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Artificial Feeding Systems for Plant-parasitic Nematodes

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Abstract: Feeding of an "obligate" plant-parasitic nematode (nonfungal feeder), *Pratylenchus scribneri,* in the absence of plant tissue was demonstrated in an artificial system consisting of liquid media and indicator dyes including amaranth and various nontoxic food colors. Among the compounds tested, sucrose, dextrose, Gamborg's B5 medium, and DL-methionine stimulated a small percentage of feeding (12–36%). A high percentage of feeding (90–100%) occurred in a filtrate from excised corn roots cultured in Gamborg's B5 medium. This feeding system has the potential to develop an artificial medium for plant-parasitic nematodes and to screen novel nematicides that are stomach poisons.

Key words: amaranth, dye, feeding stimuli, feeding system, food color, indicator, nematode bait, *Pratylenchus scribneri,* screening.

Plant-parasitic nematodes that are also fungal feeders, such as *Aphelenchoides, Aphelenchus, Bursaphelenchus,* and *Ditylenchus,* have been cultured with difficulty in artificial media (2,5,8,9); however, "obligate" plant parasites (nonfungal feeders) have not been cultured in the absence of plant tissues. There is a general consensus that a living plant cell is necessary to provide mechanical stimuli for feeding because probing on the surface of plant tissue prior to feeding is a common behavior of plant-parasitic nematodes (3). It is difficult to assess the suitability of a cultural medium for the nematodes when both physical and biochemical factors may be involved. In the past, growth or reproduction of the nematodes was usually used to determine the suitability of a cultural medium. In negative responses, however, there was always the possibility that the medium may have been suitable but the nematodes did not ingest it because of a lack of physical or chemical feeding stimuli. To study artificial feeding systems it was deemed necessary to find a simple nontoxic indicator of ingestion. If feeding occurs with a particular medium but no growth of the nematodes takes place, then modification of the medium deficiencies can be continued to screen the suitability of various nutrients. A feeding indicator might also provide a means for separating the effects of physical and biochemical factors. During our routine inspection of *Pratylenchus scribneri* Steiner in excised root cultures, stylet movement was often observed when the nematodes were some distance from the roots. The movement of the stylet could be either probing or feeding, and the use of a feeding indicator might distinguish between the two processes.

Nematode cuticle is selectively permeable when the nematode is alive (7). Using radio-labeling, Marks et al. (7) demonstrated that water and some pesticides were readily taken up and released by the nematode, whereas glucose, sodium acetate, and glycine were not. However, radio-labeling of each nutrient is not feasible for screening cultural media or a complex medium of unknown chemical composition. A dye that does not penetrate the cuticle and is not toxic to the nematode when ingested would be an ideal indicator for feeding.

The objectives of this paper were to study 1) the possibility of using dyes as feeding indicators and 2) the role of feeding indicators and feeding stimuli in the development of an artificial feeding system for the obligate plant-parasitic nematode *P. scribneri.*

MATERIALS AND METHODS

Pratylenchus scribneri was cultured on excised corn roots grown in Gamborg's B5

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medium (4). The nematode culture was obtained from R. Mankau at the University of California at Riverside.

Red food color (Iris brand, double strength, containing FD & C red #40, citric acid, and benzoate of soda), amaranth (Sigma), Phloxine B (Sigma), green food color (Schilling brand, containing propylene glycol, FD & C yellow #5, FD & C blue #1, and 0.1% propylparaben), and table beet extract were tested for their suitability as feeding indicators. Table beet extract was prepared by heating 100 g sliced beets in 150 ml distilled water in a microwave oven for 5 minutes. All the other dyes were prepared as 5% stock solutions. Their final concentration in the test solutions was 0.25%. The dyes were autoclaved separately then pipetted aseptically into the test media. The pH was adjusted to 7.0 before autoclaving.

Several compounds were tested for their effectiveness as nematode feeding stimuli. Sucrose (2%), dextrose (5%), DL-methionine (0.04%, Sigma), glycine (0.07%, Sigma), yeast extract (2%, Difco), soytone (3%, Difco), chick embryo extract (10%, GIBCO), beef extract (0.3%, Difco), Gamborg's B5 (GIBCO), and corn root culture filtrate (roots cultured in liquid Gamborg's B5) were tested separately and in different combinations. Distilled water was used as the control for all tests.

Thirty adult female nematodes were transferred aseptically into 2 ml sterilized test solution in a Petri dish, sealed with paraffin film, and incubated at 28 C for 14–21 days. There were four or five replicates of each treatment. The effect of incubation time on feeding was studied with corn root culture filtrate over 21 days with amaranth as the feeding indicator. Fourteen days was used as the optimum time interval for all other tests. At the end of each test, the nematodes were transferred to distilled water and the color of the intestine was examined under a dissecting microscope (30–40×). The *fluorescent* light was dimmed by rotating the mirror of the microscope to an angle that best emphasized the color of the intestine. The number of nematodes with colored intestines was determined and the percentage of colored nematodes was obtained by dividing this number with the total number of nematodes observed. In some treatments where amaranth was used as the feeding indicator, detailed comparison between treatments was made by estimating the length of the red portion of the intestine as ½, ⅓, ¼, ⅛, or ¹⁄₁₀ of the intestine. The percentage of red intestine were then calculated by the formula: % red intestine = $(X_1 \times 1/2 + X_2 \times 1/3 + X_3 \times 1/4 + X_4 \times 1/8 + X_5 \times 1/10)$/total no. nematodes × 100%, where $X_1, \ldots, X_5$ are the number of nematodes with the particular length of colored intestine. The intensity of the red color in the intestine also was estimated as red, pink, or faintly pink. In most tests, only the red category was used in determining the results. The feeding activity of nematodes in corn root culture filtrate was tape recorded with a video cassette time lapse recorder (GYYR, Model No. TLC 2051 T/D) to observe and confirm the feeding behavior.

Two kinds of dead nematodes, heat killed (70 C, 1 minute) and highly vacuoled nematodes which died in old cultures, were used to study the penetration of dye particles through the cuticle when the selective permeability of the nematodes was lost. Live nematodes were used as the control. To further demonstrate that ingestion was the essential passage for the dye into live nematodes, one test was conducted at 4 C to inhibit the feeding activity of the live nematodes. Corn root culture filtrate and amaranth were used as the test medium.

Results

Among the dyes tested, amaranth was the most suitable indicator of feeding during a 14-day feeding period (Table 1). Phloxine B was unsuitable as feeding indicator because the control nematodes turned red. The color of table beet extract faded in the test medium in ca. 7 days and no red color could be detected inside the nematodes. Green food color did not have as much contrast under the dissecting microscope as the red color of amaranth and

TABLE 1. Ingestion of indicator dyes by *Pratylenchus scribneri* incubated for 14 days in corn-root culture filtrate.

	Colored nematodes (%)		
	Corn-root culture filtrate	Distilled water	*t*-test
Red food color	89.4	61.8	ns
Green food color	51.1	2.4	*
Amaranth	96.2	3.5	***
Phloxine B	100.0	100.0	ns
Table beet extract	0.0	0.0	ns

Mean of five replicates, 30 individuals per replicate.
* = significant at $P \leq 0.05$; *** = significant at $P \leq 0.001$; ns = not significant.

TABLE 2. Effect of nematode viability on uptake of amaranth indicator dye by *Pratylenchus scribneri.*

	Red nematodes (%)			
	Whole nematode red		Esophagus not red, intestine partially red	
Treatment	Dark red	Pink		Total
Heat-killed	100.0	0.0	0.0	100.0
Dead in old culture	4.5	41.8	0.0	46.3
Alive, at 4 C	0.0	0.0	0.0	0.0
Alive, at 28 C	0.0	0.0	100.0	100.0

All nematodes were immersed in corn root culture filtrate plus amaranth.
Mean of four replicates, 30 individuals per replicate.

therefore was less desirable than amaranth. Red food color was not satisfactory because many nematodes in the control turned red and died during the 14-day test. It can be used in short-term feeding when the incubation period is 7 days or less. No nematodes turned red in 4 and 7 days, whereas 20.3% of them were red in 10 days. These results were confirmed in two additional experiments.

Amaranth penetrated the cuticle of heat-killed nematodes, and to a lesser degree the dead nematodes from old culture, but it did not penetrate the cuticle of live nematodes (Table 2). The route of entrance of amaranth into live nematodes was through ingestion. When feeding activity was inhibited by cold temperature (4 C), the red color did not appear in any nematode intestines. All the nematodes had red intestines when incubated at 28 C in corn root culture filtrate. Only the food granules in the intestine were stained, suggesting that the dye entered the live nematode through ingestion. This was true for both amaranth and green food color (Fig. 1A, B). In the dead nematodes, the esophagus region as well as the intestine of all 120 nematodes examined was stained by the dye (Table 2, Fig. 1C).

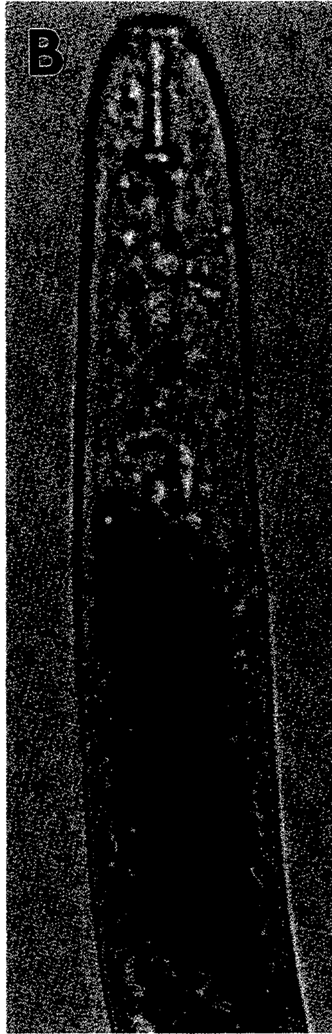
Among the feeding stimuli tested, corn root culture filtrate was the most effective (Table 3). In the presence of sterile water and amaranth, only 3% of nematodes had red intestines, whereas 97% of them had red intestines in the presence of corn root culture filtrate as a feeding stimulus. No feeding was found in beef extract, glycine, chick embryo extract, yeast extract + soytone, and the *Aphelenchoides* medium. Sucrose, dextrose, Gamborg's B5 medium, and DL-methionine stimulated a low percentage of nematodes to feed (12–36%).

As length of exposure to corn root culture filtrate increased from 7 to 21 days, the percentage of red nematodes increased from 77.3% to 100% (Table 4). After 21 days all the nematodes had ingested some dye; however, the intestine was not completely red in many of the nematodes. The average length of the red intestine was only 22.6% after 21 days.

The stylet movement during feeding was clear when observed at normal recorder speed; the movement of the medium bulb was more evident with time lapse photography at ⅙ normal speed (10 pictures/second). The nematodes assumed a particular posture when they were feeding. The lower two-thirds of the body was straight or slightly curved and motionless. The upper one-third assumed a near 90 degree angle to the rest of the body most of the time. The head moved slowly in various directions when feeding. The feeding posture is shown in Figure 1D. No feeding was observed in the nematodes that were moving actively in their normal behavior.

Nematodes in the distilled water were observed pumping their stylets in four of eight tests, with 13–27% of individual

A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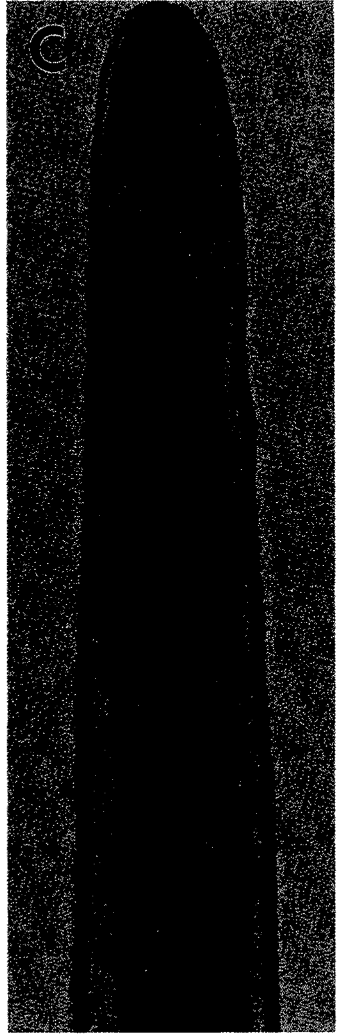
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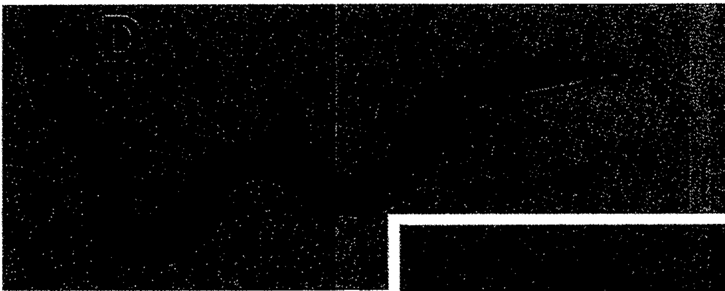

TABLE 3. Effect of incubation medium composition on ingestion of amaranth indicator dye by *Pratylenchus scribneri*.

	Feeding (%)		
	Treatment	Control	*t*-test
Sucrose (2%)	36.2	2.8	***
Dextrose (5%)	14.1	0.9	*
DL-methionine (0.04%)	12.5	3.9	*
Glycine (0.07%)	3.6	4.5	ns
Yeast extract (2%) + soytone (3%)	0.0	0.0	ns
Chick embryo extract (10%, v/v)	17.4	7.2	ns
Aphelenchoides medium†	0.0	2.6	ns
Beef extract (0.3%)	3.3	8.7	ns
Corn root culture filtrate	97.3	3.0	***
Gamborg's B5 medium	11.6	0.9	*

Mean of four replicates, 30 individuals per replicate.
* = significant at $P \leq 0.05$; *** = significant at $P \leq 0.001$; ns = not significant..
† Yeast extract + soytone + chick embryo extract (8).

TABLE 4. Effect of incubation time on the feeding of *P. scribneri* in corn root culture filtrate.

Incubation time (days)	Red nematodes (%)	Red intestine (%)
0	0.0	0.0
7	77.3	5.4
12	98.3	10.1
21	100.0	22.6

Mean of four replicates. Any nematode with red color in the intestine was counted as a red nematode regardless of the length of the red portion. Percentage of red intestine was based on the length of the red portion of the intestine.

nematodes exhibiting stylet activity in positive tests. Among the collections that did not have stylet movement, the nematodes were all moving actively, whereas the bodies of the nematodes with stylet movement were all motionless. It appeared that when the nematodes were ingesting water they assumed the same posture as when they were feeding on nutrients.

DISCUSSION

Our studies have demonstrated that obligate plant-parasitic nematodes can be stimulated to feed and ingest artificial media in the absence of plant tissues. These observations are encouraging for future efforts to culture plant-parasitic nematodes axenically and to test novel nematicides that are stomach poisons.

The culturing of mycophagous nematodes in artificial medium is possible but not very efficient. Glass wool lining over the surface of the culture tube was used to axenically culture *A. avenae* in artificial medium (5). Culturing of *Bursaphelenchus xylophilus* was improved when small volumes of medium were dispersed on filter paper strips, glass beads, or glass wool (1). Although the possibility remains that a solid substrate may be necessary for feeding and reproduction of most plant-parasitic nematodes, our studies suggest that some feeding and ingestion of liquid media by *P. scribneri* did not require a solid substrate. Probing on the root surface has been observed in our stock cultures on the corn roots; however, it appears that nematodes can learn to recognize a food-based chemical signal in vitro and respond by ingesting the surrounding media. It is interesting that the feeding posture of *P. scribneri* in the liquid media resembles that of nematodes probing and feeding on plant tissue: in most cases the body becomes immobile and the head turns perpendicular to the body.

Selective permeability has been demonstrated by the cuticle of living nematode for certain molecules (7). This difference in permeability has been used to distinguish between live and dead nematodes. Fluorescent dyes such as acridine orange, neutral red, or methyl blue have been reported to stain dead larvae of potato cyst nematodes but not live ones (6). In our study, amaranth penetrated the cuticle of dead *P. scribneri* but not the live ones. It entered the live nematodes through inges-

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FIG. 1. Entrance of dye molecules into live and dead *Pratylenchus scribneri* incubated in corn root culture filtrate. A) Green food color in live nematode. B) Amaranth in live nematode. C) Amaranth in heat killed nematode. D) Feeding posture of *Pratylenchus scribneri* in corn-root culture filtrate.

tion. When feeding activity was inhibited by cold temperature (4 C), no dye was observed inside the nematodes. Further evidence for ingestion of amaranth was the lack of staining in the esophagus region in the live nematodes. Only food granules in the intestine are stained. After the nematodes were killed by heating at 70 C for 1 minute, amaranth penetrated the cuticle and membrane freely. The dead nematodes from old cultures were less colored than the heat-killed nematodes but more colored than the live nematodes without feeding activity. In either case there was no differential location of the dye, suggesting that the control of selective permeability requires active metabolism. Among the dead nematodes from old cultures, the degree of coloring probably depended on the degree of deterioration and aging of the membrane.

Our observation of stylet movement of nematodes in distilled water can explain the occasional red nematodes in the controls of some tests. It became clear to us that nematodes may ingest water without any chemical or physical stimulus. Although corn root culture filtrate stimulated a high percentage (95%) of feeding, as indicated by the ingestion of amaranth, the intestines of the nematodes were not completely red after 21 days incubation. This suggested that the dyes may be metabolized in the lower intestine or that the quantity of nutrient ingested over time is small.

In view of safety, green food color may be more desirable to work with than amaranth because accidental ingestion of amaranth can be harmful to humans. Amaranth was banned for use in food, drugs, and cosmetics by the Food and Drug Administration in 1976 (10).

The use of dyes as feeding indicators and the use of feeding stimuli should greatly facilitate and expand the research on nematode feeding and may lead to eventual success in culturing obligate plant-parasitic nematodes in chemically defined media.

In addition to obtaining basic information on nematode nutrition there are other practical consequences of the research on feeding stimuli. One is that the feeding system can be used for screening novel nematicides such as stomach poisons that would not be detected by the conventional contact screening methods. Another possibility is the development of a poisonous nematode bait consisting of a nematicide and a feeding stimulus. The chances of the nematodes coming in contact with sufficient concentration of the nematicide could be enhanced greatly by the nematode bait. This technique could increase the efficiency of the nematicide and reduce the quantity of nematicide needed.

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