Screening Hybrid Citrus Rootstocks for Resistance to *Tylenchulus semipenetrans* Cobb

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Abstract. The response of 22 citrus hybrid rootstocks to a Mediterranean biotype of *Tylenchulus semipenetrans* Cobb was determined in a greenhouse test. Five of the 11 rootstocks with *Poncirus trifoliata* (L.) Raf in their parentage did not support nematode reproduction. However, rootstocks with Troyer citrange [*Citrus sinensis* (L.) Osbeck x *P. trifoliata* (L.) Raf] in their parentage were susceptible to the nematode.

Citrus is cultivated in 268,000 ha in the east and south of Spain, mainly for fresh consumption. Orange, mandarin, and lemon account for 99% of the hectareage, and the annual production is ≈ 5 million tons (MAPA, 1995). Sour orange (*Citrus aurantium* L.) 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) has killed more than 17 million trees on sour orange (Cambra, 1994), and forced the replacement of this rootstock. Tristeza was first recognized as a problem in 1957 in the Rivera Alta District of Valencia. Subsequently, CTV spread to most of Spain, and citrus nurseries were forbidden to propagate sweet orange, mandarin, and grapefruit on sour orange in 1968. This policy resulted in the systematic replacement of trees that died or severely declined with replants tolerant to the virus. Sour orange still remains as a rootstock in 60% to 65% of the old orchards that have not yet been affected by CTV, but could develop the disease due to its rapid spread in affected areas (Cambra, 1994). Since the early 1970s, the CTV-tolerant rootstocks Cleopatra mandarin (*C. reshni* Hort.) and Troyer citrange [*Citrus sinensis* (L.) Osbeck. x *Poncirus trifoliata* (L.) Raf.] have been used to replace dead trees and to

establish new orchards. In 1991, citrus nurseries began producing Carrizo citrange instead of Troyer citrange (Forner and Pina, 1992). Although the citranges perform better than Cleopatra mandarin, they do not grow well in Spain because soils are calcareous and salinity can be elevated (Forner and Pina, 1992). There is still a significant need to develop new rootstocks that are well adapted to Spain's conditions and tolerant to CTV, *Phytophthora*, and the citrus nematode.

Tylenchulus semipenetrans Cobb is the most frequent and abundant plant-parasitic nematode that occurs in Spanish citrus orchards (Bello et al., 1986). The nematode has been detected in more than 90% of the orchards surveyed (Bello et al., 1985; Martinez Beringola et al., 1987; Navas et al., 1992; Ortuño Martinez et al., 1969; Tuset and García, 1986; Verdejo-Lucas et al., 1995). Although damage thresholds (nematode population densities that suppress tree growth and yield) have not been precisely established for citrus, nematodes can impede tree establishment and fruit production under a wide range of environmental and edaphic conditions, particularly for trees replanted in citrus nematode-infested orchards (Barreda et al., 1988; Duncan and Cohn, 1989). The use of rootstocks resistant to the citrus nematode could be an effective, economic, and safe method of regulating nematode population densities. However, the existence of physiological races or biotypes of *T. semipenetrans* may limit the usefulness of some resistant rootstocks. For instance, a population of *T. semipenetrans* capable of circumventing resistance in Swingle citrumelo (*C. paradisi* Macf. x *P. trifoliata*) has been reported recently in Florida (Duncan et al., 1994). At present, three biotypes of *T. semipenetrans* have been identified according to the host

status of several indicator plant species (Inserra et al., 1980, 1994). Thus, the poncirus biotype reproduces on *Citrus* spp., *P. trifoliata* and their hybrids, and grape (*Vitis vinifera* L.), but not on olive (*Olea europaea* L.). The citrus biotype infects many genera in the Rutaceae, including *Citrus* spp. and Troyer and Carrizo citrange, as well as olive, grape, and persimmon (*Diospyros kaki* L.). The Mediterranean biotype is very similar to the citrus biotype, but does not reproduce on olive. This biotype of *T. semipenetrans* is the only one reported in Spain (Verdejo-Lucas, 1992; Verdejo-Lucas et al., 1997). All citrus rootstocks commercially available in Spain are highly or moderately susceptible to *T. semipenetrans* except for the Swingle citrumelo CPB 4475, which is resistant, but this rootstock is not well adapted to calcareous soils and high pH, and is only used in small areas of Spain (Forner and Pina, 1992).

A breeding program was initiated at Instituto Valenciano de Investigaciones Agrarias (Moncada, Valencia, Spain) in 1974 to identify new citrus rootstocks tolerant to tristeza virus and adapted to Spanish conditions, particularly calcareous soils and salinity. Many of the hybrids obtained in that program have *P. trifoliata* as the male progenitor because of its tolerance to tristeza (Forner and Alcaide, 1994). *Poncirus trifoliata* is also known to be resistant to most biotypes of the citrus nematode (Baines et al., 1960; Ducharme, 1948). This paper identifies optimal citrus nematode inoculum densities and reports the results of a greenhouse test designed to determine the host status for citrus nematode for the 22 citrus hybrid rootstocks.

Materials and Methods

Plant material. For Expt. 1, seeds of sour orange and Carrizo citrange were germinated in seedbeds, and the seedlings were transplanted singly to black plastic bags (1.5-L capacity) containing a steam-sterilized potting mixture of sphagnum peat moss (60%) and quartz sand (40%) (v/v) with additional superphosphate (1.47 g L⁻¹) and microelements (CuSO₄, ZnSO₄, MnSO₄, and FeSO₄ at 50, 27.5, 27.5, and 46.6 mg L⁻¹, respectively). The pH of the potting mixture was adjusted to 6.5 with CaCO₃. Plants were maintained in a greenhouse for 12 months before adding the nematodes.

For Expt. 2, hybrid rootstocks of citrus were selected that were either resistant to or tolerant of CTV. The 22 selections tested included 11 crosses of Cleopatra mandarin x *P. trifoliata* (Rubidoux), two crosses of Cleopatra mandarin x Troyer citrange, eight crosses of Troyer citrange x Cleopatra mandarin, and one cross of Troyer citrange x common mandarin (*C. deliciosa* Ten.). Sour orange and *P. trifoliata* Rubidoux were included as susceptible and resistant standards to the citrus nematode, respectively.

Expt. 1. Optimum nematode inoculum. To determine the optimal initial inoculum level of citrus nematode for greenhouse studies, the soil was inoculated with 0, 1 x 10⁴, 2 x 10⁴, 4

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Table 1. Numbers of females and eggs + J2 per g of root of *Tylenchulus semipenetrans* Cobb on sour orange and Carrizo citrange at five inoculum levels after 6 months of growth in a greenhouse.^a

Inoculum level (eggs + J2 per plant × 10 ⁴)	Sour orange		Carrizo citrange	
	Females	Eggs + J2	Females	Eggs + J2
1	11 ab ²	130 bc	27 b	248 b
2	7 b	108 c	17 b	134 b
4	17 ab	187 bc	16 b	160 b
8	17 ab	221 ab	41 ab	594 a
16	18 a	415 a	61 a	1084 a
Mean	14	211	34.8	485
cv	8.9	19.8	19.8	11.8

^aValues are means of five replicates. Mean separation within columns by Duncan's multiple range test ($P = 0.05$).

× 10⁴, 8 × 10⁴, and 16 × 10⁴ eggs + second-stage juveniles (J2) per plant. The nematode inoculum was obtained from *T. semipenetrans*-infected citrus roots collected from a 12-year-old citrus orchard of 'Washington Navel' orange on Troyer citrange. This citrus nematode population had been identified as the Mediterranean biotype of the nematode (Verdejo-Lucas et al., 1997). Citrus roots were dug, washed free of soil, and cut into 0.5-cm sections. Roots were blender macerated (McSorley et al., 1984) to estimate the number of eggs and J2 per gram of root. The experiment was arranged in randomized complete blocks, and each rootstock-inoculum level combination was replicated five times. Plants were harvested after 6 months of growth in a greenhouse, and the fresh weight of tops and roots was determined. Nematodes were extracted from all fibrous roots from each plant. Root systems were macerated in a food blender at the maximum speed for two successive 15-s intervals (McSorley et al., 1984). Nematodes collected on a 25-μm screen were subjected to centrifugation and sugar flotation (Jenkins, 1964) to remove root debris. The numbers of females, eggs, and J2 were counted and expressed per gram of root. Data were transformed to log (x + 1) and subjected to analysis of variance (ANOVA), and means were separated by Duncan's multiple range test ($P = 0.05$).

Expt. 2. Screening of citrus hybrids. The procedure described by Kaplan (1990) for screening citrus rootstocks for resistance to *T. semipenetrans* was followed in this study with some modifications as indicated below. Seeds of each selection were germinated in seedbeds, and transplanted singly to black plastic bags (1.5-L capacity) containing the steam-sterilized potting mixture described previously. Plants were maintained in a greenhouse for 12 months before adding the nematodes. The nematode inoculum was obtained and prepared as previously described. Plants were inoculated with ≈ 1 × 10⁴ eggs + J2 per plant. The experiment was arranged in randomized complete blocks, and each rootstock-nematode combination was replicated seven times. The temperature of the greenhouse ranged from 16 to 26 °C during the six months of the experiment. At harvest, roots were washed free of soil, weighed, and then frozen at -20 °C until processed. When required, roots were thawed at room temperature and nematodes extracted from the entire root system by blender maceration (McSorley et al., 1984) as indi-

cated earlier. The infectivity (females/g root) and reproductive potential (eggs + J2/g root) of *T. semipenetrans* were considered as indicators to evaluate the response of the rootstocks. To establish the host status of these rootstocks, nematode infectivity and reproductive potential were compared with those on the susceptible sour orange. Data were transformed to log (x + 1) and subjected to ANOVA, and the means were separated by Duncan's multiple range test ($P = 0.05$). A multiple comparison procedure that compares all treatments with a single control was used to identify rootstocks expressing resistance to *T. semipenetrans*. Host suitability of individual rootstocks was compared with that of the standard of resistance, *P. trifoliata*, by Dunnett's *t* tests ($P = 0.05$) (Dunnett, 1955).

Results

Expt. 1. Optimum nematode inoculum. Inoculum levels did not affect numbers of females produced per gram of root on sour orange significantly except for the initial density 2 × 10⁴ (Table 1). Numbers of eggs + J2 produced were highest ($P = 0.05$) at the initial inoculum level of 16 × 10⁴ eggs + J2. Numbers of females per gram of root on Carrizo citrange were also highest ($P = 0.05$) at this level, but there were no significant differences among the remaining initial densities (Table 1). Numbers of eggs produced were highest ($P = 0.05$) at initial inoculum levels of 8 and 16 × 10⁴ eggs, but egg production did not differ among treatments with 1, 2, and 4 × 10⁴ eggs as inoculum. The nematode populations were greater for Carrizo citrange than for sour orange ($P = 0.05$) only at the highest inoculum level tested (Table 1). Inoculation did not affect fresh mass of top or root.

Expt. 2. Screening of citrus hybrids. *Poncirus trifoliata* and the selections from crosses of Cleopatra mandarin × *P. trifoliata* 03.01.8, 03.01.31, and 03.01.42 did not support nematode reproduction (Table 2). These selections were considered as highly resistant to the Mediterranean biotype of *T. semipenetrans*. The nematode showed very low infectivity (<1–2 females per gram of root) and reproduction (6–34 eggs per gram of root) on five additional selections of the same cross (Cleopatra mandarin × *P. trifoliata*), and therefore these selections could be considered as nematode-resistant. The remaining selections tested showed several degrees of susceptibility. Thus, nematode development on the hy-

brid 03.01.9 with *P. trifoliata* did not differ from that on the susceptible sour orange. The 11 selections tested with Troyer citrange in their parentage were all susceptible to the citrus nematode. In spite of considerable variation in the numbers of females per gram of root and eggs + J2 per gram of root recovered from individual root systems, rootstocks could be classified into three groups representing low, medium, or high levels of nematode infectivity and reproduction. Thus, selections of crosses of Troyer citrange and Cleopatra mandarin 02.03.5A, 02.03.17, and 02.03.20 showed the highest levels of *T. semipenetrans*, whereas selections 03.02.9, 02.03.3, 02.03.7, and 02.03.4 supported low nematode levels.

Discussion

Based on the results of this study, we conclude that an initial inoculum level of 1 × 10⁴ eggs + J2 per plant appears to be sufficient for greenhouse screening of citrus rootstocks, since enough adult females and eggs were produced per plant after 6 months to evaluate resistance. Although the highest initial inoculum level tested, 16 × 10⁴ eggs + J2, produced the maximum numbers of females and eggs, the amount of roots needed to provide such inoculum was far too high to be of practical use. Furthermore, artificially high inoculum levels may affect plant response and may not be field relevant. Recent surveys in Spain have shown that population densities of *T. semipenetrans* were lower than 1 × 10⁴ eggs + J2 per gram of root in 84% of citrus orchards sampled (Verdejo et al., 1995, 1997). Numbers of nematodes recovered were related to those added at the higher, but not at the lower, inoculum levels. Females and eggs + J2 recovered from either rootstock inoculated with 2 × 10⁴ eggs + J2 were lower than expected for unknown reasons. The rate of nematode increase can be affected by the experimental conditions, including the method of inoculation, pot size, and potting mixture (Niles et al., 1995; O'Bannon, 1968; Van Gundy and Tsao, 1963).

The variability in nematode infectivity and reproductive potential occurring in this study has also been found in other studies, where numerous rootstocks showing different levels of susceptibility were compared (Geraci et al., 1981; Hutchinson and O'Bannon, 1972; McCarty et al., 1979; Niles et al., 1995). In general, females per gram of root and eggs + J2 per gram of root were related in this study, but there was higher variability in the lower number of females than in the eggs + J2 per gram of root (cv = 48.9 and 25.5, respectively). Both indicators are useful to evaluate resistance because some plants may not impair female development, but they may adversely influence nematode reproduction (Kaplan, 1990). Nematode extraction from roots by blender maceration allows the recovery of all stages of the life cycle of the nematode and they can be counted separately when examining the nematode suspension by light microscopy.

Poncirus trifoliata has been used as a source of resistance to CTV, *Phytophthora*, and the

Table 2. Numbers of females and eggs + J2 of a Mediterranean biotype of *Tylenchulus semipenetrans* per g of root of citrus hybrid rootstocks after exposure for 6 months to an inoculum level of 1×10^4 nematode eggs + J2 per plant in a greenhouse test.

Parentage and selection no.	Females*	Eggs + J2
<i>Poncirus trifoliata</i> Rubidoux	0 h	0 j
Cleopatra mandarin (03) x <i>P. trifoliata</i> (01)		
03.01.8	0 h	0 j
03.01.31	0 h	0 j
03.01.42	0 h	0 j
03.01.40	<1 gh	6 i
03.01.5	1 gh	11 hi*
03.01.18	1 gh	16 hi*
03.01.7	1 gh	26 h*
03.01.16	2 gh	34 h*
03.01.22	9 ef*	96 g*
03.01.13	13 def*	110 fg*
03.01.9	26 cde*	310 defg*
Cleopatra mandarin (03) x Troyer citrange (01)		
03.02.9	5 fg	120 defg*
03.02.1	31 abcd*	484 cde
Troyer citrange (02) x Cleopatra mandarin (03)		
02.03.3	10 def*	270 cdef*
02.03.7	11 def*	180 defg*
02.03.4	12 def*	230 efg*
02.03.13	22 de*	750 cdef*
02.03.32	26 bcd*	770 bc*
02.03.20	60 ab*	2990 ab*
02.03.5A	70 ab*	2725 a*
02.03.17	77 a*	2950 a*
Troyer citrange (02) x common mandarin (04)		
02.04.18	40 abcd*	720 cde*
Sour orange	83 abc*	1063 bcd*
Mean	21.6	560.9
CV	48.9	25.5
Dunnett's <i>t</i> value, lower-tailed test	3.48	3.48
MSE	0.8	1.1

*Data were transformed log ($x + 1$). Mean separation within columns by Duncan's multiple range test ($P = 0.05$).

*Difference between *P. trifoliata* and rootstock significant according to Dunnett's *t* tests ($P = 0.05$).

citrus nematode in the selection of hybrids between *Citrus* species and *P. trifoliata*. This rootstock is highly resistant to many Spanish populations of *T. semipenetrans* (Verdejo-Lucas et al., 1997), but is not grown commercially in Spain because it performs poorly in calcareous or alkaline soils, which dominate in the citrus-growing areas. Although *P. trifoliata* is a source of resistance to the citrus nematode, most of the resulting poncirus hybrids are susceptible to *T. semipenetrans* (Hutchinson and O'Bannon, 1972), and only a few hybrids inherit resistance to the citrus nematode. Therefore, large numbers of hybrids must be examined to detect segregating types with resistance. Most hybrids of *P. trifoliata* are likely to be less susceptible to infection by *T. semipenetrans* than the susceptible citrus parent cultivar if *P. trifoliata* can provide any of several different resistance genes during meiosis (Duncan et al., 1994). In this study, poor nematode reproduction occurred on most of the hybrids from crosses of Cleopatra mandarin and *P. trifoliata* (73% of the hybrids tested), despite the susceptibility of Cleopatra mandarin. The latter rootstock is comparatively less susceptible to *T. semipenetrans* than other citrus or citrus hybrid rootstocks (Lamberti et al., 1976; McCarty et al., 1979).

Resistance to *T. semipenetrans* exhibited by some crosses of Cleopatra mandarin and *P. trifoliata*, such as 03.01.5 and 03.01.18, is of great practical importance since these root-

stock selections are also CTV-resistant and CTV-tolerant, respectively. Both selections are more tolerant to calcareous soils than Carrizo citrange, and their ability to exclude chlorine is intermediate between Cleopatra mandarin and Carrizo citrange. They also provide semi-dwarfing trees, have good nursery performance, and produce 15–20 seeds per fruit, and the fruit quality of the cultivars grafted on them is high (Forner and Alcaide, 1994). Additional field testing is needed to verify their resistance and to determine if plant defenses such as the hypersensitive reaction and formation of wound periderm (Kaplan and O'Bannon, 1981; Van Gundy and Kirkpatrick, 1964) are involved in the resistant response observed in this study. Intermediate levels of resistance to the citrus nematode could be useful for field application if rootstocks possess desirable horticultural characteristics or specific resistance to a particular pathogen, because citrus trees can perform relatively well in soils with low nematode infestation levels. For instance, the selection Troyer citrange x common mandarin 02.04.18 is tolerant to tristeza virus and limits tree size when grafted with 'Navelina' scion. Both features warrant consideration of this hybrid as a promising rootstock, despite its intermediate level of resistance to *T. semipenetrans*.

Changes in production practices leading to higher planting densities per ha will be feasible by combining CTV-tolerant rootstocks

with dwarfing characteristics and citrus nematode resistance. Several of the rootstocks included in this study possess the combination of those characteristics, and further evaluations will determine their potential for commercial use in Spain and elsewhere. Hybrids of *P. trifoliata* resistant to the citrus nematode continue to be reported (Gottlieb et al., 1986; Niles et al., 1995; Spiegel-Roy et al., 1988) and they may provide additional acceptable rootstocks in the future.

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