SOIL MICROBIOLOGY[1]

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The field of Soil Microbiology was last reviewed in this journal by Lochhead in 1952. The present review is thus intended to deal with the period from 1952 to 1956 inclusive, although a few papers published before this period have been included for various reasons. A complete coverage of the literature is not possible in a review of this length, and where a subject has been recently reviewed the authors have only mentioned the subject briefly and have quoted the review as a source of references.

TECHNIQUE

Soil microbiologists have been concerned for a long time with the problem of studying the soil micropopulation in the natural solid soil as little affected by manipulation as possible. A method for demonstrating microorganisms in sections of soil has been developed by Alexander and Jackson. A core sample stained in bulk with cotton blue is vacuum-impregnated with a polyester resin and sectioned by a method similar to that used for rock specimens. This method is very promising, particularly for the observation of fungal hyphae (1).

The method initiated by Rossi and Cholodny, in which the growth of microorganisms is observed on slides buried in soil, is used in its original form as well as in various modifications (60, 182). It has always given results difficult to interpret in terms of microbial habit in the soil mass itself; but useful information has been obtained on antibiosis by modified Rossi-Cholodny methods. Dobbs & Hinson have used one to study fungistasis (47); Chinn, and also Stevenson, have placed fungal spores on slides and inserted them in soil. By subsequent examination of the preparations the percentage germination can be estimated, while by pregerminating the spores, deformed growth of the hyphae, or lysis, can be observed (35, 199). Methods of isolating *Acrasieae* from soil, by using a suspension of edible bacteria, either sprinkled on soil, or in agar, have been developed by Kitzke (105) and by Borg (19).

Most methods used for the study of soil microorganisms, however, still depend on making a suspension of the soil. In the most direct application of this method, films are made from the suspensions, and the organisms in them examined microscopically. Tchan has used fluorescence microscopy for observing and counting algae, and Tchan & Bunt have developed a method in which protozoa, fixed in the wet film with osmic acid or formalin vapour, are then stained with erythrosin and methyl green (208, 213).

[1] The survey of the literature pertaining to this review was concluded in December, 1956.

Experiments made to test the agreement between estimates of the numbers of microorganisms in replicate samples of soil, usually based on plate counts, have given very different results in different localities and soil conditions. In contrast with the good agreement obtained in past work on the very long term arable experiments of Rothamsted, Rose & Miller found very great variation in plate counts of fungi from replicate soil cores taken from virgin land and pasture in New Zealand, and have proposed a method for reducing error by bulking numerous cores (176).

In some cases plate counts of bacteria made at a range of dilutions give estimates of numbers per gram that rise with the degree of dilution. Lavergne & Augier attribute this to the retention of bacteria by the internal surfaces of the pipettes used in making and distributing the dilutions, and also to the break-up of clumps of bacteria (112). The same effect would, of course, be observed if the soil contained some factor inhibiting bacterial growth on agar, which was progressively removed by increasing dilution of the soil suspension (138). Miller and his colleagues recommend the use of oxgall as a bacterial inhibitor in agar media for isolating fungi and yeasts, as it can be sterilized in the medium (11, 143), and de Barjac proposes the use of soil humic acid to acidify media used to grow organisms from acid soils (41).

Several methods based on fractionation of soil suspensions have been used for the isolation of fungi (4, 113, 234); Chesters & Thornton have compared six different techniques for isolating soil fungi, and find that two of them, dilution plates and Warcup's soil plates, favour species which sporulate abundantly. The greatest variety of species was obtained by the screened immersion plate method of Thornton (34, 235).

Among techniques for estimating the total microbial activity in a soil, the macrorespirometer of Swaby & Passey may be mentioned (205). Pochon and his colleagues measure various types of microbial activity by comparing the rates at which a reaction takes place in media inoculated with a series of dilutions from a soil suspension (43). Tchan has proposed that nutrients should be assessed by measuring the growth of the indigenous algal flora of a soil with and without added test compounds (211).

EFFECTS OF SOIL CONDITIONS ON THE MICROFLORA

Locality and Type

Bacteria.—It is usually supposed that all species of soil bacteria have a world-wide distribution; Prévot & Moureau have shown that this is probably true of the common anaerobic bacteria, which they have isolated from a variety of soils, including some from the Antarctic (169). Curiously enough, thermophilic bacteria have been found by McBee & McBee, in an Arctic soil which is frozen for most of the year (128).

A relation between the quality of the bacterial flora of a soil and its fertility was found by Pochon & Coppier, who compared neighbouring fertile and infertile soils from two localities in France. The fertile soils contained more microorganisms in general, but fewer cellulose decomposers than in-

fertile soils; and non-symbiotic nitrogen fixers were found in the fertile, but not in the infertile soils (164). On the Broadbalk wheat field at Rothamsted, on the other hand, Skinner, Jones, & Mollison found larger numbers of bacteria, actinomycetes, and fungi in plot 2B, given farmyard manure, than in plot 7, given artificials, or plot 3, unmanured; but there was little difference in numbers between these last two plots, which differ greatly in their yield of wheat (190). Pochon and his colleagues have shown that a compost may contain a high total number of bacteria developing on agar plates, and yet be very poor in nitrifiers, nitrogen fixers, and other important groups (166).

The most striking effects of soil amelioration on the microflora are shown by reclaimed peaty soils which, like acid forest soils, are very poor in microorganisms in their natural state. De Barjac has made a detailed study of the microflora of several types of peat before and after improvement (42). Boquel, Kauffmann, & Toussaint, in a study of the tropical forest soils of the Ivory Coast, found increased numbers of nitrogen-fixing clostridia, and of cellulose-decomposers, during the rainy season. Clearing the forest decreased the numbers of the latter (18).

Fungi.—Peyronel represents the character of the fungus flora of a particular soil by dividing the species into 8 groups, and showing the frequency of each group on a compass diagram. He has compared the flora of different temperate and tropical soils by this method (163). Stenton has reported on the fungus flora of the soil of Wicken Fen (195), and Gordon on the occurrence of *Fusarium* species on cereal plots in Canada (62). Zeidberg & Ajello report on the presence of two pathogenic fungi in Tennessee soils (248). Forest and agricultural soils were compared by Welvaert & Veldeman, who found a greater variety of fungi in polder soil (pH 7.5) than in a sandy loam forest soil (pH 4.3). They suggested that, in the latter, the antagonistic action of *Trichoderma* and *Penicillium* might be an important factor (239). The short time needed for trees to affect the microflora is illustrated by work reported by Meyer, who found differences in the fungal population under pasture and under tree saplings (141). An influence of the type of tree growing on forest soils is suggested by the results obtained by Chase & Baker, who showed that the ratio of fungi to bacteria and actinomycetes was higher under conifers than under maples (33).

The soil yeasts are a little-known group that has been studied recently by Capriotti (32) in Holland and by Di Menna (46) in New Zealand. Those found in New Zealand soil included the pathogen *Candida albicans*.

Vertical Distribution of Microorganisms

The distribution of microorganisms down a sandy soil profile is reported in a paper by Blue, Eno, & Westgate. Highest numbers occurred in the top six inches. Very few microorganisms were found in the A2 horizon, nine to twelve in. down, which was deficient in nutrients. More were found between 12 and 30 inches below the surface; numbers at this level were increased by

dressings of potassium and of nitrate (15). Tchan & Whitehouse have shown that algae are confined to the top few mm. in wet soil, and that there is no evidence that they can live under the soil surface as heterotrophs (214). Garbosky reports that *Azotobacter* is found at depths down to two metres in Argentine soils, and that it may be more abundant between 50 and 100 cm. down than at the surface (58). The distribution of fungal species at different levels in sandy podsol profiles was studied by Jeffreys and co-workers, who found that plate counts decreased generally with depth but showed a secondary maximum in the B horizon, noticeable for the preponderance of *Mucor* at this level (92). Guillemat & Montegut observed, in plots from the long-term field experiments at Grignon (69), that different species of fungi occurred at different depths. Burges & Fenton conclude that the ability of fungi to live deep in the soil is a consequence, not, as is commonly supposed, of their ability to do without oxygen, but of their ability to withstand high concentrations of carbon dioxide (25).

Nutrients

Starkey has reviewed the whole subject of the need of different micro-organisms for inorganic nutrients (193). Jensen has shown that *Azotobacter* needs much more magnesium than the related genus *Beijerinckia*, which, unlike *Azotobacter*, cannot partly replace its need for molybdenum by using vanadium (95, 96). Shug *et al.* have thrown more light on the reason why some microorganisms need molybdenum, by showing that it is a cofactor for the hydrogenase of *Clostridium pasteurianum* (186).

Lochhead & Thexton found that an important group of soil bacteria required vitamin B_{12}, replaceable by an aqueous extract of soil (125). More than a quarter of the 500 isolates from soil studied by Lochhead & Burton were found to require one or more of the following growth factors: thiamin, biotin, B_{12}, pantothenic acid, folic acid, nicotinic acid, and riboflavin (122). In soil, the growth factor requirements of such bacteria can, no doubt, be supplied by other organisms; for example, Burton & Lochhead found that several species, especially of *Rhizobium*, can synthesise vitamin B_{12} (27). Soil extract has also been found by Lochhead & Burton to contain at least one growth factor other than B_{12}, and not supplied by yeast extract. Culture filtrates from a number of soil bacteria having simple nutrient requirements were able to supply a growth factor having the same effect. One of them, named *Arthrobacter pascens*, synthesises such a factor, to which a related species, *Arthrobacter terregens*, is exacting. Soil extract is therefore useful in the study of soil bacteria, not only as a source of minerals, but also of some organic nutrients (123).

Partial Sterilisation

In their early work on partial sterilisation, Russell & Hutchinson suggested that increased bacterial activity following partial sterilisation was due to the destruction of soil protozoa. Singh & Crump used the improved

dilution technique, developed by the former (Singh), to estimate numbers of amoebae in soils from forest nursery beds, untreated and partially sterilised with steam and formalin. Bacterial counts were also made by plating. In the formalin-treated soil, bacterial numbers rose above those in the untreated, and numbers of amoebae were reduced. But in steamed soil, numbers of both bacteria and amoebae increased over those in the untreated soil. These effects were found to persist over a period of six months (187). It is thus unsafe to generalise as to the effects of different types of partial sterilisation on relative numbers of amoebae and bacteria. Stout's qualitative observations of steamed glasshouse soil also showed that a number of species of protozoa survived the treatment (202). The fungal population seems to be more drastically affected by partial sterilisation of soil. Mollison followed the changes in fungi in the same forest nursery plots that were examined by Singh and Crump. Both steam.and formalin treatments almost completely eliminated the fungi and, even after later recolonisation, the plate "counts" of fungi were much reduced, an effect which lasted for the 25 months during which counts were made. The fungal population was still varied after steam treatment, but after formalin, *Trichoderma viride* appeared to be the principal species that recolonised the soil (146). This fungus is exceptionally tolerant of formalin. Wensley, in a paper giving useful data on the immediate microbiological effects of soil treatment with several fumigants, reports that after treatment with methyl bromide, soil was recolonised in 4 weeks, mainly by *Aspergillus* (240). Evans also followed the recolonisation of soil by fungi after partial sterilisation, in this case with formalin and chlorpicrin (53). Warcup showed that *Pythium* in forest nursery soils is killed by steam or formalin (233). There is also a change in the relative proportions of different groups of bacteria in soil following partial sterilisation. Thus Davies & Owen found that ammonia accumulates in steamed glasshouse soils, because the nitrifying bacteria are killed (39); and Holding found an increase in the percentage of Gram-negative bacteria after steaming. This group also increased after the addition of fresh organic matter or the growth of oat seedlings, suggesting that the effect of steaming may be partly due to release of nutrients which favour these bacteria (84).

An interesting specific effect was noted by Bromfield in some experiments with soil treated with carbon tetrachloride. After treatment these soils evolved hydrogen sulphide from ammonium sulphate added to them, apparently owing to the activity of *Bacillus megaterium*, which, when isolated from the soil, would carry out this reduction in pure culture. The action of carbon tetrachloride was to eliminate certain bacteria which prevent this reducing activity of *B. megaterium* from taking place in untreated soil (21). The practical aspects of disinfestation of soil by heat, flooding and fumigation have recently been reviewed by Newhall (151).

Weedkillers and other poisons.—More and more very poisonous substances are being added to soils all over the world to kill weeds or insects, and it is fortunate that most of them have been found, on investigation,

to have no lasting harmful effect on useful soil microorganisms. Magee & Colmer (129), for instance, found that *Azotobacter* was poisoned in culture only by much larger doses of herbicides than would ever be used in practice, and the same was found to be true of four commonly-used insecticides by Callao & Montoya (31), who worked with soil as well as liquid cultures. Gray has carried out a series of studies on the effects of hexachlorocyclohexane and its *gamma* isomer (Gammexane), and finds that the effect of both isomers is very much less in soil than in culture media; but it did reduce the count on soil-extract agar plates (66). Staněk (191) reports that Gammexane actually stimulates *Azotobacter*, however, and Koike & Gainey (108) and Jones (101) find that the total plate count of bacteria is increased by large doses of 2-4-D, CADE and DDT; and none of these three compounds affected nitrification in any dose likely to be used in practice. Surface-active agents are regularly added as spreaders to agricultural sprays; Ivarson & Pramer have found that Tween 80, a non-ionic compound, was rapidly decomposed in soil, and had little or no undesirable biological effect. The cationic spreader Ceepryn was more slowly decomposed, and in large doses it reduced the total plate count and hindered nitrification. An anionic compound, Nacconol NRSF, on the other hand, was not decomposed at all, and had serious and lasting toxic effects (89). The same may be true of radioactive phosphorus, and Goring & Clark recommend that it should not be used for experiments on plants if the soil in which the plant is growing is meant to be in its natural state (63).

ACTIVITIES OF SOIL MICROORGANISMS

Soil Structure

There is evidence that some fungi may hold the soil together by the network of their mycelium; Downs, McCalla, & Haskins found that one out of twelve cellulose-decomposing fungi improved the structure of a soil poor in organic matter (50). The gum produced by *Agrobacterium radiobacter* improved aggregation (173); Rorem suggests that bacterial gums, besides increasing aggregation, may be important to the species which produce them as a mechanism whereby they can concentrate ions that they need out of the surrounding soil (175). Hely & Bonnier found that synthetic soil conditioners increased the numbers of bacteria which produced natural gums, and so had a double effect on soil structure; the synthetic compounds were not toxic (76). Mortensen & Martin found that two synthetic soil conditioners were not toxic to microorganisms, but were very resistant to decomposition (147). In certain soils the addition of synthetic aggregating substances has been found to improve nodulation of soy beans and of lucerne (185, 78).

Breakdown of Natural Carbon Compounds

Not much interest is being taken at present in this aspect of Soil Microbiology. Reese & Levinson have compared the breakdown of cellulose by different species; Kox found that aerobic bacteria, as well as fungi, are responsi-

ble for the breakdown of cellulose and pectin in Sphagnum peat, and McBee describes a thermophilic anaerobe which can decompose cellulose (110, 127, 172).

Until recently, fungi were thought to be the only organisms capable of decomposing lignin; but Fischer, Bizzini, Raynaud, & Prévot have found that bacteria which can break down lignin are very widespread, and especially numerous in forest soils. All their strains were species of *Pseudomonas*, and most of them could attack benzoate and other aromatic carbon compounds as well as lignin (55). Henderson & Farmer found that some soil fungi could utilize aromatic compounds such as syringaldehyde, which might be breakdown products of lignin (79). Veldkamp found that a number of soil microorganisms can decompose chitin; actinomycetes seem to be the most numerous. Twenty-three different actinomycete species were found to be chitin-decomposers, and likewise 50 species of bacteria, including the myxobacterium *Cytophaga johnsonae*. Ammonia and acetic acid are the final breakdown products in cultures of this species and of a new species, *Pseudomonas chitinovorans* (220). The breakdown of pectin in fallen leaves has been studied by Wieringa; he finds that the first agent of breakdown is always the fungus *Pullularia pullulans*, which is present on the surface of leaves while they are still on the tree. Pectin-decomposers in acid soils are mostly fungi, but in well-limed soils, actinomycetes predominate (242).

The Nitrogen Cycle

Soil microbiologists have written over a hundred papers on this subject since 1951, but their interest is very unevenly distributed; over three-quarters are concerned with the fashionable subjects of *Azotobacter* and nitrification. There is practically no work on the formation of ammonia, which is overdue for investigation by modern methods. It is usually supposed that the breakdown of proteins is the source of soil ammonia; but Veldkamp has recently shown that chitin, which is abundant in soil in fungus hyphal walls and arthropod integuments, is an important ammonia source (220). He incubated chitin in soil, and found that up to 60 per cent of the nitrogen in it was recovered as nitrate, presumably formed by the oxidation of ammonia. It is also possible that nitrogen fixers supply ammonia directly to the soil (as Winogradsky thought), for Delwiche & Wijler found that most of the nitrogen fixed in their experiments could be accounted for as nitrate (44).

Nitrification.—There have been recent reviews on this subject by Lees (116, 117) and Meiklejohn (132, 134). It is becoming increasingly apparent that the agents of nitrification are the classical autotrophic nitrifiers of Warington and Winogradsky, which are ubiquitous, and which convert ammonia quantitatively to nitrite and then to nitrate. There are several recent reports of heterotrophic microorganisms which form nitrite or nitrate by oxidation; but, if the amounts are stated, they are always very small (54, 56, 85, 88). The very laborious dilution method is still the only satis-

factory way of counting nitrifiers in soil (37). Many of the colonies which develop on Winogradsky's silica-gel plates are not nitrifiers, and Millbank found that a method with agar disks, similar to Singh's method for protozoa, would not work because agar and soil together were toxic to *Nitrosomonas* (59, 86, 142). It is possible to compare the nitrifying activity of different soils by measuring the rate of nitrification in enrichment cultures, or by the Lees & Quastel percolation method, if the soil structure is good enough (133, 200). This method was used by Stevenson & Chase, who found that nitrification was less under a grass cover than under bare fallow (200). Mills (144) has also found that different African grass species depress the formation of nitrate to different degrees. In the Uganda soil that he studied, there was an accumulation of nitrate in the top 6 inches in the dry season, though the soil was acid. Meiklejohn found that this soil contained autotrophic nitrifying bacteria (133). Jacquemin & Berlier found that there were very few nitrifiers in a forest soil from the Ivory Coast, and many more in cleared land (90). Burning the vegetation over a Kenya soil was found by Meiklejohn to kill the nitrifiers (136).

There has not been much work on the physiology of the nitrifying bacteria, mainly because of the enormous difficulty of getting them to grow in pure culture. This may be partly due to their need for some growth factor which they can obtain readily in soil, and which is lacking in culture media; but Gundersen (70) tried various vitamins of the B group on pure cultures of *Nitrosomonas*, and Meiklejohn (131) tried several other possible stimulants in enrichment cultures, in both cases with entirely negative results. Though Gundersen (70) found that several amino-acids are very toxic to *Nitrosomonas*, and though Jensen & Sørensen (100) found that the same was true of some organic sulphur compounds, there does not seem to be any truth in the belief that organic matter as such has a mysterious toxic effect on nitrification.

Goldberg & Gainey (61) have studied the effect of clay minerals on ammonia oxidation, and find that ammonium ions are more readily oxidized by enrichment cultures if free in solution than if adsorbed on the clay. This is quite contrary to earlier results of Lees & Quastel with soil (see Lees, 116).

Lees (115) has found, by using very dilute solutions, that hydroxylamine is an intermediate product in the oxidation of ammonia by *Nitrosomonas* cells, and Imshenetskiĭ & Ruban (87) have shown that it is oxidized by cell-free autolysates. Hydroxylamine is also formed in the oxidation of pyruvic acid oxime by heterotrophs; further oxidation to nitrite, in these species as well as in *Nitrosomonas*, is blocked by hydrazine (120). It would be interesting to know if chelating agents such as allylthiourea, which Lees (114), and Hofman & Lees (83), found to stop ammonia oxidation at a very low concentration in suspensions, have the same effect in soil. Lees & Simpson find that the oxidation of nitrite by *Nitrobacter* is interrupted at different stages by chlorate and by cyanate. *Nitrobacter* contains more than one cytochrome,

and they are reduced as nitrite is oxidized (118, 119). In view of these results it is most puzzling that Engel, Krech, & Friederichsen conclude that neither iron nor zinc-containing enzymes are involved in the oxidation of nitrite, though *Nitrobacter* needs iron for growth (52). These workers also investigated the amino acids in *Nitrobacter*, and found the same 18 that Hofman had found in *Nitrosomonas*, and that they themselves had found in *Hyphomicrobium*. The list does not include *alpha-epsilon*-diaminopimelic acid. Hofman found four sugars, galactose, ribose, rhamnose and xylose, but oddly enough, no glucose, in his *Nitrosomonas* preparations (81).

Several workers find that the ratio of nitrogen oxidized to carbon assimilated is higher in old than in young cultures of nitrifiers. Hofman & Lees (82), looking at this from a thermodynamic point of view, think that the ratio increases because *Nitrosomonas* needs more energy to keep increaing concentrations of toxic nitrite out of its cells. But, as Engel *et al.* point out, this can hardly be true of *Nitrobacter*, which forms a less toxic compound, nitrate, from the more toxic nitrite (52). A more probable explanation is given by Bömeke, who thinks that there is a progressive loss of carbon from old cultures, as the cells break down some organic storage material to keep themselves alive (16).

Enrichment cultures have been studied by Klein, who found a way to get rid of *Nitrobacter*, which can be a very troublesome contaminant of *Nitrosomonas*, by supplying ammonia in the form of ammonium borate (106).

Imshenetskiĭ (86) and also Bisset & Grace (12) claim that there are no genera of autotrophic nitrifying bacteria other than *Nitrosomonas* and *Nitrobacter*, and that the genera *Nitrosocystis*, *Nitrosogloea*, *Nitrosospira*, and *Nitrocystis*, described by Winogradsky and his colleagues, are not nitrifiers (86, 12). They base this criticism on the observation that nitrifying cultures may be contaminated with *Myxobacteria*, whose fruiting bodies could have been mistaken for zoogloeal organisms responsible for the nitrification. More definite evidence is needed to substantiate so comprehensive a criticism. Indeed, Palleroni has claimed to have isolated *Nitrosospira* sp. from Antarctic and Argentine desert soils and found that it was a nitrifier (155, 156).

Denitrification.—Wijler & Delwiche, using isotopic nitrogen and soil, showed that denitrification is probably entirely due to microbial action, as all the nitrogen in the reduction products was derived from nitrate, which excludes the formation of nitrogen by a non-biological reaction between nitrite and amino groups. They also found that nitrous oxide, as well as nitrogen gas, was released when soil and nitrate were incubated together (243). The soil they used therefore presumably contained organisms such as the *Denitrobacillus* species studied by Verhoeven, that produce nitrous oxide as well as nitrogen in the reduction of nitrate (221). The effect of aeration on denitrification may vary with the species of bacteria present in a soil, as Marshall *et al.* found that different *Pseudomonas* strains are differently affected by aeration. One strain may only reduce nitrate under conditions of

almost complete anaerobiosis, while another may be so little affected by oxygen that it is almost impossible to stop it reducing nitrate in culture by increasing the air supply (130).

Nitrogen fixation.—There has been a recent experiment with isotopic nitrogen by Delwiche & Wijler, the results of which were almost entirely negative. In the soil that they studied they found negligible fixation in bare fallow, and very little under grass (44). In consequence of this, some microbiologists may conclude that non-symbiotic nitrogen fixation never adds any nitrogen to any soil in any circumstances. But, if one leaves the laboratory and turns to the field, it is obvious that the nitrogen supply is maintained in soils in which legumes are not growing, and in which leaching certainly, and denitrification probably, takes place. The evidence as to non-biological fixation is most contradictory, but recently Bjälfve has shown that light is not an agent of nitrogen fixation (13). Biological fixation has been reviewed by Wilson & Burris (245), by Fogg (57), and again by Wilson (244), who deals especially with the mechanism of fixation.

Azotobacter.—There is a certain irony in the fact that so much is being written about *Azotobacter*, as it is becoming more and more doubtful if this genus is of real practical importance in adding to the soil's nitrogen supply, for there seem to be many soils, in various parts of the world, where it is never found (20, 102, 135). Where it does occur, it is usually found in very small numbers, and not only in poor soils, like the Arno Atoll soils examined by Stevenson (197), for Meiklejohn (137) found less than 2000 *Azotobacter* cells per gram throughout a series of counts on Rothamsted soil. But Pochon and his colleagues record a count on the very fertile Nile silt, in which about 8000 *Azotobacter* per gram of dry matter, as well as nitrogen-fixing clostridia, were present (167). Counting *Azotobacter* in soil is difficult, however; neither Hely & Bonnier (76) nor Meiklejohn (137) were able to obtain consistent results with Winogradsky's method of "plaques moulées"; and when they tried his other method, of crumbs of soil sprinkled on silica gel, they found that a colony of *Azotobacter* developed from every crumb. Eventually they used surface inoculation on nitrogen-poor agar, as did Tchan (207), who also used a dilution method with liquid cultures. Absence or scarcity of *Azotobacter* may be due to lack of moisture, to lack of calcium or phosphate, and perhaps of other nutrients (135, 206). It might even be due to excess of oxygen, as Parker (158) has found that *Azotobacter* fixes nitrogen more efficiently under reduced oxygen tension; but lack of water is an equally probable explanation for the scarcity of *Azotobacter* near the surface of soils in Argentina, described by Garbosky (58).

Inoculation with *Azotobacter* to increase the yield of various crops is regularly practised in Russia, but it does not seem to be uniformly successful (180, 226, 228, 241). Petrenko suggests that many of the negative results may be due to the use of unsuitable cultures; it is well known to all microbiologists who have worked with *Azotobacter* that long-continued maintenance in artificial culture alters its properties, and use of an old stock culture

from a type collection may well lead to confusing results (162). On the other hand, it is possible that some positive results of inoculation are due to fertilizers added with the bacterial culture (9). Bukatsch & Heitzer claim that strains of *Azotobacter* isolated from the rhizospheres of different plant species differ in nitrogen-fixing power; it is unfortunate that they examined one strain only from each species of plant, and still more unfortunate that their experiment on *Azotobacter*-inoculated peas was carried out in open pots, so that infection with *Rhizobium* cannot be excluded as the cause of the fixation observed (24).

Parker has produced evidence that *Azotobacter* fixes nitrogen more efficiently in the presence of other bacteria than in pure culture. His freshly-isolated cultures fixed 18 mg. N per gram sugar decomposed when the *Azotobacter* was still contaminated with a small motile rod; but when this last contaminant was removed, fixation in the pure culture went down to three mg. N per gram sugar, and only gradually improved after several transfers in a nitrogen-poor medium (160).

It is generally supposed that *Azotobacter* does not grow or fix nitrogen at any pH more acid than 6.0, but recently several acid-tolerant *Azotobacter* strains have been found. Jensen (97) has described a new species, *Azotobacter macrocytogenes*, and acid-tolerant varieties of known species have been found by Tchan (209), Döbereiner (48), and Metcalfe (139). A new species, *Azotobacter halophilum*, which will only develop in saline media, has been found in saline soils in Siberia by Blinkov (14).

Beijerinckia.—Jensen has shown that there are good reasons for separating the aerobic acid-resistant nitrogen fixers of tropical soils from *Azotobacter* and placing them in the genus *Beijerinckia*. They differ from *Azotobacter* in morphology (the cells are much smaller), and also in being able to fix nitrogen at pH 3.5, in needing no calcium, and in being unable to use vanadium in place of molybdenum. Azotobacters occur in the tropics in calcareous soils, but tropical soils are commonly acid, and here Beijerinckias, which are efficient but slow nitrogen fixers, replace them (96). Many attempts have been made without success to isolate *Beijerinckia* from temperate-zone and subtropical soils (210). Derx (45) attributed the tropical distribution of this genus to a possible association with some special genera of plants, perhaps legumes which do not form nodules (e.g. *Cassia* spp.), and suggested that *Beijerinckia* is a facultative symbiont which, unlike *Rhizobium*, has not lost the power to fix nitrogen outside the plant. On the other hand, Kluyver & Becking (107) think that *Beijerinckia* may be confined to lateritic soils. There is a recent report of the occurrence of *Beijerinckia* outside the tropics, as Suto has isolated a nitrogen-fixer, which seems from his description to belong to this genus, from an acid volcanic soil at Sendai, Japan (lat. 38°N) (204). Ruinen has found a new habitat; she has discovered large numbers of *Beijerinckia* cells on the leaves of trees and epiphytes in the tropical forests of Indonesia, a fact which may explain the lavish vegetation of the forest on a soil which gives very poor yields of crops when cleared and planted (179).

Clostridium.—Though very little has been written about *Clostridium pasteurianum* (and related species) in recent years, it is quite possible that these anaerobes account for much more nitrogen fixation than does *Azotobacter*. In the first place, they are much more widely distributed than *Azotobacter;* Rybalkina (181) in Russia, Kaila (102) in Finland, Boswell (20) in England, Meiklejohn (135) in East Africa, and Tchan & Beadle (212) in Australia, found them always, or nearly always present in every soil examined. They are also far more numerous; in contrast to the thousand *Azotobacter* cells per gram, *Clostridium* cells number hundreds of thousands (74, 137). Hart found from a hundred thousand to a million per gram of garden soil, nine-tenths of them vegetative cells and one-tenth spores. Numbers in an oakwood soil were somewhat smaller (74).

It has generally been supposed that clostridia were poor fixers of nitrogen, adding only about two to four mg. per gram of sugar decomposed. But Parker (159) has recently shown that, given suitable cultural conditions, a strain of *Clostridium butyricum* can do as well as the best *Azotobacter*, fixing 27 mgm. N per gram sugar. To obtain this level of fixation it is necessary to grow the bacteria in presence of carbon dioxide as well as nitrogen, in absence of carbon monoxide, and to supply them with growth factors. Parker's strain required biotin and para-aminobenzoic acid, and a strain studied by Virtanen & Lundbom (227) required folic acid.

It might be objected that strict anaerobes could not multiply fast enough to be able to fix much nitrogen in the topsoil; but Hart found that his nitrogen-fixing clostridia were able to grow under aerobic conditions if supplied with combined nitrogen. They did not fix nitrogen aerobically; but it is quite possible that clostridia could grow in topsoil if combined nitrogen were present, and then, in local pockets of anaerobiosis, or at times of temporary waterlogging, proceed to fix nitrogen when the original supply was exhausted (74).

Other Nitrogen-fixers.—The blue-green algae are probably the most efficient of all non-symbiotic nitrogen fixers. De & Mandal estimate that, given sufficient phosphate and molybdenum, algae in flooded rice soils can fix as much as 70 lb. nitrogen per acre in six weeks (40). Blue-green algae are also able to fix nitrogen in some symbiotic systems, for Bond & Scott showed that lichens and liverworts can fix nitrogen if *Nostoc* is present as a partner in them (17). Douin showed that the nodules on the roots of Cycads contained a species of *Anabaena* (49). There is also an increasing list of organisms which can only fix very small quantities of nitrogen, in many cases so small that fixation can only be detected by the use of isotopic nitrogen. Metcalfe *et al.* (140) used this method to find that two yeasts, isolated by the percolation method from acid health soils, were able to fix nitrogen. Anderson (3) describes another poor nitrogen fixer which is apparently a *Pseudomonas*, and Brown (23) has found two nitrogen-fixing *Nocardia*, one of which could decompose cellulose. Newton & Wilson (152) report that the purple sulphur bacterium *Chromatium* can fix small quantities of nitrogen, and

Hamilton & Wilson (71) have been able to show, by using isotopic nitrogen, that *Aerobacter aerogenes*, which has long been suspected of being a nitrogen-fixer, can fix small amounts anaerobically in a well-buffered medium.

Microorganisms and Inorganic Soil Constituents

Evidence continues to grow that microorganisms can make various inorganic nutrients available to plants by bringing them into solution. Bromfield found that several common species of soil bacteria, *Bacillus circulans*, *Bacillus megaterium*, and *Aerobacter aerogenes*, could reduce ferric compounds in the presence of a suitable hydrogen donor and so increase the available iron (and manganese) in soils (22). Aristovskaya points out that the acid produced by some microorganisms may be an important agent of soil formation, especially in podzols. She found that the microflora from podzols was mostly composed of species which grew best in media poor in nutrients, and that several fungus species produce more acid in poor than in rich media (5). Uarova found bacteria in the rhizosphere of wheat plants, which could decompose calcium phosphate, and which increased the water-soluble phosphorus in a compost (219).

Butlin & Postgate have reviewed the sulphur cycle in nature, and have pointed out the economic importance of organisms which produce actual sulphur (28, 29). Quispel, Harmsen, & Otzen report on the oxidation of pyrite in newly-reclaimed marine soils; only the second stage of the process, the oxidation of sulphur to sulphate, is carried out by bacteria, but this reaction stimulates the primary chemical oxidation of the sulphide to sulphur (170). Oxidation of sulphur in Kansas soils has been studied by Moser & Olsen (149).

The Breakdown of Introduced Organic Compounds

There are few substances which are so insoluble, or so toxic, that soil microorganisms cannot dispose of them. As is well known, even such unpromising carbon sources as the straight-chain hydrocarbons can be broken down by bacteria. Ladd (111) describes a *Corynebacterium* which oxidizes such compounds, and Konovaltschikoff-Mazoyer & Senez (109) obtained several hydrocarbon-decomposing pseudomonads from the oil-soaked earth near the Marseilles refineries (111, 109). Levine & Krampitz found a *Corynebacterium* which could oxidize acetone (121).

Arnaudi, Canonica, & Treccani have reviewed the whole subject of the breakdown of hydrocarbons, and also of aromatic compounds (6). Treccani *et al.* (218) and Murphy & Stone (150) studied the breakdown of naphthalene, and Walker & Wiltshire (230) that of chloro and bromo-naphthalene, by soil bacteria. Webley *et al.* found that *Nocardia opaca* breaks down the side chain of phenyl-substituted fatty acids by *beta*-oxidation (237).

Many studies deal with the decomposition of the hormone herbicides and related compounds. Audus (7) has published a series of papers on the breakdown of 2,4-dichloro-phenoxyacetic and 4-chloro-2-methyl-phenoxya-

cetic acids (better known as the weedkillers 2,4-D and MCPA). Jensen & Petersen (99) describe two species that can break down 2,4-D, and Stapp & Spicher isolated a new 2,4-D decomposer, *Flavobacterium peregrinum* (192). Audus & Symonds (8) studied the kinetics of breakdown of 2,4-D by their previously-isolated strain of *Bacterium globiforme*, and Walker & Newman found that the same compound was attacked by a species which they tentatively identify as a *Mycoplana* (231).

Steenson & Walker have isolated, from soil, a *Flavobacterium* that can break down 2,4-D, an *Achromobacter* which attacks both 2,4-D and the related compound MCPA, and another *Achromobacter* which attacks *para*-chloro-phenoxyacetic acid (194). Rogoff & Reid (174) find that a *Corynebacterium* can break down 2,4-D, and Jensen & Gundersen (98) describe another that decomposes aromatic nitro-compounds. Walker has measured the breakdown of chlorophenols in soil by the percolation method (229).

INTERRELATIONS OF THE SOIL POPULATION

Thornton in his Leeuwenhoek lecture (216) has discussed the various possible effects which the different groups of soil microorganisms may have among and between themselves. It is of course clear that the relations between microorganisms in soil may be either mutually beneficial or harmful, but comparatively little attention has been paid to the former aspect. The process of dissimilation of a compound in soil is usually by stages so that an organism that carries out the initial stage may provide nourishment to different groups, but these food chains are usually complex, and their analysis awaits knowledge of the chemical pathways along which the compounds concerned are broken down (79, 194, 230). Some organisms benefit their neighbours by synthesising growth substances such as vitamin B_{12} (27).

Most attention has been paid to the competition between microorganisms in soil and this aspect has received increasing attention lately because of its importance to the control of root pathogens. Most of these are fungi, hence soil microbiologists have been concerned with the isolation and study of soil organisms antagonistic to fungi. These include other fungi, actinomycetes and bacteria. Morton & Stroube found that 3.5 per cent of the fungi, 1.7 per cent of the actinomycetes and 0.2 per cent of the bacteria isolated by them from soil were antagonistic in agar cultures to *Sclerotium rolfsii* (148). Luke & Connell found that 16 per cent of the fungi and 3.6 per cent of the bacteria isolated by them from sugar cane soils were antagonistic to *Pythium* (126). Soil bacteria producing antibiotics are relatively uncommon but are of interest as being possibly easier to use as inoculants for biological control. Chinn described a pseudomonad, found to predominate on a sample of wheat grain, that was strongly antibiotic to *Helminthosporium* and showed considerable antibiotic antagonism to *Fusarium* and to a wide variety of bacteria (36). Antibiotic fungi are more abundant in soil. Jeffreys *et al.* found that of 65 fungal species isolated from sandy soils, about half produced antibiotics, usually active against both bacteria and other fungi. About 45 per

cent of the species that were widespread or locally abundant antagonised other fungi, but only 15 per cent of the rare fungi did so, suggesting some selective advantage in antibiotic production. None of the ten species of Phycomycetes showed such antagonism (92).

On platings of soil suspensions, antagonism of actinomycete colonies towards nearby colonies of fungi is often noticeable so that the former group has attracted particular attention as antagonists to fungi (217). Thus Stessel, Leben & Keitt sprayed platings of soil dilutions with suspensions of the fungi *Glomerula*, *Colletotrichum*, *Helminthosporium* and *Verticillum*. Out of some 70,000 colonies developing on the plates, 170 were antagonistic and, of these, 80 per cent were of actinomycetes (196). Fungi vary greatly in susceptibility to actinomycete antibiotics and this specificity is found even amongst closely related strains. Buxton & Richards tested sixteen soil actinomycetes for activity *in vitro* against eight pathogenic strains of *Fusarium oxysporum*. Three of the former were inactive, nine inhibited all the *Fusarium* strains equally, but four of the actinomycetes showed specific differences in degree of inhibition according to strain of *Fusarium*, which they could be used to distinguish (30). One of the actinomycetes that showed specific activity was identified as *Streptomyces albidoflavus*, shown by Skinner also to be strongly antagonistic to *Fusarium culmorum* (188). Antibiotics produced by actinomycetes are also active against some other actinomycetes. Peterson studied the cross antagonisms amongst a collection of 46 actinomycetes, all of which were active against *Streptomyces scabies*. They varied greatly both in the number of the other strains that they would antagonise, and in the number to which each was susceptible. These results show the need for careful selection of antagonistic actinomycetes resistant to the attack of other species, if it is desired to establish them in soil to control a pathogen (161).

The potential usefulness of antibiotic-producing organisms for biological control in soil depends not only on the feasibility of establishing them in fresh soil, but also on their ability to produce antibiotics in effective concentration in field soil and on the activity and persistence of these antibiotics in the soil. Even in sterilised and partially sterilised soil Grossbard (68) found that *Penicillium patulum*, *Aspergillus clavatus* and *Aspergillus terrens* only produced antibiotic in detectable amounts where available carbon sources were added, while Gregory *et al.* found only traces of activity in soil cultures of *P. patulum* (67). Clear evidence for the production of a specific antibiotic in unamended soil was obtained by Gottlieb & Siminoff (65) in the case of chloromycetin and by Wright for gliotoxin (247). But attempts to show this with other antibiotics have generally been negative. Even if an antibiotic is formed in soil a variety of environmental factors may limit its activity or result in its rapid destruction. These factors have been studied in the case of several antibiotics by Gottlieb *et al.* (64) and Hessayon (80), while Jeffreys investigated the behaviour of 10 antibiotics in soil (91). The principal factors causing inactivation appear to be (a) the adsorption of basic antibiotics by the soil, (b) instability at the pH of the soil, (c) chemical reaction with some

soil component and (d) microbial decomposition. Such results have caused the view to be expressed that antibiotic action is unlikely to be important in soil. On the other hand failure to detect antibiotics in soil cultures of organisms known to be capable of their production may be due to a lack of selectivity in the methods used for their detection, most of which have involved extraction from the soil. Stevenson (198, 199) has developed a sensitive method for detecting the production of antibiotics by cultures of actinomycetes in sterilised soil. Agar-coated microscope slides seeded with spores of *Helminthosporium* were buried in the soil culture, which inhibited their germination to varying degrees as compared with a sterile soil control. Evidence that this effect was in fact due to the actinomycete antibiotic was obtained by studying the effect with pregerminated spores. Some of the actinomycetes employed produced quite characteristic types of deformation of the fungal hyphae *in vitro*, and these specific effects were also shown on the hyphae from pregerminated spores buried in soil culture. Moreover the same specific effects were produced by *Streptomyces antibioticus* and by the antibiotic actinomycin, which it produces, both *in vitro* and in soil.

Another important cause of antagonism between microorganisms in soil is competition for some limiting nutrient which may be exerted whether or not a competing organism produces an antibiotic. Skinner studied the growth of *Fusarium culmorum* in the presence of *Streptomyces albidoflavus* which produces an antibiotic very active against this fungus but one that is strongly adsorbed by bentonite. In sand culture, the actinomycete could strongly inhibit the fungus, even preventing the germination of its spores. When bentonite was added to the sand in excess, normal germination of the fungal spores took place but growth of the fungal mycelium was still much reduced by the actinomycete, and this Skinner attributed to nutrient competition. He found that competition increased with the concentration of glucose supplied (188, 189).

Antibiotic organisms may merely arrest growth of a susceptible organism but some, such as the Myxobacteria (Noren) actually lyse and can thus feed on the organisms attacked (153). Such lysis of fungal mycelium by actinomycetes has also been observed by Skinner (188). A dramatic example of direct attack of one organism on another is that of fungi that catch and destroy eelworms. In a survey of these fungi from 49 English soil samples, Duddington found that the most abundant were *Arthrobotrys dactycoides* and an unidentified species that would not form spores (51). Equally remarkable is the amoeboid organism found by Weber, Zwillenberg & van der Laan that attacks and digests nematodes (236).

Evidence is accumulating that fresh soil contains a fungistatic factor, destroyed by heating, that inhibits the germination of spores of a number of fungi. This has been found by Dobbs & Hinson (47), Chinn (35) and Jeffreys & Hemming (93) while extracts of fresh peat have been found to be strongly inhibitory to bacterial growth by Pochon & de Barjac (165).

Stover (203) and Sanford (184) have reported on the survival of differ-

ent plant-pathogenic fungi in soil, and Park (157) has found that alien species of fungi introduced into soil do not withstand competition from the indigenous microflora as well as do species native to the soil. Cuthbert *et al.* report on the survival of bacteria which are indicators of faecal pollution (38). Vilas, Tejerina & Rubio put forward the interesting hypothesis that some bacteria may be present in soil as filterable forms, perhaps less vulnerable to antibiotic attack (222).

When the difficulties of establishing an antibiotic organism in soil, of ensuring conditions therein for adequate antibiotic production, and of choosing an organism whose antibiotic is active and persistent in soil are considered, it is small wonder that attempts at biological control of root disease have so far met with very limited success, though the number of positive results has been by no means negligible. This field has been well reviewed recently by Wood & Tveit (246).

Since the root surroundings are the site at which biological control might most likely be effective, more search for antagonistic organisms in the rhizosphere would seem worth while. This is the more so since several antibiotics are known to be taken up by the roots wherein they remain active and may be protected from the hazards to which they are exposed in the soil (168, 201).

INTERACTIONS OF PLANTS WITH THE MICROFLORA

The Rhizosphere

The micropopulation of the rhizosphere has continued to attract well-deserved attention. The rhizosphere effect can be particularly well studied in soils poor in organic matter, where the population of the control soil is sparse. Sand dunes in course of reclamation, for instance, show a gradual increase in numbers of microorganisms; Milosevic (145) has shown this for dunes on the coast of Yugoslavia, and Webley *et al.* (238) showed that the plants colonizing the partly reclaimed dunes on the Scottish coast had, in their rhizospheres, very much larger numbers of microorganisms than were found in the surrounding sand. It was known from early work that numbers of organisms in soil fall off rapidly with distance from the root; some data on this point have been provided by Glathe *et al.*, who buried Cholodny slides among and near the roots of growing plants (60). The validity of the qualitative differences in bacteria claimed to exist as between rhizosphere and control soil were questioned by Wallace & King as a result of a study of cereal plots (232). This gave rise to a paper by Lochhead & Rouatt (124) who criticised the taking of the control samples by Wallace & King and gave an interesting summary of data from a considerable number of experiments showing qualitative differences between rhizospheres and control soil, in the percentage of bacteria requiring amino acids.

Compounds secreted by the roots of plants must obviously have a very marked influence on the rhizosphere. Samtsevich found the highest numbers of organisms in tree rhizospheres during the autumn, and suggests that root

secretions may be most abundant at this time (183). Kerr found that some roots secrete a substance stimulating the growth of fungi (104). Good progress in the study of the nature of the compounds secreted by roots is reported by Katznelson, Rouatt & Payne (103) and by Rovira (178). The former authors found that drying plants to wilting point and remoistening greatly increased the secretion of amino acids, and enabled that of reducing substances to be detected. They suggested that alternate drying and wetting of soil may produce a similar and possibly important effect in the field (103). The presence of growth factors in the rhizosphere can also be due to their synthesis by microorganisms, as is shown by the work of Lochhead and his colleagues referred to above (27). The specific effects on the microflora of living roots in contrast to dead plant material is shown in a paper by Rouatt & Lochhead, who found higher percentages of bacteria requiring amino acids in the rhizospheres of wheat, oats, flax, timothy, red clover and lucerne, than in the control soil. When materials from the same plants were added to and allowed to decompose in the soil, no important changes in the proportions of different nutritional groups of the soil bacteria could be detected by the same technique, with the interesting exception of lucerne. The addition of lucerne increased the proportion of bacteria requiring B_{12}, known to be produced by *Rhizobium meliloti* in exceptionally large amounts (27, 177).

Rhizobium

The wide and varied field covered by studies of legume nodules and of *Rhizobium* has been the subject of review by Wilson & Burris (245), Thornton (215), Allen & Baldwin (2), Virtanen (225), and Nutman (154), the last dealing more particularly with the relation of host plant physiology and genetics to infection and nodule behaviour. It is not therefore proposed to attempt any cover of this field in the present review. But one aspect of it has come into prominence recently and may be briefly discussed, because of its bearing on the ecology of microorganisms in soil. This is the competition between strains of *Rhizobium* in the relation to the establishment of a culture used for inoculation. The practical importance of this depends on the existence of areas where local strains of *Rhizobium* that are ineffective on the crop that is to be sown, are prevalent in the soil.

Among clover nodule bacteria there is a tendency for strains isolated from subterranean (*Trifolium subterraneum*) crimson (*T. incarnatum*) or cluster (*T. glomerulum*) to be ineffective on white clover *T. repens* and *vice versa* [Baird (10), Vincent (223)]. One cause of the prevalence of strains ineffective on the crop to be sown may thus be the natural prevalence or frequent cultivation of other clovers on which these strains are effective. Thus Vincent found that in the Lismore region of New South Wales, where *T. repens* is the commonest species, nearly all the strains of *Rhizobium* tested were ineffective on *T. subterraneum* and on *T. incarnatum* (223). It may, therefore, be necessary to introduce an effective strain where it is desired to sow a crop variety on land in which the predominant strains are ineffec-

tive, and this must be done in competition with the strains already existing in the soil. It is known that strains of *Rhizobium* differ markedly in the ability to compete with each other for growth and nodule formation in the host. In choosing a strain for use as an inoculant it may be necessary to select one not only effective on the crop but dominant in establishing itself in the crop in competition with the strains already in the soil. The study of strain establishment has been facilitated by extensive surveys that have been made of the antigen relationships of clover nodule bacteria. The use of serologically identifiable strains of *Rhizobium* has enabled the percentages of nodules produced by each of a mixture of strains or by the inoculant strain in field trials, to be determined. Read, using this method in field trials distributed over Great Britain, found that strains used to inoculate *T. pratense* differed greatly in the percentage of the total nodules that each produced in the crop and that the most successful strains in this respect differed with the locality (171). Vincent and his colleagues, using single strains and mixtures of them as inocula, also found that strains differed markedly in the percentages of the nodules that each produced, and also in their effect on crop growth. [see Vincent & Waters (224), Jenkins, Vincent & Waters (94) and Baird (10)].

The factors involved in competition for nodule formation are complex. They involve some, inherent in the host plant and the bacterial strain, that influence the process of infection. These are discussed by Nutman (154). Strains of *Rhizobium* also differ in ability to compete with each other outside the root and are variously affected by other components of the soil micropopulation. These include antagonistic organisms [Hely *et al.*, (75)], but Harris also found organisms in the rhizosphere which increased nodule numbers produced by a strain of clover *Rhizobium* in *in vitro* culture (72). These environmental factors will influence the proportions of different strains in the rhizosphere, and hence the chances of infection of each strain. Plants can receive sufficient effective nodulation by an effective strain even when also bearing ineffective nodules, as was shown by Burton, Allen & Berger with *Phaseolus* (26). On the other hand, Harris, in pot experiments in sterilised soil and sand, found considerable competition between effective and ineffective strains as judged by growth of the plants (73). The relative numbers of effective and ineffective nodules produced by competing strains is probably of importance beyond some limiting ratio, which may differ with the type of nodulation of each strain and under different conditions. It should be remembered that the number of cells of *Rhizobium* introduced by seed inoculation may be small compared with those in the soil, so that, when the latter are ineffective, a competitive inoculant strain may be needed to ensure a sufficient percentage of effective nodules.

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