Post-Infection Development and Histopathology of *Meloidogyne incognita* in Resistant Cotton[1]

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Abstract: The numbers of *Meloidogyne incognita* larvae which migrated from cotton roots declined over a 16-day period, but the difference in numbers migrating from resistant and susceptible cultivars was not significant. Larvae penetrated susceptible roots, matured, and reproduced within 14 days following inoculation, whereas nematode development in the resistant roots was greatly retarded. Three types of histological responses were observed in infected, resistant roots, and these correlated with the degree of nematode development. Some galls were examined which contained only fragments of nematodes; others contained no detectable traces of developing larvae. Formation of druses in galls, but not in healthy tissue, was noted in both cultivars 20 days after inoculation. Massive invasion of roots resulted in deep longitudinal fissures of root cortex. *Key Words: Gossypium hirsutum,* larval egression, larval degradation, syncytium formation.

Resistance in cotton (*Gossypium hirsutum* L. and *G. barbadense* L.) to the root-knot nematode, *Meloidogyne incognita* (Kofoid & White) Chitwood has been the subject of several investigations (2, 9, 13). In certain cultivars, resistance has been associated with increased root necrosis (13), failure of the nematodes to mature (2, 13), reduced fecundity of female nematodes (9), and failure of the host to develop nematode-induced galls and syncytia (2). The numbers of nematodes in the roots are known to decline following infection in at least three cultivars (13). In a previous study (12) we showed that a resistant cultivar, 'Clevewilt-6-3-5' (Clevewilt) developed fewer galls and supported fewer egg-laying females than did a susceptible cultivar, 'Deltapine Smooth Leaf' (Deltapine). This phenomenon was not a function of differential attraction to host roots or the ability of the nematodes to penetrate the resistant cultivar. Furthermore, resistant plants were characterized by the occurrence of galls which, when examined, contained no nematodes. Two explanations for these observations seemed likely: nematodes entered roots of Clevewilt but later egressed, or nematodes which entered the roots failed to become established and subsequently died and decomposed beyond recognition. Resistance of the first type is not known to occur in cotton but has been demonstrated in certain cultivars of alfalfa resistant to *M. incognita* (15). Lysis or disintegration of root-knot nematodes was reported by Radewald (14) in resistant sweet potato but Brodie et al. (2) found no evidence of nematode disintegration in 'Auburn 56', a moderately resistant cotton cultivar. Endo (5) described deterioration of second-stage larvae of *Heterodera glycines* which had penetrated roots of resistant soybeans.

The purpose of the present investigation was to follow, chronologically, the development of *M. incognita* and the histopathology of both susceptible and resistant roots and to determine whether or not reduced gall formation and the occurrence of "empty" galls could be attributed to larval egression.

MATERIALS AND METHODS

Meloidogyne incognita from a single egg mass isolation (12) was propagated in the greenhouse on chili pepper (*Capsicum frutescens* L.) and second-stage larvae were obtained by Lownsbery and Viglierchio's method (10). Post-infection development studies were conducted in an environmental chamber at 25 C. Light was provided for 12 h per day at an intensity of 20,000 lux. Cotton seeds were germinated on moist filter paper for 72 h at 25 C in the dark. Three seedlings were transferred to each plastic petri dish (100 × 15 mm) to which were added 100 g of 60-mesh quartz sand and 25 ml of distilled water containing 450 freshly hatched second-stage larvae. Lids of the petri dishes were notched to allow the stems to extend beyond the rims of the dishes. Seedlings were floated free of the sand 48 h after inoculation and rinsed in running tap water to remove adhering larvae

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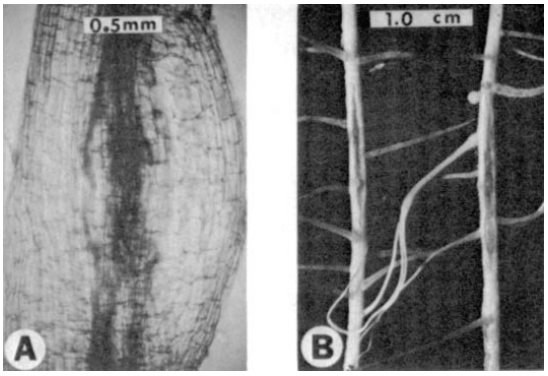

FIG. 1-(A, B). Cotton roots showing symptoms of infection by *Meloidogyne incognita*. **A.** "empty" gall on resistant 'Clevewilt' 16 days after inoculation. **B.** longitudinal splits in taproots of susceptible 'Deltapine' 8 days after inoculation.

which had not penetrated. Each seedling was transplanted to a 10-cm plastic pot containing (20-mesh) quartz sand and watered daily with Hoagland's solution. Roots of three seedlings of each cultivar were harvested at 48-h intervals, pooled, washed, and stained with acid fuchsin (11). All detectable nematodes were removed from the roots by dissection and their developmental stages were classified according to Triantaphyllou and Hirschmann (16) except that no attempt was made to distinguish between molting larval instars. In Clevewilt, a number of galls were dissected in which no nematodes could be detected (Fig. 1-A). The numbers of such galls were recorded.

Post-infection migration of larvae from roots of cotton was observed in plants grown in nutrient solution. Seedlings, inoculated as above, were removed from the petri dishes 48 h after inoculation, and any nematodes which had not penetrated were removed by washing under a jet of tap water. Single seedlings then were transferred to 250-ml Erlenmeyer flasks containing Hoagland's solution aerated with a continual flow of humidified air. Seedlings were supported in the necks of the flasks by cork stoppers split longitudinally and with a central groove four times larger than the diam of the stems. A small amount of glass wool packed loosely between the stems and the cork maintained the plants vertically yet permitted unrestricted radial growth. Each flask was wrapped in aluminum foil to exclude light and incubated at room temp under a bank of fluorescent lights with a photoperiod of 12 h. Nutrient solution was collected at intervals of 96 h, passed through a 0.45-μm (pore size) filter, and the nematodes retained on the filter resuspended in 10 ml H_2O and counted. Twelve seedlings (replicates) of each cultivar were examined and the experiment, which was conducted once, terminated 16 days after inoculation.

Roots of both cultivars were examined histopathologically at regular intervals following infection. Seedlings, healthy and infected as above, were transferred to pots (15-cm diam) of sterile soil and placed in growth chambers with temp of 29 C for day (15 h) and 24 C for night (9 h). Eight days after inoculation, and at 4-day intervals thereafter for 24 days, two plants of each treatment were harvested and the roots fixed in FAA (Formalin, 5 ml: ethanol, 20 ml: glacial acetic acid, 1.0 ml: H_2O, 40 ml). Selected portions of these roots were embedded in paraffin, sectioned at 12 μm and stained with safranin and fast green. In a separate experiment, infected roots also were examined 48 h after inoculation. These roots were inoculated by placing the terminal 2 cm of each root between strips of Miracloth® (7) and applying suspensions of freshly hatched larvae in two or three drops of distilled water. At harvest, the roots were washed, fixed, and processed for histological examination as described above.

RESULTS

Total numbers of nematodes recovered from roots of resistant Clevewilt decreased markedly following penetration and was paralleled by an increase in the number of "empty" galls (Table 1, Fig. 1-A). Twenty days after inoculation only 15% of those initially present could be found. In contrast, the numbers recovered from the susceptible Deltapine declined the first 6 days and then remained relatively constant for the next 2 wk. No empty galls were observed. The majority (65%) of those nematodes which remained in the roots of Clevewilt 20 days after inoculation had not developed past the larval stages. Adult female nematodes began to appear 8 days after inoculation in Deltapine and by the 16th day and thereafter, 90% of the developing nematodes were adults. In terms of total numbers of egg masses, reproduction of *M. incognita* on Clevewilt was considerably less than on Deltapine. This may have been due partly to delayed development and partly to reduced fecundity of the mature females.

TABLE 1. Numbers of empty galls and relative numbers of developmental stages of *Meloidogyne incognita* recovered from roots of resistant Clevewilt (CW) and susceptible Deltapine (DP) cotton. Column values are means of three replications.

Post-inoculation (days)	Nematodes recovered (total no.)		Population composition: Infective larvae (%)		Developing larvae (%)		Adults (%)		Egg masses (no.)		Empty galls (no.)	
	CW[a]	DP[a]	CW	DP	CW	DP	CW	DP	CW	DP	CW	DP
4	53	79	100	94	0	6	0	0	0	0	0	0
6	31	42	91	9	9	91	0	0	0	0	0	0
8	23	41	67	0	33	95	0	5	0	0	4	0
10	9	42	43	0	53	42	4	58	0	0	6	0
12	23	50	37	3	56	33	7	64	0	0	15	0
14	19	49	27	0	49	17	24	83	0	6	15	0
16	17	49	30	0	50	10	20	90	1	23	8	0
18	15	43	13	0	55	8	32	92	1	20	10	0
20	8	43	0	0	65	7	35	93	1	20	18	0

[a]CW = Clevewilt (which is Clevewilt-3-6-5, a breeding line); and DP = Deltapine (which is cultivar 'Deltapine Smooth Leaf').

An average of 45% of those nematodes reaching maturity between 18 and 20 days were laying eggs in Deltapine whereas only 28% were fecund in Clevewilt.

Reduced numbers of nematodes recovered from Clevewilt were not the result of migration of larvae from infected roots. Apparently a few nematodes which failed to become established were capable of egression but over a 16-day period the numbers leaving Clevewilt (4.2) were not significantly different ($P = 0.05$) from those leaving Deltapine (4,7). No nematodes were recovered from the nutrient solution 16 or more days after inoculation.

Host reactions to nematodes which penetrated susceptible Deltapine were comparable to those previously described for susceptible cotton cultivars (13) and other suitable hosts (6). However, when large numbers of larvae entered small sections of roots (100 larvae/cm), large cracks appeared which penetrated to the inner cortex (Fig. 1-B). This heretofore undescribed symptom occurred in both the susceptible and resistant cultivars. Initiation of syncytia in Deltapine could be detected as early as 48 h after inoculation (Fig. 2-A). By this time, most of the nematodes had migrated to the stele and were found lying parallel to it. Cells immediately adjacent to the head of the parasites had undergone karyokinesis without cytokinesis and the nuclei were enlarged and contained prominent nucleoli (Fig. 2-B). Eight days after inoculation, the syncytia were easily recognizable as typical multinucleate "giant cells" with dense granular cytoplasm and walls 2-4 times thicker than those of adjacent parenchyma cells (Fig. 2-C). Secondary wall thickenings began to form by the 12th day (Fig. 2-E) and in syncytia supporting mature females, these thickenings sometimes occupied a major portion of the cross-sectional area of individual cells (Fig. 2-G). By this time, 64% of the female nematodes had completed the final molt and by the 14th day after inoculation, egg masses had begun to appear. With the commencement of egg-laying, giant cells began to senesce, the cytoplasm lost its densely granular form and appeared loosely clumped; the nuclear envelope became less distinct. This condition became pronounced by the 20th day and was accompanied by development of granular, knob-like extensions of the secondary thickenings of the syncytial cell wall (Fig. 2-G).

Most larvae which penetrated the resistant cultivar, Clevewilt, failed to elicit the nuclear divisions found in infections of Deltapine. Nuclear and nucleolar hypertrophy and hyperplasia could be found; but 48 h after infection, the percentage of larvae which had evoked this reaction in Clevewilt was considerably less than in Deltapine. By the 8th day after infection, three types of host response could be distinguished in resistant Clevewilt: (i) Those larvae which had failed earlier to stimulate nuclear activity in the host were lying closely appressed and parallel to

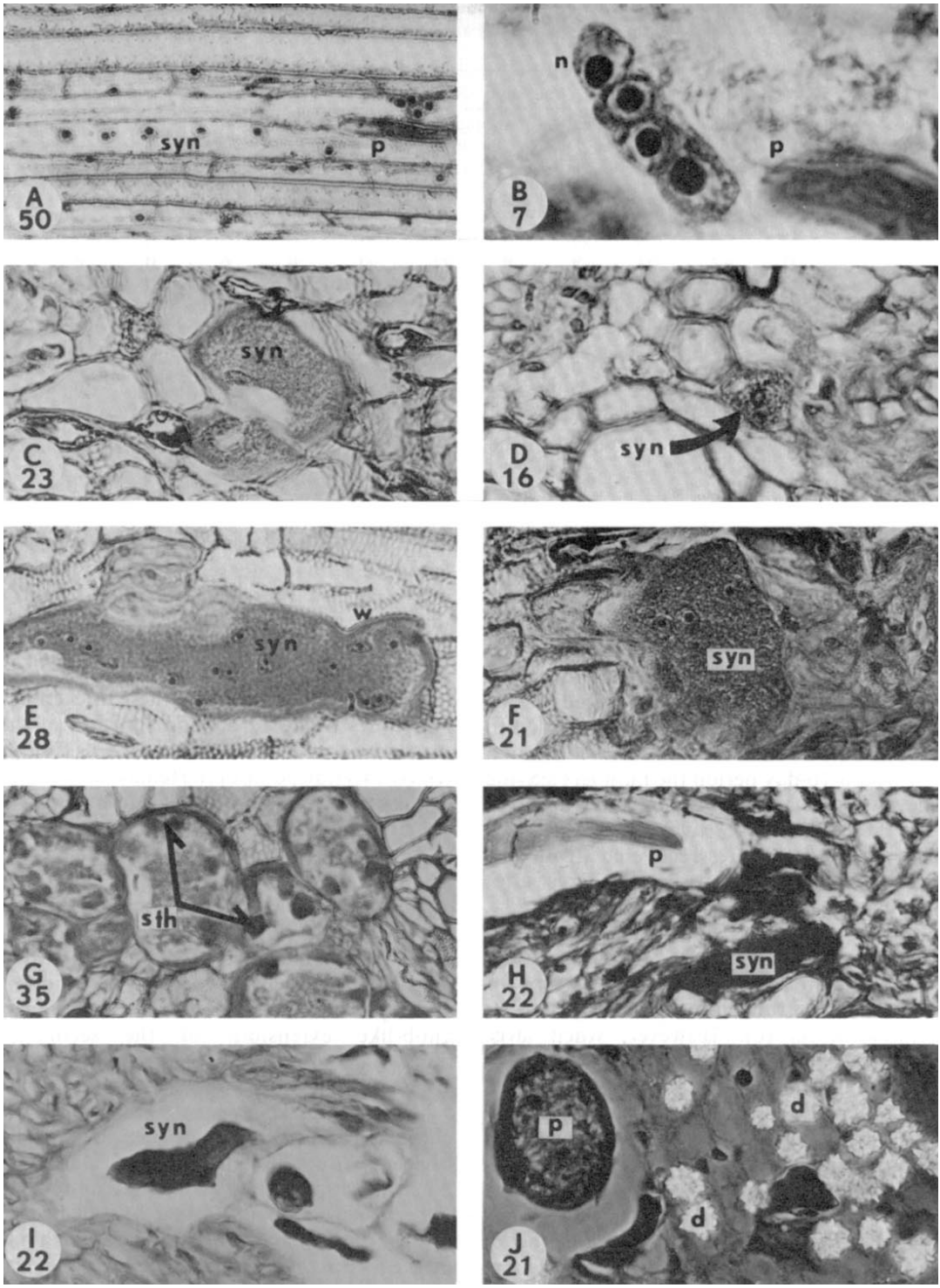

FIG. 2-(A to J). Postinoculation histopathology of susceptible 'Deltapine' and resistant 'Clevewilt' cotton roots infected with *Meloidogyne incognita*. Numbers indicate the relative diam (μm) of the white circles in which the letters and numbers appear. **A, B.** Deltapine, 48 h. **C.** Deltapine, 8 days. **D.** Clevewilt, 8 days. **E.** Deltapine, 12 days. **F.** Clevewilt, 12 days. **G.** Deltapine, 20 days. **H.** Clevewilt, 20 days. **I.** Clevewilt, 20 days; section through "empty" gall. **J.** Clevewilt, 20 days. d = intracellular druses, n = nucleus, p = nematode, syn = syncytium, sth = secondary thickening of syncytial cell wall, w = syncytial cell wall.

the stele. No cellular disturbances of the host, other than mechanical injury, occurred with these larvae, nor was there any evidence of the type of necrosis usually associated with a hypersensitive reaction. (ii) Other larvae had stimulated the initiation of syncytia (Fig. 2-D) but unlike the syncytia of Deltapine the nuclear abberations were never extensive, and the syncytial cell walls failed to develop secondary thickenings (Fig. 2-F). This type of syncytium did not support ovipositing female nematodes, and by the 20th day after inoculation it usually had disintegrated (Fig. 2-H). Sections through "empty" galls (Fig. 2-I) showed similar senescent syncytia which, upon collapse, often left large cavities in the root. Occasionally, objects interpreted as remnants of nematodes were found associated with the collapsed giant cells in the central portions of the galls. (iii) A few nematodes reached maturity and deposited eggs in a gelatinous matrix. The syncytia induced by these nematodes were indistinguishable from those produced in Deltapine and exhibited all the morphological characteristics normally associated with giant cells.

Numerous multifaceted crystals (druses) were found in galled roots of both cultivars approximately 20 days after inoculation (Fig. 2-J). These crystals were observed only in infected tissue, usually in the vascular parenchyma adjacent to the posterior end of the nematode. A few scattered crystals sometimes were observed near, but never within, the syncytia.

DISCUSSION

In contrast to earlier reports of root-knot nematode infections in resistant cotton cultivars (2, 13), resistance in *G. hirsutum* Clevewilt was not associated with increased root necrosis. Following massive invasion, longitudinal splits, which ultimately darkened upon exposure to air, appeared along the main axis of the taproot. However, this reaction occurred in resistant and susceptible cultivars alike and appeared to play no role in the mechanism of resistance. Evidence also was lacking for a hypersensitive necrotic reaction found in certain other resistant hosts invaded by *Meloidogyne* spp. (4). Dropkin (3) suggested that the nematode and the root exchange a "signal" following infection and that the type of host response is a result of this exchange which must occur very soon after the infective process begins. Cytological manifestations of resistance in Clevewilt were observed as early as 48 h after nematode penetration. Apparently, in this cultivar, many infective larvae are unable to influence host-cell nuclear division and growth. In the susceptible cultivar, Deltapine, nuclear abberations were associated with every larva which reached its feeding station.

The origin of the multinucleate giant cells is, at present, a matter of controversy. Many investigators (6) have cited the formation of multinucleate syncytia through dissolution of cell walls of adjacent cells and coalescence of their contents. However, Huang and Maggenti (8) in an ultrastructural study of galls induced by *M. javanica* found no evidence of cell wall dissolution and they concluded that mitosis without cytokinesis was responsible for the multinucleate condition of the syncytium. Although we were unable to confirm cell wall dissolution, the formation of linear tetrads of nuclei (Fig. 2-B) suggests that these nuclei do indeed arise from mitoses rather than protoplasmic amalgamation. However, within 48 h of penetration (Fig. 2-A), developing giant cells already had attained lengths of 3-5 times those of the surrounding vascular parenchyma cells while their diam was relatively unchanged. Thus, if cell wall dissolution is involved in the initial stages of giant cell formation, it would appear to operate more effectively on cell end walls than on side walls immediately adjacent to the head of the parasite. In Clevewilt, when syncytia were produced, elongate cells could be found which lacked a correspondingly high number of nuclei; indicating, perhaps, the independence of cell enlargement and mitosis.

Larvae which failed to elicit these responses in resistant cotton roots also failed to reach maturity. Unlike those which penetrate resistant alfalfa (15), however, only a few of the larvae which failed to become established migrated from the roots.

In an earlier paper (12) we proposed the hypothesis that resistance in *G. hirsutum* Clevewilt is a post-infection phenomenon acting at the cellular level. Failure of the host to comply with the nematode's "signal" for alterations in the host's nuclear metabolism could account for the observed failure of penetrating larvae to develop. Larvae not associated with nuclear hypertrophy and hyperplasia did not develop to the second molt.

Failure of larvae to develop, and lack of host response are interrelated, however, and death of the nematode is known to cause breakdown of the developing giant cell (1). Therefore, in Clevewilt production of a nematode-toxic substance could result in aborted syncytia as well as inhibited larval development. The observation that, in Clevewilt, some larvae induce partial development of syncytia while a few others reach maturity and induce syncytia comparable to those in Deltapine could be explained on the basis of rate and quantity of toxin production. It is not known whether the degradation of nematodes in resistant roots is a result of toxic action, or is the natural fate of any larva which dies from other causes.

The events leading to reduced pathogenesis in Clevewilt are envisioned as occurring in this sequence: (i) following penetration, the nematode becomes immobilized by a toxin or toxins produced locally; (ii) due to reduced viability, the nematode is unable to influence maturation of surrounding host cells and these cells rapidly develop to a point at which they no longer are capable of responding to "commands" from the nematode. During this period, a certain degree of growth of the nematode may have occurred as a result of limited feeding activities; and (iii) nematodes which fail to mature are degraded by the plant apparently resulting in reduced infections of the root system.

LITERATURE CITED

1. BIRD, A. F. 1962. The inducement of giant cells by *Meloidogyne javanica.* Nematologica 8:1-10.
2. BRODIE, B. B., L. A. BRINKERHOFF, and F. B. STRUBLE. 1960. Resistance to the root-knot nematode, *Meloidogyne incognita acrita,* in upland cotton seedlings. Phytopathology 50:673-677.
3. DROPKIN, V. H. 1969. The necrotic reaction of tomatoes and other hosts resistant to *Meloidogyne:* reversal by temperature. Phytopathology 59:1632-1637.
4. DROPKIN, V. H., J. P. HELGESON, and C. D. UPPER. 1969. The hypersensitivity reaction of tomatoes resistant to *Meloidogyne incognita:* reversal by cytokinins. J. Nematol. 1:55-61.
5. ENDO, B. Y. 1965. Histological responses of resistant and susceptible soybean varieties, and backcross progeny to entry and development of *Heterodera glycines.* Phytopathology 55:375-381.
6. ENDO, B. Y. 1971. Nematode induced syncytia (giant cells). Host-parasite relationships of Heteroderidae. Pages 91-117 *in* B. M. Zuckerman, W. F. Mai, and R. A. Rhode eds. Plant parasitic nematodes, Vol. II. Academic Press, New York, N.Y.
7. FRECKMAN, D. W., and R. A. CHAPMAN. 1972. Infection of red clover seedlings by *Heterodera trifolii* Goffart and *Pratylenchus penetrans* (Cobb). J. Nematol. 4:23-28.
8. HUANG, C. S., and A. R. MAGGENTI. 1969. Wall modifications in developing giant cells of *Vicia faba* and *Cucumis sativus* induced by root knot nematode, *Meloidogyne javanica.* Phytopathology 59:931-937.
9. JONES, J. E., and W. BIRCHFIELD. 1967. Resistance of the experimental cotton variety, Bayou and related strains to root-knot nematode and *Fusarium* wilt. Phytopathology 57:1327-1331.
10. LOWNSBERY, B. F., and D. R. VIGLIERCHIO. 1961. Importance of response of *Meloidogyne hapla* to an agent from germinating tomato seeds. Phytopathology 51:219-221.
11. MC BETH, C. W., A. L. TAYLOR, and A. L. SMITH. 1941. Notes on staining nematodes in root tissue. Proc. Helminthol. Soc. Wash., 8:26.
12. MC CLURE, M. A., K. C. ELLIS, and E. L. NIGH. 1973. Resistance of cotton to the root-knot nematode, *Meloidogyne incognita.* J. Nematol. 6:17-20.
13. MINTON, N. A. 1962. Factors influencing resistance of cotton to root-knot nematodes (*Meloidogyne* spp.). Phytopathology 52:272-279.
14. RADEWALD, J. D. 1957. The nature of resistance and susceptibility to the root-knot nematode (*Meloidogyne incognita* var. *acrita* Chitwood) in sweet potato. M.S. Thesis, Oklahoma State University. 51 p.
15. REYNOLDS, H. W., W. W. CARTER, and J. H. O'BANNON. 1970. Symptomless resistance of alfalfa to *Meloidogyne incognita acrita.* J. Nematol. 2:131-134.
16. TRIANTAPHYLLOU, A. C., and HEDWIG HIRSCHMANN. 1960. Post-infection development of *Meloidogyne incognita* Chitwood 1949 (Nematoda: Heteroderidae). Ann. Inst. Phytopathol. Benaki 3:1-11.