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Effect on resistance to *Tylenchulus semipenetrans* of hybrid citrus rootstocks subjected to continuous exposure to high population densities of the nematode

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Abstract

Experiments simulating interplanting of resistant rootstocks with susceptible rootstocks that maintain high population densities of *Tylenchulus semipenetrans* in field soil were carried out in microplots at two locations, and in an naturally infested orchard. Selections of Cleopatra mandarin (03) × *Poncirus trifoliata* (01) 03.01.5 and 03.01.13, *Citrus volkameriana* (23) × *P. trifoliata* 23.01.17, Troyer citrange (02) × Cleopatra mandarin 02.03.24, Troyer citrange × Common mandarin (04) 02.04.18, King mandarin (05) × *P. trifoliata* 05.01.7, and Carrizo citrange were exposed to continuous high population densities of a population of the Mediterranean biotype of *T. semipenetrans*. The selection 23.01.17 retained its resistance in the microplots and in the field ($<$1.2% females and eggs per gram fibrous root of those on Carrizo citrange). The selection 03.01.5 also retained its resistance in the microplots at Moncada ($<$0.5% females and eggs per gram fibrous root of those on Carrizo citrange) but numbers of females and eggs per gram fibrous root were 27% and 22% at Amposta, and 139% and 18% in the orchard of those on Carrizo citrange, respectively. The selection 05.01.7 supported equal number of females and 43% eggs per gram fibrous root of those on Carrizo citrange in the nematode-infested orchard. The remaining selections supported high populations of *T. semipenetrans*.

Introduction

Citrus cultivation occupies some 280,000 ha located mainly in coastal areas and river valleys of eastern and southern Spain. Citrus production has increased by approximately one million metric tons in the last decade. Sour orange (*Citrus aurantium*) was formerly the most widespread citrus rootstock in Spanish orchards, and more than 95% of the trees were grafted on this rootstock. However, citrus tristeza virus (CTV) caused an epidemic killing millions of trees in the 1960s and forced the replacement of this rootstock by others tolerant to the virus (Cambra, 1994). At present, more than 80% of the citrus trees produced in Spanish nurseries are grafted on the CTV-tolerant rootstock Carrizo citrange (*Citrus sinensis* × *Poncirus trifoliata*). This citrange, however, does not grow well in Spain because soils are calcareous in most areas and salinity levels can be elevated (Forner and Pina, 1992). Therefore, there is a significant need to develop new rootstocks adapted to Spanish conditions.

A programme for breeding citrus rootstocks by sexual hybridization was initiated at the Instituto Valenciano de Investigaciones Agrarias (IVIA) in 1974 to identify new citrus rootstocks tolerant to CTV and well adapted to Spanish conditions. The response of 66 hybrids from this programme to a population of the Mediterranean biotype of *Tylenchulus semipenetrans* was assessed in greenhouse tests after exposure of the hybrids to initial inoculum levels of 1 × 10^4 eggs per plant for six months. Rootstocks expressing resistance supported less than 15% females and eggs per gram of fibrous root respect to the susceptible rootstock (Verdejo-Lucas et al., 1997a; 2000a). Additional studies were needed since different conditions affect the expression and durability of the resistant response. The method developed by Esmenjaud et al. (1992; 1996) to evaluate the durability of the resistance in highly resistant plum genotypes was used with some modifications to examine the response of six selected hybrids when subjected to continuous high population densities of the citrus nematode in microplots and in the field. The source of nematode inoculum came from nematode-infected roots that yielded high population densities for several months. This provided a durable inoculum pressure (Esmenjaud et al., 1992; 1996) in contrast with single inoculations with defined nematode numbers (Verdejo-Lucas and Kaplan, 2002). Our experiments simulated interplanting of resistant rootstock with susceptible rootstocks that maintain high populations of the nematode in field soils. The selected CTV-tolerant hybrids possess agronomic characteristics and traits that are promising for commercial use. For instance, the selection of Cleopatra mandarin (03) × *P. trifoliata* (01) 03.01.5 and of *Citrus volkameriana* (23) × *P. trifoliata* 23.01.17 are resistant to the citrus nematode (Verdejo-Lucas et al., 1977a; 2000a); the selection of Cleopatra mandarin × *P. trifoliata* 03.01.13 excludes chlorine; the selection of Troyer citrange (02) (*C. sinensis* × *P. trifoliata*) × common mandarin (04) *(Citrus deliciosa*) 02.04.18 is lime tolerant and provides dwarfing trees (Forner et al., 1996); the selection of Troyer citrange × Cleopatra mandarin 02.03.24 is tolerant to calcareous soils whereas the selection of King mandarin (05) (*Citrus nobilis*) × *P. trifoliata* 05.01.7 is resistant to the citrus nematode (Verdejo-Lucas et al., 2000a), and has a good tolerance to calcareous soils and salinity (Forner et al., 2000).

Materials and methods

Microplot study

Seeds of Carrizo citrange were germinated in seedbeds in January 1998, and seedlings were transplanted into 3-l black plastic bags containing a steam sterilized potting mixture (Verdejo-Lucas et al., 1997b) in June 1998, and maintained on a greenhouse bench for nine months. They were transplanted into 27 × 32 cm diameter-round bucket-microplots (Barker, 1985) containing 12 l of the same steam sterilized potting mixture. One hundred buckets were set at 3 m spacing at the Instituto Valenciano de Investigaciones Agrarias at Moncada, Valencia, Spain. The buckets were placed inside others of similar size that were buried in the ground to their rim in order to minimize changes in soil temperature and humidity. Plants were allowed to grow in the microplots for two additional months before nematodes were added to the soil. *T. semipenetrans* inoculum was obtained from infected Troyer citrange roots collected from a 17-year-old citrus orchard of Washington Navel orange budded trees located in Moncada (Valencia). The nematode population had been identified as the Mediterranean biotype (Verdejo-Lucas et al., 1997b). Citrus roots were dug, washed free of soil and cut into 1-cm sections. Roots were macerated in 0.5% NaClO (McSorley et al., 1984). To estimate the number of eggs per gram root, the egg suspension was passed through a 74 μm to remove root debris, and dispersed eggs were concentrated in a 25-μm screen and used as inoculum. Juveniles present in the inoculum were not recorded since NaClO may have affected their infectivity. In May 1999, trees were infested with approximately 33 × 10^3 eggs per plant by adding the nematode suspension into two holes made in the soil near the base of the plant.

Seeds of the selections Cleopatra mandarin × *P. trifolita* 03.01.5 and 03.01.13, *C. volkameriana* × *P. trifoliata* 23.01.17, Troyer citrange × Cleopatra mandarin 02.03.24, and Troyer citrange × common mandarin 02.04.18 and of the susceptible Carrizo citrange were germinated in seedbeds in January 1999 and seedlings were transplanted into 3-l black plastic bags containing the same steam sterilized potting mixture mentioned above in June 1999. They were maintained on a greenhouse bench for 11 months. In May 2000, the tops of Carrizo citrange trees that were infested with the nematode in May 1999 were cut at ground level and roots were left undisturbed in the soil of the microplots. These roots served as the continuous source of inoculum of *T. semipenetrans* for the experimental selections that were immediately transplanted into the infested soil of the microplots. Fourteen replicated trees of each selection were prepared. Carrizo citrange was included as the susceptible reference rootstock. Previously, two soil cores were collected from each microplot at opposite sites of the tree with a soil auger (30 × 2.5 cm diameter) to ascertain *T. semipenetrans* infestation. Soil was mixed and nematodes were extracted from a subsample of 50 cm^3 of soil for 48 h on Baermann trays. Eighty-four microplots were selected based on nematode numbers that ranged from 298 to 375 juveniles per 50 cm^3 of soil. Three weeks after transplanting, half of the trees of each selection were transferred into their bucket-microplots to the Ebro Experimental Station of the Institut de Recerca i Tecnologia Agroalimentàries (IRTA) in Amposta (Tarragona) located 200 km north of Moncada where they remained until the end of the experiment. Buckets were set at 1 m spacing within a row at Amposta. The other half of the trees was left at Moncada. The experiment was completely randomized at each location. The diameter of the trunk (cm) was measured 10 cm above ground level at the time of transplanting the trees into the microplots and at the end of the experiment. Trees were harvested after approximately 15 months of growth in the microplots on 30 August (Moncada) and 4 September (Amposta) 2001. Tops were cut at ground level and their fresh weight determined. Trees were removed from the microplots and soil was shaken from roots. They were washed free of soil, weighed and eggs were extracted from two 10-g root subsamples by blender maceration (McSorley et al., 1984). Nematodes collected on a 25-μm screen were subjected to centrifugation and sugar flotation to remove root debris (Jenkins, 1964). Young (sausage) and mature (globose) females were counted separately. Numbers were expressed as females and eggs per gram of fibrous root tissue.

Field study

The selection of King mandarin × *P. trifoliata* 05.01.7 was included in this study in addition to the ones tested in the microplots. Seedlings of each selection and of Carrizo citrange (susceptible reference rootstock) were obtained as described previously. In May 2000, one-year-old trees were transplanted at the edge of the canopy of 17-year-old trees of Washington Navel on Troyer Carrizo infested with a population of the Mediterranean biotype of *T. semipenetrans* in Moncada (the same field population that was used for the microplot study). To ascertain nematode infestation, soil samples were collected from the planting hole and nematodes were extracted for 48 h on Baermann trays. The average soil densities were 15,120 ± 11,800 juveniles per 250 cm^3 soil. Each selection was replicated three times and trees were completely randomized. The orchard soil was sandy loam with a pH of 8.3 and received supplemental irrigation through a drip-irrigation system from March to October. After 12 months of growth, trees were dug from the ground, soil was shaken from the roots and they were washed free of soil, weighed and eggs were extracted from two 10-g root subsamples (McSorley et al., 1984).

Evaluation of the citrus rootstock response

Highly resistant rootstocks did not support nematode reproduction, Resistant rootstocks supported ≤15% females and eggs per gram fibrous root of those on the susceptible reference rootstock whereas susceptible rootstocks supported >15% females and eggs per gram fibrous root (Verdejo-Lucas et al., 1997a).

The General Linear Model procedure of SAS version 8 (SAS Institute Inc., Gary, NC) was used for statistical analyses. Data on the number of females and eggs per gram of fresh root were transformed to $[\log_{10}(x+1)]$ and subjected to analysis of variance and means were separated by the LSD test ($P < 0.05$). The proportional increase in trunk diameter of the plants was calculated (diameter at harvest divided by that at nematode infestation) and data were subjected to analysis of variance.

Results

Microplot study

The response of the selections to a continuous source of inoculum of *T. semipenetrans* in the microplots indicated that rootstock, site and the interaction rootstock–site were responsible for the significant differences in nematode numbers, whereas, only the site affected tree growth. The numbers of females and eggs per gram of fresh root were higher ($P < 0.05$) at Amposta than Moncada irrespective of the rootstock. The selection 23.01.17 supported lower ($P < 0.05$) nematode numbers than the remaining ones at both sites, and retained its relative level of resistance against the citrus nematode (<1.2% females and eggs per gram fibrous root of those on Carrizo citrange) (Table 1). The selection 03.01.5 supported lower ($P < 0.05$) numbers of females and eggs per gram fibrous root than Carrizo citrange at Moncada (12% and 6% of those on Carrizo citrange, respectively), but not at Amposta (Table 1). Therefore, the resistance to *T. semipenetrans* in 03.01.5 was retained at Moncada and reduced at

Table 1. Number of females and eggs per gram of fresh root on hybrid citrus rootstocks after 15-month exposure to a continuous inoculum source of the Mediterranean biotype of *T. semipenetrans* in microplots at two locations

Site	Parentage and selection number	Females per gram root		Eggs per gram root		Fecundity[2]
		Numbers	Percentage[1]	Numbers	Percentage	
Moncada	*C. volkameriana* × *P. trifoliata* 23.01.17	0.3 ± 0.7 c	0.5	5 ± 10 c	0.4	1 ± 0 c
	Cleopatra mandarin × *P. trifoliata* 03.01.5	6 ± 4 b	12	80 ± 50 b	6	20 ± 15 b
	Cleopatra mandarin × *P. trifoliata* 03.01.13	13 ± 18 b	23	250 ± 300 b	18	30 ± 20 b
	Troyer citrange × Cleopatra mandarin 02.02.24	9 ± 8 b	14	520 ± 290 a	40	160 ± 100 a
	Troyer citrange × common mandarin 02.04.18	17 ± 12 ab	31	1220 ± 910 a	93	85 ± 40 a
	Carrizo citrange	34 ± 44 a	100	1130 ± 1460 a	100	40 ± 25 a
Amposta	*C. volkameriana* × *P. trifoliata* 23.01.17	37 ± 50 b	1.2	110 ± 45 c	0.3	30 ± 30 b
	Cleopatra mandarin × *P. trifoliata* 03.01.5	170 ± 80 a	27	5630 ± 2620 b	22	65 ± 15 a
	Cleopatra mandarin × *P. trifoliata* 03.01.13	310 ± 230 a	52	8550 ± 5620 b	34	50 ± 10 ab
	Troyer citrange × Cleopatra mandarin 02.03.24	3490 ± 7895 a	59	33240 ± 17890 a	129	300 ± 500 a
	Troyer citrange × common mandarin 02.04.18	5730 ± 6655 a	39	8570 ± 5690 b	20	40 ± 25 b
	Carrizo citrange	460 ± 330 a	100	24580 ± 18040 a	100	85 ± 25 a

Values are mean ± standard deviation of seven replicated trees. Means separated by LSD test within each site ($P < 0.05$).
[1]Respect to the Carrizo citrange used as susceptible reference rootstock.
[2]Eggs produced per individual mature female (globose shape).

Amposta. The remaining selections showed different levels of nematode reproduction and their susceptibility to the citrus nematode was confirmed. The fecundity of mature females on the selections with *P. trifoliata* was lower ($P < 0.05$) than on Carrizo citrange at Moncada, but only 23.01.17 differed at Amposta (Table 1).

Plants of each rootstocks had similar trunk diameter at the time of transplanting and differences in rootstocks were due to inherent characteristics of the particular hybrid. The proportional increase in trunk diameter of each rootstock was similar at each site but the selections grew more ($P < 0.05$) at Moncada than Amposta (Figure 1). Continuous inoculum had no measurable effect on fresh root weight.

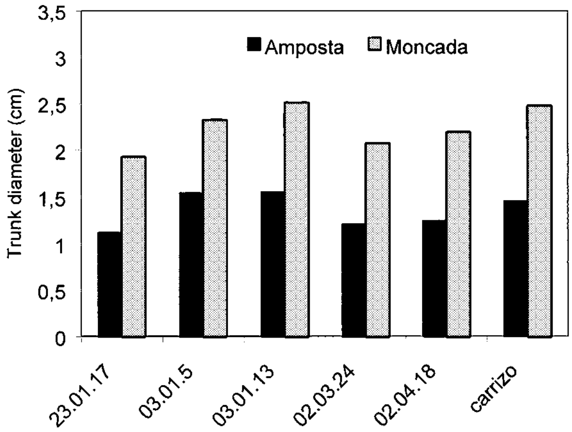

Figure 1. Proportional increase in trunk diameter (diameter after 15 months/diameter at planting) of hybrid citrus rootstocks and of Carrizo citrange after exposure to a continuous inoculum source of a population of the Mediterranean biotype of *T. semipenetrans*.

Field study

The selection 23.01.17 supported lower ($P < 0.05$) nematode numbers than the remaining ones, and retained its relative level of resistance to *T. semipenetrans* when interplanted in a citrus nematode-infested orchard (Table 2). The remaining selections supported similar levels of reproduction to that on Carrizo citrange. Number of females and eggs per gram fibrous root were 139% and 18% on 03.01.5, and 100% and 43% on 05.01.7 of those on Carrizo citrange, respectively, under the continuous source of inoculum provided by the nematode-infested roots from the mature trees.

Discussion

The selection of *C. volkameriana* × *P. trifoliata* 23.01.17 retained its relative level of resistance under

Table 2. Number of females and eggs per gram of fresh root in hybrid citrus rootstocks after 12-month exposure to a continuous inoculum source of the Mediterranean biotype of *T. semipenetrans* in a 17-year-old orchard of Washington Navel on Troyer citrange infested with the nematode

Parentage and selection number	Females per gram root		Eggs per gram root	
	Number	Percentage[1]	Number	Percentage
King mandarin × *P. trifoliata* 05.01.7	41 ± 13 a	100	6740 ± 5080 a	43
C. volkameriana × *P. trifoliata* 23.01.17	<1 b	0	49 ± 50 b	0.3
Cleopatra mandarin × *P. trifoliata* 03.01.5	57 ± 44 a	139	2850 ± 2040 a	18
Cleopatra mandarin × *P. trifoliata* 03.01.13	129 ± 112 a	315	9620 ± 8835 a	61
Troyer citrange × Cleopatra mandarin 02.03.24	22 ± 27 a	54	3370 ± 472 a	21
Troyer citrange × common mandarin 02.04.18	54 ± 71 a	132	870 ± 942 a	6
Carrizo citrange	41 ± 54 a	100	15780 ± 24890 a	100

Values are mean ± standard deviation of seven replicated trees. Means separated by LSD test within each site ($P < 0.05$).
[1]Respect to Carrizo citrange used as the susceptible reference rootstock.

a continuous source of inoculum of *T. semipenetrans* in the microplots and in the field, which confirmed the resistant response previously identified in a greenhouse test (Verdejo-Lucas et al., 2000a). In contrast, continuous exposure of 03.01.5 to the nematode increased final densities, which in turn decreased the relative level of resistance of this selection respect to the reference rootstock in the microplots at Amposta and in the nematode-infected orchard. This selection inhibited citrus nematode reproduction in repeated greenhouse tests (Verdejo-Lucas et al., 1977a; 2000a) and after exposure to increasing inoculum densities in microplots (Galeano et al., 2003). The resistant response of 05.01.7 was also reduced in the nematode-infested orchard. Because the field population of *T. semipenetrans* used was the same and came directly from the orchard, the reduced durability of the resistance in the selections 03.01.5 and 05.01.7 was most probably due to the pressure exerted by the continuous source of inoculum. Most inoculum was probably eggs in egg masses that release juveniles progressively. These provide nematode inoculum for several weeks in the microplots and throughout the one-year study in the field. The highly resistant rootstock Swingle citrumelo and *P. trifoliata* supported a certain level of parasitism when exposed to continuous high numbers of the citrus nematode in a field in Florida, although average densities were generally higher in the susceptible than resistant rootstocks (Duncan et al., 1994). In contrast, although salinity increased egg production in susceptible and resistant rootstocks, it did not change the relative levels of nematode resistance of the rootstocks (Mashela et al., 1992). Continuous inoculum did not modify the relative level of resistance of 03.01.5 in the microplots at Moncada suggesting that other conditions were involved in the reduction of the resistance. Differences in management practices between sites may explain increased reproduction at Amposta than Moncada (Verdejo-Lucas et al., 2000b).

Resistance to *T. semipenetrans* is derived from *P. trifoliata* and it seems to be dominant and oligogenic (Hutchinson, 1985). Differences in expression of resistance suggest that there is one major gene or multiple tandem genes directly or indirectly contributing to *T. semipenetrans* resistance (Ling et al. 2000). The mode of inheritance of the resistance is not understood. Their mechanisms of resistance also need to be determined (Kaplan, 1988). Several mechanisms are involved in the resistant response including the hypersensitive response and the formation of wound periderm (Van Gundy and Kirpatrick, 1964; Kaplan, 1981; Kaplan and O'Bannon, 1981). Histological examination of the response of 03.01.5 to nematode feeding showed that the feeding site established by *T. semipenetrans* was similar to the one on Carrizo citrange, but deposits of lignin-suberin like material were more abundant on 03.01.5 than Carrizo citrange. The formation of wound periderm in the cortex was only observed on the former rootstock (Galeano et al., 2003). Nematode reproduction on this selection is unfavourable because of decreased female fecundity, higher proportion of males (Verdejo-Lucas et al., 2000b), and higher accumulation of deposits of a lignin-suberin like material (Galeano et al., 2003).

The experiments described herein mimicked interplanting of young citrus trees among existing citrus nematode-infected trees, a common practice in many citrus areas of the world. This resulted in increased infection rates and egg production on the rootstocks due to continuous exposure to high numbers of *T. semipenetrans* that may eventually increase the selection pressure against resistant genes. Therefore, the present work supports the concept that resistance has to be deployed in a responsible manner to enhance durability (Starr et al., 2002) and to prevent the development of biotypes capable of circumventing resistance (Baines et al., 1974; Duncan et al., 1994), particularly when only partial resistance is available. The remaining rootstocks included in the study supported high population densities of *T. semipenetrans*, hence management tactics for nematode control will be necessary if they are going to be planted in nematode-infested soils. These rootstocks have interesting agronomic characteristics and traits besides their tolerance to CTV (Forner et al., 2000). Additional long-term studies will be needed to identify optimal conditions for their use. At present, we are evaluating the performance of the selection 03.01.5 in replanted nematode-infested orchards.

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