Journal of Nematology 27(1):42–50. 1995.

Optimal Release Rates for Attracting *Meloidogyne incognita, Rotylenchulus reniformis,* and Other Nematodes to Carbon Dioxide in Sand

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Abstract: Movement of vermiform stages of *Meloidogyne incognita, Rotylenchulus reniformis, Ditylenchus phyllobius, Steinernema glaseri,* and *Caenorhabditis elegans* in response to carbon dioxide was studied in 40- and 72-mm-long cylinders of moist sand inside 38-mm-d acrylic tubes. *Meloidogyne incognita, R. reniformis,* and *S. glaseri* were attracted to CO_2 when placed on a linear gradient of 0.2%/cm at a mean CO_2 concentration of 1.2%. When CO_2 was delivered into the sand through a syringe needle at flow rates between 2 and 130 µl/minute, the optimal flow rate for attracting *M. incognita* and *R. reniformis* was 15 µl/minute, and maximal attraction of the two species from a distance of 52 mm was achieved after 29 and 40 hours, respectively. After 24 hours, a total CO_2 volume of 20 cm^3 was sufficient to induce 96% of all *M. incognita* introduced to move into the half of the cylinder into which CO_2 was delivered and more than 75% to accumulate in the 9 cm^3 of sand volume nearest the source. Results indicate it may be possible to use a chemical or biological source of CO_2 to attract nematodes to nematicide granules or biocontrol agents.

Key words: behavior, *Caenorhabditis elegans,* carbon dioxide, chemotaxis, *Ditylenchus phyllobius, Meloidogyne incognita,* nematode, *Rotylenchulus reniformis, Steinernema glaseri.*

Carbon dioxide strongly attracts several nematode species in vitro (1,4,6,7,9,10,12) and has been proposed to mediate root finding (13), stomate penetration (10), insect finding (6), movement through root tissue (14), and the vertical distribution of nematodes within soil (12). If CO_2 gradients influence nematode movement in soil sufficiently, various inorganic or biological sources of the gas could be applied to soil to interfere with nematode behavior, and possibly attract nematodes to nematicide granules. Responses of nematodes to CO_2 apparently have been studied only in water, on transparent gels, and in saturated soil on Baermann funnels (16). The objectives of this research were to test five ecologically diverse nematodes for responses to linear gradients of CO_2 in moist sand and to determine the optimal CO_2 flow rates and gradients for attracting two root-parasitic species to a point source.

Materials and Methods

Nematode preparation: Second-stage juveniles (J2) of *Meloidogyne incognita* race 3, J3 of *Steinernema glaseri* strain NC, J4 of *Ditylenchus phyllobius,* and mixed vermiform stages of *Rotylenchulus reniformis* were obtained as previously described (15). *Caenorhabditis elegans* (obtained from J. A. Veech, USDA, College Station, Texas) was maintained on *Escherichia coli* on agar containing 10 g glucose, 8 g casein hydrolysate, and 4 g yeast extract per liter; mixed stages were separated from nutrient medium by Baermann funnel. All species were washed and transferred by membrane filter (5.0-µm-pore) to a synthetic soil solution (3 mM NaCl, 0.5 mM KCl, 0.05 mM $CaCl_2$, 0.05 mM $MgCl_2$, pH 6.8) 2–18 hours before experiments.

Behavioral arenas: Acrylic tubes (3.8-cm-d, 20 cm long) containing 40- or 72-mm-long cylinders of moist sand (12–15% moisture by weight, 25% volumetric water content) were prepared as previously described (15); however, sand was wetted with synthetic soil solution instead of deionized water and the sand used was grade-5 sandblasting abrasive (pH 7.8, estimated conductivity at saturation 30 µmho/cm, specific density 2.5, bulk density 1.7, total porosity 30%, particle size distri-

Received for publication 27 August 1994.

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The author gratefully acknowledges A. C. Bridges, Biological Laboratory Technician, USDA ARS, for innovative assistance in all aspects of this research, and Myesha Nichols, Biological Aide, USDA ARS, for preparing the figures.

bution 0.2% 0–75 μm, 23% 75–150 μm, 71% 150–250 μm, 6% 250–425 μm). Sand cylinders had 2–3% greater moisture on the downward than on the upward end during preparation. Tubes were randomly assigned to experimental treatments, and half of the replications of each treatment were oriented opposite to the other half relative to CO_2 gradients. Tubes were placed horizontally during experiments on a laboratory bench at ambient temperature (21–23 C).

CO_2 control: Linear gradients were achieved in each 40-mm-long cylinder by plugging each end of the tube with a two-holed rubber stopper and continuously purging (24 ml/minute) the space between stopper and sand on one end with breathing grade air (<0.1% CO_2) while purging the other end with air containing 2.5% CO_2. Purge air was humidified by pumping it from a 30- or 90-liter gas sampling bag containing 200 ml water. Placing the bottom of the bag in an open pan of water lowered the dew point sufficiently to prevent condensation in flowmeters.

Point sources of CO_2 were achieved in 72-mm-long sand cylinders by drilling a 1-mm-d hole in the tube wall and inserting a 23-gauge syringe needle halfway through the sand 20 mm inward from one end; this positioned the needle tip at the center of the third or seventh of nine sections (designated A-I) into which cylinders were cut at the end of experiments (Fig. 1). Carbon dioxide was humidified as described above for purge air and pumped to the needle through capillary tubing with an IPC peristaltic pump (Ismatec SA, Switzerland). A vinyl septum fastened over the hole prevented leakage from around the needle cannula. Where indicated, ends of tubes containing 72-mm-long cylinders were loosely sealed with 36-mm-d polyethylene foam culture-tube plugs to block room air currents and retard evaporation. Otherwise, tubes were equipped with rubber stoppers and continuously purged with hydrated air containing 0, 1.0, or 2.0% CO_2, as described for 40-mm cylinders. Carbon dioxide concentrations in air leaving the tube ends were measured with a CO_2 microelectrode (Microelectrodes, Inc., Londonderry, NH).

Nematode introduction and extraction: Nematodes were syringe-injected into the centers of 40-mm-long cylinders in 250 μl water 18 hours after purge air flow across the cylinder ends was started. Nematodes were applied to the surface of both ends of 72-mm-long cylinders in 250 μl water per end as 7–8 evenly spaced drops per end 6 hours before CO_2 flow through the needle was started. In most cases, 3,000–12,000 nematodes per tube were introduced. Final nematode distributions were determined by extruding each sand cylinder from the tube and slicing it into 8-mm-thick sections (9.1-cm^3) with special tools that kept sections intact, yielding five sections from the 40-mm tubes and nine sections from the 72-mm tubes. The end of the cylinder that was upward during tube preparation was extruded first and the presence of the hole made by the needle

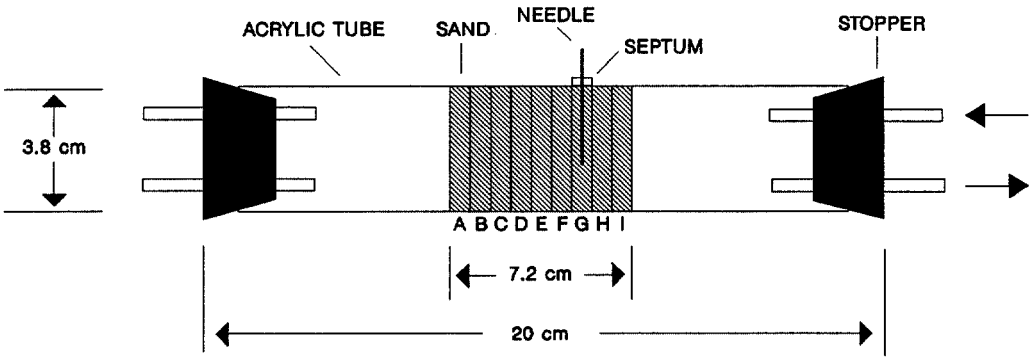

FIG. 1. Diagram of cylinder in which responses of nematodes to a point source of CO_2 were studied.

used to inject nematodes or CO_2 was verified in the center of the third or seventh section. Sand sections were placed in cups containing 20 ml water, vigorously shaken, and allowed to settle for 30 seconds. Nematodes were counted in a 2.0-ml aliquot. Counts were expressed as the percentage of nematodes in the tube, arcsine $\sqrt{\%}$ transformed, and subjected to one-way analysis of variance.

Measurement of CO_2 gradients: Sand sections from tubes prepared and equilibrated identically to those yielding nematode responses of particular interest were transferred to 24.5-ml serum bottles and sealed with septum caps less than 5 seconds after sectioning. Serum bottles were held overnight at 21 C to allow CO_2 from the water to diffuse into the air, and the CO_2 concentration in the air was measured by gas chromatography (50 µl split injection, 30 C oven temperature, thermal conductivity detector, 25-m Poraplot-U megabore capillary column; Supelco, Bellefonte, PA). Peak areas were compared with those of CO_2 concentration standards. Measurements from additional control bottles containing sand sections equilibrated 24 hours against known CO_2 concentrations were used to correct for small losses in total CO_2 content during transfer of sections from tubes to serum bottles. The sand weight and moisture content of every bottle were determined by oven drying, and CO_2 content was expressed relative to water content in the sand (cm^3 CO_2/ml water). Concentrations in four or six replicate cylinders were measured. Altogether, more than 700 CO_2 determinations were done.

Nematode responses to linear gradients: In separate experiments, the response of each of the five species to a linear gradient of CO_2 was tested in six replicate tubes. Each experiment was done twice. Tubes containing *S. glaseri, C. elegans,* and *D. phyllobius* were sectioned 3 hours after injecting nematodes. Tubes containing *M. incognita* and *R. reniformis* were sectioned 5 hours after injection.

Preliminary observations of movement toward a point source: Various combinations of flow rates and exposure times were compared in tubes with loose plugs, using two, four, or six replicate tubes per combination of flow rate and exposure time. Initially, tubes containing *M. incognita* or *R. reniformis* were sectioned at five time intervals 4–8 hours after starting CO_2 delivery, with CO_2 flow rates adjusted so that every tube received a total CO_2 volume of 10 cm^3. In a second experiment, the same exposure times were used but flow rates were increased to deliver a total CO_2 volume of 38 cm^3. In three other experiments, *M. incognita* or *R. reniformis* was exposed to four or seven of 10 flow rates between 0 and 110 µl/minute for 17 or 24 hours.

Time required for a maximal response: Four replications of tubes containing *M. incognita* or *R. reniformis* and receiving CO_2 at 15 µl/minute were sectioned after 0, 18, 42, and 66 hours (*M. incognita*) or 0, 12, 24, and 42 hours (*R. reniformis*). Ends of tubes were continuously purged with breathing-grade air. Control tubes receiving no CO_2 were sectioned at 42 and 66 hours.

Effect of elevated ambient CO_2: Tubes containing *M. incognita* or *R. reniformis* and receiving CO_2 at 14 µl/minute were sectioned 24 hours after starting CO_2 delivery. Ends of tubes were continuously purged with air containing 0, 1.0, or 2.0% CO_2 for 6 hours before starting CO_2 flow and for the remaining 24 hours. Four replications were included for each combination of nematode species and purge gas.

Optimal flow rate: Tubes with *M. incognita* were sectioned 40 hours and tubes with *R. reniformis* 29 hours after starting CO_2 delivery at seven flow rates between 0 and 130 µl/minute. Tube ends were purged with air containing 1.0% CO_2 for 6 hours before starting CO_2 delivery and for the remainder of the experiment. Four replications were included. Controls receiving no CO_2 were included for each species.

Results

CO_2 gradients: Purging the two ends of 40-cm cylinders with air containing 0 and

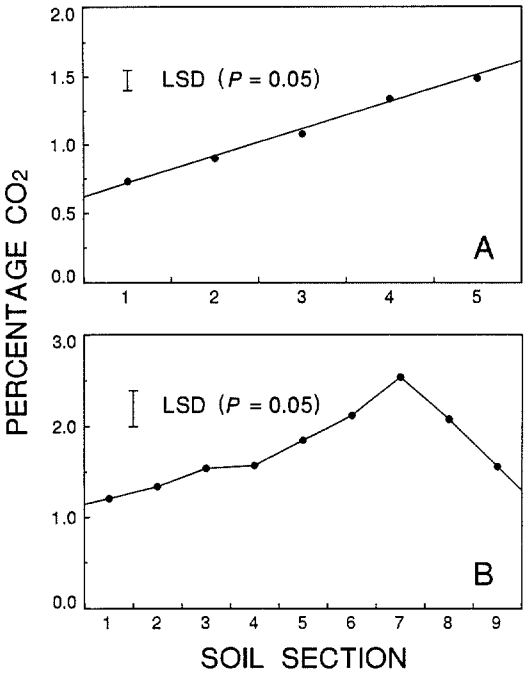

FIG. 2. Typical CO_2 gradients achieved in A) 40-mm-long sand cylinders exposed to 0% CO_2 on one end and 2.5% CO_2 on the other, and in B) 72-mm-long cylinders of sand receiving 15 µl CO_2/minute in section 7 and exposed to a 1% ambient CO_2 concentration. Cylinders were cut into 8-mm sections at the end of experiments.

2.5% CO_2 produced a stable, linear gradient from 0.7 to 1.6% CO_2 along the length of the cylinder (Fig. 2A). Delivering CO_2 into 72-mm tubes through a needle produced a stable gradient within 12 hours. The average CO_2 concentration in the section into which CO_2 was pumped varied from 2 to 10% at flow rates between 5 and 230 µl/minute. Elevating the ambient CO_2 concentration by purging tube ends with air containing 1 and 2% CO_2 elevated the average CO_2 concentration in sand correspondingly. In tubes receiving CO_2 at 15 µl/minute and purged with 1% CO_2, the average CO_2 concentration in the cylinder was 1.5%, and the gradient measured ranged from 0.65%/cm nearest the source to 0.12%/cm at the end farthest from the source (Fig. 2B).

Nematode responses to linear gradients: Movement toward the cylinder end exposed to 2.5% CO_2 after 3–5 hours was detected for *M. incognita, R. reniformis,* and *S. glaseri,* with 70–80% of the nematodes introduced recovered from the half of the cylinder exposed to 2.5% CO_2 (Table 1). The average percentages of nematodes in the section directly exposed to 2.5% CO_2 were 55%, 41%, and 35% for *S. glaseri, M. incognita,* and *R. reniformis,* respectively, with 8, 11, and 1% in the section exposed to air without CO_2.

Preliminary observations of movement toward a point source: In 72-mm cylinders receiving a total of 10 cm^3 CO_2 during 8 hours or less, significant responses by *M. incognita* and *R. reniformis* were detected only at flow rates between 23 and 35 µl/minute, 5–7 hours after starting CO_2 delivery (Table 2). No responses were detected at these

TABLE 1. Response of *Meloidogyne incognita, Rotylenchulus reniformis, Ditylenchus phyllobius, Steinernema glaseri,* and *Caenorhabditis elegans* to a 0.6%/cm linear gradient of CO_2 in 40-mm-long cylinders of sand.†

Species	Experiment 1		Experiment 2	
	Total nematodes counted	Percentage in half receiving CO_2	Total nematodes counted	Percentage in half receiving CO_2
M. incognita	73	72.0 NS‡	358	71.7*
R. reniformis	407	80.3***	238	77.3*
D. phyllobius	518	49.0 NS	384	45.3 NS
S. glaseri	258	78.0***	657	76.1***
C. elegans	396	58.6 NS	213	54.4 NS

Data are means of six replications and were arcsinc $\sqrt{\%}$ transformed before analysis. Untransformed means are presented.

† Nematodes were injected into the centers of cylinders and extracted from each of five equal sections along the tube after either 3 hours (*D. phyllobius, S. glaseri,* and *C. elegans*) or 5 hours (*M. incognita* and *R. reniformis*).

‡ NS = not significant.

*,*** indicate means differ from 50% by one LSD at P = 0.05 and P = 0.001, respectively.

TABLE 2. Movement of *Meloidogyne incognita* and *Rotylenchulus reniformis* toward a point source of CO_2 in 72-mm-long cylinders of sand when the flow rate and exposure time were adjusted so that the total gas volume delivered was 10 cm^3 or 38 cm^3 and the total time elapsed was 8 hours or less.

		10 cm^3 total CO_2			38 cm^3 total CO_2	
			Response (%)†			Response (%)
Gas	Time (hours)	Flow rate (µl/min)	*M. incognita*	*R. reniformis*	Flow rate (µl/min)	*R. reniformis*
CO_2	0	0	55.3	46.0	0	49.0
	3:50	44	54.3	56.7	150	52.7
	4:45	35	60.9**	60.0^	130	50.7
	6:00	28	56.6^	58.6	113	49.7
	7:15	23	58.6*	59.0^	85	54.0
	8:00	20	52.2	57.7	80	52.5
Air	6:00	28	48.8	51.5	113	47.3

Each value is the mean of four replications. Data were arcsine $\sqrt{\%}$ transformed before analysis, but untransformed means are presented.

† Response is defined as the percentage of nematodes recovered from the half of the cylinder into which CO_2 was injected.

^,*,** indicate that values are one LSD greater than the statistically expected value of 50% at $P = 0.10$, $P = 0.05$, and $P = 0.01$, respectively.

time intervals at flows of 80–150 µl/minute. The final distribution of nematodes in tubes receiving >100 µl/minute were similar to initial distributions, suggesting that nematodes were anaesthetized; however, nematodes extracted from soil were always highly motile when counted, several hours after sectioning. When CO_2 delivery was continued for 17 or 24 hours, stronger responses occurred than at 5–7 hours and were obtained in tubes receiving low flow rates (6–31 µl/minute) (Table 3). Responses of *R. reniformis* were greater than those of *M. incognita,* suggesting that *M. incognita* was less strongly attracted or moved more slowly. In water, *M. incognita* appeared slower than *R. reniformis* at ambient temperature.

Time required for a maximal response: In tubes purged with air containing no CO_2, maximal responses to CO_2 delivered at 15 µl/minute were achieved for *R. reniformis* after 24–28 hours and for *M. incognita* after 36–40 hours (Fig. 3). More than 80% of the nematodes of both species were recovered from the tube half receiving CO_2 and

TABLE 3. Results of preliminary experiments on movement of *Meloidogyne incognita* and *Rotylenchulus reniformis* toward a point source of CO_2 in 72-mm-long cylinders of sand after 17 and 24 hours.

17 hours			24 hours			
		Response (%)†			Response (%)	
Flow rate (µl/min)	Total volume (cm^3)	*R. reniformis*	Flow rate (µl/min)	Total volume (cm^3)	*M. incognita*	*R. reniformis*
0	0	54.0	0	0	50.0	44.0
6	6	85.0***	2	3	51.0	68.3**
15	15	75.0***	7	10	67.3*	80.0***
31	32	70.8***	17	24	72.3**	82.5***
47	48	55.8				
75	77	52.0				
110	112	54.0				

Data are means of four replications and were arcsine $\sqrt{\%}$ transformed before analysis. Untransformed means are presented.

† Percentage of nematodes recovered from the half of the cylinder into which CO_2 was injected.

*,**,*** indicate means differ from 50% by one LSD at $P = 0.05$, $P = 0.01$, and $P = 0.001$, respectively.

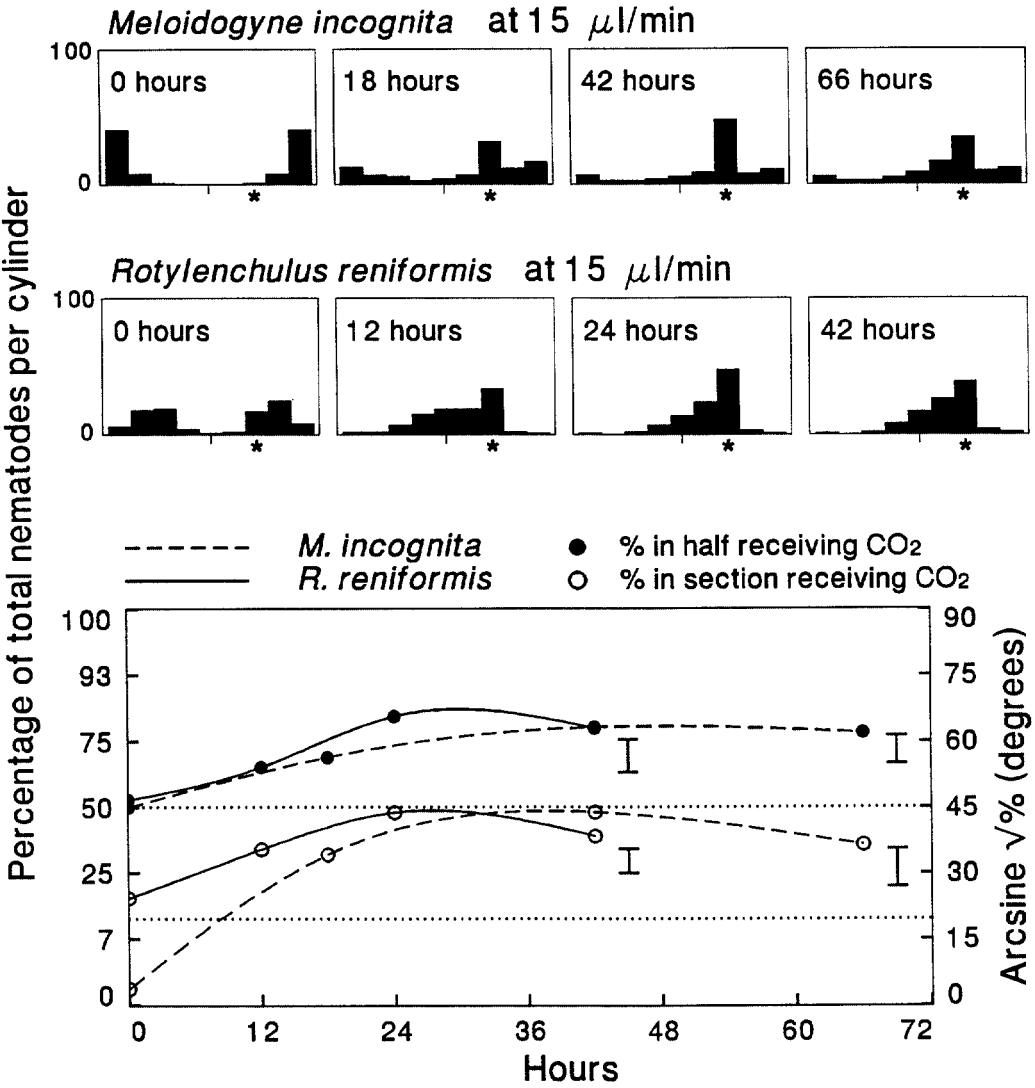

FIG. 3. Changes in the distributions of *Meloidogyne incognita* and *Rotylenchulus reniformis* through time within 72-mm-long cylinders of sand in response to a 15-µl/minute point source of CO_2. Bars (above) indicate nematodes in each of nine sections along the cylinder; asterisks (*) indicate CO_2 injection points. Brackets (below) indicate LSDs at $P = 0.05$ for arcsine $\sqrt{\%}$ transformed data. Data are means of four replications.

50% accumulated in the section into which CO_2 was injected.

Effect of elevated ambient CO_2: Purging tube ends with air containing 1.0 or 2.0% instead of 0% CO_2 increased the response of *M. incognita* to CO_2 at 14 µl/minute but did not measurably affect *R. reniformis* (Table 4). In tubes purged with air containing 1.0% CO_2, 96% of *M. incognita* were recovered from the tube half receiving CO_2 and 78% were in the section into which CO_2 was injected.

Optimal flow rate: In tubes held long enough to expect a maximal response, the optimal flow rate for both species was 15 µl/minute (Fig. 4). Thus, a maximal response was obtained for *M. incognita* with 36 cm^3 total CO_2 volume, and with 26 cm^3 for *R. reniformis*. The response of *M. incognita* again was greater than that

TABLE 4. Influence of ambient CO_2 concentration on the movement of *Meloidogyne incognita* and *Rotylenchulus reniformis* toward a point source of CO_2 emitting 14 µl/minute for 24 hours in 72-mm-long cylinders of sand.

Ambient CO_2%	Percentage in half receiving CO_2		Percentage in section receiving CO_2	
	M. incognita	*R. reniformis*	*M. incognita*	*R. reniformis*
0	84 a	86 a	46 a	60 a
1.0	96 b	86 a	78 b	53 a
2.0	93 b	90 a	66 b	67 a

Data are the means of four replications and were arcsine $\sqrt{\%}$ transformed before analysis. Means of untransformed data are presented.

† Different letters within a column indicate values differ by one LSD at $P = 0.05$. The expected value, assuming uniform dispersal, is 50% in the left two columns and 11% in the right two columns. All means differ from the appropriate expected value at $P = 0.001$.

of *R. reniformis* with more than 95% of all nematodes recovered within the tube half receiving CO_2 and more than 75% within the section into which CO_2 was injected.

DISCUSSION

Nematode movement toward CO_2 on agar and agarose has been reviewed several times (4,13,20). My results confirm that CO_2 can strongly attract some species in sand, where the physical conditions influencing gas diffusion and nematode movement can differ greatly from conditions on gel surfaces. Behaviorally effective gradients in sand (0.12%/cm to 0.65%/cm) were generally similar to those measured previously for *Ditylenchus dipsaci* (0.1%/cm) (9) and *M. incognita* (0.01–0.4%/cm) (12). Elevating the ambient CO_2 concentration to simulate soil conditions either strengthened or had no effect on the nematode response.

Comparisons between threshold gradients required to elicit a response from *M. incognita,* theoretical gradients around plant roots, and vertical gradients measured in an apple orchard led Pline and Dusenbery (12) to predict that vertical gradients are more likely to attract nematodes than gradients around roots. It is noteworthy in this regard that the optimal release rate measured in the present study (15 µl/minute) was similar to that which could be produced by 1–200 mg of various kinds of biological tissue. For example, 3×10^5 cells (<1 mg) of *Saccharomyces cerevisiae* (baker's yeast) can release 15 µl CO_2/minute during aerobic respiration (5), and a young sunflower seedling can release about 2.5 µl/minute (17). Moreover, in soil with greater diffusive resistance than the sand used, the release rate required to establish a behaviorally effective gradient would likely be lower than in sand (2). Therefore, the low flow rates required to attract nematodes in this study support the interpretation that CO_2 is commonly used by nematodes to locate food.

The results also provide a basis for considering the possibility of using a CO_2 source to attract root-parasitic nematodes to nematicide granules or to pellets containing biocontrol agents (8,11). The total volume of CO_2 needed to attract *M. incognita* and *R. reniformis* (4–40 cm^3) can be generated by 10–100 mg of HCO_3^-, and the volume of soil in the 72-mm tubes was 10^{-7} of a 7.5-cm-deep hectare slice. Therefore, similar conditions might be achievable in a crop field by applying potassium bicarbonate as granules coformulated with an acid and a nematicide at 100–1,000 kg HCO_3/ha on a broadcast basis, or 10–100 kg/ha band-incorporated over the seed furrow. Alternatively, sufficiently slow release of the gas might be achieved better through calcium alginate co-encapsulation of a yeast and nutrient substrate. Calcium alginate encapsulation, a rapidly developing technology in the fermentation industry (3,19), is undergoing considerable research as a means for storing and dispersing microorganisms for various applications, including pollutant biodegradation, oil recovery, and inundative release of biocontrol agents in row crops (18). In either case, the byproducts of CO_2 generation could alter the nematode response or affect plant growth and

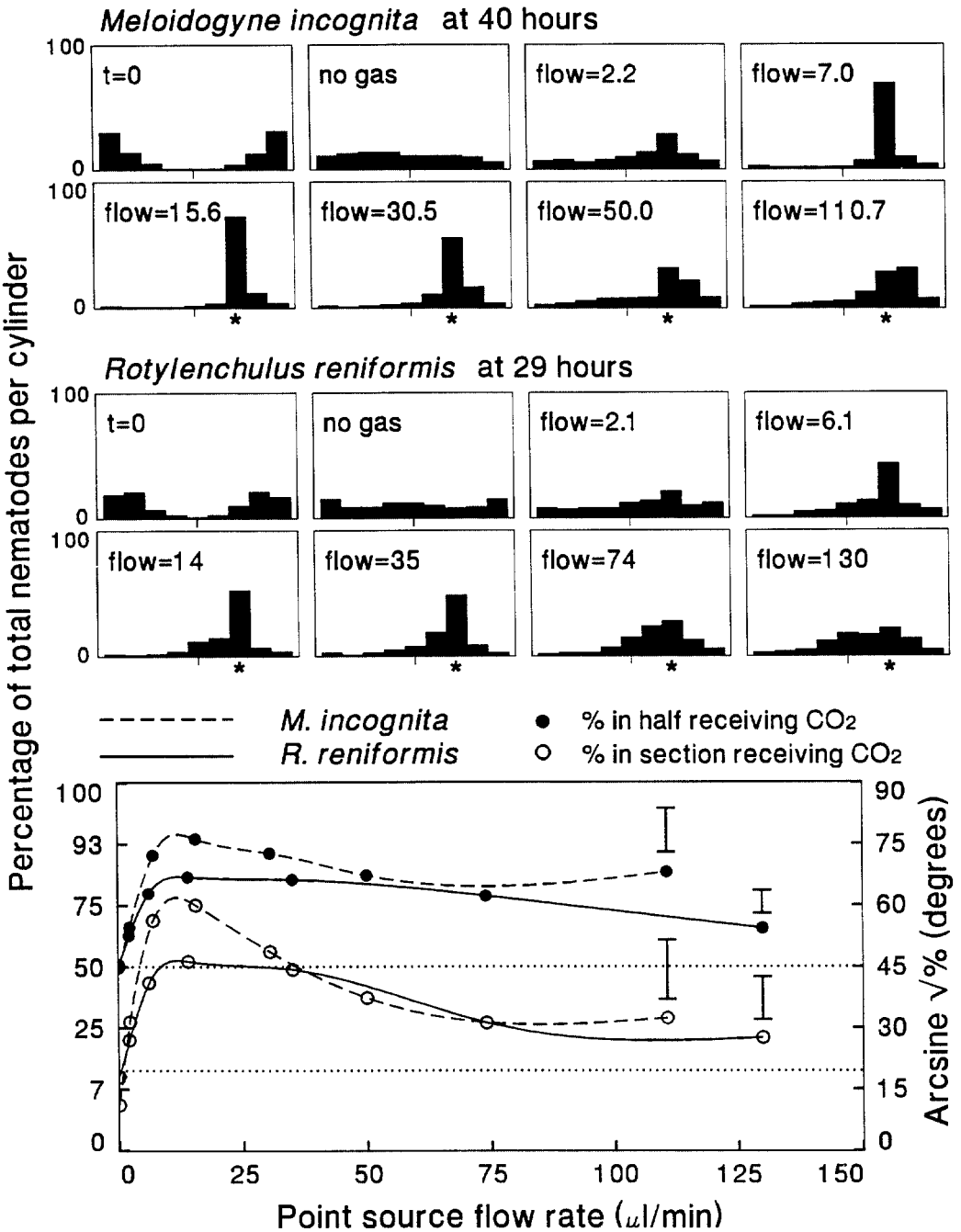

FIG. 4. Effect of CO_2 flow rate on responses of *Meloidogyne incognita* and *Rotylenchulus reniformis* to a point source of CO_2 in sand. Bars (above) indicate nematodes in each of nine sections along the cylinder; asterisks (*) indicate CO_2 injection points. Brackets (below) indicate LSDs at $P = 0.05$ for arcsine $\sqrt{\%}$ transformed data. Data are means of four replications.

would need careful study. Information is also needed regarding the attraction of nematodes to CO_2 in different kinds of soil with different physical structures and different moisture contents.

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