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Title: BIONOMICS OF THE CLOVER CYST NEMATODE,
HETERODERA TRIFOLII (GOFFART, 1932) OOSTENBRINK, 1949.
(NEMATODA: HETERODERIDAE)

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Plants were tested to determine their susceptibility to the "Clover Cyst Nematode," Heterodera trifolii (Goffart, 1932) Oostenbrink, 1949. All leguminous plants tested were suitable hosts of the nematode. Of the non-leguminous plants tested, only cucumber, pumpkin and squash were infected. These three plants are new hosts of H. trifolii.

Second-stage larvae were reared at various temperatures to learn the effect of temperature on the time needed to complete a life cycle. As the temperature increased, the time required to complete a life cycle decreased and conversely as the temperature was decreased, the time required to complete a life cycle increased. In higher temperatures, the genital primordium matured earlier than in lower temperatures. At 80° F however, the genital primordium was

fully developed before the fourth molt whereas at lower temperatures the genital primordium developed after the fourth molt. At 88 and 91° F, the larvae were killed.

Encysted larvae in wet storage conditions survived four months at 25° C. Above and below this temperature, survival time declined rapidly. Encysted larvae in dry storage conditions survived three months at 25° C, and the longevity declined above and below this temperature. Regardless of the storage condition, there was no emergence from encysted eggs incubated at 30 and -10° C. The effect of different moisture percentages upon survival of nematodes is that the viable cysts survived a range of 2.93 to 5.14 moisture percent level, with an optimum of 4.40. Above and below this level, the population declined.

The effect of pH and temperature upon larval emergence indicates that there was no emergence from the encysted eggs incubated at 15 and 30° C. At 20 and 25° C, the optimum pH for emergence was 8. Regardless of temperature, there was no emergence at pH 1-4 and 11-12. The effect of vacuum upon viable cysts is that encysted eggs and larvae require oxygen for their growth and development.

BIONOMICS OF THE CLOVER CYST NEMATODE, HETERODERA TRIFOLII (GOFFART, 1932) OOSTENBRINK, 1949. (NEMATODA: HETERODERIDAE)

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INTRODUCTION

Microscopic thread or roundworms, though devoid of eye, ear, nose and limbs, can find their hosts without much difficulty, attack them and establish themselves within and on the plant roots.

The first free-living nematode was discovered by Borellus in 1656, and named Turbatrix acetae by Miller in 1783. While examining smutted grain of wheat in 1743, Needham discovered the first plant parasitic nematode and described the first plant disease caused by a nematode. Today this pest is commonly known as the wheat nematode, Anguina tritici (Steinbuch, 1799) Filipjev, 1936.

Oregon agricultural industries export and import large quantities of plants and plant material which may contain various nematodes. Hence it was necessary to obtain further specific information about the nematodes in order to help the Oregon agriculture with plant disease problems caused by nematodes. Heterodera cysts (females) containing viable larvae have been found in the soil included with the shipment of bulbs, tubers, roots, and other market or propagation materials.

Recent findings of numerous shipments containing Heterodera cysts in the course of routine inspection of shipments of plant material from foreign countries, suggests that, other shipments containing

cysts have entered the country in the past and that many foci of infection now exist here as a potential threat to the crop production (46).

The origin of many species of Heterodera are unknown, but most of them are fairly widespread in Europe, the British Isles, some parts of Asia, Africa, and the United States. For example, the clover cyst nematode, Heterodera trifolii (Goffart, 1932) Oostenbrink, 1949, has been reported in the United States from California, Oregon, Utah, Maryland, Illinois, and also from British Columbia in Canada (5). The most important way in which an infestation is started in new localities is when cysts of a certain species accompany plant material from its favored hosts. This is particularly true if the underground portions of the plant are harvested and the material is planted immediately after arrival.

The genus Heterodera contains many economically important plant parasitic nematodes. Its species are especially adapted to a parasitic mode of life in plants. The protective cyst-like covering enables eggs and larvae to tolerate long periods of adverse conditions. Nematodes belonging to this genus are highly host specific, usually attacking only limited members of a few genera in a given plant family. The presence of suitable host plants, however, stimulates the Heterodera larvae to emerge in large numbers from the cysts.

The invasion of plant roots by larvae results in damage to the plant. The plant often responds to this injury by forming new rootlets

at or near loci where larvae have settled. Dwarfing is a common symptom and plants become unthrifty, wilt during warm weather and eventually die. Both above and below ground parts of the plants exhibit symptoms due to the attack. The internodes of the plant become short but do not increase in thickness. Root areas, where nematodes feed, become slightly corky. Natural development of the roots stops completely and as a result, partial failure has been observed in some crops.

The plant parasitic nematodes are classified mainly as ectoparasitic and endoparasitic types. Ectoparasitic nematodes remain outside the plant and feed by means of a well developed spear, whereas endoparasitic nematodes penetrate the plant, develop within and feed with a short spear. The clover cyst nematode, Heterodera trifolii, falls in the latter category. This nematode has been found repeatedly in soils of northwestern Oregon. No males have yet been found. This pest is known to attack Ladino clover, hairy vetch and other legumes. As this species of Heterodera is found in virgin areas in the coastal mountains, the clover cyst nematode probably is native to Oregon (21).

Prior to this study, very little was known about the effect of temperature on its life cycle or about the effect of changed environmental conditions upon this species of Heterodera. Hence, the investigation included studies of the following:

I. Life cycle in relation to soil temperature

II. Host range

III. Optimum environmental requirements

IV. Longevity

V. Length of time needed by larvae to penetrate a host plant

VI. Effect of pH on larval emergence

VII. Effect of vacuum (low oxygen level) on larval emergence.

LITERATURE REVIEW

Literature discussed in this review covers (i) life history, (ii) host range, (iii) effect of pH and (iv) longevity of the species in the genus Heterodera. Very little work has been done with the clover cyst nematode, Heterodera trifolii. It has been assumed that any deviation from normal condition usually affects the life cycle of the nematodes. The cyst nematodes are readily stimulated to hatch by host root diffusate. The larvae then enter just behind the growing point of the root, which is the zone of greatest attraction. The nematodes are killed or inactivated if they are subjected to high, low or prolonged temperature and moisture conditions.

Life History of Heterodera spp.

Cysts

Nematodes belonging to the genus Heterodera are commonly called cyst nematodes. The Heterodera cyst is formed as the female dies and body cuticle hardens and attains a brownish color. Cysts, according to Steiner et al. (47), are capsule-like structures formed by many lower animals at some stage of their development. This structure serves the purpose of protecting developmental stages (egg and larvae) from dessication and other unfavorable environmental conditions (12, 47). According to Thorne (51), fully matured cysts are typically ovate to spheroid in

form and usually range from 0.4 to 0.8 mm. in length, with occasional extremes as small as 0.2 mm. and as large as 1.1 mm. The old empty cysts hold no eggs, while the mature ones hold as many as 600 eggs.

Eggs

Heterodera eggs are typically jelly-bean shaped, and about three times as long as wide. Triffitt (54) reported that variations in width of 35 to 50μ and length of 75 to 125μ, with an average of 46 x 110μ. In all cases eggs are formed in the body where they may be retained or deposited in a gelatinous matrix.

Larvae

In so far as known, the first molt occurs within the egg, and in rare cases it is possible to observe molting of the first cuticle. Eggs from mature cysts may contain well developed first or second stage larvae. The second stage larvae usually complete their development in about seven days after penetration in a host root, and then the second molt occurs. Larval length usually varies from 0.3 to 0.6 mm. The genital primordium continuously develops from the first stage and reaches its maturity after the fourth stage. The sexes can be distinguished by this time. The larvae start becoming flask shaped during the second stage. In general, the larval body is distinctly robust, the spear and cephalic framework are strongly developed.

Males

Males are very common in most species of Heterodera. The males have a finely annulated cuticle, a spear, median bulb, posterior oesophageal portion and other masculine features. Some Heterodera males are found in the gelatinous matrix. Instances of males feeding on root-tissues have not been recorded (51).

Females

The female body is generally citron shaped and apparently two elevations can be observed. First, the oesophageal portion that contains both spear and oesophageal bulb and second, the caudal elevation above the genital and anal aperatures. The body is lemon shaped in Heterodera schachtii group, spherical in the H. rostochiensis group and pear shaped in the H. punctata group. In the H. rostochiensis group the vulva and anus are both located on the rounded posterior portion of the body (51). As the female developes, the larval stages increase in girth until the posterior portion of the body breaks through the root cortex. At maturity, the female body contains eggs and larvae.

Reported Life History Studies of Heterodera trifolii

The clover cyst nematode was first described as a variety of Heterodera schachtii and given the varietal name trifolii by Goffart in 1932 (33). In 1944 he designated it as H. schachtii trifolii on the basis

of further information. Franklin (13) also considered it a variety of H. schachtii. Oostenbrink referred to this nematode as H. trifolii in 1949 and Steiner et al. (47) also considered it as a separate species in discussing the various species of Heterodera present in the United States.

Cysts

Franklin (13) reported that the cysts of H. schachtii var. trifolii are typically lemon shaped and of a dark brown color. Hirschmann (17) found that the ratio between length/breadth of H. trifolii varied from 2.40 to 1.32μ. Cyst cone is prominent in this species and bullae are also present.

Eggs

The maximum number of eggs has been found to be 200. They may be deposited in the gelatinous egg sac (51, 33) or retained in the cyst body (33). The eggs were found to range from 100 to 110 in length and 40 to 45μ in width. As the embryo increases in length it becomes slender and eventually, through a progressive increase in length, forms two to three flexures within the egg shell (33).

Larvae

Mulvey (33) conducted a life cycle study at an average soil temperature of 60.0°F. He reported that the first molt occurs within the egg and spear formation takes place during the second stage. Second stage larvae varied from 475 to 490μ in length and 20 to 22μ in

width. Franklin, however, reported a length of 490 to 504μ (13) and Hirschmann (17) found a length of 443 to 547μ. Evidence of second molt was found five to six days after penetration. By the twelfth day all larvae completed the second molt. Mulvey measured the third stage larvae (within the cast skin) to be 400 to 460μ in length and 30 to 50μ in width (33). The spear was found to be 25 to 29μ long, with forward pointing knobs. The third stage larvae was measured 400 to 460μ in length and 53 to 93μ in width. Formation and development of third molt was completed by seventeenth day after penetration. Fourth stage larvae varied from 410 to 500μ in length and 125 to 160 μin width and was matured fully by 21 days. The fourth molt was completed by 26 days.

Females

Twenty-nine days after penetration, white females start producing eggs. Forty-three days after penetration, several females were filled with eggs. Many of these eggs contained first stage larvae. Fifty-six days after penetration eggs containing second stage larvae were found in the gelatinous matrix. The first brown cysts were found 64 days after the larvae had penetrated the roots.

Males

Males are not common and have only been reported by McBeth (30) and Franklin (13). Cephalic annules were found to be four to five

in number and a bidentate spicula was also present.

Morphological Characters Separating H. trifolii from Its Most Closely Related Species

The genus Heterodera contains 16 species of which some are very similar to Heterodera trifolii. In order to separate the clover cyst nematode from other closely related species, a detailed summary of differentiating morphological features for the nine most closely related species is given in Table 1 (page 12). Features frequently used to differentiate species of Heterodera are cyst shape, cyst size, larval length, presence or absence of males, cone top/underbridge and bullae.

From the literature it appears that the soybean cyst nematode, Heterodera glycines Ichinohe, 1952, is most closely related to H. trifolii. In comparing morphological differences between H. glycines and H. trifolii, Hirschmann (18) reported that surface markings of H. glycines cysts have more pronounced punctations than does H. trifolii. Hirschmann later found that the cysts of H. trifolii are slightly larger than H. glycines (length 469.6 μ and 439.6 μ respectively) (17). The stylet of H. glycines is shorter (23.0 μ) and appears somewhat stouter than that of H. trifolii (27.5 μ). In H. glycines the basal knobs are distinctly separated and are much broader than high, whereas they are closer together in H. trifolii, which gives

them a more rounded appearance. Larvae of H. glycines has a shorter, plumper tail (length 50.4μ) with a more bluntly rounded terminus and a shorter tail terminus (26.6μ). On the other hand, in case of H. trifolii, tail length is 60.3μ and tail terminus 30.7μ. Using the underbridge as a separation character, Mulvey (31) found that the depth of underbridge is greater and also is more strongly developed in H. trifolii than in H. glycines. Mulvey later reported that second generation females of H. trifolii produced abnormal second stage larvae, measuring 670μ in length (34). Abnormalities were more prominent in tail development. In cyst size and larval length, H. trifolii is larger than H. glycines. The clover cyst nematode, H. trifolii, is typically a lemon shaped cyst. Apart from all, H. trifolii is the only parthenogenetic female reported here.

Table 1. Summary of morphological features used to separate clover cyst nematodes from closely related species in the genus Heterodera.

Nematodes	Cyst Shape	Cyst Size	Second Stage Larval Length	Number of Lips (annules)	Males	Conetop Underbridge	Bullae
H. rostochiensis Wollenweber. 1923	Spherical	L. 0.5-0.8 mm.	440-460 μ	6 to 8, spear with round knobs	Present 1 mm. long		
H. schachtii Schmidt. 1871	Lemon shaped with acute vulval cone	L. 0.5-0.8 mm.	460 μ	3 to 4. spear 25-28 μ long	Present 1.3-1.6 mm.	Correlation between cyst volume and underbridge not significant	Present contains a distinct vagina
H. glycines Ichinohe, 1952	Lemon shaped	L. 439.6 μ ratio of L/B μ 2.05-1.19 μ	484 μ	6 to 8, spear round and massive	Present 1.3 mm. long		Present
H. gottingiana Liebscher. 1892	Round to lemon shaped		462 μ Leibscher 456 μ Goffart 487 μ Franklin	3 to 4	Present 1.2 mm. long	Semicircular semifenestrate	Absent
H. avenae Filipjer. 1934	Lemon shaped	L. 0.55-0.75 mm. and two thirds wide as long	575 μ Franklin 530-550 μ Schm. 560 μ Goffart	5. spear 26-29 μ long	Present 1-1.38 mm. long		
H. humuli	Small elongated	L. 0.4-0.7 mm. twice as long as wide	390-400 μ	Spear 27-31 μ long	Present 1.0 mm. long	Bifenestrate cone top and stout vulval bridge	
H. cruciferae Franklin, 1945	Spherical to rounded		418 μ	4 to 5, spear 25 μ long	Present 1.2 mm. long	Semifenestral cone tope	Absent
H. punctata Thorne, 1928	Pear shaped	L. 0.52 mm.	350-470 μ	4	Present 0.9-1.3 mm. long		
H. trifolii (Goffart, 1932) Oostenbrink, 1949	Typically lemon shaped	L. 496.6 μ ratio of L/B 2.40-1.32 μ	502 μ	Spear 25-29 μ long	Absent (extremely rare)	Present. less developed than H. schachtii	Present contains distinct vagina

Differentiation of Sex

Thorne (49) in 1935 reported that differentiation of sexes in H. schachtii occurred in the last molt, the males and females being present within the roots and in the surrounding soil. Because of the difficulty males would experience in locating females within the roots, Thorne believes that copulation probably occurs in the soil, after which the young females enter the roots. The female body lengthens but little as it becomes a distorted mass forcing its way into the hard root tissue. Mulvey (33) reported that the differentiation of sex in case of H. trifolii occurs in the fourth stage. The body is flask shaped and the body cavity is surrounded by a distinctive granular layer.

The female reproductive system bears two ovaries with single spermatheca, oviduct, uterus, vagina leading to a terminal vulva. The same system was found by Mulvey in case of H. trifolii (33).

The male reproductive system of H. schachtii on the other hand consists of a single outstretched testis, seminal vesicle, vas deferens, and a ejaculatory duct leading to the spicula.

Root-diffusates and Their Effect on Emergence

Cyst nematodes are generally readily stimulated to hatch by host root-diffusates, whereas a non-host diffusates have very little or practically no effect. Given suitable physical conditions, hatching from

mature cysts proceeds at a relatively slow rate. But addition of a chemical stimulus e.g., root-diffusate from a growing host, causes an increase in the hatching rate. Fenwick (11) and Mankau (27) reported that considerable spontaneous hatching of the clover cyst nematode, H. trifolii, occurs in the presence of active root-diffusate. Seinhorst (43) reported that in both H. rostochiensis and H. schachtii larvae and eggs react to substances diffusing from roots of host plants by hatching. Whereas in plain water most larvae remain quiescent in the eggs.

Stimulatory effect of sugar-beet root diffusates upon the emergence of larvae of H. schachtii was first described and demonstrated by Baunacke (15). In 1930, O'Brien and Prentice observed an increased effect on emergence of H. rostochiensis larvae. Wood and Serro (62) reported that root-diffusates from several plants contained some chemicals which constitute a major portion of the exudate. These chemicals might increase (in case of host plant) or decrease (in case of non-host) the larval emergence (1). Diffusates are absorbed in water which leaches through the soil around roots. These leachings may be collected and stored in refrigeration for future use.

Fenwick (10) found that activation of H. rostochiensis larvae was decreased as the potato-root diffusate was diluted.

Hatching in the absence of active root-diffusates varies among the different species of Heterodera. In H. rostochiensis emergence is

very low, but considerable spontaneous hatching can occur with H. cruciferae and H. schachtii (11).

Schmidt (1931) reported that cereal root diffusates do not stimulate the oat nematode (H. avenae) and that pea root diffusates do not stimulate the pea nematode (H. göttingiana) in laboratory conditions. But he believes that such stimulation must occur in field conditions. Thorne (50) and Winslow (61) found that good hatching responses occur with host plant leachings, whereas non-host leachings had very little effect. Shepherd and Wallace (44) reported that though the sugar beet nematode (H. schachtii) is readily stimulated by root-diffusates to give a high percentage of emergence, it still has a low invasion rate. Pea root nematodes (H. göttingiana), on the other hand, have a low rate of emergence, but a high invasion rate.

Invasion of Roots

Entrance within a plant depends upon the penetrating power of the nematode, even if it happens to be the host plant. If the cortical or epidermal cells are too thick, the nematodes may not be able to enter the plant. Mankau (27) reported that larvae of H. trifolii penetrate into epidermal cells with some difficulty, but once the head enters the epidermis, further penetration is rapid. The entire larval body often disappears within the cortex in 15 minutes. The larvae are attracted towards wounds in roots, and often enter through these avenues.

Host Range

Christie (5) reported that Heterodera trifolii is wide spread in Europe, Canada and the United States. Jensen (21) reported that H. trifolii has been repeatedly recovered from non-cultivated areas near the summit of coastal mountains, but very little information exists about injury to crops. Christie reported the maximum number of hosts of this particular nematode belong in the family Caryophyllaceae (20 species), Leguminosae (14 species) and Polygonaceae (7 species). This nematode may attack other hosts, but fails to maintain a population on these plants. For example, the larvae entered the roots of 27 varieties of soybean; but females reached maturity in only two varieties. Even in these cases very few females matured and they produced very few eggs.

The host range of clover cyst nematode, H. trifolii, as reported by different workers is given in Table 2 (page 18). It would appear that certain plants, selected as hosts by one worker are rejected by another. Few plants suspected as hosts were not selected because the larvae entered the roots but failed to develop. Mankau and Linford (26) were interested in finding soybean varieties which could be used as host, but reported that the nematode population could not be maintained in those host plants.

Parasitic Relationship

Mankau (28) studied the parasitic relationship of clover cyst nematode, H. trifolii. He reported the syncytium that develops in the steele adjacent to the head of the nematode is formed by the coalescing of adjoining cells. Hypertrophy of cells or nuclear division in this area stops. Small, tubular structures are found within the syncytium, close to and sometimes attached to the nematode's stylet. Mankau found a highly variable development in pea, where the cells often react necrotically from the feeding of this nematode (H. trifolii), or from its salivary secretions. Due to the salivary secretions produced by the developing females, the surrounding cell walls rupture and eventually are dissolved. There remains a mass of nuclei floating in that place, each governing a certain area. As this situation develops, less resistance is offered for the development of the developing females.

Table 2. Host range of Heterodera trifolii catalogued according to results of different workers.

Workers \ Plants	Trifolium repens	T. pratense	T. incarnatum	T. resupinatum	T. hybridum	Phaseolus vulgaris	Pisum sativum	Vicia sativa	V. villosa	V. harbonensis	Sesbania	Carnation	Lupinus spp.	Medicago sativa	Beta vulgaris	Stellaria media	Rumex spp.	Chenopodium spp.	Allium cepa	Lathyrus	Spinacia oleracea	Avena sativa	Dianthus barbatus	Lens esculenta	Sharp dock	Rumex crispus	Saponaria officinalis	Galeopsis speciosa	Isatis tinctoria	Glycine max	var. Earlyana	var. Dunfield	var. Hawkeye	var. Wabash	var. Capital	var. Renville
Franklin	+	+	+			0	+	+	+	+			-	-	+	+	+											+	+							
Goffart	+	+	+	+		+	0	0	+	+			-	-			+																			
Raski and Hart	+	+	+		-	0	+	+	+	+	+	+	+	+	+	+	+											+	+							
Oostenbrink		+	-	+		+	0	+	-	-							+																			
Mulvey	+	+	0	0	0	+	+	+	+				0	0				+	0	-																
Mankau and Linford																				-										+	+	+	0	0	0	-
Winslow	+	+																					+	+	+	+	+			+						
Gerdemann and Linford	+	+	-	+																	+	+	+	+		+				+						
Christie	+	+	+	-	+	+	+	+	+	+																				+	0	0				

+ = host - = non-host 0 = suspected

Longevity

(1) Effect of temperature upon development and hatching

There are numerous observations of nematode resistance to low temperatures, mortality at high temperatures and optimum temperature for development. The temperature requirement for different species often differs considerably, and lack of agreement among workers for the same species makes any generalization difficult. Mostly temperature studies are carried on in connection with moisture percentages. Jones (23) reported that temperature controls activity and, for each species, upper and lower thermal points exist. Except that upper thermal death points are important in the hot water treatment of plants to rid them of nematodes, these concepts usually are academic. Of greater ecological value are the temperature limits within which activity, and specially reproductive activity is possible. Usually tolerance of the nematode is related to and often exceeds that of its host plant. The optimum temperature for development or hatching of phytoparasitic nematodes is 20° C to 30° C (8; 58; 52; 12; 55) and the minimum temperature is 10° C to 15° C (40; 45; 56). At higher temperatures (40 to 55° C) cysts and larvae of other nematodes are killed or inactivated (24; 48; 55). The survival of nematodes at low and high temperatures is often a function of time. Santmyer (42) suggests that differences in

thermal death time between species may represent physiological differences, particularly in enzyme inactivation, because enzymes are quickly inactivated above 50° C. Although there are examples of nematodes surviving in temperature extremes for long periods (20) reproduction does not occur under these conditions. Mulvey (33) reported that temperatures ranging from 51 to 68° F, with an average of 60° F, was optimum for Heterodera trifolii. Gerdemann and Linford (14) reported that minimum temperature requirement under greenhouse condition for one generation of H. trifolii varies between 15 to 27° C.

Ferris (7) reported that the larvae of H. rostochiensis did enter the roots at 65, 75, and 85° F, but most rapid development was at 65° F. Further rise in temperature reduced the formation of new cysts and at 31° C the development and multiplication almost stopped (9). Raski (36) reported that 66.8° F is the optimum temperature for growth and development of H. schachtii. Cysts appeared to be well suited to survival not only at temperature where soil freezes in the winter, but in hot desert areas where soil temperatures during summer are frequently above 40° C (53). Ichinohe (20) reported that a temperature range from 65 to 74° F is optimum for development of H. glycines.

Bishop (3) reported that when temperature is altered between 15 to 25° C, there is a significant increase in larval emergence in

case of H. rostochiensis (2, 3) and H. schachtii. The same effect was also noted by Wallace (58), Ladigina (24), Raski and Johnson (39). Slack and Hamblin (55) reported that emergence of H. glycines larvae is nil below 16° C or above 36° C. Incubation of cysts below 12° C for one week cause no reduction of emerging larvae. Cysts or emerged larvae are killed by freezing and by incubation at 40° C for three hours.

Epps (6) reported that H. glycines cysts stored at 70 to 95° F, 85° F, and 70 to 80° F for one month without soil do not produce any larvae after they are inoculated to soybean plants. In another case, cysts stored with soil for one month, survived adequately, while those stored for two to three months failed to survive. Gerdemann and Linford (14) reported that soil with white clover roots, collected below a crust of frozen soil, still contained free larvae as well as cysts attached to roots.

(2) Effect of moisture upon development and hatching

As far as is known, nematodes move in the soil by undulatory propulsion, only when they are surrounded by water, even if this is only a thin film. Hence if water evaporates from around the nematodes, movement ceases and the nematode is subject to desiccation. The physiology of desiccation, especially between such widely differing species as the Heterodera, has not been investigated. Wallace

(59) while describing the effect of water-logging on survival of parasitic nematodes, reported that water-logging in soil has an inhibiting effect upon growth and reproduction of nematodes. He said that it is likely that the inhibiting effect of water-logged soils on nematodes is primarily an aeration effect. Cairns (4) reported that the death of nematodes, kept active in water, is possibly caused by the melting of a body constituent. He found the amount of moisture determined the mode of lethal action and the degree of resistance to heat. Mai and Mechow (29) reported that cysts lose viability at 40° F and 1.5 percent relative humidity after 327 days. This effect increases considerably at 94.8° F and 1.5 percent relative humidity.

Lewis and Mai (25) found the viability of golden nematode larvae decrease rapidly at 100, 88, 20, and 3 percent relative humidity stored for 243 days. They also found that the viability decreases more rapidly in flooded and moist soil, than in air-dried soil.

Ability of encysted larvae to survive in storage varies widely with prevailing moisture conditions (46). Nematodes stored under extreme temperatures are found dead after one month.

From decline in viability of the encysted larvae subjected to some of the treatments, one could conclude that the combination of temperature and relative humidity over a relatively long period of time plays an important role in influencing viability. There is no biological phenomenon associated with high relative humidities alone,

that would cause this more rapid death. At 0-1.5 percent relative humidity with higher temperatures, death may be caused by desiccation. At high relative humidity with high temperatures, death may be caused by starvation.

Effect of Different pH Ranges upon Larval Emergence

Robinson and Neal (41) while using distilled water and changing the pH by addition of HCl, reported that the absolute figure for larval emergence per cyst (of golden nematode) varied among assays for any given treatment. There was unquestionably higher hatching at pH values between two and three than at values below and higher of this range. The larvae hatched at pH 2 appeared fully active, even when the incubation period was extended to two weeks; however, incubation at pH 1 appeared to produce a lethal effect.

Fenwick (8) reported that when the pH of a normal root diffusate is changed by addition of sodium carbonate and hydrochloric acid solutions, the emergence of larvae reached maximum at pH 4.5 and 6.8. Significant differences in hatch between pure root diffusates and adjusted pH diffusates was not observed.

MATERIALS AND METHODS

General Procedures

(A) Obtaining and maintaining a stock culture of cysts

The gravity screening technique, described by Cobb in 1918, was adopted with some very minor modifications. The equipment required for this technique was: (1) two large dish pans, (2) two sieves of 40 and 100 meshes, and (3) a small pan. The infested soil was mixed thoroughly with tap water in one large dish pan, and the liquid was poured through the first (40 mesh) screen to the second pan. The water collected in the second pan was then poured through the second (100 mesh) screen. The first screen retains the larger particles of soil and organic matter, but allows the cysts to pass through. The second screen retains the cysts and allows the smaller particles of soil and organic material to pass. Cysts obtained on the screen were rinsed free of debris and collected. The entire technique was repeated until a large number of cysts were obtained. These cysts, kept in a glass jar half filled with water, were stored at 34° F. The water was changed every day to prevent the accumulation of bacteria, which might destroy the cysts. Cysts, kept in this way, can be stored for a few days without causing any appreciable effect upon the viability of larvae. At times, when required, they were removed for various experiments.

(B) Increasing inoculum

Ordinary greenhouse soil was placed in gallon cans (number 10). Twenty seeds of hairy vetch (the indicator plant) previously treated with "Spergon" to prevent decay, were planted in six cans. Cysts were taken from the jar with a long dropper, and were added to the cans. Forty cysts were added to each of six cans. A small amount of sand was placed over the soil surface to allow proper drainage of water and to prevent crusting. Adequate water was given every day for optimum plant growth. The temperature in the greenhouse was maintained between 75-78^{o} F. The eggs within the cysts were stimulated to hatch by the root secretions, developed to adults, reproduced and established a large population in each can. Whenever required, soil from these cans was sieved to obtain new cysts.

(C) Inoculation of indicator and test plants

Hairy vetch plants were used in all experiments as they proved to be a good indicator plant. Large viable cysts were obtained from the stock culture for inoculation. Inoculation was accomplished by placing two cysts near each seed, leaving some space between the seed and the cyst. This was done to prevent any harm to the cysts, while in close contact with "Spergon." Inoculation was also accomplished by adding a suspension of second-stage larvae to established vetch seedlings and allowing 10-12 hours for larval penetration.

(D) Hatching of cysts and obtaining infective larvae

Cysts were hatched by the following three methods.

(i) Incubation chamber: A flat bottomed circular glass container of eight inch diameter was filled to one-third full water. A wire screen was fixed about one-half inch above the water surface to support the viable cysts. A single tissue paper, cut to the size of the screen, was placed on the screen barrier. Cysts were placed on the tissue paper along with soil debris. The entire container was kept airtight by placing a lid on the top and sealing the open spaces with masking tape to provide a makeshift moist chamber. Every day, the lid was removed and water was sprayed through a jet to enable or force hatched larvae to fall into the water. The water, in the bottom of the container, was replaced by fresh water every day and was examined for the presence of larvae.

(ii) Modified Baermann funnel technique: This method was similar to the technique described in (i), above, except that in the place of a glass container, a plastic funnel equipped with a rubber tube attached to the spout was used. The open end of the rubber tube was fitted to a glass tube which ultimately led to a 80 ml. test tube. Every day, the clamp on the rubber tube was released and water was drawn off to the tube. As described previously, water was sprayed with a jet on the tissue paper containing the cysts. The larvae that hatched within the cysts, escaped from the vulval slit, dropped in the water,

and finally were collected from the bottom of the tube.

(iii) Root-diffusate: Young, healthy vetch seedlings were used for obtaining root-diffusates. The roots were first thoroughly rinsed with distilled water, and then were placed in eight 150 ml. flasks with each containing 125 ml. of distilled water. After six to twelve hours, the plants were returned to the nutrient solution, until additional root-diffusate was required. The exudate was stored in distilled water at 10° C.

Twenty-five ml. of this exudate was poured in each of eight petri dishes along with 20 mature cysts. Two other dishes were used as check, which contained tap water instead of the root-diffusate. All petri dishes were stored at room temperature (24° C). After two weeks, the dishes were examined and the larvae counted. The larvae were removed and inoculated whenever and wherever required.

LIFE HISTORY

(A) Determination of time required by larvae to penetrate a host plant

For this experiment, vetch seed was sown in rows a week prior to inoculation. As sufficient larvae were obtained from various hatching methods, they were inoculated in the root zone of the seedlings (which were about one and one-half inches tall at that time). Inoculations were accomplished by adding water containing newly hatched larvae to the root zone of vetch seedlings by hypodermic syringe. Every hour after inoculation, one or two plants were removed, washed, stained, and examined for the presence of larvae within the roots. When larvae were actually observed inside the roots, the duration of time was recorded, and further inoculation was stopped.

Goodey's staining technique, modified by Jensen (22) was used for examining the nematodes in root tissues. The staining solution, containing 0. 1 gram of acid fuchsin in 100 cc. of lacto phenol (a solution containing lactic acid, glycerine and water in equal parts) was brought to a near boiling point and the roots were placed in this solution for a couple of minutes. Excess stain was then washed off the roots by placing them in a beaker of tap water. The roots were then allowed to destain in a clear lactophenol solution, for a period of about two days. During this time, the root tissues destained while the nematodes retained most of the stain. Penetration occurred in 18 hours.

(B) Determination of temperature effects upon the life cycle

A greenhouse installation of seven thermostatically controlled temperature tanks was available for this experiment. The tanks were adjusted to maintain constant temperatures of 50, 60, 70, 75, 80, 88, and 91° F.

Hairy vetch was planted in flats and inoculated with larvae as described on page 28. After 18 hours (by this time the larvae had penetrated the host) the plants were removed and roots were washed carefully to remove any nematode that had not entered the roots. These plants were then transplanted in fruit juice cans (capacity, 46 ounces) which had been previously filled with sterilized soil. The cans were then placed immediately in the temperature tanks. Water was applied every day to maintain the most suitable moisture for the plant growth. The water level in the temperature tanks was also kept constant.

Every two days, two plants from one can in each temperature tank were removed. The roots were washed, stained, and examined with a dissecting microscope. By this method of differential staining, the developmental stages were observed easily inside the roots. Time taken to develop from one stage to another was recorded.

(C) Stages of development

It is evident from the results shown in Table 3 that as temperature is increased, the time required for larvae to complete their life

cycle is decreased and vice versa. Development did not occur at 88 and 91° F. The larvae, at these higher temperatures, were either inactivated or killed after second molt. Mulvey (33) reported that first molt occurred within the egg and the emerging larvae were in the second stage. This stage is the infective stage when larvae attack the host plants.

The second molt appeared in 12 to 14 days at 50°, eight to ten days at 60°, four to six days at 70°, two to four days at 75 and 80°, and one to two days at 88 and 91° F temperatures. Molting started from the posterior part of the body and then began in anterior region. During the molt a new spear appears, which is shorter in appearance than the previous one. The larvae increases in width and the digestive system reappears. The additional molts are similar. The genital primordium started undergoing cell division after this molt. The newly formed larvae still retain a worm-like appearance.

Third-stage larvae appeared two to four days after the second molt according to the different temperatures mentioned earlier. Two days later, development of the third-stage larvae was completed. Body annulations could only be distinguished in the head region. The larvae increased in body width about three times that of the previous stage. The genital primordium changed considerably with an increase of nine to twelve cells and also a noticeable increase in length. Towards the later part of this stage, the genital primordium rotates 90°

and becomes vertical to the body axis, the ovaries begin initial development.

The third molt appeared two to four days after the third-stage larvae appeared. The body continued to increase in width and the genital primordium increased by two oval enlargements, forming a "U" shaped structure. The spear increases in length, but the basal knobs remain about the same size as in the previous stage.

Fourth-stage larvae developed within two to three days after the third molt, at 50 to 80° F. The new recognized juvenile females were flask shaped. The ovaries increase in length and reflex at the anterior ends. The entire cuticle was marked by transverse lines. The new spear was longer and had distinct basal enlargements.

Evidence of the fourth and final molt appeared one to two days after the development of fourth-stage larvae. The ovaries continued to increase in length and reflex two to three additional times. Formation of a terminal vulva appeared as the females assumed a lemon shape.

Males were not observed and have only been reported once or twice. Eggs containing a new generation of second-stage larvae were observed within the females, seven to seventeen days after the fourth molt, depending upon the different temperatures. Two to thirteen days later, (depending upon the temperature) eggs and larvae were observed in the gelatinous matrix. The cuticle retained a chalky white color

until the fourth molt. Following the white color, the cuticle started to turn yellow. Four days later, almost all females reached the yellow phase. This yellow color, towards the later stage, becomes brown and remains brown as the females die and form cysts. Brown cysts were observed four days later at different temperatures ranging from 50 to 80° F.

The genital primordium was in a unicellular condition about twice as wide as long (Figure 1, A. 700x)(soon after the second-stage larvae invaded the host plant). Thirteen, eight, two and one days after penetration, the genital primordium was found to be in two cell stage according to the temperatures shown in Table 3 (Figure 1, B. 700x). Vertical cell development appeared at 14, 9, 3 and 2 days after penetration (Figure 1, C. 700x), and one day later cells started forming near the external margin of the genital primordium (Figure 1, D. 700x). Two days later at the temperatures mentioned in Table 3, the genital primordium doubled in length and made a 90° turn, becoming horizontal to the body axis (Figure 1, E. 700x). Eighteen, thirteen, seven and six days after penetration, the genital primordium became concave in shape with the concave surface facing the anterior end of the body (Figure 1, F. 700x). This development was more pronounced the following day (Figure 1, G. 700x). During this time rapid cell division and growth were taking place as evident by the swollen appearance of the ends. By now the genital primordium had increased in

Table 3. Development time for various stages of H. trifolii at different temperatures beginning with host penetration.

	Temperature						
Stage/Molt	50.0°F (days)	60.0°F (days)	70.0°F (days)	75.0°F (days)	80.0°F (days)	88.0°F (days)	91.0°F (days)
2nd molt	12-14	8-10	4-6	2-4	2-4	1-2	1-2
3rd stage	15-16	12-14	8-10	6-8	5-6	-	-
3rd molt	19-20	16-18	11-12	9-10	7-8	-	-
4th stage	23-24	20-22	14-16	12-14	10-12	-	-
4th molt	28-30	24-26	18-20	15-16	13-15	-	-
Eggs/larvae	47	40	32	27	22	-	-
Yellow phase	64	56	45	34	26	-	-
Brown cyst	68	60	49	38	30	-	-

Figure 1. Various developmental stages of the genital primordium of H. trifolii at 70° F soil temperature (700x).

A... Unicellular condition at time of penetration

B... Two cell condition, two days after penetration

C... Vertical cell division, three days after penetration

D... Cell formation near external margin, four days after penetration

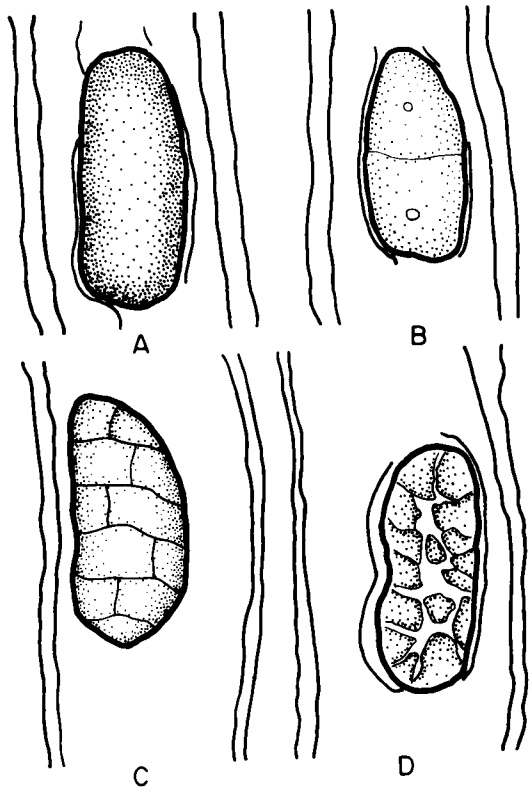
E... Horizontal rotation, six days after penetration

F... Concave appearance, seven days after penetration

G... "U" shaped appearance, eight days after penetration

H... Posterior shift and ovary development, ten days after penetration

I... Development of the uterus and elongation of ovaries, eleven days after penetration


A
B
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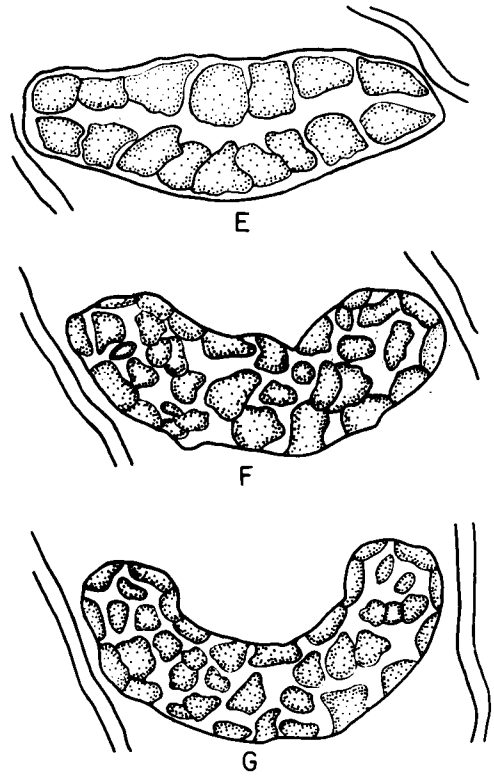
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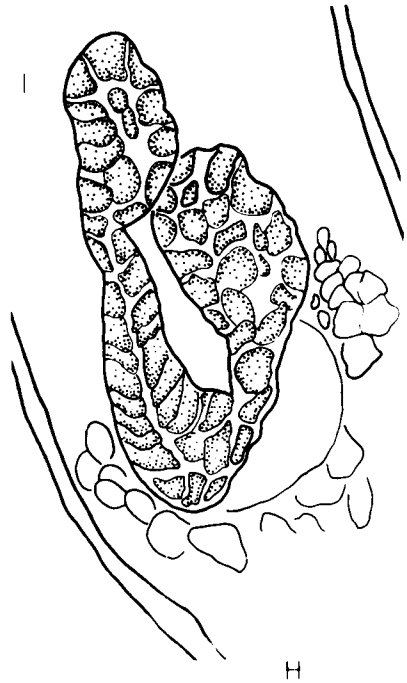
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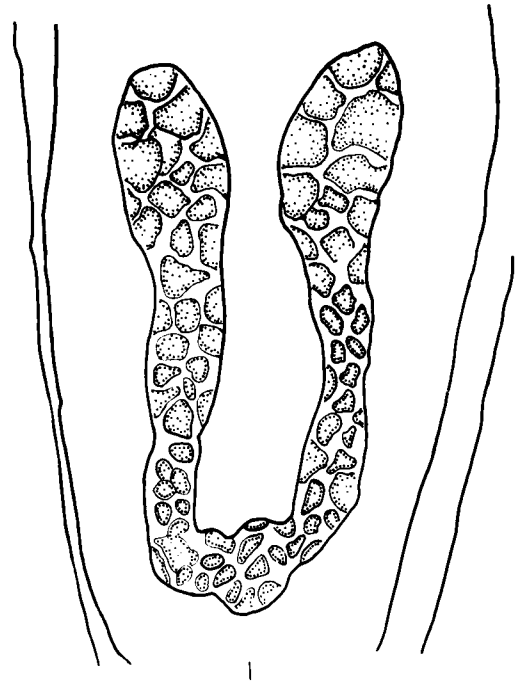

I

length and further developed, in 21, 16, 10 and 9 days after penetration at different temperatures stated before (Figure 1, H. 700x). Formation of two ovaries was obvious as the genital primordium became "U" shaped with ends directed anteriorly (Figure 1, I. 700x). The following day formation of two ovaries was distinct at all temperatures. The ovaries began to reflex rapidly increased in length (Figure 2, J-K. 300x), with each passing day. The vulva started forming about this time. Twenty-nine, 24, 18, 15 and 10 days after penetration, the ovaries half filled the body cavity, increased further in length and coiled two to three times (Figure 2, L. 300x). Formation and the maximum length of ovaries appeared to be completed by 33, 27, 21, 17 and 12 days after the initial penetration of the second-stage larvae. By this time the ovaries had coiled several times and almost filled the body cavity. Formation of vulva and vagina now were complete (Figure 2, M. 200x).

There was very little difference in relative time for the development of genital primordium at different temperatures. Only at 80° was there a slight difference when the genital primordium apparently developed before the fourth molt. The genital primordium was fully developed after the fourth molt at 60, 70 and 75° F. At 50° F the genital primordium was not completely developed until two to three days after the fourth molt. At the 75 and 80° F temperatures, growth was very similar during the first nine days after penetration, however,

maturation was five days earlier at 80°F.

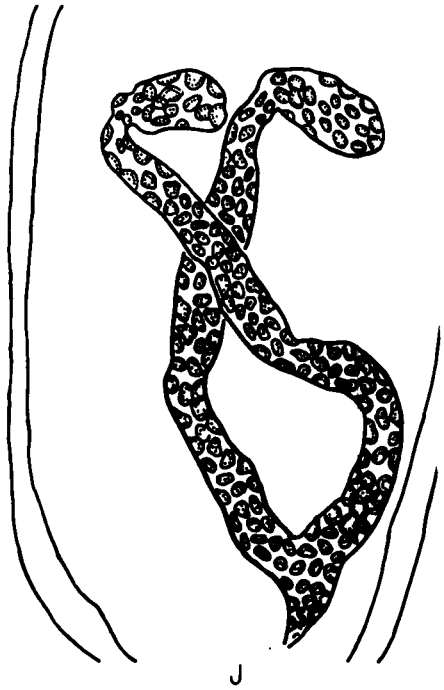
Figure 2. Various developmental stages of the genital primordium of H. trifolii at 70° F soil temperature. (continued)

J. . . Reflexing of ovaries, 12 days after penetration (300x)

K. . . Development of vagina and vulva, 13 days after penetration (300x)

L. . . Additional elongation of ovaries and unification of reproductive system, 18 days after penetration (300x)

M. . Complete reproductive system, 21 days after penetration (200x)



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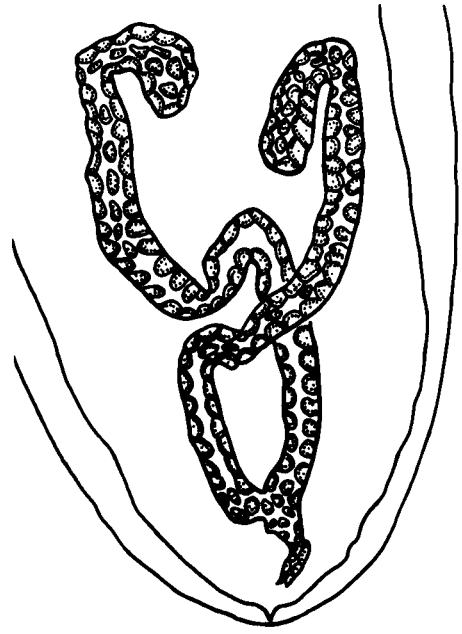

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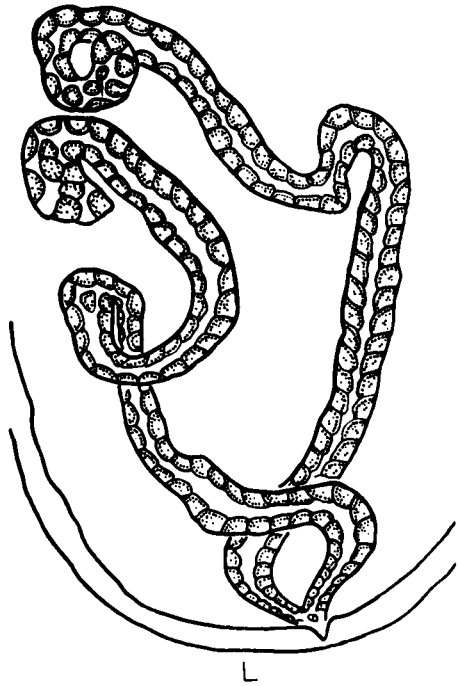

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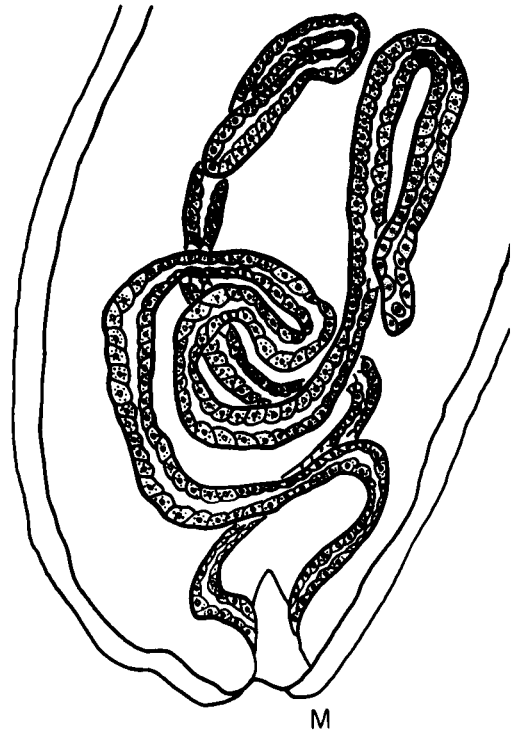


M

HOST RANGE

Seeds of 15 different plants (legumes and non-legumes) as listed on page 42 were planted in number 10 cans in the greenhouse. Hairy vetch plants were used as check. After inoculation, five replications were made and the plants (15 cans each in number) were randomized within each replication. Sufficient water was given to insure adequate moisture for growth of plants. Fish fertilizer was applied twice, during the period of growth in order to maintain healthy and normal plant growth.

The plants were maintained in this condition for approximately 50 days. After this period the plants were removed from the cans very carefully, without injuring the roots. This was accomplished by turning the can upside down, supporting the plants and soil by one hand and gently hitting the can against a dry hard surface with the other hand. By doing this the whole lump of soil with the plants was shaken out of the can. Soil with the plants was then placed in a larger container, which was half filled with water. This method was adopted to soak the soil so that it could be easily removed without damaging the root system. The plants were then placed under tap water and remaining soil particles were removed. Later on, the roots were placed in a large dish pan half filled with water to separate the roots from each other so that examination for presence of cysts could be accomplished easily.

When the presence of brown cysts attached to the roots was suspected, the root portion was cut off and examined with a dissecting microscope. When cysts were actually observed protruding from the roots, the plant involved was regarded as a host.

Results of the host range studies point out that all leguminous plants tested (six) were infected by H. trifolii. The degree of severity varied among the test plants. Results were obtained by counting the number of cysts found on the root system of each plant. Alfalfa, clover and vetch had the maximum number of cysts (80, 100 and 100 respectively). Bean and pea had 40 cysts, soybean had only 20 cysts.

Of the nine non-leguminous plants, only cucumber, pumpkin and squash were infected. But the infection was not high in comparison to some of the leguminous plants. Fifteen cysts were obtained from squash and eight from pumpkin and cucumber. Other non-leguminous plants were not infected and appeared immune.

The experimental results of host range studies are compiled in the following Table 4.

Table 4. Plants tested for the host range study of Heterodera trifolii.

Family	Plants	Variety	Binomial Name	Susceptibility
Leguminosae	alfalfa	Ranger	Medicago sativa L.	Infected
Leguminosae	bean	Bush	Phaseolus vulgaris var. humilis Alef.	Infected
Leguminosae	clover	White	Trifolium repens L.	Infected
Leguminosae	pea	Perfection	Pisum sativum L.	Infected
Leguminosae	soybean	Capital	Glycine max (L.) Merrill	Infected
Leguminosae	vetch	Hairy	Vicia villosa Roth	Infected
Gramineae	barley	Hannchen	Hordeum vulgare L.	Resistant
Gramineae	oat	Atlas	Avena sativa L.	Resistant
Gramineae	rye	Perennial	Lolium perenne L.	Resistant
Gramineae	wheat	Elmar	Triticum vulgare Vill.	Resistant
Cucurbitaceae	cucumber	Niagara	Cucumis sativus L.	Infected
Cucurbitaceae	musk melon	Casaba	Cucumis melo L.	Resistant
Cucurbitaceae	pumpkin	Conn. field	Cucurbita pepo L.	Infected
Cucurbitaceae	squash	Zucchini	Cucurbita maxima Duchesne	Infected
Cucurbitaceae	water melon	Giant Klondike	Citrullus pepo var. medullosa Alef.	Resistant

There was significant difference between the weight of infected and non-infected plants. Infected alfalfa, clover and vetch weighed 6.2, 4.1 and 5.9 grams respectively, whereas non-infected alfalfa, clover and vetch weighed 18.0, 16.2 and 17.4 grams respectively.

Figure 3. Roots of hairy vetch infected with cysts of H. trifolii.

Figure 4. Symptoms of clover cyst nematode, H. trifolii, on white clover.

LONGEVITY

Effect of Wet and Dry Conditions at Different Temperatures

Ten glass vials (3.5 x 1.2 mm.) were used as storage containers. Of the ten vials, five were half filled with water, and the remainder were kept perfectly dry. Twenty cysts were transferred to each of the storage containers with a bamboo spatula, specially made for this purpose. The water, added to the dry vials while transferring the cysts, was removed with a dropper and blotting paper. Hence the cysts were placed in dry storage within dry containers. Two vials (one dry and one with water) were tied together and placed in a small glass jar (10.2 x 2.2 mm.). The jars were then placed in different temperature chambers ranging from -10 to 5°, 15°, 25° and 30° C.

All glass jars were stored at their respective temperatures for one month. After this period, a few cysts were taken out from each vial and inoculated to vetch seedlings in the greenhouse. This was done every month for a period of five months. Another 20 cysts were stored in water at -10° C on December 1959. They were inoculated after one and one-half years of storage in two cans with hairy vetch as an indicator plant.

When vetch seedlings were three to four inches in height, a few of them were removed and stained to determine if the larvae had survived, hatched and entered the roots. The remaining plants were then

left in the greenhouse for approximately 50 days. After which they were removed, and searched for the presence of adult females, attached to the roots. The temperature and storage periods in which the nematodes survived, developed, and reproduced were carefully noted. This process was repeated every month for five months to determine the length of time nematodes could survive in different moisture and temperature conditions.

The data on Table 5 indicates that encysted eggs did not survive the storage in dry and wet conditions at 30.0° C and -10.0° C even for a single month. The cysts only survived two months in wet storage at 5.0° , three months at 15.0° C and four months storage at 25° C. Encysted eggs stored dry, only survived a storage period of one, two and three months at 5, 15 and 25° C. From these results it is evident that encysted eggs, stored in water, survived longer than those stored dry. Although one collection of cysts were stored in wet condition at -10° C for one and one-half years, the encysted eggs did not survive beyond one month of storage.

Table 5. Effect of temperatures and storage conditions upon survival of H. trifolii cysts.

Temperature	Condition	Period of Storage in Months				
		1	2	3	4	5
5.0° C	Dry	S	D	D	D	D
	Wet	S	S	D	D	D
15.0° C	Dry	S	S	D	D	D
	Wet	S	S	S	D	D
25.0° C	Dry	S	S	S	D	D
	Wet	S	S	S	S	D
30.0° C	Dry	D	D	D	D	D
	Wet	D	D	D	D	D
-10.0° C	Dry	D	D	D	D	D
	Wet	D	D	D	D	D
-10.0° C	Wet	Stored for 1-1/2 years*				D

S - Survived
D - Died

* Cysts, that had been stored in a wet condition for 1-1/2 years, were available for this study.

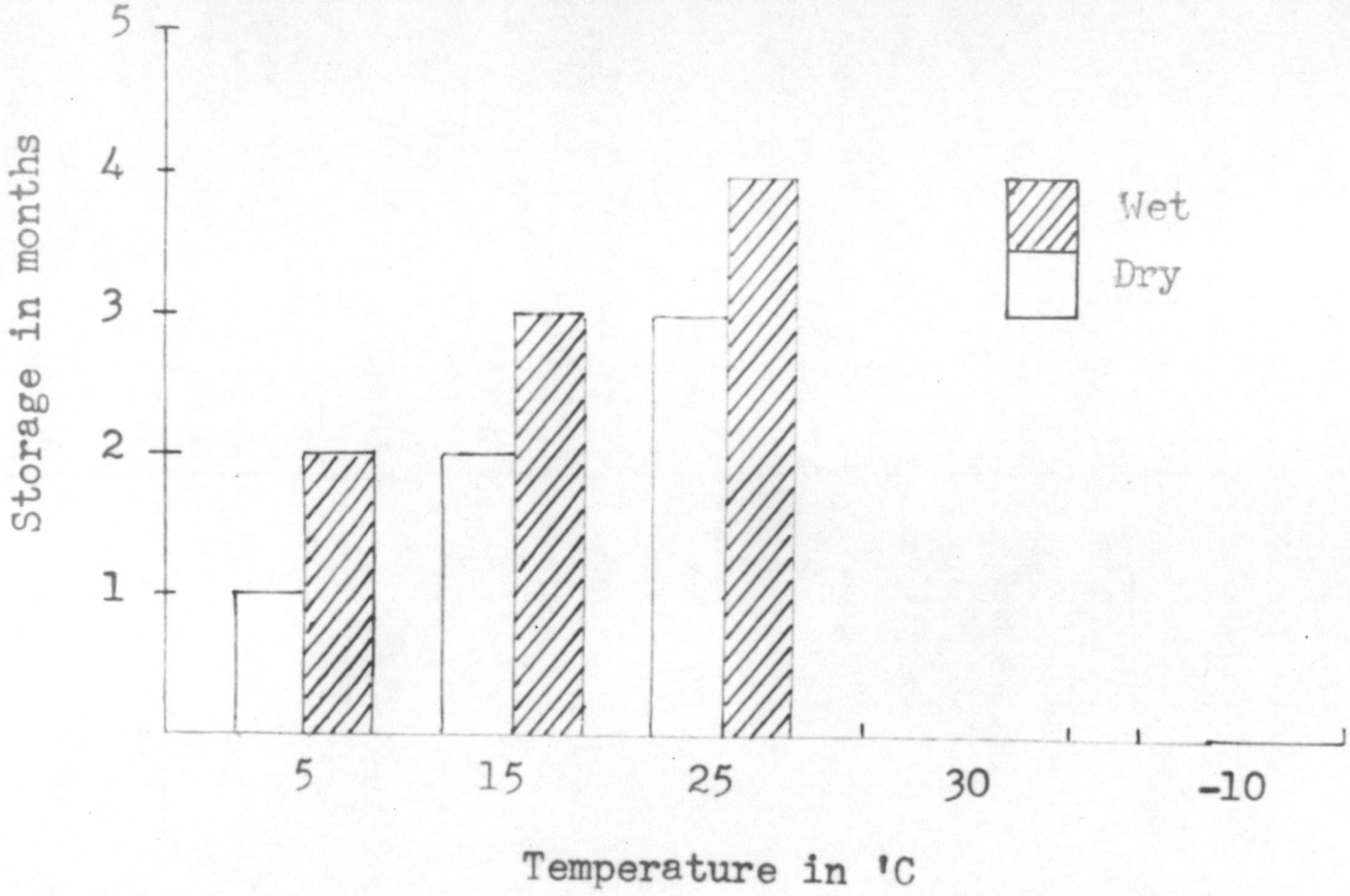


Fig. 5 Survival of encysted eggs stored dry and wet

Effect of Soil Moisture upon Development and Reproduction

Hairy vetch was planted in number 10 cans with six pounds of soil in the greenhouse and was inoculated with a suspension of nematodes. Water was added in the soil from 50-150 ml. initially and moisture percent of 1.83-5.51 was maintained. The process was carried on for a period of two months after which the roots were removed, washed free of soil, and examined for presence of adult females. The effect of different moisture percentages upon development and reproduction of nematodes was noted.

It is evident from Table 6 that no reproduction occurred at 1.83, 2.20, 2.57 and 5.51 percent soil moisture level. Multiplication and reproduction started from 2.93 percent, reached highest at 4.40 percent and then slowly declined. Apparently both high and low moisture percentages are harmful for the development and reproduction of nematodes. The higher water content (130 and 140 ml.) did not seem to have any appreciable harmful effect upon nematode survival and multiplication. Clover cyst nematode fails to develop at a soil moisture level below 2.93 percent or above 5.14 percent.

Effect of pH upon Larval Production

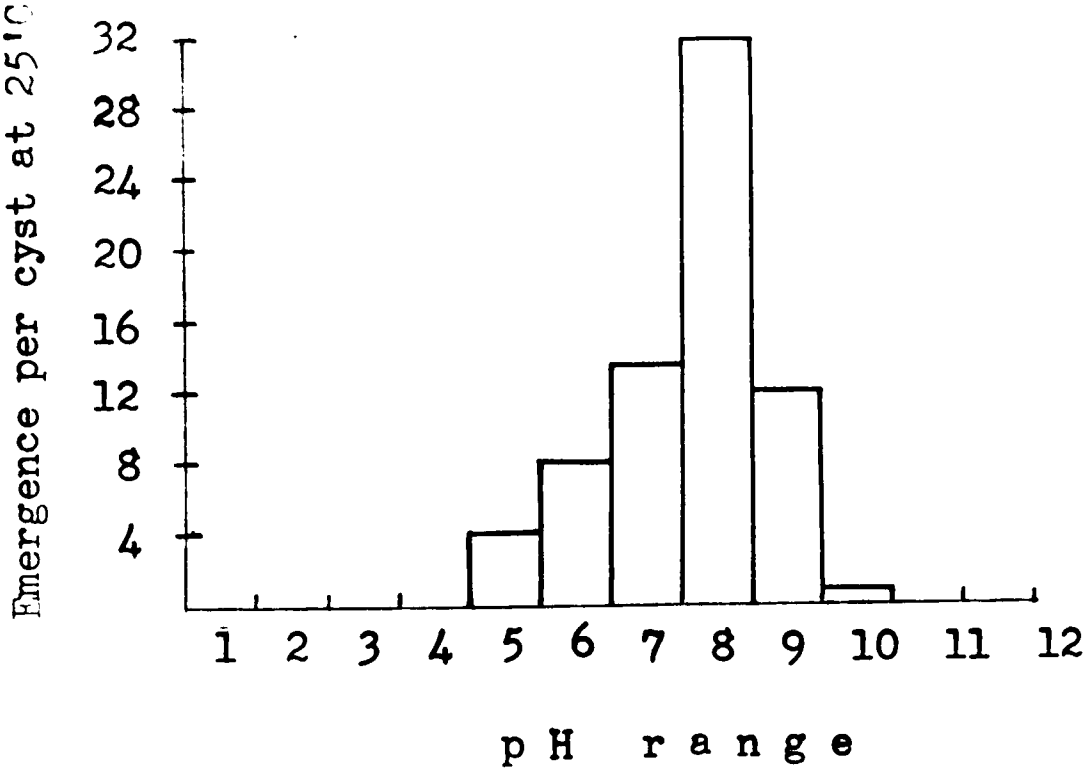
Distilled water was used for this experiment and pH was varied by adding hydrochloric acid (for lower range) and sodium hydroxide (for higher range). The range of pH was varied from 1 to 12. About 25 ml. of the adjusted solutions was poured in each of 50 petri dishes.

Table 6. Survival of H. trifolii at different soil moisture contents.

Water Content	50 ml	60 ml	70 ml	80 ml	90 ml	100 ml	110 ml	120 ml	130 ml	140 ml	150 ml
Cyst obtained	-	-	-	50	65	80	90	105	80	70	-
Moisture %	1.83	2.20	2.57	2.93	3.30	3.67	4.04	4.40	4.78	5.14	5.51

Table 7. The effect of pH and temperature upon larval emergence from H. trifolii cysts.

pH Range	1	2	3	4	5	6	7	8	9	10	11	12
Emergence/cyst at 20° C	-	-	-	-	4	8	14	32	12	1	-	-
Emergence/cyst at 25° C	-	-	-	-	5	10	15	22	15	2	-	-



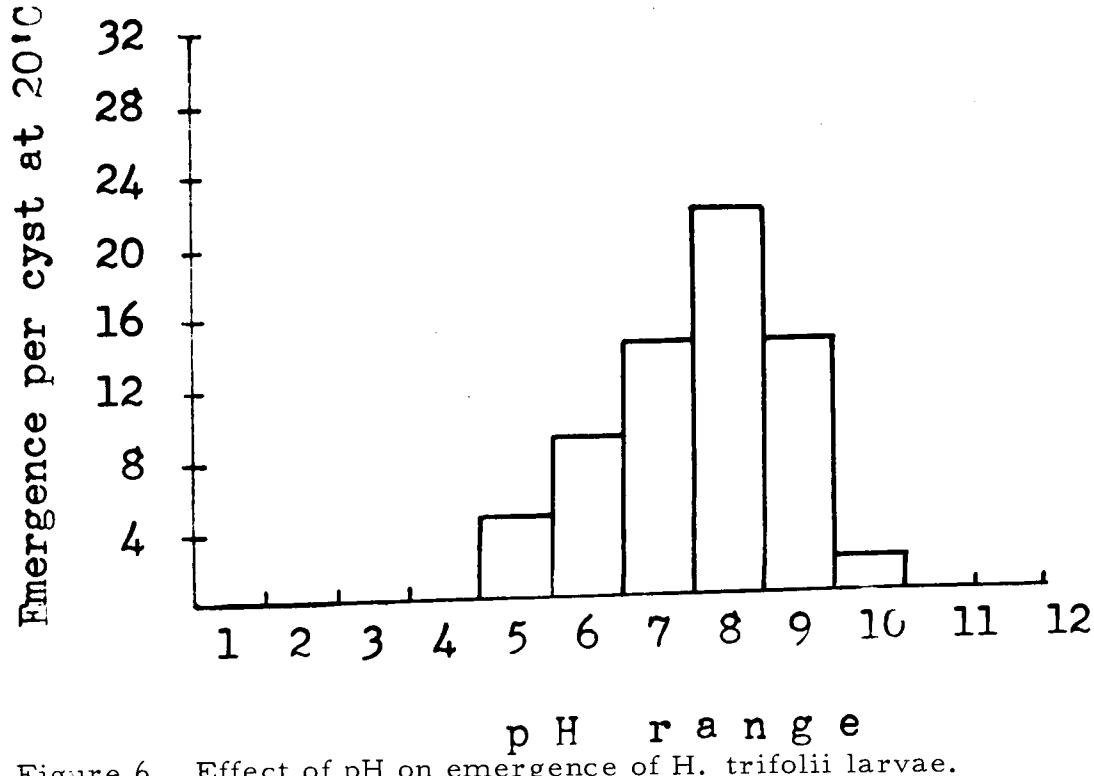


Figure 6. Effect of pH on emergence of H. trifolii larvae.

Approximately 25 cysts were transferred to each dish, then they were incubated at 15, 20, 25, 30° C, and room temperature, 24° C, for a period of 12 days. From the sixth day onwards, the emerging larvae were counted each day. The effect of pH combined with temperature, upon the emergence of larvae was recorded, and stated in Table 7.

There was no emergence from the encysted eggs incubated at 15° C or 30° C. The results indicate that the clover cyst nematodes prefer a neutral condition of soil. Optimum pH for larval emergence was at pH 8. There was no emergence at pH 1-4 regardless of temperature. Also larvae did not emerge at pH 11-12. Emergence of larvae started at pH 5, but the number of emerged larvae was only four or five per cyst. At pH 8, the hatched larvae numbered 22 to 32, which was the maximum. After this, the emergence decreased and at pH 9 was 12, at pH 10 emergence was limited to one to two in number. Regardless of the specific pH conditions, larvae failed to emerge from cysts stored at 15 and 30° C. Routine checking of the pH for adjusted solutions revealed that the pH increased. This increase was approximately 0.1 unit but never exceeded 0.2 unit. The difference was corrected immediately and the solutions were returned to the original pH level.

Effect of Vacuum on Nematodes

Twenty-four small number 2 cans (3.07 x 4.09 inches) were obtained for this experiment consisting of five treatments, four

replications and four controls. Each can was half filled with sterilized soil. Ten viable mature cysts were wrapped in tissue paper and placed in each can. This was done to aid in finding the cysts within the soil without difficulty. The remainder of the can was then filled with sterilized soil. Then the cans were vacuum sealed. The range of vacuum was varied from 5, 10, 15, 20 and 25 inches vacuum. A jet of steam was passed into the cans (for a very short time) to create the vacuum which altered the pressure inside the cans. The pressure was noted on the pressure-meter placed on the side of the canning machine. Four cans were used as controls in which pressure was not altered. After storing the cysts in this condition for 15 days, the cans were opened and cysts were removed and inoculated to hairy vetch in the greenhouse. The effect of vacuum in development and reproduction was recorded.

Cysts subjected to 10, 15, 20 and 25 inches vacuum failed to produce any larvae from the encysted eggs. The encysted larvae subjected to five inches vacuum survived, but the percentage of emergence was very low. Only two cysts were observed on the roots of the indicator plant.

DISCUSSION

Although the host range study agrees closely with those conducted by other workers (13, 14, 32, 37), there were some outstanding differences particularly in the susceptibility of non-leguminous plants (Cucurbitaceae family). Of the nine non-leguminous plants tested, only cucumber (Niagara), pumpkin (Conn. field) and squash (Zucchini) were infected. Infectivity was not high in comparison to some of the leguminous plants. Differences were very minor, however, in the susceptibility of six leguminous plants used for the host range study. The results which indicate that bean (Bush), pea (Perfection) and vetch (Hairy) are hosts of the clover cyst nematode, differ from reports of other workers (13, 37, 16, 14).

There were significant effects from nematodes feeding on the roots of the leguminous plants. The cortex became necrotic at the invasion site and giant cells were formed at the region of feeding. The formation of a large number of branch rootlets resulted in a matted appearance. Above ground symptoms were stunting and unthrifty appearance of the plant. In the non-leguminous plants, only giant cell formation was observed.

Host root diffusate is known to stimulate the encysted eggs. In this study, root diffusate was used only to hatch the encysted eggs for

larval production. Satisfactory results were obtained from the use of root diffusate, although Fuch (54) did not consider the presence of the diffusate as an important factor.

Males were not observed while examining numerous greenhouse cultures during the course of these studies. The females are apparently parthenogenetic and a new generation occurs from unfertilized eggs. Time taken for development of a new generation varies according to the soil temperatures. Mulvey (33) reported that 26 days were required for the larvae to become adults at an average soil temperature of 60° F, and 43 days for production of the second generation. In the life cycle studies 30, 26, 20, 16 and 15 days were required for the larvae to become adults at soil temperatures of 50, 60, 70, 75 and 80° F respectively. For the production of second generation larvae, 47 days were required at 50°, 40 days at 60°, 32 days at 70°, 27 days at 75°, and 22 days at 80° F. The period of time needed to complete a life cycle at 60 and 75° F agrees with Ichinohe's results for H. glycines. From the life cycle investigation it appears that a decrease in soil temperature increased the time required to complete a life cycle and vice versa, in case of H. trifolii.

Development of the genital primordium was observed from the second day after penetration of the host plant by second stage larvae. At higher temperatures, the genital primordium matured earlier than

at lower temperatures. At 80° F the genital primordium was fully developed before the fourth molt whereas at 60, 70 and 75° F the genital primordium developed to maturity as expected, after the fourth molt. The genital primordium was first observed to develop parallel to longitudinal axis of nematode body, but later, six days after penetration, turned to an angle of 90 degrees, assumed a "U" shape. The future ovaries, or the two ends of the "U" enlarged, then increased in length. As the length increased, the developing ovaries reflexed, coiled and almost filled the entire body cavity. The vulva appeared in a terminal position about the end of the fourth stage and developed completely after the fourth molt. At higher temperatures (88 and 91° F), the larvae did not continue to develop beyond the second day after penetration and apparently died. The genital primordium, in these cases, shrunk to much smaller size than the original. There was no significant difference observed in the size or shape of nematodes reared at different temperatures. At lower temperatures, more time was required for the larvae to complete a life cycle than at higher temperatures.

The survival of encysted eggs at different temperatures varied according to the period and type of storage. In this study, encysted larvae stored in water for two months at 5°, three months at 15°, and four months at 25° C survived. These results agree with those of Slack and Hamblin (45) for another type of cyst nematode, H. glycines. Clover cyst nematodes failed to survive when stored in water for

longer periods. Death of nematodes kept active in water, is probably caused by melting of a body constituent. Encysted larvae survived a dry storage condition for one, two and three months at 5, 15 and 25° C, but failed to survive when stored for one additional month at the same temperatures. Death of nematodes in dry storage was probably due to desiccation. The physiology of desiccation has not been investigated for such widely varying species as the Heterodera. Encysted eggs, under wet or dry conditions, failed to survive even one month's storage at a freezing temperature of -10° C. This agrees with the reports of other workers (12, 13).

The apparent difference of the pH study with that of Fenwick (8) was that he used root diffusates in place of the distilled water used in this study. It is well known that a root diffusate stimulates encysted eggs to hatch. Hence when Fenwick made pH solutions, the solutions probably had a stimulatory effect due to the presence of diffusate. Therefore his results for larval emergence may not be entirely due to the changed H-ion concentration of the solution, but due to the presence of a stimulatory agent, the root diffusate. It is apparent that larval emergence is dependent not only upon pH, but also upon the maturity of the cysts as well as temperatures in which the cysts are incubated. Fenwick did not notice any significant effect on emergence between pH 3.2-8.0, when hydrochloric acid was used to adjust the acidity of the potato root diffusates. Though root diffusates were replaced by

distilled water in this study, sufficient hatching of larvae occurred when hydrochloric acid was used to adjust the pH. Encysted eggs incubated at 15 and 30° C failed to produce any larvae. This could be due to the effect of temperature as sufficient larvae hatched from a number of cysts incubated at 20 and 25° C. Apparently the clover cyst nematode reproduces well in neutral soil conditions with a slight inclination towards acidity or alkalinity.

Nematodes have been reported to complicate the interpretation of soil moisture studies involving plants as indicators. The plants were used in this study only for producing root diffusates to stimulate and hatch the encysted eggs. It is likely that the inhibitory effect of a water logged soil on nematodes is a primarily a lack of oxygen. Johnston (1958) suggests that reduction in nematode population at saturation levels is primarily due to some cause other than excessive soil moisture. At low moisture level, nematodes do not reproduce. They probably die due to desiccation, starvation or some other factor. It is possible that a certain portion of added water is absorbed by the plants, and then evaporated during transpiration. This might reduce the moisture level of the soil in which the cysts were subjected, but the plants also produced root secretions which left a short margin to balance the deficit.

Most of the data on plant parasitic nematodes suggest that development is inhibited at low oxygen concentrations (60). By creating

vacuum, gaseous substances (namely oxygen) were eliminated from the pore spaces of the canned soil. A large reduction in number of cysts was observed on the roots at lower oxygen level. The reduction in number of cysts appears to be due to retarded development, hatch and invasion of emerging larvae. It is possible that lack of oxygen inhibited or retarded the hatching of the encysted eggs. Only a few larvae survived the reduced oxygen level and infected the roots of indicator plants.

The accepted assumption that the clover cyst nematode, H. trifolii, prefers leguminous plants should be amended. H. trifolii will attack non-leguminous plants (as in Cucurbitaceae family) though with lesser severity, under greenhouse conditions. The time required by larvae to become adults can be altered by varying soil temperatures. As the temperature was increased, less time was required for the larvae to become adults and conversely, more time was required when the temperature was decreased. The genital primordium reached full growth in all temperatures (except at 88 and 91° F), but at higher temperature the growth was completed before the fourth molt. At lower temperatures full growth was achieved after the fourth molt--which is the usual occurrence.

Optimum temperature for survival of encysted eggs was 25° C in a wet storage condition. Higher or lower temperatures have an inhibiting effect upon larval emergence. The optimum moisture percentage

for survival of nematodes in soil was 4.40 percent moisture content. Above and below this level the population declined. Emergence of larvae from encysted eggs reached a maximum at pH 8. Emergence declined and ultimately ceased at higher or lower pH ranges. Oxygen is required for emergence and for invasion of the host plant. Even a slight deviation from normal oxygen concentration in soil effects the survival of the nematodes.

SUMMARY

Fifteen plants were tested to determine their susceptibility to the clover cyst nematode, H. trifolii. All leguminous plants tested were infected by the nematode. The largest number of cysts were found on the root system of alfalfa, clover and vetch. Of the non-leguminous plants tested, only cucumber, pumpkin and squash were infected. These three plants are new hosts and they should be included in the host range of the clover cyst nematode.

Second stage larvae were reared at 50, 60, 70, 75 and 80° F to learn the effect of temperature on the time needed to complete a life cycle. As the temperature increased the time required to complete a life cycle decreased and vice versa. Development of the genital primordium in relation to temperature was studied. The results indicate that the genital primordium reached full growth as temperature was increased and at the highest temperature (80° F), maturity occurred before reaching the fourth molt. At lower temperatures maturity was reached shortly after the fourth molt. At 88 and 91° F, the nematodes were inactivated or killed.

Tests to determine the effect of temperature upon the survival of nematodes indicate that the encysted eggs can survive for a period of four months in wet conditions and three months in dry conditions. Beyond this time, nematodes failed to survive. The effect of different

moisture percentages (1.83-5.51) upon survival of nematodes indicate that the viable cysts survived at 2.93 to 5.14 moisture percent level with an optimum of 4.40. Above and below this level, the population declined. The effects of pH on the hatching of encysted eggs were determined by placing the cysts in different pH solutions. Emergence started with a pH of 5, reached a maximum at pH 8 and ceased after reaching a pH of 9.

Effect of vacuum upon viable cysts indicate that encysted eggs and larvae require oxygen for their growth and development. With the exception of five inches vacuum, no hatching occurred from the viable cysts.

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