Rearing Migratory Endoparasitic Nematodes in Citrus Callus and Roots Produced from Citrus Leaves

R. N. INSERRA and J. H. O'BANNON[1]

Abstract: Radopholus similis and *Pratylenchus coffeae* were reared on callus and roots developed from citrus leaves. Callus formed best when leaf petioles were immersed in Astatula fine sand and the leaves were sprayed daily with 4 ppm 2,4-D solution and maintained at 25 or 30 C. The nematodes completed one generation in 20 days at 25 C. Highest populations of *R. similis* (1,127) occurred after 50 days, and the highest for *P. coffeae* (619) after 70 days. Leaf-callus cultures from *R. similis*-resistant citrus rootstocks showed the same degree of infection as susceptible rough lemon callus after 30 days. *Key Words:* Citrus leaf-callus.

The burrowing nematode, *Radopholus similis* (Cobb) Thorne, can be reared easily on plant tissue in artificial media. Okra, citrus, and alfalfa callus tissues are excellent for rearing large populations of *R. similis* (4, 5). *R. similis* and *Pratylenchus brachyurus* (Godfrey) Filipjev & Schuur.-Stek. multiplied rapidly on carrot cultures (6) maintained in petri dishes. Gnotobiotic cultures were used successfully for life-cycle and ecological studies of *R. similis* (2, 3). These results, and the ability to induce callus and root development from stems and leaves of several citrus cultivars (1, 7, 9) prompted us to investigate the use of citrus-leaf callus tissue for rearing the migratory endoparasitic nematodes, *R. similis* and *Pratylenchus coffeae* (Zimmerman) Filipjev & Schuur.-Stek., in these tissues.

MATERIALS AND METHODS

Leaves of sour orange (*Citrus aurantium* L.), rough lemon [*C. limon* (L.) Burm. f. 'Chase' and 'Estes'], lemon (*Citrus* sp. 'Milam'), mandarin (*C. reticulata* Blanco 'Cleopatra'), orange [*C. sinensis* (L.) Osbeck 'Algerian' navel and 'Ridge Pineapple'], and citrange [*C. sinensis* × *Poncirus trifoliata* (L.) Raf. 'Carrizo'] were used to produce leaf-callus and root tissues.

Leaves with or without petioles were partially embedded in various soil substrates in petri dishes or 30-cm diam covered plastic dishes. The leaf surface was sprayed daily with an atomizer containing water or aqueous solutions of 4 μg/ml 2,4-dichlorophenoxyacetic acid (2,4-D), 4 μg/ml 1-naphthaleneacetic acid (NAA), or a mixture of 0.25 μg/ml 2,4-D + 0.5 μg/ml NAA + 0.25 μg/ml kinetin. Dishes were incubated at 15, 20, 25, or 30 C.

Nematode development was studied in rough lemon-leaf-petiole callus and roots maintained in steam-pasteurized, moist sand in petri dishes. Tissues were inoculated with 10 females and one male of either *R. similis* or *P. coffeae* obtained from infected citrus roots. Algerian navel and Ridge Pineapple orange and Milam lemon, which are highly resistant to *R. similis* under natural conditions, were used to study adaptability of the nematode to resistant rootstocks. Nematodes in a water suspension were pipetted directly on the callus or root surfaces or in the sand around the tissue.

Development and reproduction of *R. similis* and *P. coffeae* alone or in combination were evaluated in callus or root tissues 10, 20, 30, 40, 50, and 72 days after inoculation. Nematodes were dissected from callus and root tissues and their developmental stages determined. Except in temperature studies, cultures were maintained at about 25 C. All treatments were replicated a minimum of four times.

RESULTS AND DISCUSSION

Although roots and callus developed on leaves without petioles, the data reported were obtained only from callus and roots that developed on the cut surfaces of the petioles. Of various soil mixtures tested, such as peat or mixtures of peat and sand, callus and roots developed best in Astatula fine sand (94-97% sand, 4-6% silt and clay). All rough lemon-leaf callus produced roots after 30-40 days. After roots developed, the callus atrophied. In some leaves, roots emerged first and prevented callus growth. Callus tissue developed best from leaves collected from healthy 5- to 7-

Received for publication 27 September 1974.

[1] Visiting fellow, Agricultural Nematology Laboratory, National Council Research, Bari, Italy; and Nematologist, U.S. Horticultural Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Orlando, Florida 32803.

TABLE 1. Numbers of *Radopholus similis* extracted from rough-lemon leaf callus and roots inoculated with 10 females and 1 male.

Time after inoculation (days)	Callus		Root	
	Fresh wt (mg)	*R. similis* (mean no.)	Fresh wt (mg)	*R. similis* (mean no.)
10	10.3	0.3	32.8	4.3
20	41.8	66.5	52.5	81.3
40	28.0	264.0	74.7	102.7

TABLE 2. Numbers of *Radopholus similis* and *Pratylenchus coffeae* found in roots produced from rough lemon-leaf petioles.

Nematode species	Time after inoculation (days)	Numbers of nematodes in life stages:				
		L2	L3-4	Female	Male	Total
R. similis	10	1.5	0.5	2.0	0.3	4.3
	20	11.5	66.8	3.0	1.3	82.6
	30	26.5	79.8	13.3	27.8	147.4
	40	72.8	91.0	14.0	8.0	185.8
	50	133.0	642.0	176.0	176.0	1,127.0
P. coffeae	10	1.5	0.3	2.0	0.3	4.1
	20	15.7	11.2	2.7	0.3	29.9
	30	8.8	32.3	9.0	15.3	65.4
	40	6.3	27.0	5.8	10.0	49.1

TABLE 3. Mean numbers of *Pratylenchus coffeae* and *Radopholus similis* found in rough-lemon-leaf, and root callus tissue after inoculation with both nematodes.

Nematode species	Time after inoculation (days)	Mean no. of nematodes in life stages:				
		L2	L3-4	Female	Male	Total
P. coffeae	10	2.4	0.3	3.9	0.4	7.0
R. similis	10	3.2	0.5	4.4	0.3	8.4
P. coffeae	20	4.8	15.8	0.3	2.0	22.9
R. similis	20	12.5	84.5	2.3	3.3	102.6
P. coffeae	30	6.8	31.3	7.3	7.5	52.9
R. similis	30	24.3	104.0	25.0	22.8	176.1
P. coffeae	40	5.3	7.8	5.5	6.3	24.9
R. similis	40	12.0	44.5	8.8	7.3	72.6
P. coffeae	72	29.5	213.3	31.0	51.3	325.1
R. similis	72	12.6	100.5	10.2	6.0	129.3

month-old seedlings. Callus tissue was susceptible to fungal infections, but rooted leaf petioles were more resistant and could be maintained as long as 4 mo in dishes. Leaves sprayed daily with a 2,4-D solution, formed the greatest amount of petiole callus, 12.6 mg fresh wt after 11 days, compared with NAA, 5.7 mg, the mixture of 2,4-D, NAA, and kinetin, 6.1 mg, or water, 5.3 mg. At 25 and 30 C, callus formation was greater, 11.6 and 11 mg fresh wt, respectively, after 11 days, than at 20 C, 5.4 mg. No callus formed at 15 C.

Chase rough lemon, Estes and Milam lemon, Algerian navel and Ridge Pineapple orange, and Carrizo citrange formed callus more readily than sour orange and Cleopatra mandarin. They rooted in the following order: Chase rough lemon > Estes > Carrizo > Milam > Algerian navel orange. Milam, Algerian navel, and Ridge Pineapple produced no roots until after about 2 mo, and sour orange and Cleopatra mandarin did not produce roots.

R. similis and *P. coffeae* readily infected leaf-callus and root cultures of all citrus cultivars studied. *R. similis* infected and developed equally well in callus or roots (Table 1). Both species induced a brown discoloration in the callus.

Twenty to forty percent of the *R. similis* inoculum infected the roots from rough lemon leaves within 10 days. Second- and a few third-stage larvae were observed at that time (Table 2). One generation was completed in 20 days, as previously reported (3). Early in the development of the second generation, males were more numerous than females, but the male:female ratio decreased with time. Maximum recovery from callus and roots of one leaf was 1,127 nematodes after 50 days. Fewer *P. coffeae* than *R. similis* were recovered from comparable rough lemon roots after 40 days (Table 2). Populations of *R. similis* and *P. coffeae* developed equally for the first 10 days after inoculation with both, but after that *R. similis* predominated up to 40 days. After 72 days, the *P. coffeae* populations were more than double the *R. similis* populations (Table 3).

Callus from *R. similis*-resistant rootstock (Algerian navel and Ridge Pineapple orange, and Milam lemon) showed the same degree of infection after 18 days as callus from susceptible rough lemon. The number of *R. similis* extracted after 30 days ranged from 48 to 105 in Algerian navel, 40 to 115 in Ridge Pineapple, and 55 to 315 in Milam, compared to 78 to 219 in susceptible rough lemon callus. *P. coffeae* did not reproduce as well as *R. similis* in these tissues.

These data indicate that citrus-leaf callus and roots may be satisfactory substrates for single progeny or mass rearing of *R. similis* and *P. coffeae*. The simple method for inducing callus and root development from citrus leaves, and the ability to grow these tissues in this laboratory for longer than 4 mo,

provided a useful way to study ecology and biology of migratory endoparasitic nematodes from citrus.

In these studies, the life cycles of *R. similis* and *P. coffeae* were completed in 20 days, which is similar to that reported in the greenhouse and field (3), but the two species showed different population dynamics. With both species, at the beginning of the second generation, males developed earlier and were more numerous than females. *R. similis* males did not penetrate the tissues in the absence of females. When females were added, males were found inside the tissues 2 days later, indicating that males penetrated through lesions made by females.

R. similis was expected to attack callus and root cultures of resistant rootstocks, since it takes at least 9 mo for populations to decline when these resistant rootstocks are grown in infested soil (8).

LITERATURE CITED

1. CARPENTER, J. B. 1961. Occurrence and inheritance of preformed root primordia in stems of citron (Citrus medica L.). Proc. Am. Soc. Hortic. Sci. 77:211-218.
2. DUCHARME, E. P., and R. W. HANKS. 1961. Gnotobiotic techniques and the study of Radopholus similis on citrus. Plant Dis. Rep. 45:742-744.
3. DUCHARME, E. P., and W. C. PRICE. 1966. Dynamics of multiplications of Radopholus similis. Nematologica 12:113-121.
4. FEDER, W. A., P. C. HUTCHINS, and R. WHIDDEN. 1962. Aseptic growth of Radopholus similis (Cobb) Thorne on okra root callus tissue. Proc. Fla. State Hortic. Soc. 75:74-76.
5. MYERS, R. F., W. A. FEDER, and P. C. HUTCHINS. 1965. The rearing of Radopholus similis (Cobb) Thorne on grapefruit, okra, and alfalfa root callus tissues. Proc. Helminthol. Soc. Wash. 32:94-95.
6. O'BANNON, J. H., and A. L. TAYLOR. 1968. Migratory endoparasitic nematodes reared on carrot discs. Phytopathology 58:385.
7. PLATT, R. G., and K. W. OPITZ. 1973. Propagation of citrus. Pages 1-47 *in* W. Reuther, L. D. Batchelor, and H. J. Webber, eds. The Citrus Industry. University of California Press, Berkeley.
8. POUCHER, C., H. W. FORD, R. F. SUIT, and E. P. DUCHARME. 1967. Burrowing nematode in citrus. Bull. Fla. Dep. Agric., Div. Plant Ind. 7, 63 p.
9. SALOMON, E., and K. MENDEL. 1965. Rooting of citrus leaf cuttings. Proc. Am. Soc. Hortic. Sci. 86:213-219.