

Hatching of *Meloidogyne incognita* Eggs in the Neutral Carbohydrate Fraction of Root Exudates of Gnotobiotically Grown Alfalfa¹

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Abstract: *Meloidogyne incognita* eggs were hatched in soil sterilized by gamma irradiation and wetted with root exudates from alfalfa plants in different stages of development and subjected to various levels of clipping. Carbohydrate components of the exudates were identified by gas chromatography-mass spectrometry. Although significant stimulation of hatch was detected in exudates of seedling and flowering plants, the practical importance of the increase is doubtful as hatch in distilled water was always greater than 50%. Hatch did not differ among exudate samples from clipped plants. Incubation of eggs in soil moistened with 10^{-7} to 10^{-3} M solutions of glucose did not result in increased hatching over that in distilled water.

Key Words: Plant age, clipping effect, glucose effect, *Medicago sativa*, root-knot nematode.

Received for publication 15 June 1972.

¹Contribution No. 669 from the Department of Plant Pathology, The Pennsylvania Agricultural Experiment Station. Paper No. 4202 in the Journal Series, authorized for publication on 2 May 1972. Portion of a thesis submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree, The Pennsylvania State University, University Park 16802.

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Considerable information has been accumulated on the effect of various chemical substances, including those detected in root exudates, on the hatching of *Heterodera* (19, 20, 21); however, relatively little is known about the influence of root exudates on the hatching of *Meloidogyne* species. Early investigations by Ahmed and Khan (2), Chidambaranathan and Rangaswami (4), Khan and Saxena (12), Swarup and Pillai (17) and Viglierchio and Lownsbery (18) into the

relationship of root exudates and hatching of *Meloidogyne* species showed root exudates of numerous plants to be stimulatory as well as inhibitory. Exudate samples were usually from seedling plants or obtained without regard to the age or stage of plant development at the time of sampling. The stage of plant development (3, 7, 15, 16) and severity of clipping (8) have been shown to influence the release of carbohydrates in root exudates. Studies of the influence of exudates from seedling host and nonhost plants on hatch of *Meloidogyne* species indicated host exudates often stimulated hatch whereas exudates of nonhost had no effect or reduced hatching (2). Some attention has been given to the hatching influence of specific exuded substances. The alcohol soluble fraction eluted from a cation exchange column (nonamino acid fraction) of root extracts obtained from several plant species, including alfalfa, is inhibitory to hatching of *M. incognita* (5). Ahmed and Khan (2) found that amino acids were less stimulatory to hatching than were carbohydrates, whereas vitamins and plant growth hormones were inhibitory. Similar findings were reported by Khan and Saxena (12). Jones and Nirula (11) were unable to demonstrate significant differences in hatching in water, root exudates or sucrose solutions.

The objectives for undertaking this study were to determine the influence of the neutral carbohydrate fraction of root exudates of alfalfa plants on hatching of *M. incognita*, to assay the involvement of the carbohydrate fraction in the previously reported nonamino acid hatch inhibition phenomenon and to obtain information on whether the stage of plant development or severity of foliage cutting prior to and during exudate procurement would affect hatching.

MATERIALS AND METHODS

Alfalfa plants, *Medicago sativa* L. 'DuPuits', were grown and maintained gnotobiotically using techniques previously described (7, 8, 13). The methods essentially were the use of a plastic isolator equipped with sterile air locks and maintained at 21 ± 2 C. All materials were either sterilized by autoclaving or surface-disinfested with 5% peracetic acid. Four plants per 250-ml autoclavable plastic beaker were grown in washed river-bank sand and irrigated with Hoagland's Solution No. 1 (9), which was modified by reducing the amount of

KNO_3 to one-half the recommended amount and omitting the MnCl_2 . These modifications were carried out to avoid the problem of salt accumulation encountered after prolonged use of Hoagland's Solution. After autoclaving, the solutions were adjusted to a pH of 6.5 with sterile 0.1N KOH. Plants were clipped weekly to a constant height of 20 cm and exudate samples were obtained at 2-week intervals for 16 weeks by flushing the *in situ* sand-root system of two pots with 1000 ml of sterilized glass-distilled water. The water was passed through the first pot, and the leachate collected and passed through the second pot to achieve the final leachate. After 17 weeks' growth different levels of top removal were initiated. One set of plants was completely clipped except for four mature leaves with stubble height of 4 cm (severe clip); a second set was maintained at a height of 15 cm (intermediate clip); and the third was clipped to prevent flowering (light clip). Exudate samples were obtained after clipping for 1 month and again 1 month after the first sample. The pH range of the exudate solutions was 6-7 which was well within the optimum range for hatching of *M. incognita* (1, 17). Samples were concentrated to 10 ml under vacuum at 40 C, desalted using a Torbal-B.T.L. chromatographic desalter (Model CD-1), and analyzed for carbohydrates as trimethylsilyl derivatives by gas chromatography; identity of compounds was confirmed by mass spectrometry using the procedure reported previously (6, 7). Data were recorded as total milligrams of carbohydrate released in root exudates as existed in the container.

Egg masses of *M. incognita* (Kofoid and White) Chitwood were removed under 12.5X magnification from galled tomato roots (*Lycopersicon esculentum* (L.) Mill. 'Rutgers') and held at 5 C for a period not exceeding 8 hr. Hatching occurred in small containers (Fig. 1) constructed from a 5-mm-high ring of 12-mm diam glass tubing with two 2×3 mm slits cut into the sides of the ring to ensure fluid movement to the interior of the ring. A layer of Kimwipes® disposable wipers (Kimberly-Clark Corporation) was affixed to the upper rim of the ring with Duco® cement (E. I. du Pont de Nemours & Co., Inc.). The center of the tissue was depressed into the interior of the ring forming a concavity 4 mm deep. The units were sterilized by autoclaving for 1 hr in depression culture slides (3-mm concavity), which were

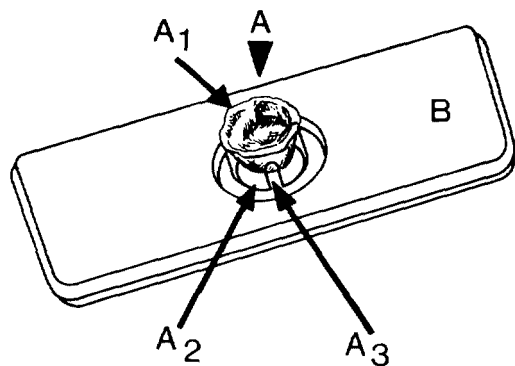


FIG. 1. Hatching container (A) showing Kimwipes® disposable wiper (A₁), 5 mm high ring of 12-mm diam glass tubing (A₂), 2 × 3 mm slit cut into the side of the glass ring (A₃) and the culture slide (B).

contained in filter paper-lined, 150-mm petri dishes.

Soil was prepared for sterilization by passage through a 40-mesh screen, placed in screw cap glass tubes (125 × 20 mm) and received a total dosage of 6.0 megarads of ⁶⁰Co gamma irradiation (14). Prior to use, the soil was cultured on various media to check for the presence of microorganisms. A predetermined quantity of soil (24 ± 5 mg) was added to the depression in the tissue, enough sterilized test solution was added to the concavity in the culture slide to moisten the soil and an egg mass (102-963 eggs/mass) was placed in the soil. The filter paper in the petri dish was saturated with sterilized water to maintain high relative humidity. Larval counts were made at 7, 14 and 21 days and the test solutions were renewed following each count. Percentage of total larval hatch was determined by the ratio of the number of larvae to the total number of larvae and eggs remaining in the egg mass after 21 days. The egg mass matrix was removed by a 12-hr immersion in a 10% solution of 5.25% sodium hypochlorite (Clorox®) to facilitate counting of the eggs. All incubation periods were carried out in the dark, to avoid the hatch-depressing influence of light (17), and at 25 C, the optimum temperature of hatching of *M. incognita* (1, 17). Studies were terminated after 21 days because preliminary and published data indicated that hatch rapidly decreased after that period (10).

To determine whether indigenous soil substances would mask the potential effect of exudate materials, hatch in gamma-irradiated

soil was compared to that in irradiated soil previously leached with 1 liter of sterilized-distilled water (12 g soil/liter). Following air-drying of the soil, nine replications of each treatment were established with only distilled water added to moisten the soil.

The neutral carbohydrate fraction of root exudate samples from 4-week-old seedlings, 16-week-old nonflowering plants, flowering plants and all clipping treatments were bioassayed. All solutions were sterilized by filtration (0.45μ pore size, Millipore Corporation) and checked for contamination prior to use. Test solutions were administered to egg masses embedded in soil in the hatching chamber. The experimental design consisted of 15 replications/treatment with exudate solutions tested at rhizosphere concentration and at 10 times that concentration. Hatching in distilled water served as the control. Rhizosphere concentration is defined as the concentration of solutes in the sand-pot system as existed within the isolator. The volume of the rhizosphere was established by determining that one saturated sandfilled pot held a mean volume of 110 ml of water. Prior to testing of solutions a 2 ml aliquot of the 10 ml concentrate was adjusted to 44 ml with distilled water. This value was used as the exudates were extracted from two pots. This concentration was tested and is referred to as the rhizosphere concentration. Ten times rhizosphere concentration was obtained by adjustment to only 4.4 ml. The effect of glucose, reported in previous studies to be released in greatest quantity in root exudates of seedling, flowering and lightly clipped alfalfa was investigated by the incubation of eggs of *M. incognita* in solutions of glucose at concentrations of 10⁻³ to 10⁻⁷ M. This range encompassed detected levels of glucose in root exudates under gnotobiotic conditions (7). Each treatment was replicated 10 times with a pH of 6.2 to 6.8. Again, hatching in glass-distilled water served as a control. Data were analyzed by analysis of variance or Student's t-test with mean values compared by Duncan's multiple range test.

RESULTS

Carbohydrates detected were arabinose, ribose, xylose, fructose, mannose, glucose, inositol, sucrose, maltose and five unidentified compounds. Quantities detected have been

TABLE 1. The effect of the neutral carbohydrate fraction of root exudates obtained from gnotobiotically grown DuPuits alfalfa at different stages of plant development on hatching of *Meloidogyne incognita*.

Stage of Plant Development	Percent Hatch ^a	
	Rhizosphere concentration	10X Rhizosphere concentration
Seedling	70.2 a	52.6 cd
Mature, non-flowering	40.1 d	40.1 d
Flowering	54.2 bc	65.5 ab
Control	51.4 cd	

^aMeans not followed by the same letter are significantly different ($P < 0.01$).

reported previously (7, 8).

Hatch in leached and nonleached gamma irradiated soil was 93.5 and 93.7%, respectively. The table $t_{0.01}$ value of 2.92 compared to a calculated t value of 0.09 indicated that substances present in the soil would not invalidate an assessment of the influence of exudate materials on hatch.

At rhizosphere concentration of exudate solutions, egg hatching differed significantly in samples from all stages of plant development. Greatest hatch occurred in samples from seedling plants with the lowest hatch in exudate samples from mature, nonflowering plants (Table 1). At 10 times rhizosphere concentration hatching in exudate solutions from seedling and mature, nonflowering plants did not differ but were significantly less than hatch in exudate samples from flowering plants. Hatch in exudates of seedling plants at rhizosphere concentration and flowering plants at 10 times rhizosphere concentration was significantly greater than that in the control.

Egg hatching in exudate samples from plants maintained under the various levels of clipping did not differ significantly at rhizosphere concentration (Table 2). Hatch in the control was greater than in exudate samples from severely clipped plants but was not significantly different from either of the other two clipping treatments. At 10 times rhizosphere concentration, exudate samples from lightly clipped plants allowed a significantly greater hatch than samples from plants clipped severely. Neither lightly- nor severely-clipped treatments differed from hatch encountered in samples from intermediately clipped plants. No treatment differed from the control. Hatch in

solutions of glucose was not significantly greater than in distilled water (Table 3).

DISCUSSION

The results indicate that the neutral carbohydrate fraction of root exudates of seedling alfalfa is more conducive to egg hatch of *M. incognita* than samples obtained from mature plants. It further appears that flowering results in a neutral carbohydrate exudate fraction that allows increased hatching in comparison to samples from nonflowering plants of the same age. Even though stimulation of hatching was observed under conditions of seedling development and flowering, the *in vivo* importance of such increases is questionable. A 51.4 - 84.4% hatch in water alone indicates a high percentage of nematodes could potentially be available for infection in the absence of stimulatory materials. This fact was especially evident by the high level of hatch (93.7%) in the leached soil. The wide range in percent hatch is conceivably a factor of the variability existing among individual egg masses. Although

TABLE 2. The effect of the neutral carbohydrate fraction of root exudates obtained from gnotobiotically grown DuPuits alfalfa maintained under different degrees of clipping on egg hatching of *Meloidogyne incognita*.

Clipping Treatment	Percent Hatch ^a	
	Rhizosphere concentration	10X Rhizosphere concentration
Light	58.4 cd	73.6 a
Intermediate	62.7 abcd	72.6 ab
Severe	53.3 d	60.0 bcd
Control	69.2 abc	

^aMeans not followed by the same letter are significantly different ($P < 0.01$).

TABLE 3. Influence of glucose concentration on hatching of *Meloidogyne incognita*.

Glucose Concentration (Molarity)	Percent Hatch ^a
10^{-3}	72.9 a
10^{-4}	64.9 b
10^{-5}	79.1 a
10^{-6}	74.6 a
10^{-7}	76.0 a
Control	84.4 a

^aMeans not followed by the same letter are significantly different ($P < 0.01$).

hatch decreased as the severity of clipping increased, clipping treatments did not result in an over-all significant change in hatch.

It was reported by Khan and Saxena (12) and Ahmed and Khan (2) that hatching of *M. incognita* was increased by submersion of eggs in carbohydrate solutions, particularly glucose. Our findings do not support their conclusion that the carbohydrate fraction or glucose are effective hatching stimulants. It also appears that the carbohydrate fraction was not involved in the hatching inhibition of the nonamino acid fraction reported earlier (5). It is possible that techniques of allowing hatching in liquid suspensions as followed in earlier studies (2, 12), did not permit a realistic assessment of the influence of the assayed materials.

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