Characterization of Citrus Rootstock Responses to Tylenchulus semipenetrans (Cobb)

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Abstract: Citrus rootstocks which significantly limited the reproduction of *Tylenchulus semipenetrans* (Cobb) "Citrus" and "Poncirus" biotypes responded to infection by producing a hypersensitive-type response in the root hypodermis, wound periderm and/or cavities in the root cortex, and/or abnormal vacuoles in nurse cell cytoplasm. Rootstocks which limited nematode reproduction also had significantly fewer nematodes in the rhizoplane within 8 d of inoculation than did rootstocks which did not limit reproduction. Germplasm sources of the cellular responses which limited citrus nematode reproduction were identified. *Key words:* histology, germplasm, rhizoplane, citrus nematode.

The citrus nematode, *Tylenchulus semipenetrans* (Cobb), is distributed throughout the citrus-growing regions of the world, and often causes significant reductions in tree growth and yield (10). Historically, citrus nematode control has depended on the integration of preplant and postplant nematicides, resistant rootstocks, and sanitation practices. However, the future of agrichemicals, specifically nematicides, is in jeopardy. They are expensive, their manufacture and application require energy, and, most important, they can be hazardous to health and environment. In addition, the perennial nature of the crop and the ability of the citrus nematode to develop biotypes (1,7) increases the difficulty of implementing effective control measures. Finally, the wide distribution of the citrus nematode (10) and grove maintenance requirements often make sanitation a difficult practice.

Ideally, rootstock germplasm would combine several traits that limit nematode reproduction. Planting trees with multiple defense systems (horizontal resistance) to combat citrus nematodes should reduce the likelihood of biotype development. Consequently, the relative importance of nematicides and sanitation could be reduced. The purpose of this study was to identify the characteristics which limited citrus nematode reproduction in various citrus rootstock germplasms. The interaction of two citrus nematode biotypes with certain rootstock germplasms was examined.

MATERIALS AND METHODS

Rootstock influence on nematode reproduction: Citrus limon (L.) Burm. f. cv. Milam, *C. reticulata* Blanco cv. Cleopatra mandarin, *Fortunella margarita* (Lour.) Swing. cv. Nagami, *Poncirus trifoliata* (L.) Raf. cv. Flying Dragon, *Severinia buxifolia* (Poir.) Ten., and the hybrid 'Swingle' citrumelo (*C. paradisi* Macf. X *P. trifoliata*) were grown at 25 C ± 1 in 460-cm^3 cups containing a steam sterilized potting medium (Astatula fine sand:peat moss:vermiculite/2:1:1; pH = 6.7). Nine 2-month-old seedlings of each rootstock variety were each inoculated with 4,000 larvae of either the "Poncirus" or "Citrus" biotype of *Tylenchulus semipenetrans* (Cobb) (2). Sixty days after inoculation, root systems were gently rinsed of debris, weighed, and incubated in jars for 5 d at 26 C. Roots were then shaken in 50 ml of 1.25 M sucrose. Larvae were collected on a 25-μm sieve and resuspended in 5.0 ml of water. Numbers of nematodes were estimated from counts of 1.0-ml sample aliquots. Data were expressed as the number of larvae per gram of root fresh weight, and results subjected to analysis by Duncan's multiple-range test (P = 0.1).

Cellular responses to nematode infection: Seedlings were grown as previously described. Seedling roots selected for inoculation were distributed in 16-cm^3 side chambers attached to the 460-cm^3 cups (Fig. 1). A 5-mm-i.d. plastic inoculation tube was attached to the side chamber. The apparatus was buried in a sandbath to within 2 cm of the top of each cup. The

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sandbaths, in turn, were placed in refrigerated temperature tanks maintained at 26 C ± 1 throughout the experiment. This technique facilitated the application of inoculum to a small portion of the intact seedling root system; it also allowed easy access to the infected roots at the time of harvest. The roots in the side chamber were inoculated with 1,500 Poncirus or Citrus biotype *T. semipenetrans* larvae 1 wk later.

The roots in the side chambers of five styrofoam cups were harvested 2, 4, 6, and 8 wk after inoculation. Infected root segments were fixed in Randolph's modified Navashins fluid (3), dehydrated through a tert-butyl alcohol series, and embedded in Paraplast-plus at 59 C. Sections (12 μm) stained with safranin and fast green were examined and photographed with a photomicroscope at 160× and 400×.

Rootstock influence on infection and rhizoplane association: To determine the effect of host germplasm on the rate of citrus nematode infection as well as on nematode association with the rhizoplane, each of six seedlings of Cleopatra mandarin, Milam, Flying Dragon, 'Swingle' citrumelo, and *S. buxifolia* was equipped with four side chambers as previously described. Each side chamber was inoculated with 3,900 larvae of the Citrus biotype of *T. semipenetrans*. Roots in each of four side chambers from four different plants were weighed, stained in acid fuchsin/lactophenol, and cleared in lactophenol (6) 3, 6, 9, 12, 16, and 18 d after inoculation. The stained root samples were mounted in glycerin between two glass plates. The number of larvae associated with organic debris adhering to roots (rhizoplane) and the number of larvae penetrating roots were counted at 30× and 60×. Data were expressed as number of larvae penetrating roots per gram of root fresh weight and total number of larvae per gram of root fresh weight (larvae penetrating roots plus larvae in the rhizoplane). Lack of sufficient Nagami seedlings prevented its comparison with other rootstocks in the experiment. Data were analyzed by linear regression.

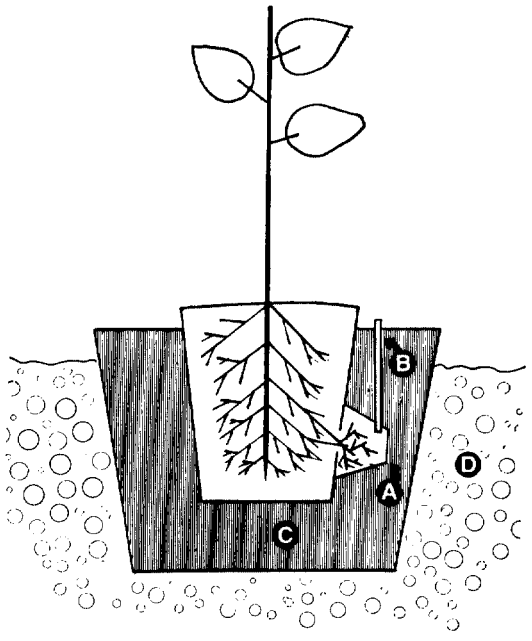


Fig. 1. Feeder roots segregated from the seedling root system were contained within a 16-cm³ sidearm and inoculated through a tube which extended to the surface of the surrounding sandbath. A = side chamber; B = inoculation tube; C = sandbath; D = water bath.

RESULTS

Rootstock influence on nematode reproduction and cellular responses to infection: Citrus nematode reproduction was lowest in rootstocks in which a hypersensitive-hypodermal response (HHR) occurred within 2 wk of inoculation (Table 1). The HHR was characterized by safranin positive staining of hypodermal cells adjacent to the anterior portion of citrus nematode larvae (Fig. 2). In general, wound periderm formation (WP), the cellular division of cortical cells (Fig. 3), also occurred within 2 wk of inoculation. WP appeared to occur in conjunction with HHR (Fig. 2) and/or cavity formation (CAV) in the root cortex (Table 1, Fig. 3). CAV occurred in all rootstocks except Nagami and Swingle in response to infection by the Poncirus biotype; it was not observed in any rootstock in response to infection by the Citrus biotype (Table 1). Cavities which had thickened boundaries were most prominent in the roots of Cleopatra mandarin. The development of numerous large vacuoles (VAC) with distinct walls in nurse cell cytoplasm was also correlated with reduced nematode reproduction in some rootstocks (Table 1, Fig. 4). VAC occurred in roots of Milam, Flying Dragon, and Swingle inocu-

Table 1. The influence of various citrus rootstocks on the reproduction of "Citrus" and "Poncirus" biotypes of *Tylenchulus semipenetrans,* and the time after inoculation of detection of various host cellular responses associated with infection.

Citrus biotype						Poncirus biotype					
Rootstock	Reproduction nema/gram	Cellular responses* (wk after inoculation) HHR	WP	CAV	VAC	Rootstock	Reproduction nema/gram	Cellular responses* (wk after inoculation) HHR	WP	CAV	VAC
Nagami	49.9 a†	–	–	–	–	Nagami	90.2 a†	–	–	–	–
Cleopatra mandarin	26.4 b	–	–	–	4	Milam	24.0 ab	–	6	2	8
Milam	15.1 b	–	–	–	8	Flying Dragon	21.7 bc	–	–	4	8
Flying Dragon	3.3 c	2	8	–	–	*Severinia buxifolia*	6.9 bcd	2	2	2	–
Swingle citrumelo	1.3 c	2	2	–	–	Cleopatra mandarin	6.5 cd	–	2	2	–
Severinia buxifolia	0.0 c	2	2	–	–	Swingle citrumelo	3.7 d	2	2	–	6

*HHR = hypodermal hypersensitive-type response; WP = wound periderm formation; CAV = cavity formation; VAC = abnormal vacuolation in nurse cell cytoplasm.

†Column means followed by the same letter are not significantly different ($P = 0.10$) according to Duncan's multiple-range test $\left(\log\left(\frac{\text{nema}}{\text{gram}}\right)+1\right)$.

lated with Poncirus biotype and in roots of Cleopatra mandarin and Milam when inoculated with the Citrus biotype.

The summary of cellular responses to citrus nematode infection listed in Table 1 were derived from numerous observations. However, some exceptions did occur. Occasional Citrus biotype larvae circumvented the HHR in roots of Swingle citrumelo (Fig. 5) and established feeding sites which led to limited nematode reproduction. Generally, Citrus biotype larvae encountered the HHR and WP in roots of Swingle citrumelo which limited nematode development.

In contrast, none of the cellular responses to infection which were correlated with reduced citrus nematode reproduction were observed in Nagami. Larvae which infected roots established feeding sites composed of several nurse cells (Fig. 6). Nurse cell cytoplasm contained central vacuoles 4–6 wk after inoculation. The granular appearance of nurse cell cytoplasm increased with time and these vacuoles disappeared by the 8th wk after inoculation.

Rootstock influence on infection and rhizoplane assocation: Citrus rootstock germplasm influenced the number of citrus nematodes which were associated with roots. Within 8 d, significantly ($P = 0.01$) larger numbers of citrus nematode larvae (Citrus biotype) were in the rhizoplane and had penetrated roots of Milam and Cleopatra mandarin than were in the rhizoplane and had penetrated roots of *S. buxifolia,* Flying Dragon, and Swingle (Figs. 7 and 8). The number of Citrus biotype larvae in the rhizoplane and subsequent infection of citrus roots was correlated ($r = 0.86$) with citrus nematode reproduction.

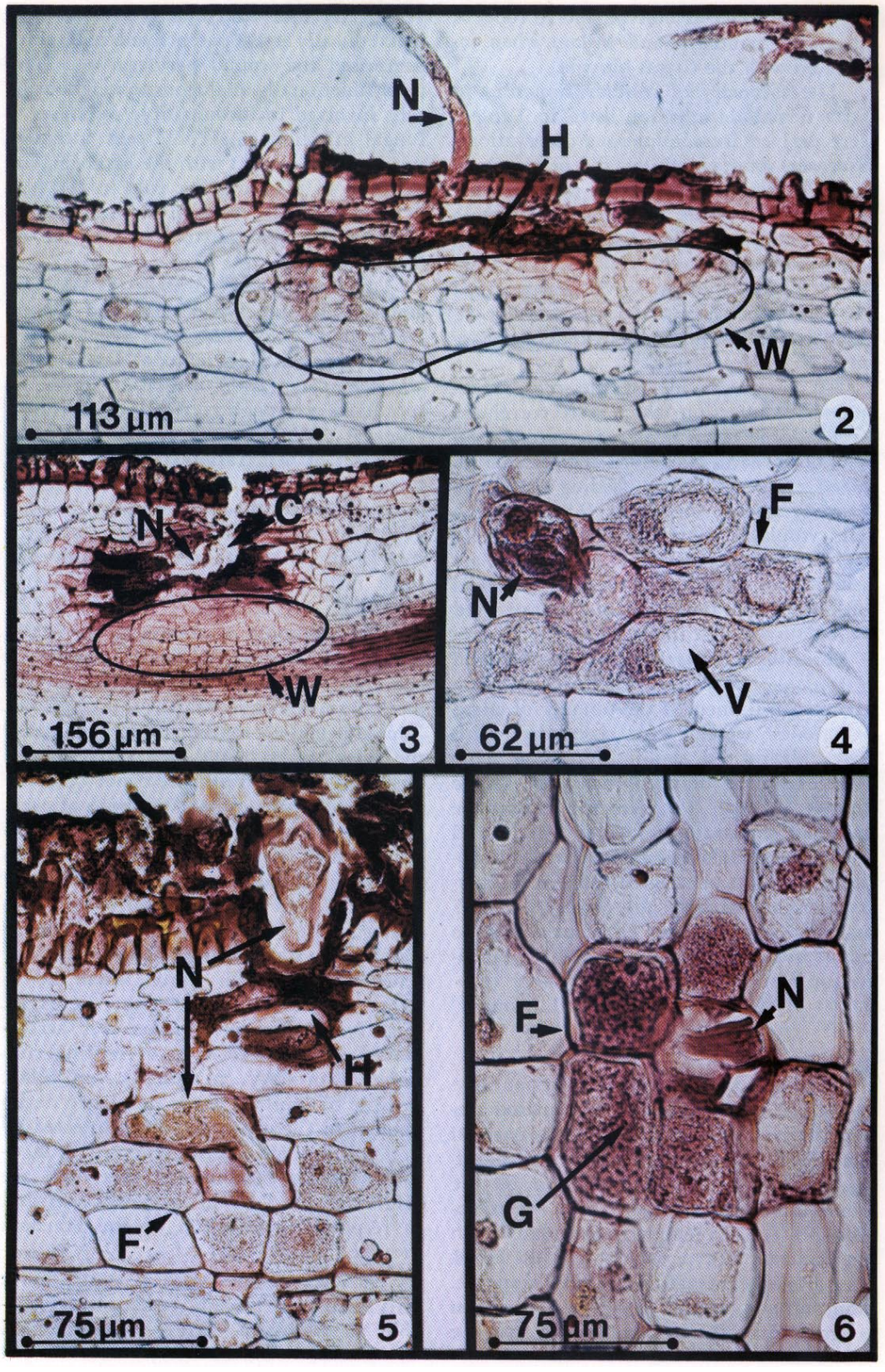
DISCUSSION

Four cellular responses to infection were correlated with reductions in nematode reproduction. The consistent occurrence of the HHR among varieties which significantly limited the reproduction of the Poncirus and Citrus biotype with the exception of Cleopatra mandarin inoculated with the Poncirus biotype suggest that the HHR was a major nematode-limiting response. Concomitant wound periderm formation suggests that the two responses may be genetically and functionally linked. The metabolically active parenchyma cells which comprised the wound periderm may have been functionally analogous to neighboring cells involved in incompatible responses to fungal infection (5). Those cells, although not in direct contact with the pathogen, are believed to play a major role in the host defense system by transporting biosynthetic intermediates and phytoalexins to the infection site (5).

Van Gundy and Kirkpatrick (11) suggested that the hypodermal and wound periderm responses to citrus nematode infection prevented nematode development and deep penetration of the root cortex, respectively. Although cortical penetration is essential to the citrus nematode life cycle, the effect of penetration depth on relative nematode success (as measured by reproduction) has not been determined. Further experimentation is needed to determine the role (if any) of the wound periderm in rootstock incompatibility to the citrus nematode.

The formation of cavities in the cortex of roots of Cleopatra mandarin infected by the Poncirus biotype may reflect a specific mechanism through which this rootstock limited nematode reproduction. In contrast, the thick cavity boundaries which stained positively with safranin were not observed in cavities which developed in roots of sweet orange (*C. sinensis* [L.] Osb.) (9), a rootstock which supported significant citrus nematode reproduction. In addition, CAV and HHR were only observed to occur together in one of the six rootstocks (*S. buxifolia*) which significantly reduced nematode reproduction in this study. When nematodes were able to circumvent the HHR (Fig. 5), cortical penetration was accompanied by the formation of feeding sites rather than CAV. In contrast, an HHR capable of limiting citrus nematode development in Cleopatra mandarin infected by Poncirus biotype was not observed. *S. buxifolia* was the exception in that both the HHR and the CAV in the cortex occurred in response to infection by the Poncirus biotype.

Vacuoles observed within young nurse cells in roots of Nagami became nondetectable as cytoplasm density increased and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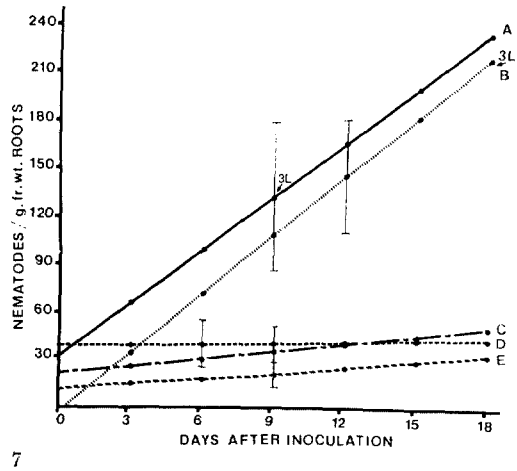

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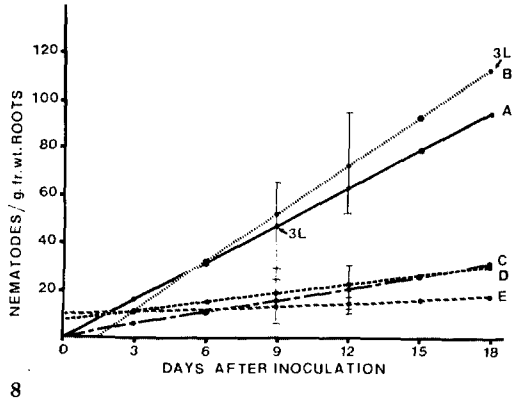

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Figs. 7–8. Rootstocks influence on infection and rhizoplane association of roots. 7) Influence of rootstock on the total number of citrus nematode larvae (Citrus biotype) associated with feeder roots (larvae associated with rhizoplane + larvae penetrating roots). A = Milam (r = 0.67), B = Cleopatra mandarin (r = 0.95), C = Flying Dragon (r = 0.26), D = *Severinia buxifolia* (r = 0.0), E = Swingle citrumelo (r = 0.42). 3L = third stage larvae. 8) Influence of rootstock on the number of citrus nematode larvae (Citrus biotype) penetrating feeder roots. A = Milam (r = 0.81), B = Cleopatra mandarin (r = 0.87), C = Flying Dragon (r = 0.50), D = *Severinia buxifolia* (r = 0.33), E = Swingle citrumelo (r = 0.68). 3L = third stage larvae.

nurse cell morphology was similar to that previously described (11). However, the large and numerous vacuoles in nurse cell cytoplasm in Cleopatra mandarin and Milam infected by the Citrus biotype and in Milam, Flying Dragon, and Swingle infected by the Poncirus biotype had not been observed previously. Cytoplasmic vacuolation probably reflects quantitative and/or qualitative alterations in nurse cell metabolism, thereby affecting citrus nematode feeding and ultimately reproduction. While VAC was the only postinfection cellular host response that could be correlated with reduced reproduction of the Citrus biotype in roots of Milam and Cleopatra mandarin, these rootstocks also reduced the number of nematodes associated with their roots. Findings of the present study indicated greater Citrus biotype association with and penetration of the Milam and Cleopatra mandarin when compared with Swingle citrumelo, Flying Dragon, and *S. buxifolia* which supported significantly lower reproduction.

Plant-regulated attraction and repulsion of nematodes has been demonstrated in many plants (8) but has not been demonstrated in citrus. Further research should elucidate the mechanism in citrus rootstock germplasm responsible for differences in rhizoplane-nematode association. Infection of all citrus varieties occurred once larvae were in the rhizoplane. Root penetration, as in other nematode-plant interactions which limit reproduction (4), did not appear to be affected by rootstock germplasm.

The correlation (r = 0.86) between nematode reproduction and the number of citrus nematodes in the rhizoplane within 3 wk of inoculation may be developed into a useful procedure for screening citrus in

Figs. 2–6. Cellular responses in roots of Swingle citrumelo (*Citrus paradisi* × *Poncirus trifoliata*), *C. reticulata* cv. Cleopatra mandarin, *C. limon* cv. Milam, and *Fortunella margarita* cv. Nagami infected by the "Poncirus" and "Citrus" biotypes of *T. semipentrans*. 2) The hypersensitive-type response in the hypodermis and outer cortex stained with safranin ('Swingle' citrumelo infected by Poncirus biotype). N = citrus nematode, H = hypodermal hypersensitive-type response, W = wound periderm. 3) Cavity formation in the cortex of Cleopatra mandarin infected with *Tylenchulus semipenetrans* (Poncirus biotype). Note the pronounced wound periderm. C = cavity, N = citrus nematode, W = wound periderm. 4) Large vacuoles with distinct walls in nurse cell cytoplasm 8 wk after inoculation of Milam with *Tylenchulus semipenetrans* (Citrus biotype). N = citrus nematode, F = feeding site, V = vacuole. 5) Citrus nematode which circumvented the hypodermal hypersensitive-type response and established a feeding site (Swingle citrumelo × Citrus biotype). N = citrus nematode, H = hypodermal hypersensitive-type response, F = feeding site. 6) Citrus nematode and feeding site in compatible feeder root 6 wk after inoculation of Nagami with *Tylenchulus semipenetrans* (Poncirus biotype). N = nematode, F = feeding site, G = granular cytoplasm.

the future. Presently, however, the USDA breeding program will continue to compare the number of citrus nematodes in the rhizoplane with nematode reproduction on roots of new germplasm in hopes of shortening the present 1–2 yr preliminary greenhouse evaluation of rootstock germplasm to a period of 2 wk.

Ideally, perennial rootstocks, such as citrus, should incorporate several nematode defense mechanisms. Five factors—HHR, WP, VAC, CAV, and reduced numbers of nematodes in the rhizoplane—were correlated with reductions in citrus nematode reproduction. However, none of the germplasm studied conferred all five characteristics. Utilizing these plant responses to citrus nematode infection as phenotypic markers, progeny of *S. buxifolia* or Swingle citrumelo × Cleopatra mandarin might be selected which possess all of the responses associated with limited citrus nematode reproduction.

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