ROTHAMSTED RESEARCH

Report for 1961

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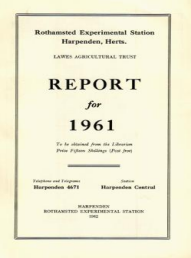

Soil Microbiology Department

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P. S. NUTMAN

R. M. Jackson left in June for two years' secondment to the Soil Bureau, Wellington, New Zealand, to work on soil fungi. G. Lim was awarded the Ph.D. degree of the University of London and returned to Singapore in April. B. Mosse spent eight weeks at the University of Pisa as an O.E.E.C. Research Fellow. Under the auspices of the Royal Society, Prof. E. N. Mishoustin, Corresponding Member of the U.S.S.R. Academy of Sciences, made Rothamsted a centre from which he visited microbiological laboratories in the United Kingdom over a period of two months. Through the International Association for the Exchange of Students for Technical Experience, Dr. H. Klaus of the Institute of Microbiology, Göttingen, spent six weeks working with N. Walker on the microbiological effects of drying soil. At the invitation of the United States Department of Agriculture, Clover and Range Division, Beltsville, and the Legume Inoculant Manufacturers' Association, P. S. Nutman visited and lectured at State Colleges in the U.S.A.

Work was started in collaboration with the Departments of Plant Pathology and Biochemistry on the moulding of hay. Because of requests for information from the Advisory Service and elsewhere, experiments were initiated on the effect of vacuum treatment on inoculation of legume seed.

Distribution of nitrogen fixers and nitrifiers in soil

The distribution of nitrogen fixers and nitrifiers in soil was further studied. A series of samples taken from Park Grass in the spring showed nitrifiers to be present except in the very acid plots. The unlimed halves of Plot 3 (no manure, pH 5·5) and Plot 9 (ammonium sulphate, pH 4) contained none, but the limed halves of the same plots contained many nitrifiers, as did the whole of Plot 14 (sodium nitrate, pH of unlimed half 6, of limed half 7).

Four plots on Broadbalk (Nos. 3, 5, 7 and 10) all contained very many nitrifying bacteria during summer; more than 6,000 ammonia-oxidisers per gram were counted from Section III, carrying the first crop after fallow. Section I, fallow, had fewer. *Azotobacter* was also more numerous on Section III than on Section I.

The grass end of Broadbalk Wilderness contains many nitrifiers, and many *Azotobacter*; the wood end also has many nitrifiers, but few *Azotobacter*. Other woodland soils had few or none of either. Two samples from Morven (Argyll), under oak and pine, had no *Azotobacter*, very few Clostridia and no nitrifiers; but an arable sample from the same place had about 1,000 Clostridia per gram, and many nitrifiers. Pine wood samples from Delamere Forest (Cheshire) had no *Azotobacter*, no nitrifiers and very few Clostridia. A series of beech-wood soils from the Chilterns were similar, except

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for a calcareous mull, which had moderate numbers of nitrifiers and nitrogen fixers.

Further counts on the long-term Bare Fallow on Highfield showed no *Azotobacter* in this soil and that Clostridia and cellulose-decomposers have become very few, whereas the numbers of nitrifiers are well maintained. (Meiklejohn.)

Methods of counting Azotobacter

The effect of the following factors on the spread-plate method of counting *Azotobacter* was examined: (1) the method of preparing the soil suspension; (2) the time agar plates were dried before adding the suspension to the surface; (3) the concentration of phosphate in the agar medium. Neither grinding the soil in a mortar before suspending in distilled water nor mixing the suspension in a M.S.E. top-macerator increased the count compared with simple shaking, but shaking the soil suspension with glass beads for only 3 minutes increased it by 80–100%; longer shaking with beads had no further effect. Adding dispersing agents ("Nonidet", "Tween 80") to the soil suspension, varying the length of time the soil was held in suspension before plating or the type of suspending fluid (tap water, distilled water or physiological saline) and the time of shaking (up to 30 minutes) had no significant effect on the count of *Azotobacter*.

Concentrations of phosphate in the medium greater than 2 g./l depressed the count when the phosphate was added separately to the autoclaved medium. Surface drying altered the concentration of salts in the medium, and drying appeared to affect *Azotobacter* colonies growing on mannitol more than those grown on glucose medium. Tests of media containing different energy sources confirmed the superiority of glucose and sucrose over mannitol. Satisfactory results were obtained with the dilution-tube method when $CaCO_3$ was added to the tubes as a buffer. (Brown, Burlingham and Jackson.)

Natural distribution of Azotobacter *in plant rhizospheres*

The distribution of *Azotobacter* was studied in the rhizospheres of 17 crops grown in 13 different soils in the field and glasshouse. *Azotobacter* were usually few in both rhizosphere and soil. In the field *Azotobacter* was present in only 1 of 60 rhizosphere samples from soil below pH 6·5, but in 168 of 177 samples from soils above pH 6·5; of these only 86 had more *Azotobacter* in the rhizosphere than the corresponding soil sample. In the glasshouse experiments (soil pH above 6·5) *Azotobacter* was in the rhizospheres of 201 samples, and more numerous in 149 of these than in the soil samples. The presence or absence of a rhizosphere effect seems to depend on plant species, age of plant at sampling and soil type. *Azotobacter* was never recovered from serial root washings, indicating that it was present in the rhizosphere only and not on the root surface. (Brown and Burlingham.)

Factors affecting establishment of inoculated Azotobacter *in the rhizosphere*

Results of inoculating seed, roots or soil in field and greenhouse experiments showed that large populations of *Azotobacter* could be

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established and maintained in the rhizosphere. Some of the factors affecting establishment were examined. Wheat seed, inoculated with the same volume of 2-, 7- or 14-day-old cultures containing $1{\cdot}5 \times 10^9$ viable cells per ml., were sown immediately after inoculation or one day later. The *Azotobacter* on the seed were counted immediately after spraying, after storing the seed for one day and in the rhizosphere after 3 weeks. Recovery of *Azotobacter* from seed and its establishment in the rhizosphere depended on age of culture, and was greatest with the 14-day-old culture. A delay of 1 day between inoculation and sowing had little effect on establishment, although the seed recovery for the 2-day-old culture decreased more than 1,000 times. *Azotobacter* establishment was not significantly affected by soil moisture over the range normally found in the field (10–20% water holding capacity). (Brown and Burlingham.)

Effect of Azotobacter *inoculation on crop yields*

Five field experiments, two with wheat and one each with barley, potatoes and beet, showed no significant increases in yield from inoculating seed with *Azotobacter*. Earlier field trials (*Rep. Rothamst. exp. Sta.* for 1960, p. 91) reported sporadic and significant effects on yield which were always associated with good establishment of *Azotobacter* in the crop rhizosphere. In the 1961 field trials *Azotobacter* failed to become established in three experiments because of low or marginal pH, and in the remaining two experiments (on wheat and sugar beet) establishment was poor. The beet experiment gave a 6·6% increase in yield of roots, but this was not significant.

Eight pot experiments in the greenhouse with six different crops all gave a small total increase in yield resulting from *Azotobacter* inoculation. None of these total increases was individually significant at the 5% level. However, as shown in Table 1, four experiments produced significant effects of inoculation in some treatments. (Brown, Burlingham and Jackson.)

TABLE 1

Crop	Method of inoculation	Increase in total yield from inoculation (not significant)	Treatment	Partial yields	Significant increase
Wheat	Seed	+ 5·6%	+ NP	Ear wt.	+21%
Tomato	Root	+11·0%	J. I. compost	Green tomatoes	+41%
Spinach	Root	+18·1%	−N, 60% W.H.C.	Top wt.	+13%
Carrot	Seed + soil	+ 1·5%	−N, seed inoc.	Root wt.	+45%

Studies on Nitrosomonas

In a search for faster-growing strains or species of *Nitrosomonas*, three further strains were isolated in pure culture from different soils or activated sludge. Altogether five such strains have now been examined and shown to differ in morphological and physiological characteristics. These organisms, however, all have roughly the same growth rate in pure culture, namely a doubling time of 10–12 hours; washed organisms of the different strains all oxidise both ammonia and hydroxylamine to nitrous acid.

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Attempts to obtain pure components from the cytochrome fraction, which can be readily isolated from *Nitrosomonas* cells, have not yet succeeded, neither has a cell-free enzyme fraction able to oxidise ammonia yet been obtained. (Walker.)

Decomposition of simazine, stilboestrol and toluene in soil

To study the persistence in soil of simazine, a synthetic herbicide, and stilboestrol, a synthetic stilbene derivative with oestrogenic activity and used in agriculture, enrichment cultures were set up. Although there are some indications that these compounds can be biologically degraded, attempts to isolate pure cultures of organisms able to break them down have so far failed.

A knowledge of how toluene is degraded by bacterial action is important in relation to the fate of a methyl group when linked to an aromatic molecule. A soil bacterium has been isolated which grows readily, under certain conditions, in a simple salts solution containing either toluene, benzene or benzyl alcohol as sole carbon source. Toluene cultures produce a bright yellow, fluorescent, acidic pigment, which has been isolated in small quantity. The oxidation of various aromatic compounds by washed toluene-grown organisms was studied in Warburg respirometers. The pathway of the bacterial decomposition of toluene remains to be established. (Walker.)

Microbial population changes in self-heated hay

Changes in bacterial and fungal populations in self-heated hay were studied in connection with the Plant Pathology Department's programme on mouldy hay and Farmer's Lung disease. Wet and dry bales, 30% and 15% moisture content respectively, were made from hay of Great Field and sampled frequently during the summer. A stack of each type of hay was also built and studied in the same way.

At each sampling time a large sample was removed from bale or stack and a sub-sample taken for analysis of the wind-blown micro flora (Plant Pathology Department). Hay from a second sub-sample was cut into 2–3-cm. lengths, thoroughly mixed, and a 10-g. portion shaken mechanically with sterile water for 10 minutes to prepare a suspension for bacteriological analysis. (This same suspension was used by the Biochemistry and Plant Pathology Departments for pH determinations and some wind-tunnel experiments.) Serial dilutions were plated with nutrient agar and replicate plates incubated at 25°, 40° and 60°. Anaerobes and micro organisms able to grow in an acid lactobacilli medium were also counted. Some of the cut hay was used for chemical analysis and moisture determinations by the Biochemistry Department.

Dry bales reached a maximum temperature of only 27° 2 days after baling, and thereafter cooled to just below 20° for most of the 90-day sampling period. The numbers of bacteria growing at 25° and 40° tended to decline after reaching a peak coincident with the temperature peak. Fungi at 25° were fairly numerous, but few grew at 40°. No growth was found at 60° in the dilutions tested, and the populations of fungi and bacteria at lower incubation

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temperatures were very mixed in species composition. This hay was sweet-smelling and remained in good condition throughout the experimental period.

In the wet bales a maximum temperature of 55° was recorded after 3–4 days, and thereafter the temperature slowly declined. Bacteria at 25° increased rapidly to a peak of $1{\cdot}9 \times 10^8$/g. dry wt. when the temperature was highest and then fell to $5{\cdot}5 \times 10^5$/g. after 15 days. Bacteria at 40° showed the same trend, although they were always fewer than at 25°. At the start thermophilic bacteria growing at 60° were few, but rose steadily from $6{\cdot}4 \times 10^3$/g. at 6 days to $1{\cdot}7 \times 10^7$/g. at 21 days. Fungi at 25° and 40° were numerous throughout. The hay was dusty with fungus spores and had an unpleasant irritating odour.

In the dry stack the character of the hay and the pattern of change in the microbial population resembled that of the dry bales. However, hay from the centre of the wet stack differed greatly from that in the wet bales. High temperature was maintained in this stack for a long period, and a maximum of 65° recorded. The hay was dark brown, and had a tobacco-like odour and an acid reaction. Bacterial populations at all three incubation temperatures remained at a high level until the 16th day, after which they fell sharply. Fungi at all incubation temperatures remained numerous throughout the period.

A second set of bales was made from hay cut from Great Knott I. The wet bales contained 35% moisture and reached a temperature of 60° three days after baling. Many bacteria and fungi were recorded at all incubation temperatures, and the hay developed an odour even more irritating than that of the wet bales from Great Field. In the dry bales from Great Knott (20% moisture content) microbial activity was greater than in the corresponding bales of hay from Great Field. Fungi growing at 25° and 40° were numerous, but in spite of this the hay was not obviously " mouldy " like the wet bales of both series. Actinomycetes were rare on the plates made from any of the hays tested, although plentiful when agar plates were inoculated on the surface with wind-blown dust from some hay samples by the Plant Pathology Department.

Anaerobes and organisms growing in the lactobacilli medium occurred in most samples, but were almost always few and showed no definite trends. Micro-organisms which appeared to predominate in some of the samples were isolated and cultivated for use in future laboratory experiments. (Skinner.)

Methane fermentations

Work on the isolation and cultivation of the methane-producing bacteria and on the behaviour of these bacteria in mixed culture with anaerobic cellulose-decomposing bacteria is continuing, and will be reported later. At the request of the Sports Turf Research Institute, the emanation of inflammable gas in quantity from cultivated land at Eckington in Derbyshire, where soil containing much pig manure had been dumped on to a badly drained soil, was investigated. Tests showed that methane was generated in the upper layers of the soil by fermentation of the abnormal amounts of

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readily decomposable organic matter. The surface soil is being removed to restore the area to its former condition. (Skinner.)

Transformation studies on Rhizobium trifolii

Work on the transformation of symbiotic virulence (Ljunggren, *Rep. Rothamst. exp. Sta.* for 1960, p. 85) was extended to a study of the transformation of symbiotic effectiveness in nitrogen fixation. A bacteria-free preparation of deoxyribose nucleic acid (DNA) from a strain of clover nodule bacteria (Strain H.K.C.) which is quite ineffective in fixing nitrogen in red clover nodules was used successfully to transfer this property to a normal effective strain (Strain A). All such transformations in response in Strain A were to complete ineffectiveness; no intermediate forms were recovered. The efficiency of the transformation (in terms of proportion of altered cells) depends on the concentration of DNA and the length of time the recipient strain is grown in the donor DNA; the optima for these factors are being determined. The transforming activity of DNA preparations declines on storage at −20°. (Kleczkowska.)

Inheritance of resistance and ineffectiveness in red clover

A single family of red clover (late-flowering Montgomeryshire) selected for sparse nodulation, segregated two kinds of abnormal plants: (1) non-nodulating plants, and (2) plants that gave an ineffective response in nitrogen fixation with a normal effective strain of bacteria (Strain A); these abnormalities were investigated genetically.

The non-nodulating seedlings were weakly, and only two survived to the flowering stage. These were crossed together (133 florets cross-pollinated) but produced no seed. Sib crosses between nodulating and non-nodulating plants gave small families with a high proportion of abortive seeds (seed set, 19%; germination, 36%). Of the seeds which germinated 21 nodulated and 2 did not. Crosses among nodulating plants were more successful (seed set, 42%; germination, 70%). Two resistant plants appeared in the 143 seedlings examined; they were normal as seedlings, but neither survived to the flowering stage for further breeding work. This form of host resistance to nodule formation, which is clearly heritable, because resistance is otherwise very rare in red clover, differs from a previously described host resistance (*Heredity*, **3**, 263, 1949) in that it is not simply inherited, and is not associated with a maternally transmitted component, but it is similar in its association with early lethal effects.

The ineffectively responding plants segregating in the same original family were recessive for a simply inherited factor, provisionally designated *n*. *n* homozygotes in segregating families formed the same number of nodules as their effective counterparts. Breeding experiments showed that the factor *n* was distinct from the ineffective host factors i_1 and *ie* previously described.

The ineffective response in *n* homozygotes was specific to bacterial Strain A. (Nutman.)

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Inoculation of legume seed by vacuum treatment

Loneragan, Moye and Anderson (*Nature, Lond.* **192**, 526, 1961) report that alternate application of a vacuum and atmospheric pressure to legume seed at the time of inoculation with *Rhizobium* improves the survival of the inocula during later storage. To study this, lucerne and red clover seeds were inoculated with ^{32}P-labelled *Rhizobium* spp. with or without vacuum applications. The residual radioactivity, and hence the number of bacteria retained by the seeds after washing, was measured by Geiger-counting the sulphuric acid digests of the seeds. Lucerne seeds inoculated without vacuum treatment and washed three times carried 5×10^3 bacteria per seed for every 10^8 bacteria per ml. of inoculum; vacuum treatment increased uptake of bacteria by 90–120%. Further washing (up to twenty washes) removed 10–20% of the bacteria per wash. The percentage difference between bacterial numbers with and without vacuum treatment was much decreased by washing, and disappeared after twenty washes. Red clover seeds retained only one-fifth as many bacteria as did lucerne seeds.

The uptake by seeds of ^{32}P compounds in solution was studied with supernatant fluid from the centrifuged cultures used to produce ^{32}P-labelled *Rhizobium*. Here also, the vacuum treatment doubled the uptake of ^{32}P and washing removed it by 10–20% per wash.

The agricultural benefit which may result from such additional retention of *Rhizobium* by legume seed after vacuum treatment depends on the way in which the bacteria are distributed between seeds. This was investigated by treating lucerne seeds with ^{32}P-labelled *Rhizobium*, with and without vacuum treatment, washing three times, drying and distributing the seeds over the surface of X-ray film. The developed film was examined for dark spots coincident with the original positions of the seeds. 100 seeds produced only 6–11 dark spots, with or without the vacuum treatment. The effect of vacuum treatment was merely to intensify 3–5 of the spots about four-fold (estimated visually). A similar picture was given by seeds treated with the labelled culture fluid. A duplicate sample of the seeds used had a germination percentage of 95.

These results suggest that vacuum treatment does not increase bacterial impregnation of normal good seed of lucerne or clover and cannot be recommended in practice. Samples of commercially produced vacuum-inoculated seed of both lucerne and red clover were examined; nodule bacteria per seed were few and could not be detected at all in some samples. (Cooper.)

The microbial transfer of bound nitrogen from agar and other sources to plants

Growth and nitrogen content of *Trifolium parviflorum* on nitrogen-deficient agar slopes were increased by inoculation with a *Pseudomonas* sp. (*Rep. Rothamst. exp. Sta.* for 1960, p. 88). Measurement of total nitrogen in plants and agars showed that nitrogen from impurities (probably protein) in the agar was transferred from agar to plant by bacterial action; less nitrogen was transferred from purer types of agar. Two other test plants, *Cucumis sativus* and *Dactylis glomerata*, responded like the *Trifolium* sp. Six soil fungi

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(species of *Cladosporium, Pullularia, Pythium, Verticillium, Gliocladium*) and a culture of *Rhizophagus* as well as another *Pseudomonas* sp. also released bound nitrogen from agar. No nitrogen was transferred when the inoculum was either an *Endogone* species producing mycorrhizal infection of the clover or *Rhizobium phaseoli* which does not form nodules with clover.

To study the mechanism of this nitrogen transfer, the clover seedlings were grown in water culture with egg albumen instead of agar as nitrogen source. Plants took up the following proportions of the added albumen N: clover alone, none; clover + *Pseudomonas*, 65%; clover + *Pseudomonas* + glucose, 15%. When the *Pseudomonas* was grown alone on this medium, 75% of the added albumen N was recovered as free ammonia in the culture fluid, but only 25% when glucose also was added. The protein without glucose may not have supplied enough carbon and energy for the assimilation of more than a small proportion of its nitrogen by the micro-organisms. (Cooper, Jackson and Mosse.)

The establishment of mycorrhizal infection under aseptic conditions

Earlier work showed that *Endogone* alone did not establish a mycorrhizal infection under aseptic conditions, but did so in the presence of a species of *Pseudomonas* from soil. Additions of either Seitz-filtered suspensions of *Pseudomonas* sp. in water, of " Pectinol " or EDTA also enable the fungus to penetrate the root. None of the sterile filtrates was as effective as the live inoculum; each induced fewer infections, acted more slowly and was sometimes without effect. Autoclaving further lowered but did not destroy the effectiveness of the " Pectinol " and bacterial filtrates. Omitting calcium from the medium or replacing it by potassium greatly decreased infection, but calcium could be replaced by sodium without affecting infection. The factors controlling fungal entry into the root remain to be explained; the results suggest that changes in the pectin compounds of the cell wall are involved in the infection process. (Mosse.)

Effects of mycorrhizal infection on the growth of Trifolium parviflorum

Mycorrhizal seedlings grown aseptically in a nitrogen-deficient mineral salt medium were the same size (total dry wt.) as control plants. Individual mycorrhizal roots on infected seedlings were often strikingly longer and more branched than uninfected roots on the same seedlings. No net gain in nitrogen was demonstrated either in the infected seedlings or in the agar.

Mycorrhizal seedlings were transplanted from aseptic culture into autoclaved soil mixtures and grown in the greenhouse. Two months after transplanting in compost containing peat, mycorrhizal seedlings weighed more than non-mycorrhizal controls, but without peat growth was unaffected. (Mosse.)

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