A necessary diapause in root-knot nematodes. Observations on its distribution and inheritance in *Meloidogyne incognita*

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SUMMARY

Under optimal conditions egg masses of *M. incognita* almost ceased to hatch after twenty days. They still contained living undifferentiated eggs which could not be stimulated to develop and to hatch in the presence of host roots or their diffusates, or after dissociation by NaClO.

Most egg masses collected on tomato roots eight weeks after inoculation contained between 10 and 20% of those undifferentiated living eggs but some egg masses contained as many as 70 or 80% (log-normal distribution). This distribution was transmitted from one generation to the other, irrespective of the percentage in the original egg mass.

Literature data show that a diapause is necessary to explain persistance of soil infestation by root knot nematodes when host plant and climatic stresses are absent. Results presented here suggest that this diapause most probably occurs in *M. incognita* eggs at an undifferentiated stage.

RÉSUMÉ

Une diapause nécessaire chez les Meloidogyne. *Observations sur sa distribution et son hérédité chez* M. incognita

Après vingt jours en conditions optimales les masses d'œufs de *M. incognita* cessent pratiquement d'éclore mais contiennent encore des œufs vivants indifférenciés. Le développement et l'éclosion de ces derniers n'ont pu être stimulés par la présence de racines de plantes hôtes ou de leurs diffusats, ni par la dissociation des masses dans NaClO.

La plupart des masses d'œufs récoltées sur des racines de tomate huit semaines après l'inoculation contiennent entre 10 et 20% de ces œufs vivants indifférenciés mais certaines peuvent en contenir jusqu'à 70 ou 80% (loi de distribution log-normale). Cette distribution se transmet d'une génération à l'autre, quel que soit le pourcentage dans la masse d'œufs dont est issue la population.

Les données de la littérature montrent qu'une diapause est nécessaire pour expliquer la persistance de l'infestation du sol par les *Meloidogyne* en l'absence de plante hôte et de contraintes climatiques. Les résultats présentés ici montrent que cette diapause se produit très probablement à un stade d'œufs non différenciés chez *M. incognita.*

Meloidogyne arenaria, M. hapla, M. incognita and *M. javanica* respond to climatic stresses, e.g. soil dryness, by quiescence usually in the egg stage (Linford, 1941) but also in larvae (de Guiran, 1979). This quiescence cannot explain the long persistance of soil infestation by these nematodes in the absence of host plants (Martin, 1951). This persistance covers periods during which climatic conditions are favourable to hatching and to activity of hatched larvae resulting in the depletion of their food reserves and exhaustion (Van Gundy, Bird & Wallace, 1967).

Another biological mechanism must allow the species to retain their infective potentialities under these favourable conditions. Such a mechanism exists in *Meloidogyne naasi*. Nearly all the eggs are blocked at the unhatched L_2 stage and hatching is stimulated by chilling or by treatment with NaClO (Watson & Lownsbery, 1970).

Ishibashi (1969) stated that old or underfed females of *M. incognita* lay dormant eggs which are resistant to environmental stresses and nematicides and hatch under the stimulation of plant root emanations. However no details are given as to which stage this "dormancy" occurs. More recently Ferris, Du Vernay and Small (1978) observed that 74% of *M. arenaria* eggs hatched fairly rapidly, whereas the remainder hatched at a lower rate and appeared to be uninfluenced by root exsudates.

De Guiran and Demeure (1978) showed that in optimal conditions not all eggs of *M. incognita* hatch ; a few remain alive undifferentiated. Diapause has been considered a possible explanation for this phenomenon.

The terms used to qualify different categories of arrest of development have been reviewed by Evans and Perry (1976). In this paper, the following definition of diapause will be used : an arrest of development that can be spontaneous or externally induced and that is removed only after a minimum of time and/or by an exogenous stimulus other than a simple return to favourable conditions that characterizes quiescence.

The present paper gives results of observations and experiments indicating the stage at which a diapause most probably occurs in *M. incognita* (De Guiran, 1975 *b*) and can explain the persistance of soil infestation in the absence of host plant and climatic stresses.

Material and methods

A strain of *M. incognita* originating from a single female collected at Adiopodoumé (Ivory Coast) was reared on tomato cv. Montfavet H 63-5 growing in 18 × 18 cm pots filled with a mixture of sandy soil and plant compost, in a glass house at ca. 28°. Plants aged two weeks were inoculated with 2 000 larvae (day old). Egg masses were hand-picked from the roots eight weeks after inoculation.

Egg masses were hatched on 0.1 mm aperture nylon sieves placed in Syracuse watch-glasses with just enough liquid to cover the mesh. Each day subjacent liquid was changed and larvae counted in it.

Emergence of larvae in the soil was studied with a control of soil water potential by the osmotic method decribed by de Guiran (1975 *a*) and de Guiran and Demeure (1978). Egg masses were introduced in and retrieved from the soil by means of small perspex and nylon sieves. Larvae were extracted from the soil by an elutriation-centrifugation method (Demeure & Netscher, 1973). The sandy soil used was fully described by de Guiran and Demeure (1978).

When necessary, egg masses were dissociated by slow mixing in 0.5% NaClO. The viability of the eggs was verified by coloration with New Blue R (Shepherd, 1962). In all experiments, each treatment included four replications of five egg masses picked at random from a homogenous sample. All tests were conducted at 28°.

Results

Arrested development in living eggs

Preliminary observations has evolved the hatching of four replicates of five egg masses each in demineralized water and the daily counting of hatched larvae. Fig. 1 shows that the daily hatch was more or less constant during the first ten days and then decreased to zero after eighteen days. This ensured that hatching was achieved after 20 days in the following experiment (confirmed by many similar observations).

Eight replicates of five egg masses each were placed in demineralized water and eight others introduced in sandy soil kept at pF 3. When hatching ceased, i.e. after twenty days, the egg masses placed in the soil were recovered, the larvae being extracted from the soil and counted. Half of the egg masses from each treatment

Table 1

Larvae emerged during a twenty day period in water or in soil at pF 3, living eggs remained in egg masses and larvae emerged in water during twenty additional days from five egg masses of *M. incognita* (mean of four replicates)

	larvae hatched from 0 to 20th day	living eggs on 20th day		larvae hatched from 20th to 40th day
		embryos	unhatched larvae	
water	5898	355	37	15
soil	2656	266	120	water 17

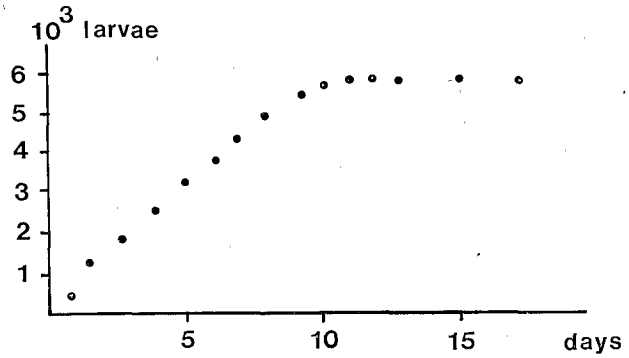

Fig. 1. Cumulative hatch in water of five egg masses of *M. incognita* during twenty days (mean of four replicates).

were stained for 12 hours in New Blue R and counted under the microscope (100 × magnification). The remaining egg masses were hatched during the next twenty days in water. Results of this experiment are given in Table 1.

As ca. 50% of the larvae are recovered by the soil extraction method used, it can be concluded that the number of eggs which hatched in water or soil was roughly the same. After twenty days, egg masses kept in water or in soil contained about the same number of living eggs, most of which were at an undifferenciated

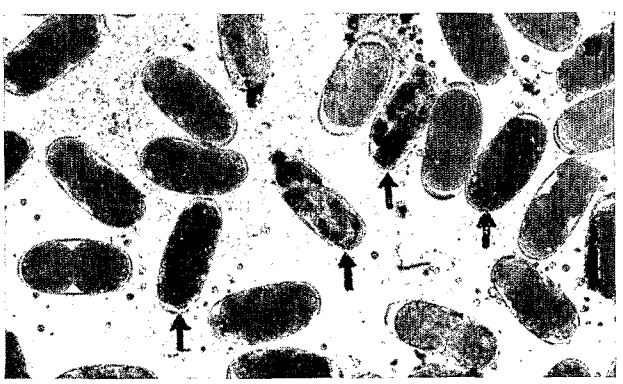

Fig. 2. Aspect of egg in an egg mass of *M. incognita* after complete hatch in water and 12 hours in New Blue R.Dark coloured eggs, shown by arrows, are dead. The others are living undifferentiated eggs presumed in diapause.

stage (embryos) (Fig. 2). After having been maintained in, or transfered to water for an additionnal twenty days, egg masses produced only a few more larvae. After 40 days egg masses from both treatments still contained about the same number of living eggs.

Effect of dissociation of egg masses

Forty egg masses were hatched in water. After twenty days twenty of them were dissociated in 0.5% NaClO, separated eggs collected on 10 µm aperture sieves and kept in water for 40 more days, like the twenty undissociated egg masses. Table 2 shows that dissociation does not stimulate the eggs to hatch at a higher rate than the non dissociated ones.

Table 2

Larvae of *M. incognita* emerged from five egg masses in water during a period of twenty days and an additional 40 days, with and without dissociation on the twentieth day (mean of 4 replicates)

larvae hatched from 0 to 20th day	larvae hatched from 20th to 60th day	
4909	12	not dissociated
5898	17	dissociated 20th day

dissociation

Effect of host plant

Twenty egg masses were hatched in water for twenty days. Subsequently they were placed in pots of denematised sandy soil planted with two-week-old tomato seedlings. Two weeks later, the seedlings were removed and their roots stained with cold cotton-blue lactophenol (de Guiran, 1966). Egg masses were recovered and stained during twelve hours with New Blue R. Examination of roots showed only traces of infection. The egg masses still contained living eggs : twelve unhatched larvae and 104 embryos per five egg masses (mean of four replicates). Thus the presence of host roots in the soil did not stimulate egg differentiation and hatching.

Resumption of development

A preliminary observation was made with egg masses collected on tobacco roots : four replicates of five egg masses were placed in demineralized water at 28°. Water was changed at daily intervals and the number of hatched lar-

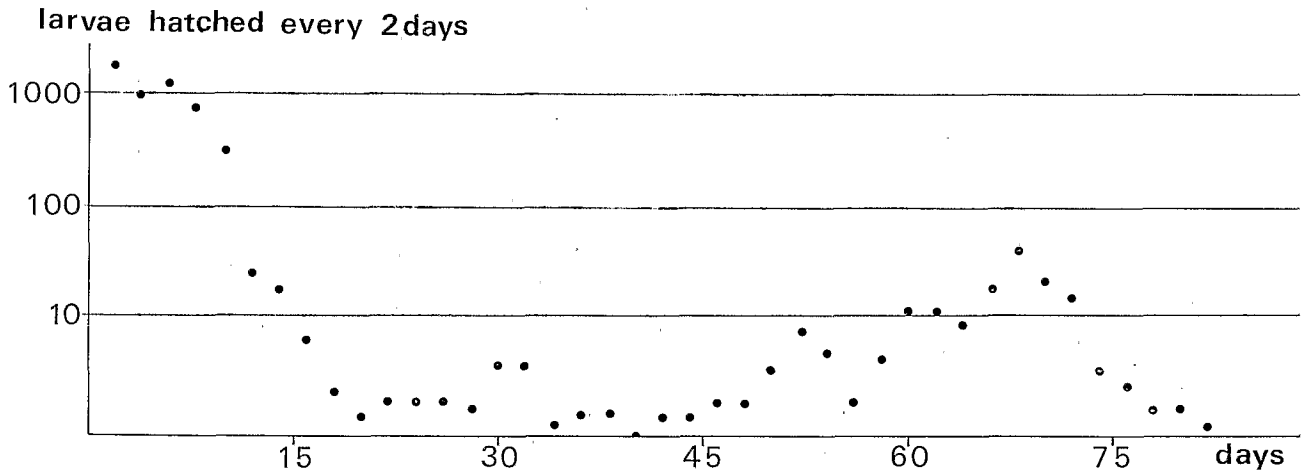

Fig. 3. Larvae of *M. incognita* that hatched every two days during 90 days from five egg masses kept in a film of demineralized water at 28° (mean of four replicates).

vae counted over a period of 90 days. Fig. 3 shows that, as in Fig. 1, hatching was sporadic after the twentieth day. But hatching resumed on the 50th day, reached a maximum on the 70th day and dropped to zero after the 80th day. At this time full eggs were not present anymore. Attempts to repeat this experiment with egg masses collected from tobacco or tomato roots were unsuccessful. Successive attempts to stimulate the resumption of development and hatch by exposing egg masses, stored under different conditions (anoxybiosis, anhydrobiosis), to growing tomato plants or to tomato root leachates also were unsuccessful.

Frequency

Thirty egg masses, collected at random from a homogenous sample, were individually hatched in water and the emerged larvae counted daily. After three weeks, when hatching had ceased, the egg masses were immersed in New Blue R and the remaining eggs counted under the microscope. Table 3 gives the number of hatched larvae, of the different categories of unhatched eggs and their variation coefficient. The "Total" is considered to be the total number of full eggs present in the egg masses at the time they were collected. The last column gives the same values as a percentage of this total. It can be seen from this table that, among unhatched eggs, the living embryos form the largest group and other categories can be neglected. Living unhatched embryos are considered to be in diapause and are expressed as a percentage of full eggs in the collected egg masses ; living unhatched larvae could represent eggs leaving the diapause state.

The frequency distribution of eggs in diapause within these 30 egg masses (Fig. 4) was fitted to distribution laws. A first approach by the method of maximal likelihood led to a log-normal distribution. The Kolmogorov-1 test gives a probability $P > 0.2$ that the observed frequencies fit this law. Fig. 5 shows that the cumulated observed frequencies (triangles) fit correctly the theoretical log-normal distribution, characterized by a zero frequency for the value zero.

Table 3

Numbers of hatched larvae and different categories of unhatched eggs in one egg mass of *M. incognita* (with their variation coefficient $\left(\text{V.C. \%} = \frac{s}{\bar{x}} \cdot 100\right)$ and corresponding percentages), after complete hatch (twenty days) in water (mean of 30 egg masses)

	Mean	*V.C.* %	%
Hatched larvae	1 075	22	78
Unhatched eggs			
Living : embryos	237	94	17
larvae	36	286	3
Dead : embryos	12	109	1
larvae	9	120	1
Total	1 369	14	100

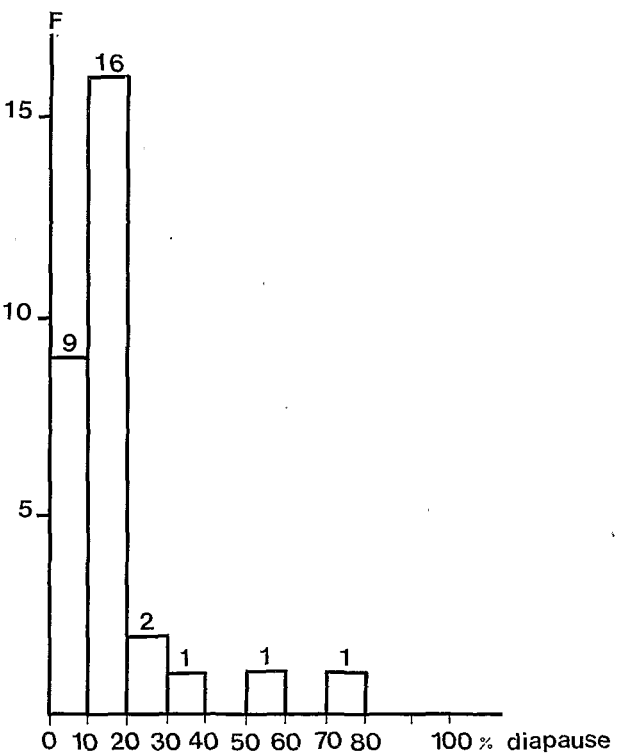

Fig. 4. Frequency distribution of percentages of diapause within 30 egg masses of *M. incognita* collected on tomato roots eight weeks after inoculation.

It can be concluded that within egg masses collected eight weeks after inoculation on tomato plants, the percentage of eggs in diapause are distributed according to a log-normal law. This means that in most egg masses, between ten and twenty per cent of the eggs are in diapause but, in a few cases, this rate will be much higher : 50%, 70% or more.

The question arises whether the above frequency distribution is found among successive generations, irrespective of the percentage of diapause in the original egg mass, or whether the percentage of diapause in an egg mass is transmitted to its offspring. To answer this, the percentages of diapause within the offspring of each of the 30 egg masses used in this experiment were compared.

Inheritance of diapause

Larvae hatched from each of the original 30 egg masses (mothers) used in the preceding experiment were inoculated onto separate tomato seedlings. Eight weeks later, ten egg masses (daughters) were collected from each plant and hatched together in water for twenty days. The unhatched eggs were counted for evaluation of the percentage of diapause within the daughter egg masses. Fig. 6 shows that there is no correlation between the percentage of diapause of mothers and daughters (r = .01019).

In Fig. 5 the data representing the daughters are the means of 10 egg masses. For a more

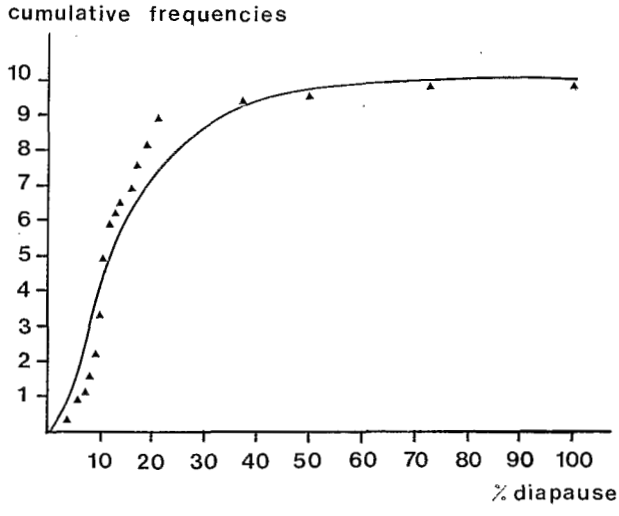

Fig. 5. Observed cumulative frequencies of percentages of diapause within 30 egg masses of *M. incognita* collected on tomato roots eight weeks after inoculation, compared with theoretical log-normal distribution having the same parameters (continuous line).

Table 4

Percentages of diapause in the offspring of three females of *M. incognita* having laid high, mean and low numbers of diapaused eggs

Mother (n = 1)	*Daughters (n = 1)*		*Grand-daughters (n = 10)*
	12,9	→	25
	75,5	→	9,1
	—	→	5,6
	20,5	→	7,9
	13,5	→	11,4
6,3%	17,3	→	31,3
	10,9	→	24,5
	18,5	→	7,6
	11	→	12,8
	85,2	→	6,8
	$\bar{x}$ = 29,5%		$\bar{x}$ = 14,2%
	6,8	→	46,4
	15,6	→	11,6
	15,4	→	18,2
	9,8	→	13,8
	11,5	→	15
17,2%	8,3	→	38,7
	10,8	→	4,8
	14,8	→	—
	6,7	→	20,6
	10,4	→	13,5
	$\bar{x}$ = 10,6%		$\bar{x}$ = 20,3%
	15,6	→	14,4
	13,7	→	29,2
	14,7	→	24,6
	6,9	→	25,6
	28,6	→	11,4
73%	16,5	→	8,9
	40,4	→	14,5
	17,5	→	10,6
	5,9	→	12,5
	10,2	→	9,4
	$\bar{x}$ = 16 %		$\bar{x}$ = 16,1%

precise evaluation three mother egg masses were chosen, one with a low (6.3%), one with a medium (17.2%), and one with a high (73%) percentage of eggs in diapause. Larvae hatching from each of these three egg masses were inoculated on separate tomato plants and from each line thus established, ten daughter egg masses were selected and hatched separately. Larvae hatching from each of these daughter egg masses were also inoculated on separate tomato plants. From each of the 30 different lines obtained, ten grand-daughter egg masses were hatched together to evaluate the percentage of eegs in diapause. Examination of Table 4 does not suggest any relation between the percentage of diapause in an egg mass and that of egg masses issued from it. Kruskal-Wallis test applied to the data of Table 4 demonstrates a significant difference between the three groups of daughter egg masses and Dunn's test (Dunn, 1964) differentiates daughter egg masses originated from the egg mass with 6.3% eggs in diapause from the other daughter egg masses. This implies that larvae emerging from egg masses with few eggs in diapause would develop into females which produce egg masses with a high percentage of eggs in diapause. This conclusion may be due to chance as the number of egg masses examined was rather small. There is no correlation between levels of diapause of daughter and grand daughter egg masses and a Kruskal-Wallis test did not demonstrate any difference between the three groups of grand daughter egg masses where each percentage of eggs in diapause was determined on a composite sample of 10 egg masses. From this experiment one may conclude that no indication exists that the percentage of eggs in diapause in an egg mass is transmitted to its offspring ; the frequency distribution shown in Fig. 3 is obtained in any single egg mass line, irrespective of the percentage of eggs in diapause in the egg mass from which the line was issued.

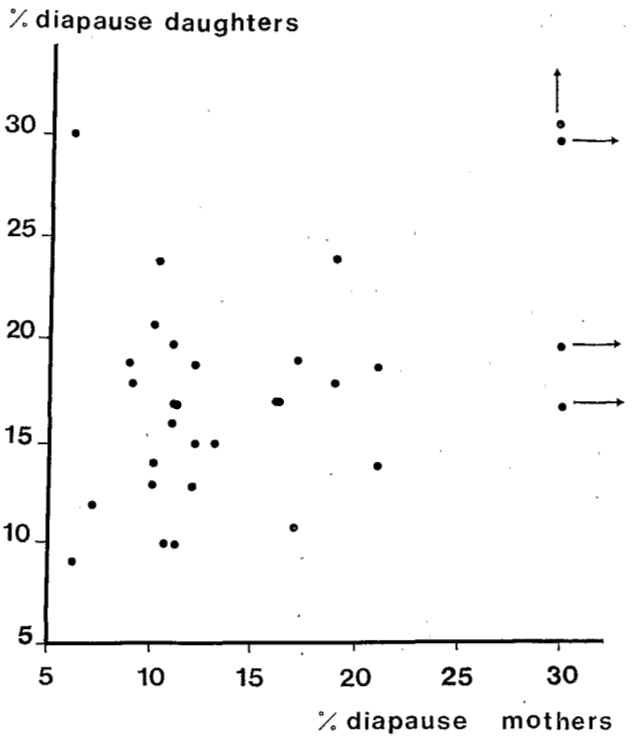

Fig. 6. Relationship between the percentages of diapause of 30 mother egg masses of *M. incognita* (abscisses) and, for each, the mean of the same value of ten of their daughter egg masses (ordinates).

Discussion

As stated in the introduction a diapause is an ecological necessity to maintain soil infestation by *Meloidogyne* when host plant and climatic stresses are absent. Such a situation frequently occurs in areas where these nematodes are found, for example between harvest and the begining of low temperatures in temperate climates or dry season in the tropics. The living undifferentiated eggs which do not hatch in optimal conditions most probably represent the stage at which this diapause occurs.

One may discuss the reliability of the New Blue R (N.B.R.) method to demonstrate the viability of eggs. Although other methods have been proposed (Jatala, 1975 ; Ogiga & Estey, 1974) there is probably no means to determine the viability of eggs with 100% accuracy, apart from sophisticated and time-consuming methods. However, the N.B.R. method is useful when high numbers of eggs are involved. Those eggs shown by N.B.R. to be alive should promptly decay if they were actually dead : it has been

shown (de Guiran, 1979) that they can remain intact for months in poor conditions like water-saturated soil. Another proof of their viability is their ability to hatch after 70 days in a constant and favourable environment. The unability to repeat this result most probably was due to the developing conditions of the egg masses, always difficult to control in a pot and glasshouse environment. Experimental attempts to remove diapause, needed to fully prove its existence, will always be faced with the difficulty of maintaining for long periods a sub-microscopic organism in a soil in which it has not developed naturally. During such attempts, the egg masses were frequently destroyed by various predators (collembolas, enchytraeids, etc.) and fungal parasites.

As noted above, many references suggest the existence of a diapause in *Meloidogyne spp.* and the work summarized by Ishibashi (1969) clearly describes such a phenomenon but the hatching of "dormant" eggs in response to plant root emanations may not be as positive as stated. From the work of Ferris, Du Vernay and Small (1978) it seems more likely that development of undifferentiated eggs in diapause resumes little by little over time. The constant presence of some unhatched larvae beside the diapausing eggs is a strong indication of this process of removal which is shown by other species: Merny (pers. comm.) has observed that cysts of *Heterodera oryzae* in soil slowly release larvae during more than three years. Ogunforowa and Evans (1977 *a* & *b*) have found that about 10% of the eggs laid by *Meloidogyne naasi* females fail to hatch in spite of chilling treatment and release larvae much later. It is noticeable that dissociation of *M. incognita* eggs by NaClO doesn't remove the diapause as in *M. naasi*. As most of the diapause occurs in this latter species on the unhatched L_2 stage this strongly oxydative substance perhaps only ruptures the egg shell and so releases the larva.

External mechanisms may control the hatching of nematode eggs in soil but the arrest of development of living *M. incognita* eggs has been also observed in monoxenic cultures on excised tomato roots (to be published).

The presence of a diapause in *M. incognita* as well as in *M. naasi* or many species of *Heterodera*, by balancing their highly specialized parasitism, ensures the perenniality of these organisms with its important consequences in agriculture : Martin (1967) has found that infestation of soil by *M. javanica* persists over 51 months of clean fallow in Rhodesia.

It is also important, when experimenting with egg masses of *Meloidogyne*, to be aware that most egg masses will contain ten to twenty per cent of unhatched eggs in diapause and even much more in some egg masses.

Many questions remain to be solved with regard to diapause in nematodes, a phenomenon widely overlooked until now. Investigations now in progress will perhaps help answer some of them.

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