

Resistance of Hybrid Citrus Rootstocks to a Mediterranean Biotype of *Tylenchulus semipenetrans* Cobb

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Abstract. The response of 52 citrus hybrid rootstocks to a Mediterranean biotype of *Tylenchulus semipenetrans* Cobb was determined in greenhouse tests. Seven selections of the cross 'Cleopatra' mandarin [*Citrus reshni* Hort. × *Poncirus trifoliata* (L.) Raf], and one of *Citrus volkameriana* Pasq. × *P. trifoliata* did not support nematode reproduction and were considered as highly resistant to the citrus nematode. The nematode showed very low infectivity and reproductive potential on seven additional selections of 'Cleopatra' mandarin × *P. trifoliata*, one of 'King' mandarin × *P. trifoliata*, and two *C. volkameriana* × *P. trifoliata*. These selections were considered as nematode resistant. All the selections with 'Troyer' citrange [*Citrus sinensis* (L.) Osbeck × *P. trifoliata* (L.) Raf] in their parentage supported nematode reproduction but showed different levels of susceptibility.

The citrus nematode infects citrus worldwide and is associated with poor growth of young citrus trees planted in infested groves and with poor performance of mature citrus trees (Duncan and Cohn, 1990). The host range of *T. semipenetrans* includes all *Citrus* species and most hybrids of citrus with other members of the Rutaceae family, such as trifoliolate orange [*Poncirus trifoliata* (L.) Raf]. Nonrutaceous plants, such as grape (*Vitis vinifera* L.), olive (*Olea europea* L.) and persimmon (*Diospyros* sp.), are also hosts of the citrus nematode. The only germplasm source of citrus nematode resistance that has been incorporated into commercially acceptable citrus rootstocks is derived from *P. trifoliata* (Kaplan, 1990). Some selections of *P. trifoliata* have a high level of resistance to populations of *T. semipenetrans*, whereas others are moderately susceptible (Baines et al., 1969; Cameron et al., 1954; Crozzoli and González, 1989; Ducharme, 1948; Feder, 1968; McCarty et al., 1979; O'Bannon and Ford, 1977; Reddy

and Agarwal, 1987). Trifoliolate orange hybridizes readily with most *Citrus* sp., and many of the resulting hybrids inherit resistance to the citrus nematode (Cameron et al., 1969). Of these, the hybrid rootstock 'Swingle' citrumelo (*Citrus paradisi* Macf. × *P. trifoliata*) is highly resistant to the citrus nematode in Florida (Kaplan and O'Bannon, 1981), Italy (Lo Giudice and Inserra, 1980), and Venezuela (Crozzoli and Funes, 1992). Duncan et al. (1994) have reported a population of *T. semipenetrans* capable of overcoming resistance in 'Swingle' citrumelo in Florida, but this population appears to be confined to the nursery site where it was detected. Other potential sources of citrus nematode resistance among noncultivated Rutaceous plants have been reported (Baines et al., 1960; Kaplan and O'Bannon, 1981), but these genera are not horticulturally acceptable as rootstocks and their hybridization with *Citrus* sp. has had limited success.

Although *P. trifoliata* and 'Swingle' citrumelo are resistant to *T. semipenetrans*, both rootstocks have important limitations to use in Spanish soils because of their poor performance in calcareous and alkaline soils. Therefore, new rootstocks are still needed to satisfy regional demands. A breeding program was initiated at the Instituto Valenciano de Investigaciones Agrarias (IVIA), Moncada, Valencia, 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 tristeza tolerance (Forner and Alcaide, 1994). In a previous study, the response of 22 citrus hybrid rootstocks to a Mediterranean biotype of *T. semipenetrans* was determined in a greenhouse test (Verdejo-Lucas et al., 1997a). This study reports the response of 44 additional citrus hybrid rootstocks that had not been tested previously to the same biotype of the citrus nematode. Eight rootstocks evaluated previously as hosts of citrus nematode were also included.

Materials and Methods

Procedure. The procedure described by Kaplan (1990) for screening citrus rootstocks for resistance to *T. semipenetrans* was followed in this study, with some modifications as previously reported (Verdejo-Lucas et al., 1997a). Briefly, germinated seeds of each selection were transplanted singly to black plastic bags (1.5-L capacity) containing a steam-sterilized potting mixture, and plants were maintained in a greenhouse for 12 months before adding the nematode inoculum. The latter was obtained from *T. semipenetrans*-infected citrus roots collected from a 14-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 using a differential host test (Verdejo-Lucas et al., 1997b). Citrus roots were blender macerated (McSorley et al., 1984) to estimate the number of eggs and second-stage juveniles (J2) per g root. Plants were inoculated with $\approx 1 \times 10^4$ eggs + J2 per plant. The rootstocks assessed were selected because of their tolerance to citrus tristeza virus. The selections included in each of the three experiments performed are indicated in Tables 1, 2, and 3. Selections without *P. trifoliata* in their parentage were also included in the experiments to determine their relative susceptibility to *T. semipenetrans*. The selections 03.01.40, 03.01.8, 03.01.42, 03.01.5, 03.01.18, 03.01.46, 02.04.18, and 02.03.24 included in the third experiment had been tested previously but were retested again to verify their response (Verdejo-Lucas et al., 1997a, and Table 2).

The experiments were 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 6 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) using a 0.5% sodium hypochlorite solution. Nematodes collected on a 25-mm screen were subjected to centrifugation and sugar flotation to remove root debris (Jenkins, 1964). The infectivity (females/g root) and reproductive potential (eggs + J2/g root) of *T. semipenetrans* were considered as indicators of the response of the rootstocks to the nematode.

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Table 1. Numbers of females, second stage juveniles, and eggs of a Mediterranean biotype of *Tylenchulus semipenetrans* per gram of root for citrus hybrid rootstocks. Expt. 1.^z

Parentage and selection no.	Females ^y	Eggs + J2
<i>Poncirus trifoliata</i> Rubidoux	0 g	0 i
'Cleopatra' mandarin (03) x <i>P. trifoliata</i> (01)		
03.01.23	0 g	0 i
03.01.24	0 g	0 i
03.01.41	0 g	0 i
03.01.34	0.04 g	2 h
03.01.5	0.47 fg	3 h
03.01.14	2 d-f	11 g*
03.01.38	3 b-e*	83 b-e*
03.01.43	4 a-d*	56 ef*
03.01.9	6 ab*	109 b-e*
'Cleopatra' mandarin (03) x 'Troyer' citrange (02)		
03.02.15	1 e-g	18 g*
03.02.26	2 c-f	48 f*
03.02.06	0.5 fg	87 b-f*
03.02.11	5 a-d*	72 c-f*
03.02.07	4 b-e*	150 a-c*
03.02.24	10 ab*	232 a-d*
'Troyer' citrange (02) x 'Cleopatra' mandarin (03)		
02.03.34	4 a-e*	78 d-f*
02.03.02	7 a-c*	198 a*
02.03.22	7 ab*	233 a*
02.03.44	8 a*	248 a*
Common mandarin (04) x 'Troyer' citrange (02)		
04.02.02	10 a*	157 ab*
Sour orange	7 a-c*	218 a*
Mean	3.5	91
CV	62	18.60
Dunnnett's <i>t</i> value, lower-tailed test	2.96	2.96
MSE	0.41	0.37

^zRootstocks were exposed to an inoculum level of 1×10^4 nematode eggs + J2 per plant for 6 months in a greenhouse.

^yData were transformed $\log(x + 1)$. Means separation within columns by Duncan's multiple range test, $P \leq 0.05$.

*Difference between *P. trifoliata* and rootstock tested significant according to Dunnnett's *t* tests, $P \leq 0.05$.

Statistical analysis of data. The host status of the rootstocks was established by comparing nematode infectivity and reproductive potential with those on the susceptible sour orange. Data were transformed to $\log(x + 1)$ and subjected to analysis of variance, and the means were separated by Duncan's multiple range test ($P \leq 0.05$). 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 Dunnnett's *t* tests ($P \leq 0.05$) (Dunnnett, 1955). Data on number of females per plant and eggs produced per female were transformed to $\log(x + 1)$ and subjected to regression analysis to determine the relationship between fertile females and their fecundity in rootstocks that differed in susceptibility to the citrus nematode. Individual plants with no females were excluded from the analysis. Analysis of variance indicated that nematode reproduction on susceptible sour orange was similar in the three experiments. Hence, nematode infectivity and reproductive potential on selections of 'Troyer' citrange x 'Cleopatra' mandarin (02.03) were compared with those on selections of 'Cleopatra' mandarin x 'Troyer' citrange (03.02) by Student's *t* test ($P \leq 0.05$) to determine if there was any cytoplasmic inheritance.

Results

The Mediterranean biotype of *T. semipenetrans* did not reproduce on *P. trifoliata*. In Expt. 1, the selections from crosses of 'Cleopatra' mandarin x *P. trifoliata* 03.01.23, 03.01.24, and 03.01.41 did not support nematode reproduction (Table 1). These selections were considered as highly resistant to *T. semipenetrans*. A very low number of females and of eggs + J2 were recovered from two additional selections of the same cross (03.01.34 and 03.01.5), and these selections could be considered as nematode-resistant. The remaining four selections of 'Cleopatra' mandarin x *P. trifoliata* supported nematode reproduction. All selections from crosses with 'Troyer' citrange supported various levels of nematode reproduction (Table 1). In Expt. 2, the citrus nematode did not reproduce on the selections from crosses of 'Cleopatra' mandarin x *P. trifoliata* 03.01.27, 03.01.28 and 03.01.33, or of *C. volkameriana* x *P. trifoliata* 23.01.98 (Table 2). As in the previous experiment, these selections were considered as highly resistant to the population of the Mediterranean biotype of *T. semipenetrans* tested. The nematode showed a very low infectivity (< 1 female per gram root) and reproductive potential (< 15 eggs + J2 per gram root) on three additional crosses of 'Cleopatra' mandarin x *P. trifoliata* (03.01.16, 03.01.20 and 03.01.26),

and on two of *C. volkameriana* x *P. trifoliata* (23.01.17 and 23.01.29). The remaining selections included in this experiment supported nematode reproduction in varying degrees. Numbers of females and of eggs + J2 per gram of root were higher ($P \leq 0.05$) on three selections of 'Cleopatra' mandarin x 'Troyer' citrange, two of 'Troyer' citrange x 'Cleopatra' mandarin, and on 'Mineola' tangelo x *P. trifoliata* than on the susceptible sour orange (Table 2). In Expt. 3, the selection 03.01.40 of 'Cleopatra' mandarin x *P. trifoliata* was considered as highly resistant to the citrus nematode, whereas the selections 03.01.8, 03.01.42, 03.01.5, and the selection of 'King' mandarin x *P. trifoliata* 05.01.24B were resistant (Table 3).

A total of 20 selections from crosses of 'Troyer' citrange and 'Cleopatra' mandarin were tested, and each of them supported nematode reproduction (Tables 1, 2, and 3). However, the numbers of females per gram of root in some of these selections without *P. trifoliata* parentage, such as 03.02.15, 03.02.26, 03.02.6 (Table 1), 02.03.24 (Table 2), 03.02.12 and 02.03.18 (Table 3) did not differ from those on *P. trifoliata*. This was probably due to the great variability among individual plants (Fig. 1). The numbers of females produced per plant and their fecundity was negatively correlated, that is, rootstocks supporting higher infectivity showed lower reproductive potential. This relationship was also found in rootstocks that expressed resistance to *T. semipenetrans*. Cytoplasmic inheritance was not evident in the resistance of progeny from crosses of 'Troyer' citrange and 'Cleopatra' mandarin. Overall, the average numbers of females and of eggs + J2 per gram of root produced by 'Troyer' citrange x 'Cleopatra' mandarin (02.03) and 'Cleopatra' mandarin x 'Troyer' citrange (03.02) were similar (Table 4).

Discussion

The rootstocks included in our studies had shown tolerance to CTV in previous studies (Forner, personal communication). The citrus nematode is the most frequent and abundant plant-parasitic nematode that occurs in Spanish citrus orchards, but other pest nematode species are also present (Bello et al., 1985). The resistance found in trifoliolate orange and 'Swingle' citrumelo have shown a useful level of durability (Roberts, 1992). At present, these two rootstocks are the only commercially available rootstocks with effective resistance to *T. semipenetrans*; however, horticultural characteristics preclude their use in Spanish soils.

In this study, we have only considered rootstocks to be resistant to *T. semipenetrans* if citrus nematode infectivity and reproductive potential (females and eggs + J2 per gram of root, respectively) were similar to those of trifoliolate orange. These resistant rootstocks included 14 hybrids with 'Cleopatra' mandarin, three with *C. volkameriana*, and one with 'King' mandarin, which represented 64% of the selections from crosses with *P. trifoliata*. Some of these hybrids possess other characteristics that are of interest for citrus produc-

Table 2. Numbers of females, second stage juveniles, and eggs of a Mediterranean biotype of *Tylenchulus semipenetrans* per gram of root for citrus hybrid rootstocks. Expt. 2.²

Parentage and selection no.	Females ^y	Eggs + J2
<i>Poncirus trifoliata</i> Rubidoux	0 f	0 i
'Cleopatra' mandarin (03) x <i>P. trifoliata</i> (01)		
03.01.27	0 f	0 i
03.01.28	0 f	0 i
03.01.33	0 f	0 i
03.01.16	0.4 ef	1 hi
03.01.26	0.2 ef	2 hi
03.01.20	0.03 f	15 h
03.01.47	1.4 d-f	37 g*
03.01.21	1.7 d-f	71 fg*
03.01.46	5 de	283 fg*
<i>Citrus volkameriana</i> (23) x <i>P. trifoliata</i> (01)		
23.01.98	0 f	0 i
23.01.29	0.4 ef	0 i
23.01.17	0.4 ef	0.5 hi
23.01.95	12 c*	57 g*
'Cleopatra' mandarin (03) x 'Troyer' citrange (02)		
03.02.30	10 c*	275 de*
03.02.27	13 c*	666 b-d*
03.02.25	21 bc*	1016 bc*
03.02.32	77 a*	1492 b*
'Troyer' citrange (02) x 'Cleopatra' mandarin (03)		
02.03.24	6 d	249 fg*
02.03.41	13 c*	352 cd*
02.03.23	10 c*	369 c-e*
02.03.20	43 ab*	4168 a*
Tangelo 'Mineola' x <i>P. trifoliata</i>	20 bc*	656 cd*
Sour orange	7 d*	128 ef*
Mean	10	397
CV	56.30	26.9
Dunnett's <i>t</i> value, lower-tailed test	2.99	2.99
MSE	0.53	0.79

²Rootstocks were exposed to an inoculum level of 1×10^4 nematode eggs + J2 per plant for 6 months in a greenhouse.

^yData were transformed $\log(x+1)$. Means separation within columns by Duncan's multiple range test, $P \leq 0.05$.

*Difference between *P. trifoliata* and rootstock tested significant according to Dunnett's *t* tests, $P \leq 0.05$.

Table 3. Numbers of females, second stage juveniles, and eggs of a Mediterranean biotype of *Tylenchulus semipenetrans* per gram of root for citrus hybrid rootstocks. Expt. 3.²

Parental and selection no.	Females ^y	Eggs + J2
<i>Poncirus trifoliata</i> Rubidoux	0 c	0 h
'Cleopatra' mandarin (03) x <i>P. trifoliata</i> (01)		
03.01.40	0 c	0 h
03.01.8	0.003 c	0.3 h
03.01.42	0.03 c	1.5 gh
03.01.5	0.06 c	5 fg
03.01.18	1 bc	11 ef*
03.01.46	2 b*	30 de*
'King' mandarin x <i>P. trifoliata</i>		
05.01.7	0.8 bc	19 ef*
05.01.24B	1 bc	6 fg
'Cleopatra' mandarin (03) x 'Troyer' citrange (02)		
03.02.12	1.4 bc	42 cd*
'Troyer' citrange (02) x 'Cleopatra' mandarin (03)		
02.03.18	1.4 bc	20 de*
02.03.24	2 b*	60 cd*
'Troyer' Citrange x common mandarin		
02.04.18	10 a*	372 a*
X-639	14 a*	216 ab*
Sour orange	7 a*	143 bc*
Mean	2.7	61.8
CV	81.10	39.10
Dunnett's <i>t</i> value, lower-tailed test	2.87	2.87
MSE	0.33	0.84

²Rootstocks were exposed to an inoculum level of 1×10^4 nematode eggs + J2 per plant for 6 months in a greenhouse.

^yData were transformed $\log(x+1)$. Means separation within columns by Duncan's multiple range test, $P \leq 0.05$.

*Difference between *P. trifoliata* and rootstock tested significant according to Dunnett's *t* tests, $P \leq 0.05$.

tion in Spain. For instance, the selection 'Cleopatra' mandarin x *P. trifoliata* 03.01.5 has been recently released to nurseries because its resistance to CTV, higher tolerance to calcareous soils than 'Carrizo' citrange, and its ability to exclude chlorine is intermediate between 'Cleopatra' mandarin and 'Carrizo' citrange (Forner and Alcaide, 1994). This selection and 'Troyer' citrange x common mandarin 02.04.18 (Table 3) have been registered for the European Union. New citrus rootstocks adapted to local conditions will contribute to diversification of the Spanish citrus industry which now relies on a few CTV-tolerant rootstocks (Forner and Pina, 1992).

Five of the 28 crosses with *P. trifoliata* studied differed from trifoliata orange in their reproductive potential but not in their infectivity. These crosses were 03.01.14 (Table 1), 03.01.47, 03.01.21, 03.01.46 (Table 2), 03.01.18, and 05.01.7 (Table 3). Females were not recovered from two to four of seven replicated plants of these crosses, which could explain their similarity in infectivity to the resistant standard. Overall, data on number of females were more variable than those on eggs + J2 per g of root in the three experiments, probably because of the low number of females recovered from some individual plants. The range of host susceptibilities for *T. semipenetrans* for the rootstocks tested in this study is probably similar to the variation reported previously (Geraci et al., 1981; Hutchinson and O'Bannon, 1972; McCarty et al., 1979; Niles et al., 1995). The rootstock selections that were included in the third experiment provided results similar to those obtained previously. Their host suitability to a population of the Mediterranean biotype of *T. semipenetrans* was confirmed. Further studies are needed to determine the performance of these resistant rootstocks under different field conditions. Also, the effect of inoculum pressure, heat sensitivity, disease complexes, and salinity should be determined (Mashela et al., 1992; Roberts, 1992). At present, we are evaluating the performance of the selection 03.01.5 in replant situations and the effect of initial and continuous inoculum pressure of the citrus nematode on several selections from crosses of "Cleopatra" mandarin x *P. trifoliata* that showed resistance in the greenhouse tests. Although the Mediterranean biotype of *T. semipenetrans* has been the only citrus nematode biotype detected thus far in Spain (Verdejo-Lucas, 1992; Verdejo-Lucas et al., 1997b), the resistance of these new rootstocks to the citrus and *Poncirus* biotypes of *T. semipenetrans* should be assessed.

Tylenchulus semipenetrans has been the target nematode in screening tests of citrus and citrus relatives in California (Baines et al., 1960; Cameron et al., 1954; McCarty et al., 1979; Niles et al., 1995), Florida (Hutchinson and O'Bannon, 1972; O'Bannon and Ford, 1977), Italy (Geraci et al., 1981; Lo Giudice and Inserra, 1980), India (Chabra and Bindra, 1974; Reddy and Agarwal, 1987; Reddy et al., 1987), Israel (Spiegel-Roy et al., 1988), Venezuela (Crozzoli and González, 1989), China (Zhu et al., 1992), Brazil (Salibe and Jaehn,

1996), and Spain (Verdejo-Lucas et al., 1997a). New rootstocks will be screened as they become available. Because *T. semipenetrans* is a highly specialized plant parasitic nematode, planting highly resistant rootstocks may exert selection pressure on the nematode, resulting in development of new biotypes that can reproduce on the resistant rootstocks. Nematode populations able to reproduce on trifoliolate orange have been reported only from areas with a high incidence of *P. trifoliolata* and its hybrids (Baines et al., 1969; Duncan et al., 1994). In practice, rootstocks with moderate levels of resistance would be desirable if they could provide adequate protection from nematode damage in the field; citrus trees can perform relatively well in soils with low nematode infestation levels.

This study used conventional plant breeding procedures to obtain the citrus hybrids. These procedures could be coupled with molecular techniques to expedite the plant breeding process. Currently, genetic markers linked to nematode resistance loci have been identified in other crops (Gebhardt, et al., 1993; Kreike et al., 1993; Salentijn et al., 1995; Weisemann et al., 1992; Williams et al., 1994; Williamson et al., 1994). Unfortunately, inheritance mechanisms for resistance to *T. semipenetrans* have not been determined. A dominant and oligogenic gene may be involved given the high frequency of F₁ hybrids of *P. trifoliolata* that show resistance. (Cameron et al., 1969; Hutchinson, 1985). Histopathological studies have shown that the resistance to the citrus nematode derived from *P. trifoliolata* is based on several mechanisms including a hypersensitivity response to early nematode feeding and subsequent formation of wound periderm (Kaplan, 1981; Kaplan and O'Bannon, 1981; Van Gundy and Kirkpatrick, 1964). Additional research is needed to determine mechanisms involved in the identification of molecular markers that link to the gene for *T. semipenetrans* resistance in *P. trifoliolata*. Recently, random amplified polymorphic DNA fragment markers tightly linked to genes for resistance to citrus nematode and citrus tristeza virus have been identified (Gmitter et al., 1996; Mestre et al., 1997) in *P. trifoliolata*. This finding is encouraging because both pathogens, the virus, and the nematode, appear to share the same source of genetic resistance.

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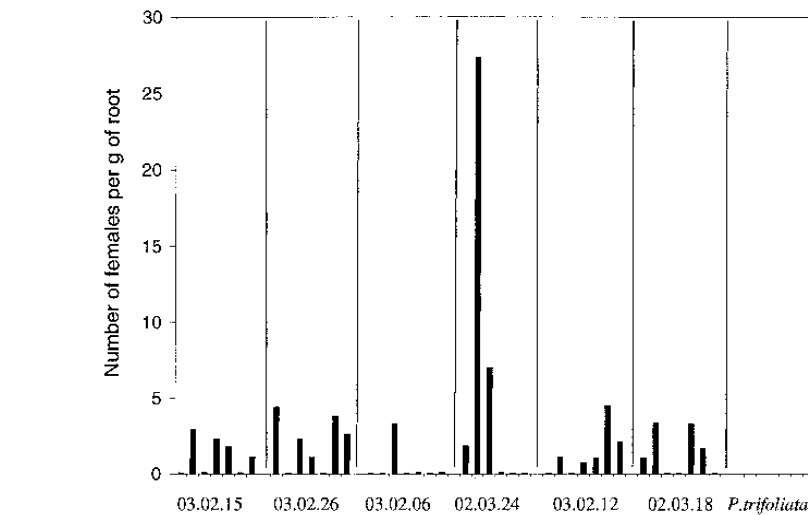


Fig. 1. Number of females per gram of root of a Mediterranean biotype of *Tylenchulus semipenetrans* 6 months after inoculation of 1×10^4 nematode eggs + J2 per plant in a greenhouse test. Each bar represents one of seven plants of selections from crosses of 'Troyer' citrange (02) with 'Cleopatra' mandarin (03).

Table 4. Root fresh weight and numbers of females and eggs + J2 per gram of root of a Mediterranean biotype of *Tylenchulus semipenetrans* on selections from crosses of 'Troyer' citrange and 'Cleopatra' mandarin.

Expt.	Cross	No. selections ²	Root fresh wt (g)	Females /g root	Eggs + J2 /g root
1	'Troyer' x 'Cleopatra'	4	18.0	6.4*	196 ^c
	'Cleopatra' x 'Troyer'	6	20.6	3.7	101
2	'Troyer' x 'Cleopatra'	4	16.6	18.4*	1323
	'Cleopatra' x 'Troyer'	4	15.0	31	884
3	'Troyer' x 'Cleopatra'	2	24	1.8	40
	'Cleopatra' x 'Troyer'	1	20	1.4 ^{ns}	42
Mean	'Troyer' x 'Cleopatra'	10	18.8	611	10.2
	'Cleopatra' x 'Troyer'	11	18.6	373 ^{ns}	13.1

²Seven replicate plants per selection.

^{ns}. *Nonsignificant or significantly different from reciprocal cross by Student's *t* test, *P* ≤ 0.05.

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