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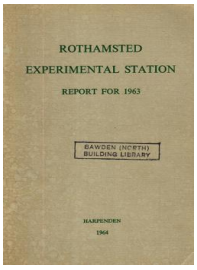
Rothamsted Report for 1963

Full Table of Content



Soil Microbiology Department

P. S. Nutman

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SOIL MICROBIOLOGY DEPARTMENT

P. S. NUTMAN

R. M. Jackson returned from secondment to the Soil Bureau, Wellington, New Zealand. Shoshana Bascomb joined the department to reorganise the culture collection of Rhizobium. Visiting workers included J. Patel of Gujarat University, Ahmedabad, India, Dr. H. Jacks of Massey Agricultural College, Palmeston North, New Zealand, and H. Reichenbach of the Botanisches Institut, Karlsruhe, Germany.

Barbara Mosse and P. S. Nutman contributed to the 13th Symposium of the Society of General Microbiology at London on "Symbiotic Associations", R. M. Jackson and P. S. Nutman to the Symposium at Berkeley, California, on "The Behaviour of Plant Pathogens in Soil", Margaret Brown to the Nottingham Symposium of the British Society of Soil Science on "The Estimation of Biological Activity in Soil", R. Cooper to the John Innes Symposium on the "Comparative Biochemistry of the Leguminosae" and Barbara Mosse to the Royal Microscopical Society's Symposium at Oxford on "Botanical Applications of Electron Microscopy".

Persistence of simazine in soil. Samples from small bare patches of soil on Great Field 1 and 2 and Great Knott 3, thought to be where accidental overdoses of simazine were applied in 1961, were taken and residual simazine estimated by biological methods. Tests of effects on germination or seedling growth (oats, barley, radish, melon) confirmed the presence of phytotoxic material of simazine type, but were not sensitive enough for quantitative work. Tests with *Chlorella* were more rapid, accurate and sensitive; they indicated that the bare patch in Great Field 2 contained 6 lb simazine/acre in the top 3 in. soil and in Great Knott 3 ½–1 lb/acre, close to the value of ½ lb/acre measured chemically by Fisons Ltd. The use of *Chlorella* and other algae for the assay of simazine and atrazine is being further investigated.

Soil from the bare patches respired less than soil from nearby areas carrying crops, probably because it contained less readily decomposable plant material rather than because of the presence of simazine. The numbers of bacteria, including clover, pea and lucerne nodule bacteria, fungi or actinomycetes, were similar in the patches and the soil that carried crops. (Dr. H. Jacks and Walker)

Decomposition of β-naphthol by a soil organism. An organism corresponding to the description of *Pseudomonas fluorescens* was isolated from soil enriched with β-naphthol. It grows on several organic substances, including salicylic acid, but cultures grown on β-naphthol do not oxidise naphthalene, α-naphthol or salicylic acid. It oxidises gentisic acid and catechol, but

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only after a short lag. It differs metabolically from naphthalene-using Pseudomonads, earlier studied by Treccani, Walker and Wiltshire (*Rothamsted Report* for 1954, p. 61), which oxidise but do not grow on either α- or β-naphthols. (Walker)

Nitrifying organisms. Isolation procedures for nitrifiers were facilitated by the use of highly purified agar, now obtainable commercially, and Soriano's technique for picking micro-colonies. A new ammonia-oxidising bacterium, obtained in pure culture from a Ghana soil, differs morphologically from *Nitrosomonas europea* in being larger and multi-flagellate.

Nitrosococcus nitrosus (a strain from Prof. S. Soriano) was grown in continuous culture. Its growth rate—doubling time 12 hours—is similar to that of *Nitrosomonas europea*. *Nitrosococcus* cells in quantity are deep red and show strong cytochrome *c* absorption bands. (Prof. S. Soriano and Walker)

Development of "farmer's lung hay" (F.L.H.) antigen in inoculated sterile hay and in culture media. Thermophilic actinomycetes from mouldy hays were grown on sterile hay pretreated with ammonia vapour to raise the pH to about 7·0. After incubation at 40° or 60° C for one or more weeks, the cultures were defatted with acetone and their extracts tested by Dr. J. Pepys and Mr. P. A. Jenkins of the Institute of Diseases of the Chest, Brompton, London, against sera of patients with farmer's lung, using the methods of double-diffusion and immuno-electrophoresis. Sterile hay inoculated with a mixture of thermophilic actinomycetes and incubated at 40° C yielded an extract that gave precipitin reactions with 19 out of 20 sera. These reactions were of the same type as those given by the non-fungal antigens contained in mouldy hays associated with cases of farmer's lung disease. This antigen complex (defined by the pattern of precipitin arcs in electrophoresis tests) was named "farmer's lung hay" (F.L.H.) antigen.

Growth of the actinomycete *Thermopolyspora polyspora* on ammoniated hay yielded antigens of the F.L.H. type that reacted with nearly all the sera. This organism produced the same antigens when grown on hay treated with chalk to raise the pH, and on hay that had previously supported growth of *Mucor* sp. Extracts of hay on which *Micromonospora vulgaris* had grown reacted with fewer sera and contained only one component of the F.L.H. antigen complex.

F.L.H. antigen was also produced by *T. polyspora* grown on nutrient (peptone–meat extract) agar, vegetable juice agar and on agar medium containing glucose and hydrolysed casein. Extracts from cultures on nutrient agar were used in skin reaction and aerosol inhalation tests by our medical colleagues.

For the preparation of pure *T. polyspora* antigen a synthetic medium free from peptones, hydrolysed casein and agar is preferred. The best synthetic medium so far devised, although giving slower growth than nutrient agar, consists of a mineral salts solution containing glutamate and glucose. (Skinner with Gregory and Lacey, Plant Pathology Department, and Festenstein, Biochemistry Department)

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Field experiments on Azotobacter inoculation. Winter wheat was sown on Great Field in November 1962 to test the effect of Azotobacter inoculation in a soil naturally infested with take-all; fertiliser nitrogen was applied to half the plots. The seed was inoculated with an 11-day culture of *Azotobacter chroococcum* strain A6 giving an average of 132,000 bacteria per seed. The wheat did not emerge before the snow came and was not visible until March. 23,000 Azotobacter were recovered in early spring per gram of dry rhizosphere soil from inoculated roots and 163 from control rhizosphere soil. Yields were small and replicate uniformity poor, and Azotobacter inoculation had no obvious effect on yield or on the amount of roots infected with *Ophiobolus graminis*.

Seed potatoes, variety "King Edward", inoculated by dipping in a 14-day-old culture of Azotobacter A6, were planted in April in the Garden Plots. An average of 190,000 Azotobacter were recovered per gram dry rhizosphere soil from the young plants. A second sample in August gave 630,000 Azotobacter in the rhizosphere and 62,000 per gram of scrapings from the new tubers. In August the haulms were killed by blight. Yields were small and variable. Inoculation increased the number of stems but not yield of tubers.

Seedlings of "Velocity" cabbage, raised individually in potting compost and inoculated at the 2-leaf stage with 2 ml of a 14-day-old culture of Azotobacter, were planted out at 5 weeks, when 39,000,000 Azotobacter were recovered per gram of rhizosphere soil. Two strains of Azotobacter were used—A6, and a strain isolated from soil from Broom's Barn—and, as with the wheat and potato experiments, the effect of inoculation was examined in conjunction with fertiliser nitrogen. Fertiliser nitrogen had no effect on yield, and Azotobacter inoculation did not influence yield in plots without added nitrogen. In plots with nitrogen the Broom's Barn inoculum increased yield by 23% and the A6 strain by 5%.

In collaboration with the Lee Valley Horticultural Station, inoculation experiments were done with lettuce, cucumber and tomato. In the first lettuce experiment young plants were transplanted into the open after inoculation with a 6-day-old culture of A6. The rhizospheres of inoculated plants contained 505,000 Azotobacter per gram dry soil, whereas controls had none. Inoculation increased yield of the variety "Borough Wonder" by 7·8%, but had no effect on the other four varieties. In a second field-inoculated experiment with the lettuce variety "Cobham Green", using 8 ml of a 9-day-old culture per plant, inoculation increased yield by 17·8%. The rhizosphere population of Azotobacter at harvest was 2,000,000 per gram dry soil.

Pot-grown plants of the cucumber varieties "B.D.R." and "Sporü" were inoculated with a 14-day-old culture of A6 and planted out, at the flower-bud stage, into straw bales. Establishment of Azotobacter around new roots in the soil near the crown was satisfactory—70,000 per gram dry rhizosphere soil. More fruits were set on the inoculated vines, but whereas batch pickings of "B.D.R." in June and August showed a 14·9% increase from inoculation, they showed a 19·7% decrease with "Sporü". "Sporü" is an early flowering variety, and these differences are of interest because Azotobacter tends to promote earliness.

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In 1961 inoculated tomato plants flowered and fruited a little earlier than controls. This was confirmed in experiments started in March and in June 1963 with plants of "Moneymaker" tomato, stopped at the third truss. Weekly gradings of flowers and fruits showed that inoculation advanced maturity of the first truss by about 10 days and of the second and third truss by less than a week; inoculation did not affect yield.

These experiments confirm earlier work in demonstrating sporadic effects of inoculation on crop yield or maturity and in showing that the rhizosphere of plants grown in neutral soil can be readily colonised by Azotobacter. (Brown and Burlingham)

Azotobacter and plant disease. The effect of Azotobacter inoculation on diseased crops (*see* Abstracts of Papers, No. 4·3) was further investigated in experiments with naturally infected seed or soil and by double-inoculation experiments.

Experiments with agar-grown wheat inoculated with *Azotobacter chroococcum* and *Ophiobolus graminis* were inconclusive. In one, wheat plants inoculated with *Ophiobolus graminis*, or treated with culture filtrate, responded to Azotobacter inoculation; seedlings were bigger than controls, and subsidiary infection from seed-borne *Fusaria* sp. was decreased. Further experiments with wheat grown on agar or in vermiculite medium confirmed an Azotobacter–Fusarium interaction. Lesions occurred on all plants inoculated with Fusarium, but were less in the presence of Azotobacter, and root growth was improved. Similar results were obtained in experiments with oats grown in vermiculite inoculated with *Helminthosporium avenae*. Three amounts of inoculum were used. Azotobacter improved root growth at the two smaller amounts of fungus but not with the largest.

Azotobacter diminished the effect of seed-borne *Ascochyta imperfecta* on *Medicago lupulina*, and its ameliorating effect was again related to the amount of pathogen on the seed; it has no effect when seed is severely infected.

A systematic investigation on the effect of Azotobacter inoculation on the rhizosphere microflora has been started with special reference to the root surface fungal flora. (Brown and Burlingham)

Azotobacter in sand-dunes. The Azotobacter populations (per gram) in young sand-dunes at Sizewell, Suffolk, ranged from 0 to 47,000 in the rhizosphere and from 0 to 17,000 in sand. Most were under *Ammophila arenaria* and *Carex arenaria* and few under *Euphorbia paralias*, *Festuca rubra* and *Glaucium flavum*. Azotobacter was found only in sand collected at or just above spring high-tide line, and was confined to samples collected in the autumn. Three distinct colony forms of Azotobacter were isolated. (Burlingham)

Rhizobium culture collection. We maintain the largest collection of nodule bacteria in the United Kingdom. Strains are subcultured twice a year on a yeast–mannitol medium, and fresh subcultures are made when required. Because of increasing demand for cultures from research workers, and the

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impracticability of regularly testing so many strains on plants the collection is being dried. Each strain is examined bacteriologically before drying and tested on its principal host plant; some strains will be tested on a range of host plants.

The single-stage drying procedure used follows the method of Annear (*Aust. J. exp. Biol. med. Sci.* (1962), **40**, 1–8) as modified by R. A. Lelliott for the National Collection of Plant Pathogenic Bacteria. Batches of 60, 30, 15 or 10 ampoules are being dried, depending on likely demand.

Before starting the routine drying, six suspending media and two storage temperatures were tested; more survived drying in 10% sucrose or 10% sucrose and 5% peptone than in other media, and more survived when stored at 2° C than at 26° C. The average survival immediately after drying in sucrose–peptone was 40%, or 5×10^8 bacteria per ampoule, and there was no decline during 4 months storage at 2° C.

The 14 *Rh. meliloti* strains were tested on *Medicago sativa* before and after drying and no difference was found in virulence or nitrogen-fixing ability. The 135 strains of the *Rh. trifolii* collection are currently being dried and tested on *Trifolium pratense* and *Trifolium glomeratum*. It is hoped to complete the reorganisation and testing of the collection during the coming year and to prepare a catalogue. (Shoshana Bascomb)

Genetic studies on *Rhizobium trifolii*. Transformation studies continued with the effective nitrogen-fixing strain A, the stable ineffective mutant f12 and two unrelated ineffective strains HKC and Coryn.

With strain HKC as donor, ineffectiveness was transferred to strain A, using the standard method for DNA extraction and identifying the isolates immunologically and by phage susceptibility tests (*Rothamsted Report* for 1962, p. 77). Three attempts to do the reciprocal transformation with strain A as donor and strains f12, HKC and Coryn as recipients failed, because all 500 isolates tested failed to produce nodules on red clover. Serological tests against strain A antisera confirmed that sample isolates were nodule bacteria, related to strain A, and not contaminants that had overgrown the recipient strain. This result was unexpected, because with HKC as donor to the effective strain A all transformants were virulent. Furthermore, although strain A has given stable avirulent mutants, their virulence was restored by transformation procedures. (Kleczkowska)

The infection of clover root hairs by Rhizobium. Uninfected root hairs of *T. glomeratum* elongate at a uniform rate of 8–15 μ per hour for 1–2 days after their first appearance as small epidermal papillae. Throughout the period of extension most of the cytoplasm of the cell is retained near the growing apex; elsewhere in the hair it forms a very thin lining to the cell wall with a few strands traversing the central vacuole. Protoplasmic streaming is conspicuous and regular. The spherical nucleus, which has one clearly defined nucleolus, moves into the hair soon after the initial papilla develops and remains within the apical mass of cytoplasm throughout the hair's growth. One or two days after the root hair reaches its adult length (300–400 μ) the apical mass of cytoplasm disperses and the nucleus moves down to a position in the basal half of the root hair, becomes smaller

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and elongated, and its nucleolus cannot be seen. Lateral protrusions may develop anywhere along the hair surface, although they usually develop on the basal half of the root hair. Lateral branching is preceded by accumulation of cytoplasm and enlargement of nucleus.

Infection threads originate from the growing region of the root hair, either apical or lateral. The earliest stages of infection cannot be observed with the light microscope, but about 3 hours before the infection thread is visible the apical mass of cytoplasm becomes more opaque and the cytoplasm stops streaming in this region. The infection thread grows away from the site of infection, with its tip closely associated with the root-hair nucleus and surrounding cytoplasm. The infection thread grows from 5 to 8 μ per hour. When the hair nucleus and infection thread tip are separated for 1 day, and perhaps even less, the infection aborts.

Infection-thread formation is a growth-dependent process and may be influenced by a supplementary supply of growth hormones. The effect of indolyl-3-acetic acid, tryptophan, caffeic acid, kinetin, gibberellic acid, tri-4-chlorophenoxyacetic acid, 2,6-dichlorophenoxyacetic acid, 2,4,6-trichlorophenoxyactic acid, ethylenediaminetetraacetic acid, colchicine and polymixin β sulphate, all of which affect cell growth or nuclear behaviour, were tested on *T. glomeratum* seedlings.

At concentrations above their usual physiologically active range most of these substances severely limited infection and hair growth. At lower concentrations no compound promoted earlier infection or increased the rate of infection, but two compounds, kinetin (at 10^{-7} *M*) and EDTA (at 10^{-4} *M*), delayed nodule formation and prolonged the initial rapid phase of infection. This pattern of stimulation is similar to that previously reported with trace amounts of nitrate and nitrite salts (at 10 μg N/seedling) (*Rothamsted Report* for 1962, p. 78). A pectinase preparation (Pectasin 100D at 0·4 μg/plant) similarly stimulated infection by delaying nodulation. No explanation can be offered for these effects.

The effect of carbohydrate supply on the normal and nitrate-stimulated pattern of infection was examined. The nitrate effect was unresponsive to large changes in the host's carbohydrate status produced by added glucose, or by different light intensities (600–1300 ft-candles) or day length (4–24 hours), but in darkness the few infections were further reduced by nitrate. (Darbyshire)

The fine structure of the clover-root nodule. The fine structure of effective nitrogen-fixing nodules of *Trifolium parviflorum* (inoculated with *Rhizobium trifolii*, SU297) was studied, particular attention being given to the structure of the infection thread, the emergence of bacteria from the thread, changes in host and bacterial structure during nodule development and the exact position of the infection within the host cell. Nodules were fixed in potassium permanganate or osmic acid and embedded in araldite.

The mature infection thread has a wall continuous with that of the host cell, surrounded by a plant membrane continuous with the plasmalemma. Near the host nucleus and at the thread tip the enclosing membrane is fragmentary, and the region that will develop into thread wall is indistinguishable from the host cytoplasm. Bacteria emerging from thread tips,

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from mature threads or from vesicles, immediately become surrounded by a plant membrane formed as an extension of the existing thread membrane. The bacteria in the thread are suspended in a dense matrix distinct from the middle lamella of the cell wall; they are also surrounded individually by a transparent region (increasing with prolonged retention in the thread), which may be a polysaccharide secretion. The structural changes leading to bacteroid formation do not begin until the bacteria emerge from both the matrix and the electron-transparent zone. The fate of emerged bacteria—division or rapid swelling—is controlled by the "state" of the host cytoplasm. In the active cytoplasm of meristematic cells the bacteria divide, but in the vacuolated dispersed cytoplasm of expanding cells they swell rapidly. During the transition to the bacteroid changes occur in the nuclear region and in the spatial arrangement of the bacterial and enclosing plant membranes. The final stage in bacteroid formation is preceded by the enclosing membrane swelling greatly. The typical bacteroid-containing cell has a central vacuole, its cytoplasm is largely replaced by bacteroids and its cytoplasmic organelles are lost or compressed near the cell periphery. Bacteroids contain several discrete nuclear regions, some internal vesicles with double membranes and a ground cytoplasm like that of the host cell. Bacteroids from nitrogen-fixing nodules of *Vicia faba*, *Vicia hirsuta* and *Trifolium repens* are also enclosed individually by well-defined plant membranes, but not those from soya-bean nodules.

In clover nodules the unit membrane around the recently emerged bacteria connects with portions of the endoplasmic reticulum. The bacterial infection therefore seems to lie within the paired membranes of the endoplasmic reticulum, i.e., the intra-reticular space. This space provides a continuous passage from the nuclear membrane through the cytoplasm and cell wall to adjacent cells.

Confirmatory evidence for some of the above interpretations was provided by examining two types of ineffective nodules. In *T. parviflorum*, inoculated with *R. trifolii* Cl.F., symbiotic development of the bacteria is restricted because the host cytoplasm becomes vacuolate prematurely. In *T. repens* inoculated with *Rh. trifolii* SU297, the initially over-developed membrane system of the host cell collapses as the emerged bacteria lyse. (Mosse in collaboration with Nixon, Woods and Welch, Plant Pathology Department)

The fine structure of vesicular–arbuscular mycorrhiza. Roots of clover seedlings inoculated with *Endogone* spores were examined. In contrast to the condition in clover nodules, no evidence was found that the intracellular infection branches (arbuscules) cause plasmalemma to invaginate. The arbuscule wall breaks down and collapsed fungal organelles, fat globules and cytoplasm are released into the host cell, which nevertheless retains some apparently normal organelles. (Mosse, in collaboration with Nixon, Woods and Welch, Plant Pathology Department)

Metabolism of root nodules. Study of reducing processes that may be important in the nitrogen-fixing system of root nodules has led to experiments on the metabolism of elemental hydrogen by nodule extracts. A gas,

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probably hydrogen, was produced when pea nodules were crushed in a mortar, briefly filtered through coarse sintered glass to remove plant debris but not bacteroids and immediately incubated under argon in Warburg flasks. Gas was produced rapidly at first, but ceased within the first 20 minutes of incubation; to confirm that the gas is hydrogen will be difficult unless more can be obtained. Adding reduced nicotine adenine dinucleotide increased hydrogen production above the endogenous, but only by 10% of that theoretically possible. All of this increment appeared in a rapid burst completed within 5 minutes of adding the substrate. Several other hydrogen donors had no effect.

Another approach is to compare nodule metabolism with that of free-living nitrogen-fixing anaerobes. A nitrogen-fixing strain of *Clostridium butyricum* was isolated from Rothamsted soil by dilution, followed by isolation of surface-grown colonies on a nitrogen-deficient medium incubated in anaerobic jars filled with nitrogen. To remove traces of oxygen and supply carbon dioxide, an actively growing yeast culture was placed in each jar. The *Clostridium* was grown in bulk in the continuous-culture apparatus of Skinner & Walker (*Arch. Mikrobiol.* (1961), **38**, 339), modified so that nitrogen instead of air was continuously passed through the culture. Growth initiation was much delayed unless the inoculation was large and actively growing, probably because it then rapidly removed traces of oxygen. Acetone powders and partially autolysed suspensions of cells grown in this way were active in the phosphoroclastic oxidation of pyruvate to acetyl phosphate, a reaction known to be closely integrated with nitrogen fixation in clostridia. However, no such reaction was detected in various extracts from pea nodules. (Cooper)

Nitrogen is fixed in the nodule probably under low oxygen tensions, but not by free-living *Rhizobium* in air. However, the possibility that the free-living bacteria may fix nitrogen under this condition has been little studied. Glutamate-grown *Rhizobium meliloti* was incubated overnight in a nitrogen-deficient medium and then resuspended with either sucrose or succinate, with or without small additions of yeast extract or glutamate, under atmospheres containing $^{15}N_2$ and either 0·5% or 5% O_2. No enrichment by ^{15}N was detected in any vessel after 16 hours incubation. (Cooper and Dr. C. A. Parker)

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