bp DNA ladder.

MVC locus amplification on DNA extracted from draining water samples

PCR reactions carried on water samples showed that amplification of MVC-F3/R2 and MVC-VF/R2 molecular markers occurred in draining-water samples from NFW and 5 μm-FW, but not in 0.2 μm-FW. Therefore, DNA amplification occurred in all samples (Fig. 1a and b), except in samples that were passed through a 0.2 μm cellulose filter, that retained bacteria and fungal spores. To determine whether the MVC locus had been amplified from DNA of any microorganism(s) associated to the GM of the egg mass, molecular analyses were performed in an aqueous suspension of individual egg masses. DNA amplification with co-dominant and dominant markers for the MVC locus occurred in both the suspension resulting from incubation of a single *M. javanica* egg masses and the pellet containing J2 and fragments of the GM. Amplification with co-dominant primers MVC-F3/R2 occurred in all the

supernatant samples (Fig. 2a; lanes 1 to 8) and in six out of eight samples containing the pelleted J2 and remains of the egg mass (Fig. 2a; lanes 10, 12 and 13 to 16).

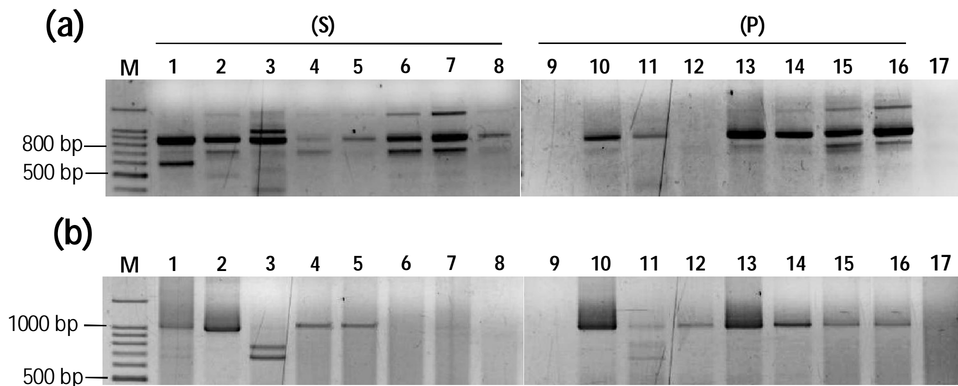

Figure 2. MVC marker profile of egg masses (EMs) of the virulent *Meloidogyne javanica* population (MJON-VI). **(a)** Co-dominant molecular marker MVC-F3/R2. **(b)**. Dominant molecular marker MVC-VF/R1. (S) Indicates samples supernatant phase obtained from the aqueous suspension of an egg mass, and (P) samples obtained from the sediment containing the juveniles and the gelatinous matrix of the egg. Lanes: (1 and 9) EM_3, (2 and 10) EM_4, (3 and 11) EM_5, (4 and 12) EM_6, (5 and 13) EM_7, (6 and 14) EM_8, (7 and 15) EM_9, (8 and 19) EM_{10}, (17) MiliQ sterilized water. Lane M indicates 100 bp DNA ladder.

In addition to the expected fragment of approximately 832 bp, several additional bands appeared in the gel, which indicated that the microorganism(s) species present in the egg masses were highly polymorphic for this marker, or that different species of microorganisms were amplified by the MVC marker. In PCR performed with MVC-F1/R2 marker four supernatant samples showed the expected genomic fragment of 1Kb (Fig. 2b; lanes 1, 2, 4 and 5); amplification occurred in six of the pelleted samples and polymorphism was also found for this marker (Fig. 2b; lanes 10 to 16). Digestion of the MVC-F3/R2-PCR products with restriction enzyme *Nde*I did not produce any restriction fragment for any of the samples analyzed, including single virulent J2 from MJON-VI population (Fig. 3). Molecular experiments were repeated twice, with similar results.

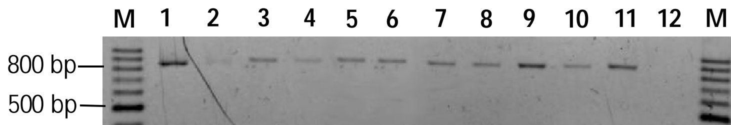

Figure 3. Digestion of MVC-VF/R1-PCR products with Nde I restriction enzyme. Samples correspond to the virulent population *M. javanica* (MJON-VI). Lanes: (1) juvenile no. 1 ($J2_1$) of egg mass no.1 (EM_1), (2) $J2_2$ from EM_2, (3) Pellet of EM_4, (4) Pellet of EM_5, (5) Supernatant of EM_4, (6) Supernatant of EM_5, (7 to 8) 5 μm-FW, (9 to 10) non-filtered draining-water, (11) reaction without Nde I (positive control), (12) water. Lane M indicates 100 bp DNA ladder.

MVC locus amplification on DNA root-knot nematode juveniles and females

DNA amplification with the co-dominant MVC-F3/R2 marker in ten individual J2 obtained from two independent egg masses (10 J2/EM) or a pool of DNA extracted from ten single juveniles of the MJON-VI population, did not occur. No amplification from nematode DNA occurred either from individual females of the MIYN-VI population (Fig. 4). An identical result was obtained when amplification with MVC-F3/R2 and MVC-VF/R1 molecular markers was performed using the selected virulent populations from north-eastern Spain PG-76-P3, Brigeor-P3, Monika-P3, MJ-27, and Durinta-P3 as DNA template (Fig. 5a and b).

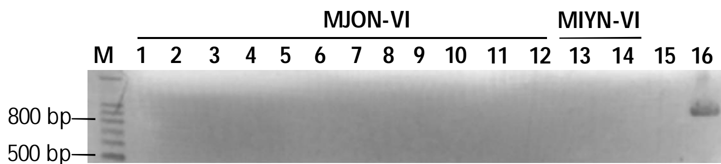

Figure 4. MVC-F3/R2 marker profile of the selected virulent populations *Meloidogyne javanica* (MJON-VI) and *M. incognita* (MIYN-VI). Lanes: (1 to 10) individual juveniles (J2) from one egg mass EM_1 of MJON-VI population, (11) Pool of DNA from 10 J2 of EM_1, (11) Pool of DNA from 10 J2 of EM_2, (13-14) individual females of the MIYN-VI population, (15) MiliQ sterilized water, and (16) Supernatant of EM_3. Lane M indicates 100 bp DNA ladder.

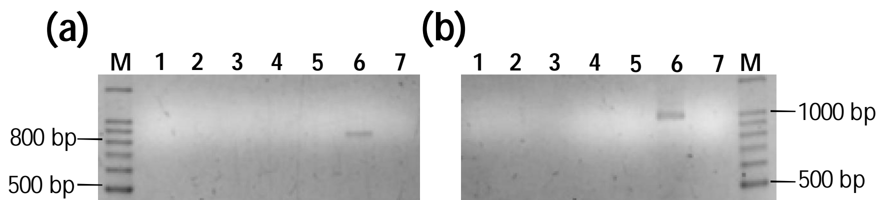

Figure 5. MVC marker profile of the selected virulent *Meloidogyne javanica* populations (PG-76-R3, Brigeor-P3, Monika-P3) from north-eastern Spain, naturally virulent *M. javanica* population (MJ-27), avirulent *M. javanica* (Durinta-P3), and the selected virulent *M. javanica* population (MJON-VI) from Japan. **(a)** Co-dominant molecular marker *MVC-F3/R2*. **(b)**. Dominant molecular marker *MVC-VF/R1*. Lanes: (1) PG-76-R3, (2) Brigeor-P3, (3) Monika-P3, (4) MJ-27, (5) Durinta-P3, (6) Pellet of egg mass EM_4, (7) MiliQ sterilized water. Lane M indicates 100 bp DNA ladder.

In order to determine that the lack of amplification of nematode DNA was not related to a DNA degradation, amplification of the ITS region was successfully performed (Fig. 6).

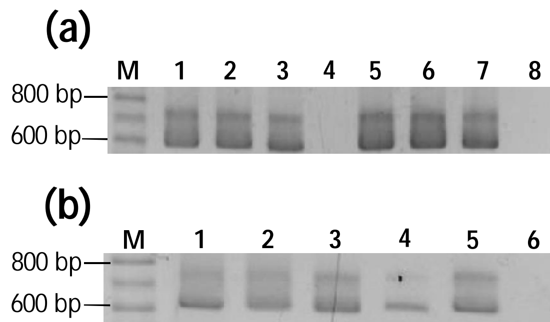

Figure 6. ITS profile of the *Meloidogyne javanica* populations. **(a)** Individual juveniles from two independent egg masses (EM1 and EM2) from the Japanese virulent MJON-VI population. Lanes: (1 to 3) $J2_1$, $J2_2$ and $J2_3$ from EM_1, respectively, (5 to 7) $J2_1$, $J2_2$ and $J2_3$ from EM_2, respectively, (4 and 8) Water. **(b)** Pool of females from virulent selected (PG-76-R3, Brigeor-P3, Monika-P3), naturally virulent (MJ-27), and avirulent (Durinta-P3) populations from north-eastern Spain). Lanes: (1) PG-76-R3, (2) Brigeor-P3, (3) Monika-P3, (4) MJ-27, (5) Durinta-P3, (6) MiliQ sterilized water. Lane M indicates 100 bp DNA ladder.

Sequence characterization of the MVC-F1/R2-1-Kb alleles

The MVC-F1/R2 PCR-products obtained from the supernatant phase of the EM_4 water suspension were sequenced. Results showed that the fragment sequenced by the MVC-VF/R1 dominant marker from water samples in the absence of nematodes is closely related to a group of bacterial enzymes. Using BLAST searches of public databases, bioinformatics analyses revealed that the 1,032 bp DNA fragment obtained was highly homologous to the seven MVC-allelic sequences (GenBank accession no. AB047761 to AB047767), but also to a ribosome small sub-unit dependent GTPase A (RsgA) and a Ste24 endopeptidase (Ste24) from *V. eiseniae* EF01-2 (Score =9 51, E value = 0), to a RsgA and a pterin-4-alpha carbinolamine dehydratase (PCD) from *A. avenae* subsp. *citrulli* AAC00-1 (Score = 771; E value = 0), and to a Ste24 and a PCD from *Diaphorobacter* spp. TPSY (Score = 767, E value = 0) (Fig. 7). An alignment of the MVC-allelic sequences and the MVC/S-EM_4 revealed a mutation in this sequence in the target site of the restriction enzyme Nde I (Fig. 8) which would explain why no restriction fragments were found after digestion of MVC-F3/R2 PCR-products.

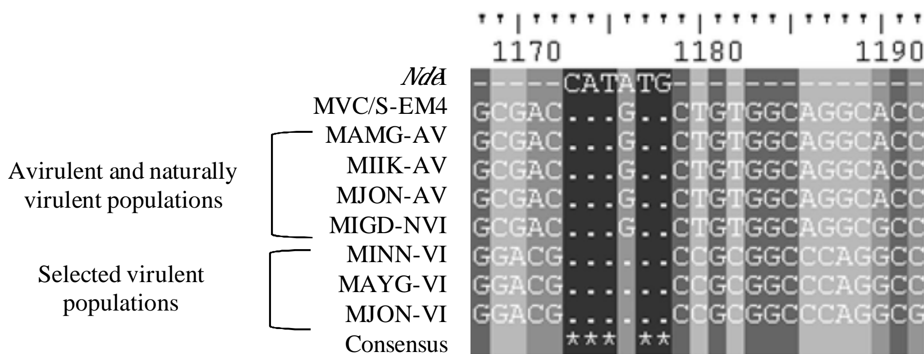

Figure 7. Alignment sequences of the seven alleles from the MVC locus from the *Mi*-selected virulent (VI), naturally virulent (NVI) and avirulent (AV) populations of *Meloidogyne arenaria*, *M. incognita* and *M. javanica* from Japan, the genomic sequences of the MVC/S-EM_4 corresponds to the genomic sequence amplified from the supernatant phase from an egg mass suspension using the MVC-F1/R1 dominant marker, with the target sequence of the restriction enzyme Nde I. MAMG-AV, MIIK-AV and MJON-AV are avirulent populations of *M. arenaria*, *M. incognita* and *M. javanica*, MIGD-VI is a naturally virulent population of *M. incognita, and* MAYG-VI, MINN-VI and MJON-VI are selected virulent populations of *M. arenaria*, *M. incognita* and *M. javanica*, respectively.

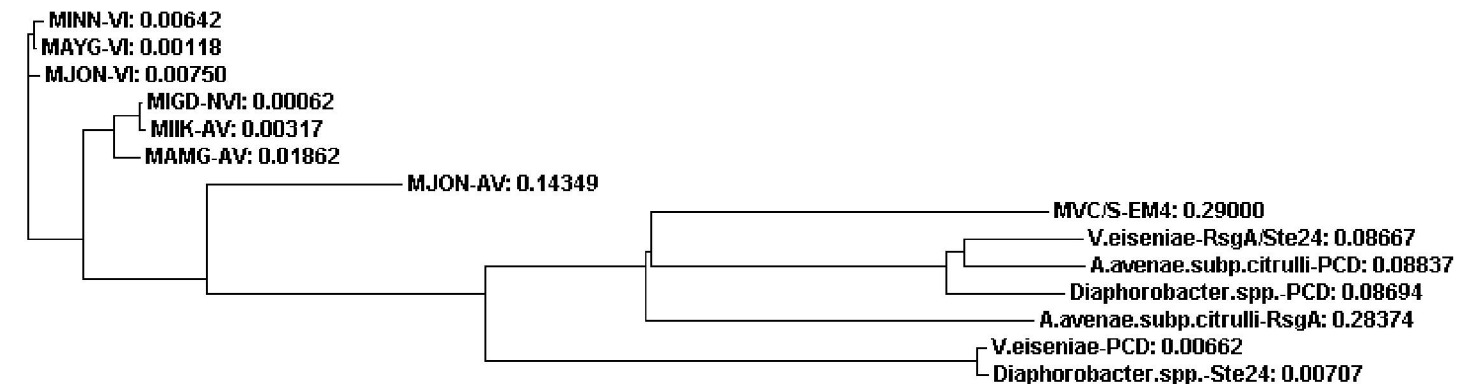

Figure 8. Neighbor-joining tree showing the relationship among the seven alleles from the MVC locus from the Mi-selected virulent (VI), naturally virulent (NVI), and avirulent (AV) populations of Meloidogyne arenaria, M. incognita and M. javanica from Japan, and the genomic sequences of the betaproteobacteria species Verminephrobacter eiseniae EF01-2, Acidovorax avenae subsp. citrulli AAC00-1, and the genus Diaphorobacter spp. TPSY. MAYG-VI, MINN-VI and MJON-VI are selected virulent populations of M. arenaria, M. incognita and M. javanica, respectively; MIGD-VI is a naturally virulent population of M. incognita; MAMG-AV, MIIK-AV and MJON-AV are avirulent populations of M. arenaria, M. incognita and M. javanica; MVC/S-EM_4 corresponds to the genomic sequence amplified from the supernatant phase from an egg mass suspension using the MVC-F1/R1 dominant marker; RSgA is a ribosome small sub-unit dependent GTPase A, Ste24 is a Ste24 endopeptidase, and PCD corresponds to a pterin-4-alpha carbinolamine dehydratase.

Discussion

Experimental evidence indicates that the MVC locus is not related to any root-knot nematode gene but to a microorganism(s) genome. The MVC molecular marker related to a (a)virulence locus from *Meloidogyne* spp. populations (Xu et al., 2001), failed to amplify DNA from single juveniles of a selected-virulent populations from Japan. It should be mentioned that the Japanese populations used in this paper were the original populations used to develop the MVC marker. Indeed, amplification did not occur either when molecular analyses were performed increasing the amount of DNA extracted from *Meloidogyne* juveniles, nor when selected virulent females from Japanese and Spanish populations were analyzed. In silico analyses, indicated that the molecular markers MVC-F3/R2 and MVC-F1/R2 are closer to a betaproteobacterial enzymes than to any other eukaryotic gene. All together, these results reveal that MVC locus dominant marker is not related to a nematode *avr*-gene, but most likely to a bacterial genome. DNA amplification of bacteria and not of the nematode would explain why the results reported by Xu et al. (2001) have been considered as non-reproducible with virulent isolates of *Meloidogyne* (Bleve-Zacheo et al., 2007).

Xu et al. (2001) did not find any significant matches at the nucleic acid level for the MVC-allelic sequences in public databases. The recent sequencing of the genome of *Acidovorax* spp., *Diaphorobacter* spp. and *V. eiseniae* in 2006 hampered previous detection of the close homology between the MVC allelic sequences and some proteobacterial enzymes. Results are consistent with those obtained by Xu et al. (2001), as amplification only occurred in DNA samples obtained from pelleted egg masse but not from individual J2. Most likely, no amplification occurred in J2 as bacteria(s) adhered to the cuticle surface of the nematodes would have been washed away, while hand-picking from water suspension; and adult females lying inside the root tissue might be protected from bacteria(s) attachment. Therefore, characterization of the MVC locus by Xu et al. (2001) could have been originated by the presence of some bacteria(s) in the samples used. Although data presently available, could not identify the bacteria(s) species associated to the Japanese populations, the closest similarity was established with the species of betaproteobacteria *V. eiseniae* EFO1-2, recently described as an endosymbiont of the earthworm *Eisenia foetida* (Pinel et al., 2008). Despite the similarity with *V. eiseniae*, the MVC-amplified DNA does not correspond to endosymbiont bacteria as no amplification occurred on

individual juveniles. A high sequence homology was also found to the species *Acidovorax avenae* subsp. *citrulli* AAC00-1 and *Diaphorobacter* spp. TYPS. Bacteria of this genus are frequently present in waste water treatment plants, aquatic environments, soil, sludge and some species can be found as a group of plant pathogens (Kardenavis et al., 2007; Pinel et al., 2008) and on siderurgic-polluted environments (Freitas et al., 2008). The bacteria enzymes showing the highest homology with the sequence amplified from the supernatant samples (MVC/S-EM_4) present a zinc-binding motif and have been described in bacteria and plants.

Whether the bacteria(s) species were present in the GM of the egg mass or in the rizhosphere, has not been solved. Bacteria play an important role in the egg mass ecosystem and are involved in the protection of nematode eggs against microorganisms (Orion et al., 2001). Up to 70 isolates of bacteria have been found in the gelatinous matrix of the egg masses of *M. hapla* from infected tomato roots (Kok et al., 2001), being *Acidovorax delafieldii* the second most abundant species. The bacterial composition of egg masses differ from that of the rizhosphere and the abundance of bacteria is three times higher in egg masses than in the surrounding soil (Kok et al., 2001). Whatever it may be the species of bacteria amplified, it must be present at high densities in the soil, considering that DNA amplification occurred in all samples from egg masses suspension or draining-water from the potted plant. Microbiological and genetic analyses will be necessary to determine whether the DNA amplified by MVC molecular marker corresponds to the *Acidovorax, Diaphorobacter* or *Verminephrobacter* genus, or to an undescribed species.

Results obtained from the neighbor-joining tree are similar to those obtained by Xu et al. (2001). Nevertheless, the wide genetic distance between the MVC/S-EM4 and the MJON-VI has to be remarked. Despite that the MVC/S-EM4 sequence was obtained from a MJON-VI infected plant, results indicate that this sequence is genetically closer to the betaproteobacteria proteins than to the MJON-VI sequence itself (accession no. AB047762). Isolation and identification of the bacterial species present in the egg masses of the Japanese population MJON-VI would be necessary to perform new phylogenic trees. Mutations on the genomic DNA sequences have been detected in the amplified fragment. The fact that *Nde*I was able to digest the genomic sequences of MJON-VI populations in Xu et al. (2001) experiments, but not in the ones described in this paper, can be explained by the mutation in the target sequence of the restriction enzyme. The maintenance and replication of this population from a single egg mass, for the last 10 years, may have

selected a different strain of bacteria from the nematode population MJON-VI through several mutations.

Virulent populations overcame all the resistance plant mechanisms and reproduce successfully on *Mi* tomato cultivars. Apart from the MVC molecular marker, the (*a*)*vir* candidates protein *MAP-1* and *Cg-1* cannot be used for general detection of virulence. No functional evidence correlating the *avr* candidate protein *MAP-1* to virulence or avirulence has been established, as this protein candidate did not reveal genetic polymorphisms between a virulent and an avirulent isolate of *M. javanica* (Gleason et al., 2008). The *Cg-1* marker has been detected in the three most important species of *Meloidogyne* affected by *Mi-1* mediated resistance, indicating that this gene was probably present in a common nematode ancestor (Gleason, 2003; Gleason et al., 2008). No similarities have been found to date between *Cg-1* and other public sequences on the GenBank although silencing experiments performed wit dsRNA indicated that *Cg-1* is required in the nematode for *Mi*-mediated resistance. Nevertheless, *Cg-1* has not been tested with more virulent isolates from different geographic origins. Thus, with the refuting of the MVC marker as a molecular virulence related locus, no reliable molecular markers are able at the present moment for the easy identification of virulent populations of root-knot. Therefore, virulence still needs to be determined by pathogenicity assays, until new molecular markers will be created.

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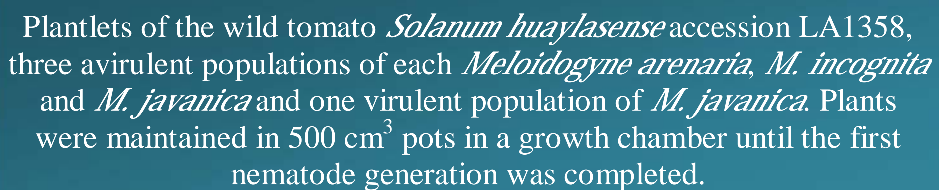

Plantlets of the wild tomato *Solanum huaylasense* accession LA1358, three avirulent populations of each *Meloidogyne arenaria*, *M. incognita* and *M. javanica* and one virulent population of *M. javanica*. Plants were maintained in 500 cm^3 pots in a growth chamber until the first nematode generation was completed.

6 Analysis of the resistance response of *Solanum huaylasense* to *Meloidogyne spp*

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Pathogenicity test were performed in order to determine resistance of *Solanum huaylasense* to four populations of *Meloidogyne* spp. The *S. huaylasense* accession LA1358, the root-knot nematode resistant tomato cultivar Anairis and the susceptible tomato cultivar Bodar were assessed against three *Mi* avirulent populations of *Meloidogyne arenaria*, *M. incognita* and *M. javanica* and to one naturally *Mi* virulent population of *M. javanica*. The number of eggs per plant, referred to the number of infective juveniles inoculated, was used as the dependent variable to determine variability in the reproduction of the four *Meloidogyne* populations tested. Results indicate that *S. huaylasense* accession LA1358 was able to reduce reproduction of the *M. arenaria* population at similar levels as the resistant tomato cultivar Anairis. Reproduction of the *M. incognita* population in accession LA1358 was highly variable and it did not differ from reproduction on resistant and susceptible tomato cultivars used as controls. Nevertheless, the *S. huaylasense* accession LA1358 did not reduce reproduction of the avirulent and the naturally virulent *M. javanica* populations tested. This is the first report on a nematode-species specific resistance in the newly described species *S. huaylasense*. Identification of novel root-knot nematode resistance genes in wild *Solanum* species is the first step for the deployment of new resistance genes in tomato cultivars to preserve durability of plant resistance to root-knot nematodes.

Keywords: Durability; Mi-gene; Mi-homologues; resistance genes, Solanum species, wild tomatoes.

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Introduction

Root-knot nematodes of the genus *Meloidogyne* are important pests that cause millionaire economic losses in agriculture worldwide (Sasser and Freckman, 1987). In tomato (*Solanum lycopersicum* Mill.) the *Mi-1* gene confers resistance to *Meloidogyne arenaria* (Neal) Chitwood, *M. incognita* (Kafoid and White) Chitwood and *M. javanica* (Treub) Chitwood (Roberts and Thomason 1989). It was first identified in *Solanum peruvianum* L. accession PI 128657 in the 1940s and introduced through embryo rescue in the commercial *S. lycopersicum* (Smith, 1944). All resistant tomato cultivars commercialized nowadays originate from that single resistant hybrid (Williamson, 1998). The *Mi-1* gene is referred to as *Mi* in this text. The *Mi* mediated resistance is phenotipically expressed only when soil temperature is below 28˚C (Dropkin, 1969). Resistance conferred by the *Mi* gene considerably reduces nematode reproduction, although variability in the efficiency of resistant cultivars to control different *Meloidogyne* populations has been observed (Netscher, 1976; Roberts and Thomason, 1989; Tzortzakakis et al., 1998). Despite these few limitations, plants carrying the *Mi* gene are considered a reliable, economical and environmentally friendly method to control *Meloidogyne* spp. in infested fields (Sorribas et al., 2005). Moreover, specific rotation sequences with resistant tomato cultivars have been suggested to prevent the emergence of virulent populations (Talavera et al., 2009).

The use of a single genetic source of resistance (*R*) genes in monoculture can lead to the defeating of valuable resistance genes (Pedersten and Leath, 1988). Race-specific resistance associated to monogenic genes that provide a hypersensitive response (HR) has been proved to be non-durable (Lindhout, 2002). Despite the fact that *Mi*-mediated resistance in tomato has remained durable for a long time (Roberts, 1995; Castagnone-Sereno, 2002), the appearance of virulent root-knot nematode populations after repeated exposure to the *Mi* gene has already been reported in field conditions (Kaloshian et al., 1996; Eddaoudi et al., 1997; Ornat et al., 2001; Xu et al., 2001; Tzorzakakis et al., 2005; Verdejo-Lucas et al., 2009). In this sense, gene pyramiding has been proposed as a strategy to reduce the chances of appearance of virulent populations and to preserve the durability of plant resistance (Pink, 2002). Simultaneous deployment of *R* genes has been implemented to control nematodes (Sacks and Robinson, 2009), bacteria (Kousik and Ritchie, 1999; Singh et al., 2001), virus (Pérez de Castro et al., 2008; Vidavsky et al., 2008)

and fungi (Richardson et al., 2006). Wild *Solanum* species have been widely explored as a source of new *R* genes to control root-knot nematodes in the tomato crop. Several *Mi*-homologues (from *Mi-2* to *Mi-9*), that present resistance either to virulent nematode populations or to high temperatures, have been found in accessions of the species that integrate the *Solanum peruvianum* Marañón complex (Ammati et al., 1986; Cap et al., 1993; Yaghoobi et al., 1995; Veremis and Roberts, 1996a, 1996b, 2000; Jablonska et al., 2007). This complex has been recently split into four new species (Peralta et al., 2005; Zuriaga et al., 2008): *S. arcanum* Peralta (formerly *S. peruvianum*), *S. corneliomulleri* J.F.Macbr. (formerly *S. glandulosum*), *S. huaylasense* Peralta and *S. peruvianum* L. s.str. Both *S. peruvianum* and *S. arcanum* have been widely screened for *Meloidogyne* spp. resistance (Ammati et al., 1986; Veremis and Roberts, 1996a, 1996b, 2000). However, no information is available about the spectrum of resistance of *S. corneliomulleri* and *S. huaylasense* to root-knot nematodes. Only resistance to the fungi *Alternaria solani* Sorauer and *A. tomatophila* Simmons has been described in *S. huaylasense* (Foolad et al., 2007).

Therefore, searching, identification and characterization of new nematode *R* genes would be the first step towards the use of "pyramided" cultivars, with the aim to preserve plant resistance on a long term basis. In a previous screening for resistance to root-knot nematodes carried out over several accessions of *S. chilense*, *S. habrochaites*, *S. peruvianum* and *S. huaylasense*, pathogenicity assays indicated that the *S. huaylasense* accession LA-1358 was able to reduce nematode reproduction at the levels of a resistant tomato cultivar. This paper presents the results of pathogenicity tests conducted to determine the response of the wild tomato *S. huaylasense* accession LA-1358 to three avirulent populations of *M. arenaria*, *M. incognita* and *M. javanica* and one naturally virulent population of *M. javanica*.

Material and Methods

Nematode Screens

Tomato cultivar seeds were germinated in seedling trays filled with an organic planting mix (Sun Gro Horticulture). The tomato cultivars used as controls were

Anairis [De Ruiter Seeds; highly resistant to: *Tomato mosaic virus* (ToMV); *Tomato spotted wilt virus* (TSWV); *Fusarium oxysporum* f.sp. *lycopersici* races 0 and 1 (Fol:0,1); *Verticillium albo-atrum* (Va); *V. dahliae* (Vd); *Meloidogyne arenaria* (Ma), *M. incognita* (Mi); *M. javanica* (Mj)] and Bodar F_1 [Seminis Royal Sluis; highly resistant to: *Tobacco mosaic virus* (TMV); *Tomato spotted wilt virus* (TSWV); *Fusarium oxysporum* f.sp. *lycopersici* races 1 and 2 (Fol:1,2); *Verticillium albo-atrum* (Va); *Verticillium dahliae* (Vd)]. Cuttings from the wild tomato *S. huaylasense* accession LA-1358 were treated with Inavarplant-IV growing hormone (3-indolbutiric acid at 0.4%; 1-naphthaleneacetic acid at 0.4%; Captan 15%; Inbar) and rooted in seedling trays in vermiculite. Cuttings were maintained in a growth chamber at 25˚C until new leaves were produced. Five-week-old rooted cuttings and plantlets of tomato cultivars at the three-true-leaf stage were transplanted singly into 500 cm^3 pots containing a mixture of steam-sterilized river sand and peat (v/v) and used for nematode assays a week later.

Four *Meloidogyne* populations were used for nematode screening: one avirulent population of each *M. arenaria* (code MA-68), *M. incognita* (code MI-CROSS) and *M. javanica* (code MJ-05), and one virulent population of *M. javanica* (code MJ-27). The (a)virulent condition of each population had been previously tested (Ornat et al., 2001; Cortada et al., 2009). The identity of the *Meloidogyne* populations was confirmed by SCAR-PCR reaction (Ziljstra et al., 2000). Nematode inoculum was obtained from infected tomato plants of the susceptible cultivar Roma maintained in a glasshouse. Roots were macerated for 10 min in a blender containing a 0.5% NaOCl solution and eggs collected (Hussey and Barker, 1973). Infective second-stage juveniles (J2) were obtained from hatched eggs as described by Martínez de Ilarduya and Kaloshian (2001). Each plant was inoculated with 130 J2 collected after 72 h. Every cultivar-nematode population combination was replicated 12 times whereas the accession LA-1358 was replicated 10 times. Pots were maintained for 6 weeks in a growth chamber at 25 ± 1.5˚C until the first nematode generation was completed. Plants were fertilized with a slow release fertilizer (NPK: 17-7-10; Osmocote® Pro, Sierra Chemical).

At harvest, the tops were cut at ground level and the root systems washed free of soil and weighed. The number of eggs per plant was determined by maceration of the entire root system, as described previously for the nematode inoculum.

Statistical analyses

Fresh root weight of *S. huaylasense* accession LA-1358 had to be checked for differences with cultivars Anairis and Bodar, in order to avoid data distortion caused by the low root weight of *S. huaylasense* cuttings that differed from both tomato cultivars (factorial ANOVA; factors: nematode population and tomato variety; $F_{(6,\ 121)} = 5.384$; $P < 0.001$) (HSD test; Fig. 1). The reproduction rate (RR) was calculated as the number of eggs per plant obtained at the end of the experiment in relation to the initial inoculum (number of eggs per plant per J2 inoculated). The RR was fourthroot transformed to comply with test assumptions, and then subjected to factorial ANOVA, where nematode population and tomato variety (cultivars and accession) were included as factors.

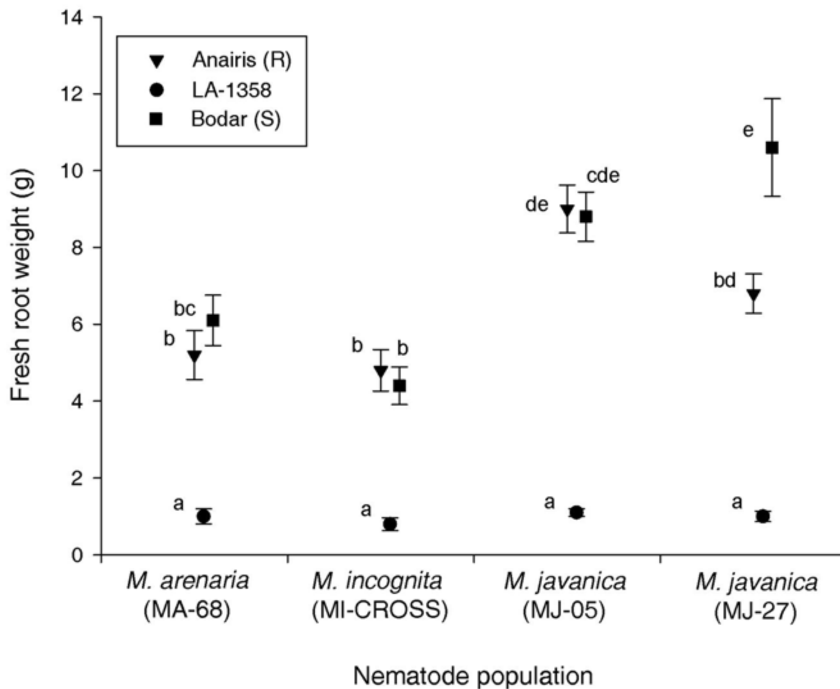

Figure 1. Mean fresh root weight (g) of *Solanum huaylasense* accession LA-1358, resistant tomato cultivar Anairis and susceptible tomato cultivar Bodar six weeks after nematode inoculation with four *Meloidogyne* populations. Different letters indicate significant differences (HSD test). Error bars depict the standard error of the mean.

For each nematode population, the post-hoc Tukey test procedure was used to determine differences in reproduction between *S. huaylasense* accession LA-1358 and the resistant or susceptible cultivars. The *S. huaylasense* accession LA-1358 was considered resistant when its RR did not differ statistically from the resistant cultivar Anairis. The reproduction index (RI), calculated as the number of egg per plant on the *S. huaylasense* accession LA-1358 or the resistant cultivar divided by the number of eggs per plant on the susceptible cultivar ×100, was used to categorize the level of resistance as highly resistant (RI < 10%), intermediate resistant (10% RI< 25%) or moderately resistant (25% RI< 50%) (Hadisoeganda and Sasser 1982). Statistica software (StatSoft, Inc., 2004) was used for the statistical analyses.

Results

The plant materials analyzed had a differential resistance response across the four different nematode populations tested ($F_{(6,121)}$ = 3.468; *P*= 0.003) (Fig. 2).

A multiple comparison test performed for all the populations upon the RR data on the susceptible control Bodar indicated that the four *Meloidogyne* populations presented a similar ability to reproduce in the absence of the *Mi* mediated resistance. The RR values were significantly lower on the resistant cultivar Anairis than on the susceptible Bodar, for *M. arenaria* MA-68 (*P*< 0.001), *M. incognita* MI-CROSS (P< 0.01) and *M. javanica* MJ-05 (*P*< 0.01) (Fig. 2), thus confirming the avirulent character of these populations.

The RR value of the *M. arenaria* MA-68 population on *S. huaylasense* accession LA-1358 was similar (*P*= 0.07) to that of the resistant cultivar Anairis, and lower (P< 0.001) than on the susceptible cultivar Bodar (Fig. 2). For the *M. incognita* MI-CROSS population, reproduction differed neither from the resistant control Anairis (P= 0.70) nor the susceptible cultivar Bodar (P= 0.13) (Fig. 2). Reproduction of the avirulent population *M. javanica* MJ-05 was higher (P= 0.02) on the *S. huaylasense* accession LA-1358 than on the resistant cultivar Anairis and did not differ from reproduction on Bodar (P= 0.07) (Fig. 2). As expected, the virulent population *M. javanica* MJ-27 reproduced equally on the *S. huaylasense* accession LA-1358 and on both tomato cultivars (P> 0.60) (Fig. 2). Calculation of the *RI* for the *S. huaylasense* accession LA-1358 indicated that it showed intermediate resistance to *M. arenaria* MA-68 (RI=

20%), moderate resistance to *M. incognita* MI-CROSS (RI= 38%), and susceptibility to both *M. javanica* populations (Table 1). The resistant control Anairis responded as highly resistant (RI = 10%) to *M. arenaria* MA-68, and intermediate resistant to *M. incognita* MI-CROSS (RI= 12%) and *M. javanica* MJ-05 (RI= 22%) (Table 1).

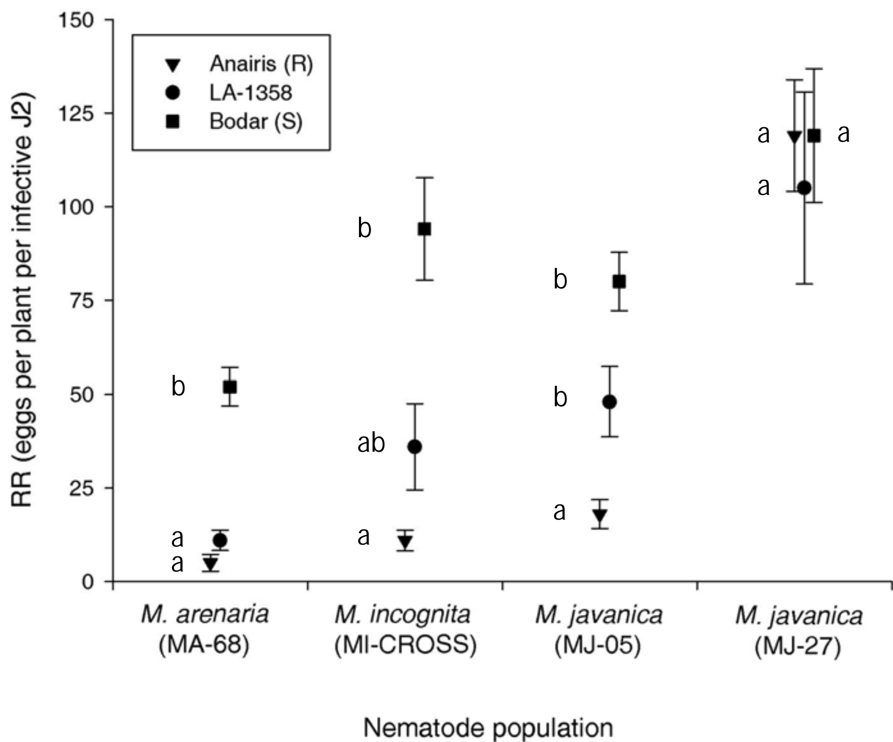

Figure 2. Mean reproduction rate (RR) of four *Meloidogyne* populations on *Solanum huaylasense* accession LA-1358, resistant tomato cultivar Anairis and susceptible tomato cultivar Bodar six weeks after nematode inoculation. Error bars depict the standard error of the mean. For each nematode species, values in the same column followed by different lower-case letters are significantly different according to Tukey's studentized range test ($P < 0.05$).

Table 1. Reproduction index (RI) calculated as the number of eggs per plant on *Solanum huaylasense* accession LA-1358 and on the resistant tomato cultivar Anairis, expressed as a percentage of the number of eggs per plant on susceptible tomato cultivar Bodar in plants inoculated with *Meloidogyne arenaria*, *M. incognita* or *M. javanica* six weeks after plant inoculation.

Population	(A)Virulence status	Tomato	RI[1]	Response
M. arenaria (MA-68)	Avirulent	LA-1358	20 ± 16	Intermediate resistant
		Anairis (R)	10 ± 15	Highly resistant
		Bodar (S)	100 ± 34	
M. incognita (MI-CROSS)	Avirulent	LA-1358	38 ± 39	Moderately resistant
		Anairis (R)	12 ± 10	Intermediate resistant
		Bodar (S)	100 ± 44	
M. javanica (MJ-05)	Avirulent	LA-1358	60 ± 37	Susceptible
		Anairis (R)	22 ± 17	Intermediate resistant
		Bodar (S)	100 ± 34	
M. javanica (MJ-27)	Virulent	LA-1358	88 ± 61	Susceptible
		Anairis (R)	100 ± 44	Susceptible
		Bodar (S)	100 ± 52	

[1]For each nematode-plant combination, valid n=12, except for *S. huaylasense* accession LA-1358, where valid n=10.

Discussion

Results presented in this paper indicate that the accession LA-1358 of the newly described species *S. huaylasense* shows root-knot nematode resistance, although this resistance is species-specific and only effective against *M. arenaria* and *M. incognita* but not against *M. javanica*. As only one avirulent population of *M. arenaria*, *M. incognita* and *M. javanica* were tested, it cannot be asserted that the resistance response of accession LA-1358 is nematode-isolate specific.

Among the *Meloidogyne* populations, the RR values remained similar when they were inoculated on susceptible Bodar indicating that the tested populations had the same reproduction ability in absence of the *Mi* gene. Resistance to root-knot nematodes in tomato has been generally characterized by the failure of the nematodes to induce the formation of giant cells in the host after infection, to molt and to develop into adult gravid females (Williamson and Kumar, 2006). As the RR value for *M. arenaria* MA-68 on accession LA-1358 did not differ statistically from RR on the resistant cultivar Anairis, but it was significantly lower than on susceptible cultivar Bodar, it was assumed that a resistance mechanism in accession LA-1358 either prevents J2 from penetrate into the root, settle down and/or become adult fertile females. A similar phenomenon seems to occur with *M. incognita* MI-CROSS population, but to a lesser extent. Reproduction of the virulent *M. javanica* MJ-27 population was similar among all the plant materials tested, confirming that when an incompatible interaction occurs between a virulent nematode and a resistant plant (*vir- R*-gene), no differences are found between resistant and susceptible cultivars regarding infection or female fecundity (Ornat et al., 2001).

Solanum peruvianum is the wild *Solanum* species that has provided most of the *R* genes to root-knot nematodes (Ammati et al., 1986; Veremis and Roberts, 1996a, 1996b). From the formerly species included in the *S. peruvianum* Marañón complex, *S. huaylasense* is the closest to *S. peruvianum* according to molecular data (Moyle ,2008). The fact that these species derive from an original *Solanum* ancestor, supports the hypothesis that all of them share a common pool of *Mi*-homologues (Seah et al., 2007) and hence, it would not be surprising to find a resistant *Mi*-homolog in *S. huaylasense*. Specificity in the resistance response of some *Mi*-homologues has been reported in other *Solanum* species by Roberts et al. (1990) and Huang et al. (2004). In these works, the resistance response of the wild *Solanum Mi*-homologues presented a variable phenotype according to the *Meloidogyne* isolate tested. In tomato, Cortada et al. (2009) reported similar results for the hybrid rootstocks Beaufort and Maxifort. This phenomenon has also been reported for resistant soybean (*Glycine max*) (L.) Merr. (Luzzi et al., 1987), resistant tobacco (*Nicotiana tabacum*) L. (Bowman et al., 1990; Noe, 1992), the *Me-3* resistance gene in pepper (*Capsicum annuum*) L. (Castagnone-Sereno et al., 2001), and for other families of race-specific monogenic *R* genes (e.g. *Ty*-homologues) (Pérez de Castro et al., 2008). Even within tomato cultivars, genotype differences have an effect on the effectiveness of *Mi* gene (Roberts and Thomason, 1989; Jacquet et al., 2005). Understanding this phenomenon will be necessary for the successful transfer of new root-knot nematode *R* genes into cultivated tomato. Despite that

the main obstacle to obtain new resistant hybrids is the incompatibility between the germplasms of wild *Solanum* species and cultivated tomato (Veremis and Roberts, 2000; Ammiraju et al., 2003), transference of the *Mi*-resistance gene to susceptible tomato plants has been achieved using transgenic techniques (Williamson, 1998; Goggin et al., 2006; Williamson and Kumar, 2006). In addition, interesting works have been performed to introduce new *Mi*-resistance genes from wild *Solanum* species into *S. lycopersicum* through "bridge lines" or embryo rescue (Williamson and Hussey, 1996). Unfortunately, putting new resistance genes from wild *Solanum* species at disposal of new tomato cultivars is not a trivial matter. So far, it seems that the most feasible alternative to overcome germplasm incompatibility is the use of tomato hybrid rootstocks (*S. lycopersicum* × *Solanum* spp.) (Santos et al., 2004).

Naturally and selected resistance-breaking populations of *Meloidogyne* spp. have been reported worldwide from agricultural fields where *Mi*-resistant tomato cultivars are cropped (Kaloshian et al., 1996; Eddaoudi et al., 1997; Ornat et al., 2001; Xu et al., 2001; Tzorzakakis et al., 2005; Verdejo-Lucas et al., 2009). Therefore, the identification of new root-knot nematode *R* genes in tomato opens a door to preserve the efficiency of the *Mi*-mediated resistance through combination of different *Mi*-homologues into one single genotype. Although this is the first step towards the identification of *S. huaylasense* as a new source of root-knot nematode *R* genes, a deeper characterization of the resistance spectrum of this species will be necessary, including additional *Meloidogyne* populations from several geographic origins and their evaluation under different agronomic and environmental conditions (e.g. high temperatures). Comprehension of the species-specific resistance response of the *S. huaylasense* accession LA-1358 will also provide insights into host mechanisms underlying specific plant-nematode interactions.

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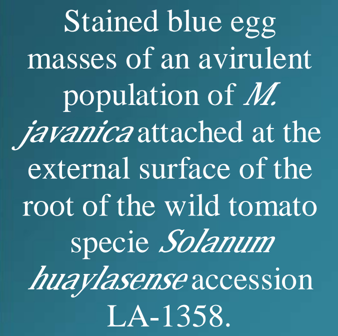

Stained blue egg masses of an avirulent population of *M. javanica* attached at the external surface of the root of the wild tomato specie *Solanum huaylasense* accession LA-1358.

Plantlets of tomato rootstock Maxifort at the three true-leaf stage ready for grafting at a commercial nursery in Níjar (Almería)

General discussion

Based on the experiments performed along this thesis, the objectives of characterization of the resistance response of tomato rootstocks to *Meloidogyne* spp., and the evaluation of the durability of the *Mi*-resistance have been achieved. Data indicates that the resistance response of the *Mi-1* tomato hybrid rootstocks is highly variable (Chapter 1) and some rootstocks show a nematode-isolate specific resistance response (Chapter 2). Abiotic factors like soil temperature also influenced the phenotypic expression of the *Mi*-mediated resistance in tomato rootstocks (Chapter 1). The molecular markers presently available failed to characterize the *Mi* locus in tomato hybrid rootstocks, which lead us to design a new molecular marker for detection of the *Mi-1.2* gene (Chapter 3). Field experiments demonstrated for the first time that selection of virulence occurred after repeated cultivation of resistant tomato rootstocks in the same experimental plots, and that selection occurred faster in resistant rootstocks than in resistant tomato cultivars (Chapter 4). Thus, *Mi*-tomato rootstocks were considered a non-durable strategy to control *Meloidogyne* spp. in nematodes infested soils. In addition, it was proved that selected-virulent populations of *Meloidogyne* cannot be traced at the molecular level using the MVC molecular marker, as this marker is related to proteins of betaproteobacteria species but not to a nematode virulence effector (Chapter 5). The wild tomato species *S. huaylasense* was assessed to search for new *Nem-R* genes that could be a new source of resistance in tomato breeding programs (Chapter 6). The results presented in this dissertation are relevant for the use of resistant tomato rootstocks as a management strategy to control *Meloidogyne* spp.

Variability of the resistance response of *Mi-1* tomato rootstocks

The resistance response of the *Mi-1* tomato rootstocks tested was highly variable compared to the resistant tomato cultivars used as controls (e.g. Monika and Caramba). When rootstocks were confronted to a *Mi*-avirulent population of *M. javanica*, the resistance phenotype ranged from highly

resistant to fully susceptible, although intermediate resistance responses were also found (Chapters 1 and 2). The susceptible phenotype of rootstock Beaufort (Graf et al., 2001; López-Pérez et al., 2006) has been confirmed in this thesis, and the susceptible phenotype of Maxifort (Chapter 1 and 2) and the moderately resistance of Big-Power and He-man is reported for the first time. The phenotype of the rootstocks in the glasshouse tests involving one nematode generation were validated in the field indicating that the resistance response of tomato rootstocks can be early characterized.

The resistance response of *Mi-1* tomato rootstocks also varied according to the soil temperature. Although most of the studied hybrid rootstocks (*S. lycopersicum* × *S. peruvianum* × *Solanum* spp.) were susceptible when temperatures rose above 28 ˚C, rootstocks PG-76 and He-man were able to inhibit root-knot nematode reproduction (Chapter 1). This suggests that there is, at least, one MiGH in the genome of PG-76 and He-man different from *Mi-1.2* gene which is heat-stable resistant to the *Mi*-avirulent nematode population tested. Most likely this heat-stable MiGH(s) was introgressed from the wild *Solanum* parental species. Therefore, some resistant tomato rootstocks have an advantage over tomato cultivars, as they express resistance during the hottest time of the year. This heat-stable resistant phenotype turns these rootstocks into a suitable method to inhibit nematode reproduction in areas where summer temperatures frequently exceed 28 ˚C (e.g. Mediterranean basin). The resistant tomato cultivar Caramba reduced more effectively the reproduction of the nematode than the resistant cultivar Monika when temperatures above 28 ºC (Chapter 1). Although no heat-stable MiGHs are present in the *S. peruvianum* introgression of Caramba, the higher resistance of this cultivar points out the influence that the genetic background of tomato cultivars has on the expression of the *Mi*-mediated resistance (Jacquet et al., 2005). In the light of the results presented in this thesis, this can be also applicable for resistant tomato rootstocks.

Tomato rootstocks Beaufort and Maxifort responded as susceptible to *M. javanica* but showed a nematode-isolate specific resistance response when they were infected with different *Mi*-avirulent populations of *M. arenaria* and *M. incognita* (Chapter 2); this phenotypic variability was not observed in the resistant tomato cultivars: Monika (Chapter 2) and Anairis (Chapter 6). Nematode-isolate specific resistance responses have been mainly described in MiGHs from *S. peruvianum* (e.g. *Mi-3*, *Mi-7*, *Mi-8*) (Ammati et al., 1986; Roberts et al., 1990; Veremis and Roberts, 1996a, 1996b) but also for the *Mi-1.2* gene (Sorribas and Verdejo-Lucas, 1999). Thereby, the question that rose

from these results was which is the mechanism associated to the nematode-isolate specific resistance response is of these rootstocks.

The plant-nematode pathosystem is influenced by the nematode isolate and the genetic background of the resistant plant (Jacquet et al., 2005). In this sense, the use of wild *Solanum* species in tomato breeding programs increases the genetic variability of rootstocks compared to cultivars, because of the inherent variability of the *Solanum* accessions used as parentals. The gen-for-gen model (Flor, 1971) has been accepted to explain the interaction between root-knot nematodes and the *Mi-1* gene. So far, this theory has not been completely demonstrated as no Mendelian analyses can be performed to study the *Avr-R* gene interaction for the parthenogenetic species *M. arenaria*, *M. incognita* and *M. javanica* (Roberts, 1995; Bakker, 2002; McK Bird et al., 2009). In contrast to this theory, the *guard model* postulates that the initial pathogen recognition occurs between of a host protein (e.g. *Rme1*) of the resistant plant and the nematode *Avr* effector. This first interaction induces a conformational change of the targeted protein that allows the union to the *R* protein (e.g. *Mi-1.2*) and the subsequent resistance response of the infected cell (Kaloshian, 2004) (Fig. 1). According to this model, the recognition of the infection by the *R* gene would be modulated by both the genetic background of the resistant plant and the specific *Avr* determinant of each nematode population. The nematode isolate-specific resistance response of Beaufort and Maxifort could be associated to the introgression of different genes (e.g. host genes and/or *R* genes) from *S. habrochaites* in their genetic background (Chapter 3). Therefore, the recognition of the nematode infection in resistant tomato rootstock would be more specific than previously thought.

As opposed to the resistant tomato rootstocks, the resistant tomato cultivars available nowadays originate from a single F1 hybrid (*S. lycopersicum* × *S. peruvianum*) obtained by Smith in 1944; in addition breeders have done many efforts to reduce the *S. peruvianum* introgression in chromosome 6 (Ho et al., 1992), which overall might have reduced the genetic variability of *Mi*-tomato cultivars. Thus, in the light of the presented results it can be concluded that the resistance response of the tomato hybrid rootstocks is more variable than that of resistant tomato cultivars as it is depends on the genetic background of the rootstock, the nematode isolate tested and the soil temperature.

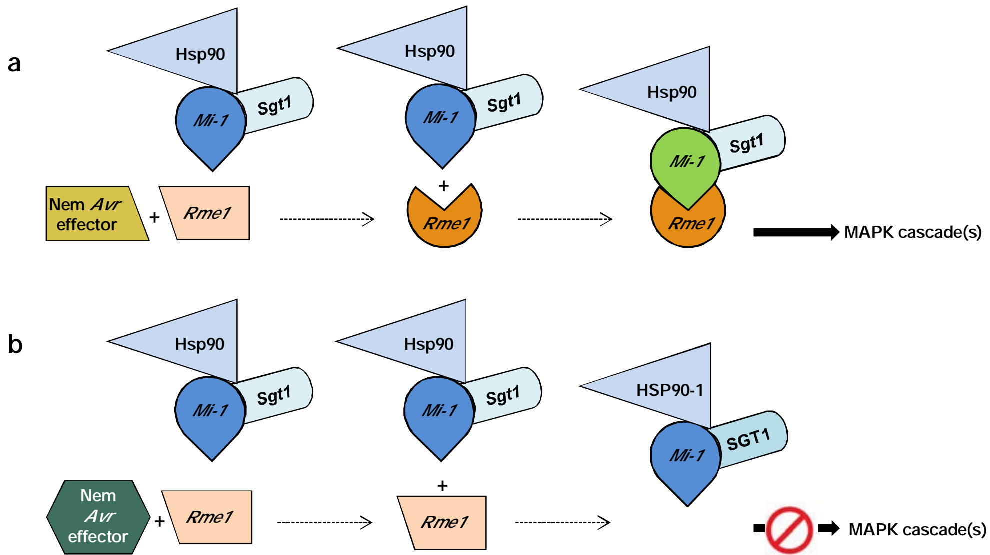

Figure 1. Predicted interaction between the host proteins of a *Mi*-resistant tomato and the *Avr* effectors of two nematode populations (a and b), based on the guard model. The *R* proteins Hsp90 (heat shock protein 90), and the *Stg1* (suppressor of the G2 allele of SKP1) form a functional cluster of *R* proteins to assist in the resistance response of the *Mi-1* gene. **(a)** In an incompatible interaction (resistant response), the nematode *Avr* effectors interact with a NBS-LRR *R* proteins (*Rme1*) to form an *R*-signaling complex to trigger the downstream *Mi-1* resistant pathway (e.g. MAPK cascades). **(b)** In a compatible interaction (susceptible response), the lack of recognition of the nematode *Avr* effector by the host protein inhibits the conformational change of the host protein and prevents the subsequent resistance response mediated by the *Mi-1* gene like MAPK cascade (adapted from Bent and Mackey 2007 and Bhattarai et al., 2007).

The characterization of the *Mi-* locus in tomato hybrid rootstocks

The molecular characterization of the *Mi*-locus in tomato rootstocks was initially done using the conventional marker REX-1 but the phenotypic resistance response of a subset of the rootstocks (e.g. Beaufort and Maxifort) was in disagreement with genotyping data (Chapter 1 and 2). Therefore PCR markers for detection of root-knot nematode resistance in tomato lines with introgressions from wild *Solanum* species were used for the characterization of the *Mi*-locus. Although the Mi23 marker was initially used to characterize the *Mi*-locus in tomato rootstocks (Chapters 1 and 2) further analyses indicated that this marker was polymorphic for the species *S. habrochaites*, *S. peruvianum* and

S. lycopersicum (Chapter 3). Similar results were obtained for PMi marker. The PM3 marker was only suitable to trace the *Mi*-locus in hybrid rootstocks of *S. lycopersicum* × *S. habrochaites* (e.g. Brigeor, He-man, Beaufort, Maxifort). As the PM3 marker was designed to anneal at the 3' end of the UTR of the gene, the *Mi*-locus could not be characterized either in any hybrid of *S. lycopersicum* × *S. habrochaites* hybrids; only the presence of a *Sp-Mi*-like region could be confirmed. Characterization of the *Mi*-locus was not possible for those rootstocks of unknown parental origin (PG-76, Gladiator, and MKT-410). Thereafter, the allelic condition of the *Mi-1* locus could not be determined for any of the tomato hybrid rootstocks, and the question on whether the *Mi*-homozygous tomato rootstocks are more resistant than the *Mi*-heterozygous ones could not be answered.

As in silico tests performed with *Mi-1.2* specific primers (Mint-up/do, C1/2, C2S4, IMO.F1/R1, VIGS-F) indicated that none of the available molecular markers were able to distinguish the *Mi-1.2* gene, the new marker Pau-Do was designed. At the moment, Pau-Do and C2S4 (Milligan et al., 1998) are the only existing markers that allow the amplification of the *Mi-1.2* gene in resistant tomato cultivars (*S. lycopersicum* × *S. peruvianum*). Nevertheless the specificity of both markers should be tested in wider pool of rootstocks, cultivars and accessions of wild *Solanum* species to ensure their suitability for amplification of the *Mi-1.2* gene. As it was mentioned in Chapter 3, the hypothesis that a homolog different than *Mi-1.2* gene was amplified in this two tomato rootstocks remains possible, and cloning of this candidate gene would be required to identify its functional role.

The parental species of the resistant tomato rootstocks studied in this thesis were not available, as seed breeders refused to share this information. Therefore, the *Mi-1* locus could not be traced by resistance and marker screening of an F_2 population and the derived F_3 lines. The traditional molecular markers used in breeding (e.g. REX-1, PM3, PMi, Mi23) to identify the introgression of the *S. peruvianum Mi-1.2* gene, are not suitable for tomato hybrid rootstocks. The lack of knowledge about the sequence of the MiGHs present in wild tomato species also hampered the characterization the *Mi*-locus in resistant tomato rootstocks. As far as the number, sequence and function of the MiGHs present in the wild *Solanum* species used in breeding programs remains unknown, pathogenicity assays will still be essential to test resistance to root-knot nematodes in tomato rootstocks.

Durability of the *Mi*-mediated resistance in resistant tomato rootstocks

The second objective of this thesis was to determine the durability of the *Mi*-mediated resistance in tomato rootstocks to control *Meloidogyne* spp. The initial hypothesis considered was that the durability of the *Mi*-resistance would be similar to that of resistant tomato cultivars, although it did not. Virulent populations were rapidly selected from a *Mi*-avirulent nematode population of *M. javanica* after repeated cultivation of resistant tomato rootstocks during three cropping cycles in the field (Chapter 4). The resistance level of the rootstocks decreased as the frequency of cropping increased. This was particularly evident with rootstock PG-76 that became fully susceptible after the third cropping cycle. Virulence was also selected in plots planted with Brigeor and Monika, although at much lower speed. At the end of the third year, cultivar Monika provided similar resistance responses to those reported in previous studies (Sorribas et al., 2005; Verdejo-Lucas and Sorribas, 2008). The cropping frequency of the resistant plants was crucial, although the rapid selection of virulent nematodes in plots cultivated with PG-76 compared to Brigeor and Monika suggested the existence of a different resistance mechanism to root-knot nematodes in their genome.

The question that rose from the field experiments was why selection of virulence occurred so rapidly in rootstock PG-76. Avirulence (*Avr*) is considered a genetically dominant character and only homozygous individuals with recessive alleles (*avr*) can become virulent (Block et al., 1997). Therefore, it was initially considered that rootstock PG-76 had induced several mutational events in the genome of the population leading to acquisition of virulence. It is known that the durability of the resistance conferred by a *R* gene depends on the evolutionary potential of the pathogen population (McDonald and Linde, 2000). Experimental data indicates that *Meloidogyne* can accumulate a large amount of genetic variability in the offspring from one generation to the next (Jarquin-Barberena et al., 1991; Castagnone-Sereno, 2002). Mechanisms such as transposable elements, which are very abundant in the genome of some species like *M. incognita* (McK Bird et al., 2009), allow root-knot nematodes to exhibit high mutation rates compared to other organisms with asexual reproduction; polyploidy, aneuploidy or gene conversion could also play an important role in genetic plasticity (Roberts, 1995; McK Bird et al., 2009). In population genetics, creation of new genetic material through mutagenesis is considered the ultimate source of genetic variation (Hartl, 2000). In nematode populations where *avr* alleles are initially absent it is unlikely that these alleles will increase their frequency dramatically in a short period of time. If we consider that the

original nematode population was nearly fixed for the wild type allele for avirulence (*Avr*), non-lethal mutations were first needed for the acquisition of virulence; and in second term, the frequency of the *avr* alleles must increase in the genetic pool of the population in order to allow the appearance of homozygous virulent (*avr*/*avr*) individuals. As the spontaneous forward mutation rates are typically small (10^{-4} to 10^{-6} per allele and generation), the changes in the allele frequency as a result of recurrent mutation (mutation pressure) are very small over the course of a few generations (Hartl, 2000). Hence, the hypothesis of selection of virulence caused by direct mutagenic events favored by rootstock PG-76 was rejected due to the short time elapsed in the field experiments and the progressive appearance of virulence.

On the contrary, it has been proposed that selection for increased virulence will occur in populations that already contain virulent individuals that are able to reproduce on *R* plants (Block et al., 1997). This seems to be the case of the population of *M. javanica* infesting the field soil that became virulent following a gradual fashion as a result of the selection pressure exerted by the repeated cultivation of the resistant tomatoes in the same plots. Apparently, rootstock PG-76 produced a stronger directional selection pressure over the avirulent *M. javanica* population than rootstock Brigeor or cultivar Monika, as virulent individuals did not appear after three consecutive cropping cycles. From the different evolutionary forces described, selection pressure has been considered the main force that drives changes in the frequencies of mutant alleles (*avr*) at a faster pace than the other causes of evolution (e.g. genetic drift, gene flow, reproduction mating system or mutations) (Hartl, 2000; McDonald and Linde, 2002). The higher the selection pressure is, the faster the adaptation of the pathogens to the new environment and the appearance of individuals that are able to overcome the *R* genes. Rootstock PG-76 consistently showed the highest resistance response among all the rootstocks analyzed towards several *Meloidogyne* populations in pot experiments and in field conditions (Chapters 1, 2 and 4), and also in the hottest time of the year. Under these statements, we concluded that the repeated cultivation of the highly resistant PG-76 rootstock favored the reproduction of the virulent individuals already present in the population, resulting in a rapid change on the frequency of the *Avr* alleles.

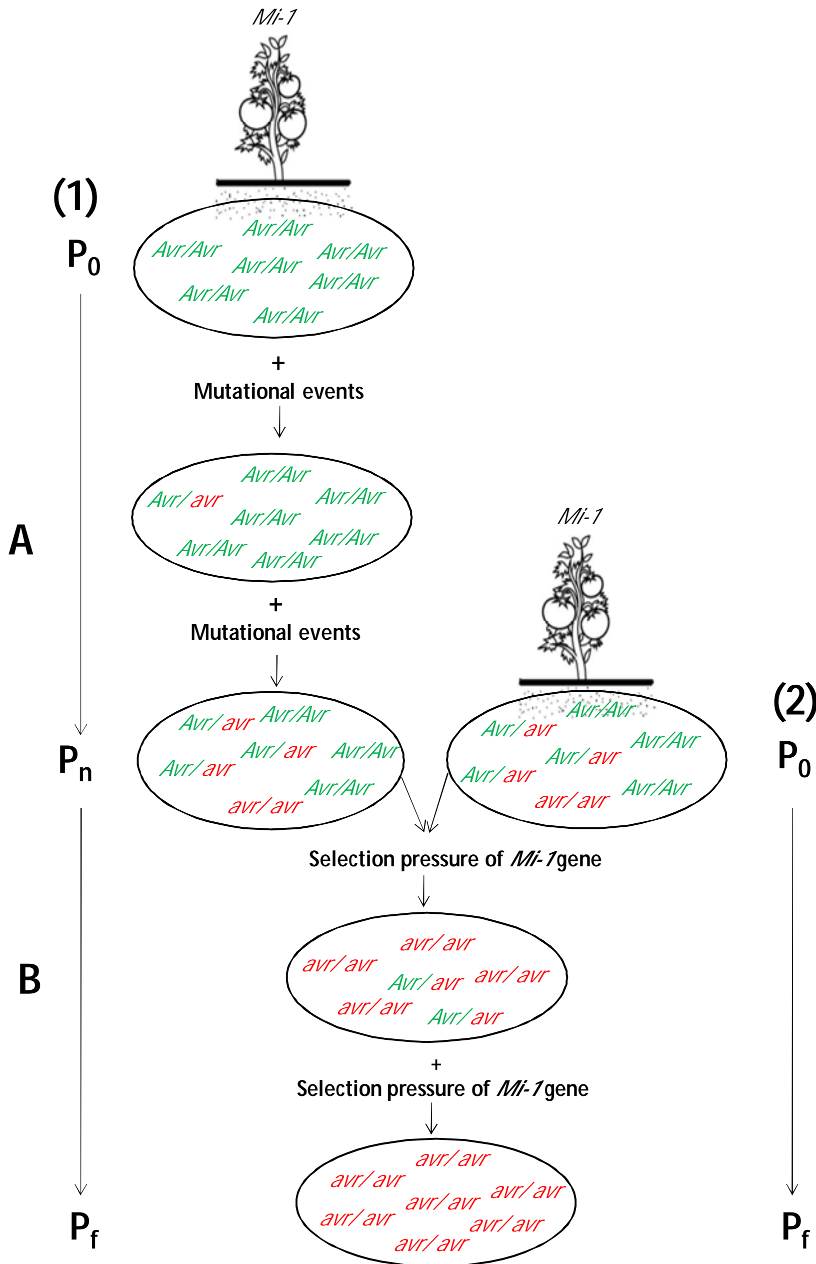

Figure 2. Variation on the frequency of the alleles of avirulence ***Avr*** (green) and virulence ***avr*** (red) in the genetic pool of two nematode populations (1) and (2). **(A)** Changes in the allelic frequency in the avirulent population **(P_0)** as a result of recurrent mutation (mutation pressure) leads to the acquisition of virulence after *n* nematode generations **(P_f)**. **(B)** Positive selection of the virulent alleles (*avr*) already present in the genetic pool of the population (P_0) by selection pressure of the *Mi-1* gene.

Noteworthy, selection for virulence has always been tested in resistant tomato cultivars. For the first time, it was demonstrated that not only the cropping frequency of the *R* plants has an effect upon the frequency of the *Avr* alleles of the population, but also that the genetic background of the resistant tomatoes is a critical factor for the selection of virulence. Thus, acquisition of virulence can be explained by the interaction between plant genotype, nematode isolate and cropping frequency but not by either factor alone. This close interaction between root-knot nematodes and the genetic background of tomato rootstocks has important implications in nematode management.

Molecular characterization of virulent populations of *Meloidogyne* spp.

Acquired virulence invalidates the use of the *Mi*-mediated resistance as a management strategy to control root-knot nematodes, since virulence is genetically stable and transmitted to the progeny (Castagnone-Sereno et al., 1993). Little is known about root-knot nematode *Avr* effectors and the reason why some isolates can reproduce on resistant plants, whereas others never overcome resistance (Jarquin-Barberena et al., 1991). In this thesis, it was demonstrated that the molecular marker MVC (Xu et al., 2001) described for characterization of selected-virulent populations of *Meloidogyne* spp. it is not related to root-knot nematodes but to betaproteobacteria proteins (Chapter 5). Virulence markers like *MAP-1* and *Cg-1* (Semblat et al., 2001; Gleason et al., 2008) have been tested with a limited number of nematodes populations which compromises their reliability as a molecular tool for identification of virulent root-knot nematodes. In the same way that the genetic background of tomato rootstocks is a key element in the selection of virulent populations, the genetic pool of the nematode populations is also a crucial factor that determines the durability of the *Mi*-mediated resistance. At the moment, molecular characterization of virulence in nematode populations is not feasible, and the potential virulence (frequency of *Avr*/ *avr* alleles) of the nematode population which is intended to manage will remain unknown. Consequently, the characterization of virulence still requires specific pathogenicity assays.

Management of *Meloidogyne* spp. with resistant tomato rootstocks

The differences found in the resistant responses of the tomato rootstocks have important implications in root-knot nematode management. High reproduction levels of the nematode were reached on some resistant tomato rootstocks just

after two nematode generations in spring and a single generation in summer. In addition, some resistant tomato rootstocks showed a nematode-species specific resistant phenotype; such variability has implications on rootstocks management as the effectiveness of the *Mi*-mediated resistance may be compromised by local isolates of root-knot nematodes infecting the soil.

As tomato rootstocks produce very vigorous plants, nematode infection might be initially go unnoticed before nematode population levels increase. Yield reduction of resistant tomato rootstocks was related to an increase on the reproduction of the nematode populations. Besides the deep and massive root systems of PG-76 and Brigeor, yield production was reduced on both resistant tomato rootstocks in the same way than on resistant cultivar Monika after the third cropping cycle (Chapter 4). Although the use of tomato rootstocks can be profitable and are considered an ecological alternative to chemical control, particularly for organic farming, nematode management should include rotation of resistant rootstocks with susceptible genotypes. For resistant tomato cultivars, rotation sequences involving one cropping cycle with a susceptible cultivar followed by two consecutive cropping cycles with resistant cultivars have been proposed for effective suppression of *Meloidogyne* spp. on tomato cultivated in nematode-infested soils in plastic houses (Talavera et al., 2009). The optimum cropping sequence of resistant rootstocks needs to be determined to preserve the durability of the resistance and prevent the selection of virulent populations.

As it has been shown in Chapter 4, the use of a single genetic source of resistance in monoculture is a thread for the durability of valuable resistance genes (e.g. *Mi-1.2* gene). For this reason, wild *Solanum* species have been largely explored as novel sources of *R* genes and improved agronomical traits. Preliminary studies revealed the resistance of the accession LA-1358 of *S. huaylasense* to *M. arenaria* and *M. incognita* but not to *M. javanica* (Chapter 6). This resistance, however, was nematode-species specific, a similar situation found in rootstocks Beaufort and Maxifort (Chapters 1 and 2). Screening larger numbers of accessions along with a deeper characterization of the resistance spectrum of wild *Solanum* species will be necessary in order to identify novel nematode resistance in wild tomatoes.

The management of the *Mi*-mediated resistance in tomato rootstocks should be particularly careful compared to that of *Mi*-resistant tomato cultivars, due to the variability of their effectiveness and the potential selection of virulent populations. The genetic background of the resistant tomato rootstocks is

complex and unknown, and the molecular tools currently available are inadequate for their characterization it. The existence of several MiGHs in tomato hybrids rootstocks that provide resistance to different nematode populations and environmental conditions have been demonstrated in this thesis. For this reason, the selection pressure that resistant tomato rootstocks can exert on field populations may be superior to that of resistant tomato cultivars. Emergence of virulent nematode populations results in a point of no return on the use of plant resistance as a management method to control root-knot nematodes.

Glass-house facility at IRTA where bioassays where performed. This picture corresponds to the pathogenicity test performed to determine the resistance response of tomato rootstocks to *M. javanica* (Chapter 1).

Conclusions

1.- The phenotypic resistance response observed in *Mi-1* tomato rootstocks ranged from highly resistant to susceptible.

2.- A subset of *Mi-1* tomato rootstocks showed a nematode-isolate specific resistance response.

3.- Susceptible and intermediate resistance responses in tomato rootstocks cannot be detected through molecular techniques.

4.- The molecular markers Mi23, PMi and intron-1 used in tomato breeding programs are unable to characterize the *Mi* locus in resistant tomato rootstocks.

5.- The molecular marker PM3 can only be used to trace the *Mi* locus in *S. lycopersicum* × *S. peruvianum* × *S. habrochaites* hybrids.

6.- The molecular markers C1/2, C1/2Do, C2S4, VIGS-F, and IMO-F/R specifically designed to detect the *Mi-1.2* gene are unable to distinguish this gene from other *Mi*-homologs from the *S. lycopersicum* or *S. peruvianum* species.

7.- The newly designed molecular marker Pau-Do, in combination with C2S4, can specifically detect expression of the *Mi-1.2* gene in *S. lycopersicum* × *S. peruvianum* hybrids.

8.- Several *Mi*-homologs present in the genetic background of hybrid tomato rootstocks are responsible of the variability in the resistant phenotype.

9- Molecular analyses to determine the resistance response of tomato rootstocks cannot replace pathogenicity assays.

10.- Repeated cultivation of resistant tomato rootstocks selected virulent populations of *M. javanica*.

11.- Acquisition of virulence is influenced by the frequency of *Avr/avr* alleles in the genetic pool of the nematode population, the genetic background of the resistant rootstock, and the frequency of cropping.

12.- The durability of the *Mi*-gene mediated resistance was lower in tomato rootstocks than in resistant tomato cultivars.

13.-The highly vigorous root-system of resistant tomato rootstocks does not prevent yield losses if infected by a virulent population of *Meloidogyne* spp.

14.- Careful management of resistant tomato rootstocks will be needed to preserve the durability of *Mi*-gene mediated resistance.

15.- The molecular marker MVC is unable to characterize virulent selected populations of *Meloidogyne* spp.

16.- The newly described species *S. huaylasense* can be a source of new resistance genes to control *Meloidogyne* spp.

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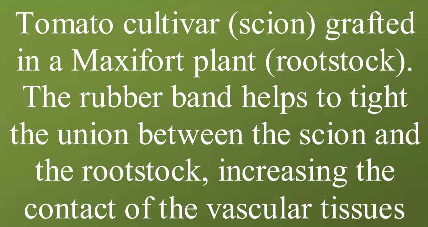

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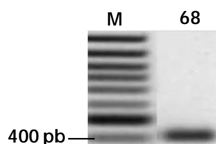
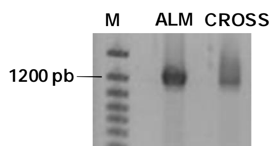
Annex 1: Identification of *Meloidogyne* populations using molecular markers

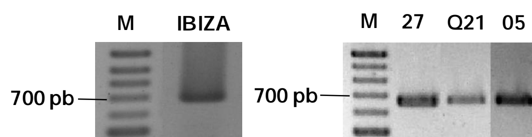
Meloidogyne arenaria **(MA-68)**



Meloidogyne incognita **(MI-ALM and MI-CROSS)**



Meloidogyne javanica **(MJ-IBIZA, MJ-27, MJ-Q21, and MJ-05)**



All the populations are avirulent except the population *M. javanica* (MJ-27), which is naturally virulent. PCR analyses were performed according to protocol described by Ziljstra et al. (2000). For all PCR reactions, 20 μL of the PCR products were resolved on 1.5% agarose gel in 1×TAE buffer and stained in ethidium bromide. Lane M indicates 100 bp DNA ladder.

Table 1. Primers used in PCR reactions for identification of *Meloidoyne* species (Ziljstra et al., 2000).

Nematode specie	Primer	Sequence (5'-3')
M. arenaria	**F**ar	TCGGCGATAGAGGTAAATGAC
	Rar	TCGGCGATAGACACTACAACT
M. incognita	**F**inc	CTCTGCCCAATGAGCTGTCC
	Rinc	CTCTGCCCTCACATTAAG
M. javanica	**F**jav	GGTGCGCGATTGAACTGAGC
	Rjav	CAGGCCCTTCAGTGGAACTATAC

Annex 2: List of publications related to this thesis

Papers

Cortada L., Sorribas F. J., Ornat C., Kaloshian I., Verdejo-Lucas S. (2008). Variability in infection and reproduction of *Meloidogyne javanica* on tomato rootstocks with the *Mi* resistance gene. Plant Pathology. Plant Pathology 57: 1125-1135. Doi: 10.1111/j.1365-3059.2008.01906.x

Cortada L., Sorribas F. J., Ornat C., Andrés, M. F., Verdejo-Lucas S. (2009). Response of tomato rootstocks carrying the *Mi*-resistance gene to populations of *Meloidogyne arenaria*, *M. incognita* and *M. javanica*. European Journal of Plant Pathology 124: 337-343. Doi 10.1007/s10658-008-9413-z.

Talavera, M., Verdejo-Lucas, S., Ornat, C., Torres, J., Vela, M. D., Macias. F. J.; **Cortada, L.**, Arias., D. J., Valero, J., Sorribas, F. J. (2009). Crop rotations with *Mi* gene resistant and susceptible tomato cultivars for management of root-knot nematodes in plastic-houses. Crop Protection 28: 662-667. Doi:10.1016/j.cropro.2009.03.015.

Verdejo-Lucas, S., **Cortada, L.**, Sorribas, F. J., Ornat, C. (2009). Selection of virulent populations of *Meloidogyne javanica* by repeated cultivation of *Mi* resistance gene tomato rootstocks under field conditions. Plant Pathology 58:990-998. Doi: 10.1111/j.1365-3059.2009.02089.x

Technical reports

Cortada, L., Sorribas, F. J., Ornat, C., Andrés, M. F., Verdejo-Lucas, S. (2009). Variabilidad de la respuesta de resistencia en función de la población del nematodo. Horticultura: Revista de frutas, hortalizas, flores, plantas ornamentales y de viveros. In press

Cortada, L., Sorribas, F. J., Ornat, C., Kaloshian, I., Verdejo-Lucas, S. (2009). Patrones de tomate: Resistencia variable frente al nematodo Meloidogyne. Horticultura: Revista de frutas, hortalizas, flores, plantas ornamentales y de viveros, N° 212, 12-17. ISSN 1132-2950

Book chapters

Verdejo-Lucas, S., **Cortada, L.** (2009). Root-knot nematodes in Tomato. In: *Tomatoes: Agricultural procedures, pathogen interactions and health effects*. Nova Science Publishers Hauppauge. Editors: Eric D. Aube and Frederick H. Poole (New York). ISBN: 978-1-60876-869-1.

Seminars and Congress

Cortada, L., Verdejo-Lucas, S., Ornat, C., Sorribas Royo, Xavier; Viera, A. Influència del nombre de còpies del gen *Mi* en tomàquet sobre el control de poblacions de *Meloidogyne* spp. In: VII Jornadas de Protecció Vegetal. Barcelona, 17th de February 2006.

Cortada, L; Verdejo Lucas, Soledad; Ornat Longarón, Cèsar; Sorribas Royo, Xavier; Viera, A. Influencia del Locus *Mi* en manejo de la durabilidad de la resistencia a *Meloidogyne* en tomate. In: XIII Congreso de la Sociedad Española de Fitopatologia (SEF). Murcia, 18th- 22nd September 2006

Cortada, L., Verdejo-Lucas, S., Ornat, C., Sorribas, F.J., Viera, A. Influencia del número de copias del gen *Mi* en tomate sobre el control de poblaciones de *Meloidogyne* spp. In: VII Jornadas de Protección Vegetal. Barcelona, 17th February 2006.

Cortada, L., Sorribas F. J., Ornat, C., Kaloshian, I., Verdejo-Lucas, S. Avaluació de portaempelts de tomàquet per al control de *Meloidogyne javanica*. In: VIII Jornades de Protecció Vegetal. Barcelona, 22nd February 2008.

Cortada, L., Sorribas F. J., Ornat, C., Kaloshian, I., Verdejo-Lucas, S. Variability in infection and reproduction of *Meloidogyne javanica* on tomato rootstocks with the *Mi* resistance gene. In: 5th International Congress of Nematology. Brisbane (Australia), 13th-18th July 2008.

Cortada, L., Verdejo-Lucas, S., Mantellin, S., Kaloshian, I. Marcadores moleculares para la detección del gen *Mi-1.2* de resistencia a *Meloidogyne* en patrones híbridos de tomate. In: XIV Congreso de la Sociedad Española de Fitopatología (SEF). Lugo, 15th-19th September 2008.

Cortada, L., Ornat, C., Sorribas, F. J. y Verdejo-Lucas, S. Evaluación de la respuesta de resistencia de los patrones híbridos de tomate Beaufort y Maxifort frente a distintas poblaciones de *Meloidogyne*. In: XIV Congreso de la Sociedad Española de Fitopatología (SEF). Lugo, 15th-19th September 2008.

Talavera, M., Verdejo-Lucas, S., Ornat, C., Torres, J., Vela, M. D., Macias. F. J.; **Cortada, L.**, Arias., D. J., Valero, J., Sorribas, F. J. Secuencias de rotación con tomate resistente en el invernadero para preservar su durabilidad en el manejo de *Meloidogyne*. In: XIV Congreso de la Sociedad Española de Fitopatología (SEF). Lugo, 15th-19th September 2008.

Verdejo-Lucas, S, **Cortada, L.**, Sorribas F. J., Ornat, C. Selection of virulent populations of *Meloidogyne javanica* by repeated cultivation of *Mi*

resistance gene tomato rootstocks in the field. In: 5th International Congress of Nematology. Brisbane (Australia), 13th-18th July 2008.

Cortada L. The use of tomato rootstocks to control *Meloidogyne* spp. in Spain. In: XVII Meeting of the Japanesse Nematological Society. Kumamoto, 3rd to 5th September 2009.

In the past decades, the use of pesticides like methyl bromide in agriculture has led a deep damaging footprint in the environment and ecosystems that will be difficult to erase in the next century. The use of non-chemical methods to control pests and diseases is becoming a main objective for sustainable production. In this sense, plant resistance conferred by the *Mi-1* gene has been adopted as an economically and non-harmful alternative for the control of *Meloidogyne* spp. In this thesis studies were conducted to determine the ability of tomato rootstocks to control root-knot nematodes and the implications of their long-term use on the durability of the resistance conferred by the *Mi-1* gene. Results show that, resistant tomato rootstocks are a good method to control root-knot nematodes in tomato, although not all of them are equally effective as their resistance phenotype depends on the nematode isolate and soil temperature. In addition, some tomato rootstocks are able to select virulent nematode populations in a very short period of time. In the light of these results, resistant rootstocks should be carefully supervised and alternated with other control methods to avoid their use in monoculture systems.