The Pin Nematode, Paratylenchus neoamblycephalus, on Myrobalan Plum and Other Hosts[1]

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Abstract: Elimination of *Paratylenchus neoamblycephalus* from soil by fumigation with 1,2-dibromoethane stimulated the growth of Myrobalan seedlings grown in it. Addition of a suspension of *P. neoamblycephalus* to Myrobalan seedlings inhibited their growth as compared to noninoculated controls. When nematodes were removed from the suspension by settling, and the supernatant liquid was used as inoculum, no stunting occurred. Roots of Myrobalan seedlings inoculated with surface-sterilized *P. neoamblycephalus* were smaller, darker, and had fewer feeder roots than those of noninoculated controls. Nematodes were observed feeding ectoparasitically, but with heads embedded in roots as deep as the cortex. They were associated with small lesions and dead lateral roots. Clusters of nematodes were common at ruptures in the epidermis, and where lateral roots emerged. Limitation of Myrobalan growth by *P. neoamblycephalus* was greater at 20 and 27 C than at 30 C, and was not affected by pH over the range 4.5 to 6.5. Rose, apricot, peach, and all selections and hybrids of *Prunus cerasifera* tested were hosts for *P. neoamblycephalus*. The nematode could not be cultured on various herbaceous plants nor on Myrobalan callus tissue. *Key Words:* pathogenicity, temperature, hydrogen-ion, feeding sites, culture, host-range, rose, apricot, peach.

Pin nematodes, *Paratylenchus* spp., are the most commonly occurring plant-parasitic nematodes in California prune orchards (15). They were found in 65 of 97 orchards sampled. *P. neoamblycephalus* Geraert, the species most often encountered, was found in all four of the important prune growing districts in the state. Although this species was described recently (7), it has been reported from Europe, Africa, North and South America, and Australia around apple, cherry, apricot, peach, grape, and some herbaceous plants (6, 7, 23, 24, 27).

We investigated the effects of *P. neoamblycephalus* on plum, *Prunus cerasifera* Ehrh. 'Myrobalan', which is used as a rootstock for prunes. Effects of soil temperature and soil pH on this nematode and its host were also investigated. Various woody and herbaceous plants were tested as hosts for this nematode.

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MATERIALS AND METHODS

General: Dormancy of Myrobalan seeds (obtained from Herbst Bros., Brewster, N.Y.) was broken by incubation for 3-4 mo at 2.2 C in a shallow layer of distilled water containing

10 μg/ml thiram (75% by wt tetramethylthiuramdisulfide). These seeds were planted and maintained for experimental use in a mixture containing equal parts by volume of peat moss, vermiculite, and sand.

P. neoamblycephalus was obtained from a prune orchard in the Napa Valley at Oakville, California. Colonies for experimental use were obtained by hand-picking specimens and increasing them on Myrobalan seedlings. Jenkins' (10) sugar-flotation method was used to obtain nematode inoculum. Inoculations were made by pouring nematode suspensions on the roots at time of planting.

Unless otherwise indicated, the soil used was a sandy loam (2) with field capacity 5.8%, porosity 50.9% and pH 6.5. It was autoclaved for 4 h at 118 C at 1.0 atmosphere pressure (15 psi) and aired in wooden boxes at least 1 mo to regain chemical stability. Experimental containers were 15-cm clay or plastic pots with a volume of 1,200 ml. Pots were sunk in wood shavings (13) or in a temperature controlled box (14) or water bath (5) to keep soil temperature from reaching levels too high for the nematodes.

Plants were irrigated with distilled water and fertilized regularly with either half-strength Hoagland's solution (9), or a solution of Ortho-Gro® (Chevron Chemical Co., San Francisco, Calif.) liquid plant food (12% N in the form of urea, 6% P, 6% K, and 4% ethylenediaminetetraacetic acid). Mites and white flies were controlled with a mixture containing 15 ml malathion (33% by wt 0,0-dimethyldithiophosphate of diethyl mercaptosuccinate) and 0.8 g of miticide decachlorobis [50% bis-penta-chloro-2,4-cyclopentadiene-1-yl (PENTAC, Hooker Chemical Co., Niagara Falls, N.Y. 14302) per liter of water.

At harvest, roots were carefully freed from soil, washed, and dried with paper towels and weighed. Only in the soil fumigation experiment were roots oven-dried at 105 C for 24 h before weighing. Soil from each pot was hand-mixed for homogeneity, and nematodes were recovered from a 50-cc aliquot under a heated, intermittent mist (16) unless otherwise indicated.

Effects on Myrobalan plum: To test association of *P. neoamblycephalus* with disease, soil infested with this plant-parasitic nematode species only was mixed for uniformity. One-half of this soil was treated with an EDB fumigant (83% by wt 1,2-dibromoethane) in closed containers (11) at a rate of 16 μl/liter, the experimentally determined lowest rate required to eliminate *P. neoamblycephalus*. The other half was not treated. Plants were weighed and nematode populations determined after 5 mo of growth in the treated and nontreated soils.

To test its involvement (17) in disease, *P. neoamblycephalus* was added to Myrobalan seedlings in a logarithmic series of numbers (Table 1). Nematodes were removed from a portion of the suspension containing the highest concentration of nematodes by settling the suspension 8 h at 5 C and removing any remaining specimens by hand-picking. This nematode-free material was used to test the effect of microorganisms accompanying the nematodes in the suspension.

To further test the role of this nematode, approximately 2,000 nematodes, surface-sterilized by the method of Moody et al. (22) were added to Myrobalan seedlings grown from seeds surface-sterilized using the method of Hildebrandt et al. (8). The efficacy of the surface-sterilization procedures was checked using N.I.H. thioglycollate broth (Difco Laboratories, Detroit, Mich.). Germinated seeds were grown 3 wk in steam-sterilized sand before use in this experiment. After 5

TABLE 1. Growth of Myrobalan plum seedlings 4 mo after inoculation with *Paratylenchus neoamblycephalus*, supernatant water from nematode inoculum, or distilled water.

Treatment	Final no. nematodes/pot[z] (1,000's)	Height (cm)[z]	Fresh weight (g)[z]	
			Top	Root
1. Distilled water	0	68.8 a	10.4 a	15.4 a
2. 100 nematodes	150.1 a	44.8 b	6.3 b	11.0 b
3. 1,000 nematodes	122.0 ab	43.0 b	5.0 b	10.7 b
4. 10,000 nematodes	105.6 b	39.8 b	4.7 b	8.7 b
5. Supernatant from treatment 4	0	72.0 a	11.2 a	16.4 a

[z]Average of six replications ± standard error. Averages followed by the same letter do not differ significantly, $P = <$ 0.01 by Duncan's multiple range test.

TABLE 2. Number of *Paratylenchus neoamblycephalus* after growth of various plants in soil from an Oakville, California prune orchard.

Plant	Final no. of nematodes (1,000's)	
	Per pot[x]	Per g of root[x]
Test 1[y]		
Peach, *Prunus persica* 'Lovell'	119.6 a	12.9 a
Plum, *Prunus cerasifera* 'Myrobalan 3 J'	27.9 b	3.0 b
Pea, *Pisum sativum* 'Early Perfection'	0 c	0 c
Sweet pea, *Lathyrus odoratus*	0 c	0 c
Bean, *Phaseolus vulgaris* var. *humilis* 'Stringless Green Pod'	0 c	0 c
Tomato, *Lycopersicon esculentum* 'Rutgers'	0 c	0 c
Sunflower, *Helianthus annuus*	0 c	0 c
Cantaloupe, *Cucumis melo* 'PMR 45'	0 c	0 c
Barley, *Hordeum vulgare* 'California Mariout'	0 c	0 c
Oats, *Avena sativa* 'California Red'	0 c	0 c
Carrot, *Daucus carota* 'Imperator'	0 c	0 c
Test 2[z]		
Rose, *Rosa multiflora*	70.7 a	.8 a
Plum, *Prunus cerasifera* × *P. munsoniana* 'Marianna 2624'	43.4 b	.4 b
Apricot, *Prunus armeniaca* 'Blenheim'	18.6 c	.3 b
Fig, *Ficus carica* 'Kadota'	.8 d	.01 c
Walnut, *Juglans hindsii* 'Northern California Black'	.4 d	.01 c
Cowpea, *Vigna sinensis*	.1 d	.01 c
Fallow	.3 d	

[x]Averages and standard errors, in test 1 of two replicates, and in test 2 of six replicates. Averages followed by the same letter do not differ, $P = < 0.01$, by Duncan's multiple range test.
[y]Plants grown 14 wk. Original infestation, 6,960 ± 500 *P. neoamblycephalus* per pot.
[z]Plants grown 4 mo. Original infestation, 8,190 ± 165 *P. neoamblycephalus* per pot.

TABLE 3. Number of *Paratylenchus neoamblycephalus* after growth of various plants in sterilized soil to which this nematode was added.

Test and plant identification	Final no. nematodes/g fresh root (1,000's)
Test 3[x]	
Plum, *Prunus cerasifera* × *P. munsoniana* 'Cody Marianna'	37.0 a
Plum, *Prunus cerasifera* 'Myrobalan'	24.6 b
Plum, *Prunus cerasifera* var. *atropurpurea* '*Prunus moseri*'	16.8 bc
Plum, *Prunus cerasifera* 'Myrobalan 3 J'	15.5 bcd
Plum, *Prunus cerasifera* hybrid 'Corotto Marianna'	12.0 cde
Plum, *Prunus cerasifera* 'Myrobalan 29 C'	8.8 cde
Plum, *Prunus cerasifera* × *P. munsoniana* 'Marianna 2624'	8.5 cde
Plum, *Prunus cerasifera* × *P. munsoniana* 'Marianna 2623'	6.2 cde
Plum, *Prunus cerasifera* × *P. munsoniana* 'Marianna 4001'	4.2 cde
Plum, *Prunus cerasifera* × *P. munsoniana* 'Marianna F'	2.3 e
Carnation, *Dianthus caryophyllus* 'Scania 3 C'	0 f
Test 4[y]	
Apple, *Malus sylvestris* 'Bittenfelder'	0 a
Plum, *Prunus cerasifera* 'Myrobalan'	11.8 b

[x]Averages and standard errors, in test 3 of three replicates, and in test 4 of six replicates. Averages followed by the same letter do not differ, $P = < 0.01$, by Duncan's multiple range test.
[y]Plants grown 6 mo after inoculation with 1,400 *P. neoamblycephalus* per pot.
[z]Plants grown 4 mo after inoculation with 1,000 *P. neoamblycephalus* per pot.

mo, plant growth and nematode populations were assayed and an attempt was made to isolate fungi from the roots by dipping them in 1% sodium hypochlorite solution for 2 min, rinsing twice in sterile water, and inserting them into either solidified corn meal agar or modified PV medium (21), selective for *Pythium* and *Phytophthora*.

Feeding sites of *P. neoamblycephalus* were observed after killing nematodes in situ on the roots. Four months after inoculation with 1,000 nematodes, the roots and soil ball were removed from pots and immersed in a water bath at 90 C for 1 min. Much of the soil fell into the bath; more was removed by a subsequent dip in cold water. Then the roots

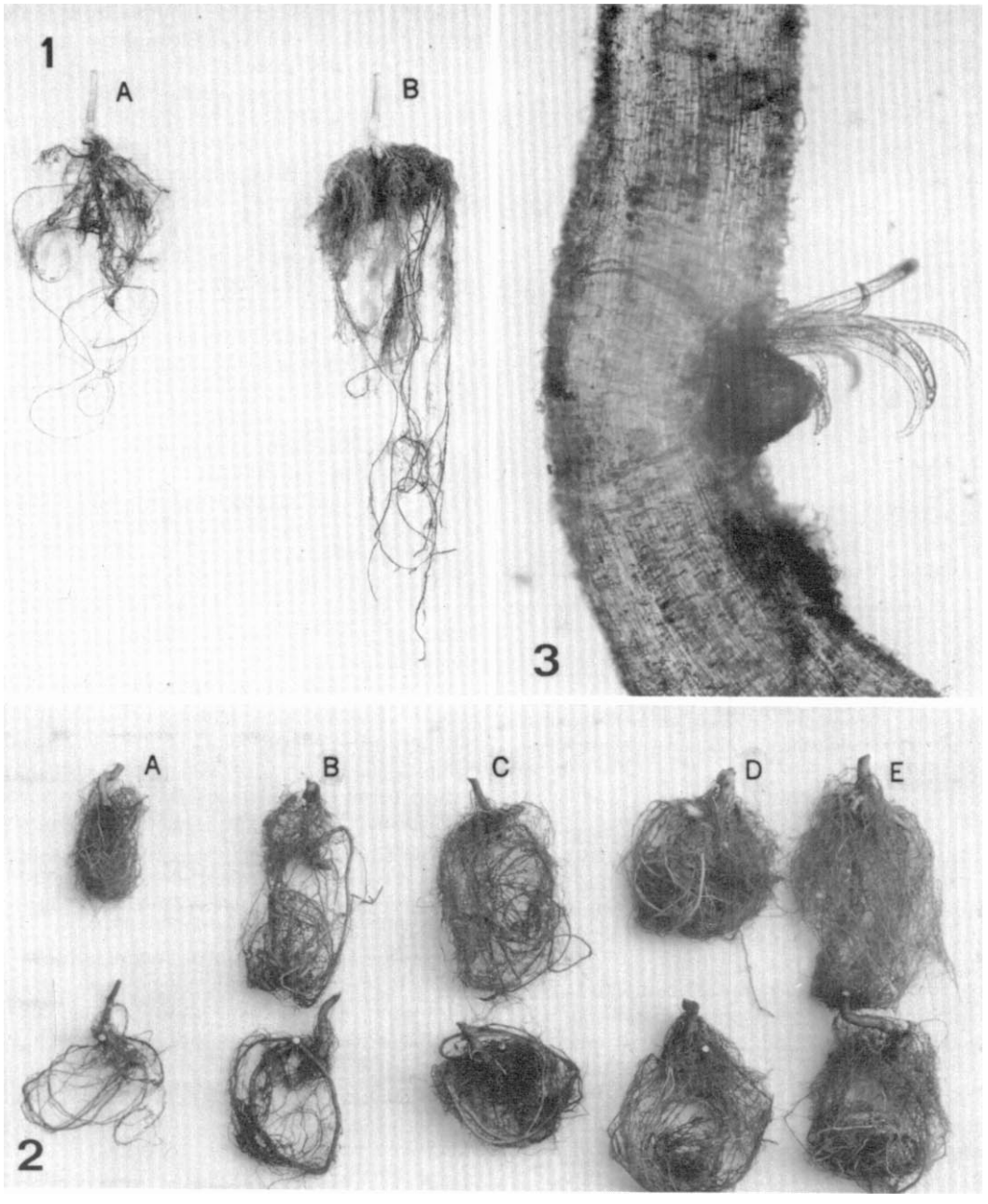

FIG. 1-3. **1-(A, B)**. Roots of Myrobalan plum seedlings grown 5 mo in soil infested with *Paratylenchus neoamblycephalus* and **A)** nontreated, or **B)** treated with an EDB fumigant (83% by wt 1,2-dibromoethane) at the rate of 16 μl/liter. **2-(A to E)** Roots of Myrobalan plum seedlings grown 4 mo after inoculation with **A)** 10,000, **B)** 1000, or **C)** 100 *P. neoamblycephalus*, **D)** supernatant liquid after settling out the 10,000 *P. neoamblycephalus* from that inoculum level, or **E)** distilled water. **3)** *P. neoamblycephalus* associated with the death of a young lateral root.

were immersed in hot lactophenol-acid fuchsin solution for 1 min and cleared in lactophenol (19). Sections of roots were removed with a razor blade and examined microscopically.

Factors affecting nematode pathogenicity: The effects of temperature on the nematode and its pathogenicity were tested by growing nematode-inoculated and uninoculated Myrobalan seedlings at 15, 20, 27, and 30 C for 4 mo. Water-tight ceramic crocks, containing the plants, were placed in water

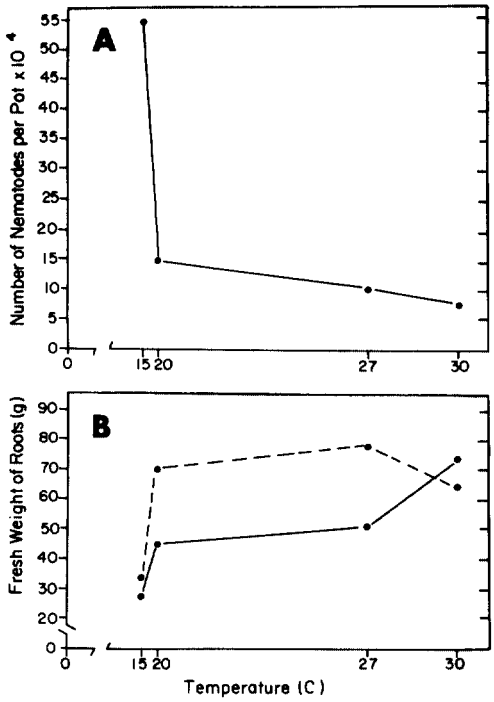

FIG. 4-(A, B). A) Numbers of *Paratylenchus neoamblycephalus* and B) fresh weights of roots of Myrobalan plum resulting from 4 mo growth of seedlings at three temperatures after inoculation with zero (● ---●) and 1,400 (● - ●) nematodes.

baths at the desired temperature (5). Six replicates were arranged in a randomized complete block design. Results in terms of root weight and nematode numbers, were subjected to analysis of variance. Cobb's (3) method was used to recover nematodes in this experiment. Some Myrobalan seedlings died at 15 C. These were replanted, and the replants allowed to grow 4 mo. Because the 15 C planting was not synchronous, data from it were analyzed separately, using Student's *t*-test. For easy comparison, all results are presented together (Fig. 4).

The effects of hydrogen-ion concentration were determined by growing Myrobalan seedlings at three levels of soil pH, and inoculating them at planting time with 7,928 ± 352 *P. neoamblycephalus,* or leaving them uninoculated. The six replicates of the six treatments were arranged in a Latin square design in a controlled temperature box (14). Three parts sterilized clay loam (2) and one part sand were mixed in a cement mixer producing a mixture whose pH was 6.5. Additional pH levels (5.5 and 4.5) were obtained by application, using a household sprayer, of 1 N H_2SO_4, followed by mixing. Treated soil was stored 2 wk before use. The pH of the pH 6.5 series increased slightly with time, and was adjusted by addition of small amounts of 1 N H_2SO_4. The other two pH levels required no adjustment. No fertilizer amendments were made during the experiment. After 4 mo, plants were weighed, numbers of *P. neoamblycephalus* determined, and pH values measured.

Nematode host range and culture: Various plants (Tables 2 and 3) were tested as hosts for *P. neoamblycephalus* in four experiments. For the first two experiments an Oakville, California, prune orchard soil was used in which *P. neoamblycephalus* was the predominant plant-parasitic nematode species. Soil was mixed for uniformity before planting, and numbers of *P. neoamblycephalus* were determined at planting and at harvest time. In host range tests 3 and 4, the numbers of nematodes indicated in Table 3 were added at planting to sterilized soil.

In an attempt at gnotobiotic culture, *P. neoamblycephalus,* surface-sterilized using the method of Moody et al. (22), was added to callus tissue cultures of Myrobalan plum. To obtain these callus tissues, Myrobalan seeds and shoots were surface-sterilized using the method of Hildebrandt et al. (8). Then callus tissue was grown from sections of cotyledons and shoots placed in test tubes containing Bradley and Dahmen's (1) medium. Fifty surface-sterilized nematodes were added to each of 24 cultures. Twelve cultures, six of cotyledon, and six of shoot callus, were incubated in darkness at 15 C, and 12 at 20 C. After 3 mo, culture contents were incubated under a heated, intermittent mist (16) for 2 wk, and the number of *P. neoamblycephalus* recovered were counted.

RESULTS AND DISCUSSION

Effects on Myrobalan plum: Elimination of *P. neoamblycephalus* from the soil with EDB stimulated ($P = < 0.01$) the growth of Myrobalan seedlings (Fig. 1). After 5 mo of growth, the dry weight of seedlings in treated soil was 19 ± 1 g, and in nontreated soil 13 ± 0.5 g. Soil fumigants have chemical effects on soils (18, 25) which are the result of their action on microorganisms other than

nematodes, but EDB has fewer such effects than most fumigants. At the dosage used here, its most important effect is the nematicidal one.

Addition of *P. neoamblycephalus* to Myrobalan seedlings at time of transplanting resulted in smaller ($P = < 0.01$) plants (Table 1 and Fig. 2) after 4 mo of growth. Stunting was not proportional to the nematode inoculum level. The inverse relation of final to initial nematode population levels suggests an early limitation of root growth at higher nematode levels. The supernatant liquid from the highest level of nematode inoculum did not cause stunting. However, removal of nematodes by settling at 5 C provided a less than perfect control for the effect of organisms accompanying the nematodes, since some bacteria may have settled out with the nematodes. Many would not have settled. Inoculum was obtained by the sugar-flotation method (10) and was essentially free of fungal spores and mycelium as determined by microscopic examination. *Paratylenchus* larvae are too small to be effectively separated from other particles by sieving a suspension. The results of this experiment support the hypothesis that *P. neoamblycephalus* is involved (17) in the stunting which was observed. The magnitude of stunting does not appear diminished after surface-sterilization. Plants inoculated with surface-sterilized nematodes weighed 6.8 ± 0.7 g and their roots weighed 5.2 ± 0.5 g. For paired, noninoculated controls these weights were 18.8 ± 5.8 and 13.0 ± 3.2 g, respectively. Nematode-infected roots were darker, and had fewer feeder roots than noninoculated controls. Neither *Pythium* nor *Phytophthora* was isolated from Myrobalan roots from this experiment on the selective medium. A *Fusarium* sp. and an *Alternaria* sp. were isolated from roots of nematode-inoculated and noninoculated plants. These are believed to be air-borne contaminants of no importance. The thioglycollate broth test indicated that the surface-sterilized nematodes did not carry contaminants.

When acid-fuchsin stained Myrobalan roots were examined, nematodes were found feeding ectoparasitically, either isolated or in groups of 5-15, with their heads in the cortex. No preference for a particular region of the root was noted, but group feeding occurred at ruptures or where lateral roots emerged (Fig. 3). A similar feeding habit was observed by Linford et al. (12) for *P. minutus* on pineapple roots. Death of lateral roots and small lesions, probably caused by oxidation of phenolic compounds in the root, were associated with feeding. The nematodes observed were predominantly second and third-stage larvae. Eggs were found on the root surface. Males and fourth-stage larvae do not possess a stylet.

Factors affecting nematode pathogenicity: The best temperature of those tested for increase of *P. neoamblycephalus* was 15 C (Fig. 4), and the best temperature for growth of noninfested Myrobalan plants was 27 C. Limitation of Myrobalan growth by *P. neoamblycephalus* was greatest at 20 and 27, and least at 30 C. Fisher (6) found that Australian *P. neoamblycephalus* also had a relatively low optimum temperature (20 C) for increase.

Lowering pH by acid amendment from 6.5 to 4.5 inhibited ($P = < 0.05$) the reproduction of *P. neoamblycephalus*. The numbers of nematodes per gram of root were 15,688 ± 5,166 at pH 6.5 and 2,612 ± 527 at pH 4.5. At pH 5.5 this number was 7,034 ± 2,505, which did not differ significantly from the other two pH levels. Nematode pathogenicity was not altered by pH (i.e. the interaction between nematode effect and pH effect on plant weight was not significant). When data for all pH levels were pooled, inoculation with approximately 8,000 *P. neoamblycephalus* resulted in a final plant weight after 4 mo of 46 ± 2 g, compared to 68 ± 3 for controls ($P = < 0.01$). Plant height was linearly correlated ($P = < 0.01$) with pH for both non-infested controls ($r = 0.61$) and nematode-inoculated plants ($r = 0.77$). For pH vs. leaf nitrogen, r for the same treatments was 0.64 and 0.61 ($P = < 0.01$), respectively. Root weight was not affected significantly by pH. Infection by *P. neoamblycephalus* did not result in lowered leaf nitrogen in this test. The lower number of nematodes with low pH is probably a result of acid inhibition of nitrification resulting in some accumulation of ammonia, toxic to nematodes (4), rather than a direct effect of low pH on the nematodes. This is consistent with the observed nitrogen deficiency effect in leaves. Ammonia is a poor source of nitrogen for plants at low pH (20).

Nematode host range and culture: Rose, apricot, peach, and all *Prunus cerasifera* selections and hybrids were good hosts for *P. neoamblycephalus* from Oakville, California, (Tables 2 and 3). The two rootstocks used

most commonly for prunes in California, cultivars Myrobalan 29C and Marianna 2624 are included among these good hosts. Apricot is also reported to be a host for *P. neoamblycephalus* in Australia (6) as is apple; and the species is also found around cherry, peach, and grape in the field. *P. neoamblycephalus* occurs around apples and grape in Western Europe (7) and around grape in Algeria and California (7, 23). None of the herbaceous plants tested were hosts for this nematode, and some trees (e.g. walnut and fig) were either non-hosts or very poor hosts. The only inconsistency between the results reported here and those reported previously is the reaction of apple, which apparently is a host for *P. neoamblycephalus* in Western Europe and Australia (6, 7), but does not seem to be a host for the Napa Valley, California, population. This may indicate the existence of races of this species.

P. neoamblycephalus did not multiply on callus tissues cultured from shoot and cotyledon tissue of Myrobalan plum, although callus tissue was formed. Surface-sterilization of the nematode apparently was not the reason for lack of reproduction in culture since reproduction was not altered in another test.

After 3 mo, 15 ± 4 nematodes were recovered from the cultures grown at 15 C, and 8 ± 2 from the cultures grown at 20 C. The 20 C temperature was more favorable than 15 C for culture of the callus tissues. Reasons for failure of the method are not known. Success at culture of ectoparasitic plant nematodes such as reported by Upadhyay and Swarup (26) recently, is exceptional.

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