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Report for 1962

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ROTHAMSTED
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REPORT FOR 1962

Soil Microbiology

Rothamsted Research

Rothamsted Research (1963) *Soil Microbiology ;* Report For 1962, pp 77 - 84 **- DOI: https://doi.org/10.23637/ERADOC-1-95**

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SOIL MICROBIOLOGY DEPARTMENT

P. S. NUTMAN

J. Kleczkowska spent six months at the University of California, Berkeley, as visiting Professor of Microbiology in the Department of Soils and Plant Nutrition. J. Meiklejohn left in October on secondment to the University College of Rhodesia and Nyasaland to lead a research group in Microbiology. Mr. V. Radulovic of Sarajevo University worked four months in the department assisting with legume-inoculation experiments, and Dr. G. Fåhraeus of the Department of Microbiology, Agricultural College, Uppsala, studied the growth response of clover-root hairs to growth substances, bacterial secretions and pectic enzymes. H. Hippe of the Institute of Microbiology, Gottingen, assisted with the work on toluene decomposition. Other temporary workers included Prof. S. Soriano of the University of Buenos Aires and La Plata, Dr. C. A. Parker, University of Western Australia, Dr. Ugo Laudani, of University of Pavia, and Mr. H. Kanyike, of Namulonge Experimental Station, Uganda.

Margaret Brown, R. Cooper, N. Walker, Jane Meiklejohn and P. S. Nutman read papers at the 8th International Congress for Microbiology at Montreal in August; P. S. Nutman also attended the Commonwealth Culture Collection Conference at Ottawa, and lectured at Guelph.

Genetic studies with *Rhizobium trifolii*. The transfer of symbiotic ineffectiveness using preparations of deoxynucleic acid was confirmed, and it was further shown that the number of transformed colonies recovered from an effective parent strain after treatment was influenced by the quantity of donor nucleic acid used and the incubation time before plating. Best results were obtained when the recipient strain was grown for 14 days at 28° in the presence of 10% nucleic acid. Ineffective transformants were identical serologically to the recipient strain, but more resistant to phage. This change in phage susceptibility is of particular interest because resistant variants after phage treatment contain a high proportion of ineffective mutants. Experiments are now in progress on the reciprocal transformation. (Kleczkowska)

The deformation of clover root hairs by nodule bacteria and by their culture-filtrates. Infection of the root hairs of leguminous plants by nodule bacteria is accompanied by a characteristic deformation or curling of the hairs, which can also be brought about by bacterial filtrates. Work done 20 years ago (*Rep. Rothamst. exp. Sta.* for 1939–45) seemed to show that the active substance in the filtrates was indoleacetic acid (IAA), but Sahlman and Fåhraeus (*LantbrHögsk. Ann.* (1963), **28**, 261) recently reported that various concentrations of IAA had no distinct effect on the root hairs of clover plants.

To elucidate this question further, seedlings of *Trifolium parviflorum*, grown on ordinary agar slopes or on slides in water culture, were inoculated

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with living clover or lucerne bacteria, or treated with bacteria-free filtrates from the same strains or with some pure auxins. To produce filtrates, the bacteria were grown on standard liquid or solid media containing organic substrates, or in purely inorganic salt solutions together with clover seedlings. Bacterial suspensions were filtered through ceramic filter candles or Pyrex glass filter funnels.

Root-hair development was observed daily in the microscope. The first and most common effect of filtrates was frequent branching of the root hairs, which is rare in untreated plants. Hairs on young roots were curled only by living bacteria, but on older ones some filtrates caused curling, though usually only in restricted areas. Filtrates from some strains were more active than others. Filtrates from bacteria grown on the plant rhizosphere were more active than those from bacteria grown in culture on sucrose or mannitol media, although they contained fewer bacteria, suggesting that some necessary factor comes from the root. The active substance from the rhizosphere was heat-stable and non-dialysable. L-tryptophan or IAA did not augment the effect of filtrates. α-naphthalene-acetic acid (at 10^{-8} *M*) increased the branching of hairs, but indoleacetic acid and 2,4-dichlorophenoxyacetic acid did not. (Fåhraeus)

Clover root-hair infection. Earlier work (*Rep. Rothamst. exp. Sta.* for 1958, p. 72) showed that very small concentrations of nitrate (10–200 μg N per seedling) delay the onset of nodule formation in white clover and lucerne. The progress of root-hair infection was followed on seedlings of *T. glomeratum* and *T. repens* given 0, 10, 100 and 1,000 μg N as KNO_3, $NaNO_2$, $(NH_4)_2SO_4$ or urea. As in the earlier experiments, nitrate and nitrite nitrogen, but not ammonium nitrogen or urea, delayed the beginning of nodulation by one or two days.

On control plants and those with 10-μg additions, infection of root hairs were first observed at the same time and their numbers increased exponentially at the same rate. The change in rate, which is correlated with the time of appearance of the first nodule, was delayed by nitrate and nitrite, and infection continued at the high initial rate for longer with 10 μg NO_3 or NO_2 nitrogen. More nitrate and nitrite nitrogen (100 and 1000 μg N per plant) delayed the start of infection and depressed its rate. Ammonium N did not affect nodulation at any level below 1,000 μg/plant, but urea at 1,000 μg/plant caused severe stunting and stopped infection.

The effect of nitrate was further investigated with *T. glomeratum* by adding the nitrate to the plants at different times after starting an experiment. Additions at any time up to day 7 delayed the onset of nodulation and increased the number of infections produced later. The addition of nitrate after day 7 had no effect on root-hair infection or nodulation. Nodule formation occurred on the control plants on day 9. There was no gradual transition from many to few infections as time of adding nitrate was delayed. The results all show that the specific effect of nitrate and nitrite in delaying nodule formation acts on the inception of the nodule primordium or on its early growth and not on hair infection. (Darbyshire)

The way in which infections appear to spread along the root from a few well-separated centres suggests that substances stimulating infection may

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be produced at these centres and then diffuse into the medium and affect nearby root hairs. To examine this possibility, infection was studied on plants grown singly or in pairs, and on plants grown in medium already planted with young seedlings. The presence of another plant did not increase infections; on the contrary, infected hairs were fewer on paired plants or after preplanting the medium.

The effect of nodule formation in slowing infection rate was studied using similar techniques. The slight lessening of infections in paired or preplanted systems was not correlated with the time or rate at which nodules or lateral roots formed. When one plant of a pair nodulated much earlier than the other it did not prevent infection of its neighbour. Any stimulating or inhibiting substances responsible for the pattern of infection on the young root must therefore move within the root's tissues; stimulating substances possibly come from the cotyledons and leaves, as first suggested by Thornton, and inhibiting substances from the growing point of the root. Experiments to investigate these possibilities are in progress. (Nutman)

Tests for polygalacturonase formation in sweet peas inoculated with *Corynebacterium fascians*. When roots of leguminous plants are being infected by nodule bacteria they secrete polygalacturonase (PG) (Fåhraeus & Ljunggren, *Physiol. Plant.* (1959), **12**, 145–154). Whether this is a reaction specific to this infection or general to bacterial infection is unknown, so experiments were made with *Lathyrus odoratus* infected with *Corynebacterium fascians*, the cause of fasciation in various legumes.

The plants were grown in sand, and PG was tested for by the viscometric technique in water extracts of the sand. PG was not detected, so its production is not a general phenomenon in infection by bacteria. (Fåhraeus)

Field experiments on legume inoculation. These experiments were initiated to determine the conditions for successful nodulation of legumes in the short-term green-manuring experiments at Woburn, where there is a history of poor establishment of trefoil (*Medicago lupulina*), whether or not the seed was inoculated at customary rates. Counts made in 1960 showed that the appropriate nodule bacteria occurred sporadically in reasonable numbers in the trefoil plots 5 months after sowing, but not in the ryegrass plots (*Rep. Rothamst. exp. Sta.* for 1960, p. 86). Establishment might have been poor because acidity prevented the bacteria multiplying rapidly in the rhizosphere. Measurements over the site on 40 0–1-in. soil samples gave pH values between 5·2 and 6·6, with a mean value of 5·8, acid enough to prevent rapid colonisation of the rhizosphere by the very few nodule bacteria present originally, or from an added inoculum.

Microplots of trefoil (*Medicago lupulina*) were inoculated with nodule bacteria (*Rh. meliloti*) at Woburn (Lansome field, pH 6·2–6·9) and at Rothamsted (Sawyers I field, pH 4·8–5·7). Large inocula were applied to the seed immediately before sowing. All plots were given basal P and K and half were limed to raise the soil pH to 7·0. The effects of inoculating beans and clover, and the effects of liming were also studied on the acid soil at Rothamsted. At Woburn inoculation greatly increased early growth of the trefoil, but lime had no effect. At first cut (12 weeks) inoculation

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increased yield by 77% and at the second cut (22 weeks) by 8%. The total yield from inoculated plots (without making allowance for large edge effects) was equivalent to 6,500 lb dry matter per acre of 2·8% nitrogen content. Increased yields were associated with better nodulation.

Rh. meliloti in the rhizosphere were counted at intervals. At 4 weeks the inoculated rhizospheres had more than 10 times as many nodule bacteria as uninoculated plots; there was no effect of lime. After a further 4 weeks these differences disappeared, and all plots had about 50,000 bacteria per g dry rhizosphere soil.

An identical experiment with *Medicago lupulina* on the acid site on Sawyers field also showed benefit from inoculation, but only when lime was also added. Inoculation combined with liming encouraged good early establishment and growth; growth on the uninoculated and inoculated–unlimed plots was equally poor. At 12 weeks liming with inoculation increased top yield by 170%. Treatment differences increased during the season; the second cut yielded seven to eight times as much fresh herbage from inoculated and limed plots as from the uninoculated ones, and the other treatments gave intermediate yields. The best yielding plots produced about 4,600 lb dry matter per acre with a nitrogen content of 2·8%. Large yields were associated with abundant nodulation, intermediate yields with irregular nodulation (often with few coralloid nodules of unusually large size) and small yields with very few or no nodules.

Rhizosphere populations of *Rh. meliloti* followed the yield pattern, increasing rapidly in limed and inoculated plots, slowly and irregularly on inoculated and unlimed plots and hardly at all on uninoculated and unlimed plots. At 12 weeks nodule bacteria were not recovered from the control plots, whereas there were about 100 per g dry rhizosphere soil in the limed but uninoculated plots, from 0 to 1,000 in the inoculated and unlimed plots and from 64,000 to 700,000 in the inoculated and limed plots. The need for inoculations is evident at both sites and for lime with inoculation on the acid soil on Sawyers.

The long-term liming experiment on beans, and the magnesium experiment on clover (see Chemistry Department report), both on Sawyers I (unamended pH of no-limed plots, 4·8–5·2), were also examined for nodulation. The plots in the bean experiment were split and half of each inoculated. The *Rh. leguminosarum* in the rhizosphere were counted on three occasions for each of four replicated plots given basal PK and with lime at 0, 2, 4 and 6 tons/acre. Bacteria were few (0–400/g dry soil) in soil before planting and the rhizosphere effect was very large. The rhizosphere numbers increased on all limed and uninoculated plots from an average of 4,000 at 7 weeks to 10,800 at 10 weeks to >2,000,000 at 14 weeks. Except at the last sampling, inoculation increased the rhizosphere count by factors smaller than 10; bacteria were fewest on unlimed plots and most on the 4 tons/acre treatments. Effects were also evident in the final yields, but the average effect of inoculation in increasing yield by 1·1 cwt grain/acre was not significant.

This experiment confirms other work in showing that *Rh. leguminosarum* tolerates acidity better than *Rh. meliloti* and multiplies more in the host

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rhizosphere, particularly in clayey soil. A simple comparison made in the clover trial between inoculated and uninoculated plots, with and without lime, showed similar results. From an initial soil population of about 450/g dry soil the rhizosphere numbers of *Rh. trifolii* reached more than 700,000/g dry soil by early June, regardless of liming or inoculation, and no effects on yield were noted. (Radulovic and Nutman)

Metabolism of root nodules. One of the functions of leghaemoglobin in root nodules may be to carry electrons (reducing power) from bacteroid dehydrogenase systems to the sites of nitrogen fixation, which may be outside the bacteroids (Bergersen, *Bact. Rev.* (1960), **24**, 246). A recording spectrophotometer was used to detect the first step in such a process, the reduction of leghaemiglobin to leghaemoglobin. Soya-bean nodules were crushed gently with buffer, filtered and centrifuged to remove bacteroids. When the supernatant fluid was incubated with succinate and phenazine methosulphate (an artificial electron carrier) its proportion of leghaemoglobin to leghaemiglobin increased, but not when any component of the mixture was omitted.

Thus, the supernatant fluid contained succinic dehydrogenase, which reduces leghaemiglobin via the artificial electron carrier. Further work is needed to determine whether bacteroids or their extracts have similar activity and whether the reduction of leghaemiglobin can be detected without an artificial electron carrier. (Cooper)

The role of nitrogen in the establishment of mycorrhizal infection in agar culture. The effect of soil micro-organisms on the establishment of *Endogone* in aseptically grown clover seedlings was further studied. Fungal entry into the root can be mediated by several other soil bacteria in addition to the *Pseudomonas* sp. originally used, for example by species of *Arthrobacter*, *Agrobacterium*, *Bacillus*, *Alcaligenes* and *Cytophaga*, but not by species of *Rhizobium* (effective or ineffective) or by *Azotobacter*. All the bacterial species that promoted fungal entry, but not those that failed to do so, had some ability to make nitrogenous impurities in the agar available to plants. Small amounts of nitrogen added without the bacteria did not stimulate infection. When more than 30 μg of nitrogen/10 cc medium was added to cultures containing Pseudonomonas fungal infection was delayed until the extra nitrogen was used in plant growth. When extra nitrogen was supplied to the plant via nitrogen-fixing nodules mycorrhizal infection was not inhibited.

The ultra-structure in symbiotic associations. Techniques were studied for preparing material for electron-microscope examination of intracellular structure in leguminous nodules and in mycorrhizal roots. Technically satisfactory results were obtained with tissues fixed in potassium permanganate and embedded in araldite. Sections of white clover nodules showed that the bacteroids in the nitrogen-fixing tissue are surrounded individually by one double and one single membrane, and the single membrane may be of plant origin. (Mosse)

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Field experiments on Azotobacter inoculation

1. ***Cappelle winter wheat on Long Hoos.*** Seed freshly inoculated with Azotobacter was sown by combine-drilling with PK fertiliser. No Azotobacter was recovered from the seedling rhizosphere, and the experiment was later reinoculated by applying culture to the soil along the plant rows. This method established a large rhizosphere population of Azotobacter that persisted until harvest. The crop was graded at intervals and yield taken at harvest, but inoculated and control plots did not differ.

Pot tests, simulating the conditions of combine-drilling with fertiliser, were made by planting Azotobacter-inoculated seed and fertiliser granules separately and together. Ten times as many Azotobacter were recovered at 3 weeks from the rhizospheres of plants grown without fertiliser granules as from those grown in contact with granules, indicating that combine-drilling with fertiliser was the probable cause of failure in establishing the initial inoculum.

2. ***Jufy 1 spring wheat on Fosters Corner.*** Seed inoculation gave satisfactory establishment of the inoculum and its maintenance until harvest. Grain weight was significantly increased by inoculation on plots receiving 0·2 cwt/acre of nitrogen fertiliser, but not at zero and higher rates of fertiliser application.

3. ***Velocity summer cabbage on Garden Plots.*** Seedlings roots were treated at transplanting with suspensions of Azotobacter. Two isolates were used, and both significantly increased (14%) cabbage top weight, one with a large dressing and the other with a small dressing of nitrogen.

Pot experiments on Azotobacter inoculation. Further pot experiments on inoculation with and without fertiliser nitrogen were made with lettuce grown in soil from Broom's Barn Experimental Station, with wheat grown in limed Rothamsted clay-loam, with spinach grown in Rothamsted clay-loam and barley grown in soil from Sleaford, Lincs, where barley grows poorly. In all these trials the plants responded to inoculation, but only barley did so significantly. These and other experiments indicate that the nitrogen-fixing property of Azotobacter is probably not the cause of its beneficial effect on plant growth. (Brown and Burlingham)

Experiments were also made on the effect of Azotobacter inoculation on nitrogen fixation in the rhizosphere. Three-week-old wheat seedlings growing in a Rothamsted soil in large test-tubes were incubated for 5 days under an atmosphere containing $^{15}N_2$. After digestion and mass-spectrometric analysis the rhizosphere soils, but not the whole plants or the non-rhizosphere soils, were slightly enriched with ^{15}N, showing that nitrogen fixation was more active in the rhizosphere than in the non-rhizosphere soil. However, there was no more fixation in rhizosphere containing more than 10^6 Azotobacter per gram of soil (from inoculation) than in uninoculated controls with fewer than 10^3/g of soil. (Brown and Cooper)

The effect of Azotobacter inoculation on take-all infection. That Azotobacter may in some way ameliorate the harmful effects of root pathogenic fungi was investigated in two pot experiments with wheat sown in take-all infected soil from Great Field I, and in one experiment with wheat grown

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in potting compost inoculated with *Ophiobolus graminis*. In both kinds of experiment Azotobacter inoculation significantly increased yields by 11 to 35%, but examination of the root systems of the test plants showed that this inoculation did not affect the amount of root affected by the fungus. The experiment was repeated with the same soil after half of it was steam-sterilised. In the steam-sterilised soil plants grew well and were unaffected by Azotobacter inoculation, whereas in the untreated soil Azotobacter inoculation increased plant yield by 38%. If the harmful factor in the soil that was counteracted by Azotobacter is not the take-all fungus, it is obviously heat-labile and probably microbiological.

The fungal and bacterial floras of the rhizosphere of wheat growing in soil with and without the take-all fungus and with and without Azotobacter inoculation were examined and differences noted. Some fungi and bacteria prevalent in inoculated rhizospheres were used in experiments with wheat grown under conditions of aseptic culture. All seedlings inoculated with these isolates had better roots than uninoculated seedlings, particularly when Azotobacter was also present.

Other fungi, notably *Penicillium* spp., are decreased in take-all infested soil by Azotobacter inoculation, and effects of inoculation with *Penicillium nigricans* and Azotobacter are being further studied. Experiments have also started on the effect of Azotobacter inoculation on fungal seed-borne diseases, and some degree of control was achieved with trefoil seed naturally infected with *Ascochyta imperfecta*, providing infection was not too great. (Brown and Burlingham)

Nitrifying organisms. Nitrosomonas organisms labelled with ^{14}C were produced for Jenkinson (see Chemistry Department report) in connection with studies on the turnover of organic matter in soil. These bacteria were grown in continuous culture supplied with ^{14}C-containing sodium carbonate. Difficulty was encountered in securing continued growth of Nitrosomonas at small CO_2 concentrations. (Walker)

Decomposition of toluene by soil bacteria. A toluene-decomposing *Achromobacter* sp., isolated from Rothamsted soil, has been grown on a range of aromatic substrates related to toluene and its oxidising abilities studied by Warburg respirometry. There is evidence that the oxidation of the methyl group of toluene to carboxyl may not be the only pathway of metabolism, but that hydroxylation of the benzene ring may also occur involving the intermediate formation of 3-methyl-catechol. The toluene-decomposing bacterium is not identical with any species described in *Bergey's Manual of Determinative Bacteriology* (7th edition). (Walker)

Mouldy hay and Farmer's Lung disease. Samples of mouldy hay made in 1961 (*Rep. Rothamst. exp. Sta.* for 1961, pp. 76–77) were tested by Dr. J. Pepys and Mr. P. A. Jenkins at the Institute of Diseases of the Chest, London, against sera from patients suffering from Farmer's Lung disease. The results of these tests, together with the microbiological and biochemical data for the corresponding hay samples, were analysed statistically. For one series of hay samples the presence of antigen, thought to be

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characteristic of the sera from patients (Farmer's Lung antigen), was positively correlated with pH, soluble and volatile nitrogen, and with actinomycetes, bacteria and fungi. Hay samples of a second series were less mouldy than those of the first series and gave less definite results in immunological tests.

Mouldy hay was produced in small quantities to study the various effects of heating and of growth of different micro-organisms on the hay, and to attempt to establish the conditions necessary for the formation of the Farmer's Lung antigen. 50-g samples of good chopped hay made from Great Field in 1961 were sterilised in 2-lb Kilner jars by adding 1 ml of propylene oxide to each jar and leaving the lids sealed for 48 hours. A series of experiments using *Absidia reflexa* as test organism was made to determine the conditions most suitable for growth on the hay after sterilisation. It was found essential to allow all the propylene oxide to escape by leaving the jars unsealed for a further 48 hours, and to incubate jars with the lids loose to ensure adequate aeration. Each jar was inoculated with a suspension of selected organisms in sterile water sufficient to bring moisture content up to about 40%. Jars were incubated at 40° or 60°. After growth had apparently ceased the contents of the jars were dried, tested immunologically and the pH noted.

Some jars were inoculated with a mixture of organisms by adding water in which mouldy hay had been shaken. Hay inoculated in this way with one 1961 sample became mouldy, and extracts of it reacted with 11 out of 16 sera tested. The extract from the original hay reacted with 13 out of 16 sera. Similar results were obtained with mixed inocula from farm-made hays associated with clinical cases of Farmer's Lung. Typically, fungi grew first and actinomycetes with white spores later appeared.

Inoculation of hay with thermophilic fungi isolated from mouldy hay, e.g., *Mucor pusillus*, *Absidia ramosa*, *Aspergillus niger*, *A. fumigatus* and *Paecilomyces* sp., increased pH from 6·0 to about 7·0, but did not increase Farmer's Lung antigen. Previous heating of the hay by autoclaving, or by incubation at 60°, had no effect on antigen production.

Actinomycetes cannot be easily grown on hay because the pH (*c.* 6·0) is too low. Hay was therefore inoculated with various thermophilic fungi to raise the pH, and subsequently inoculated with mixtures of known actinomycetes. Incubation was at 40° or 60° according to the temperature of isolation of the actinomycetes. At both temperatures production of antigen was increased. Whether this is the true Farmer's Lung antigen remains to be seen. Recently, actinomycetes have been grown directly on hay previously treated with ammonia to raise the pH. Chopped, moistened hay allowed to heat spontaneously in wide-mouthed vacuum flasks also became mouldy, and those in which white actinomycetes developed extensively gave extracts that reacted strongly with Farmer's Lung sera.

These results suggest that thermophilic actinomycetes are responsible for the formation of the Farmer's Lung antigen in mouldy hay. (Skinner, in collaboration with Gregory and Lacey, Plant Pathology Department, and Festenstein, Biochemistry Department)

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