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   Experimental data if available. For UNFs to be had to meet all of the following criteria:
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Fitness cost but no selection for virulence in Meloidogyne incognita after two consecutive crops of eggplant grafted onto Solanum torvum

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Department of Agri-Food Engineering and Biotechnology, Universitat Politècnica de Catalunya, Esteve Terradas 8, 08860 Castelldefels, Barcelona, Spain

The eggplant Solanum melongena ‘Cristal’, either ungrafted or grafted onto the Solanum torvum ‘Brutus’ rootstock, was cultivated for two consecutive years in the same plots in a plastic greenhouse to assess the level of resistance to Meloidogyne incognita and crop yield. At the end of the second crop, the putative selection for virulence of the nematode subpopulations coming from infected ungrafted and grafted eggplant was assessed in the eggplant and in S. torvum in a pot experiment. Nematode population densities at transplantation in 2017 ranged from 2 to 378 100 cm⁻² of soil and did not differ between ungrafted and grafted eggplant. At the end of each crop, higher galling index and number of nematodes in soil and in roots were registered in ungrafted than grafted eggplant. The grafted eggplant was categorized as resistant in 2017 and as highly resistant in 2018. Eggplant yield did not differ irrespective of grafting in 2017 after being cultivated for 135 days, but did differed after 251 days of cultivation in 2018. In the pot experiment, S. torvum was categorized as resistant to both M. incognita subpopulations. However, the M. incognita subpopulation obtained from roots of S. torvum produced 49.4% fewer egg masses and 56% fewer eggs per plant in the eggplant than the nematode subpopulation obtained from roots of the eggplant cv. Cristal. The results of this study reveal that the infective and reproductive fitness of the nematode decreased without having been selected for virulence.

Keywords: eggplant yield, grafting, resistance durability, root-knot nematode, rootstock, Solanum melongena

Introduction

Eggplant, Solanum melongena, is one of the most cultivated solanaceous crops with an estimated worldwide production of c. 52 million tonnes in 1.8 million ha (FAOSTAT, 2017). Root-knot nematodes (RKN), Meloidogyne spp., are one of the most damaging soil-borne pathogens in solanaceous crops, especially under protected cultivation (Hallman & Meressa 2018). Maximum eggplant yield losses of 95% have been reported (Greco & Di Vito, 2009). The use of resistant plants is an effective and economically profitable management strategy to control RKN (Sorribas et al., 2005) that is more environmentally friendly than the common soil nematicides (Nyczepir & Thomas, 2009). In nematology, plant resistance is defined as the ability of a plant to suppress infection development and/or reproduction of plant-parasitic nematodes (Roberts, 2002). Grafting onto resistant rootstocks has become a common method to control soilborne pathogens when no commercial resistant cultivars are available (Lee & Oda, 2002; Thies et al., 2015). That is the case for eggplant, which is usually grafted onto resistant tomato or interspecific hybrids such as S. lycopersicum × S. habrochaites (Daunay, 2008). However, the expression of resistance can be limited by several factors such as constant soil temperatures above 30 °C (Araujo et al., 1982), and the genetic background of the rootstock along with that of the nematode species (Cortada et al., 2008). Moreover, the repeated cultivation of plant species carrying the same resistance gene can select virulent nematode populations capable of overcoming the plant defence mechanisms (Verdejo-Lucas et al., 2009; Giné & Sorribas, 2017; Expósito et al., 2019). Thus, other resistance sources have been assessed, including S. melongena lines, interspecific hybrid of S. integrifolium × S. melongena, and the wild related species S. integrifolium, S. sisymbriifolium and S. torvum (Daunay, 2008). Nonetheless, S. torvum is currently the only wild species commercially available for use as rootstock for eggplant worldwide (Uehara et al., 2017; Öcal et al., 2018). Several S. torvum accessions and cultivars have been previously described as resistant to M. incognita (Dhiyya et al., 2014), M. luci (Öcal et al., 2018), and to some populations of M. arenaria and M. javanica (Tzortzakakis et al., 2006; Uehara et al., 2017; Öcal et al., 2018), but

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susceptible to M. hapla (Öcal et al., 2018). Some S. torvum rootstocks have also been recently described as valuable tools for managing M. incognita and M. javanica populations from Spain, irrespective of their (a) virulence status to the resistance N and Mi-1.2 genes in pepper and tomato, respectively (García-Mendivil et al., 2019a). Nevertheless, no information has been previously reported about the effect of continuous cultivation of S. torvum on selecting for virulence and on the nematode fitness. Therefore, experiments were conducted to estimate the effect of two-year cultivation in the same plot under plastic greenhouse conditions on M. incognita population densities, disease severity, eggplant yield, selection for virulence and nematode fitness.

Materials and methods

Experimental plots
The experiment was conducted in a 700 m² plastic greenhouse located at Viladecans (Barcelona, Spain). The soil texture was sandy loam with 83.8% sand, 6.7% loam and 9.5% clay, pH 8.7, 1.8% of organic matter (w/w) and 0.5 dS m⁻¹ electrical conductivity. The soil was infested in 2014 with the virulent Mi-1.2 gene isolate Agropolis from M. incognita coming from a single egg mass and multiplied in the susceptible tomato cv. Durinta (Expósito et al., 2019). The plots used in this experiment were previously cultivated with the rotation lettuce-French bean-eggplant. The experiment consisted of two treatments: eggplant cv. Cristal (Semillas Fitó) grafted onto the S. torvum ‘Brutus’ (Semillas Fitó), and ungrafted eggplant cv. Cristal as standard for comparison. Each treatment was replicated 10 times in plots with a narrow variation in nematode densities between treatments at transplantation. Crops were grown from 16 June to 29 October 2017 (135 days) and from 20 March to 26 November 2018 (251 days) and plots maintained in black fallow between cropping seasons. Individual plots consisted of a row of 2.5 m with four plots spaced 0.6 m apart and plots within a row were spaced 1 m apart. Plants of each treatment were cultivated in the same plot each year to determine the effect on M. incognita population densities, the disease severity, the crop yield and the durability of the resistance. Soil of each plot was prepared in a greenhouse, and between species per each planting date.

Results

Selection for virulence and nematode fitness
At the end of the plastic greenhouse experiment in 2018, two nematode subpopulations were considered according to the plant species in which they were produced, i.e. eggplant or S. torvum. The eggs extracted from roots of the ungrafted and grafted plants were incubated in Baermann trays at 27 ± 2 °C to allow J2 emergence to determine the putative selection for virulence in a pot experiment. The J2 emerged in the first 24 h were discarded. Nematodes were collected daily for 10 days using a 25 μm sieve, and stored at 5 °C until inoculation. Seeds of S. torvum ‘Brutus’ were pretreated with a KNO₃ solution to improve germination (Ranil et al., 2015), transferred to vermiculite filled trays and incubated in a growth chamber at 25 ± 2 °C and 16:8 h (light-dark) photoperiod for 4 weeks. Afterwards, the seedlings were transplanted to 200 cm³ pots containing sterile sand and maintained in a growth chamber at 25 ± 2 °C with a 16:8 h (light-dark) photoperiod for 1 week, and inoculated with one J2 per cm³ sand. Each treatment was replicated 10 times. Plants were maintained in the growth chamber for 55 days, watered as needed throughout the experiment and fertilized with a slow-release fertilizer (15% N, 9% P₂O₅, 12% K₂O, 2% MgO₂, microelements; Osmocote Plus). Soil temperatures were recorded daily at 30 min intervals with PT100 probes (Campbell Scientific Ltd).

At the end of the experiments, the roots were carefully washed, the GI evaluated, the number of egg masses and eggs per plant determined, the number of eggs per egg mass calculated as well as the RI, and the level of resistance categorized, following the procedures previously stated.

Statistical analysis
Statistical analyses were performed using r statistical software v. 3.5.1 (R Foundation for Statistical Computing). The data were not normally distributed according to the normal Shapiro–Wilk W-test. The nonparametric analyses Mann–Whitney U-test was then used for paired comparisons between plant species per cropping season, between plant species per each M. incognita subpopulation and between M. incognita subpopulations per each plant species.

Effect of S. torvum on M. incognita reproduction, disease severity and eggplant yield
The minimum and maximum soil temperatures during the cropping season in 2017 ranged from 21.1 to 29.9 °C,
and from 14.6 to 31.3 °C in 2018. The nematode population densities in soil at transplantation in 2017 ranged from 2 to 378 J2 per 100 cm³ of soil and did not differ (P < 0.05) between treatments. At the end of the crop, a higher (P < 0.05) GI, and number of J2 in soil and eggs per plant were registered in the ungrafted than the grafted eggplant, but fruit yield did not differ (Table 1). In 2018, the nematode population densities in soil at transplantation were between 94% and 98% less than those registered at the end of the crop in 2017 but differed (P < 0.05) between treatments. At the end of the crop, a higher (P < 0.05) GI, number of J2 in soil and eggs per plant were also registered in ungrafted than grafted eggplant. Grafted eggplant yielded 2.1 more kg of fruit per plant (P < 0.05) than ungrafted. The S. torvum rootstock performed as resistant (1% ≤ RI < 10%) to M. incognita in 2017, and highly resistant (RI < 1%) in 2018 (Table 1).

**Virulence selection**

The S. torvum ‘Brutus’ was resistant (1% ≤ RI < 10%) to both M. incognita subpopulations obtained from roots of the ungrafted eggplant cv. Cristal or grafted onto the S. torvum ‘Brutus’ after being cultivated for two consecutive years in the same plots in a plastic greenhouse. Both M. incognita subpopulations caused between 53% and 69% lower (P < 0.05) GI and produced 97% less egg masses and eggs per plant, and between 21% and 31% fewer eggs per egg mass in S. torvum than in eggplant (Table 2). M. incognita subpopulation from roots of S. torvum ‘Brutus’ produced 49.4% less (P < 0.05) egg masses and 56% less eggs per plant in the eggplant cv. Cristal than the nematode subpopulation from roots of eggplant cv. Cristal, but these parameters did not differ between subpopulations when inoculated in S. torvum (Table 2).

**Discussion**

This study demonstrates for the first time that two consecutive crops of S. torvum in the same plots do not select for virulence in M. incognita but have an infective and reproductive fitness cost for the nematode in the susceptible eggplant. The resistance of S. torvum seems to

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**Table 1** Meloidogyne incognita population densities in soil at transplantation (Pi) and at the end of the crop (Pi), galling index (GI), number of eggs per plant, reproduction index (RI) and eggplant, Solanum melongena ‘Cristal’ yield ungrafted or grafted onto the Solanum torvum ‘Brutus’ rootstock cultivated from June to October 2017 (135 days) and March to November 2018 (251 days) in the same plots in a plastic greenhouse

<table>
<thead>
<tr>
<th>Plant</th>
<th>J2 per 100 cm³ soil</th>
<th>PI</th>
<th>PI</th>
<th>GI</th>
<th>Eggs per plant (x10⁶)</th>
<th>RI (%)</th>
<th>Yield (kg per plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First crop</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ungrafted</td>
<td>51 ± 36</td>
<td>29054 ± 8626</td>
<td>4.6 ± 0.3</td>
<td>12323 ± 2408</td>
<td>0.8 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grafted</td>
<td>28 ± 12</td>
<td>2061 ± 818*</td>
<td>1.0 ± 0.1*</td>
<td>228 ± 76*</td>
<td>2.0 ± 1.00</td>
<td>0.8 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Second crop</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ungrafted</td>
<td>686 ± 302</td>
<td>821 ± 179</td>
<td>7.5 ± 0.2</td>
<td>36422 ± 5895</td>
<td>1.9 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grafted</td>
<td>127 ± 54*</td>
<td>124 ± 44*</td>
<td>0.9 ± 0.1*</td>
<td>883 ± 138*</td>
<td>0.1 ± 0.01</td>
<td>4.0 ± 0.4*</td>
<td></td>
</tr>
</tbody>
</table>

Data on nematode population densities in soil are the mean ± standard error of 10 replicates. Data on GI, eggs per plant, RI and yield are the mean ± standard error of 40 replicates.

Data followed by * in the same column and year indicate significant differences (P < 0.05) between germplasms according to the nonparametric Mann-Whitney U-test.

GI: galling index on a scale from 0 to 10, where 0 = complete and healthy root system and 10 = plant and roots dead (Zeck, 1971).

RI (reproduction index) = 100 x [(number of eggs/plant in the rootstock)/(number of eggs/plant on the eggplant cv. Cristal)].

**Table 2** Galling index (GI), number of egg masses per plant, number of eggs per plant, reproduction index (RI) and number of eggs per egg mass of Meloidogyne incognita subpopulations obtained from roots of ungrafted eggplant, Solanum melongena ‘Cristal’, and grafted onto the Solanum torvum ‘Brutus’ rootstocks in Cristal and Brutus 55 days after cultivation in 200 cm³ pots inoculated with 1 J2 cm⁻³

<table>
<thead>
<tr>
<th>Plant</th>
<th>Galls per plant per plant</th>
<th>Eggs per plant</th>
<th>RI (%)</th>
<th>Eggs per egg mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cristal</td>
<td>3.8 ± 0.2*</td>
<td>2.9 ± 0.2*</td>
<td>79 ± 9</td>
<td>40 ± 3*</td>
</tr>
<tr>
<td>Brutus</td>
<td>1.8 ± 0.3*</td>
<td>0.9 ± 0.2*</td>
<td>2 ± 1*</td>
<td>1 ± 0*</td>
</tr>
</tbody>
</table>

The nematode inoculum was obtained after cultivating the ungrafted and grafted eggplant cv. Cristal onto the rootstock cv. Brutus over two consecutive cropping seasons in the same plots in a plastic greenhouse.

Data are mean ± standard error of 10 replicates. Values followed by * in the same column show significant differences (P < 0.05) between germplasms according to the nonparametric test Mann-Whitney U-test. Values of each parameter followed by * show significant differences (P < 0.05) between nematode subpopulations per each plant according to the nonparametric test Mann-Whitney U-test.

GI: galling index on a scale from 0 to 10, where 0 = complete and healthy root system and 10 = plant and roots dead (Zeck, 1971).

RI (reproduction index) = 100 x [(number of eggs/plant in the rootstock)/(number of eggs/plant on the eggplant cv. Cristal)].
be more stable than other resistance genes in fruiting solanaceous crops such as tomato and pepper. In tomato, the selection for virulence to the Mi-1.2 gene can be acquired progressively crop by crop of resistant tomato cultivars (Giné & Sorribas, 2017) or rootstocks (Verdejo-Lucas et al., 2009), or suddenly just after one tomato crop grafted onto the resistant rootstock cv. Aligator (Expósito et al., 2019). Regarding pepper, the selection for virulence to the Me3 gene has been reported after two consecutive pepper crops grafted onto the rootstock cv. Atlante (Ros-Ibáñez et al., 2014). In relation to the N gene, virulence has been reported in the USA but without any information on the selection process (Thies, 2011). Yang et al. (2014) consider that the entire disease resistance pathway is amplified in S. torvum compared with tomato and potato, enhancing plant defence mechanisms and resistance durability.

The acquisition of virulence to a resistance gene can have a fitness cost for the nematode in susceptible cultivars of the same plant species (Castagnone-Sereno et al., 2007; Djian-Caporalino et al., 2011; Expósito et al., 2019) after a minimum number of exposures to this resistance gene. For example, three resistant tomato crops were needed to affect the infectivity, reproduction and fecundity of a partially virulent M. incognita subpopulation in susceptible tomato compared to the avirulent subpopulation (Expósito et al., 2019). Surprisingly, the results of the present study revealed that the infective and reproductive fitness of the nematode decreased without having been selected for virulence after two years of repeated cultivation. The causes for this loss of fitness as well as the stability of this characteristic should be investigated. In a nematode field population, a certain proportion of infective J2 can counteract the S. torvum resistance in a proportion that is maintained in the offspring, irrespective of the plant resistance status in which they were originated, as observed in the experiment here. Nonetheless, the proportion of the offspring originated in S. torvum has a fitness cost manifested in susceptible eggplant. This finding can have important consequences for managing M. incognita by agronomic methods because the nematode reproduction in susceptible eggplant decreased by about 56%. Therefore, the use of different resistant sources in rotation with susceptible ones will decrease the risk of selecting virulent nematode populations. Solanaceae and Cucurbitaceae are the two most common botanical families used in rotation under potato crops; it reduces the infective and reproductive fitness of the nematode in susceptible eggplant after two consecutive crops without selecting for nematode virulence. However, special attention should be given in relation to variants of the nematode able to overcome resistance in S. torvum. Recently, the genotype A2-J of M. arenaria from Japan has been reported as virulent to S. torvum, but not the A2-O. Interestingly, the distribution area of the genotype A2-J overlaps with the cultivation area of eggplant (Uehara et al., 2017). Additional long-term studies will be necessary to determine the resistance durability.

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References


in the parthenogenetic nematode *Meloidogyne incognita*. *Evolutionary Ecology* 21, 259–70.


