A method for the detection and analysis of growth patterns of microorganisms in soil

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A fluorescence-staining technique using the magnesium salt of 8-anilino-1-naphthalene sulfonic acid is described and used to follow the changes in the distribution patterns of microorganisms in soils. A statistical procedure was used to determine the occurrence of significant differences in clumping of bacteria (i.e., production of colonies) in different regions of artificial soil-aggregate systems treated with nutrient solutions and also with a herbicide, Linuron. The response of soil microorganisms to glucose amendment was most marked in the aerobic, outer zone of aggregates. Linuron inhibited colony formation in aggregates treated with the herbicide. The method allows continued observations to be made on the same soil sample at intervals during incubation and so can be used to determine growth rates, inhibitory effects of chemicals, distribution patterns in soils, effects of added nutrients, and other effects where growth in situ is important.

Polonenko, D. R., D. J. Pike et C. I. Mayfield. 1978. Une technique de marquage à la fluorescence utilisant le magnésium de l’acide 8-anilino-1-naphthalène sulfonique et utilisée pour suivre les changements des profils de distribution des microorganismes dans le sol. Nous avons utilisé un cheminement statistique pour déterminer l’apparition de différences significatives dans la formation d’amas de bactéries (i.e. production de colonies) dans différentes couches de systèmes artificiels d’agréats de sol traités au moyen de solutions nutritives ainsi qu’avec un herbicide, le Linuron. La réponse des organismes du sol à un traitement au glucose était plus importante dans la couche aérobie en périphérie des agrégats. Le Linuron a inhibé la formation de colonies dans les agrégats ayant subi le traitement avec l’herbicide. La méthode permet d’effectuer une série d’observations sur le même échantillon de sol à des intervalles donnés au cours de l’incubation; elle permet ainsi de déterminer des taux de croissance, les effets inhibiteurs de produits chimiques, des profils de distribution dans les sols, les effets d’un ajout de nutriments, et les autres effets où l’évaluation de la croissance in situ est importante.

Introduction

The influence of environmental chemicals on soil microorganisms has been investigated using many different methods. Most have involved monitoring changes in the numbers or types of microorganisms in soil (Casida 1968) in response to applied chemicals. Simpler methods using pure cultures of microorganisms can provide only limited information on the probable responses of these same organisms in situ in soils.

Fluorescent-staining methods using the magnesium salt of 8-anilino-1-naphthalene sulfonic acid (Mg-ANS) have been described (Mayfield 1975) and these methods allow colony formation from single organisms to be followed microscopically in soil samples (Mayfield 1977).

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This latter technique has a number of disadvantages: the staining agent may selectively inhibit growth and colony formation, and the exposure to ultraviolet light during examination may have the same effect. In addition, the method is time-consuming, particularly since it is necessary to examine the same field at intervals to follow the development of the colonies. Many of the original fields are disturbed during observation and only a fraction of the total can thus be observed throughout the period of the experiment.

The present technique was developed to minimize most of the disadvantages listed above. Periodic microscopic examination of stained microorganisms in incubated soil samples were made at intervals and the results from different areas of the soil sample were compared by statistical pattern analysis methods. Continued observation of the same field, with the attendant disadvantages,
was not required. The technique was applied to studies on the response of microorganisms to nutrients and to studies on inhibition of microorganisms by a herbicide.

Materials and Methods

Apparatus

A plexiglass slide-perfusion apparatus was designed which allowed both the fluorescent stain (Mg-ANS) and the nutrients or chemicals under investigation to be percolated through a slide. The overall length of the slides was 80 mm, the overall width 33 mm, and each compartment was 3 mm deep (Fig. 1). Eighty-millimetre strips of Whatman GF/C glass fibre filter material were placed across the three compartments and a layer of acid-washed silica sand was placed on top of the filter strips to a depth of 2 mm in the central compartment. The slides were autoclaved for 15 min at 121°C. A soil sample was placed on top of the silica sand to a depth of 3 to 4 mm and the substrate under investigation was placed in the outer two compartments with two or three drops also added to the soil. A coverslip was placed on top of the soil sample (Fig. 2). Since air could diffuse into the peripheral region of the soil, this soil-incubation system approximated natural soil aggregates. The filter strips acted as a type of perfusion mechanism allowing the liquid in the outer wells to percolate into the centre compartment at a controlled rate. The layer of silica sand acted as a drainage system preventing excess water accumulating in the soil sample.

Staining and Observation

A modified version of the Mg-ANS staining technique (Mayfield 1975) was used where the stain was prepared at a concentration of 3 mg ml⁻¹ in distilled water and added to the outer compartments for 4 h before microscopic observation. A Nikon Apath microscope with epifluorescence equipment was used for observation and photography with a Nikon HPM automatic camera. The filter combination was used in the violet excitation, dichroic mirror, and eyepiece barrier system on the microscope.

If the microorganisms in the microscope field of the 70 × lens normally used were counted manually, long periods of exposure to ultraviolet light would have been encountered. Tests showed that exposure to the illumination longer than 20 s inhibited some soil microorganisms. To minimize this exposure, photographic records of the fields were made with high speed film (Kodak Tri-X rated at 800 ASA). Photographs of a 20 × 20 square grid pattern (Zeiss Canada Ltd.) in the microscope eyepiece were made and printed onto sheets of film the same size as the photographs of the soil microorganisms. Exactly the same conditions were used for enlarging this grid pattern, so that when it was placed over the photographs the number of microorganisms in a particular area could be determined.

Two different soil-amendment systems were studied. The first was a control amendment and was designed to see whether growth occurred (as indicated by the formation and expansion of distinct colonies) and if so, to determine the growth rates in the slides. The second system was designed to determine the effect of a herbicide, Linuron, on the growth of soil microorganisms. Two control and two experimental slides were prepared as described above. Both were percolated with a sterile straw extract (1 kg wheat straw autoclaved in 1 L of water) and the experimental slides were also treated with Linuron at the field application rate (1 mg ml⁻¹). The slides were observed at daily intervals for the first 3 days of each of four successive weeks.

Sampling and Analysis of Distribution

Microbial distribution was determined by observing six fields in each of two planes superimposed on each slide. Each plane extended over the width of the soil sample. Since the average width was 18 mm, the six fields were placed 3 mm apart (Fig. 3). This spacing provided two observations in each of the three main zones designated in the soil aggregates (zone 1 = aerobic zone; zone 2 = transition zone; zone 3 = anaerobic zone). The two planes were separated by 1 mm and were equidistant from the centre of the soil sample. Since there were two duplicate soil slides, there were four replicate planes and therefore eight replicate sets of the three zones.

Five block sizes were used to partition the data for analysis (Fig. 3). Block 1 consisted of the individual squares in each microscope field. Block 2 was formed by joining the two squares (i.e., square 1 and 2; square 3 and 4) to form two rectangles in the microscope field, and block 3 was formed by joining the two rectangles in each field. Block three was therefore the sum of all observations in a particular field. Each field was placed in a specific zone. Block 4 was the plane made up of the six fields (two of which came from each of the three zones) and block 5 consisted of the two planes taken from one soil slide.

Results

The results from both systems showed that the amended soil slides were markedly different from the control slides with respect to the number of microorganisms present, colonies formed, and the overall extent of colonization. Distinct zones were present in the soil samples since only the outer zone
I was heavily colonized. Zones 2 and 3 were very similar and were colonized to a lesser extent. The following data are summaries of observations of 240 individual squares from the glucose-amended soil system (Fig. 4) and 576 individual squares from the Linuron-treated system (Fig. 5). There was no large difference in the counts of microorganisms in zone 1 between the control and the glucose-amended soil until between the 3rd and 7th day of incubation. During this time the organisms in zone 1 of the amended soil exhibited a rapid increase. The glucose appeared to have no stimulatory effect on the organisms in zones 2 and 3 during the period of the experiment. After 7 days' incubation, about 12% of the individual squares in zone 1 of the control slides contained a colony (or part of a colony), whereas 78% of the squares in zone 1 of the glucose-amended soil contained colonies. In addition, the percentage of organisms giving rise to colonies in zone 1 of the control slide was about 3.5%, but this percentage in the glucose-amended soil was about 15%.

Observations in the Linuron-treated system were collected over a 4-week period and the numbers of organisms per zone are summarized in Fig. 5. There was no marked difference between the control and the experimental zone 1 until the 3rd week, when there was a substantial and rapid increase in colonization after which the numbers remained relatively constant. There was no such in-
(a) Block 1: individual squares

area = 214.92 \mu m^2

(b) Block 2: rectangles = paired squares

area = 429.84 \mu m^2

(c) Block 3: paired rectangles = microscope field = zone

area = 859.68 \mu m^2

(d) Block 4: plane

area = 5158.08 \mu m^2

(e) Block 5: slide

area = 10316.16 \mu m^2

FIG. 3. Arrangement of sampling blocks 1 to 5 on the soil.
crease in the Linuron-treated soil; in fact a small decrease in numbers was noted.

The colonization during the 4-week period was extensive in the control slides, with 62 to 81% of the squares in zone 1 containing colonies during the 3-day period in week 4. About 14% of the original organisms formed colonies and the average doubling time of the organisms in these colonies was 2.8 days. The colonization of the Linuron-treated soil was completely different; at the end of week 2, about 16% of the individual squares in zone 1 contained colonies with about 7% of the organisms forming colonies. However, by week 4, there was no increase in colonization with respect to both numbers and colony size; a decrease was in fact observed.

Both systems had been designed so that analysis of the degree of clumping present in each system and comparisons between the treatments could be carried out by the method of Newman and Bowen (1974). Their technique is a modification of Greig-Smith's pattern-analysis technique (1952), but is based on percentage cover rather than raw numbers. Newman and Bowen used a simple transformation between raw numbers and percentage cover, in which case the scale of measurement should have no effect. On performing the analysis on the raw counts obtained here, the Newman Bowen paired t-test failed to show up significant differences even when photographic records showed marked differences in the degree of clumping (Fig. 6.). At the same time the reason became clear. The presence of clumping caused the variation of the variance/mean (V/M) ratio between replicates to be so large as to mask any average differences between the V/M ratios in different treatments—even when such differences were very marked. Assumptions on the distribution of V/M ratios may be particularly suspect when the data are highly clumped as was the case in this study. A discussion of the statistical problems raised by Newman and Bowen, together with a broader investigation of indices of dispersion, is in preparation.

It is sufficient here to observe that the problems involved in performing significance tests on data obtained from application of Greig-Smith's method have been outlined by Mead (1974). He points out that it is generally accepted that a valid test of significance for such data does not exist. In an effort to overcome this impasse, he develops a fully valid two-within-four randomization test for data obtained by the quadrat method. It is this test which has been applied to the data from the exper-
Fig. 6. Typical appearance of soil from zone 1 of the control slides in the Linuron experiment. A, soil before colony formation. B, C, and D, typical colony types observed during incubation (scale = 1 μm).

Discussion

The direct observation technique has provided a method for long-term examination of microorganisms in soil samples. The increases in microorganisms can be monitored over a time period and the effect of various substrates on microbial growth can be determined (Figs. 4 and 5). The colonization dynamics of the system can be determined by examining the increases in number of organisms, the increases in the number of colonies, and the percentage of organisms giving rise to colonies. The degree of colonization that occurs is indicated by the number of sampling units that contain a colony or part of a colony. The doubling time of organisms in colonies can be calculated by counting the number of cells in colonies at different time
TABLE 1. Two-within-four randomization test results for glucose system

<table>
<thead>
<tr>
<th>Time, days</th>
<th>Slide</th>
<th>Soil plus distilled water</th>
<th>Soil plus glucose solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range*</td>
<td>Value†</td>
<td>Prob≥‡</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>18-86</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8-114</td>
<td>98</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>16-62</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>17-83</td>
<td>79</td>
</tr>
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<td>2</td>
<td>1</td>
<td>25-85</td>
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<td>2</td>
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<td>354</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>16-68</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>105-191</td>
<td>181</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>29-87</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24-88</td>
<td>66</td>
</tr>
</tbody>
</table>

*Randomization distribution range; all possible combinations of the pair differences.
†Observed value; sum of pair differences for observed data.
‡Probability of getting a value equal to or greater than the observed value.
§Probability of getting a value equal to or less than the observed value.

periods. In most cases the colonies observed contained only one morphological type of bacterial cell, but occasionally colonies with two or more different morphological types were found.

The use of the two-within-four randomization pattern-analysis technique also provides insight into the clumping characteristics (i.e., growth, extent, and position of colonies) of a system. The
extent of clumping is indicated by the randomization range (Tables 1 and 2). When there is no clumping present, the numbers of microorganisms per square sampled will be low. When the pair differences for this type of situation are randomized the range will be small with relatively low numbers (Table 1; range column for soil and distilled water). However, when colonies are present, the numbers of microorganisms will be larger in some of the squares sampled. When the pair differences for this type of data are randomized, the range will be large. This type of range indicates that clumping occurred, in some, but not all, of the squares sampled (Table 1; day 7, soil plus glucose). Since the sampling scheme was set up to test for differences in clumping between zone 1 and 2, the range obtained indicated that a difference in clumping occurred. It is explained in the Appendix that the randomization test as it is set up is particularly sensitive to differences in clumping between zones 1 and 2. If the range obtained was fairly small, but the upper and lower values were large, the inference would be that most, if not all, of the squares sampled were colonized.

The randomization range is composed of all possible combinations of the pair differences obtained from the sets of four numbers (see Appendix). The pair differences from the actual recorded data are tallied and are referred to as the observed value. The type of microbial activity (or non-activity) present in the system is determined by the location of the observed value in the randomization distribution. If the probability of getting a value equal to or greater than the observed value is 5% or less, then the assumption is that clumping occurred. The probability of getting a value equal to or less than the observed value is representative of uniformity, while probabilities between these two values are the result of random distribution. It should be noted that there are two factors of interest in the results. The first is the level of significance of the non-randomness of the data and the second factor is the actual extent of the clumping obtained. This can be demonstrated using data from Table 2. In the soil plus sterile straw extract, slide 1 at 8 days and slide 2 at 21 days show similar levels of significance indicating the level of non-randomness of the counts. It is clear from Table 2 that the level of clumping at 21 days is much greater than at 8 days. In the light of these facts the results are now discussed.

One general point which should be noted is the extent of the variation between slides. This highlights one possible deficiency in the experiment as performed. The sampling scheme was set up in the same way as Newman and Bowen, not using
exactly contiguous quadrats. If, in fact, the colonies of bacteria which are formed are tightly knit and very dense, with large gaps between them (Fig. 6), then it is possible to get large variation between replicate measures. More replicates and a sampling scheme more nearly akin to a line transect of contiguous quadrats might help to reduce this level of between replicate variation (Kershaw 1957).

Table 1 for the glucose system indicates that for the first 3 days the data appeared to show similar levels of departure from randomness, and also similar levels of clumping as exhibited by the range of scores obtained. However, at 7 days the picture changes dramatically. The level of clumping for the glucose-amended slides was markedly greater whilst that for distilled water remained static. Also the difference between the counts in the three zones is highly significant for the glucose-treated soil whereas for the distilled water treatment the differences are much less marked.

The results for the Linuron system are also interesting although rather different. From Table 2 it can be seen that the replicate slides show the same variability as has been noted for the glucose-amended system. There are, however, patterns to the results which should be noted. The control slides in the 1st week indicated that the bacterial counts were comparatively low and there was no evidence of clumping. By week 2 the level of counts had not changed very much, but there was evidence that the bacteria were appearing in clumps. Through weeks 3 and 4 the counts were at a much higher level but were fairly stable. They also exhibit general evidence of clumping. This change occurred somewhere between day 9 and 14. From the results for the treated slides it was clear that Linuron inhibited bacterial growth. At the same time it should be noted that the two-within-four randomization test showed inconsistent results regarding the pattern distribution of the counts. For example, the result for day 8, slide 1, suggested that the counts appeared more uniformly distributed than would be the case for Poisson data (this tendency was also found in day 22, slide 1 and day 23, slide 2). On the other hand, a number of cases (for example, days 16 and 21) showed strong evidence of clumping.

The technique used in these studies could provide a rapid way to examine the effects of nutrients on soil microorganisms on relatively intact soil samples. Because these samples can be maintained for long periods in an undisturbed state, the effects of pesticides or other chemicals on growth rates (even slow growth rates) and colony formation can be examined. The number of microorganisms able to form colonies under the conditions of incubation can be determined and the morphological types predominating under the conditions can also be detected.

The photographic method of data collection reduces the time required and also minimizes the exposure of the soil sample to adverse conditions such as ultraviolet light and the attendant heat. Permanent records of spatial relationships are also obtained and they can be analysed at a later date using a variety of statistical procedures (Kershaw 1957; Mead 1974; Pielou 1977).

The adaptation of the two-within-four randomization test to the soil systems provided a valid method of analysing data gathered from quadrats on a linear transect. The variation between replicates would probably be reduced if contiguous quadrats were used, but this would entail considerably more effort in data collection in order to examine the same soil systems.

Appendix

The arrangement of blocks obtained with the sampling method used resulted in an agglomerative hierarchy. A typical set of results from day 7, soil + glucose, slide 2, is given in Table 3. The numbers of individuals found in the 48 quadrats (i.e. squares from Fig. 3) are in the first row, the 24 totals for the pairs of quadrats are in the second row, the totals for the fours are in the third row, the totals for eights in the fourth row. The 6 sets of 8 are then grouped into 2 sets of 24 in row 5 rather than 3 sets of 16. This was necessary to maintain the integrity of the zones described earlier.

The question, "are the pair totals of the counts, at any particular scale, compatible with random pairing?" can be asked at each block size, although in this case we required a form of test which would be sensitive to differences between the zones. If there is no pattern at a particular block size, the pairings should be random and a randomization test can be run using the variance of group pair totals as a test criterion. The distribution of the variance over the random pairings of the group totals can be evaluated. Mead (1974) pointed out that the problem with this approach is that pattern at a larger scale implies that the set of group totals available for pairing at all lower levels consists of at least two populations and thus invalidates the randomization test. He then modified the test in such a way that the two-population difficulty was overcome by looking at the group totals in sets of four instead of as one entire set. Thus, each set of four totals produces two-pair totals and then the absolute differences between the two-group pair totals are used as a basis for the test criterion instead of the variance of the group pair totals.

Although this reduces the randomization distribution to only three values, consideration of the sum of the absolute differences between group pair totals over N sets of four group totals results in a randomization distribution of 3\(^N\) values which is fairly easy to compute.

To illustrate the adaptation of the two-within-four randomization test to provide a test for the soil systems which is sensitive to differences between zones, consider row 2 in Table 3. The six sets of four are (34, 22, 7, 4) (11, 5, 12, 13) (21, 11, 49, 37) (31, 53, 15, 18) (3, 4, 16, 19) (75, 39, 292, 261). The sets of possible differences between the pairs are:
The range of the set totals was 43–657 with the sum of the observed pair difference (i.e. the actual recorded data) being 657. Thus the observed value of 657 was the largest value in the permutation distribution and the possibility of getting an observation at least as large as the observed value was 1/729. An observed value at the upper end of the permutation distribution is an indication of clumping, whereas an observed value at the lower end suggests that the data follows a uniform distribution. Formally, we can say that when the probability of getting a pair difference greater than or equal to the observed difference was 5% or less, it is an indication that clumping had occurred. When the probability of getting a pair difference less than or equal to the observed difference was 5% or less, the indication was that the distribution was uniform. When either probability ranges between these values, the distribution was not considered to be significantly different from random. Mead (1974) pointed out that one possible disadvantage with this technique was that a set of scores that showed a large range might tend to dominate the randomization distribution. We found that when there was strong evidence of clumping or uniformity, sets with large ranges would tend to dominate the randomization distribution, regardless of the form in which they were analysed (i.e. raw data or distribution-free data). Indeed, such sets with large ranges serve to indicate the presence of pattern.

Acknowledgments

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