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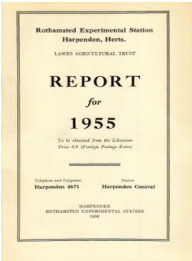

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THE HATCHING OF CYST-FORMING NEMATODES

By

D. W. Fenwick

The genus *Heterodera* contains a number of species of cyst-forming plant-parasitic nematodes. They are highly specialized morphologically and biologically, and one of their adaptations to parasitism is their selective response to substances which are produced by the roots of host plants and which are generally referred to as root diffusates. Before considering the work of the Nematology Department on this subject it is opportune to summarize the life history of a typical member of this genus. Soil infested with *Heterodera* spp. contains round or lemon-shaped bodies known as cysts about ½ mm. in diameter and brown in colour; these cysts may contain up to 700 eggs, each of which may contain a larva. In the absence of host plants larvae emerge only slowly—in the case of the potato-root eelworm *Heterodera rostochiensis* Woll. about 40–50 per cent in one year; the cysts can therefore remain infestive for several years. When a host plant is present diffusates produced by the growing roots stimulate the larvae to emerge rapidly—with the potato-root eelworm up to 80 per cent of the contained larvae emerge from cysts in the vicinity of plant roots in the first few weeks of the plant's life. Following penetration of the roots, development of the larvae results in the production of a new generation of flask-shaped females which protrude from the roots as white, rounded bodies containing large numbers of eggs. They lose their white colour and become brown, the body hardens and dies, the dead body acts as a protective envelope for the viable eggs and later becomes detached from the roots to lie freely in the soil. This is the cyst stage referred to above.

Hatching from beet-eelworm cysts (*Heterodera schachtii* Schmidt) was shown by early German workers to be stimulated by diffusates produced by the roots of host plants. Triffitt (1930) investigated this in detail. Working with *H. rostochiensis*, she concluded that potato roots would produce a diffusate capable of stimulating larval emergence only as long as root growth was actively occurring; the stimulatory factor was heat resistant and active in high dilutions; its breakdown was rapid under non-sterile conditions. Triffitt also confirmed the findings of Morgan (1925) that the diffusate from mustard roots was antagonistic to potato-root diffusate and did not stimulate potato-root eelworm; the substance was present in shoots and, unlike potato diffusate, was comparatively stable under non-sterile conditions. She also reported a dormancy period during the winter when larval emergence was extremely slow. In later papers (1932, 1934) Triffitt presented data showing that certain grasses produced diffusates which stimulated the hatching of potato-root eelworm. Franklin (1937) detected the same effects with diffusates of white mustard and yellow maize.

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Our present understanding of the problem throws considerable doubt on the reliability of a great deal of this work, which was based on the assumption, now known to be erroneous, that all cyst-forming species of *Heterodera* were biological strains of *Heterodera schachtii*; the very high level of variability exhibited by eelworm cysts, and the absence of sound statistical design, make it difficult to assess the validity of the findings. The main conclusions to be drawn appear to be that root diffusates can induce the emergence of larvae from cysts; their action is specific, i.e., a given species of eelworms can only be stimulated by diffusates from certain plants.

Work carried out in the Nematology Department on larval emergence of *Heterodera rostochiensis* can conveniently be divided into three main sections :

(*a*) The development of satisfactory techniques for the conduct of hatching tests.

(*b*) An examination of the factors influencing hatching in the laboratory and in the field.

(*c*) An attempt to elucidate the chemical nature of the active principle in potato-root diffusate.

Techniques

When hatching tests were commenced it was found that a number of factors rendered their conduct very difficult : the number of eggs per cyst within any population was very variable, which resulted in tests being subject to large and unpredictable errors : cysts from different populations differed considerably, both in egg content and also in their response to hatching stimuli, so that it was difficult to obtain results capable of generalized application. Nothing was known about the " quality " of the diffusate used, since the method for its collection was not amenable to rigid control, and the chemical constitution of the active factor was unknown.

Preliminary investigations involved isolating the larvae hatched from single cysts, for this a new slide was designed (Fenwick, 1943) as a refinement of the single-cyst techniques of Gemmell (1940) and Ellenby (1943). Using this slide, 50 cysts could be accommodated individually in glass cups on a 6 × 6-cm. glass plate. Using cysts from a number of populations and exposing them individually to diffusate drawn from a single bulk stock, it was possible to investigate the variability exhibited within and between different populations. The results of these investigations (Fenwick, 1949) showed that if batches of 100 cysts were used as units in hatching tests a basic minimum error of ±10 per cent was to be expected.

The use of units of this magnitude introduced new problems of technique, the most important of which was the counting of the thousands of larvae produced. Two modifications (Fenwick, 1951*c*; Peters, 1952) of the McMaster slide (Gordon & Whitlock, 1939) enabled this to be done by dilution. To overcome the tedium and labour in setting up replicate batches of cysts, a weighing technique (Fenwick & Reid, 1952) and later a sucking technique (Hesling, 1952) were developed. The use of either of these methods in conjunction with the mechanical purification methods developed by Hesling (as yet unpublished) have made it possible to conduct

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hatching tests on a larger scale than could previously have been contemplated.

The investigations of Elizabeth Reid (afterwards Widdowson) into problems of production and storage of root diffusates have led to a more standard and reliable product which can be preserved for several months with little loss of activity.

A difficulty associated with hatching tests has been the fact that larval emergence in the laboratory is a protracted business occupying up to 3 or 4 months. Preliminary investigations into the form of the hatching curve (Fenwick, 1950*a*) showed it to be sigmoidal in character when hatch was plotted against log time. Use was made of this relationship to forecast, at the end of 10–12 days, what the final hatch would be (Fenwick, 1951*b*). Although this shortened the total duration of a test, the need for daily counts during this period increased rather than decreased the total labour required on a test. Later work has shown this labour to be unnecessary, and it is now standard practice to run a test for 21 days, the hatched larvae being counted at 7 and 21 days. By this method 85 per cent of the hatchable larvae are recovered.

In any hatching tests, two classes of larvae are recognizable—those which hatch and those which do not. The former are counted when they emerge, but the latter are frequently of equal importance, and it is often necessary to estimate them quantitatively. Two techniques for estimating the unhatched larval content of cysts have been developed (Reid, 1952 and 1955) and are of great importance in potato-root eelworm research; they form the basis of the egg-hatching experiments referred to later, and are in general use for infestivity investigations outside Rothamsted.

As a result of the development of the above techniques it has been possible to lay down standard hatching procedures giving results of predictable accuracy: even so, a very high degree of replication is necessary if a reasonable degree of precision is to be obtained. In hatching tests conducted by the writer over several years, using weighed batches of 150–200 cysts in five-fold replication, the overall errors have been of the order of ± 20 per cent. This has been shown to be due, primarily, to the variability in the egg content of individual cysts. A new technique has been developed in which eggs, after removal from the cysts, are exposed to the action of diffusates. There is every reason to believe that the errors resulting from cyst variability can be significantly reduced by this method, with a substantial saving in material and effort.

The " dormancy period " experienced by Triffitt, when hatching was very slow, has been investigated, and techniques have been described (Fenwick & Reid, 1953) for the storage of cysts, which have enabled hatching tests to be conducted throughout the year, with no apparent diminution of hatching in winter.

The techniques so far described have all been concerned with measuring and reducing the errors resulting from the variability in egg content of *Heterodera* cysts. Variations in potency of natural diffusates are of equal importance, and a technique has been developed for standardizing a given sample (Fenwick, 1952*b*). If a sample of diffusate be diluted in a logarithmic series, larval emergence in each successive sample decreases in a linear manner. It was found possible, by plotting a curve, to calculate a

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" threshold " value of concentration beyond which a sample of diffusate was inactive. A strength of 1 arbitrary unit of concentration was ascribed to this " threshold ", and original concentration was expressed in terms of it—i.e., if the " threshold " for a sample corresponded to a 1/1,000 dilution, then the strength of the original sample was 1,000 arbitrary units. In practice a new term was used—the L.A. value (the logarithm of the concentration of the hatching factor)—which was proportional to the hatching ability of a given sample of diffusate. Using this technique, it has been possible to secure by dilution diffusates of known L.A. values and to measure quantitatively root-diffusate production and breakdown.

All the experiments so far described have been carried out on apparently " normal " cysts as recovered from naturally infested soil, i.e., they have not been treated with nematicidal fumigants. As far as the writer is aware, the relatonships so far mentioned in this review have never been tested on cysts exposed to nematicides. This point is worthy of emphasis, since many workers have sought to utilize hatching responses as a method of estimating kill following nematicidal treatment. In the absence of data showing the effect of chemical pretreatment on the parameters of the hatching dilution curves, interpretation of these workers' data is at most conjectural. Even if the same or new relationships for hatching can be applied to cysts treated with nematicides, the " hatchability " of larvae may not be synonymous with their " viability ". To regard the results of hatching tests as more than a mere indication of viability is an unjustified assumption. A thorough investigation into the relationship of hatchability to viability is an essential preliminary to the use of hatching tests in nematicidal work.

Larval emergence in the laboratory and in the field

Diffusate for hatching tests on *H. rostochiensis* is usually obtained from potatoes grown in 6-inch pots of 3 : 1 loam and sand, and knowledge is now available of some of the factors influencing its production. Elizabeth Reid has carried out experiments on the relationship of the age and variety of potato and tomato plants on root-diffusate production, and has investigated the effect of infestation with potato-root eelworm on root-diffusate production. Her work indicates that root-diffusate production is closely correlated with rate of increase of root growth measured by weight : total root weight plays a subsidiary but significant part. In the case of potatoes, diffusate production is at a maximum 5–6 weeks after planting, and this is the period of maximum rate of growth under experimental conditions. A large number of potato varieties has been tested, but there is little evidence of marked differences in the potency of the diffusates produced. Comparisons between potatoes and tomatoes showed that in the early stages of growth, diffusates from the latter were less active, but in older plants diffusate production in both was comparable; as potatoes require less attention than tomatoes, they are more suitable plants for root-diffusate production. Increased root-diffusate production resulted when potatoes were exposed to a low to medium rate of infestation with eelworm, which stimulated root proliferation. Heavy attacks stunted root growth and reduced the production of diffusate. Root-diffusate production appeared to be a function of growth. Elizabeth

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Reid has shown that root diffusate is produced equally well in soil, in sand with the addition of a plant nutrient or in sand watered only with distilled water; in the last case, however, production was not maintained for so long.

Root-diffusate production by plants other than potatoes and tomatoes has received attention. J. J. Hesling has repeated the work of Triffitt and of Franklin on the effects on *H. rostochiensis* of root diffusates of Gramineae. Fourteen species of grasses and five varieties of cereals were tested. Diffusates collected after three weeks growth did not stimulate the hatching of *H. rostochiensis*. He also compared diffusates produced by *Solanum andigenum* Juz. and Buk., *Solanum demissum* Lindl. and potato. All three species produced active diffusates, but as no account was taken of root size or growth rates comparisons among them were difficult.

Investigations by C. C. Doncaster showed that the nightshade *Solanum nigrum* L., which is not a host of potato-root eelworm, although it can be invaded, produced a very active diffusate. In the presence of even light infestations, diffusate production in young plants was reduced, but this effect was not so marked with older plants. Experiments on trap-cropping with this plant gave disappointing results.

Doncaster (1955) has also worked on the hatching responses of *Heterodera cruciferae* Franklin by diffusates produced by sprouts, swedes, rape kale and mustard. They are all active, although mustard is less so than the other three. There is no evidence that the addition of mustard diffusate to the others has any inhibitory effect. He finds that the hatching curves for these diffusates are sigmoidal and that their dilution curves are similar to those for potato-root diffusate.

Attempts by J. J. Hesling to obtain a diffusate which stimulates the hatching of *Heterodera major* (O. Schmidt) have been unsuccessful.

Experiments on the factors influencing the hatching of *H. rostochiensis* in the laboratory have been carried out (Fenwick, 1951*a*). Presoaking of dry cysts for 7–12 days in tap water before exposure to root diffusate increases the rate of hatching, but has no effect on the total number of larvae emerging from replicate batches of cysts. A constant temperature of 25° C. appears to be the optimum, but 30° C. inhibits hatching. Within wide limits larval emergence is not influenced by the volume of diffusate used nor by the number of cysts in that volume. Direct sunlight inhibits hatching, and this effect is permanent; cysts having been exposed to it, still do not hatch when transferred to the dark; diffused light is without effect. Hatching is unaffected by pH between 3·2 and 8·1. Hesling has found that if cysts of different sizes are exposed to potato-root diffusate there is a tendency for larvae to emerge from smaller cysts more readily than from the larger.

The hatch in water varies between the different species of *Heterodera*. In *H. rostochiensis* emergence is very low, but considerable spontaneous hatching can occur in the case of *H. cruciferae*, *H. trifolii* and *H. schachtii*; *H. göttingiana* and *H. major* do not normally hatch in water. The hatching of *H. major* has been investigated by Hesling, who found that it occurred in spring and summer as a result of a rise in temperature, the effect of which lasted

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6–10 days. Hatching from the cysts of *H. major* was inhibited by drying. When cysts were exposed to different relative humidities he found that those exposed to R.H. 98–100 per cent "hatched" equally well as cysts which had not been dried. Exposure to R.H. 87 per cent and lower inhibited hatching for a considerable time.

Larval emergence in soil and sand has not been investigated in such detail as it has in fluid media. J. J. Hesling found that hatching of *H. rostochiensis* in sand saturated with root diffusate was very similar to that observed in watch-glasses—the hatching curve was sigmoidal and its parameters did not differ substantially from those in fluid media. Other work on this subject has been done by Elizabeth Reid. She used shallow seed pans 12 inches in diameter filled with damp sand which had been uniformly infested with potato-root eelworm cysts. Known volumes of root diffusate were added to the centre of each pan twice daily for 8 weeks. After this time the larval content of cysts near the application point had fallen by 80 per cent, compared with a fall of 40 per cent near the perimeter.

Fenwick (1950*b*) investigated the effect of root diffusates from different varieties of potatoes in natural infestations of cysts on three different soils. In the absence of diffusate larval emergence over a season was about 50 per cent : in the presence of diffusate, 84 per cent of the larvae emerged. There was evidence of differences in response to diffusates from different varieties of potato and in the response of cysts from different localities to a single diffusate.

Hatching of *H. major* in soil under natural conditions has been shown by Hesling to occur at a steady rate from March to July during which time about 50 per cent of the cyst contents emerge. After July, hatching is considerably reduced.

Interesting results have been obtained by comparing hatching from free eggs of *H. rostochiensis* with eggs within their cysts. The general relationships were similar in both cases : the hatching curve was sigmoidal and the dilution curve was linear, although in general the rate was slightly faster in the case of free egg hatching. An interesting effect has been observed as a result of presoaking experiments. Eggs from cysts presoaked in water for 7 days hatched faster in root diffusate than those from dry cysts. If eggs from dry cysts were soaked, soaking appeared to inhibit subsequent hatching. This was the case whether particles of cyst wall were present during soaking or not—merely squashing cysts before soaking was sufficient to inhibit hatching of eggs. Soaking of whole cysts for as little as 1 hour counteracted this effect. No convincing hypothesis has been advanced to explain this curious result.

A factor which must influence larval emergence in soil is the degree of persistence of diffusate. The stability of potato-root diffusate under storage conditions has been investigated by Elizabeth Reid : she has confirmed Triffitt's findings that at room temperature it breaks down fairly rapidly; at 0–5° C. no appreciable loss in activity occurs over 12 months.

Fenwick has investigated its breakdown in soil. In a naturally infested heavy loam, breakdown was very rapid—90 per cent of the active principle was lost in 4–6 days, and the effect of a single application on the egg content of cysts was very small. When root diffusate was applied to horticultural peat, clay, sand and

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gravel its breakdown in peat was less rapid than in the other three media. When repeated applications of root diffusate were made to soil the breakdown of later applications was more rapid than of the earlier. Breakdown in partially sterlized soil was less rapid than in unsterilized soil. The author believes that in soil, breakdown is due to the action of micro-organisms which utilise root diffusate as a substrate. The results of these experiments cast considerable doubt on the practicability of using root diffusate as a method of eelworm control; it would appear unlikely that root diffusate can persist in soil long enough to have any appreciable effect in causing larvae to hatch in the absence of a host plant.

Chemical investigations

In 1951 a project for research into the chemical nature of the potato-root eelworm hatching factor was financed by the Agricultural Research Council to be carried out jointly at Reading, Cambridge and Rothamsted. At Reading, Professor R. H. Stoughton grew potatoes in order to produce the active diffusate; at Cambridge, Professor Sir A. R. Todd and his collaborators worked on the analysis of the active principle; at Rothamsted, the Nematology Department undertook the assay of "fractions" produced at Cambridge.

Preliminary isolation of the factor (Johnson, 1952) was accomplished by adsorption of the active principle from crude leachates on animal charcoal which was subsequently eluted with aqueous acetone. The hatching factor was found to be relatively stable between pH 2 and 7, but was rapidly deactivated at pH 8 and over. It was acidic, of low molecular weight and probably contained a lactone group. Treatment of the active brucine salt with acid followed by exaustive extraction with ether gave an acidic resin, active at concentrations of 1×10^{-7} to 1×10^{-8} and the name "eclepic acid" was proposed. Because of the heavy losses of active principle involved in this process, physical methods of purification were examined. Extraction of the crude concentrate with ether proved useful, as also did partition chromatography in silica gel buffered at pH 6. Titration of the product indicated an equivalent of 250–290; there was evidence of at least one double bond in the compound, and an approximate formula of $C_{19}H_{26}O_8$ was proposed.

Armitage (1955) attempted concentration by vacuum distillation. Phenol extraction of the product resulted in negligible recovery of the active principle. He then resorted to ion-exchange techniques. After preliminary passage through a cation-exchange resin (Zeokarb 225) the activity was retained on an anion-exchange column (Deacidite FF) and recovered from this by elution with 3*N*–HCl: the product had considerable activity, but phenol extraction of it resulted in considerable losses. Paper-strip chromatography of the organic acid fraction using a *n*-butanol–water–formic acid developer disclosed six acids. All attempts to obtain a solid derivative failed, as also did attempts to separate the water-soluble organic acids, whether by counter-current distribution, ion-exchange chromatography or partition chromatography.

After Armitage's departure from Cambridge in August 1955 the

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joint work with Cambridge and Reading ceased, but a series of joint small-scale experiments was commenced by D. W. Fenwick and G. N. Wiltshire at Rothamsted. As a result of cation exchange by charcoal adsorption and acetone elution, these workers reduced the dry weight of substances to 0·5 per cent of the original, with only a small loss of the active factor; this corresponded to a purification factor of ×184. Further work on this is in progress, but is not as yet far enough advanced for comment.

This review is a summary of the work carried out in the Nematology Department, Rothamsted. Valuable research is being carried on at several other centres, but there is not space to describe them in this review.

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