Quantitative Evaluation of a Leaching Model System for Soil Fungistasis

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ABSTRACT

Filter paper disks, each containing 45 μg glucose, were incubated on smoothed soil in petri dishes. Loss of glucose from such disks was linear with logarithm of time from 30 to 240 minutes. Glucose loss was more rapid with natural than with autoclaved soils, with the difference detected within 8 minutes. A model system designed to mimic this microbial glucose sink through continuous aqueous leaching of a bed of glass beads was calibrated by measuring glucose loss from disks incubated on the beads. At low flow rates (2-5 ml/hr) glucose loss characteristics were similar to those for soil. As flow rates increased up to 60 ml/hour, early losses exceeded those on soil, resulting in progressively decreased glucose half-lives; however, slopes after 15 minutes steepened only slightly. Germination of conidia of *Curvularia lunata, Helminthosporium sativum,* and *H. victoriae* in the leaching system decreased as flow rates increased. High flow rates (> 20 ml/hour) were required to reduce germination to the level occurring on soil.

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Additional key words: microbial energy sink.

The role of nutrient deprivation in restricting fungal spore germination in soil has been emphasized by Lockwood and co-workers (5, 6, 10), and others (1, 3). Evidence supporting the importance of nutrient-deprivation is the fact that fungal propagules which germinate poorly in soil are also inhibited when incubated on sand or glass beads where nutrients are continually removed by leaching with water or dilute salt solutions. The propagules inhibited include many whose germination is nutrient-independent; i.e., they germinate well on such substrates when not undergoing leaching. In a recent study, 15 out of 17 such propagules tested were inhibited similarly in the soil and in the leaching system (5). Incubation of nutrient-independent spores in leaching systems or on soil, for periods up to 48 hours, in neither case resulted in loss of subsequent ability to germinate without added nutrients when the stress was removed (5, 6). Moreover, spores which had taken up ^{14}C glucose during germination, but which had not yet produced germ tubes, lost label at similar rates during subsequent incubation on soil or the leaching system (10). The soil's inhibitory specificity may also be simulated by the leaching model. Activated ascospores of *Neurospora tetrasperma,* whose germination is also nutrient-independent, but which germinate freely on soil also germinated in the leaching system (5, 6). Thus, in several respects the leaching model appears to reproduce the fungistatic effect of soil.

However, it was not known whether the leaching system at the flow rates used, generally 5-30 ml/hour in a 15-cm diameter dish, quantitatively reflects the efficiency of the energy sink imposed in soil through microbial activity. Therefore, a method was sought by which the natural microbial energy sink of soil could be characterized quantitatively, and by which the model system could be calibrated.

MATERIALS AND METHODS.—The approach used was to measure, through time, the loss of glucose from an inert carrier incubated on soil. The rate of glucose loss was expected to be greater on natural (nonautoclaved) than on autoclaved soil due to imposition of a steepened diffusion gradient via microbial activity.

Glucose and carriers.—Disks prepared from various kinds of agar all contained soluble carbohydrates which could complicate the assay, and which were removed

only with difficulty. Silica-gel disks were carbohydrate-free, but tended to crumble and shrank readily. Membrane filter disks were unsuitable because they lost glucose to the soil too rapidly for convenient handling; 85-90% was lost in 30 minutes and half-life was approximately 2 minutes, even on sterile soil. Whatman filter disks (13 mm in diameter) were found to be suitable carriers in that glucose was retained long enough for convenience, but was readily leached out for assay.

Soils.—Three soils were used: (i) a clay loam from the Cambridge Botany School Field Station [pH 7.8, free $CaCO_3$ 8%, moisture holding capacity (MHC) 52 ml/100 g air-dry soil, organic matter 2.2%, total nitrogen (N) 0.12%, available phosphorus (P) 90 μg/g, available potassium (K) 400 μg/g] (ii) a sandy loam from the Cambridge Botanic Garden [pH 7.5, free $CaCO_3$ 7.5%, MHC 45 ml/100 g soil, organic matter 4.9%, total N 0.28%, available P 95 μg/g, available K 415 μg/g]; and (iii) Kettering loam [pH 6.0, free $CaCO_3$ nil, MHC 66 ml/100 g, organic matter 5%, total N 0.33%, available P 6 μg/g, available K 45 μg/g].

Soil was sieved (2 mm), and stored moist for several weeks in glass jars. Fifteen to 20 g were placed in petri dishes (15-cm diameter), the soil moisture was adjusted to 75-80% of MHC, and the surface smoothed with a spatula. For sterilization, the soil was autoclaved for 60 minutes in the petri dish, the moisture content was adjusted by weight, and the surface smoothed aseptically. Soil was used for assay the same day the dishes were prepared.

Model system.—The leaching system described earlier was used (5). Briefly, it consisted of a separatory funnel equipped with a sealed-in dripping tip to maintain a constant head. The funnel stem was connected to a petri dish (15 cm diameter) with an inlet at one side of the lid, and an outlet in the bottom on the opposite side. The petri dish contained 180 g of #9 glass beads (0.32 - 0.42 mm in diameter) through which the leaching fluid was percolated. Flow rates were adjusted with a needle valve. The petri dishes were slanted with the upper (inlet) side 6 mm higher than the lower, and were leveled laterally.

Assay.—Glucose (usually 45 μg) was applied in 90 μl aqueous solution to paper disks using a disposable micropipette. The disks were placed on the surface of soil or on glass beads in the leaching system. Duplicate disks were placed randomly on soil plates. For the leaching system, disks were usually arranged in horizontal rows across the plates, with one disk of a duplicate pair for each time interval placed on the left, and the other on the right of the midline. At designated times, the disks were removed and placed in 2 ml water in vials and assayed immediately. Disks from soil were placed on an equivalent volume of ice to inhibit microbial activity.

Glucose concentrations were determined by means of the glucose-oxidase reaction (Glucostat reagent, Worthington Biochemical Corp.). Five ml of the reagent were added to vials placed in a water bath at 37 C for 30 minutes. The reaction was stopped with 4N HCl. Optical density was determined with a colorimeter with a blue (425 nm) filter, using glucose as the standard. Glucose, as percent of that present initially, when plotted against log of time, gave essentially straight lines after the first 15 minutes. Slopes of lines and glucose half-lives were

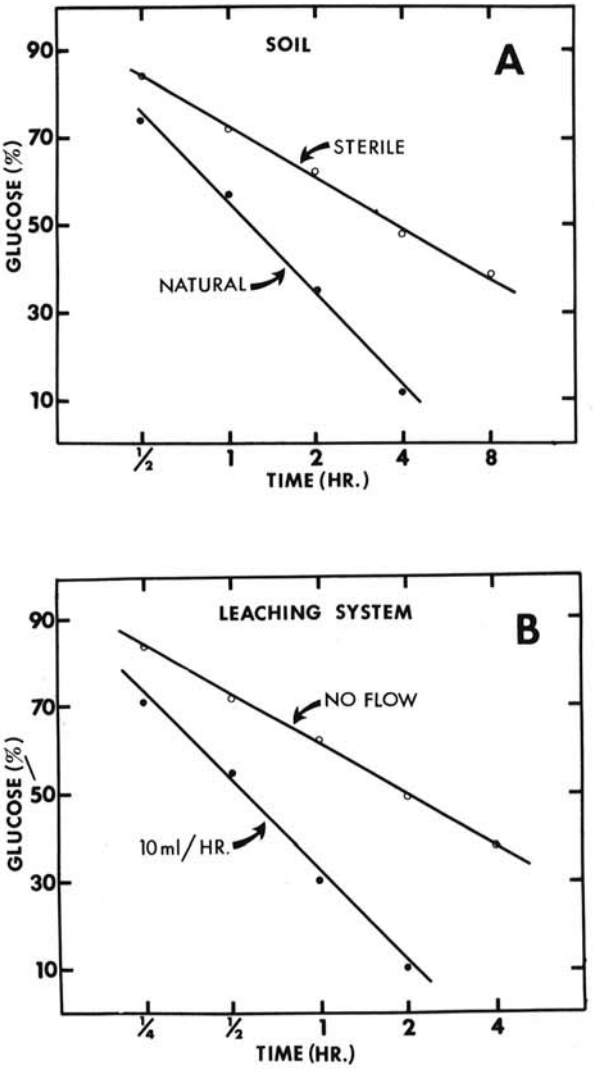

Fig. 1-(A, B). Loss of glucose, as % of original amount, from filter paper disks incubated on **A)** natural and autoclaved (sterile) clay loam soil, and **B)** a bed of water-saturated glass beads continually leached at the rate of 10 ml/hour, or without leaching.

TABLE 1. Loss of glucose from filter paper disks incubated on the surface of three soils[a]

Soil	Slope[b]		Glucose half-life (min)	
	Natural	Autoclaved	Natural	Autoclaved
Clay loam	−0.70	−0.41	90	230
Sandy loam	−0.50	−0.36	98	224
Loam	−0.66	−0.41	65	264
Mean	−0.65	−0.40	84	239

[a]Results are means of six experiments for clay loam and of three each for sandy loam and loam.

[b]Slope of line (b) for % loss of glucose as a function of log of time after 15 minutes.

TABLE 2. Rates of loss of glucose from filter paper disks during different intervals of incubation on clay loam soil, or on glass beads continually leached at various flow rates

Substrate	% loss/minute for indicated interval (min)[a]					
	0-8	8-15	15-30	30-60	60-120	120-240
Soil						
Natural	1.1	1.0	0.6	0.8	0.6	0.5
Autoclaved	[	0.5	]	0.4	0.2	0.1
Leached glass beads						
0 ml/hour	[	1.3	]	0.5	0.6	0.2	0.2
1-4 ml/hour	1.5	1.5	1.1	0.7	0.7	0.6
5-20 ml/hour	2.4	1.8	1.4	1.4	1.5	1.1
20-60 ml/hour	3.0	1.6	2.2	1.5	1.3	

[a]Percent loss was calculated with the amount remaining at the end of the preceding interval taken as 100%. Figures in brackets indicate that the samples were not taken until 30 minutes (autoclaved soil) and 15 minutes (leached glass beads, 0 ml/hour).

TABLE 3. Loss of glucose from filter paper disks incubated on glass beads continually leached with water at three different flow rates, or on water-saturated beads without leaching

Flow rate (ml/hour)	Slope[a]	Half-life (minutes)
0	−0.37	110
2	−0.58	53
10	−0.64	34
50	−0.70	22

[a]Slope of line (b) for % loss of glucose as a function of log of time after 15 minutes.

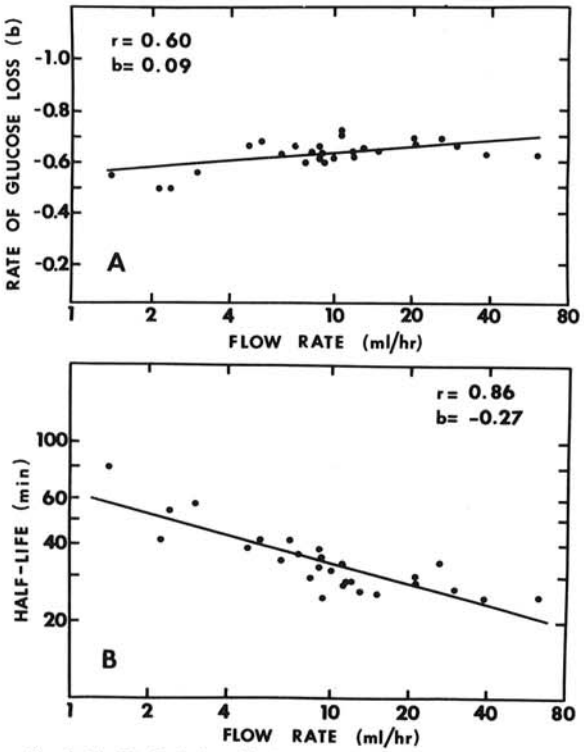

Fig. 2-(A, B). Relationship between rate of glucose loss from filter paper disks incubated on glass beads continually leached with water, and flow rate of water. **A)** Slope (b) × flow rate. **B)** Half-life × flow rate.

calculated mathematically. Rates of glucose loss per minute for the various incubation intervals on soil and on the model leaching system were determined, using the amount remaining in the disks at the beginning of each interval to calculate percentage loss during that interval.

Spore germination tests.—Germination of conidia was determined on clay loam soil, and on the leaching system, at different flow rates using 0.025 M phosphate buffer (pH 7.0) as the leaching medium. Fungi used were *Curvularia lunata* (Wakker) Boedijn, *Helminthosporium sativum* Pam., King and Bakke, and *H. victoriae* Meehan & Murphy. Spores of *C. lunata* were produced on V-8 juice agar (6), and those of the other two fungi on sterilized wheat straws. Germination of spores washed three times by refrigerated centrifugation was determined on Nuclepore membrane filters (General Electric Co., 0.5μm). After being incubated for 16 hours, propagules were stained with rose bengal, destained on water-saturated sand, and air-dried on slides. The membranes were then dissolved by adding 2-3 drops of chloroform; when dry, the preparation was transparent. At least 100 spores of each fungus were counted on each of duplicate membranes

RESULTS.—*Soil.*—Loss of glucose from paper disks was much more rapid with natural than with autoclaved soil (Fig. 1-A), as shown by slopes of glucose loss curves and half-lives (Table 1), and rate of loss per min (Table 2). Soils sterilized with propylene oxide gave slopes and half-lives similar to those for autoclaved soils. Glucose loss from disks on natural soil was most rapid during the first few minutes, then declined to a more or less constant rate thereafter (Table 2). By contrast, loss of glucose on autoclaved soil began at a slower rate than that on natural soil, then declined even more. The difference in rate of glucose loss from disks on natural and autoclaved soils was easily detectable within 30 minutes (Fig. 1-A). In other tests using lower concentrations of glucose (9 and 18 μg/disk) it was detected within 8 minutes. The greater sensitivity with lower glucose concentrations is presumably because that portion of the glucose utilized by micro-organisms is greater in proportion to the total amount entering the soil. With more sensitive methods, it may be shown to occur even earlier.

The model system.—In numerous experiments, leaching systems were run at different flow rates ranging

TABLE 4. Germination of conidia of *Curvularia lunata, Helminthosporium sativum,* and *H. victoriae* on Nuclepore filters incubated on potato-dextrose agar, clay loam, or on sand leached with 0.025 M phosphate buffer (pH 7.0) at different flow rates

Substrate	Flow rate (ml/hour)	Germination (%)[a]		
		C. lunata	*H. sativum*	*H. victoriae*
Sand	0	79	30	76
Sand	4-6	68	17	54
Sand	15-18	37	11	41
Sand	45-55	30	6	23
Clay loam		16	7	17
Agar		89	87	88

[a]Mean of four experiments in which at least 200 spores of each fungus were counted.

from approximately 2 to 60 ml/hour to determine which rates would most closely approximate the glucose uptake characteristics of natural soils. Loss was more rapid with water flowing through the bed of glass beads than on a saturated bed without flow (Fig. 1-B). At low flow rates, the leaching system closely resembled soil in terms of half-life and slope (Tables 1 & 3) and rate of loss per min (Table 2). However, increased flow rates resulted in much greater early losses of glucose from paper disks than occurred at flow rates or on natural soil, although these also declined with time. This had little effect on slopes over the linear portion of the curve (Fig. 2-A), but resulted in increasingly shortened half-lives (Fig. 2-B), in effect displacing the curve to the left.

To determine whether a prior addition of glucose would result in a more rapid loss of a subsequent application, disks containing 450 μg glucose alone, glucose + NH_4NO_3 (C/N ratio, 12/1), or glucose + 180 μg peptone, were applied to clay loam soil, left for 16 hours, then replaced with fresh disks which were assayed for glucose loss. Slopes and half-lives for such disks did not differ from those of control disks assayed without a prior addition of glucose.

Spore germination.—Germination of conidia of *C. lunata, H. sativum,* and *H. victoriae* was determined on potato-dextrose agar, on clay loam soil, and on the leaching system operated at the following rates of flow: 0 (buffer-saturated sand), 4-6 ml/hour, 15-18 ml/hour, and 45-55 ml/hour. Increased flow rates resulted in decreased germination, but the fastest flow rate was required to reduce germination to levels approaching those occurring on soil (Table 4).

DISCUSSION.—The more rapid loss of glucose from paper disks on natural soil than on autoclaved soil is attributed to the general activity of soil microbes in providing a sink for energy-yielding nutrients. Such nutrients, including those in spore exudates, are readily utilized by microorganisms in soil (2, 7). The onset of the microbial energy sink is early, probably less than 8 minutes, which is sufficiently fast for its participation in the inhibition of the rapidly germinating nutrient-independent propagules (8). Increased rates of glucose loss similar to those occurring on soil were produced by the leaching system over a wide range of flow rates. However, glucose loss characteristics on the leaching system most closely resembled those on soil at the lower flow rates, at which germination was not strongly suppressed. Suppression of germination to the low levels occurring on soil has been reported to occur at relatively low flow rates (5, 6), but even these rates of flow would give somewhat shorter half-lives for glucose than seem to occur on soil.

The low level of fungistasis of nutrient-independent spores in the leaching system at low flow rates may suggest that factors other than nutrient deprivation are involved in the inhibition of such spores. Volatile (4) and non-volatile (9) fungal inhibitors have been shown to exist in some soils, and these have been proposed to have a role in fungistasis.

However, it is also possible that the rate of loss of glucose from paper disks may not be a quantitatively appropriate means of calibrating the leaching model system. The leaching system probably removes glucose by two means: by imposition of a steepened diffusion gradient from the disks, and by direct leaching of glucose by passage of some of the leaching solution through the disks. In soil, loss of glucose would occur only through diffusion, enhanced by microbial assimilation of glucose from the soil solution. Moreover, loss of metabolizable materials from paper disks may not be analogous with loss from spores themselves, which would involve passage through living membranes. Loss of metabolites from living cells may be effected to a greater extent by the increased diffusion gradient than by direct effects of leaching. Hence, a high rate of flow may be required to impose upon spores a diffusion stress equivalent to that occurring in soil. In a previous experiment (10) in which spores rather than filter paper disks were carriers of glucose, results with soil and model system were in close agreement: spores which had taken up ^{14}C glucose for several hours prior to germination required about 6 days for subsequent loss of half the label when transferred either to soil or the leaching system.

The failure of a previous addition of glucose to soil to result in an enhanced rate of uptake of a subsequent application conflicts with an earlier report that a cellulose amendment increased the rate of utilization of a later addition of glucose (1). The difference may be related to the fact that in the former case glucose was mixed with the soil and was thus in intimate contact with the microbial population, whereas in the present work the contact of microorganisms with glucose was restricted to the area near the paper disks, and was limited by the rate of glucose diffusion from the disks.

The leaching system reproduces the fungistatic effect of soil, it imitates the glucose-loss characteristics of paper disks on soil in a general way, and provides an effective

method for experimentally isolating the metabolic energy-sink component of soil. However, further evaluation is required before quantitative aspects of the system as a model for soil fungistasis can be completely assessed.

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