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# Report for 1969 - Part 1

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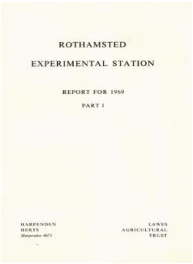
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PART 1

# Soil Microbiology Department

## P. S. Nutman

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# SOIL MICROBIOLOGY DEPARTMENT

P. S. NUTMAN

Of the many agriculturally significant microorganisms that occur in soil this department studies only a few, namely the bacteria associated with some aspects of the nitrogen cycle, those concerned with decomposing natural and artificial organic substrates in soil, and some symbiotic and rhizosphere organisms that influence crop growth and health.

Studies of the ecology of *Ophiobolus graminis* in the cereal rhizosphere and in soils naturally or artificially infested were extended. Work began on the formation of gaseous hydrocarbons (other than methane) that may affect growth of plants in waterlogged soils. Other work also began on the ecology of nitrogen fixing soil algae and on the pectic enzymes of the clover rhizosphere that may be important for infection by nodule bacteria.

Some continuing programmes not reported on this year, include the ecology and analysis of DNA of nitrifiers, inoculation of field beans with *Rhizobium leguminosarum*, selection for symbiotic effectiveness in red clover, and fine structure and biochemistry of nodules.

## Effect of *Endogone* mycorrhiza on plant growth

***In irradiated soils.*** Sixteen soils containing little available phosphate were freed from indigenous mycorrhizal fungi by gamma irradiation (0·8 Mrads), and were used to measure response of plants to mycorrhizal infection and additional phosphate. Mycorrhizal infection was re-established by adding yellow vacuolate *Endogone* sporocarps to the soil (treatment A); phosphate was added as calcium dihydrogen phosphate, 0·4 g/1000 g soil, approximately equivalent to 17 cwt superphosphate per acre (treatment B); controls were given leachings from sporocarps (treatment C). All pots were given N, K and Mg weekly and those in treatments A and C calcium (as $CaCl_2$), equivalent to that given for treatment B. Table 1 summarises results with *Coprosma robusta* and onion. All untreated plants were very small and results are given as the ratios of fresh weights of treated to untreated plants.

All soils except Nos 7 and 20 were more acid than pH 4·6, and without lime mycorrhizal infection failed to develop in them (*Rothamsted Report for 1968*, Part 1, 84). In unlimed soil containing more than 1·0 $\mu$mole available phosphate ($CaCl_2$ soluble), *Coprosma* did not respond to phosphate or, in soil No. 20, to mycorrhiza. In unlimed soils containing 0·7–1·0 $\mu$moles P, *Coprosma* responded greatly to phosphate and in soils with less P than 0·7 $\mu$moles still more; in soil No. 7, plants also responded to mycorrhiza. Phosphate gave very large growth increases (up to 27-fold with *Coprosma* and 15-fold with onions) in limed soil. Mycorrhiza produced nearly as large increases, up to 20-fold with *Coprosma* and 16-fold with onions. This effect was further studied in soil No. 7 with onion, *Coprosma*, *Liquidambar styraciflua*, fuchsia, tomato, maize, ryegrass and *Nardus*



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**TABLE 1**

*Effects of phosphate and mycorrhiza on the growth of* Coprosma robusta *and onion* (Allium cepa *var. James Keeping*) *in sixteen different soils sterilised by irradiation*

| | Properties of original soils | | Increase in total fresh weight* | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lime added | | | |
| Soil | pH | μmoles $CaCl_2$ soluble phosphate | No lime added Coprosma (6 months old) B/C | A/C | Coprosma (14 weeks old) B/C | A/C | Onion (10 weeks old) B/C | A/C |
| 16 | 4·1 | 19·8 | 1 | n.i. | 1 | 1 | 2 | 1 |
| 15 | 4·3 | 8·6 | Ø | n.i. | 27 | 20 | 9 | 4 |
| 1 | 3·4 | 8·2 | 1 | n.i. | — | — | — | — |
| 17 | 4·4 | 8·0 | 1 | n.i. | — | — | 11 | 7 |
| 20 | 5·3 | 5·5 | 1 | 1 | — | — | — | — |
| 12 | 4·1 | 2·4 | 1 | n.i. | — | — | 3 | 3 |
| 9 | 3·4 | 2·2 | 1 | n.i. | — | — | 15 | 16 |
| 10 | 3·6 | 2·0 | Ø | n.i. | — | — | 9 | 8 |
| 3 | 4·1 | 1·3 | 1 | n.i. | — | — | — | — |
| 8 | 3·8 | 0·8 | 8 | n.i. | 4 | 14 | 4 | 10 |
| 11 | 3·7 | 0·7 | 6 | n.i. | 5 | 8 | 8 | 8 |
| 6 | 3·3 | 0·7 | 3 | n.i. | — | — | — | — |
| 7 | 7·0 | 0·6 | 44 | 34 | — | — | 10 | 7 |
| 19 | 4·6 | 0·4 | 37 | n.i. | — | — | 12 | 3 |
| 13 | 4·6 | 0·3 | 35 | n.i. | — | — | — | — |
| 4 | 3·5 | 0·2 | 17 | n.i. | — | — | — | — |

A = +mycorrhiza
B = +$PO_4$
C = control

* = ratio of treatment/control
Ø = controls died
+ = final pH approximately 7
— = no observation
n.i. = not infected

*stricta*. The first three plants responded to both phosphate (× 10, × 44 and × 6 respectively) and mycorrhiza (× 6, × 34 and × 5 respectively); the others to neither, but fuchsias grew a little better with mycorrhiza than without. Only ryegrass failed to become infected in this soil. (Mosse and Hayman)

***In unsterilised field soil.*** The literature suggests that inoculating field soils increases the growth of plants very little, but we now find that growth increases can be considerable in unsterilised soil, provided seedlings are mycorrhizal when planted. When inoculated and uninoculated seedlings of onions were planted in sterilised and unsterilised No. 7 soil, after 10 weeks their fresh weights in unsterilised soil were 3·9 g when uninoculated and 9·4 g when inoculated, and in sterilised soil 2·3 g and 13·2 g. After 10 weeks the uninoculated seedlings in the unsterilised soil had acquired some mycorrhizal infection from the indigenous *Endogone* population. Similar results were obtained with three other soils (Nos 8, 10, 11) in which inoculated seedlings grown in the unsterilised soil weighed respectively, 5, 3 and 12 times as much as uninoculated ones. Relative effects were larger in these soils than in No. 7 because fewer of the uninoculated seedlings became infected, indicating a smaller indigenous population of mycorrhizal fungi.

An alternative method of infecting seedlings, by sowing the seed on a cushion of inoculum, produced very strongly mycorrhizal plants. In the



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four soils tested (Nos 7, 8, 10 and 11) inoculated plants were respectively 2, 12, 6 and 7 times heavier than uninoculated ones. In the soil (No. 7) in which inoculation had least effect, the uninoculated seedlings had become as infected as the inoculated ones after 10 weeks. (Mosse and Hayman)

**Culture of *Endogone*.** The need for large amounts of inoculum in plant-growth studies stimulated renewed attempts to obtain pure cultures of *Endogone*. Spores can be induced to germinate by using special methods, but the mycelium grows only while attached to the spore, and stops growing when food in the spore is exhausted. The effect was examined on germinated spores of the yellow-vacuolate type of supplementing soil-extract agar with the following substances: freeze-dried ground roots of *Coprosma* and hemp; the sugars trehalose and mannitol; the growth-promoting substances ethylene, ethrel and morphactin; the amino acids cystein, cystine, methionine and some of their derivatives; casein hydrolysate; peptone; sodium sulphate; bovine serum albumin (BSA). Only BSA (which also stimulates the growth of those rust fungi not yet cultured *in vitro*), consistently improved growth of the germ tubes. In soil extract agar containing 0·1% BSA vigorous hyphae with first and second order branches and vegetative spores regularly grew up to 5 cm. Without BSA growth was both less and more erratic, although occasional hyphae, usually unbranched, extended up to 3 cm. However, as in previous attempts, hyphae detached from the parent spore produced only a short, weak, thin hypha that always grew from the cut end rather than from the tip. (B. Mosse)

**Structure of honey-coloured, sessile *Endogone* spores.** Before they germinate honey-coloured resting spores form a series of peripheral compartments from which the germ tubes arise. There are indications that the resting spore may be a zygospore; if it is, this very unusual method of germination suggests possible taxonomic links between the *Endogonaceae* and the *Ascomycetes*.

When the honey-coloured, sessile spores enter dormancy, a bacterium-like organelle they contain, which divides by fission, increases in numbers and size, whether it is an actinomycete living symbiotically in the *Endogone* spores and hyphae is being investigated. (B. Mosse)

**Influence of temperature and light intensity on *Endogone* mycorrhiza.** Mycorrhizal infection and plant-growth responses were compared at different temperatures and light intensities in controlled environment cabinets. In sterilised soil containing little phosphate, infection with inoculated *Endogone* was greater at 24 000 lx than at 12 000 lx, and greater at 23°C or at 14–23°C on a 12–12 hour cycle, than at 14°C. Infection also occurred faster at 23°C and 14–23°C; entry points were observed within a week of transplanting onion and tobacco seedlings into the soil. The type of infection was affected by light intensity, and arbuscules developed more at 24 000 lx. Onions with mycorrhiza grew better than those without except at 14°C with 12 000 lx. (Hayman)



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**Growth of the take-all fungus in soil and in the wheat rhizosphere.** The colonisation of the root zone of wheat seedlings by the take-all fungus (*Ophiobolus graminis*), and the earliest stages of infection, were observed by placing small fragments of washed mycelium grown in 0·4% malt solution on slides coated with distilled-water agar, which were buried in pots of field soil sown with wheat, so that the roots grew down in contact with them, and the slides were later lifted and examined. By the time the seed germinated (3 days), new hyaline hyphae had grown from the inoculum towards the roots, and by 6 days hyphae were ramifying amongst the root hairs and formed large aggregates of cells of distinctive morphology, especially near the roots. The root was penetrated by hyphae that entered and filled the root hairs and also by hyphae arising from the inoculum. These hyphae had light-brown walls, not the characteristic black of 'runner' hyphae. Between 6 and 9 days the fungal aggregates and infected root hairs started to lyse, and by 12 days the roots had necrotic lesions and carried a few runner hyphae.

Microconidia frequently formed on the new hyphae close to the inoculum but only rarely on those in the root-hair region. These conidia later lysed without seeming to develop further.

This sequence of events was confirmed by following the growth of inocula labelled with the fluorescent brightener 'photine' (the sodium salt of 4,4′-bis[4-anilino-6-bis (2-hydroxyethyl) amino -s-triazin -2-yl-amino]-2-2′ stilbene disulphonic acid).

Increasing the temperature at which the plants were grown from 8° to 20°C, while keeping soil moisture constant, increased the amount of mycelium, but at 25°C the amount was about the same as at 12°C. The form of the mycelium was similar at all temperatures.

Without wheat seedlings in the pots, the inoculum either failed to grow or formed only a few hyaline hyphae.

The work is a preliminary to studying how colonisation and infection are affected by differences in soil type and fertility, and to gain information about the reason for take-all becoming less severe when wheat and barley are grown intensively in the same land. (M. Brown with Hornby, Plant Pathology Department)

**The production of growth-regulating substances by soil bacteria.** The bacteria used were isolated by culturing at random from root-free soil and from the root zone of wheat plants, 6, 40 or 90 days old. They were grown in a mineral medium poor in nitrogen and rich in carbohydrate, containing soil extract and trace amounts of yeast extract but no tryptophan. After 10 to 14 days on a rotary shaker, the supernatant fluids were extracted using techniques developed with *Azotobacter*. Gibberellin in the extracts was assayed by measuring the growth of the internode of dwarf peas and of the lettuce hypocotyl. Indolyl-3-acetic acid (IAA) was assayed measuring the extension of wheat coleoptiles. The culture medium alone was completely inactive in all tests.

Eighty-four per cent of isolates from seedlings 6 days old produced substances with auxin activity equivalent to 0·02 to 0·3 $\mu$g IAA/ml of culture; of these, about half produced more IAA activity when 1 mg/l of



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L-tryptophan was added to the medium. Three-quarters of the soil isolates produced auxins.

Forty-five per cent of the root isolates and 48% of the soil isolates produced substances that promoted growth of pea internodes, and 48% and 33% respectively of root and soil isolates produced substances that inhibited this. Chromatographic separation gave three different fractions with gibberellin-like activity, one of which corresponded to gibberellin $GA_3$. Amounts produced ranged from 0·001 to 0·01 $\mu$g $GA_3$ equivalent/ml. Comparing isolates from soil and root zone shows that the young root selectively stimulates organisms that produce plant growth inhibitors.

Analysis of results with isolates from rhizospheres of older plants is incomplete, but shows differences from those of young plants. (M. Brown)

**Microbial degradation of aromatic compounds.** In further work on the fate in soil of various aromatic compounds, including some related to or components of commercial herbicides, a *Pseudomonas* sp., resembling *Ps. oleovorans*, was isolated that grows with aniline as its sole carbon source. Aniline oxidation depends on an induced enzyme system and washed, aniline-grown, cells oxidise aniline with release of ammonia. Catechol, *p*-aminophenol and *o*- and *m*-toluidine are also oxidised.

*Azotobacter vinelandii* and a salicylate-utilising strain of *A. chroococcum*, when grown on benzoate, were found to oxidise 3-chlorobenzoate to 3-chlorocatechol. Neither species can use 3-chlorobenzoate as an energy source for growth. Thus aromatic halogen compounds, not occurring naturally, may under suitable circumstances be degraded by common soil microbes (see Walker, *Rothamsted Report for 1951*, 56), without their being used to support their growth.

A *Rhodotorula* sp., which metabolises phenol, and a soil actinomycete species able to grow with 3-chloroaniline as carbon source were isolated from soil and their metabolism is being studied. (Walker)

**Hydrocarbons from waterlogged soils.** Except for methane, the gases found in waterlogged soils have been little studied, though their presence, especially of ethylene, could affect the growth of roots. Samples of Gault soil provided by Dr. K. Smith, Letcombe Laboratory, who is studying toxicity of waterlogged soils to plants, were incubated at 25°C in screw-capped bottles (28 ml), containing 20 ml of mineral-salts medium with glycerol (5 g/l) and peptone (2·5 g/l). After 48 hours, gas chromatography showed that all contained methane, ethylene, ethane and acetylene, in amounts that differed in different soils.

The occurrence of methane and ethylene in control bottles of autoclaved medium or water suggested release of these hydrocarbons from the rubber liners of the screw caps. Bottles containing water and sealed with black, white or butyl rubber liners, or Suba-Seals, always yielded methane and ethylene in the gas phase after incubation or autoclaving.

Release of hydrocarbons from the liners could be prevented by interposing a sterile disc (25 mm diameter) of thin aluminium foil between the



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screw cap liner and the bottle. The foil was easily penetrated by the hypodermic needle used to take the gas sample for analysis.

For critical work, an all-glass culture vessel was designed consisting of a 100 ml conical flask with a B 24 ground glass neck socket into which fits a cone carrying a female Luer outlet. During incubation, the outlet is connected by Luer male glass joint to a delivery tube dipping under water to allow excess gas to escape. Samples of gas for GLC analysis are taken by connecting the Luer taper of an all-glass syringe directly to the culture vessel, removing an excess of gas, substituting the needle and adjusting the gas volume for injection. With this apparatus, the occurrence of trace amounts of hydrocarbon gases in the gas phase above waterlogged soil was confirmed. (Skinner)

**Nitrogen-fixing soil clostridia.** Anaerobic soil bacteria able to fix nitrogen are usually detected and enumerated by their growth in media deficient in combined nitrogen, and by their staining reaction with iodine (the granulose reaction). However, people disagree about the most suitable type of nitrogen-deficient medium to use, and about the reliability of the granulose reaction. Therefore observations were made on strains of *Clostridium pasteurianum* (probably the principal species of nitrogen-fixing anaerobe) and eight strains of the closely related *Cl. butyricum*. All strains were inoculated into either a liquid, nitrogen-deficient, medium (mineral salts, sucrose trace elements and yeast extract) or a potato medium (tubes containing fragments of potato, water and calcium carbonate), and then incubated aerobically or anaerobically, i.e., in an atmosphere of nitrogen with *c.* 10% $CO_2$.

Only one of four strains of *Cl. pasteurianum* grew in potato medium, and then only little; it did not digest starch. Seven of eight *Cl. butyricum* strains grew vigorously in the aerobic potato medium, digested the starch and produced much gas and fatty acid. No strain of either species produced cells in this medium (or in the N-rich stock culture medium) that gave the granulose reaction. Anaerobic and aerobic cultures in potato medium behaved similarly, indicating that this medium easily becomes reduced enough to permit growth of these anaerobes.

Five strains of *Cl. butyricum* grew, though poorly, in the nitrogen-deficient liquid medium but no strain grew when transferred from it to tubes of fresh medium. Hence growth in this medium probably depended on the presence of some factor in the original inoculum that was too dilute to permit growth when sub-cultured. Four strains yielded some cells that stained violet-brown with iodine. No culture grew when sub-cultured into the nitrogen-deficient medium. Inocula of small amounts of soil also gave good growth in this medium, producing gas and fatty acids, but grew less well in second transfer.

The nitrogen-deficient medium was also used, with and without soil extract and sodium thioglycollate, which changes the Eh of the medium from *c.* +200 mV to *c.* −230 mV. No strain of either species grew in medium lacking thioglycollate. Three strains of *Cl. butyricum* grew in medium without soil extract but with thioglycollate and produced some cells able to stain darkly with iodine. Adding soil extract enabled one



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strain of *Cl. pasteurianum* to grow, though this did not give cells able to stain with iodine. Cells able to stain violet with iodine occurred mostly in cultures that grew without thioglycollate; the occurrence was independent of the presence of soil extract. Growth in nitrogen-deficient medium is favoured by soil extract and sodium thioglycollate, so these substances should be in medium for MPN counts, as shown by the following counts, obtained using media with or without yeast extract and thioglycollate (— SE, — thioglycollate), 490/g; (+ SE, — thioglycollate), 1300/g; (+ SE, + thioglycollate), 3500/g. Cells giving the granulose reaction with iodine were more frequent in nitrogen-deficient medium, i.e. when the culture is actively fixing nitrogen, than in potato medium, but the granulose reaction, once thought to be characteristic of nitrogen-fixing anaerobes, is too uncertain for diagnosis. (Skinner)

**Nitrogen fixation in Broadbalk soil.** The reduction of acetylene to ethylene by the nitrogenase enzyme in soil cores was used to monitor its activity. In the section of Broadbalk sown to wheat, most nitrogenase activity was on the soil surface, which at the time of sampling (May–October) was covered by a crust of blue-green algae. The crust was less developed on plot 3 (unmanured) than elsewhere, but seemed otherwise little affected by manuring. Neither the fallow sections nor those growing potato had any surface activity, but the section growing beans had slight activity at the end of the season. The blue-green alga crust fixed more nitrogen than was fixed at other levels in the soil profile; drying the soil surface greatly decreased fixation, which was much less at soil water tensions of 15 atmospheres than at 5 and 10 atmospheres. Fixation increased rapidly when the surface crust was again made wet, and was substantial within 12 hours. A surface crust of 15 $cm^2$ in the wheat crop had the same order of activity when incubated with acetylene as of a nodulated vetch plant growing nearby.

Several species of the blue-green algae isolated from Broadbalk fixed nitrogen rapidly when grown in liquid culture. Nitrogenase activity of these cultures was little affected by temperature change from 10 to 40°C, and acetylene was reduced at 2 and 45°C. (Dart and Roughley)

**Growth of *Rhizobium trifolii* in batch culture.** *R. trifolii* TA1 was grown in a liquid basal medium containing yeast extract (Difco or Oxoid or Vegemite) at 0·1, 0·35, 0·5 or 1·0%, or with casein hydrolysate at these concentrations and 0·01% of yeast extract (Difco). The inoculated flasks were incubated, with or without shaking on an orbital shaker at 200 rev/min. Most growth was with Vegemite at all concentrations in shaken cultures and at three concentrations in static culture. Except with Oxoid yeast extract at 0·1%, the shaken culture contained more cells, sometimes many more, than the corresponding static culture. The ratios of numbers in shaken to static cultures after 48 hours ranged from 1·48 with 1% casein hydrolysate to 97·7 with 0·5% Vegemite.

In static culture, yeast extracts at 0·1% gave most cells, except with Difco extract, which gave most at 0·35%. In shaken culture, Difco and Oxoid extracts gave more at 0·35%, and Vegemite at 0·5%, than at 0·1%;



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only the casein hydrolysate gave more at 0·1% in both shaken and static culture.

Most cells were viable with the smallest concentrations of yeast extracts. Percentage viability was usually greatest at 18 hours (early logarithmic phase); the largest obtained (Oxoid at 0·35%, shaken) was 89·5%, dropping to 56·9% at 24 hours. The smallest per cent viabilities were all with yeast extract at 1·0% (from 0·06% to 0·16% at 48 hours). Aeration favoured cell growth but had a much smaller effect on percentage viability.

At 0·1%, all three yeast extracts yielded normal rod-shaped cells. Swelling and distortion, apparent at 0·35%, became greater as the concentration increased; Oxoid extract caused the least distortion. With casein hydrolysate at 0·1%, normal cells were formed but at 0·35%, all cells were greatly distorted. In a medium containing yeast extract at 0·01%, the casein hydrolysate was replaced by a mixture of 18 amino acids in approximately the proportions they occur in casein hydrolysate. The total number of cells with the amino-acid mixture equivalent to casein hydrolysate 0·1% at 24 and 48 hours was only a tenth of those in hydrolysed casein. Some cells were swollen and distorted. Larger concentrations of amino acids inhibited growth almost completely. Low surface tension, which decreases with increasing concentrations of yeast extract, is an improbable cause of severe distortion, because casein hydrolysate at 0·35% had a surface tension of 66·2 dynes/cm which, though slightly less than that of the medium with 0·1% (67·8 dynes/cm), was considerably greater than the three media with yeast extract at 0·1%, which did not give distorted cells.

To take phase contrast photomicrographs of normal and distorted cells, Brownian movement of the cells had to be prevented during the long film exposures (*c.* 10 seconds). To do this, a loopful of molten 0·5% agar was spread over a warmed slide in a steam-laden atmosphere to prevent the very thin film from drying before spreading completely. The slides were dried in air and a drop of cell suspension placed on the agar film, the cover slip applied and pressed firmly under filter paper to squeeze out excess water. Brownian movement ceases as the agar swells and gently locks the cells against the underside of the cover slip. (Skinner and Roughley)

**Sensitivity of *Rhizobium* to antibiotics.** With the object of developing a selective medium, 48 strains (16 of *R. trifolii*, 10 of *R. meliloti*, 7 of *R. phaseoli*, 6 of *R. leguminosarum*, 2 of *R. lupini* and 7 of *R. japonicum*) were tested for their sensitivity to the following antibiotics: penicillin, streptomycin, tetracycline, chloramphenicol, erythromycin and the inhibitor sulphapyrazole (Oxoid Mattodisks 30-1H), on yeast extract mannitol agar (YMA) with and without Congo Red at 1/40 000.

Tetracycline inhibited strongly the growth of 43 strains but did not inhibit four strains of *R. japonicum*. Chloramphenicol and sulphapyrazole also inhibited many strains but fewer than tetracycline. Streptomycin and erythromycin were both weakly inhibitory to about half the strains; susceptible strains occurred in all species. Only five strains (all *R. trifolii*) were strongly inhibited by penicillin (zone width $> 5$ mm), though six



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more, three *R. trifolii*, two *R. phaseoli*, one *R. leguminosarum*, were slightly inhibited. Congo Red did not appreciably affect the results.

Three strains most sensitive (*R. trifolii* 0404, *R. phaseoli* 3938, *R. leguminosarum* 1001) were inoculated on plates of YMA and Congo Red media containing 1·0, 2·5, 5·0 and 10·0 I.U. Benzylpenicillin (Glaxo)/ml. The least sensitive of these strains (1001) was inhibited only at 10·0 I.U./ml, 3938 and 0404 grew poorly with all concentrations of penicillin.

Forty-six strains out of 48 strains tested will grow on medium with 5·0 I.U. of penicillin/ml. (Skinner)

***Rhizobium* bacteriophage.** Further work with a range of *Rhizobium* strains and their phages confirmed that virulent phages could change to temperate ones in conditions that allow the virulent forms to coexist for periods of weeks with resistant mutants of the bacteria. Resistant mutants can still combine with virulent phages, whereas killed cells do not. As with susceptible cells, specific absorption of phages seems the first step in infecting resistant mutants, which the phage cannot lyse and in which they ultimately become incorporated in the bacterial genome. This formation of lysogenic cells would explain temperate and virulent phages occurring in the same environment.

Neither field soils sterilised by irradiation nor potting mixtures readily become colonised by phage in usual glasshouse conditions unless they contain not only bacteria susceptible to the phage but also a legume susceptible to the bacterium. Seven phages, isolated from a single pot of soil (J.I. compost) containing red clover, were classified by their serological relationships, their virulence to ten test strains of bacteria, and their morphology. None of the seven lysed the bacterial strain with which the soil in the pot was initially inoculated, and only two were alike in all the above tests. (J. Kleczkowski)

**Chemical and structural studies on the polysaccharide of *Rhizobium trifolii*.** Analysis of the extracellular polysaccharide purified from four infective and two noninfective strains of *R. trifolii* indicated the presence of a keto acid, which chromatography of derivatives identified as pyruvic acid. It usually amounted to 8–9% of the dry weight of the polysaccharides. Dr. W. F. Dudman, C.S.I.R.O., Canberra, has similar results (personal communication). Acid releases pyruvic acid from the polysaccharide of strain 0401, this takes 4 hours to go to completion in 1 N or 0·1 N hydrochloric acid. Aqueous solutions of polysaccharide also slowly release pyruvic acid at 100°C. Polysaccharides obtained from effective and ineffective strains of *R. leguminosarum* also contained pyruvic acid.

Qualitative analysis for sugars and quantitative analysis for uronic acids show differences between polysaccharides from various strains of *R. trifolii* that are not correlated with differences in their capacity to infect red clover. Small differences in sedimentation between polysaccharides from different strains were also not related to symbiotic properties.

The polysaccharide from strain 0401 is not degraded by $\alpha$-amylase, $\beta$-galactosidase or pectinase under conditions in which these enzymes are active. (C. Hepper)



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**Pectic enzymes of the clover rhizosphere.** The possible role of pectic enzymes in the infection of root hairs of red clover seedlings by strains of *R. trifolii* is being investigated. A viscometric assay technique was used to measure pectolytic activity in exudates and extracts from roots of seedlings grown for various periods after inoculating with rhizobial strains. Exudates of seedlings inoculated 6 to 10 days previously with *R. trifolii* strain 0403 were slightly active, whereas exudates from uninoculated seedlings, or seedlings inoculated with strains unable to infect them, were inactive. However, the activity was small (25% decrease in viscosity in 22 hours) so that its significance was uncertain. None of the extracts from seedling roots had any pectolytic activity. No changes in the pectin methyl esterase activity could be detected. (Bonish)

**Scanning electron microscopy of clover plant roots inoculated with *Rhizobium*.** The bacteria coated the root surface prolifically and some seemed firmly attached. Some areas of root hairs had a fibrillar surface, with small granules on both root hairs and epidermal surfaces. Parts of the root surface were covered by an amorphous film, thought to be the mucigel layer, described by Jenny and Grossenbacker (*Proc. Soil Sci. Soc. Am.* (1963), **27**, 273–277). (Dart)

**The effect of cold pretreatment on infection of clover by *Rhizobium*.** To make germination less erratic, clover seed to be used in infection experiments is routinely surface-sterilised, soaked in water and then left at 3–4°C for 1 or 2 days before sowing. When germination is still erratic, some day-old seedlings may also have to be left at 3–4°C until enough others have emerged for the experiment. Keeping seeds or seedlings of *Trifolium subterraneum*, *Trifolium glomeratum* and *Trifolium parviflorum* at 3°C for up to 6 days, increased the rate at which the root hairs became infected during the first week the plants were growing at 20°C, but after this infection occurred at similar rates as in plants not previously chilled. (Subba Rao, Dart, Roughley and Nutman)

**Temperature, nodulation and nitrogen fixation.** The nitrogenase activity of root nodules of *Trifolium pratense* (red clover), *T. subterraneum* (subterranean clover), *Medicago truncatula* (barrel medic), *M. sativa* (lucerne), *Vicia fabia* (field bean), *V. atropurpurea* (purple vetch), *V. hirsuta* (hairy vetch), *Vigna sinensis* (cowpea) and *Glycine max* (soya bean) was examined over the temperature range 3–45°C, using the reduction of acetylene to ethylene by the enzyme to measure activity. It was affected greatly only at the extreme temperatures, and differed little in the range 10–30°C for all plants; it was irreversibly lost at 45°C. Activity was small at 40°C with all plants except cowpea and lucerne, and with clover plants was less at 35°C than colder.

Although nitrogenase activity is less at 3° and 5°C than at 10°C with all the plants, it is still substantial, and nodulated roots of the *Trifolium* sp., *Medicago* sp. and *Vicia* sp. at 3°C continued to reduce acetylene to



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ethylene for at least 48 hours; during this period as much total ethylene was evolved as in 8 hours at 20°C when activity had ceased. Nodulated roots of the *Trifolium* sp. and *Medicago* sp. held at 3°C in the dark for 3 days lost little of their nitrogenase activity, but soya bean and the *Vicia* sp. lost most of their activity.

Nitrogenase activity of isolated bacteroids from soya bean is similar from 20–35°C; at 5°C, or 40°C, they were slightly active. (Dart)

The effects of cold on the growth of and nitrogen fixation of subterranean clover described in test-tube culture (*Rothamsted Report for 1968*, Part 1, 88) were examined in sand culture. The pattern of response to temperature was similar for both methods of culture, but growth and nitrogenase activity were restricted by tube cultures. Preliminary results indicate that the decline in nitrogenase activity in test-tube cultures is peculiar to this method of growth and may be partly caused by the roots being directly illuminated. (Day and Dart)

**Light intensity, nodulation and nitrogen fixation.** The effects of light intensity on nodulation (*Rothamsted Report for 1968*, Part 1, 87) were further studied in a range of legumes that differ in their nodulating habit. The following species were grown in different light intensities and their nitrogenase activity estimated by measuring acetylene reduction: hairy vetch (*Vicia hirsuta*), purple vetch (*V. atropurpurea*), field bean (*V. faba*), subterranean clover (*Trifolium subterraneum*), red clover (*T. pratense*), barrel medic (*Medicago truncatula*) and lucerne (*M. sativa*).

The number of nodules on the primary roots of barrel medic, lucerne and red clover was little affected by light intensity (15 000–30 000 lx), whereas more were produced in field bean, hairy vetch and subterranean clover as light intensity increased. The response of purple vetch was exceptional in that early growth and nodulation was much restricted by a light intensity of 30 000 lx. Few primary nodules formed on the root above 25 000 lx, but many formed later on the secondary roots, and at 50 days such plants had more nitrogenase activity than those grown at 15 000 lx. Plant growth and nitrogen fixation was greater with more light for all species.

The total nitrogenase activity per plant was related directly to leaf area and to the aggregate amount of light received by the plant. The total nodule weight of all species at 40 days also increased with increasing light. In subterranean clover and barrel medic, nitrogenase activity per mg of nodule tissue was uniformly large over the first 40 days and was unaffected by light intensity, and total plant nitrogenase activity was proportional to nodule weight. Activity per mg of nodule in the *Vicia* species increased with light intensity. In all species, the primary and the secondary root nodules had about the same activities per mg of nodule tissue. (Day and Dart)

**Estimation of nitrogen fixed by nodulated legumes (IBP).** In the second year of the experiments with lucerne and ryegrass at Rothamsted and Woburn, responses of lucerne inoculation with *Rhizobium* were smaller



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than in 1968, especially at Woburn, where the experiment was abandoned at the second cut because weeds infested the plots after flooding in 1968. Lucerne inoculated with effective strains of *Rhizobium* but without nitrogen fertiliser yielded 29·6 cwt/acre at Rothamsted (two cuts) and 16·4 cwt at Woburn (two cuts). Comparing N uptake by lucerne and grass shows that about 62·3 lb N/acre was fixed by the lucerne at Rothamsted and 35·6 lb at Woburn.

Another experiment with lucerne and grass was started on a nitrogen-rich (0·68% N in the top 6 in.) calcareous soil at Stopsley, Bedfordshire. In contrast to Rothamsted and Woburn, neither inoculation with *Rhizobium*, either effective or ineffective strains, nor nitrogen fertiliser, had much effect on yields, but P and K increased it greatly because plants on unfertilised plots were deficient in K. The largest yield of lucerne was 55·6 cwt DM/acre from three cuts, and the relevant comparisons of N-uptakes showed that the lucerne nodulated by the naturally occurring strains, or by the introduced effective strain fixed 130 and 146 lb N/acre respectively. The large N-uptake by uninoculated plants is attributable to the soil being rich in N which also restricted the amount fixed symbiotically. Although the calcareous soil allowed the indigenous *Rhizobium meliloti* in the plots of inoculated lucerne to multiply more than in the soils at Rothamsted and Woburn, the increase was not large; from *c.* 50/g dry soil before sowing to *c.* 5000/g dry soil in October. In the grass plots nodule bacteria remained very few. In plots with the effective strain the increase was much larger, to more than 100 000/g dry soil, but the ineffective strain established itself poorly and the autumn count was *c.* 5000/g dry soil. Consequently inoculation with the ineffective strain had a smaller and briefer effect on yield than in the earlier experiments. The largest yield of lucerne without nitrogen fertiliser was 45·7 cwt DM/acre and of grass with nitro chalk 56·2 cwt DM/acre.

Two experiments, one at Rothamsted and one at Woburn, were begun with beans and spring wheat cut green twice. The dry matter of wheat increased much faster than beans, especially at Rothamsted. Neither crop was affected by lime or PK; wheat responded much more than beans to fertiliser nitrogen and, relatively more than the ryegrass in the other experiments. Inoculating beans with effective nodule bacteria, did not increase their yield or N content. Inoculation with the ineffective strain was without effect at Woburn but slightly reduced N-uptake at Rothamsted. *Rhizobium leguminosarum* was fairly abundant (initially 10 000/g at Rothamsted and 5000/g at Woburn) and increased rapidly at both sites to more than 1 million/g. All bean roots examined, irrespective of treatment, were well nodulated with pink nodules presumably fixing nitrogen. Because the minimum uptake (and rate of uptake) of N by wheat exceeded the minimum by beans, and because the beans yielded well, whether inoculated or not, the amount of N fixed by them could not be estimated.

These results, and those with lucerne at Stopsley, show that some of the objectives of the experiment are not met when the indigenous nodule bacteria are numerous and effective, and when the experiment is too brief to allow the legume and non-legume to assimilate all, or most, of the soil nitrogen. (Bell)



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## Staff and visiting workers

Mabel Dunkley retired after 51 years work at Rothamsted, first in the Botany Department and since 1919 in this department. Her father was employed by Sir John Lawes and her departure broke the staff's last link with its founder. J. J. Patel left to take an appointment at Plant Chemistry Division, D.S.I.R., Palmerston North, New Zealand. Kathleen Gibbs was appointed to take charge of the *Rhizobium* Culture Collection.

R. J. Roughley was awarded the degree of Ph.D. of London University and returned to Division of Science Services, N.S.W. Department of Agriculture, Rydalmere, N.S.W., Australia; N. Subba Rao returned to the Division of Microbiology, Indian Agriculture Research Institute, New Delhi, India.

Visiting workers included Dr. Hana Marcková of the Central Research Institute for Plant Production, Ruzyne, Prague, Czechoslovakia, Barbel Rosenthal and H. Lehmann of Göttingen University, and Karen D. Jacob of Darmstadt University.



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