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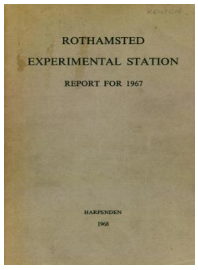

Soil Microbiology Department

P. S. Nutman

P. S. Nutman (1968) *Soil Microbiology Department ;* Rothamsted Report For 1967, pp 84 - 91 **- DOI: https://doi.org/10.23637/ERADOC-1-120**

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SOIL MICROBIOLOGY DEPARTMENT

P. S. NUTMAN

The department continued to study nitrogen transformations in the soil, the decomposition by microbes of cellulose (anaerobic), herbicides and pesticides, and the interactions between soil microbes (other than plant pathogens) and crop plants. Work on the microflora of root surfaces of plants inoculated with *Azotobacter* and on the actinophages of thermophilic actinomycetes in mouldy hay ended. A programme of field experiments was started to measure nitrogen fixation by nodulated lucerne, using a combination of agronomic and bacteriological methods.

Collaborative work in microbiology and fine structure continued with the departments of Biochemistry, Plant Pathology, Insecticides and Nematology and some outside institutes, as detailed below.

The coexistence of *Rhizobium* and its phage in culture. Susceptible strains of *Rhizobium* treated with phage produced phage-resistant mutants that can, in some instances, coexist with the phage indefinitely. The problem of coexistence, especially of phage maintenance, was examined using two unrelated strains of *Rhizobium trifolii* and one of *R. leguminosarum*. These strains had been subcultured over long periods on an agar medium without showing evidence of lysogeny or genetic instability, and tests made shortly before the experiments began confirmed their freedom from the lysogenic condition and their morphological and physiological homogeneity.

Each strain was inoculated with a different virulent phage that produced lysis, and over a period of about 7 days surviving resistant cells started to multiply. During the next 5 weeks or so (second stage) the three strains differed in their response to the presence of phage. The resistant cells of two of the strains (numbers 6 and 1020 Rothamsted Culture Collection) multiplied, and at the end of their logarithmic growth reached greater numbers than in the control cultures. In contrast, the third strain (0403) did not adjust to the presence of phage, slowly declined and was eventually extinguished.

After the secondary growth of bacterial strains Nos. 6 and 1020 had produced maximal numbers the ratio of phage particles to bacterial cells remained constant (third stage). In such cultures the decline in bacterial numbers was the same as in the control cultures. During the whole period of coexistence the phages remained virulent, and presumably maintained their titres by the lysis of susceptible back mutants. Such cultures can be considered as reservoirs of virulent phage.

To investigate further the behaviour of phage in coexistent cultures, 30 such cultures were examined. All contained live bacteria, and phage ranging in concentrations from 10^4 to 10^8 particles/ml in the third stage. Four bacterial strains isolated from these were carrying phages, i.e. they

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were lysogenic. This seems to be the first record in any bacterium of the induction of lysogeny by treating bacteria with virulent phage. (Kleczkowska)

Measurement of nitrogen fixed by lucerne. Professor J. M. Vincent and P. S. Nutman proposed for the International Biological Programme four types of experiment to assess nitrogen fixation by nodulated lucerne in the field. One combined fertiliser and inoculation treatment in a design that minimised the danger of contamination between plots.

Experiments of this kind were done at Rothamsted and Woburn in fields where lucerne had not been grown before. Both sites were acid (pH Rothamsted 6·0, Woburn 5·5) and contained fewer than 1 cell of *Rhizobium meliloti* per g of dry soil. Four replicated strips were sown with lucerne (Du Puits) inoculated with an effective strain of nodule bacteria, with lucerne inoculated with an ineffective strain of bacteria (an additional treatment not in the original IBP proposals), with uninoculated lucerne or with ryegrass (S22). The seed was inoculated with many more bacteria than in commercial practice, especially with the ineffective strain, to swamp any effect of bacteria already in the soil. The strips were split for fertiliser nitrogen (0, 60, 120 and 180 lb N/acre as "Nitro-Chalk" applied before sowing and after the first and second cuts), lime (to bring the pH of soil to 7·0) and PK (applied as 0–14–28 to give 0·6 cwt P_2O_5 and 1·2 cwt K_2O/acre). The combined lime and PK treatment was to provide optimal nutritional conditions for nitrogen fixation. Each combination of fertiliser treatment was replicated twice, and some second- and third-order interactions were confounded with whole-plot or block effects. Sample cuts were taken in July, August and October using a 3-ft cut across the rows after discarding outside rows. Dry-matter and nitrogen contents were determined on tops only.

Response to effective inoculation, and to nodule formation by naturally occurring nodule bacteria was assessed against the responses to nitrogen fertiliser of the ineffectively inoculated lucerne and of the ryegrass.

At Rothamsted smallest yields (dry matter) were from unfertilised ryegrass (17 cwt/acre) and lucerne inoculated with ineffective bacteria (14 cwt/acre). Grass responded most to fertiliser nitrogen (60 cwt/acre); the uninoculated and ineffectively inoculated lucerne also responded, but the effectively inoculated lucerne did not. Effectively inoculated lucerne responded most to lime and PK, and grass did not respond. Maximum yields of lucerne and grass did not differ significantly, but lucerne contained most nitrogen.

At Woburn experimental results were similar, except that yields were about 10% less and the effectively inoculated plants without lime and PK responded significantly to the smallest amount of nitrogen fertiliser (44 compared with 28 cwt/acre).

The ryegrass and ineffectively inoculated lucerne gave useful bases for assessing nitrogen fixation, especially by uninoculated plants. During the season the naturally occurring bacteria fixed 89·2 lb N/acre at Rothamsted and 50·5 lb N/acre at Woburn, with lime and PK, compared with 206·6 lb N/acre and 98·6 lb N/acre by effectively inoculated plants with lime and

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PK. The nitrogen fixed by the natural small population of nodule bacteria was negligible without lime and PK, and with lime and PK was noticeable only in the second and third cuts, when patchy greening showed that effective nodules had formed. This happened more at Woburn, and soil samples showed that effective bacteria established themselves more there than at Rothamsted. (Bell and Nutman)

Selection for increased symbiotic nitrogen fixation. Earlier work showed that symbiotic effectiveness in a single variety of red clover is affected by a few genes having major effects and by many genes having minor effects. To investigate this more widely, with the object of selection for increased effectiveness, samples of about 250 plants from each of four varieties of red clover—two commercial samples and two bred cultivars (S123 and late-flowering Montgomeryshire)—were examined in 1964 in a sterile culture system inoculated with the effective bacterial strain 0403. Symbiotic effectiveness differed widely over the same range in each sample. The three main classes of plants selected for further breeding were (1) highly effective plants (2) averagely effective plants (modal effectives) and (3) ineffectives. In 1965 about 800 crosses (inter and intra varietals and mostly in diallels) were made between plants showing similar responses and between ineffectives and modal effectives. All crosses involving ineffective plants were examined in 1966. Some of those between ineffectives were wholly ineffective or segregated ineffectives, and will provide material for further work on the inheritance of the ineffective response. Crosses between effective and ineffective plants confirmed earlier work in showing that ineffectiveness is a recessive character. In 1967 crosses between highly effective plants were compared with modal effectives. Breeding from highly effectives significantly increased effectiveness, giving about 5% extra dry weight. Larger yields were distributed very irregularly between families; more than half of those raised from highly effective plants were indistinguishable from crosses between the modal class. When inoculated with a bacterial strain of outstanding effectiveness (Strain 5) with unselected red clover the crosses between highly effectives were indistinguishable from those between modal effectives.

Strain 0403 is more effective with red clover than most strains occurring naturally in the United Kingdom. These results suggest that nitrogen fixation by red-clover crops could be improved by breeding for greater effectiveness or by replacing the indigenous population of nodule bacteria by more effective strains. (Mareckova and Nutman)

Effect of temperature on nitrogen fixation in nodules of subterranean clover. The influence of root temperature, and changes in temperature, on symbiosis in selected genetic lines of subterranean clover (*Trifolium subterraneum*) was studied in controlled conditions of illumination, day length and temperature of the top of the plants (22° C). The clover lines tested were selected for abundant and sparse nodulation (Nutman, *Aust. J. Agr. Res.* (1967), **18**, 381). Roots grown at 7° and 11° C nodulated less than roots grown at 15° and 19° C, and temperature greatly influenced the initial stages of infection of the root hair, the time taken to form a nodule

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and the subsequent nodule development and internal structure. Nitrogen fixation at different temperatures depends on the leaf-area index and dry weight produced by the host plant. (Roughley and Dart)

The characteristics of leg-haemoglobin of nodules. The leg-haemoglobin of soya-bean and cowpea nodules was compared, especially the haem-iron hyperfine structure, using electron spin resonance (in collaboration with Dr. J. Gibson and Dr. R. Dowsing, Chemistry Department, Imperial College, London) and Mossbauer spectroscopy (with Dr. G. Lang and Dr. A. Thompson, Atomic Energy Research Establishment, Harwell). The fluoride and azide derivatives of cowpea leg-haemoglobin have small but significantly different "g" values from the soyabean leg-haemoglobin derivatives. The two also differ in electrophoretic mobilities, and elution patterns from DEAE-cellulose columns. (Dart)

Biochemical tests for *Rhizobium*. Various tests were applied to a selection of strains of *R. trifolii*, *R. leguminosarum*, *R. phaseoli* and *R. japonicum* and to three species of *Agrobacterium*, to see whether improved biochemical methods could be used to identify species.

Agrobacterium radiobacter, *A. rhizogenes* and *A. tumefaciens* gave very similar reactions in all biochemical tests employed, and formed a well-defined group. All three grew quickly on yeast–mannitol agar (YMA), absorbed congo red from YMA containing 1/40000 of the dye, and grew on YMA containing 2% (w/v) of NaCl. Some strains of *R. trifolii* and one of *R. phaseoli* absorbed a little congo red, and the *R. meliloti* strains and two strains of *R. japonicum* grew with 2% NaCl. No strain of *Rhizobium* (unlike *Agrobacterium*) gave a positive result in both tests.

R. meliloti strains resembled each other and *Agrobacterium* spp. in that they grew well and quickly on glucose peptone agar, produced HgS on bismuth sulphite agar and gave a precipitate in calcium glycerophosphate agar.

Reduction of nitrate, use of citrate, formation of H_2S and precipitation in calcium glycerophosphate agar were not greatly changed by adding yeast extract to supply growth factors, though when yeast extract was present the result of the test could usually be read earlier.

Growth factors were not needed by any *Agrobacterium* strain for growth in any medium tested, but *A. rhizogenes* used citrate only when given with yeast extract. Three strains of *R. meliloti* reduced nitrite as well as nitrate. Other strains of *Rhizobium* reduced nitrate to nitrite, except one of *R. leguminosarum* and one of *R. meliloti*. *Rhizobium* strains usually reduced nitrate to nitrite without needing added growth factors. *R. meliloti* and *Agrobacterium* spp. were less dependent on growth factors than the other species of *Rhizobium*. Results indicate the need to provide growth factors for some biochemical tests.

Agrobacterium and *R. meliloti* were readily identifiable by these tests, but the other *Rhizobium* species could not be separated from each other. (Skinner)

The classification and ecology of *Endogone* spores. A system of classifying *Endogone* spores was developed, using descriptive names for nine distinct types, five of which (designated yellow vacuolate, laminate, honey-

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coloured sessile, white reticulate and bulbous reticulate) are common in arable soils. These have been selected for more intensive study of life histories, type of infection produced in the mycorrhizal root and effect on plant growth.

The yellow vacuolate and honey-coloured sessile spores were established as single-strain cultures on suitable hosts in pots, but the white and bulbous reticulate strains are still mixed. Laminate spores failed to infect onion, tomato, hemp or clover. They are now being tested on wheat because they occurred abundantly as the only *Endogone* spore type in some experimental plots which have carried wheat for three years.

An improved method was developed with Mr. G. W. Jones (of Reading University) for recovering *Endogone* spores quantitatively from soil, based on the differential sedimentation of organic debris and spores in gelatin solutions of different concentrations. This was used to assess spore populations in plots on Little Knott field, designed to test the effect of formalin and nitrogen fertiliser on incidence of "take-all" disease in spring wheat (Widdowson, Penny and Salt, *Rothamsted Report* for 1966, pp. 59 and 124). Plots treated with formalin in 1966 only, or consecutively in 1965, 1966 and 1967, and not receiving extra nitrogen, contained most spores (41–67/50 g air-dried soil). "Take-all" was also most prevalent after formalin treatment in 1966, and the plant growth was poorest on these plots. With nitrogen fertiliser *Endogone* spores were fewer (5–15/50 g soil), and plant growth improved. The presence of "take-all" complicates assessment of the direct effects of soil sterilisation and nitrogen on *Endogone* spore numbers; further work on these effects is in progress. Only laminate spores were found in these plots, whereas these are rare on the neighbouring Broadbalk plots, in which three other spore types are abundant.

Work on the fine structure of *Endogone* spores continued. The most satisfactory fixative was acrolein. Germination of the bulbous and white reticulate spore types is apparently preceded by degeneration of most nuclei, whereas in vacuolate types nuclear division occurs before germination. (Mosse)

Effects of mycorrhizal infection on plant growth. Plants of *Coprosma robusta* (a New Zealand shrub) grew better in a heat-sterilised soil containing little nitrogen and phosphorus when they were mycorrhizal than when not, and reached nearly twice the height and more than twice the leaf spread. Uninoculated plants grew as well as mycorrhizal plants when calcium phosphate was added to the soil, but not with sodium nitrate or a combination of calcium phosphate, sodium nitrate and potassium sulphate. In the same soil tomatoes showed no growth response to infection. This, together with some observations on the infectivity of different spore types, shows that the effects of vesicular–arbuscular infections by one fungus can differ in different host plants. (Mosse)

Thermoactinophages. Two more strains (M_1 and M_{12}) of *Thermoactinomycetes vulgaris* were found to be lysogenic. The phages were obtained from lysed cultures on an agar surface; the method of enrichment in broth culture failed to increase the final titres of the infective particles on a

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susceptible host (strain A 64). The preparations of phage were purified by differential centrifugation. Electron micrographs of negatively stained partly purified preparation showed phage M_1 to be tadpole-shaped. The polyhedral head is 62 mμ long and 50 mμ wide, the tail 90 mμ long and 8 mμ across, and the proximal end of the tail bears a knob-like structure. This morphology resembles that described for several known phages parasitic on mesophilic actinomycetes. The phages isolated from M_1 have the same host range as the earlier isolate M_3 (*Rothamsted Report* for 1965, p. 88). The phages isolated from M_1 and M_3 are similar morphologically and serologically. (Patel)

The production of plant hormones by *Azotobacter* in shake culture and fermenter. Shake cultures of *Azotobacter chroococcum*, strain A6, were examined for gibberellins and indolyl-3-acetic acid. Supernatant and cell fractions were extracted and examined by paper-partition chromatography using two solvent systems that separate authentic GA3 and IAA, namely, isopropanol:ammonia:water 10:1:1 parts by volume and benzene:acetic acid:water 4:2:1 parts by volume. Growth substances were not detected on chromatograms treated with chromogenic reagents and examined under U.V. light, but were detected using plant bioassays. Chromatograms were cut into 10 equal strips representing a sequence of *Rf* values 0·1–1·0 and eluates tested on dwarf peas and lettuce hypocotyls. Significant growth-promoting activity was shown in both tests by eluates corresponding to *Rf* 0–0·2 and 0·4–0·6, and with dwarf peas also by eluates from between *Rf* 0·8–0·9. Eluates from chromatogram sections were applied to roots of tomato seedlings at the cotyledon stage of development. Those from between *Rf* 0·4–0·6 and 0·7–0·8 significantly increased growth of stems and leaves until six internodes had formed. From then until flower buds were showing only eluates from 0·4 to 0·6 produced responses. The time between bud appearance and petal-fall was also shortened by these eluates. Substances between *Rf* 0·4–0·6 were probably GA1 or GA3, and those between *Rf* 0·8–0·9 contained three components with gibberellin activity which were separated in the second solvent system. The amount of GA1 or GA3 produced per ml of supernatant fluid was equivalent to about 0·03 μg GA3. Cell extracts contained the same quantity of gibberellins as the supernatant fraction. Crushing cells in a Hughes press at 20° and hydrolysing with crude ficin gave no evidence that they contained "bound" gibberellin (Brown).

In six experiments we failed to detect gibberellins by chemical methods in concentrated extracts from cultures of *Azotobacter* grown in a fermenter. In bioassays concentrated extracts of the supernatant fraction of fermenter cultures showed less gibberellin per ml than in extracts from shaken cultures, either because less active substance was produced or because more substances were produced which interfered with the bioassay. In fermenter cultures, enough IAA was formed to produce a pink spot on chromatograms when treated with $FeCl_3$:H_2SO_4:methanol reagent. IAA reacted in the Avena coleoptile section bioassay; approximately 0·1 μg was produced per ml of original culture.

The effect of tryptophan on gibberellin and indolyl-3-acetic acid synthesis

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was studied. In Warburg experiments with washed cells no additional oxygen was taken up in the presence of tryptophan, nor was IAA formed. The conversion of tryptophan to IAA is therefore not enzymic.

In collaboration with J. Carpenter (Plant Pathology Department), the amino-acid composition of *Azotobacter* proteins was studied using electrophoresis and chromatographic methods. The cell proteins appeared to contain about 1% tryptophan, which is a possible precursor of IAA.

Phenolic acids were also detected in *Azotobacter* cultures. (Brown and Walker)

Nitrifiers. Two more strains of autotrophic nitrifiers were isolated in pure culture. Quantities of *Nitrosomonas* and *Nitrobacter* cultures were produced for the Soil Science Section of the N.A.A.S. at Cambridge to use in experiments on the removal from soil of some substance toxic to seedlings, apparently either ammonia or nitrite. Ammonia or nitrite can be removed from soil by inoculation with the appropriate nitrifying bacteria. (Walker and Glaeser)

Microbial decomposition of chemicals used in plant protection. Work continued with triazine herbicides, especially the decomposition of atrazine. Using gas chromatography to estimate atrazine, cultures of the thermophilic *Thermoactinomyces vulgaris* degraded atrazine in 6 days at 50° C. *Aspergillus fumigatus*, which had been reported by Kaufman *et al.* (*Science* (1963), **142**, 405) to decompose simazine, caused some decomposition of atrazine in our experiments, but seven other Aspergillus species did not.

Parathion, estimated by bioassays using Collembola and by gas chromatography, was decomposed biologically when aqueous solutions were percolated through soil, but the organisms responsible have yet to be isolated. (Walker and Chandra)

Soil anaerobes. Populations of obligate and facultative anaerobes in soil incubated in different conditions were measured to assess to what extent their multiplication and the amount of their fermentation products indicate anaerobic conditions. The response of such populations to anoxic atmospheres is also being investigated.

When Broadbalk (plot 2) soil was incubated in hydrogen for 4 days at 25° C spore-forming obligate anaerobes (using the DRC medium referred to in *Rothamsted Report* for 1966, p. 83) were twice as many as in aerobic soil, but were still very few, only $1{\cdot}4 \times 10^5$/g dry soil. Fatty acids were extracted from the soils with dilute ammonium hydroxide, subsequently transferred to ether, and analysed by gas chromatography, which showed that whether incubated in air or in hydrogen the soils contained acetic, propionic and iso-butyric acid. Extraction can also be made more quickly and more accurately by extracting directly with ether or ether–acetone mixtures. Non-volatile ether-soluble material from soil does not adversely affect the gas-chromatographic analysis.

The few obligate anaerobes found in soil incubated in hydrogen, or in water-logged soil with added carbohydrate, threw doubt on the counting method. The DRC medium contains ferric iron and sulphite and becomes

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black when spore-forming obligate anaerobic clostridia grow in it. Most probable number (MPN) counts using this medium were therefore compared with those with Jensen potato tubes incubated in nitrogen, and with anaerobic colony counts in deep nutrient agar. These two other media each gave, for a Broadbalk soil, bacterial counts of the order of 150–200 $\times$ 10^3/g soil, all of spore-formers, whereas the DRC medium gave only 800. All the potato-tube cultures contained spores, and many produced fatty acids and butanol. Thus, facultatively anaerobic bacteria, in addition to obligate types, probably contribute to the bacterial fermentation products that accumulate in soils. (Skinner)

Staff and visiting workers

F. Bell joined the department in May to work on the fixation of nitrogen by nodulated legumes in the field (International Biological Programme), and D. Hayman (Science Research Council Post-doctoral Fellow) in December to work with Barbara Mosse on ecological and physiological problems of *Endogone* infections.

Visiting workers included Mr. H. Glaeser, Institute of Microbiology, Göttingen University, Germany (under the auspices of IAESTE), Professor Chandra, University of New Brunswick, Canada (NRC Canada and Nuffield scholar), Dr. H. Mareckova (under the auspices of the Czechoslovakian International Biological Programme) and Dr. R. Barrueco, Salamanca University, Spain.

J. J. Patel was awarded the degree of Ph.D. of London University.

P. S. Nutman and R. J. Roughley attended the second conference on "Global Impacts of Applied Microbiology" and a UNESCO and IBP symposium on soil and seed inoculation at Addis Ababa, Ethiopia, in November.

https://doi.org/10.23637/ERADOC-1-120