Influence of Changes in the Nurse Cell System (Syncytium) on Sex Determination and Development of the Cyst Nematode *Heterodera schachtii*: Total Amounts of Proteins and Amino Acids

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ABSTRACT

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The nutrition of seedling roots of *Brassica rapa* var. *silvestris* f. *campestris* 'Stielmus' was manipulated to test whether sex determination in the beet cyst nematode, *Heterodera schachtii*, is controlled by environmental or genetic factors. Single J2 juveniles were added to germinated roots in water agar drops under aseptic conditions. Half of the test plants were decapitated by removing root tips and shoots. Two days after adding the nematodes, nutrient solution containing minerals and various concentrations of sucrose was poured over the agar drops. Twelve days after inoculation, nematode development in the 10 treatment combinations was examined, and the total amounts of protein and amino acids in the syncytia, adjacent root segments, and uninoculated control roots were determined with sensitive micromethods. Seven treatment combinations supported and three inhibited female development. The experiments provided evidence that under favorable conditions, most juveniles develop as females. In treatments that inhibited juvenile development, the high proportion of juveniles still at the J2 or J3 stage resulted from changes in the nutrient supply. Changes in the total amount of proteins and amino acids in the syncytia, as well as in the control root segments, did not obviously influence female development.

Since the beginning of the century, the question of sex determination in cyst nematodes has been a subject of controversy. The basic question is whether certain environmental or purely genotypic factors affect sex determination. Environmental control has been known for some time, in particular for insect parasites of the order Mermithida (6,7), but also for some parthenogenetic species of sedentary root nematodes in the family Heteroderidae, such as *Meloidogyne incognita* (Kofoid & White) Chitwood and *Meloidodera floridensis* Chitwood et al (22,25,27).

Parthenogenetic reproduction is believed to be a prerequisite for phenotypic sex determination (26). However, this kind of sex determination also may occur in several amphimictic cyst nematodes. Due to different methods of nematode inoculation, culture, extraction, and examination, diverging results supporting either phenotypic sex determination (19–21,28) or genotypic sex determination (4,15) are difficult to compare.

It is essential to apply methods under defined, standardized conditions that allow a reliable interpretation of the results. Manipulation of the host plant under defined conditions in monoxenic culture may be the most suitable procedure. In the case of phenotypic sex determination, the manipulation of host plant nutrition, affecting the nurse cell system of the nematode, should result in alterations in the sex ratio. In the case of genotypic sex determination, the sex ratio should remain stable. Favorable conditions in the nurse cell system should either lead to high percentages of females among adult individuals (phenotypic) or support the development of both sexes in a balanced ratio (genotypic) with a minimal mortality of the juveniles. Unfavorable conditions should lead to high percentages of males (phenotypic) or a high mortality of the developing juveniles (genotypic).

Knowledge of biochemical changes in the nurse cell system (syncytium) of cyst nematodes will help to support the interpretation of the population data. As proteins and amino acids doubtlessly play a key role in the maintenance of the metabolically active nurse cells, we concentrated our investigations on the assessment of quantitative changes that occur in seedling roots of a host plant of *Heterodera schachtii* Schmidt after manipulation of both the plant (decapitation of the shoot and root tip) and the nutrient solution bathing the root.

MATERIALS AND METHODS

Aseptic inoculum of *H. schachtii*. Aseptic second-stage juveniles (J2) were obtained by transferring aseptic cysts of *H. schachtii* from monoxenic stock cultures on *Sinapis alba* L. 'Albatros' to water agar containing 3 mM $ZnCl_2$ (11). The cysts were crushed, and about 2 days later freshly hatched juveniles were picked from the agar surface with a fine needle and added singly to seedling roots.

Aseptic agar drop fluid culture of host seedlings. From various cruciferous host plants tested, *Brassica rapa* L. var. *silvestris* Lam. f. *campestris* 'Stielmus' proved to be the most suitable (11) for the culture method described below. Seeds were surface-sterilized

for 30 min in a 1% streptomycin sulphate and 4% chloramine-T solution, washed several times in sterile distilled water, and placed onto 0.6% water agar in petri dishes. After 1 day of germination at 25 C in the dark, 3- to 6-mm-long seedlings were transferred singly into distilled water agar drops (eight drops of about 1 cm in diameter in 9-cm-diameter petri dishes) and kept at 4 C. Each seedling root was inoculated with a single J2 juvenile 24 hr later. Two days after inoculation, the nutrient solutions were added.

To influence the nutrient conditions of the seedlings and, thus, nematode development, the sucrose content of the nutrient solution, as recommended by Dropkin and Boone (9), was varied at four concentrations: 0, 1, 2, and 3%. In another treatment, seedlings were placed in distilled water only to include mineral deficiency as an additional stress factor.

To obtain a higher degree of nutrient depletion, 50% of the seedlings in each treatment were decapitated 1 day after nematode inoculation by cutting off root tips and shoots. Inoculated and uninoculated seedlings were kept in the dark at a constant temperature of 25 C. Treatments and numbers of replicates are summarized in Table 1.

Evaluation of nematode development. Twelve days after inoculation, the roots were examined under a dissecting microscope for well-developed, typically lemon-shaped females. The subsequent preparation of roots with females for biochemical analysis is described below. Seedlings without well-developed females were treated as follows: The nutrient solutions were decanted and the shoots, if present, were excised. The root systems (still in the agar drops) were covered for 24 hr with a combined fixation/staining solution consisting of acetic acid/96% ethanol (50:50, v/v) with 17 mg of acid fuchsin per liter. After washing with water, the stained material was kept in a solution of glycerine/lactic acid/water (20:20:60) for differentiation of stain and storage of the material. The agar and most parts of the roots stained slight pink, whereas the nematodes and their nurse cell systems, as well as injured root parts and root tips, stained deep red.

The roots were carefully examined for nematodes under the dissecting microscope by squashing them with a special glass spatula (11). This allowed the detection of juveniles still at the J2 or J3 stage ("stagnated juveniles"), which in some cases stained only faintly. The nematodes were classified into sexually differentiated females and males (including those J3 stages, which could be distinguished according to their genital primordia) and undifferentiated or undistinguishable stagnated J2/J3 juveniles.

Preparation of feeding sites and extraction. Twelve days after inoculation, roots with well-developed females were drawn out of their agar drops and washed in distilled water. The females were carefully removed without injury. All lateral roots were cut off and root segments about 5 mm long, containing the syncytium as well as adjacent segments distal and proximal (in reference to the root tip) to the syncytium, were dissected and frozen in liquid nitrogen. Control root segments (15 mm long) of the same age were cut from uninoculated plants at identical positions. Samples, consisting of the segments of four or eight roots (see Table 1) were collected, lyophilized, and weighed. Buffer solution (25.9 mM KH_2PO_4/40.7 mM $Na_2HPO_4 \cdot 2H_2O$, pH 7.0) was added in the ratio of 0.17–0.25 μl/μg dry weight. Samples >400 μg were homogenized with a Wheaton Micro Tissue Grinder and given subsequent ultrasonic treatment (Vetter KG) in Eppendorf reaction tubes. Smaller samples were homogenized only by ultrasonic treatment. The extraction (15 min at 4 C) was followed by centrifugation (20 min at 4,800 *g*). The supernatants were removed and stored at −20 C until further use.

TABLE 1. Experimental conditions and numbers of samples

Sucrose in nutrient solution (%)[a]	Seedling treatment	Number of replicates: Petri dishes[b]	Inoculated segments	Uninoculated segments
3%	Intact	55	3	4
	Decapitated	38	4	3
2%	Intact	69	6	4
	Decapitated	65	3	4
1%	Intact	55	4	4
	Decapitated	39	4	4
0%	Intact	61	4	5
	Decapitated	64	1	4
Water	Intact	71	6	5
	Decapitated	66	2	5

[a] The nutrient solution was that of Dropkin and Boone (9).
[b] Each petri dish contained eight *Brassica rapa* var. *silvestris* f. *campestris* 'Stielmus' seedlings. Each inoculated sample consisted of four root segments; each uninoculated sample consisted of eight root segments.

Protein and amino acid determination. The protein content was determined according to Butcher and Lowry (5) by acid hydrolysis followed by measurement of the amino acids so released. The hydrolysis of the extracts (2 μl, two replicates) with 6 N HCl (10 μl) was carried out for 4 hr at 120 C in oil wells under light mineral oil (17). The hydrolysates were transferred into test tubes with constriction pipettes and neutralized with 50 μl of 1 N NaOH. One hundred and fifty microliters of OPT-reagent (100 mM $Na_2B_4O_7 \cdot 10\ H_2O$ containing 85 mM NaOH and 2 mM β-mercaptoethanol) was added. After 30 min (room temperature), the samples were diluted with 1 ml of 0.5 N NaOH and measured fluorimetrically (340-nm excitation, 455-nm emission wavelength).

Extract blank values were estimated by hydrolysis with distilled water. Instead of NaOH, 50 μl of water was added to the hydrolysate. The subsequent procedure was the same as described earlier. Water and buffer blank measurements were also performed. The protein values were calculated by means of regression curves made with bovine serum albumen. For any further details, see Betka (2).

A first determination of the total amino acid content of root segment samples was performed with the slightly modified method of Roth (23). The measurements were parallel to the protein determinations. Two microliters of the extracts (three replicates) were diluted with 60 μl of water and 150 μl of OPT-reagent were added. Subsequent steps were the same as described for the protein determination. Alanine served as a standard for calibration curves. Although some amino acids are not fully recorded with this method (23), it was used to derive approximate values, which were essential for the exact determination of single amino acids (3). The summation of values for singly determined amino acids gives more precise results, and these are shown in Figures 1C and 2B.

Statistical evaluation. In the population investigations, each petri dish containing eight seedlings represented one replicate, in which the number of all detected nematodes was set at 100%. The percentages of females, males, and stagnated juveniles of each replicate were added to obtain mean values of treatment. Variance analysis was made by means of the SPSS program package (ONEWAY proceedings). The variance homogeneity of the protein and amino acid mean values was tested by the Bartlett Box *F* Test. The mean values were then compared ($P = 0.05$) by the Student Newman-Keuls method (ONEWAY proceedings of SPSS). In the case of nonhomogeneous variances, the values were transformed (24): $x' = \log_{10}(x + 3/8)$.

RESULTS

Percentages of sexually differentiated nematodes and stagnated juveniles. The mean percentages of roots invaded by the J2 juveniles showed a clear dependence on the seedling treatment (intact = 55.4 ± 3.9%, decapitated = 35.6 ± 5.1%) but, as expected, did not correlate with the nutrient solutions. The lower values in decapitated seedlings resulted most likely from the removal of juveniles that had invaded the seedlings at the root tip. Recovery of nematodes was not reduced in variants, which inhibited nematode development.

Three different types of nutrient solution were tested: 1) nutrient solutions with sucrose, 2) nutrient solutions without sucrose, and 3) distilled water only. In all the variants that contained sucrose,

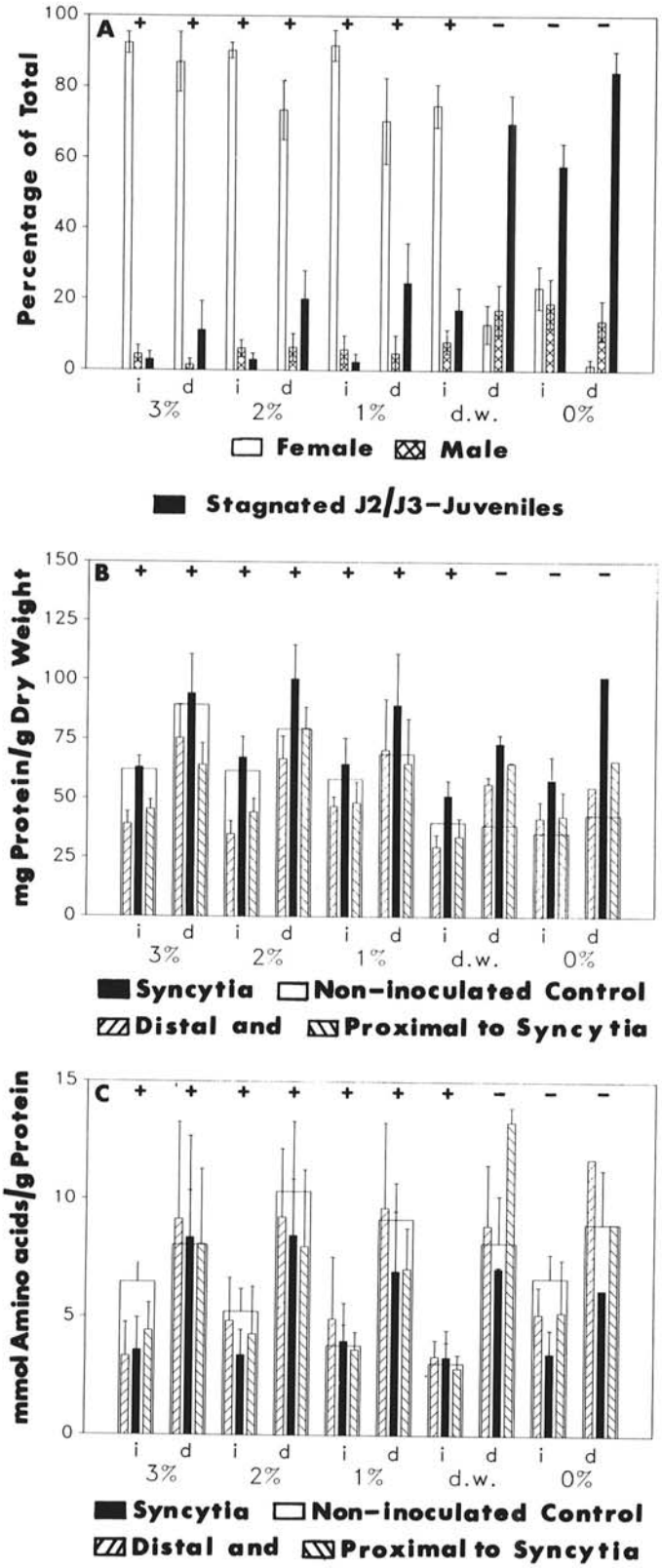

Fig. 1. A, Percentages of sexually differentiated nematodes and stagnated J2/J3 juveniles of *Heterodera schachtii*, reared at 25 C in the dark on seedling roots of *Brassica rapa* var. *silvestris* f. *campestris* 'Stielmus' following nutrient manipulation in an aseptic agar drop fluid culture system, 12 days after nematode inoculation. The values are means ± limits of confidence (95%). **B,** Protein content and **C,** amino acid content of 'Stielmus,' either inoculated with *H. schachtii* or uninoculated in different nutrient media in an aseptic agar drop fluid culture system (25 C in the dark), 12 days after nematode inoculation. The values are means ± standard deviation. Number of replicates are given in Table 1. Abbreviations are as follows: i = intact seedlings; d = decapitated seedlings; + = "+" variants; − = "−" variants; d.w. = distilled water; 0, 1, 2, and 3% = sucrose concentration in the nutrient solution.

70–90% of the nematodes that invaded roots developed into females (Fig. 1A), with no significant differences between intact and decapitated seedlings but always with higher values in the intact variants. The percentage of males never exceeded 10%, and there were no significant differences between the variants. In the intact variants, only about 5% of the nematodes stagnated in an early juvenile stage. In the decapitated variants, the values ranged between 11 and 24%, with a tendency for the lower values at the higher sucrose concentrations.

In the two variants without sucrose but with mineral and vitamin supply, only 23 and 1.6% of the nematodes developed into females in the intact and the decapitated seedlings, respectively. The percentage of stagnated juveniles increased considerably to 58 and 84%, respectively. Again, there were more females and fewer stagnated juveniles in the intact compared to the decapitated variant. Males (19 and 14%, respectively) were more abundant than in the variants supplied with sucrose. The intact variant of distilled water without any nutrients supported female development in a way similar to the sucrose-containing variants. In the decapitated variant, however, the population pattern was similar to that in variants without sucrose.

The 10 variants can be classified on the basis of their suitability for female development into two groups: 1) seven "+" variants, with high percentages of females and few stagnated juveniles and, 2) three "−" variants with significant lower percentages of females

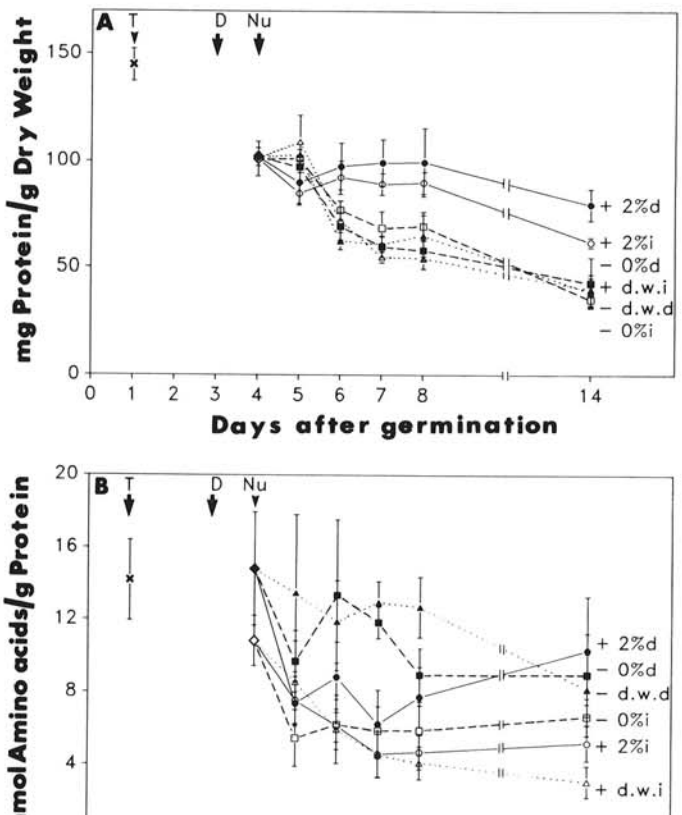

Fig. 2. Changes in **A,** the protein content and **B,** the amino acid content in uninoculated root segments of *Brassica rapa* var. *silvestris* f. *campestris* 'Stielmus' seedlings within the first 2 wk after germination. Fifty percent of all seedlings were decapitated on the third day (D), hence two values on the fourth day. The nutrient solutions were added on the fourth day (Nu), hence six values from the fifth day on. T = transfer of 1-day-old seedlings into distilled water agar drops (hence one value only). N = 5 except 14th day, where n = 4 for 2%i, 2%d, and 0%d variants. Other abbreviations are as follows: i = intact seedlings; d = decapitated seedlings; + = "+" variants; − = "−" variants; d.w. = distilled water; 0, 1, 2, and 3% = sucrose concentration in the nutrient solution.

and highly increased numbers of stagnated juveniles. The arrangement of the variants in the figures is according to this classification, marked with a "+" for conditions supporting and a "−" for conditions inhibiting female development.

Protein and amino acid content. In the seedling roots containing nematodes that developed into females (i.e., in the "+" variants and a few of the "−" variants), the protein concentration (mg/g dry weight) was always higher in the syncytia than in the adjacent root segments and the segments of uninoculated control roots (Fig. 1B). In the three "−" variants, the protein content in uninoculated roots was lower than in the control segments (distal and proximal to the syncytium) of inoculated roots, whereas in the seven "+" variants, the opposite was the case. In uninoculated control roots of six "+" variants, the protein content was significantly higher than in the three "−" variants.

The changes in protein concentrations in uninoculated control roots within the first 2 wk are represented in Figure 2A. The two "+" variants with 2% sucrose showed high protein concentrations throughout with only a slight decrease toward the end. In the four variants without sucrose (three "−" variants and one "+" variant) significant lower concentrations were found from the sixth day on, compared with the 2% sucrose variants.

Total amounts of amino acids (Fig. 1C) showed a tendency toward higher concentrations in the decapitated seedlings than in intact seedlings, but the differences were not statistically significant in all cases. Correlations between high percentages of females ("+" variants) or stagnated juveniles ("−" variants) and the amino acid concentration in the nurse cell systems were not evident. The concentration changes in uninoculated control roots within the first 2 wk (Fig. 2B) also showed higher amino acid contents in decapitated seedlings from the sixth day on and no clear correlation with either a positive or negative influence on nematode development.

DISCUSSION

Plant metabolism and nematode development were clearly affected in the different variants, mainly in relation to the supply of sucrose. However, a distinct correlation between the sucrose concentrations and protein or amino acid contents in inoculated root segments was not evident. The lowest concentration tested (1%) was obviously sufficient to maintain root growth in the culture system used. In the case of protein, significant differences between variants with or without sucrose were found only in uninoculated root segments. The syncytia, as well as their adjacent segments, in the "−" variants showed a protein content comparable to that in the "+" variants. This is not surprising considering the generally high metabolic activity in syncytia. It is possible that the few females that were able to develop in the "−" variants may have encountered favorable conditions because of the genetic variability of some of the seedlings. Because the control segments adjacent to the syncytia in these variants also had a high protein content, it may be possible that relatively high protein levels prevailed even at the beginning of the host-parasite interaction. However, root segments in the immediate neighborhood of syncytia are doubtlessly influenced by the metabolism of the syncytium, and, in addition, it is possible that some of them contained syncytial tissue, because in the cruciferous host used, syncytia sometimes exceed 5 mm in length. Because many females developed in intact seedling roots without any additional nutrient supply in which the protein pattern was similar to that in the variants, it can be concluded that the protein content before and/or during the parasitic interaction is not a limiting factor for female nematode development.

The most striking phenomenon in the case of amino acids was the high content in decapitated variants compared to intact ones. On the other hand, no correlation with the sucrose supply was detectable. The total amino acid levels were generally lower in syncytia than in the adjacent segments and uninoculated roots. This finding contrasts with that of a number of reports in which amino acid concentrations in nematode-infected roots were higher compared to controls (8,10,12,13,18). In our investigations, roots were examined after the nematodes had been carefully removed, a prerequisite for a reliable analysis. Jones (14) and Krauthausen and Wyss (16) have shown that nematodes within root samples greatly influence the amounts of biochemical constituents (e.g., proteins and amino acids). Changes in the total amount of amino acids caused by the 10 variants did not obviously influence nematode development. In fact, it has been claimed that the quality, rather than the quantity, of the amino acid pool is a decisive factor in this respect (2,3).

Considering the influence of changes in the nurse cell system on sex determination, the average of the penetration rates into intact and decapitated seedlings was high enough to provide representative data about the whole population. However, it could still be argued that penetration rates of 55.4 and 35.6%, respectively, are not high enough to exclude the possibility that mainly genetically female juveniles were able to invade the relatively large seedling roots. As far as is known, there is no sex-specific penetration behavior, and doubts can be set aside by the fact that in roots of similar or even greater thickness of a resistant cultivar of *Raphanus sativus* L. var. *oleiformis* Pers. 'Pegletta', nearly all invaded juveniles developed into males (11).

The high percentage of nematodes stagnating at an early juvenile stage in "−" variants corresponded to that of females developing in roots under favorable conditions ("+" variants). The nematodes were obviously not able to adapt to the artificially induced deterioration in nutrient supply. This pattern appears to support genotypic sex determination. On the other hand, a balanced ratio of males and females was never reached under favorable conditions, where up to 92% of the J2 juveniles in invaded roots developed into females. Thus, the results support phenotypic sex determination, but the high proportion of females demands a further explanation. From the biochemical examination of uninoculated roots during the first 8 days of seedling development, a drastic change in total amount of proteins and amino acids was observed from the fifth day on. This change occurred at a time when invaded juveniles were still in the J2 developmental stage but probably in a phase when sex already was determined. On the day of inoculation (second day of seedling growth), J2 juveniles would initially be exposed to favorable conditions independent of the different treatments. The large numbers of stagnated J2/J3 juveniles found under unfavorable conditions were probably female juveniles whose further development was inhibited by the change in food supply and quality. Male development, expected under these unfavorable conditions, was prevented by the initially favorable conditions in the growing seedlings. As stated in Materials and Methods, the invaded nematodes were classified into sexually differentiated and stagnated J2/J3 juveniles. In the routine evaluation, it was not possible to determine the sex of J3 juveniles that stagnated at an early phase. A detailed examination of the genital primordia of 118 J3 juveniles with the aid of video-enhanced contrast microscopy showed that 85% of the juveniles were females (*data not shown*), which gives additional strong support for phenotypic sex determination.

The slight increase of males in the "−" variants may be attributable to individuals that established their feeding sites later than the majority of inoculated juveniles. Under these circumstances, the biochemical changes in the root may have favored sex determination toward male.

The whole pattern presented here was found under experimentally manipulated conditions and is thought to be an exception from nature. Normally, the nematodes develop without extreme changes during their lifespan and should not be exposed to an evolutionary selection pressure that favors a quick adaptation to changing conditions. The adaptation to stable favorable or unfavorable conditions provided by the host can be considered as an advantage for amphimictic nematodes. An example of this mode of adaptation in *H. schachtii* is the high proportion of males developing in resistant plants or in small lateral roots (1,11,21). This mode of sex determination ensures, under unfavorable conditions, rich genetic variability within a population through the development of many males and few females and, under favorable conditions, a high reproductive potential.

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