ROTHAMSTED RESEARCH

Rothamsted Experimental Station Report for 1965

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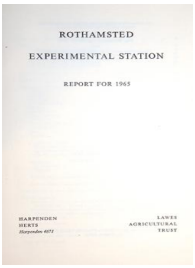

Soil Microbiology

P. S. Nutman

P. S. Nutman (1966) *Soil Microbiology ;* Rothamsted Experimental Station Report For 1965, pp 82 - 88 **- DOI: https://doi.org/10.23637/ERADOC-1-60**

SOIL MICROBIOLOGY DEPARTMENT

P. S. NUTMAN

Jane Meiklejohn, who joined the Department in 1927, retired. During the war she served in the meteorological branch of the Royal Air Force, and in recent years spent periods of secondment in East and West Africa. She is now at University College, Salisbury, Southern Rhodesia. Barbara Mosse was away for nine months working at the C.S.I.R.O. Soils Laboratory at Adelaide, South Australia, and at Dunedin University, New Zealand. Shoshana Bascomb left to take an appointment at the National Type Culture Collection Laboratory, Colindale. P. J. Dart was appointed to work on legume nodulation.

P. S. Nutman attended the International Biological Programme Meeting in Prague in April, and Margaret Brown, Susan Burlingham, R. M. Jackson and F. A. Skinner contributed to the Symposium on "The Ecology of Soil Bacteria" at the University of Liverpool. F. A. Skinner gave a course in soil microbiology at Wye College.

Visiting workers included Mr. J. Whiteway, microbiologist to the Agricultural Research Council of Central Africa. Dr. R. S. Dwivedi of Benares University, Professor Abdel-Ghaffar of the University of Alexandria, Egypt, and Mr. F. Blackkolb of the Microbiology Institute of the University of Göttingen.

Work described in previous years was continued except that the programme on the freeze-drying of *Rhizobium* was completed, and work on mycorrhiza, was temporarily suspended. We are concerned with a limited range of microbiological problems and processes of agricultural importance, viz., nitrogen fixation, nitrification, plant-soil microbe interrelations and the decomposition of natural and synthetic substances in soil.

Nitrification. Three more strains of ammonia-oxidising autotrophic bacteria were isolated in pure culture from soils or from activated sludge; and their properties are being compared. Two strains of *Nitrosomonas* and one strain of *Nitrosococcus* were lyophilised by the procedures described last year for Rhizobium. All were viable after storage dry for a year at 2° C. (Walker)

Decomposition of aromatic compounds, pesticides and herbicides. Two more strains of *Pseudomonas* that can grow with β-naphthol as sole carbon source were isolated from soil and found to differ from the *Pseudomonas fluorescens* strain already described (*Rothamsted Report* for 1963, p. 70). The new isolates tolerate more concentrated β-naphthol, grow optimally at higher temperatures, are not adapted to gentisic acid and grow on neither naphthalene nor α-naphthol. It seems therefore that strains of *Pseudomonas* can metabolise β-naphthol by different pathways.

https://doi.org/10.23637/ERADOC-1-60

Another *Pseudomonas* strain able to grow on α-naphthol but not on β-naphthol was isolated from soil, It also grew on naphthalene or α-chloro-naphthalene, from which it produces the respective dihydroxydihydro compounds. The early stages of α-naphthol dissimilation in this organism are still obscure.

A strain of *Nocardia*, which metabolises various liquid aromatic hydrocarbons causes hydroxylation in the ring of toluene. After growing on toluene it oxidises catechol, 3-methyl- and 4-methyl-β-catechol equally well. Ethylbenzene-grown cells also oxidise these catechols, but can oxidise phenylacetic acid only after a long lag period. Whether it also causes hydroxylation in ethyl benzene is being tested. Rough and smooth colonial forms of this organism were observed and separated.

The great stability of phenoxyacetic acid herbicides substituted by chlorine in the meta-position is noteworthy, and we have not yet found any micro-organisms able to degrade either 3-chloro- or 2,4,5-trichloro-phenoxyacetic acid. However, Audus (*Pl. Soil* (1951) **3**, 170) reported that 2,4,5-T was decomposed by a column of soil previously percolated with a solution of MCPA, so attempts were made to repeat this, but so far without success. Other soils will be tested, but the one used from Geescroft field decomposed MCPA and *p*-chloro-phenoxyacetic acid.

In collaboration with D. C. Griffiths of the Insecticides Department methods of assaying parathion in soil were studied as a preliminary to work on the possible decomposition of this insecticide on soil percolators. (Walker)

The soil clostridia. Soil is the principal habitat of the clostridia, spore-forming anaerobic bacteria that decompose many carbohydrates and proteins. Some features of specialised clostridia able to digest cellulose were given in the *Rothamsted Report* for 1960, p. 89. Clostridia are difficult to count because they require strict anaerobic conditions and because the gas they produce disrupts solid media. Clostridia in Rothamsted soils were counted using various media, including Oxoid reinforced clostridial agar (RCA), but cellulose-digesting types do not grow readily on this. The soil dilutions are inoculated to tubes containing liquid RCA, which are then incubated in air, anaerobiosis being ensured by reducing substances in the medium. Counts were of the order of $2{\cdot}0 \times 10^5$/g dry weight soil, but populations 100 times this number develop quickly in soils supplied with readily decomposable carbohydrate.

Clostridium welchii, generally regarded as common in soils, was not abundant in samples so far examined. Most isolates were saccharolytic clostridia of the *Clostridium butyricum* type. *C. butyricum* is closely related to *C. pasteurianum*, long regarded as the principal anaerobic nitrogen fixer. Some clostridia may be more important than *Azotobacter* as non-symbiotic nitrogen fixers because they are much more numerous in most cultivated soils. Growth factors (containing combined nitrogen) are required by almost all clostridia, and the use in the past of entirely nitrogen-free media to estimate numbers of N-fixing clostridia may have given misleading results. Reports that adding peptone and yeast extract to nitrogen-free media increases the numbers of anaerobic nitrogen fixers were not

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confirmed in our experiments using RCA and other basal media. Tubes of different media inoculated with soil dilutions were incubated in McIntosh & Fildes jars filled with nitrogen containing 10% carbon dioxide, at 25° C for 6 days. Almost identical counts were given by RCA with or without peptone and yeast extract, but a nitrogen-free medium without growth factors gave very much smaller counts. Almost all the isolates from the richer media failed to grow on a nitrogen-free medium, but a few grew outstandingly well even without growth factors. Thus, rich non-selective media have not satisfactorily indicated the numbers of nitrogen-fixing clostridia in soil. (Skinner)

The effect of *Azotobacter* on plant growth. Experiments at Rothamsted and Lee Valley Horticultural Experimental Station on treating tomatoes with *Azotobacter* confirmed and extended last year's results. Tomato stem height and leaf length were increased and flowering was advanced by the inoculation of seeds, or seedling roots, with cultures of *Azotobacter*. These effects, which are possibly caused by the production of growth factors (*Rothamsted Report* for 1964, p. 92), occur only after inoculation with whole cultures of *Azotobacter* or cells washed free from medium; cell-free culture filtrate and washed cells killed by boiling are ineffective. This suggests that tomato growth is affected by some activity of the living bacteria in the rhizosphere, and not by any substance produced or released into the culture medium and contained in the inoculum. (Brown, Burlingham and Jackson)

Establishing *Azotobacter* in the rhizosphere. *Azotobacter* inoculated on to seeds before sowing can be recovered from the plant rhizosphere at all stages of the plant's development from germination until harvest, provided the inoculum contains a minimum of about 10^4 bacteria per seed. Inocula of young vegetative cells or mature cysts seem to move down from the seed to the root; the number on the seed decreases as the number on the root increases. The maximum number of *Azotobacter* per root system is usually attained during the first 3 weeks' growth and maintained at later stages of plant development.

That multiplication occurs mostly during the early stages of seedling growth was confirmed: (1) by mapping the distribution of *Azotobacter* along the growing root, which was done by placing roots from seed-inoculated plants of different ages on nitrogen-deficient agar and noting where colonies developed, and (2) by counting a sequence of root washings, which showed that *Azotobacter* is closely associated with roots of both young and mature plants.

The stimulation of *Azotobacter* by growing roots was also shown in experiments with peas sown in unsterile soil above slides coated with water agar containing *Azotobacter* cysts. The slides, when later removed and examined, showed that *Azotobacter* multiplies as the seeds germinate. Cysts germinate within 72 hours, and micro-colonies of *Azotobacter* develop close to the seed and root; away from the roots, they do not germinate. Routine staining procedures and fluorescent-antibody techniques were used in this work. In other experiments peas were sown in small pots

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of soil and a suspension of *Azotobacter* added to the radicles as they emerged. Daily counts of *Azotobacter* in the soil confirmed the observations with slides; from 24 hours the number significantly increased.

The roots of peas and cereals excrete mainly sugars in the first 24 hours after seeds germinate, and many other substances during the next 10 days; excretion thereafter is less. Most of these substances can be used by *Azotobacter*. This pattern of excretion may explain the early colonisation by *Azotobacter*, and effects on the plant's development or final yield may reflect this early activity of *Azotobacter* in the root zone. (Brown and Jackson)

***Azotobacter* in sterile and partially sterilised soil.** In raw soil free from plants an inoculum of *Azotobacter* does not multiply, but in sterile (autoclaved) soil it increases from 10 to 100 times during the first week; thereafter numbers remain constant for two further weeks, during which the presence of a sterile wheat seedling has no effect. A John Innes potting compost was partially sterilised with aerated steam at 140° F for 40 minutes and inoculated with *Azotobacter* 48 hours later. In contrast to sterile soil, *Azotobacter* numbers remained constant in this soil without plants for up to 8 days; even with plants numbers of *Azotobacter* slowly diminished after 8 days. (Patel)

The effect of *Azotobacter* inoculation and the presence of plants on the microflora of partially sterilised soil. Work described last year showed that *Azotobacter* inoculation affected neither the flora of the rhizosphere of plants grown in field soil or potting compost nor the soil flora between the 4th and 12th week after inoculation. The rhizosphere of younger plants and of plants grown in partially sterilised soil has now been studied. John Innes compost mixture with appropriate basal fertilisers was treated with a steam–air mixture at 150° F for 40 minutes and then transferred aseptically to sterile, plugged boiling tubes kept in the glasshouse and inoculated with *Azotobacter* and planted with tomato as required.

Samples were taken immediately and 2, 6, 14 and 50 days after partial sterilisation, which decreased total bacteria and actinomycetes to about $\frac{1}{200}$ the number originally present. Spore formers fell to about $\frac{1}{4}$ and *Nitrosomonas* and *Nitrobacter* disappeared. In uninoculated soil without plants the spore formers increased rapidly, actinomycetes very slowly and the nitrifiers did not reappear. Fifty days after treatment spore formers were about 200 times as many as in soil not partially sterilised and actinomycetes had nearly reached their original numbers. Total bacteria were slightly fewer than in untreated soil.

The numbers of different types of bacteria in the rhizosphere soil were estimated using the plate count after 6, 12 and 18 days. Viable bacteria, actinomycetes, bacterial spores and anaerobic bacteria occurred in similar numbers with and without *Azotobacter* inoculation. The number of *Azotobacter* cells slowly diminished.

Two isolates each of *Fusarium oxysporium*, *Cylindrocarpon* sp. and *Mortierella* sp. were tested to see whether they affected *Azotobacter chroococcum* (Strain A_6). The fungi were first grown on a range of media

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for different periods of time, and tests were made with and without root exudates collected from aseptically grown wheat and pea plants (10–12 days old). One isolate of *Fusarium oxysporium* and one of *Cylindrocarpon* strongly inhibited growth of the *Azotobacter*. Root exudates had no effect. (Patel)

Production of gibberellin-like substances by *Azotobacter* in culture. Cultures grown on a shaker for 3, 5, 7, 10 and 14 days were extracted and tested by the barley endosperm bioassay, which showed that gibberellin-like substances increased with culture age. The amount of medium used per 500 ml flask influenced their production probably by affecting aeration. Gibberellin-like substances were assayed in whole and disrupted cells of *Azotobacter*. Treatment with lysozyme, with or without EDTA, and hemicellulase lysed only half of the cells, even in 24-hour-old cultures. Ultrasonic treatment (Mullard 60-watt, 20 kc/sec) disrupted 63% of cells of 3-day-old cultures after 5 minutes treatment, 90% after 10 minutes and 99·8% after 60 minutes treatment. Older cultures containing encysted cells were less sensitive to disruption, and treatment for 60 minutes had no effect on the amount of gibberellin-like substances estimated in a 14-day-old culture by the bioassay. Hydrolysing the whole culture with ficin to release "bound" gibberellin (McComb, *Nature, Lond.* (1961) **192**, 575) increased gibberellin-like activity, which was increased further when ficin and ultrasonic treatments were combined. This indicates that some of the gibberellin-like substances produced by *Azotobacter* are bound inside the cell and are not released by usual methods of extracting whole cultures or cells. (Burlingham)

***Rhizobium* in Park Grass.** *Rhizobium trifolii*, *Rh. leguminosarum*, *Rh. meliloti* and *Rh. lupini* were counted in 17 sub-plots of the Park Grass experiment by adding dilutions of soil to sterile-grown plants susceptible to each species of bacterium. The count is subject to much error, some of which seems to be caused by microbial interactions, but the differences between plots are so large that the main results are clear.

Each species of nodule bacterium occurred only on plots containing its host plant (viz., either *Trifolium repens*, *T. pratense*, *Lathyrus pratense*, *Vicia sepium* or *Lotus corniculatus*). Medicago and Melilotus species do not occur on Park Grass, although *M. lupulina* does elsewhere on the farm; *Rhizobium meliloti* was not found in any sample.

The numbers of nodule bacteria in the soil of the Park Grass plots depend partly on host abundance, but more on soil reaction. Between pH 5 and 7·3 they range from 10^4 to 10^8/g dry soil, independently of species; they do not occur in plots more acid than pH 4·5, and are many fewer in plots with pH above than below 7·5. Nodule bacteria are fewer in the 3–4-inch layer and lower horizons than in the 0–1-inch layer. (Nutman)

The infection of clover root-hairs by *Rhizobium*. The numbers of root-hairs infected were compared on roots of sparsely and abundantly nodulating lines of *Trifolium subterraneum*, selected from hybrids between the varieties Cranmore (CR) and Tallarook (TA), and Northam Second Early

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(N2E) and Mount Barker (MB). At 12 days from germination the abundantly nodulating selections already had twice as many nodules as the sparse lines, but the average number of infected root hairs was the same for all four selected lines. There were small differences in the times nodules were first formed on individual plants, and the early nodulating plants bore fewer infected hairs than those that nodulated later.

Previous work (*Rothamsted Report* for 1962, p. 78, and for 1963, p. 75) showed that very small amounts of nitrate ions delay nodulation and stimulate infection, and this effect is not abolished by large changes in the carbohydrate status of seedlings of *Trifolium glomeratum* produced by feeding glucose through the roots. These experiments were extended by using a range of concentrations of glucose and nitrogen-containing salts applied either to roots or cotyledons. The results confirmed that glucose does not affect the stimulation of infection by nitrate fed through the roots. Potassium nitrate, ammonium sulphate and glucose when added in microgram quantities to the cotyledons were rapidly taken up, but applied in this way neither compound affected nodulation or hair-infection. Nitrate given to the root medium is absorbed by the plant before nodulation is affected so that the failure to obtain an effect with nitrate applied to the cotyledons suggests either than nitrate does not move into the root or is reduced and rendered inactive before it reaches the root. (Nutman)

Imperfect phages in *Rhizobium trifolii*. Electron microscopy of sections of clover nodules shows that some strains of the bacteroids of *Rhizobium trifolii* contain bodies morphologically resembling particles of bacteriophages. In attempts to gain further information about their nature, electron micrographs were made of *Rh. trifolii* strain 0403 (formerly A121111), grown in shake culture. This strain is an effective nitrogen fixer, susceptible to the phage 5000 (formerly phage A121111) and is thought to be lysogenic. When plated, the strain 0403 occasionally forms plaques, but attempts to isolate phage from these failed.

Young 24-hour-old liquid cultures of strain 0403 were incubated for 2 hours with and without phage 5000, at a ratio of about 5 phage particles per bacterium. Electron micrographs showed that both the inoculated and uninoculated bacteria contained phage-like bodies indistinguishable in appearance. Most of the uninoculated cells each contained one, two or three such particles, and a few contained none, whereas those inoculated with phage all contained many such particles. (Kleczkowska and Mosse)

Eight strains of *Rh. trifolii* suspected to be carriers of lysogenic phage were exposed to ultra-violet irradiation, filtered and each filtrate tested separately against all strains by plating or inoculation into liquid cultures. Five strains formed plaques and lysed liquid cultures, and phages from these plaques completely lysed young susceptible bacterial cultures to which they were inoculated. When again inoculated to cultures of the same bacteria, however, results were inconsistent. Most phage preparations failed to produce lysis, and of those that did, some formed plaques whereas others did not. Third and later passages did not cause lysis in either liquid or solid media. All efforts to find substrates on which these phages

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could be maintained and propagated failed, and the phages obtained by ultra-violet irradiation seem to be imperfect. (Kleczkowska)

Thermoactinophages. During the work on "Farmer's lung diasese" many thermophilic actinomycetes were isolated from hay bales. One of the isolates, *Thermoactinomyces vulgaris*, carried a phage that lysed young mycelia and produced plaques 1–2 mm in diameter on spread plate cultures of *T. vulgaris*. Tests with phage purified by single-plaque isolation showed that many isolates of the genus *Thermoactinomyces* were susceptible. (Patel)

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