New citrus selections from Cleopatra mandarin × *Poncirus trifoliata* with resistance to *Tylenchulus semipenetrans* Cobb

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Summary – The response of four resistant selections of Cleopatra mandarin × *Poncirus trifoliata* to increasing inoculum densities of a population of the Mediterranean biotype of *Tylenchulus semipenetrans* was determined in microplots established at two sites. One-year-old trees of the resistant selections and of a susceptible Carrizo citrange were inoculated with 0, 1, 5 or 10 × 10^4 eggs per plant (*Pi*). Nematode reproduction on the resistant selections was consistently less than on Carrizo citrange. Increasing initial inoculum densities had no effect on the relative resistance in three selections (03.01.42, 03.01.5 and 03.01.16). Selection 03.01.42 was highly resistant (no females and eggs per g fresh root), and 03.01.5 and 03.01.16 both had good resistance (⩽15% as many females and eggs per g fresh root as on Carrizo citrange). The fourth selection (03.01.18) expressed resistance at *Pi* 1 and 5 × 10^4 eggs per plant but was moderately susceptible at 10 × 10^4 eggs per plant (> 15% females and eggs per g fresh root). Deposits of a lignin or suberin-like material were more abundant in resistant selections 03.01.5 and 03.01.18 than in susceptible Carrizo citrange. The proportional increase in trunk diameter of inoculated and uninoculated trees of each rootstock was similar at both sites.

Keywords – citrus hybrid, citrus nematode, histology, rootstock, susceptibility.

Citrus cultivation in Spain occupies about 280000 ha with a production of approximately 5 m t per year, half of which is exported mainly to the European Union. The citrus nematode, *Tylenchulus semipenetrans* Cobb, is the most common and abundant plant-parasitic nematode in Spanish citrus orchards (Martínez Beringola *et al.*, 1987; Navas *et al.*, 1992; Verdejo-Lucas *et al.*, 1995). The nematode feeds on the root cortex. Females become semi-endoparasitic and sedentary following infection of feeder roots of susceptible rootstocks. They establish feeding sites within the cortex composed of nurse cells that surround the female nematode head. The nurse cell cytoplasm becomes dense and granular as feeding sites mature (Cohn, 1965). Cellular responses to citrus nematode infection that correlate with reductions in *T. semipenetrans* reproduction include the hypodermal hypersensitive-type response, formation of wound periderm, cavity formation in the cortex and abnormal vacuolation in nurse cell cytoplasm (Van Gundy & Kirkpatrick, 1964; Kaplan, 1981; Kaplan & O'Bannon, 1981).

Rootstocks used in Spain are all susceptible to *T. semipenetrans* except for Swingle citrumelo, which is resistant (Kaplan & O'Bannon, 1981; Inserra *et al.*, 1994) but has limitations for use in Spain because of its poor performance in calcareous and alkaline soils. Resistant rootstocks could be an effective, economic and environmentally safe means for regulating nematode population densities. The only germplasm source of citrus nematode resistance that has been incorporated into commercially acceptable citrus rootstocks is derived from *Poncirus trifoliata* (L.) Raf (Verdejo-Lucas & Kaplan, 2002). Resistance to *T. semipenetrans* seems to be dominant and oligogenic (Hutchinson, 1985), and this conclusion is supported by the results reported by Ling *et al.* (2000).

Resistance to a Mediterranean biotype of *T. semipenetrans* has been identified recently in new hybrid citrus rootstocks in glasshouse tests (Verdejo-Lucas *et al.*,

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1997a, 2000). In these tests, rootstocks supporting nematode reproduction similar to that on the standard for resistance *P. trifoliata* cv. Rubidoux were considered resistant. These resistant rootstocks supported nematode densities ⩽15% those on a susceptible control cultivar. Additional studies were needed to confirm the resistance response identified under controlled conditions and to examine its likely durability. The objective of this study was to determine whether the resistant response in selections of Cleopatra mandarin (03) × *P. trifoliata* (01) was retained after exposure to increasing initial inoculum densities of *T. semipenetrans* in microplots established at two locations. Histological studies were made to examine the response of two of the selections (03.01.5 and 03.01.18) to nematode feeding.

Materials and methods

Resistant selections of Cleopatra mandarin × *P. trifoliata*, 03.01.42 and 03.01.5 in the first experiment and 03.01.16 and 03.01.18 in the second, were exposed to increasing inoculum densities of *T. semipenetrans* in microplots. The selections came from the citrus breeding programme of the Instituto Valenciano de Investigaciones Agrarias (IVIA) that supplied and prepared the materials for the study. Experiments were established at two locations 200 km apart at IVIA in Moncada (Valencia), the main citrus growing area of the country, and at the Ebro Experimental Station of the Institut de Recerca i Tecnologia Agroalimentàries (IRTA) in Amposta (Tarragona), at the northernmost limit for citrus growth. A susceptible rootstock, Carrizo citrange (*Citroncirus webberi* Ingram & Moore × *P. trifoliata*), was included in both experiments as a control. Experiments were performed in a similar way unless otherwise indicated.

Seeds of the hybrid rootstocks were germinated in seedbeds, transplanted 6 m after germination into 3 dm^3 black plastic bags containing a steam sterilised potting mixture (Verdejo-Lucas *et al.*, 1997b) and maintained for 1 year on a glasshouse bench. They were then transplanted into round buckets, 27 cm high × 32 cm diam. (Barker, 1985) containing 12 dm^3 of the same steam sterilised potting mixture. Buckets were set at 1 m spacing within a row, with 1.5 (Amposta) or 3 m (Moncada) between rows. Each bucket was placed inside another of similar size that had been buried up to the rim in order to minimise changes in soil temperature and humidity. Plants were allowed to grow in these microplots for 2 months before nematodes were added to the soil. *Tylenchulus semipenetrans* inoculum was obtained from infected Troyer citrange roots collected from a 16-year-old citrus orchard of Washington Navel orange budded trees in Moncada. 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 a 0.5% NaOCl solution in a food blender at *ca* 1000 rpm for two successive 15 s intervals (McSorley *et al.*, 1984). The egg suspension was passed through a 74 μm aperture sieve to remove root debris and the dispersed eggs collected on a 25 μm sieve for use as inoculum. Juveniles present in the inoculum were not recorded since NaOCl solution may have affected their infectivity. Fourteen-month-old trees of each rootstock were inoculated with *ca* 0, 1, 5, or 10 × 10^4 eggs per plant by adding the nematode suspension into two holes made in the soil near the base of the plant. Uninoculated plants received only water. These densities were within the ranges that occur naturally in Spanish orchards. The experiments were randomised and each rootstock-inoculum density combination was replicated seven times at each location.

Trees in Experiment 1 were inoculated in July 1998 and harvested in November 1999 and those in Experiment 2 in May 1999 and November 2000. Tree trunk diameter was measured 10 cm above ground level with a digimatic caliper at inoculation, at 12 months later and at the end of each experiment. *Tylenchulus semipenetrans* densities in the microplot soils were monitored 4 and 6 months after inoculation in Expt 1 and 2, respectively. Individual samples consisted of two soil cores collected with a soil auger from the top 20 cm of each plot from opposite sides and about 15 cm from the base of the trunk. Nematodes were extracted from a 125 cm^3 soil subsample using Baermann trays. At the end of each experiment, tops were cut at ground level and their fresh weight determined. Trees were then removed from the microplots, soil was shaken from the root system and nematodes extracted from a 500 cm^3 soil subsample by Baermann trays. Nematodes migrating into the water were collected after 1 week and concentrated on a 25 μm pore sieve. Second-stage juveniles (J2) and males were counted and soil populations expressed per 250 cm^3 soil and as J2 and males as proportions of the total nematode population. Root systems were washed, feeder roots cut and weighed and eggs extracted from two 10 g feeder root subsamples as described earlier. Nematodes collected on a 25 μm sieve were separated by centrifugation and sugar flotation

(Jenkins, 1964) and numbers expressed as females and eggs per g fresh root.

EVALUATION OF CITRUS ROOTSTOCK RESPONSE

The criterion to evaluate the response of the rootstocks was based on females (infectivity) and eggs (reproductive potential) per g fresh root because these numbers represented individuals that had infected, developed, completed their life cycle and produced offspring on the rootstock. The index of reproduction of *T. semipenetrans* on the experimental selections was expressed as a percentage that on susceptible Carrizo citrange (Triantaphyllou, 1975). It was considered that rootstocks were resistant if they supported $\leqslant 15\%$ females and eggs per g fresh root.

For histological study, feeder root samples from resistant selections 03.01.5 and 03.01.18, and from susceptible Carrizo citrange collected from both sites, were examined using a stereomicroscope. Selections 03.01.42 and 03.01.16 were not studied because of the scarcity of females. Root segments infected with females with egg masses were cut with a scalpel, placed in a small vial, and fixed in cold 3% formaldehyde or 3% glutaraldehyde in 50 mM Sörensen buffer (pH 7.2) at room temperature. After several washings with buffer solution (50 mM Sörensen buffer, pH 7.2), samples were dehydrated in a graded ethanol series to 100% ethanol, and embedded in LR White acrylic resin (Tadeo *et al.*, 1994). Cross and longitudinal serial sections 2-10 μm thick were obtained with a Reichert Ultracut ultramicrotome. Sections were stained with toluidine blue O (CI 52040) that stains the nucleus and cytoplasm blue-green, the mucopolysaccharides violet, and lignin and suberin green-blue. Sections were examined and photographed with a microscope (Zeiss Axiophot) at 200, 400 and 1000×.

The General Linear Model procedure of SAS version 8 (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses. Previously, final population densities (*Pf*) of *T. semipenetrans* in soil and roots were transformed to $\log(x + 1)$. Analysis of variance was performed to determine the effect of rootstock, initial inoculum densities, site, and their interactions on the response of the resistant selections of Cleopatra mandarin × *P. trifoliata.* Then, data on final population densities were analysed per rootstock and inoculum densities within each site, and means were separated by the LSD test ($P < 0.05$). Fresh top and root weights were subjected to analysis of variance. The proportional increase in trunk diameter of the trees was calculated (diameter at harvest divided by that at nematode infestation), and data were subjected to analysis of variance.

Results

EXPERIMENT 1

Rootstock, initial inoculum density and site were responsible for significant differences in nematode reproduction on the resistant selections of Cleopatra mandarin × *P. trifoliata* 03.01.42 and 03.01.5 exposed to increasing inocula whereas only rootstock and site affected plant growth. Final population densities of *T. semipenetrans* in soil and roots were less ($P < 0.05$) on the resistant selections than on Carrizo citrange at both sites, irrespective of initial densities. Females on susceptible Carrizo citrange at initial inoculum densities (*Pi*) of 1, 5, and 10×10^4 eggs per plant ranged from 7 to 78, 13 to 93, and 35 to 110 per g root at Moncada and from 6 to 180, 104 to 335, and 190 to 567 at Amposta.

Selection 03.01.42 did not support nematode reproduction at Moncada, and only a few females were recovered from one of 42 inoculated plants at Amposta (Table 1). This response was considered as highly resistant to *T. semipenetrans* (0%) (Table 3). There was more ($P < 0.05$) nematode reproduction on selection 03.01.5 at Amposta than at Moncada. When data were analysed per rootstock and inoculum density, 03.01.5 supported more ($P < 0.05$) J2 plus males per 250 cm^3 soil, and females and eggs per g fresh root at *Pi* of 10×10^4 than 1×10^4 eggs per plant at Moncada but there was no difference at Amposta (Table 1). This selection was rated resistant to the citrus nematode ($\leqslant 15\%$ females and eggs per g fresh root) (Table 3). A trend toward increased percentage of males was observed on selection 03.01.5, clearer at Amposta than Moncada. Thus, the male and J2 proportions in the soil population in plots of selection 03.01.5 were 69 and 31% at Amposta and 47 and 53% at Moncada. In plots with Carrizo citrange, these proportions were 42 (male) and 58% (J2) at Amposta and 37 and 63% at Moncada, respectively.

EXPERIMENT 2

Rootstock selection and initial inoculum density were responsible for significant differences in nematode reproduction on 03.01.16 and 03.01.18 exposed to increasing inoculum, and both rootstock and site affected plant growth. Final population densities of *T. semipenetrans* in

Table 1. *Numbers of a Mediterranean biotype of* Tylenchulus semipenetrans *in soil and roots of resistant selections 03.01.42 and 03.01.5 of Cleopatra mandarin* × Poncirus trifoliata *and on susceptible Carrizo citrange, at 16 months after inoculation with three densities in field microplots at two sites in Experiment 1 (means* ± *sd of seven replicates).*

Site	Rootstock	Initial inoculum (eggs/plant)	J2 + males/ 250 cm^3 soil	Females/g root	Eggs/g root
Moncada	03.01.42	1×10^4	0	0	0
		5×10^4	0	0	0
		10×10^4	0	0	0
	03.01.5	1×10^4	114 ± 120 b	0.4 ± 0.8 b	8 ± 9 b
		5×10^4	320 ± 350 b	3 ± 5 ab	74 ± 90 a
		10×10^4	1240 ± 680 a	5 ± 3 a	60 ± 40 a
	Carrizo citrange	1×10^4	6410 ± 6730 a	113 ± 239 a	590 ± 370 a
		5×10^4	4260 ± 2570 a	33 ± 26 a	890 ± 600 a
		10×10^4	7870 ± 5240 a	56 ± 21 a	1060 ± 380 a
Amposta	03.01.42	1×10^4	0	0	0
		5×10^4	0	0.3 ± 0.8	0
		10×10^4	0	0	0
	03.01.5	1×10^4	1360 ± 1010 a	12 ± 9 a	570 ± 350 a
		5×10^4	1310 ± 1116 a	15 ± 13 a	280 ± 270 a
		10×10^4	1990 ± 1040 a	19 ± 11 a	470 ± 280 a
	Carrizo citrange	1×10^4	12640 ± 9810 a	77 ± 53 b	3900 ± 2870 b
		5×10^4	9640 ± 8230 a	208 ± 70 a	8420 ± 4130 a
		10×10^4	10570 ± 7950 a	177 ± 83 a	5990 ± 1450 ab

Within each rootstock site combination, means with the same letter do not differ ($P < 0.05$).

soil and roots were less ($P < 0.05$) on resistant selections than on Carrizo citrange at both sites. Numbers of females per g of root on Carrizo citrange at *Pi* of 1, 5 and 10×10^4 eggs per plant, respectively, ranged from seven to 100, 15 to 108 and five to 164 at Moncada and from 17 to 201, 37 to 114 and 15 to 165 eggs per plant at Amposta.

Selection 03.01.18 supported more ($P < 0.05$) nematodes in soil and root than 03.01.16 at both sites. When data were analysed per rootstock and inoculum density, increasing inoculum did not affect soil or root *Pf* values on 03.01.16 (Table 2), which was rated resistant (Table 3). Selection 03.01.18 supported more ($P < 0.05$) females and eggs per g root at *Pi* of 10×10^4 than 1×10^4 eggs per plant at both sites (Table 2). This selection retained its resistance at *Pi* of 1 and 5×10^4 but was moderately susceptible at 10×10^4 eggs per plant when females/g root were 18 and 30% of those on Carrizo citrange at Moncada and Amposta, respectively (Table 3).

Individual citrus nematodes that circumvented the resistant response in selections 03.01.5 and 03.01.18 successfully established permanent feeding sites in the cortical parenchyma, similar to those on Carrizo citrange and consisting of five to six nurse cells surrounding a cavity void of content that housed the nematode (Fig. 1A-F). These cells stained dark blue, had a dense cytoplasm with granular appearance and contained vacuoles of different sizes. Deposits of a lignin or suberin-like material of yellow-green colour and granular appearance were observed in cells adjacent to the tunnels made by the nematode in the three rootstocks: these deposits were more abundant on resistant rootstocks than on the susceptible one. The formation of wound periderm in the cortex was observed in resistant selections 03.01.5 and 03.01.18 (Fig. 1E) but not in Carrizo citrange.

Trees of each rootstock had similar trunk diameters at inoculation and differences in rootstocks were due to the growth characteristics of the particular hybrid. The proportional increase in trunk diameter of inoculated and uninoculated trees of each rootstock was similar at both sites; however, the increase was more ($P < 0.05$) at Moncada than Amposta for both resistant and susceptible rootstocks. In Expt 1, the average trunk diameter of the trees was initially 6.5 ± 0.7 cm growing to 20.5 ± 3.2 cm at Moncada and to 12.2 ± 3 cm at Amposta, 16 months after

Table 2. *Numbers of a Mediterranean biotype of* Tylenchulus semipenetrans *in soil and roots of resistant selections 03.01.16 and 03.01.18 of Cleopatra mandarin* × Poncirus trifoliata *and on susceptible Carrizo citrange, at 17 months after inoculation with three densities in field microplots at two sites in Experiment 2 (means ± sd of seven replicates).*

Site	Rootstock	Initial inoculum (eggs/plant)	J2 + males/ 250 cm^3 soil	Females/g root	Eggs/g root
Moncada	03.01.16	1 × 10^4	150 ± 160 a	0.4 ± 1 a	2 ± 3 a
		5 × 10^4	780 ± 1200 a	3 ± 4 a	21 ± 23 a
		10 × 10^4	780 ± 810 a	2 ± 3 a	17 ± 16 a
	03.01.18	1 × 10^4	1050 ± 580 a	1 ± 2 b	14 ± 15 b
		5 × 10^4	2680 ± 2970 a	6 ± 7 ab	90 ± 60 a
		10 × 10^4	1395 ± 1495 a	8 ± 5 a	115 ± 80 a
	Carrizo citrange	1 × 10^4	4480 ± 5550 a	50 ± 79 a	2700 ± 4390 a
		5 × 10^4	6050 ± 4340 a	39 ± 26 a	1310 ± 1145 a
		10 × 10^4	8910 ± 7520 a	46 ± 30 a	2190 ± 2660 a
Amposta	03.01.16	1 × 10^4	240 ± 235 a	1 ± 1 a	4 ± 3 a
		5 × 10^4	520 ± 660 a	1 ± 1 a	6 ± 6 a
		10 × 10^4	410 ± 300 a	3 ± 2 a	5 ± 4 a
	03.01.18	1 × 10^4	380 ± 230 a	4 ± 4 b	37 ± 53 b
		5 × 10^4	980 ± 910 a	5 ± 4 ab	57 ± 32 ab
		10 × 10^4	1390 ± 1250 a	28 ± 32 a	378 ± 643 a
	Carrizo citrange	1 × 10^4	6450 ± 8650 a	110 ± 110 a	2200 ± 2260 a
		5 × 10^4	11645 ± 18230 a	71 ± 21 a	4630 ± 2110 a
		10 × 10^4	2640 ± 2600 a	93 ± 43 a	5660 ± 3440 a

Within each rootstock site combination, means with the same letter do not differ ($P < 0.05$).

inoculation. In Expt 2, the initial average trunk diameter of the trees was 7.9 ± 1 cm, growing to 24 ± 5 cm and 14.6 ± 2 cm at Moncada and Amposta, 17 months after inoculation. The highest inoculum of 10 × 10^4 eggs per plant decreased fresh weight of top and root of selection 03.01.42 at both sites and of 03.01.16 at Moncada, but increased that of 03.01.5 at Moncada.

Discussion

The selections of Cleopatra mandarin × *P. trifoliata* significantly limited reproduction of a Mediterranean biotype of *T. semipenetrans* after exposure to a range of inoculum densities in field microplots at two locations. They retained their resistance with the exception of 03.01.18 that became moderately susceptible when inoculated with 10 × 10^4 eggs per plant. The moderate susceptibility of 03.01.18 at the highest inoculum level and its resistance at 1 and 5 × 10^4 eggs per plant was consistently shown at both sites. The low multiplication of the nematode on the experimental selections confirm the resistance identified under controlled conditions (Verdejo-Lucas *et al.*, 1997a, 2000). These selections and Carrizo citrange are hybrids with *P. trifoliata* as the male progenitor but Carrizo citrange supported a high reproduction rate of *T. semipenetrans* in comparison with the experimental selections in both experiments and sites. Females were frequently found in groups of three to five individuals on Carrizo citrange but were observed singly on 03.01.5 and 03.01.18, indicating greater host-parasite compatibility level in the former rootstock. Nematode development on selection 03.01.5 indicated the unfavourable environment in the rootstock because more individuals tended to develop as males than on Carrizo citrange. This trend, however, was not observed in the other selections.

The intracellular deposition of lignin and suberin, a host response to pathogens providing plants with a general mechanism to resist infection, have been observed in *Citrus* species (Schneider & Baines, 1964; Cohn, 1965; Kaplan, 1981; Doucet & Ponce de Leon, 1992), but was greater in the resistant selections than Carrizo citrange. There is often a correlation between the degree of plant resistance and phenolic compounds (constituents

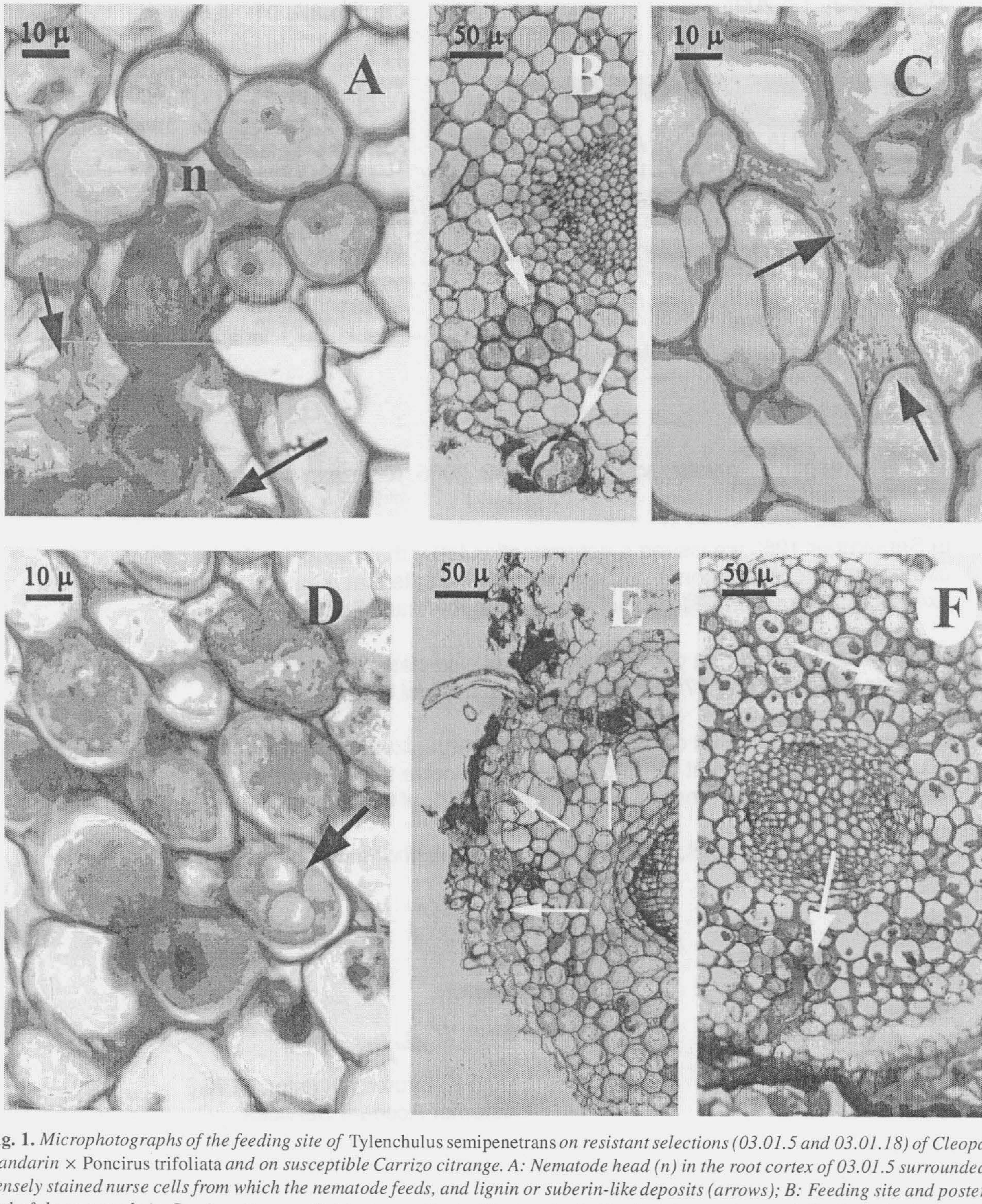

Fig. 1. *Microphotographs of the feeding site of* Tylenchulus semipenetrans *on resistant selections (03.01.5 and 03.01.18) of Cleopatra mandarin* × Poncirus trifoliata *and on susceptible Carrizo citrange. A: Nematode head (n) in the root cortex of 03.01.5 surrounded by densely stained nurse cells from which the nematode feeds, and lignin or suberin-like deposits (arrows); B: Feeding site and posterior end of the nematode in Carrizo citrange; C: Lignin or suberin-like deposits in Carrizo citrange (arrows); D: Vacuoles (arrows) and granular cytoplasm in 03.01.18; E: Wound periderm and lignin or suberin-like deposits (arrows) around a young female in 03.01.18; F: Suberised cells and feeding site on selection 03.01.18. (Scale bars A, C, D = 10 μ; B, E, F = 50 μ.)*

Table 3. *Reproductive index (as % females or eggs on susceptible Carrizo citrange) of a Mediterranean biotype of* Tylenchulus semipenetrans *on four resistant rootstocks selected from Cleopatra mandarin* × Poncirus trifoliata *after exposure to increasing inoculum densities in field microplots in two experiments at two sites.*

Rootstock	Site	Initial Inoculum (eggs/plant)	Reproductive index Females/g root	Eggs/g root
Experiment 1				
03.01.42	Moncada	1×10^4	0	0
		5×10^4	0	0
		10×10^4	0	0
	Amposta	1×10^4	0	0
		5×10^4	0	0
		10×10^4	0	0
03.01.5	Moncada	1×10^4	0.4	1
		5×10^4	9	8
		10×10^4	9	6
	Amposta	1×10^4	15	14
		5×10^4	7	3
		10×10^4	11	8
Experiment 2				
03.01.16	Moncada	1×10^4	0.9	0.07
		5×10^4	8	2
		10×10^4	5	8
	Amposta	1×10^4	1	0.18
		5×10^4	1	0.13
		10×10^4	3	0.09
03.01.18	Moncada	1×10^4	2	0.5
		5×10^4	15	7
		10×10^4	18	5
	Amposta	1×10^4	3	2
		5×10^4	7	1
		10×10^4	30	7

of suberin) present in plant tissue (Giebel, 1974). The formation of wound periderm, associated with the resistant response (Van Gundy & Kirpatrick, 1964; Kaplan, 1981; Kaplan & O'Bannon, 1981) was observed in resistant selections but not on Carrizo citrange. The resistant response is more severe on highly resistant (*P. trifoliata* and Swingle citrumelo) or non-host plants (*Severinia buxifolia*) than in resistant hybrids of *P. trifoliata* with *Citrus* species (Van Gundy & Kirkpatrick, 1964).

The results from the microplots and histological studies suggest that the growth potential of the nematode on the resistant rootstocks will be less than on the susceptible one. Therefore, these resistant rootstocks can be useful for replant situations in nematode-infested soils following careful soil preparation to remove old citrus roots, because nematode densities will probably increase more slowly than on susceptible rootstocks. The differences among the selections in expression of resistance require additional long-term studies to determine their practical implications and the likely durability of the resistance under a wide range of conditions.

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