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OBSERVATIONS ON THE DEVELOPMENT OF *HETERODERA ROSTOCHIENSIS* WOLL. IN STERILE ROOT CULTURES

BY

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The use of a tissue culture technique in studying plant parasitic nematodes enables detailed observations to be made on a variety of problems. BERLINER & BUSCH (1914) attempted to culture *H. schachtii* on seedlings in agar and in the same year BYARS kept a root-knot nematode through one generation in aseptic culture. TYLER (1933) succeeded in carrying root-knot nematodes through several generations on tomato roots grown in nutrient agar and observed variations in the ratio of the sexes under different conditions. The susceptibility or immunity of host plants to nematode attack was investigated by LINFORD (1939). He was interested in the attractiveness of growing root and excised shoot tissue to a number of nematode species and found a more marked reaction in damp sand than in soil. His observation that the area behind the growing root tip was the most attractive was later confirmed by WIESER (1955, 1956). MOUNTAIN (1954) achieved successful infestations of sterile root tips of maize, tobacco and red clover grown in nutrient agar in Petri dishes with *Pratylenchus minyus*.

In a previous paper FENWICK (1956) dealt with the production of sterile viable larvae of the potato-root eelworm *H. rostochiensis*. The present paper is a record of observations on the penetration and development of sterile larvae in tomato roots grown under *"in vitro"* conditions.

TECHNIQUE

The methods of culturing the root tissues were those of WHITE (1943). Tomato seeds (variety Moneymaker) were sterilized by agitating for twenty minutes in a 5% bleaching powder solution and were

then washed twice in sterile water. They were sown singly in culture tubes containing approximately 15 ml of White's 0.75% agar medium per tube. After seven to ten days the radicle had grown sufficiently for the seedling to be removed, the root tip excised, and transferred to White's fluid medium, where growth was normal. Lateral root tips were removed weekly and transferred to fresh culture tubes of agar. The experiments described were made on excised tips from root cultures produced in this way, and kept at a temperature of 24° C.

Sterile eggs of *H. rostochiensis* were obtained by opening cysts and treating the free eggs with 20 volume hydrogen peroxide for from eight to sixteen hours, (FENWICK 1956). Although it is possible to hatch larvae from sterile eggs in sterile root diffusate, satisfactory results were obtained by introducing the sterile eggs directly to the root culture, where hatching was rapid. It did not matter whether the eggs were rubbed into the surface of the agar with a platinum loop or merely pipetted onto the surface; in the latter case the agar surface softened sufficiently for the eggs to sink down a few millimetres. The eggs were never introduced into the deeper layers of the agar, so that the later presence of larvae more than 2.5 cm below the surface must have been the result of hatching and active migration. Inoculation tests were made in this way in White's nutrient agar or in cultures of silver sand. When sand was used it was washed, flooded with the nutrient fluid and allowed to drain before being transferred to 6 × $^5/_8$ in. culture tubes for sterilizing.

Inoculation was accomplished by transferring a root tip, 1.0-1.5 cm long, to the surface of the sterile agar medium. When growth had commenced and the root tip had penetrated 2-3 cm, a suspension of sterile eggs was introduced to the surface of the medium. Successful inoculation could also be accomplished by adding the eggs to a young germinating seed. Hatching and subsequent migration always occurred whether or not the cotyledons and growing point had been removed.

In agar cultures it was possible to obtain rough estimates of hatch and migration by visual examination, but in sand cultures this was obviously impossible. Estimates of percentage hatch were obtained by shaking the contents of a tube with warm water to dissolve the agar, making up to a known volume and counting eggs and free larvae present in an aliquot.

The degree of penetration and development of larvae within roots was investigated by staining in acid fuchsin or cotton blue in lactophenol (GOODEY 1957).

RESULTS AND COMMENTS

Using these methods, large numbers of larvae usually hatched out of the sterile eggs a few days after inoculation. Counts of eggs and of free larvae in the agar showed that up to 70% of all larvae present had emerged in the presence of a growing root in about 4-7 days. Comparative figures for inoculations of tubes without roots were in the neighbourhood of 20-30% : it is therefore probable that root diffusate was being produced by the excised roots. Larval emergence in sand cultures was lower, due probably to variations in water content of the sand.

Four or five days after inoculation, concentrations of larvae in an active condition were usually visible at some of the root tips (Fig. 1). If very heavy concentrations of eggs were introduced into the tubes, the majority of the liberated larvae appeared to become inactive and die. As this apparent mortality was more pronounced at high densities, it may have been associated with local anaerobiosis. Fig. 2 shows a culture about seven days or more after inoculation. The concentration of larvae (many motionless) at root tip A is clearly visible and the path taken by them in reaching this point is marked by the trail of motionless larvae extending from the egg mass at the agar surface to the root tip. Such trails of larvae were frequently found, and an interesting feature is the apparent preference shown by larvae for certain root tips; thus, although the trail illustrated passes very close to the tip of root B, the larvae seem to have ignored it and to have concentrated at tip A. For no obvious reason some root tips appear to be less attractive than others to the hatched larvae. WHITE (1943) mentions that excised root tips are frequently encountered which are normal in appearance but which sink in the fluid medium and that these tips never grow well and are best discarded.

Minor concentrations of larvae were often found at isolated points along the main roots. Almost invariably, within a few days, lateral rootlets appeared at these points, indicating that larvae were anticipating the production of new endogenous rootlets (cf. WIESER 1955). In general larvae were attracted to lateral rootlets as well as to the main root tip which rapidly grew down to the bottom of the tube where oxygen concentration was presumably low; however after 14 days in culture some active larvae could still be found in these lower levels. With entire seedlings, the larvae congregated at the root tips, but with excised roots they concentrated a few millimetres behind the tip. This effect has also been observed by WIESER (1955) who found that larvae

of *Meloidogyne hapla* were repelled by excised tips 1-2 mms in length but attracted in increasing numbers as tip length increased to 8 mms; beyond this length attractiveness did not increase but remained at a high level.

Despite the apparently high mortality of larvae in agar, some remained viable long enough to penetrate the root tips. Penetration was frequently accompanied by localized swelling as seen in Fig. 3, although the effect is somewhat exaggerated in this photograph because the root has been crushed to expose the developing larvae within. Fig. 4 is a typical picture; the developing root tissue has hypertrophied to form a small gall containing the developing larvae. Although swellings sometimes occurred in the absence of invasions and conversely some invasions did not result in swelling, the association between the two was sufficiently good to serve as an indication of invasion when selecting roots for subculturing or staining. Galling also occurs under natural conditions when *H. rostochiensis* attacks tomato. Cyst production on this host is lower for any given initial level of infestation than on potato where no galling occurs (JONES 1957). *Meloidogyne* spp. cause more intense galling on tomato than does *H. rostochiensis* and are not themselves cyst formers. It is therefore probable that galling may be the result of specific interactions between parasite and host. The factors influencing it can at this stage be based only on conjecture, but should it be possible to isolate and culture galled tissues, then comparisons between their physiology and that of normal tissues might yield valuable information on physiological effects of parasitic nematodes on host plants.

Although developing larvae were found in all parts of the root system, the heaviest concentrations were in areas behind the root tips (O'BRIEN & PRENTICE 1931) or at the junctions of main and lateral roots. The area of root elongation behind the apical meristem is possibly more easily penetrable than other portions of the root system. Little is known about the mechanism of attractiveness. It is highly probable that attractive substances, which may or may not be identical with the hatching stimulus, are produced in this region, because it is around here that the greatest concentration of larvae is found in excised root tip cultures (c.f. GODFREY & OLIVEIRA 1932, GADD & LOOS 1941). At root junctions larvae may be able to penetrate more easily as the lateral roots break through the cortex of the primary root.

The larvae which penetrated the roots usually did so within about 14 days from the time of maximum hatching, which was about 4-7 days

after inoculation. Moving larvae were sometimes found up to 40-42 days after inoculation but it is doubtful whether they were still capable of invading the root tissues.

The development of larvae inside the tissues appeared to proceed normally, and the time taken for the completion of each larval stage corresponded closely with that given by RASKI (1950) for the development of *H. schachtii* at the same temperature, and with CHITWOOD & BUHRER (1946) for *H. rostochiensis* under Long Island conditions. Some experiments were stopped at different times to estimate the level of larval emergence and also to investigate the degree of penetration and subsequent development. At the end of 14 days, all stages up to the third moult and including some early fourth stage larvae were found. Fig. 5 shows a second stage larva just before the moult, the pointed tail of the second stage being visible. A third stage larva is shown in Fig. 6. This photograph shows a feature which was frequently present, namely a brown patch in the cortex surrounding the head of the larva (c.f. O'BRIEN & PRENTICE 1931). This also occurred in non-sterile soil cultures of tomatoes (DONCASTER 1953). It would appear from these observations that the patches are in some way due to the nematodes and are not a secondary result of bacterial invasion. The head of the worm can be seen embedded in the root tissue.

Fourth stage larvae were usually visible about 14 or more days after inoculation and Fig. 7 shows a typical fourth stage larva photographed *in situ* through the wall of a culture tube. Fig. 8 shows a third stage larva (arrow) beside a young adult female photographed *in situ* approximately 24 days after inoculation, the shreds of the fourth stage cuticle are seen attached to the developing adult. Fig. 9 is a fully developed male worm still ensheathed within the third and fourth stage cuticles. The spicules are visible (arrow). This specimen was stained and mounted *in situ* in lactophenol. Fig. 10 shows a fully developed female approximately 42 days after inoculation. Shreds of the fourth stage cuticle can again be seen and the root is typically thickened just above the nematode.

Although the majority of larvae that succeeded in penetrating the roots developed normally into adult males or virgin females, some undeveloped second stage larvae were visible in the roots as late as 42 days after inoculation. These may have resulted from late invasion but it is more likely that they were early penetrators which had failed to develop due possibly to some abnormal conditions in the roots.

No free males were seen on the surface of the roots and it is assumed

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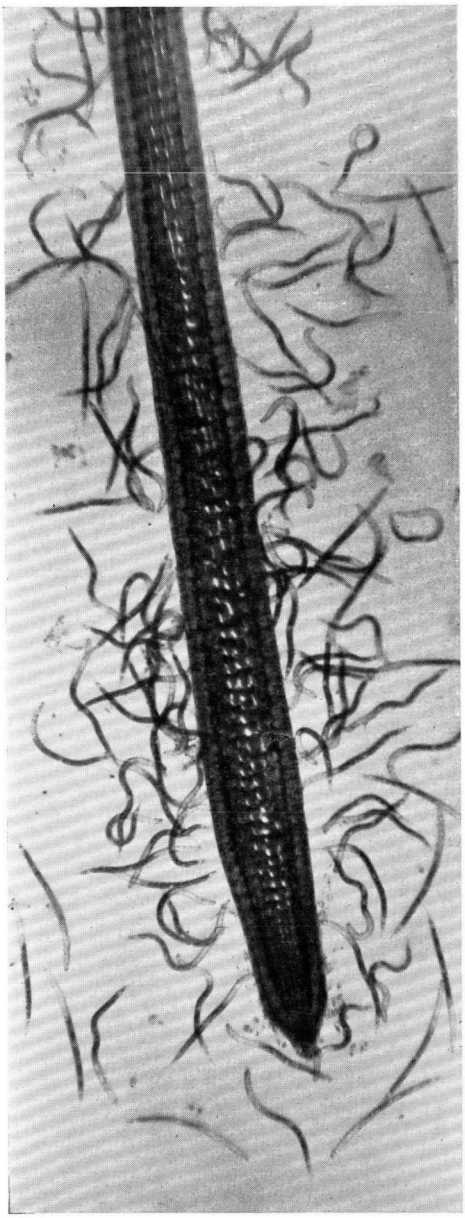

Fig. 1. Tip of excised tomato root in sterile agar, with a concentration of newly hatched *H. rostochiensis* larvae.

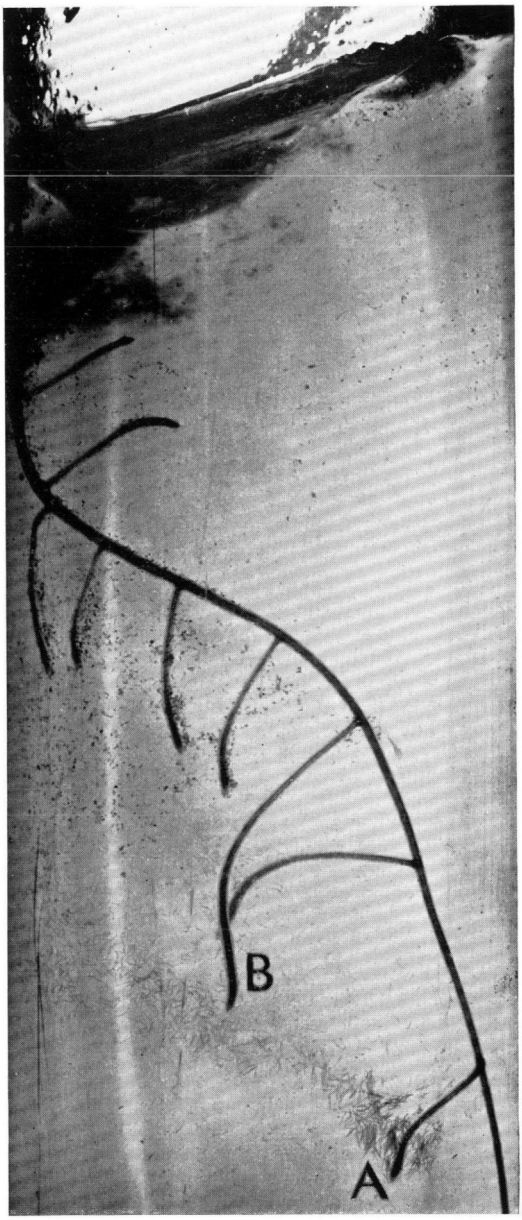

Fig. 2. Excised tomato root system in sterile agar culture. The distribution of nematode eggs and newly hatched larvae is shown.

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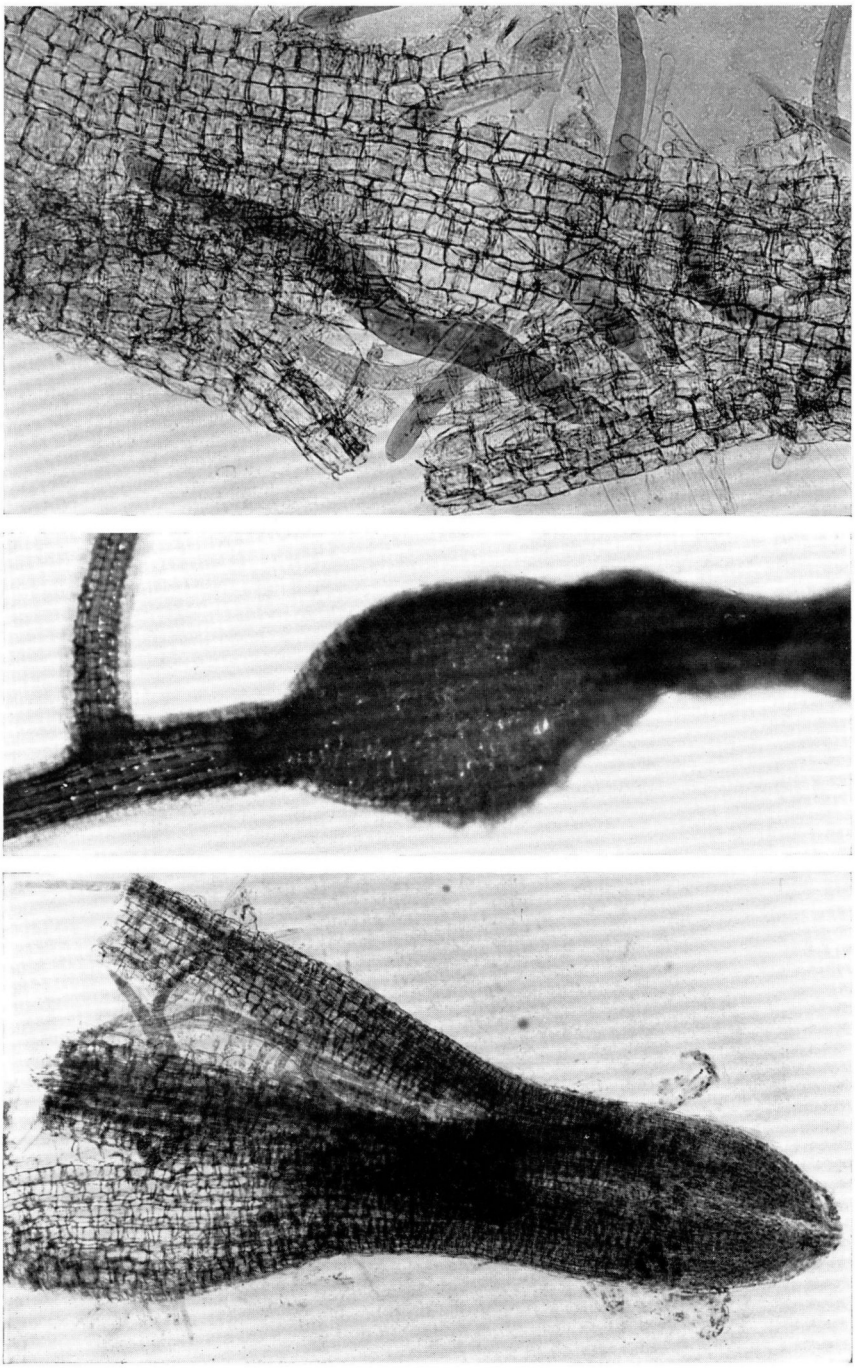

Fig. 3. Tomato root crushed to show a concentration of infesting *H. rostochiensis* larvae and associated swelling behind the tip. (Stained preparation.)

Fig. 4. Infested tomato root showing gall (Photographed in sterile agar culture.)

Fig. 5. Crushed tomato root with infesting larvae, two of which are in the advanced second stage. (Stained preparation.)

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Fig. 8. Adult female and a third stage larva of *H. rostochiensis*. (Photographed in sterile agar culture.)

Fig. 7. Tomato root with local swelling associated with a fourth stage *H. rostochiensis* larva. (Photographed in sterile agar culture.)

Fig. 6. Tomato root showing cortical necrosis associated with a third stage *H. rostochiensis* larva.

Fig. 9. Adult male *H. rostochiensis in situ* in tomato root. (Stained preparation.)

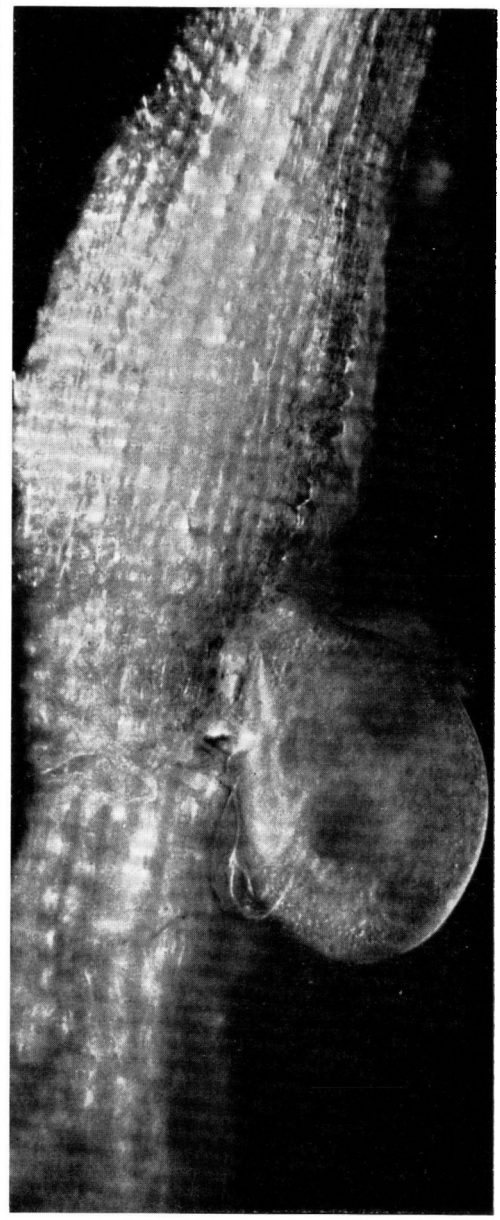

Fig. 10. Mature female *H. rostochiensis* in sterile agar culture, 42 days after inoculation

that on emergence they moved into the agar. A number of adult females were opened but contained no eggs. This may be due to conditions being unsuitable for fertilization or to inability of the host to maintain an infestation for so long a period under the experimental conditions. RASKI (1950) found fully developed cysts of *H. schachtii* 21 days after penetration. Here, none was in a suitable position for direct microscopic examination before about 35 days after inoculation.

ZUSAMMENFASSUNG

Einzelheiten über die Technik des Ansetzens von Infektionsreihen mit sterilisierten Larven des Kartoffelnematoden in Wurzelgewebe werden mitgeteilt. Die Wurzeln wachsen in 0.75% Nähragar und Nematodeneier, deren Oberfläche mit 20 Vol.-% H_2O_2 sterilisiert sind, werden hinzugesetzt. Vier oder fünf Tage nach der Infektion wurden Gruppen aktiver Larven rund um die Wurzelspitzen gesehen. Der stärkste Befall tritt gewöhnlich innerhalb von 21 Tagen nach der Infektion auf.

Die Parasiten scheinen sich innerhalb des Gewebes zu erwachsenen Männchen und jungen Weibchen normal zu entwickeln. Keine freien Männchen wurden gefunden und keines der erwachsenen Weibchen, die geöffnet wurden, enthielt Eier.

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ERRATUM

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Seite 218, 10. Zeile von unten muss es „kein" (anstatt „klein") heissen.
In Tab. VI ist die Überschrift der Spalten folgendermassen abzuändern:

	Unbehandelt absolut	Behandelt	Diff.
Welke-Transp., mg/dm² in 20 Min.	109,7	88,2	-19,6%

usw.
Auf S. 225, 6. Zeile von unten muss es 80 ml/qm (anstatt 89 ml/qm) heissen.