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Biology and soil fertility

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GENERAL CONSIDERATIONS

Soil fertility is the capacity of the soil to support the growth of agricultural plants. It depends on the activity of several physical, chemical and biological factors which are often closely associated in providing a favourable growth medium for the plants.

Although higher plants can complete a normal growth cycle in a sterile medium containing only inorganic salts, living organisms (microbes as well as higher plants and animals) are indispensable to adequate plant growth under natural conditions, i.e., for providing soil fertility. The biological activities are mostly of chemical (biochemical) nature, but physical characters of the soil may also be involved.

One of the most important features of biological action in relation to plant growth concerns the conversion of essential elements from organic into inorganic compounds in which form these elements are normally assimilated by the higher plants. This applies particulary to elements such as carbon and nitrogen which occur in large quantities in living organisms and their debris. Without degradation of organic carbon compounds by animals and micro-organisms in the soil and in natural waters, the carbon dioxide content of the atmosphere would be exhausted within a few years. Plant production would decrease and ultimately come to a standstill, soon followed by a cessation of animal and human life. Maintenance of the carbon cycle, therefore, is one of the most important functions of micro-organisms in nature. In spite of its essential function in plant growth, CO_2 is usually not included in studies concerning soil fertility, because it is taken up mainly from the atmosphere. Therefore, its supply to the plants is much less associated with a particular soil than is the case with nutrient elements like nitrogen, phosphorus, etc.

Microbial activities similar to those involved in the conversion of carbon compounds occur in nature in the transformation of nitrogen and sulphur compounds (N and S cycles, respectively), and to a lesser extent in the conversion of other elements.

Without the activity of micro-organisms there would be no decomposition of plant and animal residues in the soil and thus no supply of inorganic N and S compounds to the plants unless fertilizers had been used. Animals may break down part of the organic nitrogen to urea or uric acid and part of the organic sulphur compounds to tauric acid but without further decomposition by micro-organisms these compounds cannot or can only poorly be used by plants.

Since micro-organisms are active in different phases of the nutrient element cycles, microbial processes may affect soil fertility in different ways. This applies particularly to nitrogen which is required in large amounts by living organisms and whose supply therefore is closely associated with soil fertility. In addition to various types of nitrogen-fixing micro-organisms which may contribute materially to soil fertility, organisms which are active in some phase of the degradation process of organic N-containing compounds are also beneficial to the nitrogen supply of higher plants. However, their direct effect on plant growth depends on various other soil factors. In the presence of relatively large amounts of readily decomposable carbon compounds (i.e., at a high C/N ratio of the disintegrating plant residues), the liberated amino acids or ammonia may be utilized by the soil micro-organisms for the synthesis of their cellular material (turnover of nitrogen compounds) and as a consequence these N compounds become, at least temporarily, unavailable for plant growth.

Although after the death and the decomposition of the microbial cells most of the organic N will be liberated, part of it will be bound in organic compounds which are resistant to microbial decomposition. This material, together with resistant plant residues, will contribute to the humus fraction of the soil. Its nitrogen is almost inaccessible to plant growth.

Further microbial reactions in the nitrogen cycle which may affect the nitrogen supply of the crops, are nitrification and denitrification. Although a ready nitrification indicates a good fertility level of the soil, losses of nitrogen as a result of this process may occur: (a) on slightly acid soils where accumulation of nitrous acid may give rise to volatilization; and (b) on well-drained soils where under wet conditions a considerable part of the nitrate may be leached out; this would not have been lost if no nitrification had taken place. Denitrification, the microbial conversion of nitrate to gaseous N compounds (N_2, N_2O), may give rise to serious losses of nitrogen and thus always has an adverse effect on soil fertility.

Although from a microbiological point of view the sulphur cycle is as interesting and important as the nitrogen cycle, the direct effect of the former cycle on soil fertility is much less spectacular than that of nitrogen. This depends on the following physiological and agricultural factors.

1. The much lower requirement of plants for sulphur as compared to that for nitrogen.
2. Since this is also true of micro-organisms, immobilization of sulphur in cell constituents involves much lower amounts than may be found for nitrogen.
3. In several soils sulphates or sulphides may be released from the parent rocks by weathering.
4. Considerable amounts of volatile S compounds, particularly SO_2, derived from the combustion of coal, oil, etc., may be brought to the soil in the precipitation. Direct uptake from the atmosphere by the plants or by the soil is also possible.
5. Although under anaerobic conditions sulphate, like nitrate, may serve dissimilatory reactions, sulphate reduction requires much more reduced soil conditions than denitrification. It occurs only in submerged soil, whereas denitrification may also be found in normal soil containing more or less excessive amounts of water.

Factors 1 to 5, together with the fact that considerable amounts of sulphate are introduced into the soil with certain N, P and K fertilizers, are responsible for the fact that sulphur deficiency is seldom the cause of serious reductions in yields of agricultural crops. Investigations into the relationship between soil fertility and conversion of S compounds hardly exist in the literature. It may be expected, however, that the sulphur supply through N, P or K dressings in the future will decrease considerably as the result of increased use of sulphate-free fertilizers. Utilization of gas and soil instead of coal brings about a decreased contamination of the atmosphere with SO_2 and may cause a further reduction of the S supply. It may therefore be expected that, in the future, S deficiency will become a more serious problem in agriculture (Whitehead, 1964). This undoubtedly will result in studies of the relationship of the S cycle and soil fertility.

The influence of biological processes on the availability of most other nutrient elements in soil is less spectacular than that on nitrogen and sulphur. The reason for this is the relatively loose binding of most of these elements in the plant material so that, upon the death of the plant, they are easily liberated without the assistance of micro-organisms. This does not apply to phosphorus, however, which may be contained in a number of organic compounds. A further reason for the reduced effect of micro-organisms on the availability of most of these elements is that they do not occur in the volatile phase.

In some instances, however, specific microbial reactions in soil are involved in rendering nutrient elements unavailable to higher plants. This concerns, for instance, the oxidation of manganous compounds to manganic oxides by certain soil fungi and soil bacteria, and the fixation of copper by hydrogen-sulphide-producing bacteria. The oxidation of ferrous to ferric iron also belongs to this group of microbial reactions.

Chelating substances and humus compounds which are products of microbial activity may bind metal ions and therefore affect the availability of the latter to the plants.

A further way in which micro-organisms may alter the availability of plant nutrients is by affecting the oxidation-reduction potential or the pH of the soil.

Humus formation is a further example in which soil fertility is improved in various ways by the association of various types of higher organisms and microbes. It prevents leaching of plant nutrients, affects the physical properties of the soil by improving its water-holding capacity and structure, buffers the soil reaction and, by its slow decomposition, may supply the plants with a certain amount of nutrients.

Fungal hyphae, earthworms, living roots, undecomposed plant material and products of microbial metabolism, in addition to humus, are thought to play a part in producing a good soil structure, i.e., a high degree of stability of the soil aggregates.

Nitrogen cycle and soil fertility

INTRODUCTION

Of all the nutrient elements taken up by the higher plants from the soil, nitrogen is required in the largest amounts. This is due to the fact that proteins and nucleic acids, the building blocks of living material, consist of more than 15 per cent nitrogen. Therefore, large amounts of this element are required for optimal plant growth and maximal crop yields. For example, the production of 10,000 kg (dry matter) of herbage, a yield which may easily be obtained per hectare annually under the moderate climatic conditions of Western Europe, requires approximately 300 kg of nitrogen (Mulder, 1949). A yield of 50,000 kg of sugar beets requires the uptake of some 200 kg of nitrogen and that of 5,000 kg of wheat 120 kg. Since declining amounts of available nitrogen give proportionally declining crop yields, it is evident that nitrogen supply of the plants is one of the fundamentals of agricultural production.

Although on fertile soils containing adequate amounts of decomposable organic matter a considerable part of nitrogen required for optimal yields is derived from the soil, a supplementary amount has often to be supplied in the form of fertilizer. The lower the mineralization capacity, the higher the amount of nitrogenous fertilizers to be added. This does not apply, of course, to those plants which are able to fix elementary nitrogen, and applies to a lesser extent to non-nitrogen-fixing plants growing in association with nitrogen-fixing plants. This occurs in many pastures, particularly those of short duration, the so-called 'leys'.

The amount of soil nitrogen annually supplied to the growing crops, although widely varying on different soils or following different crops, are more or less constant under fixed conditions of agricultural use and of climate. This depends on the fact that under such constant conditions most soils tend to an equilibrium between mobilization of organic nitrogen and immobilization of inorganic nitrogen (including fertilizer N). However, when the conditions are changed by, for instance, liming of acid soils, introduction of organic material having a high C/N ratio, ploughing up of a pasture, etc., a pronounced shift in the equilibrium may occur, resulting in a change in the supply of soil organic nitrogen to the plants.

A good example of improved soil fertility due to an increased supply of soil nitrogen upon liming of an acid soil was obtained by the senior author in a field experiment on a low moor peat soil of pH 4.2 (Mulder, 1950*a*). This soil contained approximately 60 per cent organic matter and 40 per cent clay. About 60 tons of $CaCO_3$ were required to raise the pH to neutrality. The amount of soil nitrogen liberated by increased microbial activity after liming was determined by estimating the response of potato and cereal crops to increasing amounts of fertilizer nitrogen on both the acid and neutralized plots. In the first year after liming the improvement in nitrogen supply was slight, but in the three subsequent years quantities of soil nitrogen amounting to approximately 60 kg were liberated annually. Microbiological analyses revealed that the liming had increased the numbers of actinomycetes to a much larger extent than those of bacteria, indicating that particularly the former micro-organisms were responsible for the decomposition of the soil organic matter.

Although higher plants take up nitrogen mostly in the inorganic form (nitrate or ammonia), certain amino acids can also readily be taken up (Ghosh and Burris, 1950; Mulder, 1948). However, under natural conditions nitrate and ammonia (i.e., ammonium ions) are the common N sources. Since in most fertile soils of neutral or alkaline reaction ammonia is readily converted to nitrate by the nitrifying bacteria, nitrate would be expected to be the normal N compound taken up by the plant roots from such soils. Nevertheless, in several instances the uptake of nitrogen may be expected to occur as NH_4 ions rather than as nitrate. This will be the case in acid soils or in soils covered with a dense vegetation (pastures) where nitrification proceeds less readily and a large part of the added ammonium salts is absorbed by the plant root before nitrification has taken place.

In addition to the direct effect of added ammonium compounds and nitrates on plants growing in soils, an indirect effect is sometimes observed which may interfere with nitrogen nutrition (different effect of ammonium compounds and nitrates on soil pH, different effect of the nitrogen compounds on the uptake of other nutrients, etc.). A good example of such an indirect effect is the relationship between nitrogen and magnesium nutrients on the growth and yield of agricultural crops. In an elaborate study the senior author showed that nitrates exert a highly stimulatory effect on the uptake of magnesium ions by plants, even on soils poor in magnesium (Mulder, 1956). Owing to this effect, cereal plants showing serious symptoms of magnesium deficiency may resume normal growth after a top dressing with a mixture of nitrate and a soluble magnesium salt and often with nitrate only.

In contrast to nitrates, ammonium salts exert a highly antagonistic effect on the uptake of magnesium ions under soil conditions. Therefore, on soils moderately poor in available magnesium on which excellent crops may be obtained with nitrate as the N fertilizer, detrimental results often will be seen when ammonium salts are applied as the nitrogen source.

In addition to this direct effect of N compounds on magnesium nutrition, these compounds may exert an indirect effect by altering the soil pH. Ammonium salts, in contrast to nitrates, render the soil more acid and this has an adverse effect on the content of plant-available magnesium (Smit and Mulder, 1942).

The ammonium ions derived from microbial decomposition of organic matter or from fertilizer dressing may be subjected to various types of immobilization, fixation and volatilization reactions, in addition to nitrification and uptake by the plant roots. Immobilization in microbial cell material may be temporary but chemical fixation in humus compounds is much more stable and is highly resistant to microbial attack. Fixation of NH_4 ions by clay minerals may render the nitrogen almost unavailable for plant growth. In alkaline soils part of the ammonium compounds may be lost by volatilization. On slightly acid soils volatilization losses of nitrogen may occur after the conversion of the ammonia to nitrite by bacteria of the genus *Nitrosomonas*.

Similarly to ammonia, nitrate may be subjected to a number of processes causing more or less serious losses of nitrogen. In contrast to ammonia which is readily utilized as the N source by the heterotrophic soil microflora, nitrate is only used as the nitrogen source by the micro-organisms after all the ammonia has been consumed. However, under anaerobic conditions, nitrate may be readily used as the terminal hydrogen acceptor in dissimilatory reactions by denitrifying bacteria, resulting in the formation of gaseous products like N_2 and N_2O which are lost into the atmosphere. Nitrate, in contrast to ammonia, is not adsorbed by the soil colloids and, therefore, it is easily leached when precipitation exceeds evaporation.

FIXATION OF ELEMENTARY NITROGEN BY MICRO-ORGANISMS

Nitrogen fixation is carried out by two types of biological systems: (a) the free-living micro-organisms which fix the elementary nitrogen without the aid of other organisms; and (b) the plant-bacteria symbioses in which the nitrogen is fixed by close co-operation between higher plants and micro-organisms, particularly in root or leaf tissues, the so-called root and leaf nodules. A special type of N_2 fixation is found in the phyllosphere (microbial flora on leaves) and possibly in the rhizosphere, where free-living nitrogen fixers may be active in fixing N_2 in co-operation with the higher plants which excrete the necessary carbon compounds.

NITROGEN FIXATION BY FREE-LIVING MICRO-ORGANISMS

Three types of free-living nitrogen fixers may be distinguished, namely: (a) aerobic bacteria, mainly those of the *Azotobacter* type; (b) anaerobic bacteria, particularly of the genus *Clostridium*; and (c) blue-green algae.

Aerobic nitrogen fixers

Aerobic bacteria of the *Azotobacter* type occur in many soils throughout the world. The most frequently occurring species is *A. chroccoccum* which is found only in neutral and alkaline soils. *A. agilis*, an aquatic bacterium forming large spherical cells (3 to 4 μ broad), occurs in neutral or alkaline waste water containing adequate amounts of carbon compounds but poor in nitrogenous compounds. We found them in large numbers in waste water of straw board factories in Groningen.

Jensen (1955) has isolated an azotobacter from acid soils, *A. macrocytogenes*, which is able to grow and fix nitrogen in acid media. However, this organism is very rare in acid habitats so that almost all acid soils in temperate and cold climates contain no azotobacters. This is in contrast with many acid soils in tropical and subtropical areas where a different azotobacter type (*Beijerinckia*) is found (Becking, 1961*a*, *b*; Derx, 1950; Starkey, 1939).

In addition to azotobacters, many other types of aerobic bacteria have been found to be able to fix small amounts of elementary nitrogen, for instance representatives of the genera *Pseudomonas*, Anderson (1955), *Achromobacter*, Proctor and Wilson (1959) and *Bacillus*, Grau and Wilson (1962). Relatively large amounts of nitrogen were found to be fixed by a number of *Arthrobacter* strains (Smyk and Ettlinger 1963). In Russian soils the occurrence of N-fixing mycobacteria has been recorded (Fedorov and Kalininskaya, 1961). They may be the same type of organism as described by Smyk and Ettlinger since the mycobacteria in Krasil'nikov's nomenclature include the corynebacteria according to Bergey's nomenclature (Jensen 1952; Krasil'nikov, 1958).

Anaerobic nitrogen fixers

In addition to *Clostridium pasteurianum* many other representatives of the saccharolytic clostridia are able to fix moderate amounts of elementary nitrogen. Certain strains of methane-forming bacteria (Pine and Barker, 1954), and many representatives of the Rhodobacteriineae, when growing anaerobically in the light, are also able to fix N_2.

Blue-green algae

The majority of this group of micro-organisms form long filaments consisting of chains of large cells surrounded by a common envelope. Several of these algae, among them the well-known *Anabaena* and *Nostoc*, are able to fix atmospheric nitrogen. A close correlation between the nitrogen-fixing ability and the presence of heterocysts (thick-walled colourless empty-looking cells) is recorded by Fogg and co-workers (see Stewart, 1966). The ability to fix N_2 in addition to performing photosynthesis confers on a number of blue-green algae the capacity to pioneer bare mineral soils where nitrogen compounds are lacking.

Nitrogen-fixing blue-green algae are found in many soils throughout the world. They are particularly abundant in the moist tropics where, under certain conditions, they may contribute appreciably to the nitrogen supply of plants (wet rice fields, De and Mandal, 1956; Singh, 1961; Watanabe, 1966).

Nitrogen fixation may also occur in associations of blue-green algae with fungi (lichens) and with higher plants (*Azolla*, *Cycas*, *Gunnera*, etc.).

Contribution of free-living nitrogen fixers to the nitrogen economy of natural communities and to the fertility of soils

Nitrogen fixation by azotobacters and, presumably, also by other free-living nitrogen fixers is carried out only by growing cells. The amount of nitrogen fixed is mainly used for the synthesis of the bacterial cells. This applies particularly to the aerobic bacteria of the *Azotobacter* type which synthesize moderate amounts of cell material per unit of carbon compound consumed (100-150 mg of dry weight cells corresponding to 10-15 mg N_2 fixed per gramme of sugar consumed). In the anaerobic organisms the amount of cell material formed per unit of substrate consumed is much lower and here a considerable part of the fixed nitrogen is excreted.

From the above-mentioned data it will be seen that the amount of N_2 fixed by azotobacters depends on the amount of N-containing cell material synthesized per unit of carbon compound consumed. The efficiency of this synthesis is generally low because large energy losses occur, presumably owing to the very high respiration rates of azotobacters (QO_2-values as high as 2,000 occur). In addition to these energy losses, relatively large amounts of substrate are converted by azotobacters to extracellular slimy compounds which presumably are of low value in nitrogen fixation. Both phenomena may explain the small amount of cell material synthesized per unit of substrate consumed by nitrogen-fixing azotobacters. Since, theoretically, considerably larger amounts of cell material could be synthesized per unit of substrate consumed, the amount of N_2 fixed by these bacteria could be considerably increased. This was achieved under laboratory conditions (Meyerhof and Burk, 1928; Parker and Scutt, 1960) by reducing the oxygen tension of the atmosphere. This presumably decreases the growth rate of the azotobacters but it increases the efficiency of cell synthesis.

The beneficial effect of reduced O_2 concentrations on N_2 fixation by aerobic bacteria has also been found in other types of nitrogen fixers, for instance in the facultative anaerobic *B. polymyxa* (Grau and Wilson, 1962; Hino and Wilson, 1958). It is thought to be directly related to the process of N_2 fixation.

Becking (private communication) obtained an increased efficiency of the N_2 fixation in *Beijerinckia* by reducing the concentration of carbonaceous substrate in the nutrient medium.

Although the free-living nitrogen-fixing bacteria undoubtedly contribute to the nitrogen economy of natural vegetation and thus to soil fertility, their contribution to the nitrogen nutrition of agricultural plants in general is thought to be of little impotrance. This is concluded from the following facts.

1. The above-mentioned low efficiency of the fixation process under laboratory conditions. Large amounts of carbonaceous substrates must be available to the nitrogen fixers to bring about any significant increase of nitrogen in the soil.
2. Several nitrogen-fixing bacteria, when supplied with combined nitrogen, utilize the latter and fix hardly any N_2.
3. Under such conditions (fertile soils) the nitrogen fixers will be easily depressed by non-nitrogen fixers which utilize part of the carbonaceous substrates and of the N compounds.
4. The numbers of *Azotobacter* and *Beijerinckia* cells counted in soil are generally low (less than 1,000 per gramme of soil).

In such cases it is hard to believe that any nitrogen fixation of importance takes place. Clostridia are found in many soils in much larger numbers, but this may be due to the high percentage of spores which do not participate in nitrogen fixation.

In spite of these negative conclusions there are some indications that, under particular soil conditions (high water content and copious supply of plant residues), free-living nitrogen-fixing bacteria of the *Azotobacter* type contribute in a more substantial way to the nitrogen content of the soil. Since a high water content of soil is accompanied by a poor oxygen supply, these results are in agreement with the above-mentioned laboratory experiment which showed the important effect of a reduced oxygen supply to azotobacters and some other aerobic and facultative anaerobic bacteria on the efficiency of nitrogen fixation.

A number of such examples of increased nitrogen fixation by azotobacters under soil conditions is given by Jensen in his review of non-symbiotic nitrogen fixation (Jensen, 1965). Gains of nitrogen, calculated on the basis of organic matter consumed, in soils mixed with beech or oak leaves were sometimes higher in water-saturated soil than in moderately moist soil (Olsen, 1932). Similar results were obtained by Jensen (1940) in nitrogen-poor soil or in sand-kaolin mixtures supplied with straw or root organic material. Abundant growth of azotobacters and important gains of soil nitrogen were obtained when the water content of the soil was raised to saturation level. In addition to a direct effect of the reduced oxygen supply on nitrogen fixation, an incomplete breakdown of cellulose and other insoluble constituents of the straw in the waterlogged soil by the microflora, supplying the azotobacters with more adequate carbon compounds, may have been responsible for the increased nitrogen fixation. The maximum numbers of azotobacter counted in this experiment were nearly 10^7 per gramme of soil, numbers which are comparable with two other exceptionally high values reported in the literature, namely 10^8 (Abd-el-Malek and Ishac, 1962) and 10^{11} (Rouquérol, 1962). The former were found in irrigated clay soils of the Nile Valley, the latter in rice fields. In both cases large amounts of plant residues were available. In earlier investigations with rice fields (Uppal *et al.*, 1939) a maximum number of 1.6×10^6 azotobacters was counted.

In Jensen's experiments maximum amounts of 6 mg atmospheric nitrogen were found to be fixed by the azotobacters per gramme of straw added. When 1 per cent straw was given, as in Jensen's experiments, this corresponds to approximately 100 kg N per hectare. It should be kept in mind, however, that the addition of such large amounts of nitrogen-poor organic matter for a prolonged period of time will bring about immobilization of soil nitrogen, so that serious symptoms of nitrogen deficiency may be expected to occur in the crops grown on the treated soil. Application of fertilizer nitrogen may eliminate the deficiency symptoms, but it also depresses the fixation of elementary nitrogen.

Barrow and Jenkinson (1962) recently found no N_2 fixation in mixtures of soil and straw of moderate water content, but obtained gains of almost 3 mg nitrogen per gramme of straw in waterlogged soil. No bacteriological investigations were made by these authors but the fact that no nitrogen was fixed when the soil was incubated in a nitrogen atmosphere strongly indicates that aerobic nitrogen fixers were involved.

More evidence of the importance of reduced oxygen content in the soil is provided by the experiments of Delwiche and Wyler (1956) employing labelled nitrogen ($N^{15}{}_2$). No nitrogen fixation was found in non-enriched soils or in soils supplied with straw, grass or grass roots. However, in decaying grass sods with a restricted oxygen supply, maximum amounts corresponding to 13 kg N per hectare were found to be fixed in one month. Similar results were obtained by Chang and Knowles (1965) in experiments with labelled nitrogen. Under anaerobic conditions annual gains of 20 to 70 kg N per hectare were found, whereas only 10 kg was fixed under aerobic conditions.

Large amounts of fixed nitrogen were claimed for grassland soils by Whitt (1941) and Parker (1957). The former estimated annual nitrogen gains of more than 100kg/ha, the latter of 65 to 75 kg/ha. The validity of these and other data has been thoroughly discussed by Moore (1966).

Blue-green algae and soil fertility

Nitrogen-fixing blue-green algae may contribute to the nitrogen economy of the soil when light, water and supply of nutrient elements enable their undisturbed growth for a prolonged period. In such cases large amounts of algal material are formed which, after bacterial decomposition, may supply considerable amounts of available nitrogen to the plants.

Optimal conditions for the growth and the nitrogen fixation of blue-green algae are found in flooded rice fields. Undisturbed soil surfaces, particularly in humid tropical regions, represent a further example where these algae may contribute to the nitrogen content of the soil.

Although under laboratory conditions considerable amounts of nitrogen are fixed by several species of blue-green algae, large differences exist in the literature between the calculated values (kg N per hectare) derived from the results of laboratory experiments and the values found in field experiments with rice crops. Some authors (recorded by Jensen, 1965) calculated gains for fixed nitrogen as high as 2 and 10 kg/ha/day. These very high values are in contrast with the gains derived from field experiments with rice in Japan (Watanabe, 1966). In a large number of such experiments, carried out by various Japanese workers, a comparison was made between the yield of rice of uninoculated control plots and that of plots inoculated with the algae *Tolypothrix tenuis* (belonging to the family Scytonemataceae, order Nostocales) which was found by Watanabe to give high values for nitrogen fixation. Increases in rice yield on different experimental fields varied between 0 and 8 per cent in the first year and between 0 and 56 per cent in the third year of the experiments. In one field experiment the amount of N_2 fixed per hectare upon inoculation with *Tolypothrix tenuis* was found to be similar to the effect of a dressing with 72 kg/ha of ammonium sulphate. In a further experiment, in which $N^{15}{}_2$ was used, approximately 27 kg N per hectare was found to be fixed under field conditions during the growing period of the rice.

Considerably higher values for yield increase after inoculation with a nitrogen-fixing blue-green alga (*Aulosira fertilissima*, belonging to the Nostocaceae) are given by Singh (1961) for rice grown in India, namely 114 per cent in a field experiment and 368 per cent in a pot experiment. Similar results are recorded by Rao *et al.* (1963). These very high responses as compared with those of the Japanese experiments were partly due to the much lower yields of the control plots. Using a rather approximative method, Singh reports a gain of nitrogen of nearly 100 kg N per hectare within 75 days on a moist undisturbed Indian soil covered by a growth of blue-green algae. This value seems to be high compared with the moderate gains of the Japanese workers and further study is needed to obtain more data on the amounts of N_2 fixed under field conditions.

Nevertheless, the large increase in rice yield in India due to the nitrogen-fixing activity of blue-green algae in the paddy fields may be considered as promising for the application of selected species of these algae as inoculum. The favourable climatic conditions and the low level of nitrogen fertilization in India and other southern-Asiatic countries will promote the efficiency of this type of biological contribution to soil fertility and plant growth.

In addition to the climatic factors (humidity, light, temperature) and favourable nutritional conditions (adequate phosphate and molybdenum supplies, neutral or slightly alkaline pH), the presence of bacteria growing in association with the blue-green algae may have a pronounced effect on N_2 fixation and growth of the algae (Bjälfve, 1962; Wieringa, Laboratory of Microbiology, Wageningen, unpublished results). The presence of plants is a further factor which may favour N_2 fixation by algae. This effect was shown by De and collaborators (1956, 1950) in experiments with rice plants. Since aeration with CO_2 gave the same result, the beneficial effect of the plants was thought to be due to an increased CO_2 supply, stimulating photosynthesis and, indirectly, also N_2 fixation. Such an effect of increased CO_2 supply has also been observed by Wilson (1946) in leguminous plants. However, a direct effect of CO_2 on N_2 fixation, as was observed in the present authors' laboratory (Mulder and Veen, 1960*b*) with clover, pea, and bean plants, is also possible.

Nitrogen-fixing blue-green algae, in collaboration with other types of algae, may improve the fertility of uncultivated alkaline soils in arid regions by forming a dense layer of algal material during the periods of rainfall. This depends on their ability to grow on saline soils at alkaline reactions and to survive a drought period. During such a period many algal cells die, but none of the species represented will disappear entirely and with continued desiccation the population remains fairly constant for several years (Shields and Durrell, 1964). When the wet period starts, the algae immediately resume growth.

In addition to supplying nitrogen and organic matter which have a highly favourable effect on soil fertility, the presence of the algal crust protects the soil against erosion (Singh, 1961).

NITROGEN FIXATION BY MICROBIAL ASSOCIATIONS AND BY BACTERIA-PLANT ASSOCIATIONS (RHIZOSPHERE AND PHYLLOSPHERE SYSTEMS)

Microbial associations

Increased nitrogen fixation by free-living bacteria as a result of associated growth with non-nitrogen fixers can be attained in different ways. Organisms decomposing polysaccharides unavailable to the nitrogen-fixing bacteria to readily available compounds like sugars and organic acids may have a highly stimulating effect on nitrogen fixation. A good example is the degradation of cellulose by some types of cellulose-decomposing micro-organisms. Jensen (1965) describes the association of a facultative aerobic *Corynebacterium* with *Azotobacter* and *Beijerinckia* spp. which fixed amounts of N_2 from 10 to 14 mg per gramme of cellulose decomposed. Restricted aeration is reported to have increased the efficiency of the N_2 fixation, partly because more degradation products of the cellulose-decomposing bacteria accumulated, partly because of the higher efficiency of the N_2 fixation process.

Several aerobic cellulose-decomposing bacteria are recorded by Jensen to have no influence on N_2 fixation when growing with cellulose as the substrate in association with azotobacters. However, with alternating aerobic and anaerobic cultivation such associations sometimes may give moderate gains with cellulose as the carbon source (Vartiovaara, 1938). Associations of anaerobic cellulose-decomposers and *Clostridium butyricum* were found to assimilate 7-10 mg of N_2 per gramme of cellulose fermented (Fedorow, 1960; Imschenezki, 1959).

In addition to cellulose, other polysaccharides may serve as substrates for nitrogen fixers (azotobacters, clostridia) when growing in association with particular organisms.

A somewhat comparable type of nitrogen fixation by associations of micro-organisms has been described by Kobayashi and collaborators (Katayama *et al.*, 1965; Kobayashi *et al.*, 1965). A *Rhodopseudomonas* strain, unable to fix nitrogen when growing as a pure culture in a glycerol or starch medium, fixed moderate amounts of N_2 in the presence of *B. megaterium* or *B. subtilis.* A further example of associated growth is the beneficial effect of several bacteria, including *Pseudomonas*, *Flavobacterium* and *Chromobacterium* strains, on the growth and N_2 fixation of a *Mycobacterium* strain (Fedorov and Kalininskaya, 1961).

A different type of associated growth, increasing the over-all efficiency of the nitrogen fixation process, is the mixed cultivation of a nitrogen fixer and a heterotrophic organism unable to fix nitrogen. Dommergues and Mutaftschiev (1965) found much higher values for the amount of N_2 fixed per gramme glucose consumed by mixed cultures of *Beijerinckia indica* and the yeast *Lipomyces starkeyi* than were obtained with pure cultures of the nitrogen fixer, namely 10 and 2 mg, respectively. The value for the pure culture of *Beijerinckia* in this experiment is abnormally low and it could be suggested that the yeast eliminated some abnormal growth factor responsible for the low efficiency of the N_2 fixation. However, Ruinen (Laboratory of Microbiology, Wageningen, unpublished results) in mixed cultures of *Azotobacter* and a *Rhodotorula* sp. also found a large increase in amount of N_2 fixed in spite of the fact that her control cultures fixed the normal amount of 7.0 mg N per gramme of glucose consumed; in the mixed culture she found values of 22 mg. The beneficial effect of this type of association is thought to be due to the excretion by the azotobacters of nitrogenous products which in pure cultures would be reabsorbed by the growing cells, thus reducing the fixation of further amounts of N_2. In mixed cultures, however, the excreted nitrogenous compounds are taken up by yeast cells so that further growth of *Azotobacter* is only possible when additional amounts of N_2 are fixed.

Nitrogen fixation in the rhizosphere

Plant roots excrete small amounts of various organic substrates which bring about a pronounced increase of microbial activity in the medium surrounding the roots, the rhizosphere. As the excreted substrates represent an almost complete, albeit dilute, nutrient medium including sugars, organic acids, amino acids and vitamins, it stimulates the development of a flora of non-nitrogen-fixing micro-organisms rather than that of azotobacters and beijerinckias which require a selective nitrogen-free medium for enrichment. It may be assumed, however, that the root exudates of different plants differ considerably in their carbon/nitrogen ratio; leguminous plants, for instance, excrete relatively large amounts of N-containing compounds, particularly amino acids, whereas sugar-cane and sugar-beets excrete relatively large amounts of sugars. In addition to the type of plant, their nitrogen supply is a further factor which may be expected to affect the C/N ratio of the exudates.

In spite of the probability that certain root exudates might constitute a favourable nutrient medium for the enrichment of azotobacters, few indications have so far been reported in the literature in support of this view (see Jensen, 1965). The fact that these organisms are not, or practically not, enriched in the rhizosphere, even when the carbon/nitrogen ratio of the exudates is high, is ascribed by some authors to acidification of the root surface and the soil immediately surrounding the root after the excretion of carbon compounds. If this assumption were correct, it might be expected: (a) that azotobacters respond to root exudates having a high C/N ratio when the roots are growing in heavily buffered alkaline soils; and (b) that on other types of soil nitrogen fixers which are not repressed by an acid reaction (beijerinckias, clostridia and perhaps *Pseudomonas* and *Aerobacter* spp.) are selected in the rhizosphere. This hypothesis is confirmed by the results recorded by Döbereiner (1961) who found increased numbers of *Beijerinckia* in the rhizosphere of sugar-cane and by those of Katznelson (1946) who found much larger numbers of clostridia in the rhizosphere of mangel roots than in the soil.

The possibility that part of the rhizosphere flora consists of nitrogen-fixing bacteria other than azotabacters, beijerinckias and clostridia is not excluded, but has so far not been proved.

The beneficial effect on plant growth of introducing particular strains of *Azotobacter* (azotobacterin) into the soil, as frequently observed by Russian workers, has been explained as a rhizosphere effect (Fedorow, 1960; Rubenchik, 1963). Although some authors have recorded the occurrence of large numbers of azotobacters in the rhizosphere after the application of azotobacterin, others have found no, or only slight, response. It is not known whether there is a correlation between the alkalinity of the soil and the growth of the introduced azotobacters in the rhizosphere. Several authors, although not denying that azotobacterin treatment in some cases may have a favourable effect on plant growth, do not believe that this effect depends on an improved nitrogen supply to the plants owing to increased *Azotobacter* activity. According to these authors, production of some growth factor (gibberellic acid?) or of a fungistatic compound by the inoculated azotobacter strain is responsible for the observed effect (Mishustin and Naumova, 1962; Rubenchik, 1963).

Nitrogen fixation in the phyllosphere

Excretion of organic compounds, providing a substrate for micro-organisms, is not confined to the roots. Leaves may also excrete such compounds and under certain conditions even large amounts of sugars and other substances (amino acids) have been found as exudates. In rainy weather the major part of the these compounds is washed off the plants into the soil where it favours the microbial activity.

Of much more interest, however, is the development on the surface of the leaves of a microbial flora which thrives on the leaf exudates. This microflora and its milieu, in analogy with the rhizosphere, is called the phyllosphere (Last, 1955; Ruinen, 1956).

The development of a phyllosphere flora, in addition to the type and the nutrient status of the plant, depends on climatic conditions. High temperatures, in combination with an abundant dew formation, as occur in the humid tropics, are highly favourable.

In an extensive investigation carried out in Indonesia and in Surinam, with leaves of cacao, citrus, coffee, and many others, Ruinen found large numbers of free-living nitrogen-fixing bacteria of the *Beijerinckia* type to occur in the phyllosphere. Azotobacters and nitrogen-fixing bacteria of the non-*Azotobacter* type were also found. On the upper surface of mature cacao leaves 10^7 azotobacters and 2.10^7 beijerinckias per square centimetre were counted (Ruinen, 1961). In addition to the common nitrogen fixers, several other types of bacteria (belonging to the genera *Aerobacter*, *Pseudomonas*, *Xanthomonas*, *Arthrobacter*), fungi, yeasts, and algae were shown by Ruinen to occur. Earlier investigations by Wieringa (1955) had revealed the occurrence of considerable numbers of pectin-decomposing micro-organisms on the leaves of various plants growing at moderate temperatures under the humid climatic conditions of Western Europe. The senior author (Mulder, 1940), studying the microflora of wheat glumen, found large numbers of various strains of the yellow *Pseudomonas trifolii* (*Bact. herbicola*). Grainger and Keddie (1963) isolated many strains of coryneform bacteria from grass.

From the fact that nitrogen-fixing bacteria occur in considerable numbers in the phyllosphere of many plants, Ruinen (1961) suggested that these organisms might contribute to the nitrogen supply of the plants. She considered that the rather large amounts of nitrogen which according to various reports (see Ruinen, 1961) accumulate in tropical soils under vegetation free from symbiotic nitrogen fixers might be explained by such a phyllosphere effect.

So far no quantitative data are available as to the contribution of the phyllosphere to the nitrogen nutrition of higher plants under natural conditions. In laboratory experiments with detached leaves of *Coffea*, *Gossypium* and *Phaseolus*, floating on a nitrogen-free mineral medium and exposed to light, large amounts of N_2 were found to be fixed by the phyllosphere flora (Ruinen, 1965). Inoculation with *Beijerinckia* or *Azotobacter* strains sometimes increased this amount. The leaves apparently provided the substrates required for the microbial N_2 fixation.

Nothing is known of the effect of the associated growth (N_2-fixing bacteria and plants or N_2-fixing and non-fixing bacteria) on the efficiency of the fixation process in the phyllosphere. As to the latter type of association, Ruinen (1966) stresses the presence in the phyllosphere of large numbers of yeasts, particularly *Rhodotorula* spp., which are able to decompose cutin, thus eliminating the cuticula. This promotes the exchange of organic compounds between the leaf cells and the phyllosphere microflora. Furthermore, it may be assumed that the degradation products of cutin serve as additional substrates for the nitrogen fixers. A different effect of the presence of the yeasts could be the increased efficiency of the nitrogen-fixation process as observed in the above-mentioned mixtures of yeasts and azotobacters. On the other hand, it must be stated that the leaf washings, although in general containing an excess of carbohydrous material, also contain moderate amounts of amino acids, which may be considered as inhibitors of N_2 fixation. In spite of the latter, a clear N_2 fixation took place when dew collected from cacao leaves was left standing at room temperature for two days.

A somewhat different type of phyllosphere activity was recently found by Ruinen (personal communications) to occur in Guatemala grass growing in the humid coastal area of the Ivory Coast. Nitrogen fixation in these plants occurs particularly in the liquid contained in the spaces between the stem and the leaf sheath surrounding the stem. This solution may contain up to 15 mg carbohydrous material per millilitre and it therefore represents an excellent medium for nitrogen-fixing bacteria. Non-azotobacter types of bacteria were found by Ruinen to be the most frequently-occurring nitrogen fixers.

SYMBIOTIC NITROGEN-FIXATION

Symbiotic nitrogen fixation is one of the most important biological processes affecting plant production and soil fertility. This is due to the large amounts of elementary nitrogen fixed by this system. These amounts, when calculated per unit of nitrogen-fixing cell material, are 100-200 times higher than those calculated for free-living N fixers such as *Azotobacter* (see Tabel 1).

Table 1. Fixation of N_2 by symbiotic and free-living nitrogen fixers

Plant	Nitrogen (mg) fixed per day		Nitrogen (g) fixed per 40 days[2] per dry weight gramme of N_2-fixing material
	Per gramme dry weight of nodule	Per gramme dry weight of N_2-fixing material	
Horsebean	38	190	7.6
Pea	98	490	19.6
Bean	67	335	13.4
Lupin	65	325	13.0
Vetch	80	400	16.0
Lucerne	67	335	13.4
Red clover	55	275	11.0
Azotobacter	—	100[3]	0.1

1. Figures taken from Wilson (1940), p. 88.
2. Average time of functioning of a nodule.
3. During the entire growing period.

The reason for these pronounced differences is that the average root nodule maintains its nitrogen-fixing capacity for 40 (30-50) days. The nitrogen fixed during this period is transported from the nodules to the other tissues of the plant where it is used for the synthesis of proteins, nucleic acids, etc. The free-living N_2 fixers, however, particularly those of the aerobic *Azotobacter*, generally fix no more nitrogen than is required for synthesis of their cell material, i.e., seldom more than 10-12 per cent of the dry-cell weight. No further nitrogen fixation takes place when the cell growth has ceased. For more details about the free-living nitrogen fixers and about the possibilities of raising their efficiency see page 167.

Two groups of symbiotic nitrogen-fixing systems are known, bacteria of the genus *Rhizobium* living in symbiosis with leguminous plants, and micro-organisms of a different type, presumably actinomycetes, living in symbiosis with a number of bushes and trees like

Alnus, *Myrica*, *Casuarina*, etc. (Bond, 1959, 1963). Leguminous plants are by far the most important symbiotic nitrogen fixers grown in agriculture; the non-leguminous nitrogen fixers are important in silviculture for improving the nitrogen economy of forest soils.

In addition to the above-mentioned systems in which the symbiotic nitrogen fixation takes place in root nodules, a third type may be found in the leaf nodules of certain tropical plants (*Pavetta*, *Psychotria*, etc., see Stewart, 1966).

The symbiosis leguminous plants-Rhizobium

The family of the Leguminosae is one of the largest families of the higher plants, comprising four sub-families (Mimosoideae, Caesalpinioideae, Swartzioideae, Papilionaceae), and approximately 500 genera and 1,300 species (Allen and Allen, 1958).

Members of this family can be found in the humid and semi-arid, in the tropical, sub-tropical, temperate and even in arctic regions (Allen and Allen, 1958; Allen *et al.*, 1964). Of a number of legumes, several lines adapted to particular environments exist, derived by natural selection and, in the case of economically important plants, obtained by breeding. From an agricultural point of view this is a very important phenomenon. It means that a better use could be made of leguminous crops for improving the fertility of the soil if symbiotic systems (species or lines) suitable for certain purposes and adapted to climatic and ecological conditions could be found. It should be kept in mind that this adaptation could involve the plant, the bacterium (*Rhizobium*) or both.

Not all leguminous plant species tested so far have been found to form nodules. Approximately 10 per cent of the species has been examined. Particularly the members of the Caesalpinioideae were found to be devoid of root nodules (Allen and Allen, 1958). This may have been due either to the absence of the appropriate bacteria or to the genetic constitution of the plant.

The genus *Rhizobium*, belonging to the family of the Rhizobiaceae, comprises a number of bacterial species (*Bergey's Manual*, 1957) which are able to form nodules and to fix nitrogen in symbiosis with certain leguminous species but not with others. *Rhizobium leguminosarum*, for instance, is able to form effective nodules on the roots of pea (*Pisum sativum*), *Vicia* and *Lathyrus* plants, but not on clover (*Trifolium*), lucerne (*Medicago sativa*) and many other legumes. *R. trifolii* forms nodules on various clover species, but not on pea, bean, etc. Groups of different plant species which may form nodules with the same *Rhizobium* species are called 'cross-inoculation groups'.

Bacteria of the genus *Rhizobium* can be distinguished from those of the genus *Agrobacterium* only by their ability to nodulate roots of leguminous plants. Similarly, the classification of the rhizobial species depends on their nodule-forming capacities.

Each *Rhizobium* species comprises a number of different strains which may differ in nodule formation, effectiveness of nitrogen fixation, etc. However, effectiveness of a nodule not only depends on the bacterium, but also on the legume and on the environmental conditions. The effect of the host plant on the symbiosis may particularly be seen from the work of Nutman and collaborators. *Rhizobium trifolii* strains isolated from subterranean clover were found to form effective nodules with this plant, but ineffective ones with red and white clover (Nutman, 1959, 1965). Certain strains of *R. leguminosarum* formed ineffective nodules on clover roots. This, however, did not alter their genetic character since upon re-isolation of the bacterial strain from the clover nodule and inoculation of pea plants they formed effective nodules. That bacterial mutations occur under natural conditions, so that certain *Rhizobium* strains may alter their host range, is probable but has not so far been proved. Kleczkowska (1950) obtained phage-resistant rhizobial mutants after treatment of sensitive *Rhizobium* strains with their specific bacteriophages. Such mutant strains were sometimes also altered in their nitrogen-fixing capacities. Mostly loss of this property was observed, but in some cases effective phage-resistant mutants were obtained from ineffective phage-sensitive strains.

Recently mutant strains of *Rhizobium* spp. have been obtained by transformation (see Nutman, 1965).

The effect of internal and external conditions on nodulation and nitrogen fixation of leguminous plants

Nodulation and nitrogen fixation by the nodules depend on the co-operation of a number of different factors, namely (a) the presence in the rooting medium of cells of an effective *Rhizobium* strain; (b) the increase in numbers of the *Rhizobium* cells in the rhizosphere; (c) the infection of the roots by the bacteria; (d) the growth; (e) the activity; and (f) the longevity of the nodules.

In order to obtain optimal N_2 fixation, the above-mentioned factors (a) to (f) should be optimal. An inadequate functioning of the over-all N fixation process always results from the inadequate functioning of one of the constituent processes. Boron deficiency, for instance, inhibits nitrogen fixation by preventing the growth of the nodule tissue; it does not seriously affect the process of nitrogen fixation (Mulder, 1948). Molybdenum deficiency, however, primarily affects the nitrogen fixation, but it has only a slight effect on the development of the nodules (Mulder, 1954; Mulder *et al.*, 1959).

The factors affecting N_2 fixation of the symbiotic plant-microbe system may be divided into internal and external ones. The former concern either the host plant or the microbe. The external factors include nutritional as well as climatic conditions. Nitrogen fixation and thus soil fertility can be improved by agricultural measure since several of these conditions can be altered.

Internal factors.

Effect of the host plant. The important effect of the host plant on the symbiosis is clearly shown in those cases where pronounced differences in nodulation and nitrogen fixation are observed between associations of one *Rhizobium* strain with different plant species or even plant varieties (lines).

A good example of the species effect is found in the clover group where most strains of *R. trifolii*, effective on the white-clover subgroup, are ineffective on the subterranean-clover subgroup. Similar effects by the species of the host plants have been observed in the *Medicago* and *Lupinus* groups.

Examples of differences in nodulation and nitrogen fixation between lines of red clover differing only in one gene have been given by Nutman (1959). In one line bacteroid formation and haemoglobin synthesis were prevented as a result of depressed multiplication of the *Rhizobium* rods in the host cells, in second line as a result of depressed transformation of rod-shaped cells into bacteroids. With both types of plants all the earlier stages of nodule development were normal. So far it is unknown which factor or complex of factors is responsible for the observed phenomena.

A non-nodulating line of soybean was found by Elkan (1961, 1962) to produce a factor able to decrease the number of nodules of plants of a nodulating line.

Substances toxic to rhizobia were obtained by Thompson (1961), Bowen (1961), and Masterson (1962) from the coat of several leguminous seeds, particularly from that of subterranean clover. Such substances may be harmful to the rhizobia when the latter are introduced into the soil by seed inoculation.

Root exudates of leguminous plants have a beneficial effect on the multiplication of rhizobia in the soil (rhizosphere effect). This was clearly shown in a pot experiment in which the numbers of *R. trifolii* were counted in a neutralized acid soil planted with red clover, and left unplanted (Table 2, see also Mulder and Veen, 1960*a*).

TABLE 2. Effect of planting on numbers[1] of *R. trifolii* in neutralized acid soil

Soil	Days after sowing				
	10	23	39	60	79
Unplanted	0	—	0	—	1
Planted	0	17	490	13 000	28 000

[1] Calculated per 0.1 g soil containing approximately 16 per cent water.

In addition to the nutritional effect on the rhizobia root exudates of leguminous plants may affect the symbiotic processes in other ways. The excretion of tryptophan which in the rhizosphere is readily transformed by micro-organisms into the growth substance indoleacetic acid (Kefford *et al.*, 1960), the excretion of gibberellin (Radley, 1961) and that of pectic enzymes (Fåhraeus and Ljunggren, 1959) are thought to contribute to root-hair infection and nodule growth.

A further effect of root exudates was studied by Lie (1964) in culture-solution experiments with bean plants (*Phaseolus vulgaris*). These plants were found to nodulate very poorly when grown during hot, sunny weather unless small amounts of an unknown growth substance excreted by young nodulated leguminous plants had been supplied. The active compound of the pea exudate which was soluble in water and in ether, but insoluble in chloroform, was inactivated above 80°C. In subsequent experiments a relationship was found to exist between the effect of the composition of the light (light quality) and the response to added root exudates of *Phaseolus*.

Effect of the bacterial strain. For successful nodule formation and nitrogen fixation *Rhizobium* cells capable of inducing nodules of the effective type must be present. These rhizobia must be able to survive in the soil, to multiply in the rhizosphere and to compete successfully with other root-nodule bacteria and other micro-organisms for sites on the roots. Often a high proportion of the rhizobial population of a soil belongs to the intermediate or even to the ineffective types. This is particularly true of acid soils (Harmsen, 1954; Holding and King, 1963). To raise the nitrogen-fixing ability and thus the yield of the leguminous crops, effective rhizobial strains able to compete successfully with the indigenous strains should be introduced into the soil.

Effective nodules are usually large and they have a pink colour in contrast to small yellow-white ineffective ones. The latter occur in much larger numbers on the root system than the former. Bacterial strains ineffective on certain legume species may sometimes form effective nodules on other species.

The presence in the soil of rhizobial strains capable of performing effective symbiosis with one host but ineffective with another must be considered when new leguminous plants are introduced into a certain area.

External conditions. External conditions may affect the growth and the yield of leguminous crops in two different ways, namely by exerting a direct influence on the growth of the plants, independently of the nitrogen fixation, or by affecting some part of the symbiosis. To decide which type of reaction is involved, the response of the plants to a particular factor is ascertained (a) in the absence, and (b) in the presence of combined nitrogen. If the factor under consideration affects nitrogen fixation, a response will be observed in (a) and not in (b).

External conditions often affect only one stage of the plant-rhizobia association (molybdenum and borium supply). In other instances two or even more partial processes of the symbiosis may be involved (temperature, nitrogen supply).

Climatic factors.

Temperature. Temperature may affect the survival of the rhizobia in the soil as well as their growth in the rhizosphere. Of a more specific nature is its effect on nodule growth and nitrogen fixation.

Differences in resistance to high temperature between different types of rhizobia are recorded by Vincent (1965), the *Medicago* strains being more resistant than the others. This may be of importance for the survival in tropical and subtropical soils exposed to radiation where very high temperatures can be attained.

Moderately high temperatures (28°C or higher), which are favourable for the growth of the host plant when supplied with combined nitrogen as well as for the growth of the rhizobia, may depress the formation of root nodules. This was found for several legumes, including pea, vetch and *Medicago tribuloides* (Pate, 1961, 1962). Using isolated-root cultures of beans, Barrios, Raggio and Raggio (1963) demonstrated that exposure of the roots to 30°C for three days after inoculation inhibits nodulation to the same degree as permanent cultivation of the roots at that temperature. Similar results were obtained by Lie with intact pea plants (unpublished results).

In addition to nodulation, nitrogen fixation of nodulated plants (nodules formed at lower temperature) may be reduced by moderately high temperatures. The degree of inhibition is dependent on the host plant and the rhizobial strain (Gibson, 1963, 1966; Pate, 1962).

Many warm regions are supposed to have a day-time temperature high enough to affect adversely nodule formation and nitrogen fixation of certain legume-rhizobia associations. Looking for plant species or varieties and bacterial strains giving associations with high nitrogen-fixing power at high temperature may be considered as worth while from an agricultural point of view.

Low soil temperatures such as occur in arctic regions do not prevent survival of rhizobia and formation of nodules on the roots of leguminous plants (Allen *et al.*, 1964). Plants as well as bacteria are presumably adapted to low temperatures. This was shown by Vartiovaara (1937) for the growth of rhizobia from Finland and Sweden as compared with those from the Mediterranean.

The introduction of rhizobial strains and legume varieties from cold regions into the agriculture of more temperate zones might have significance for obtaining earlier growth of leguminous plants. This would be important for grass-legume mixtures where the nitrogen supply depends largely on symbiotic nitrogen fixation. The legumes at present grown in such mixtures have a higher temperature optimum than the grasses so that application of fertilizer nitrogen is required for obtaining an early production of herbage (mainly grass) in spring.

Light. Nodulation and nitrogen fixation of leguminous plants depend on photosynthesis for the supply of carbohydrates. A decreased light intensity therefore has an adverse effect on both processes. This effect is more serious when the temperature is high, owing to an enhanced respiration which causes depletion of carbohydrates. According to some reports, very high light intensities would also have an inhibitory effect on nitrogen fixation (Wilson, 1940).

However, the effect of light on plant growth is not restricted to photosynthesis. A number of plant responses to light are ascribed to the action of phytochrome, a pigment system extremely sensitive to small amounts of red light (660 mμ) and far-red light (730 mμ; see Mohr, 1962).

Evidence has been provided that nodulation is also controlled by phytochrome (Lie, 1964). Under conditions of equal dry-matter production (photosynthesis), nodulation of bean plants (*Phaseolus vulgaris*) in red light was found to be superior to that in blue light. Further experiments of Lie showed that far-red light applied for only a few minutes either to the shoots or to the roots reduced nodulation whereas subsequently applied red light eliminated the inhibitory effect of far-red. These results were obtained with intact plants, decapitated plants, and rooted leaves. In the latter two systems no shoot growth occurs. The hypothesis was put forward by Lie that under the influence of red light a growth substance, presumably related to kinetin, is formed which is necessary for nodule formation.

Soil conditions

Soil reaction. Soil pH is one of the most important factors determining the symbiotic nitrogen fixation of leguminous plants growing under natural conditions. Many symbiotic systems require a neutral or a slightly alkaline reaction for optimal functioning, but many soils, particularly those of humid regions, have pH values well below those optimal for nitrogen fixation.

Plants belonging to the *Medicago* group (lucerne, black meddick) are very sensitive to acid soil conditions in contrast to those of the lupin group (lupin, serradella) and those of the soybean-cowpea group which can be grown on acid soils. The poor tolerance to acid conditions of the former plants can be largely overcome by supplying combined nitrogen, showing that the growth of the host plants is not seriously affected by the acid medium.

To explain the acid-sensitivity of the symbiotic systems, the following phenomena, corresponding to different stages of the symbiosis, have to be taken into consideration: (1) the poor survival of the rhizobia in the acid soils; (2) the inadequacy of the conditions for the growth of rhizobia in the rhizosphere, depending on either the acidity of the medium or on the lack of nutrients secreted by the legume roots on acid soils; and (3) alteration of the structure of the root surfaces, so that the entry of rhizobia is inhibited. Evidence of these three factors is available, albeit for different plant-rhizobia associations.

(1) Several authors have recorded the poor survival of rhizobia, particularly *R. metiloti*, in acid soils (Mulder *et al.*, 1966; Vincent, 1958, 1965).

A dense suspension of *R. trifolii* added to an acid soil of pH 5.0 decreased from 2.3×10^8 to 3.3×10^3 per 0.2 g of moist soil within two months. Under the same conditions a reduction from 3.3×10^8 to 2.2×10^5 occurred in a similar type of soil of pH 7.5 (Mulder and Veen, 1960*a*).

(2) That the conditions for the growth of *Rhizobium trifolii* in the rhizosphere of red clover on acid soils are inadequate was shown by counting the numbers of this bacterium in planted pots containing acid soil. A very slight increase in number of *R. trifolii* was observed in contrast to the very large increase in a similar set of planted pots, the soil of which had been neutralized with $CaCO_3$ (Mulder and Veen, 1960*a*).

Whether the poor growth of the rhizobia in the rhizosphere on the acid soil was due to the inhibitory effect of soil acidity, or to an inadequate excretion of nutrients by the roots is undecided. Experiments with /‾ strain of *R. trifolii* which were able to grow and nodulate red clover at a low pH might indicate that low soil pH rather than reduced excretion of nutrients had prevented the multiplication of rhizobia in the rhizosphere (Mulder *et al.*, 1966). On the other hand, the fact that occasionally, by cross-pollination or by mutation, well-nodulated plants were obtained in a population of poorly nodulated red clover growing in acid soil, might indicate that the former enabled the rhizobia to grow in the rhizosphere owing to the excretion of adequate amounts of substrate.

Owing to the low multiplication rate of rhizobia in planted acid soils, at least 6.10^4 *R. trifolii* had to be introduced into pots containing 500 g soil in order to obtain normal nodulation (Mulder and Veen, 1960*a*).

(3) Alteration of the structure of the root surface was presumably the reason for the inhibited nodulation in an experiment with pea plants growing temporarily in a culture solution of pH 4.5. When after inoculation with *Rhizobium* the plants were kept for four days in the acid medium and subsequently transferred to a neutral nutrient solution, nodulation was largely depressed for at least two weeks, in spite of the presence of active rhizobia in the acid solution. Inoculation of the acid cultures with much larger numbers of *R. leguminosarum* had no effect on nodule formation (Mulder *et al.*, 1966). These results indicate the existence of a damaging effect by acidity on the root surfaces. So far it is unknown whether this effect occurs also under soil conditions.

The results of the above-mentioned experiments indicate that low pH affects particularly the earlier stages of the plant-rhizobia association. That nodulation and N_2 fixation proceed normally was shown in a culture solution experiment in which pea plants were transferred at different times after inoculation from a neutral to an acid nutrient medium (pH 4.5.) If the association was kept for four days after inoculation in the neutral medium and subsequently cultivated in the acid nutrient solution practically normal nodulation and nitrogen fixation occurred (Mulder *et al.*, 1966).

Further evidence of the insensitiveness of the nitrogen fixation of leguminous plants to low pH may be derived from the above-mentioned experiment with red clover in which a heavy inoculation with *R. trifolii* (more than 60,000 cells per pot, containing 500 g soil of pH 5.0) gave normal nodulation and N_2 fixation.

In addition to a direct effect, soil acidity may have a secondary effect on the legume-*Rhizobium* association. This includes damage by excessive amounts of certain elements which are much more soluble under acid conditions (Fe, Mn and Al) or shortage of a second group of nutrients which are absent or less available under acid conditions (Ca, P, Mo).

A further aspect of soil acidity relative to symbiotic nitrogen fixation is the occurrence of ineffective strains of various types of *Rhizobium*. With clover bacteria it was found that in the acid hill pastures in Great Britain and in certain waterlogged acid soils in the Netherlands more than 60 per cent of the nodules formed are of the ineffective type in contrast to the neutral and slightly alkaline soils where effective rhizobial strains are predominant (Harmsen, 1954, 1965; Holding and King, 1963; Jones *et al.*, 1964; Mulder *et al.*, 1966; Singer *et al.*, 1964).

Phosphorus supply. Legumes require relatively large amounts of phosphorus. In addition, their ability to utilize soil phosphate is often less pronounced than that of cereals and grasses. Therefore spectacular responses of legumes like clover to added phosphatic fertilizers on pasture soils poor or moderately poor in phosphate may be observed. Since the improved clover growth brings about an increased nitrogen fixation there is a general rise in fertility of such pastures (Donald, 1964-65; Donald and Williams, 1954). This is particularly true of those pastures where no fertilizer nitrogen is used.

Certain legumes (e.g., lupins) are able to take up considerable amounts of soil phosphate in their tissues. Since these plants in addition fix large amounts of nitrogen, they are particularly suited to improve soil fertility by way of green manuring.

Although it is sometimes stated that phosphorus has a specific function in nitrogen fixation, it is more probable that the increased N_2 fixation upon the application of P fertilizers depends on a general improvement of plant growth.

Trace elements. Of the trace elements required for plant growth boron and molybdenum are of particular

/‾a mutant

importance for the symbiotic nitrogen fixation. Boron affects nodulation (Brenchley and Thornton, 1925; Mulder, 1948); when insufficient amounts of this trace element are present, necrotic nodules are formed which fix no nitrogen. Nitrogen deficiency of leguminous plants as a result of boron deficiency was found by the senior author in culture solution experiments with pea plants as well as in pot experiments with boron-deficient soil. Although boron deficiency of various types of leguminous plants growing under natural conditions has been described in the literature, almost no attention has been paid to its influence on nodulation and nitrogen fixation.

Molybdenum is required for the functioning of the root nodules. In all the other stages of the symbiosis it is not essential. This may be concluded from the fact that in molybdenum-deficient plants nodulation proceeds normally. However, as in ineffective plant-rhizobium associations the nodules are more numerous than on plants supplied with molybdenum and their colour is yellow instead of the pink of normal nodules (Mulder, 1954). When molybdenum-deficient plants bearing such nodules are supplied with small amounts of molybdate, nitrogen fixation starts within a few hours (Mulder *et al.*, 1959).

Molybdenum deficiency of leguminous plants is found particularly on soils containing a certain amount of iron stones or bog ore. Such soils are common in many regions of the world. In western Europe they occur as low moor peat soils or sandy soils in the neighbourhood of brooklets rich in iron. The excessive amounts of iron and the moderately acid reaction of such soils cause precipitation of phosphorus and molybdenum. Application of a small amount of a molybdate (about 1 kg per hectare) may give a pronounced increase in leguminous crops and of several other crops which require traces of molybdenum for nitrate reduction (Mulder, 1954). In addition application of phosphatic fertilizers will often be necessary for obtaining optimal yields.

Further trace elements involved in symbiotic nitrogen fixation are cobalt, iron and presumably copper (Halsworth, 1958; Vincent, 1965). So far very little is known of a curtailed nitrogen fixation owing to an inadequate supply of these trace elements under natural conditions.

Organic compounds. Unknown organic compounds present in stable manure, dead *Rhizobium* cells, and yeast extract were found to have a favourable effect on nodulation and nitrogen fixation of red clover growing in acid soil (Mulder and Veen, 1960*a*). Since the numbers of rhizobia of the clover type in the acid soil were very low, both without and with clover, the added organic matter is supposed to have favoured bacterial multiplication and infection of the clover roots.

Carbon dioxide. It is a well-known fact that the CO_2 requirement of green plants is not confined to photosynthesis. Roots, like heterotrophic micro-organisms, need small amounts of CO_2 for certain metabolic processes. A similar requirement for carbon dioxide was found to exist for nodule formation and symbiotic N_2 fixation of leguminous plants. To demonstrate the effect of CO_2 on the latter processes, red clover, peas and beans (*Phaseolus vulgaris*) were grown in a culture solution of pH 4.8 which was continuously freed from CO_2 by aeration with air free from CO_2 (Mulder and Veen, 1960*b*). A number of pots were aerated with air containing 4 per cent carbon dioxide. Several measures were taken to prevent the aeration gases from being taken up by the leaves so that photosynthesis was not affected. Pronounced differences in nodulation and nitrogen fixation occurred between plants growing without and with CO_2 in the culture medium. The former plants suffered from severe nitrogen deficiency due to the low nitrogen-fixing capacity of their nodule system. When the pH of the nutrient medium was 6.4 the symptoms of CO_2 deficiency were less pronounced since part of the carbon dioxide was present as the bicarbonate which was not removed by aeration. So far it is not known whether CO_2 deficiency belongs to the factors affecting symbiotic nitrogen fixation under natural conditions.

Combined nitrogen. The effect of combined nitrogen on symbiotic nitrogen fixation depends on several factors including type of plant, *Rhizobium* strain (Pate and Dart, 1961), stage of development of the plant when the nitrogen compound is supplied, climatic conditions affecting carbohydrate content of the plants (temperature, light supply), type of nitrogen compound supplied, pH and type of nutrient medium (culture solution or soil), way in which the legume is grown (monoculture or in association whith a non-legume such as grass).

When leguminous plants, particularly those grown from small seeds, are cultivated in a medium poor in available nitrogen, symptoms of nitrogen deficiency will appear at an early stage. This is particularly true of plants growing under conditions of high illumination (in summer as compared with early spring or autumn). Although nitrogen deficiency promotes the initial stages of nodulation, it may cause a serious growth depression of the legume seedling which at a later stage when symbiotically fixed nitrogen is available can often not be recovered. The presence of small amounts of combined nitrogen prevents the early occurrence of nitrogen deficiency of the seedlings. Nodulation may be retarded but in the long run it mostly benefits from the added combined nitrogen as a result of a more vigorously developed root system providing more sites for nodule formation.

When large amounts of combined nitrogen are present in the culture medium of a plant-*Rhizobium* association, nodulation may almost entirely be depressed. This is thought to be due to the exhaustion of carbonaceous material within the plant by the added inorganic nitrogen compound. Since NH_4 nitrogen within the plant reacts more readily than nitrate with carbon compounds (for instance α-ketoglutaric acid), a more pronounced interfering effect on nodulation by ammonium compounds would be expected. This is in contrast to the results of Gibson and Nutman (1960) as far as concerns nodule initiation. These authors found small amounts of nitrate or nitrite much more effective in retarding nodulation than ammonium compounds.

Tanner and Anderson (1964) believe that nitrogenous compounds inhibit nodulation by inactivating indoleacetic acid which is assumed to be essential in nodule formation. This was concluded from the inactivation of IAA by rhizobia in the presence of nitrate or ammonium compounds. Nitrate is thought by these authors to react with IAA after its bacterial reduction to nitrite. Ammonia reacts in a different way.

Nodulation of leguminous plants growing in soil under field conditions is less depressed by large amounts of added nitrogenous compounds than that of plants growing in culture solution. This is presumably due to the fact that, in the soil, the added combined nitrogen is less evenly distributed so that part of the root system may be present in an environment which is almost free from combined nitrogen.

In pots containing small amounts of soil, moderate quantities of combined nitrogen mixed through the soil may completely depress nodulation of leguminous plants.

On acid soils inoculated with a rhizobium at the same time as sowing the leguminous plant, the retarding effect of added combined nitrogen on the infection and nodulation of the plants may have serious consequences. The survival of the rhizobial cells in such soils is much poorer than in neutral soils so that most of the rhizobia have died by the time the combined nitrogen has been utilized and the plant roots have become liable to infection. Since in acid soils large numbers of rhizobia have to be present to enable nodulation moderate amounts of added combined nitrogen may curtail or completely exclude the retarded formation of nodules so that after the utilization of the combined N symptoms of serious nitrogen deficiency occur (Dilz and Mulder, 1962*b*).

In addition to inhibiting root infection and nodulation, combined nitrogen may depress the fixation of N_2 when added to nodulated plants. This was shown in experiments with labelled N_2 (Allos and Bartholomew, 1959).

From an agricultural point of view it is important to know if the application of fertilizer nitrogen, in addition to the effect on seedlings, may improve leguminous crops. In general it is believed that the symbiotically-provided nitrogen is unable to give yields as high as those obtained with optimal amounts of inorganic nitrogen (Allos and Bartholomew, 1959; Pate and Dart, 1961). However, symbiotic N_2 fixation depends on many internal and external factors, so that it is often difficult to decide whether the symbiosis under consideration is proceeding under optimal conditions. Improving these conditions, rather than adding supplementary amounts of combined nitrogen, may be considered to be the best policy to improve leguminous crops. In this connexion it may be stressed that the presence of combined nitrogen during the period of N_2 fixation depresses the latter so that the main effect of the fertilizer nitrogen would be the substitution of combined nitrogen for fixed N_2.

In general it may be stated that a profitable use of fertilizer nitrogen in improving a leguminous crops may be made: (a) by applying small amounts (20 to 30 kg N per hectare) during the early stages of plant growth, the so-called period of 'nitrogen hunger' (Pate and Dart, 1961), when symbiotic N_2 fixation has not yet started; (b) in some instances by applying moderate amounts during the stage of N_2 fixation to supplement the inadequate amounts of fixed nitrogen. Such a dressing may be carried out on soils where the conditions for symbiotic nitrogen fixation are less favourable, for instance due to a low pH. Under such conditions the combined nitrogen should be applied as nitrate, ammonium salts being badly assimilated (Mulder, 1948).

Application of fertilizer nitrogen on grass-clover associations (leys or permanent grasslands) represents a special case of improving a legume-containing crop by combined nitrogen. Such associations are generally not dressed with nitrogenous fertilizers. The clovers are supposed to supply the grasses with nitrogen derived from symbiotic N_2 fixation. Since the leguminous plants may comprise 20 to 50 per cent of the mixture, they are unable to supply the grasses with optimal amounts of nitrogen. This is particularly the case in the spring when the grasses start growing at lower temperatures, i.e., considerably earlier than the legumes. To raise the yields of such grass-clover associations, in the Netherlands large amounts of fertilizer nitrogen are supplied, particularly in spring. Since the grasses respond more readily to the added fertilizer nitrogen than the clovers, the latter are depressed by the grasses. This is an indirect effect mainly resulting from competition for light and nutrients. During the summer months when the growing conditions are more favourable to the clover and smaller amounts of combined N are supplied, the clover mostly regains its lost territory. That the depressing effect of large amounts of nitrogenous fertilizer on the growth of clover in the above-mentioned associations is entirely due to competition with the grasses may be easily shown by growing the clover without grass, with the same nitrogen dressings (Dilz and Mulder, 1962*b*).

Interaction between rhizobia and other soil organisms. The growth of a particular rhizobium may be favoured or inhibited by other micro-organisms while in other instances no interaction occurs (Vincent, 1965). The adverse effect of microbial antagonists on rhizobial growth in the rhizosphere is thought to be the reason for the failure of nodulation in certain Australian soils. This was concluded from the fact that a thousandfold dilution of such soils interfered with the nodulation of clover plants growing in the presence of *R. trifolii* in agar tubes. Autoclaving the soils eliminated the interfering factor (Hely *et al.*, 1957). Further examples of micro-organisms antagonizing the development of rhizobia have been given by Cass Smith and Holland (1958).

In addition to aerobic spore-forming bacteria (Abdel-Ghaffar and Allen, 1950) and fungi, actinomycetes are thought to be of particular interest in the antagonism of rhizobia in soil. Wieringa (1963*a*) isolated a number of actinomycetes highly active against rhizobia. When these antagonists were added to sterilized soil before or simultaneously with *R. trifolii*, nodulation of clover plants was largely prevented. In bean plants nodulation was not inhibited, but the N_2 fixation was reduced (Wieringa, 1963*a*). By repeatedly exposing root-nodule bacteria from clover and lucerne to the action of actinomycetes in agar media, van Schreven (1964) obtained several mutant strains. Some of these mutants had lost the ability of N_2 fixation or even of nodule formation. Others formed smaller numbers of nodules than the parental strains. It would be of interest to investigate whether similar results could be obtained under rhizosphere conditions. Wieringa's observations (personal communication) that large pink and small white nodules were present on his clover plants growing in sterilized soil and inoculated with *R. trifolii* and its antagonist, is an indication in that direction.

Leguminous plants and soil fertility

The important contribution of leguminous plants to soil fertility is easily seen from the large amounts of nitrogen fixed by these plants when grown as agricultural crops. Under optimal conditions of nutrient supply, soil pH, strain of *Rhizobium*, etc., red clover and lucerne under the temperate climatic conditions of Western Europe may fix as much as 300 kg N per hectare annually. Under more favourable conditions of light and temperature, these values may be considerably higher.

With arable crops of the legume type a considerable part of the fixed nitrogen is removed with the yield; the roots and part of the lower leaves, however, remain and enrich the soil as plant residues rich in nitrogen. Since these nitrogenous compounds are gradually decomposed by microbial activity, they benefit the growth of the subsequent crop.

When the legume is grown in association with grass plants (grazed pastures) and still more so in the case of green manuring, a considerably larger part of the fixed nitrogen is left in the soil, thus improving soil fertility more extensively. The beneficial effect of legumes (clover, lucerne) on the nitrogen supply of the grass plants of these associations is presumably not due to the excretion of nitrogenous compounds by the living legume roots (Dilz and Mulder, 1962*a*), but mainly to the decomposition of dead roots and nodules.

Although the building up of a store of soil organic matter, which is one of the characters of soil fertility, can also be achieved by the supply of fertilizer nitrogen to a grassland vegetation free from leguminous plants, the application of legumes has some important advantages: (a) it utilizes elementary nitrogen, so that the method can be used also in areas where no fertilizer nitrogen is available; (b) the plant residues accumulating in the soil contain larger amounts of decomposable N compounds than those of grass residues.

A good example of soil improvement by leguminous pastures is the improvement of large areas of poor South Australian soils to fertile soils by introducing subterranean clover (Donald, 1964-65; Donald and Williams, 1954; Russell, 1960). The clover pastures are dressed with P, S and K fertilizers and eventually with trace elements, but nitrogen and carbon are introduced into the soil by the leguminous plants. The improved fertility level of such soils after a pasture period of approximately 25 years is shown by the rise of the N content of the upper 10 cm soil layer from 0.06 to 0.18 per cent and by the large increase in yield of cereals when grown after the pasture period. However, it should be kept in mind that part of the increased soil productivity was due to an improved nitrogen supply to the cereals which might also have been attained by the application of fertilizer nitrogen. That the latter is used only on a limited scale on these Australian soils apparently depends on: (a) the excellent growth of subterranean clover under the prevailing climatic conditions; (b) the importance of the pasture period in sheep grazing; (c) the wish to build up soil organic matter which may provide adequate amounts of nitrogen to the arable crop grown after the ploughing in of the pasture; and (c) the relatively high prices of nitrogenous fertilizers in these areas.

Ploughing up of a crop of a leguminous plant, a grazed leguminous pasture, a ley, or a grass pasture well dressed with fertilizer nitrogen for a varying number of years, is also done by certain farmers in areas where the application of large amounts of nitrogenous fertilizers is common practice, as for instance in the Netherlands. The introduction into the soil of plant residues high in organic nitrogen compounds give a more even supply of nitrogen to the subsequently grown arable crops. In combination with low or moderate amounts of fertilizer nitrogen it often produces higher yields than are obtained with high amounts of fertilizer N in the absence of plant residues.

The highest contribution to the soil content of organic matter comes from long-term pastures, either consisting of a grass-legume association, or of grasses adequately dressed with fertilizer nitrogen. Under grassland conditions the microbial degradation of plant residues is much less pronounced than in arable land. This results in the building up of a valuable store of nitrogen in the form of organic compounds which is almost unavailable to the grass plants of the pasture but is gradually liberated by microbial activity after ploughing up the pasture.

Legume fields or grass-clover associations a few years old, as occur in several crop rotations, in spite of bringing in smaller amounts of plant residues, are nevertheless of much importance in improving or maintaining soil fertility. The same is true, albeit to a less extent, of legumes, grass-legume associations, and fertilizer-nitrogen-dressed quickly growing grasses, sown as an after-crop (secondary crop) under cereals and ploughed up in the following spring for growing potatoes or sugar-beets. This procedure has the additional advantage of retaining the soluble N compounds which after the ripening of the cereals are formed in the soil by microbial activity. Unless vegetation is present these compounds are often leached during the wet autumn and winter periods of temperate regions.

In certain tropical countries some progress has recently been made in introducing suitable leguminous plant into the nitrogen-deficient grassland which occurs in large areas (see, for reviews, Anon, 1962, 1964). In Nigeria good results have been obtained with *Centrosema pubescens* under humid conditions and with *Stylosanthes gracilis* in more arid regions (McIlroy, 1962). With an application of superphosphate and normal grazing a sward consisting of 50 per cent grass and 50 per cent legumes can be maintained. In North Rhodesia good results were obtained with a mixture of Rhodes grass and leguminous plants like *Stylosanthes*, *Stizolobium* and *Cajanus cajan* (Smith, 1962).

From an economical point of view improvement of the production of grassland in the tropics is of major importance. This would be mainly a matter of nitrogen supply and to a smaller extent of phosphorus supply. Improvement of the nitrogen supply would mean introduction of symbiotic nitrogen fixers.

In plantations of tea, rubber, coffee, etc., in the humid tropics, the use of cover crops is an important factor for preventing erosion and leaching of nutrients, and maintaining a good soil structure. For this purpose quick-growing legumes capable of growing at a low light intensity can be used successfully. The beneficial effect of legumes as compared with natural ground cover was shown by rubber trees which, in the presence of the former, had a greater girth and came earlier into production (Watson, 1963).

Non-legume angiospermous root nodule plants

In addition to the leguminous plants, considerable numbers of non-leguminous angiosperms, all trees and shrubs, are able to fix molecular nitrogen by way of root nodules. So far more than 108 species of 13 genera belonging to 7 families have been found to form nodules. In three of these families (Coriariaceae, Myricaceae, Casuariniaceae) only one genus exists (*Coriaria*, *Myrica*, and *Casuarina*, respectively). The Betulaceae is composed of two genera, one of which, *Alnus*, forms root nodules. All of the three genera belonging to the Elaeagnaceae (*Eleagnus*, *Hippophaë* and *Shepherdia*) may be nodulated. Of the Rhamnaceae and Rosaceae only a few of the many genera belonging to these families are nodule-bearing (Allen and Allen, 1965; Bond, 1963).

The majority of these genera comprise large numbers of species, many of which apparently have not been examined for the presence of nodules. Several of the genera are widely distributed. *Coriara* species, for instance, occur in New Zealand, Japan, Central and South America, and Spain. *Alnus* species are common in most northern countries; they even occur in arctic regions. *Casuarina* species occur in tropical Asia, Australia and on the Pacific islands.

In contrast to the root nodule bacteria of the leguminous plants which can easily be isolated and kept in pure culture, the endophyte of the non-legumes so far has not been isolated and identified. From microscopical studies it is concluded that the micro-organism is an actinomycete (see, for instance, the large number of electron microscope photographs in the paper of Becking *et al.*, 1964). Although actinomycetes have been isolated from *Alnus* by several workers, inoculation experiments with the isolated micro-organisms have not been successful.

By using crushed alder nodules containing the endophyte, Quispel succeeded by way of an indirect counting method in obtaining multiplication of the unidentified symbiont in a nutrient medium fortified with a root extract of alder roots (Quispel, 1960).

Effect of internal and external factors on nodulation and nitrogen fixation of non-leguminous plants

Internal factors. In comparison with the large number of papers published on the effect of internal and external conditions on symbiotic nitrogen fixation of leguminous plants, the number of reports on these subjects in the non-legumes is very small. The results of experiments on cross-inoculation between species and genera have been summarized by Bond (1963). Reciprocal cross-inoculation between a number of species has been reported for *Alnus* and in a more restricted way for *Casuarina* and *Myrica*. Reciprocal cross-inoculation is also possible between the three genera of the Elaeagnaceae. The *Alnus* symbiont apparently does not nodulate *Casuarina*, *Myrica*, *Hippophaë* or *Elaeagnus*, and a *Casuarina* inoculum was found to be inoperative on *Myrica* and *Coriaria*.

External factors.

Soil reaction. Very little is known about the effect of external conditions on nodulation and N_2 fixation in non-leguminous plants. The pH of the rooting medium has a marked effect (Quispel, 1958; Stewart, 1966). In *Alnus*, *Myrica*, *Hippophaë* and *Casuarina* the best results are obtained at neutrality. In the last two genera nodulation is very sensitive to lower pH values but in *Alnus* and *Myrica* it is still extensive as far down as pH 4. These plants, owing to their nitrogen-fixing capacity, grow well on acid waterlogged bog soils.

Phosphorus. Quispel (1958), in culture solution experiments with *Alnus glutinosa*, found that the number of nodules was particularly reduced when the phosphate was omitted for approximately one week after inoculation.

Trace elements. Of the trace elements molybdenum has been tested by Bond and Hewitt (1961; Hewitt and Bond, 1961) in culture solution experiments with *Casuarina*, *Alnus* and *Myrica* and by Becking (1961*c*) in pot experiments with *Alnus*. In the latter case molybdenum-deficient soil was used. Similar to the legumes, N_2 fixation of the non-legumes was found to require small amounts of molybdenum. Without this element nodules were formed but fixed very little N_2 so that the plants showed symptoms of severe nitrogen deficiency and grew very poorly. With small amounts of molybdate added, normal dark-green plants were obtained.

So far it is not known whether molybdenum deficiency of non-leguminous plants occurs under natural conditions. Factors rendering molybdenum unavailable are sometimes more active in the upper layers than in the subsoil so that deep-rooted plants might be able to collect adequate amounts of molybdenum from the subsoil.

Cobalt is a further micronutrient element found by Bond and Hewitt (1962) to be required for N_2 fixation by some non-legumes (*Alnus* and *Casuarina*). This is in agreement with the requirement of this element for leguminous plants. So far it is not known whether this deficiency occurs under natural conditions.

Combined nitrogen. The effect of combined nitrogen on nodulation and N_2 fixation of a number of non-legumes has been studied by Bond and collaborators (1963, 1954; MacConnell and Bond, 1957; Stewart and Bond, 1961) and by Quispel (1958). Similar to leguminous plants, small amounts of NH_4 nitrogen or nitrate had a favourable effect on plant growth during the earlier stages of growth when no fixed nitrogen was available. Nodule development and as a consequence N_2 fixation also benefited by this improved growth. Large amounts of nitrogen depressed nodulation and N_2 fixation but *Alnus* and particularly *Myrica* were much less sensitive than *Hippophaë*.

Effect of non-legume nitrogen fixers on soil fertility

Root-nodule-bearing non-leguminous plants contribute to the fertility of the soil by enriching it with organic residues high in nitrogen. This is of particular importance on soils poor in nitrogen where agriculture has not modified the original flora. Non-legumes are thought to have been the main original source of symbiotic nitrogen in the post-glacial era when the barren mineral soils permitted hardly any plant growth.

A good picture of such a development of soil fertility was obtained in Alaska by studying the development of the vegetation on an area where the glaciers had receded (Crocker and Major, 1955; Lawrence, 1958). The original vegetation on the barren, stony soil substrate grows poorly until non-leguminous N_2 fixers appear, namely *Dryas drummondii*, a rosaceae, and particularly *Alnus crispa*. Extensive alder thickets are formed by this rapidly growing deciduous shrub, enriching the soil with litter rich in nitrogen. After a considerable number of years Sitka spruce gains entry into this vegetation and, owing to the fertility of the soil, it grows profusely and eventually may suppress the alder.

The beneficial effect of alder and other non-legume N_2 fixers on soil fertility in arctic regions is in accordance with the use made of the former plant in planting conifer forests on infertile soils in the temperate regions of Western Europe. Having improved the soil by its litter, the shade intolerant alder is subsequently overgrown by the conifers and disappears.

Further examples of the important role of non-legumes in improving the fertility of the soil in various parts of the world are given by Allen and Allen (1965).

It is to be expected that in the future nodulation and N_2 fixation will be found to occur in other genera and even families of the Angiospermae. Recently two nodulated species belonging to the Rosaceae, namely *Purshia tridentata* (bitterbrush) and *Cercocarpus betuloides* (Western mountain mahogany) have been added to the list of nitrogen-fixing non-legumes (Allen and Allen, 1965; Vlamis *et al.*, 1964; Wagle and Vlamis, 1961). The former plant covers large areas in the Western states of the U.S.A.; mountain mahogany is a dominant member in the California chaparral (Allen and Allen, 1965).

Gymnospermous root nodule plants

Large numbers of the Gymnospermae are known to bear root nodules. Knowledge of these nodules, particularly of their nitrogen-fixing abilities, is restricted. In some cases (Cycadaceae) blue-green algae are present as the endophyte, in other cases (Podocarpaceae) the endophyte is presumably a fungus. Evidence is available that nodules of the former type fix molecular nitrogen. However, this may be due to the nitrogen-fixing ability of the blue-green alga which presumably fixes nitrogen also when living in the absence of the higher plant.

As to *Podocarpus* nodules, recent investigations by Bergersen and Costin (1964) and Becking (1965) using labelled nitrogen have shown that a small but significant N_2 fixation takes place. In agreement with the poor fixation rate, nodulated *Podocarpus* plants growing in culture solution were severely nitrogen-deficient. When growing in soil poor in available nitrogen the plants developed much better, suggesting that some factor necessary for nitrogen fixation was lacking in the culture solution (Becking, 1965). To verify this hypothesis a comparison of nodulated and unnodulated plants growing in soil should be made. This comparison has not been made owing to the fact that plants free from root nodules were not obtained (Becking, 1965).

Leaf nodules

The occurrence of leaf nodules, assumed to fix molecular nitrogen, has been reported in the families Rubiaceae (genera *Psychotria*, *Pavetta* and *Chomelia*), Myrsinaceae (genus *Ardisia*) and Dioscoreaceae (genus *Dioscorea*). The symbiosis apparently consists of an association between higher plants and nitrogen-fixing bacteria. Evidence of the nitrogen-fixing ability of the endophytes of *Pavetta* and *Psychotria* has been provided long ago by von Faber (1912, 1914) and of that of the bacteria of *Pavetta* and *Chomelia* by Rao (1923). These findings were recently confirmed for the endophyte of *Psychotria* by Silver and co-workers (Centifanto and Silver, 1964; Silver *et al.*, 1963) using the labelled-nitrogen technique. The bacterium was identified as a *Klebsiella*. When growing in a nitrogen-free medium it fixed moderate amounts of molecular nitrogen under anaerobic conditions (Centifanto and Silver, 1964). In agreement with other workers on leaf nodules, it was assumed that the bacteria, in addition to fixing N_2, were required for supplying some growth substance which enabled normal development of the host plant (Silver, Centifanto and Nicholas, 1963).

Much less evidence is available of the ability of the endophyte of *Ardisia* to fix N_2 (Jongh, 1938; Miehe, 1919; Nemec, 1932). However, it should be kept in mind that, according to the above-mentioned experiments of Silver *et al.* (1964), nitrogen fixation by the bacteria of *Psychotria* requires anaerobic conditions. It is highly probable that so far the endophyte of *Ardisia* has been tested for N_2 fixation only under aerobic conditions. The negative results obtained by a number of authors may, therefore, have been due to an imperfect testing technique.

Leaf-nodule-bearing plants contribute to soil fertility by enriching the soil with litter rich in nitrogen.

TRANSFORMATION OF NITROGEN COMPOUNDS IN THE SOIL

MINERALIZATION-IMMOBILIZATION MECHANISMS

Introduction

The mineralization of N-containing organic compounds and the immobilization of inorganic N-compounds in soil occur principally as a result of microbial activity. Although the numbers of micro-organisms, counted by conventional techniques, vary between approximately 10^6 and 10^8 for soils of different fertility, several authors are of the opinion that these values are too low and should be 10-20 times higher. This is mainly concluded from direct microscopic counts (Conn, 1928; Jones and Mollison, 1948) which, however, do not differentiate between dead and living cells. According to these values 10-50 kg of nitrogen would occur in microbial cells per hectare of a fertile soil.

When the number of micro-organisms in soil remains constant, the cells are supposed to be in the stationary phase. In this phase, according to Mandelstam and Halvorson (1960) 2 to 3 per cent of the cell proteins is broken down per hour and replenished by newly formed protein (protein turnover). As a result of this process amino acids and ammonia are liberated which subsequently may be reincorporated in the newly formed proteins when sufficient energy material is available (energy of maintenance). This turnover process, which does not necessitate the continuous death and growth of microbial cells, can be easily demonstrated by mixing the mineralized ammonia with labelled ammonia ($N^{15}H_3$) and measuring the incorporation of N^{15} in the organic material.

Some authors (Jansson, 1958; Chu and Knowles, 1966) explained the nitrogen turnover in soil in terms of a continuous cycle of death and formation of new microbial cells. By adding labelled ammonium nitrogen to the soil and leaching the soil at different times after incorporation of the label, Jansson observed incorporation of the $N^{15}H_3$ in soil organic matter, followed within some days by remineralization. From this result it was concluded that the life of the soil organisms is relatively short. Since this hypothesis was not supported by microbiological examination of the soil the nitrogen turnover in the soil may also be explained as protein turnover, not necessarily involving the death of many organisms.

By means of the above-mentioned leaching technique, Jansson measured the ratio of mineralized native organic soil nitrogen to remineralized incorporated N^{15}. By comparing this ratio with the total amount of native soil nitrogen, the existence of an active and an inactive pool of organic nitrogen was demonstrated. By assuming that all the immobilized label was incorporated in the active fraction, and that unlabelled and labelled nitrogen in the active fraction were mineralized to the same extent, Jansson calculated that in most soils this fraction should account for 10-15 per cent of the total amount of organic nitrogen.

The occurrence in the soil organic matter of a fraction more active in mineralization-immobilization processes has also been recorded by other investigators (Chu and Knowles, 1966; Nommik, 1961; Stewart, Johnson and Porter, 1963; Stewart, Porter and Johnson, 1963; Tyler and Broadbent, 1958). Freshly introduced plant residues or microbial compounds formed within the soil are much more readily decomposed than the majority of the native soil organic matter (humus). It must be assumed, however, that during the breakdown of the active fraction, part of the nitrogen is incorporated into the inactive (humus) fraction.

Effect of carbon compounds on the mineralization-immobilization process

The immobilization of mineralized soil nitrogen or added fertilizer nitrogen depends on the presence of available carbon compounds. When such compounds are not introduced into the soil, a continuous mineralization of soil organic nitrogen occurs. This process may be very slow so that even in soils containing 25 per cent of humified peat the plants may show serious symptoms of nitrogen deficiency (unpublished observations of the senior author).

When carbon compounds are introduced into the soil, it depends on the ratio of available carbon nitrogen whether or not nitrogen is left in the inorganic form. Pure culture studies with different types of micro-organisms have shown that under aerobic conditions 20-40 per cent of the added carbon source may be incorporated into cell material, the remainder being used for dissimilatory processes. With fungi, higher values (up to 60 per cent) have been obtained. In general, the carbon/nitrogen ratio of microbial cell material is between 6 and 10 to 1. As a consequence it may be expected that, at available carbon/available nitrogen ratios above 20 : 1 to 30 : 1, immobilization of inorganic N in microbial material will take place.

However, exceptions to this general rule may occur. For instance, the rate of energy (ATP) production by dissimilation of the carbon source may be higher than the rate at which the generated energy is utilized for the synthesis of essential cell compounds like proteins, nucleic acids, etc. (unbalanced growth). Under such conditions part of the energy may be wasted or used for other purposes like the synthesis of nitrogen-free reserve materials. An example of such a type of growth was cited by Senez (1962) in an experiment with *Aerobacter aerogenes* with glucose as the carbon source and nitrate or ammonium sulphate as the nitrogen source. The synthesis of cell material was determined by the rate of incorporation of the nitrogen into organic compounds, ammonium nitrogen being assimilated much faster than nitrate. However, both types of cells dissimilated glucose at the same rate. The energy derived from glucose dissimilation which was not used for assimilatory purposes appeared to be lost. As a result of this the cell yield obtained with ammonium nitrogen was twice as high as that with nitrate. Twice as much nitrogen from ammonia than from nitrate was immobilized though equal amounts of the carbon source were consumed. Similar results have been obtained by Woldendorp (1963*b*) with an *Arthrobacter* species. However, in experiments with *Aspergillus niger*, the senior author obtained a somewhat higher cell yield with nitrate than with ammonia as the nitrogen source (unpublished results).

Synthesis of large amounts of nitrogen-free reserve substances instead of protein, nucleic acid, etc., an unbalanced type of growth, may be obtained with several micro-organisms when adequate amounts of carbon compounds are available but nitrogen is exhausted. Under such conditions bacteria of the *Arthrobacter* type may form more than 50 per cent glycogen within their cells (Mulder *et al.*, 1962; Zevenhuizen, 1966). Phosphorus deficiency of the nutrient medium or a low pH have a similar effect (Zevenhuizen, 1966).

It may be assumed that these principles also hold for soil conditions. Therefore, the form in which the carbon and nitrogen sources are available in the soil and the way in which micro-organisms are degrading the carbon compounds will affect nitrogen immobilization.

In the case of plant residues added to soil, resistance of both C and N compounds to microbial degradation are important factors in predicting the effect of the added material on the availability of nitrogen, i.e., on one of the most important soil fertility factors. Sugars and starch are much more readily degraded by micro-organisms than cellulose and particularly lignin. The latter compound is practically resistant to the majority of soil organisms so that its presence hardly affects the availability of soil nitrogen.

In general it is assumed that plant residues with a C/N ratio above 20 : 1 to 25 : 1, corresponding to approximately 1.5 to 2 per cent N, bring about a depletion of the available soil nitrogen by microbial immobilization (Jensen, 1931; Dubber, 1955). A well-known example of such a phenomenon is the temporarily detrimental effect of straw on plant growth as a result of nitrogen deficiency. To overcome this effect, fertilizer nitrogen should be added to the soil.

Priming effect

This is the beneficial effect of adding small amounts of fresh organic matter (mostly plant residues) on the microbial decomposition of resistant organic compounds in the soil (Bingeman *et al.*, 1953; Broadbent, 1948; Broadbent and Bartholomew, 1949; Broadbent and Norman, 1947; Hallam and Bartholomew, 1953; Hiltbold *et al.*, 1951; Norman and Werkman, 1943). Several authors studied this effect by adding C^{14}-containing organic matter to the soil and subsequently measuring the C^{12}/C^{14} ratio of the carbon dioxide evolved upon incubation. In this way it was demonstrated that, in the presence of the added material, more unlabelled carbon dioxide, originating from the decomposition of the soil organic matter, was produced by microbial activity. However, in a number of cases the priming effect was quite small, whereas some authors were unable to detect this effect at all (Becking, 1961c; Pink and Allison, 1951; Stotzky and Mortensen, 1957). So far it is undecided whether the observed priming effect was due to a stimulated breakdown of humus compounds or of 'active' soil organic matter, including microbial cells.

NITRIFICATION

Nitrification, the microbial transformation of ammonium compounds to nitrite and nitrate, is carried out mainly by two types of chemoautotrophic bacteria. The first type, belonging to the genus *Nitrosomonas*, converts NH_4 ions to nitrite, the second type, belonging to the genus *Nitrobacter*, converts nitrite to nitrate. These organisms can form nitrate in concentrations up to 2 g per litre of culture medium. Both types of bacteria have their optimal activity at an alkaline reaction. Below pH 7 the nitrification rate declines and at pH 5, for instance, it proceeds only slowly.

In addition to the autotrophic nitrifiers, several heterotrophic micro-organisms have been shown to be able to form nitrite from ammonia (Alexander *et al.*, 1960; Cutler and Crump, 1933). The fungus *Aspergillus flavus* was found to be able to convert ammonia to nitrate (Schmidt, 1954). The amounts of nitrite formed are small and do not exceed a few parts per million; *A. flavus* attained a value of 26 ppm nitrate nitrogen. These amounts are very low in comparison to the amounts converted by the autotrophic bacteria. It is therefore doubtful if the heterotrophic organisms play any significant role in nitrification under natural conditions.

From the point of view of plant nutrition, nitrification is often a superfluous, and even a harmful process, as may be seen from the following considerations. In neutral and alkaline soils ammonium ions may be readily taken up and assimilated by many plants. However, in such soils the nitrifying organisms are very active and, unless an ammonium salt is given to fast-growing plants, a large part of the ammonium ions will have been converted to nitrate before the nitrogen is taken up by the plants. The conversion brings about a severe acidification (2 moles of nitric acid and 1 mole of sulphuric acid are formed per 1 mole of ammonium sulphate) and large amounts of lime (2 moles of $CaCO_3$ for each mole of $(NH_4)_2SO_4$ nitrified) will be required to keep the pH at the original level. A further drawback is that nitrate, when it is not taken up by the vegetation, is liable to leaching out as well as to volatilization by denitrification. NH_4 ions are generally adsorbed by soil colloidal matter so that they are protected against these losses (for losses by leaching see Harmsen and Kolenbrander, 1965).

In acid soil, ammonium salts generally are a poorer nitrogen source for higher plants than nitrate. Nitrification, although proceeding much less readily than in alkaline soil, may have a favourable effect on the uptake and the assimilation of the nitrogen. However, the drop in pH resulting from nitrification may sometimes have a detrimental effect on plant growth. The best policy on acid soil, therefore, would be to add the nitrogenous fertilizers in the form of nitrate or to raise the soil pH by the addition of lime before the application of NH_4 fertilizers.

In order to prevent loss of nitrogen from the soil, stopping the formation and accumulation of nitrate by using specific inhibitors of nitrifying bacteria has been tried. Goring (1962*a*) in laboratory experiments with soil found 2-chloro-6 (trichloromethyl) pyridine to be highly toxic to the bacteria converting ammonium ions to nitrite. Bacteria of the *Nitrobacter* types and a number of other autotrophic and heterotrophic micro-organisms as well as seedlings of many plants were found to be only slightly sensitive. However, in greenhouse experiments (Goring, 1962*b*) and in field trials (Turner *et al.*, 1962) this compound was less effective in inhibiting nitrification, apparently owing to decomposition and volatilization of the inhibitor (Redemann *et al.*, 1964).

An excellent measure to prevent the nitrogen losses owing to leaching of the nitrate, formed in many soils of Western Europe during the autumn after the harvest of cereals, is to grow an after-crop for green-manuring. In addition to preserving the nitrogen during the wet winter period, the green manure enriches the soils with organic matter which generally results in an improved soil structure. Addition of carbonaceous materials, like straw, has also been recommended as a means of preventing nitrate losses by leaching (Jansson, 1958).

Formation and immobilization of nitrate during nitrogen turnover in soil

Under soil conditions favourable for mineralization of organic soil nitrogen and for nitrification, the mineralized NH_3 will be converted to nitrate. Since liberation of ammonia is the rate-limiting step, only nitrate can be detected as the final product of mineralization.

Although nitrate can be used as a nitrogen source by part of the soil microflora, this will only happen when no ammonia is available (Boischot and Sylvestre, 1951; Jansson, 1958; Jansson *et al.*, 1955; Shrikhande, 1936; Winsor and Pollard, 1956; Woldendorp, 1963*b*). Therefore, the nitrate fraction in the soil constitutes a storage pool which is practically excluded from the turnover mechanism, and whose presence indicates that the demand of the heterotrophic microflora for mineral nitrogen is fulfilled.

The preferential utilization of ammonia as a nitrogen source by soil micro-organisms when both this compound and nitrate are available has also been observed in studies with a number of pure cultures (Chang and Morris, 1962; Marshall *et al.*, 1953; Woldendorp, 1963*b*). It is thought that, in the presence of ammonia, the assimilation of nitrate is inhibited owing to the repression of the assimilatory nitrate reductase.

EFFECT OF SOME SOIL CONDITIONS ON NITROGEN TRANSFORMATION

Oxygen supply

The decomposition of organic material by aerobic micro-organisms generates much more energy (ATP) than that by anaerobic bacteria. This results in the synthesis of much higher amounts of cellular material. Since the quantity of immobilized nitrogen is proportional to the amount of cell material synthesized, immobilization of inorganic nitrogen may be expected to occur more readily on well-aerated than on anaerobic waterlogged soils. Breakdown of amino acids is mostly not inhibited under anaerobic conditions so that it contributes to the nitrogen supply under these conditions. However, denitrification is highly favoured by anaerobic soil conditions and it is often the cause of nitrogen deficiency of young crop plants well dressed with nitrate on soils waterlogged by heavy rainfall. Replacing the lost nitrate as soon as the excess water has disappeared may save a considerable part of the crop.

Nitrification may be completely suppressed in waterlogged soils; as a result, ammonium nitrogen sometimes accumulates in large amounts (van Schreven, 1963).

Drying and remoistening

Drying of soil, followed by remoistening, often increases mineralization of organic matter, resulting in an enhanced liberation of inorganic nitrogen compounds (Birch, 1958, 1959, 1960). Upon desiccation a rapid decline of the majority of soil micro-organisms takes place, followed by a pronounced rise of microbial activity upon rewetting. The nitrogen liberated during the moist period is thought to be derived partly from the decomposition of dead micro-organisms, killed during the preceding drying, and partly from the decomposition of part of the humus fraction. This phenomenon is of importance in tropical regions with pronounced dry and wet seasons. It should be kept in mind, however, that upward movement of nitrate during the dry period and leaching out of this compound during the wet season also play an important part in the nitrogen supply of the crops (Wetselaar, 1961*a*, 1961*b*, 1962).

The effect of plants

Higher plants affect the nitrogen transformation in the soil: (a) by taking up N compounds; and (b) by excreting organic compounds which serve as nutrients for micro-organisms. The presence of such root exudates is shown by the greatly increased microbial activity in the medium surrounding the roots (the rhizosphere).

The effect of (a) is clearly shown in grassland soils where the presence of a dense plant cover brings about a ready uptake of added nitrogenous fertilizers, thus preventing to a large extent the leaching-out of nitrate, and, in the case of ammonium fertilizers, the nitrification of ammonia.

The exudation of organic compounds by the roots may cause immobilization of inorganic soil and fertilizer nitrogen. The fact that, after the addition of fertilizer nitrogen, higher numbers of micro-organisms were found on and near the roots than without added nitrogen (Macura, 1961; Woldendorp, 1963*b*) clearly shows that inorganic nitrogen compounds are taken up and immobilized by rhizosphere organisms. This conclusion is confirmed by results of van Schreven (1958) who, in a number of soils during a seven weeks' incubation period, found more nitrogen mineralized than was taken up by spinach plants. The difference was ascribed to immobilization of mineralized nitrogen by rhizosphere organisms. Several other investigators came to the same conclusion (Goring and Clark, 1949; Bartholomew and Clark, 1950; Legg and Allison, 1960). A more decisive proof of the transformation of inorganic fertilizer nitrogen into organic soil nitrogen compounds under the influence of living plants was obtained by Walker *et al.* (1954) and by Dilz and Woldendorp (1960) in pot experiments with sown perennial rye-grass to which labelled fertilizer was applied.

It will be clear that the immobilization of inorganic nitrogen by rhizosphere organisms depends on the C/N ratio of the root exudates. It may be expected that leguminous plants which excrete relatively larger amounts of nitrogen-containing compounds (amino acids) than grasses, immobilize smaller amounts of added inorganic N compounds. Evidence is available that considerable amounts of organic compounds are excreted by plants growing in soil (Harmsen and Jager, 1962; Woldendorp, 1963*b*). This is in contrast to plants growing in sterile nutrient solution (Rovira, 1956, 1959).

It should be kept in mind, however, that a second mechanism may be responsible for the immobilization of fertilizer nitrogen in soil organic matter, namely the uptake of inorganic N compounds by the plant and the excretion, in a late phase of growth, of organic nitrogenous compounds (Dilz and Woldendorp, 1960; Goring and Clark, 1949; Woldendorp, 1963*b*; Woldendorp *et al.*, 1966).

The immobilization of fertilizer nitrogen in the rhizosphere in addition to the immobilization, or perhaps fixation, in plant residues is thought to be partly responsible for the low recovery of added fertilizer nitrogen (Mulder, 1949) in permanent grassland. In such soils the organic matter content increases during several decades (Richardson, 1938; 't Hart, 1950; Harmsen and van Schreven, 1955) and the immobilized nitrogen is poorly available to the grassland vegetation. Upon ploughing up the grassland, a ready mineralization of the accumulated organic material takes place, resulting in a much improved nitrogen supply for the subsequent crops.

FIXATION OF AMMONIA BY SOIL CONSTITUENTS

FIXATION BY SOIL ORGANIC MATTER

Special attention has been paid by various research workers to the interactions between ammonia and the soil constituents. In laboratory experiments it was found that ammonia may react chemically with soil organic matter to form compounds of unknown structure which are very resistant to microbial attack (Bremner and Shaw, 1957). Extensive experiments on the fixation of nitrogen in soil organic matter have been carried out by Wieringa (1963*b*, 1964) working in the authors' laboratory. Black high-moor peat of low pH, after exposure to frost, was supplied with a fertilizer mixture consisting of N (as urea), P, K and various trace elements. After incubation for about one year, two series of 10 litre containers were filled with 4 kg samples of the treated peat and to one series different amounts of glucose only were added, to the other the glucose and 2 per cent N (as urea). The containers were incubated at room temperature (18° to 20°C) for nearly two years and samples were analysed for non-exchangeable N in the organic matter at different times after starting the experiment. In the samples treated with urea the non-exchangeable nitrogen rose from 1.8 to 2.2 per cent during an incubation period of 14 months. The incorporation of such large amounts of ammonium ions which, during the incubation period, were readily formed from urea, was not due to biological immobilization since the easily decomposable carbon compounds needed for such a process were not present. Chemical reaction of ammonia with humus compounds, formed by certain types of soil microbes from the *Sphagnum* residues, was believed by Wieringa to be responsible for the incorporation of the nitrogen.

Fixation of ammonia by soil organic matter was observed by Mattson and Koutler-Andersson (1943), Sohn and Peech (1958) and Nommik and Nilsson (1963). According to these authors, incorporation of the NH_4 nitrogen takes place during oxidation processes at an alkaline reaction. Wieringa, in his experiments with *Sphagnum* peat described above, found low values for the respiration quotient during the incubation period and high pH values occurred only very seldom.

Although nitrogen incorporation in soil organic matter under field conditions has not yet been reported, it is highly probable that this process can be the cause of nitrogen deficiency of plants on certain types of soils.

FIXATION BY CLAY MINERALS

A different type of ammonia fixation takes place in the lattices of some clay minerals like illite, vermiculite and montmorillonite. In the surface layers of various soils 2 to 8 per cent of the total nitrogen was found to occur in the form of fixed ammonia (Bremner, 1959; Nommik, 1957; Stevenson and Dhariwal, 1959). The exchange of interlayer cations against K or NH_4 ions results in a contraction of the crystal lattice. This fixation of ammonium is reversible only with difficulty and must, therefore, be considered a loss of directly available nitrogen. When potassium or ammonium ions are added to the soil, the fixation of a subsequent ammonium dressing is considerably lessened (Jansson, 1958). On the contrary, by drying and heating the soil or by adding potassium immediately upon the ammonium addition, the fixation is highly enhanced (Nommik, 1957). These results can be explained in terms of differential effects on the distances between the layers of the crystal lattice.

A good example of the effect of layer distance of the crystal lattice on ammonium fixation was given by van Schreven (1963). The particles smaller than 2μ in the newly reclaimed clay soils in the Zuiderzee polders contain approximately 80 per cent Fithian illite (d = 10.8 Å). The ammonium-fixing capacity of this clay mineral is only 3 to 10 per cent. Upon ageing of Fithian illite, a process which may take several hundreds of years, open illite (Ammersooiet, d = 15.6 Å), will be formed. This transformation has taken place in old clays in the Netherlands and here the NH_4-fixing capacity was found to be much higher (20 to 60 per cent of the added NH_4 nitrogen).

The capacity of autotrophic (nitrifying) bacteria, heterotrophic micro-organisms, and higher plants to release this fixed ammonia seems to depend on the effect of these organisms on the layers of the crystal lattice. In general, when ammonium fixation has not been enhanced by a subsequent potassium addition, a good deal of the fixed ammonium seems to be released, although at a lower rate, by the micro-organisms and the higher plants (Axley and Legg, 1960; Walsh and Murdoch, 1963; Welch and Scott, 1960; Legg and Allison, 1959).

LOSSES OF INORGANIC NITROGEN FROM SOIL BY VOLATILIZATION

Volatilization of inorganic nitrogen compounds may give rise to considerable losses of soil nitrogen, and therefore may seriously affect soil fertility. Chemical, biochemical or microbial factors may be responsible for this phenomenon.

VOLATILIZATION OF AMMONIA

Such losses are of chemical nature; they occur on alkaline soils. Since clay minerals and humus compounds bind the ammonia, this type of volatilization mainly concerns ammonium fertilizers applied on tod of fairly light alkaline soils (for instance, those of some of the newly reclaimed polders of the former Zuiderzee which may contain up to 10 per cent $CaCO_3$ (Lehr and Wesemael, 1961). Mixing the ammonium fertilizers with the soil reduces or entirely prevents the losses.

Considerable losses by volatilization of ammonia may occur upon the application of urea as a fertilizer. This compound is readily decomposed to ammonia and carbon dioxide by micro-organisms with urease activity (for instance, *Bacillus pasteurii*) and even on non-alkaline soils considerable amounts of ammonia will be lost to the atmosphere, unless the urea is mixed with the soil.

Furthermore, very large amounts of nitrogen are lost by volatilization of ammonia from stable manure and liquid manure when incorrectly applied by farmers. The application of the latter should take place under humid weather conditions when rainfall may be expected. Stable manure should be ploughed in as soon as possible after spreading.

LOSSES BY CHEMICAL MECHANISMS

At the beginning of this century Wagner (1903) stated that, during nitrification in the soil, part of the nitrogen may be lost. This statement received little attention until in recent years the original findings of Wagner were confirmed by Gerretsen and De Hoop (1957). Subsequently, similar results were obtained by other investigators (Soulides and Clark, 1958; Clark *et al.*, 1960). Nowadays it is the general opinion that nitrification in soil—although often proceeding with 100 per cent efficiency—may be accompanied by considerable nitrogen losses, particularly if ureum is the nitrogen source.

It is generally assumed that the nitrogen losses are the consequence of the chemical decomposition of nitrite under more or less acid conditions, as the highest losses were found in soils which accumulated nitrite and which were originally acid, or became acid during incubation. However, there is no direct proof that other nitrogenous compounds in a stage of oxidation intermediate between nitrite and hydroxylamine are not likewise involved in the chemical decomposition.

It was found that N_2, N_2O and NO were formed as the result of chemical decomposition. Several mechanisms were suggested to explain the occurrence of these gases.

1. The decomposition of nitrous acid, leading ultimately to the formation of nitric acid:

$$3\,HNO_2 \longrightarrow 2\,NO + HNO_3 + H_2O$$
$$2\,NO + O_2 \longrightarrow 2\,NO_2$$
$$2\,NO_2 \longrightarrow N_2O_4 \xrightarrow[1/2\,O_2]{H_2O} 2\,HNO_3$$

2. The reaction between nitrous acid and amino acids (Van Slyke reaction):

$$RNH_2 + HNO_2 \longrightarrow ROH + H_2O + N_2$$

3. The decomposition of ammonium nitrite:

$$NH_4NO_2 \longrightarrow N_2 + 2\,H_2O$$

By none of these mechanisms could the results be explained satisfactorily, as in soil different results were obtained as compared with pure solutions of reactants. In soil, considerably higher quantities of gaseous nitrogen were formed which originated from nitrite exclusively, as was shown with labelled compounds (Jones, 1951; Wyler and Delwiche, 1954). The latter results excluded the mechanisms 2 and 3. Consequently, some soil factor must be involved. This may be the reducing effect of metallic ions like Fe and Mn (Wullstein *et al.*, 1963; Chao and Kroontje, 1966), or the reaction with organic compounds like phenols and quinones, which are susceptible to nitrosation and subsequent decomposition (Bremner, 1957; Stevenson and Swaby, 1964).

LOSSES BY DENITRIFICATION

Introduction

Denitrification is the microbial process in which nitrate, instead of oxygen, is used in the respiration of certain types of soil microbes, the so-called denitrifying bacteria. In biochemical terms this means that nitrate is used as the terminal electron acceptor in the dissimilation of organic and sometimes inorganic compounds. The process, also called dissimilatory nitrate reduction, gives rise to the formation of a number of reduction products, namely NO_2^-, NO, N_2O, N_2 and NH_3. Most investigations of denitrification in soil concentrate on the microbial reduction of nitrate to the gaseous products N_2O and N_2 which are responsible for considerable losses of nitrogen.

The microbial reduction of nitrate to nitrite can be carried out by many bacteria. However, far fewer species are able to form gaseous products from nitrate, i.e., to bring about denitrification. Two main types of denitrifying bacteria can be distinguished, the non-spore formers of the *Pseudomonas*, *Micrococcus* and *Achromobacter* type and the spore formers of the *Bacillus licheniformis* and *B. cereus* type. The former are obligate aerobic bacteria which, however, can live under anaerobic conditions in the presence of nitrate. Under such conditions there is a complete breakdown of the organic substrates; the tricarboxylic acid cycle and the cytochrome system function as in the presence of oxygen and large amounts of cell material are synthesized per unit of substrate consumed. This is in sharp contrast to the denitrifying bacilli which are facultative anaerobic bacteria, but which are unable to carry out an aerobic type of breakdown of organic substrates under anaerobic conditions in the presence of nitrate. The latter compound is indispensable for these bacteria under anaerobic conditions only when reduced compounds like glycerol or lactate are the substrate. Nitrate is used under these conditions as a hydrogen acceptor in fermentative reactions. Therefore, the amount of energy liberated, and thus the amount of cell material synthesized, per unit of substrate consumed under anaerobic conditions in the presence of nitrate are much lower than in the case of non-spore formers. This undoubtedly has been the reason for the results obtained by Woldendrop (1963*b*) with sods from permanent grassland which were supplied with nitrate and exposed to anaerobic conditions. Although spore formers of the *B. cereus* type were the most numerous denitrifying bacteria in the original soil, rods of the genera *Pseudomonas* and *Achromobacter* readily increased in numbers in the nitrate-treated sods and were found to be responsible for the denitrification.

Effect of oxygen supply

It is a well-established fact that denitrification proceeds only under conditions of oxygen deficiency. Skerman and McRae (1957*a*, 1957*b*) found that even in vigorously aerated nutrient solutions oxygen deficiency and denitrification might occur if the cultures had a high requirement for oxygen. Concentrations of dissolved oxygen above 0.2 ppm were found by these authors to suppress denitrification. Other workers observed that not only was the activity of the enzyme system, required for the dissimilatory nitrate reduction, inhibited by oxygen, but also the formation of these enzymes (Sacks and Barker, 1949; Kluyver and Verhoeven, 1954; Pichinoty and d'Ornano, 1961*a*; 1961*b*).

Under soil conditions the oxygen supply is even more complicated because several factors may be responsible for the oxygen content of the soil solution which ultimately determines whether or not denitrification takes place. Oxygen consumption by the higher plants and by the microflora and microfauna, diffusion rate of oxygen in the soil, partial pressure of oxygen in the soil atmosphere and soil temperature are among the most important factors. The partial pressure of oxygen in the soil atmosphere is decreed by its partial pressure in the free atmosphere, by its rate of diffusion through the soil, and by its consumption. The depth of the oxygen-consuming layer, moisture level, packing of the soil and the occurrence of soil aggregates affect the oxygen pressure of the gas phase.

This large number of soil factors affecting oxygen supply, and thus denitrification, may explain the controversial results obtained by various authors studying the influence of oxygen on denitrification in soil. Broadbent, (1951), for instance, observed denitrification at all oxygen levels in the gas phase above the soil, while Broadbent and Stojanovic (1952) reported that denitrification was inversely related to the partial pressure of oxygen above the soil but still occurred at the highest oxygen levels. On the contrary, Nommik (1956), Bremner and Shaw (1958) and Carter and Allison (1960) showed that under their experimental conditions, denitrification in well aerated soils was negligible.

Influence of substrates (hydrogen donors)

Although oxygen deficiency is the first prerequisite, no denitrification will occur when oxidizable substrates (hydrogen donors) are lacking. It may be assumed that in the soil the reducing effect is mainly supplied by organic compounds. These substrates are not only required by the denitrifying bacteria themselves but also by other types of organisms to bring about anaerobic conditions in the soil. The latter effect may be seen in the experiments of Jansson and Clark (1952), in which lucerne promoted denitrification to a larger extent than straw.

Most soils presumably contain adequate amounts of organic compounds to bring about denitrification when oxygen is lacking. Bremner and Shaw (1958), found denitrification to be absent only when the content of soil organic matter was less than 1 per cent. It should be stressed, however, that the organic soil material should be readily decomposable by the denitrifiers; humus compounds, for instance, are practically resistant to microbial attack and therefore are useless as substrates for denitrification. Woldendorp (1962, 1963*a*, 1963*b*) found that amino acids are an excellent substrate for denitrifying organisms of the *Pseudomonas* type. The favourable effect of root excretions of pea plants on denitrification was ascribed by this author to the quite large amounts of amino acids contained in the pea root exudates. Grasses, which excrete considerably smaller amounts of amino acids, were much less effective in stimulating denitrification.

Effect of plants

In general it is believed that, in soils covered with plants, losses by denitrification are low because the nitrate is readily taken up by the vegetation (Allison, 1955; Walker *et al.*, 1954). This situation does not hold for the permanent grassland occurring on many humid soils in the Netherlands where large amounts of fertilizer nitrogen (mostly in the form of ammonium nitrate) are applied. Although high yields of herbage are harvested from such soils, the nitrogen balances generally are low (mostly no more than 50 per cent of the applied fertilizer nitrogen is accounted for in the harvested herbage; Mulder (1949). Since leaching of nitrogen on grassland soils in general is only of minor importance, denitrification was thought to be one of the main reasons for the low nitrogen yields. Immobilization of the added fertilizer nitrogen in resistant organic compounds is presumably a second reason.

In pot experiment with sods and with sown grass, supplied with labelled nitrate nitrogen, Woldendorp (1963*b*) and Dilz and Woldendorp (1960), found denitrification losses ranging from 10 to 40 per cent. The losses were much smaller (3 to 9 per cent) when the grass of the sods had been killed by clipping the tops several times. These results were interpreted by assuming that the root systems of living plants exert a stimulating effect on denitrification: (a) by creating oxygen deficiency in the rhizosphere; and (b) by the excretion of organic compounds functioning as hydrogen donor in denitrification. More evidence of the latter phenomenon was obtained by Woldendorp (1963*b*) in Warburg experiments with mixed cultures of plants and various types of denitrifying bacteria. The results of Woldendorp have recently been repeated and confirmed by Mishustin and collaborators (1965).

Soil biology and phosphorus supply of higher plants

INTRODUCTION

Phosphorus may exist in the soil in different compounds depending on the type of soil, pH, organic matter content, and presumably a number of further soil characters. Most of these compounds are practically insoluble, so that losses from the soil resulting from leaching under humid climatic conditions are far less important than those of nitrogen, particularly when the latter occurs as nitrate.

Although phosphorus deficiency of higher plants may be the result of the absence of sufficient amounts of this nutrient element from the soil, it is often due to poor availability. In fertile soils, adequate amounts of phosphorus for optimal plant growth are rendered available from the soil phosphates, either by the plant roots themselves (by excretion of carbon dioxide and eventually organic acids) or by micro-organisms. However, in many soils the phosphorus compounds are very resistant to these actions, so that the plants may suffer from phosphorus deficiency, and serious yield depressions occur, unless phosphatic fertilizers are applied in sufficient amounts. But in many instances considerable amounts of the added phosphorus are fixed by soil constituents in difficultly available compounds so that heavy applications of phosphatic fertilizers have to be applied regulerly. On such phosphorus-fixing soils, the type of fertilizer added, the time and manner in which the phosphatic fertilizers is applied, and measures to promote the liberation of fixed phosphorus are important tools to enhance the phosphorus supply of the plant and thus to raise soil fertility.

OCCURRENCE OF PHOSPHORUS IN THE SOIL

Phosphorus exists in the soil in inorganic and organic compounds. In the inorganic form it is combined largely with Ca, Fe, Al and F; furthermore it may be adsorbed by clay minerals. In many soils, particularly in the lower layers, a part of the phosphorus is found as fluor apatite, the mineral from which most of the soil phosphorus was once derived (Pierre, 1948). It is highly resistant to solubilization by plant roots and micro-organisms, and its phosphorus, therefore, is practically unavailable to the plants. The same is true of the phosphorus in hydroxyapatite and in several Fe- and Al-containing minerals.

In neutral or alkaline soils the soluble monocalcium phosphate, derived from microbial breakdown of plant and animal residues, or introduced with certain phosphatic fertilizers, is converted to di- and tricalcium phosphates which are precipitated. These finely precipitated phosphates, owing to their large surface, are readily solubilized by plant roots and micro-organisms, so that they are excellent sources of P supply to the higher plants.

In acid soils which are in general poor in calcium ions, the phosphate is mostly precipitated as the ferric or aluminium compounds which are often very resistant to solubilization and, therefore, are poor sources of phosphorus supply to plants. This is particularly true of soils rich in iron, as is the case in lateritic soils in the tropics and subtropics and in low-moor peat soils along iron-containing brooklets in temperate regions. On such soils the plants often show severe symptoms of phosphorus deficiency and relatively large amounts of phosphatic fertilizers have to be applied regularly to obtain healthy crops.

From the standpoint of P availability, a soil pH of 6.5 to 7 is desirable because this is above the range of minimum solubility of iron and aluminium phosphates while it is below the pH of minimum solubility of calcium phosphates.

In addition to inorganic phosphates, organic P compounds contain a relatively large part of the soil phosphorus. This does not apply only to peat soils which for the larger part consist of organic matter but also for the surface layers of the soils. In many of the latter. one-third and in prairie soil even two-thirds of the total phosphorus occurs in organic compounds (Pierre, 1948), In Finnish soils where accumulation of organic matter is favoured by the humid and cold climate, 20 to 60 per cent of the phosphorus in sandy, loam and clay soils and 20 to 70 per cent of that in soils with a high content of organic matter was found to occur in organic compounds (Kaila, 1963).

The organic phosphorus is partly derived from plant residues and partly synthesized by micro-organisms from inorganic phosphates. In contrast to agricultural plants which contain relatively small amounts of phosphorus (0.1 to 0.5 per cent in the dry matter), micro-organisms are rich in this element (fungal mycelia contain 0.5 to 1.0 per cent, bacteria 1.5 to 2.5 per cent P in the dry matter). The ratio of organic C to organic P of mineral soils is in general considerably lower (100 : 1 to 200 : 1) than that of peaty soils (200 : 1 to 1900 : 1). With the latter type, the cultivated topsoil has a clearly lower value than the uncultivated subsoil. These values indicate that the organic soil phosphate (either derived from the higher plants, or from micro-organisms) is considerably more resistant to microbial breakdown than the organic carbon (Pratt, 1963).

There are three main groups of P-containing compounds in plants and micro-organisms, namely nucleic acids, phospholipids and phytin. Nucleic acids are found in the largest amounts in both plants and micro-organisms, phospholipids occur in low concentrations in higher plants, in moderate amounts in micro-organisms. Phytin, the Ca and Mg salt of inositol hexaphosphoric acid, is the most important P compound in seeds.

Nucleic acids and particularly phytin have been found to be important P constituents of the soil organic matter. However, the sum of these compounds generally does not account for the amount of phosphorus found to be present in the organic form. It therefore must be assumed that other still unknown organic P compounds occur in the soil.

Although both phytin and nucleic acids when applied in sterile nutrient solutions are readily utilized as P sources by higher plants, they are far less available to plants and micro-organisms when present in soil. This depends on their reaction with soil constituents which may bring down their solubility to a very low level. Phytin behaves like soluble inorganic phosphate in its reaction with calcium, ferric or aluminium ions. In acid soils it forms salts with ferric and aluminium ions wich are even less soluble than the inorganic ferric and aluminium phosphates. Under such conditions the phosphorus is unavailable to plants and it is also resistant to phytase, the enzyme produced by phytin-decomposing micro-organisms.

Phytin may also be fixed tightly by certain clay minerals like bentonite (Goring and Bartholomew, 1950). This fixation is strongest at pH 3.5 to 4.5; above pH 6.5 it decreases rapidly. Nucleic acids or nucleoproteins are fixed in a similar way by bentonite. This bond is also strongest at low pH values.

EFFECT OF MICRO-ORGANISMS ON THE TRANSFORMATION OF PHOSPHORUS COMPOUNDS

In spite of the relatively large amounts of phosphorus taken up by living organisms (N/P ratio in higher plants 15 : 1 to 10 : 1, in micro-organisms 5 : 1 to 10 : 1), the phosphorus cycle is a much less spectacular phenomenon than is the case with the C, N, and S cycles. This is mainly due to the fact that phosphorus is considerably less mobile in nature than are the other three elements.

Although phosphorus ions may exist in different states of oxidation, no change in valency occurs during the assimilation of inorganic phosphate by living organisms and during the breakdown of organic P compounds by micro-organisms. Volatilization of P compounds, therefore, does not take place during these processes. There are a few statements in the literature (Alexander, 1961; Rudakow, 1929) concerning the biological reduction of orthophosphates (H_3PO_4) to phosphite (H_3PO_3), hypophosphite (H_3PO_2) and phosphine (PH_3), involving a valency change of the P atoms from +5 to —3, but so far no convincing evidence of the existence of such a scheme is available.

The phosphorus cycle in nature, therefore, is limited to the following processes: (a) uptake of phosphatic ions ($H_2PO_4^-$) by the plant roots, preceded by solubilization effects of root excretions and micro-organisms; (b) synthesis of organic compounds (including nucleic acids, phospholipids, phytin, phosphorylated sugars, etc.) by plants, micro-organisms and animals; (c) conversion of organic P compounds derived from plant and animal residues by micro-organisms in the soil; and (d) fixation of organic and inorganic P compounds by soil constituents.

Micro-organisms may affect the P supply of higher plants (soil fertility) in different ways, namely: (a) by immobilizing available phosphates in cellular material; (b) by decomposition of organic P compounds, releasing soluble inorganic phosphate ($H_2PO_4^-$); (c) by promoting the solubilization of insoluble phosphates.

IMMOBILIZATION OF PHOSPHORUS

Immobilization of available soil phosphates may occur when relatively large amounts of available carbon and nitrogen are present in the decomposing organic material in the soil, whereas available phosphorus is low (C/P ratio $>$ 2-300 : 1). Since the phosphorus content of microbial matter is rather high (see the values mentioned above) substantial amounts of P may be immobilized when the amounts of synthesized microbial cell material are high.

Although this phenomenon is much less well known than the immobilization of nitrogen, it may be observed when straw containing a low percentage of P ($<$0.2 per cent) is added to a soil moderately poor in phosphorus. Nothing is known of the velocity of remobilization of the microbial phosphorus when the available carbon has largely been consumed. It may be expected that the type of soil (pH ,Fe and Al contents, etc.) as well as the type of micro-organisms may affect the immobilization.

DECOMPOSITION OF ORGANIC PHOSPHORUS COMPOUNDS

Although most organic P compounds derived from decaying organisms are readily mineralized by micro-organisms when incubated in an artificial medium, these compounds may be much more resistant to further microbial degradation when formed in the soil. This applies particularly to acid soils rich in ferric and aluminium oxides, and to acid clay soils. As stated above, such soils fix organic P compounds like phytin and nucleotides as the Fe and Al salts. In spite of the presence of phytase- and nuclease-producing soil micro-organisms, degradation of these compounds is very slow. Raising the pH of the soil improves the solubility of the organic P compounds and this promotes the release of soluble inorganic phosphates by microbial activity.

Organic P compounds in peat or in humus are generally highly resistant to microbial degradation. This may be due to their occurrence within plant particles protected against microbial degradation by the presence of resistant organic matter. A further explanation would be the formation of resistant complexes of organic P compounds and other organic substances. So far very little is known about these resistant organic P compounds.

SOLUBILIZATION OF INORGANIC PHOSPHORUS COMPOUNDS

Micro-organisms are able to promote the solubilization of inorganic P compounds in different ways: (a) by the formation of carbon dioxide and particularly of organic acids which may solubilize certain insoluble phosphates ; (b) by their reducing effect which may result in the reduction of ferric to ferrous compounds which are more readily taken up by the plants; (c) a special case of (b) is the production of H_2S which also favours the solubility of ferric phosphates.

Effect of CO_2 *and organic acids*

The solubilizing effect of micro-organisms on a number of inorganic phosphates was clearly shown by Gerretsen (1948) in experiments with oat and mustard plants growing in sand that was either sterile or infected with soil micro-organisms. In the latter case the plants absorbed considerably higher amounts of phosphorus than under sterile conditions. This resulted in a pronounced increase in the yield of dry matter.

Rock phosphate and ferrous phosphate, which were almost unavailable to oat plants in the sterile sand cultures, were slightly more available in the non-sterile medium. In the case of di- and tricalcium phosphates and bone meal, the solubilizing effect of micro-organisms was much more pronounced. These phosphates were moderately available in the sterile cultures, obviously as a result of carbon dioxide excretion by the plant roots. When micro-organisms were present in the rooting medium, much larger amounts of phosphate were taken up by the plants. This was presumably due to the microbiological formation of organic acids from the carbohydrous substrate excreted by the plant roots. That the solvent action of soil organisms on insoluble phosphate mainly occurred in the rhizosphere was shown by growing the oat plants in glass vessels containing a glass bottom covered with an agar film in which tricalcium phosphate had been precipitated. Clear solubilization zones were observed in the neighbourhood of the roots.

From the results obtained with some isolated bacteria, Gerretsen concluded that the solvent action on phosphates was not restricted to some typical soil organisms but would be a general character of several types of organisms.

This conclusion is partly at variance with the suggestion of subsequent investigators that the type of organic acid formed and therefore the type of organism is important in explaining the above-described phenomenon. It should be stressed, however, that most of these investigators studied the solubilization of phosphates and other minerals in nutrient media having a high content of glucose and a low content of nitrogenous nutrients. It is a well-known fact that such conditions are highly favourable for the microbial production of organic acids. Results obtained in this way should, therefore, be confirmed by carrying out experiments with higher plants growing in P-deficient soil or sand supplied with different P compounds in the absence and presence of micro-organisms.

Sperber (1957), using a modified Gerretsen technique, isolated large numbers of phosphate-dissolving bacteria from soil and particularly from the rhizosphere. Tested under laboratory conditions, formic, acetic, propionic, lactic, glycollic, fumaric, adinic and succinic acid were found to be formed by these bacteria. All these acids solubilized tricalcium phosphate, the α-hydroxy acids being among the most reactive. In subsequent papers (Duff *et al.*, 1963; Louw and Webley, 1959*a*, 1959*b*; Webley and Duff, 1965) stress was laid on the importance of 2-ketogluconic acid as a solvent for poorly soluble phosphates. This acid as well as a number of hydroxy acids may form complexes (chelates) with cations like Ca^{++} and Fe^{+++}, thus enhancing the solubility of the phosphate. Bacteria forming 2-ketogluconic acid usually belong to the pseudomonads. Under laboratory conditions this acid may be readily formed from sugars and under such conditions its solubilizing effect on a number of minerals was shown by Duff *et al.* (1963).

Although bacteria producing 2-ketogluconic acid are less abundant in soil than those producing lactic and acetic acids, the former were found by Webley and Duff (1965) on the root surface and in the rhizosphere of barley plants in larger numbers than in the rest of the soil, namely 1.3, 1.23 and 0.025 per cent of total counts, respectively. Furthermore, these authors found a pronounced increase in numbers around starch-containing pellets buried in soil. In spite of these results, no experimental evidence has been provided in the literature so far showing the difference in phosphate-dissolving activity of different types of micro-organisms under soil conditions.

Special cases of microbial phosphate solubilizatoin by acid production are the formation of nitric acid from ammonia by nitrifying bacteria and that of sulphuric acid from sulphur by bacteria of the genus *Thiobacillus.* Long ago Lipman (Lipman *et al.*, 1916) proposed applying the latter reaction in agriculture for the transformation of rock phosphates into soluble phosphate.

Effect of reduced conditions on the availability of phosphate in the soil

When soils containing appreciable amounts of unavailable ferric phosphate are kept for a prolonged period under anaerobic conditions, a considerable part of the phosphate may be converted to a soluble form. This is particularly the case in the presence of decomposable organic matter. Anaerobic degradation of this material by micro-organisms decreases the redox potential of the soil which affects the reduction of the iron from the ferric into the ferrous state. This brings about a pronounced increase of the solubility of the phosphate.

The beneficial effect of anaerobic conditions on the availability of soil phosphate is well-known from the cultivation of rice on flooded soils. The response to phosphatic fertilizers, which may be very pronounced under upland conditions, may almost completely disappear when the soil has been flooded for some months (Shapiro, 1958*a*, 1958*b*; Savant and Ellis, 1964). These authors showed that the addition of organic matter like paper pulp increased the liberation of soil phosphate. By adding different types of phosphate to the soil it was shown that the reduced conditions (flooding) did not enhance the solubility of tricalcium phosphate, affected slightly the solubility of $AlPO_4$, but had by far the strongest effect on $FePO_4$.

This type of phosphorus solubilization has also been studied in some detail by Bromfield (1960). Top growth from subterranean clover was supplied with different types of phosphate, including ferric and aluminium phosphates similar to those occurring in soils, and incubated under anaerobic conditions. The solubility of the ferric phosphate increased steadily during the incubation period of 26 days at 25°C while the pH remained at the same level. Aluminium phosphate did not dissolve under these conditions.

Formation of H_2S

A special case of mobilization of ferric phosphate under anaerobic conditions depends on the formation of H_2S as may occur in flooded soils. This compound may be formed either from sulphate by sulphate-reducing bacteria of the genus *Desulfovibrio* or by the decomposition of S-containing amino acids by several heterotrophic micro-organisms.

INOCULATION OF SOILS BY PHOSPHATE-DISSOLVING BACTERIA

Inoculation of soils with 'phosphobacterin', a so-called bacterial fertilizer containing large numbers of phosphate-dissolving bacteria, particularly strains of *Bacillus megaterium,* is common practice on many soils of the U.S.S.R. Several Russian papers give the impression that such a treatment may be comparable to a treatment with phosphatic fertilizers. However, the critical review by Mishustin and Naumova (1962) of the results of a large number of field experiments carried out with 'phosphobacterin' clearly shows that this not so. These authors report that added 'phosphobacterin' in many cases did not affect plant growth or gave only insignificant increases in yield. Nevertheless, in about 30 per cent of a large number of experiments, and in the case of soils of the chernozem type even more frequently, small but often statistically significant increases in yield (mostly varying between 5 and 10 per cent) were obtained. The response to the 'phosphobacterin' treatment in general is higher on rich soils (for instance, those of the chernozem type) than on poor soils. Furthermore, vegetables give higher growth and yield responses than field crops.

So far, no satisfactory explanation has been given of the beneficial results sometimes obtained with 'phosphobacterin'. That the added phosphobacteria, would decompose organic soil phosphates, or perhaps solubilize difficultly available inorganic soil phosphates with the aid of root exudates, seems rather improbable for the following reasons (Mishustin and Naumova, 1962).

1. Treatment of seeds with 'phosphobacterin' containing large numbers of *B. megaterium* did not result in an abundance of these bacteria on the root surface or in the rhizosphere.
2. In the rhizosphere of plants growing in natural soils several bacteria were found which were able to dissolve soil phosphorus when tested in pure culture under laboratory conditions. If the soil conditions were favourable to the phosphorus-solubilizing activity of the added phosphobacteria, such an activity would undoubtedly also be displayed by the original soil microflora.

In view of these considerations, Mishustin and Naumova ascribe the beneficial results of 'phosphobacterin' treatment only partly to its effect on P supply of the plants. A fungistatic activity in the rooting zone of the higher plants by the added phosphobacteria, and the supply of amino acids, vitamins and other plant-growth-promoting compounds are suggested to be responsible for the observed effect (Mishustin and Naumova, 1962).

EFFECT OF DEEP-ROOTED PLANTS ON TRANSFER OF PHOSPHORUS FROM THE SUBSOIL TO THE SURFACE SOIL

There are strong indications that the fertility of P-deficient surface soils can be raised by the cultivation of deep-rooted legumes which bring phosphorus from the deeper layers into the biological cycle of the surface soil. This holds for those soils which contain large reserves of subsoil phosphate (Pierre, 1948).

Soil biology and trace element supply of higher plants

The normal development of living organisms, plants and micro-organisms as well as animals, requires the uptake of small amounts of several trace elements. Iron, manganese, copper, zinc, boron, molybdenum and presumably cobalt are essential for higher plants, and, probably with the exception of boron, for micro-organisms. Animals, in addition, need small amounts of iodine and fluor.

Although the quantities of these elements required for optimal growth are small, and sometimes very small in comparison with those of the major nutrient elements, a reduced supply brings about serious deficiency symptoms which may bring down crop yields almost to zero. The reason for this pronounced effect of trace elements on living organisms is their function as co-factors in enzymatic reactions. An inadequate supply of these elements, therefore, affects vital metabolic processes.

From the foregoing considerations it will be clear that fertile soils should be able to supply the plants with adequate amounts of the essential trace elements. This does not apply only to the elements required for plant growth, but also to those required by the animals utilizing the plants. Excessive amounts of available trace elements as occurring under certain soil conditions may give rise to toxicity phenomena in higher plants or animals and therefore should be eliminated.

Trace element deficiencies may be due either to the absence of sufficient amounts of the element concerned, or to specific soil conditions which render the element unavailable to higher plants or prevent its uptake by the roots. The former may occur with poor sandy soils, the latter with soils containing certain types of organic matter or excessive amounts of certain inorganic compounds fixing the trace element or exerting an antagonizing effect. Biological processes sometimes contribute to the fixation as well as to the solubilization reactions. Furthermore they are responsible for the formation of the soil organic matter.

When the deficiency symptoms are due to the absence of sufficient amounts of some trace element, the addition of small amounts of the element suffices to give healthy plants. This is in contrast to the shortage due to secondary reactions when relatively large amounts of the trace element have to be added to the soil to correct the deficiency symptoms.

To demonstrate that secondary reactions are involved, the divided-root system technique is used by the authors. Glass jars are divided into two halves by vertical glass plates; one half is filled with the deficient soil and the other with purified sand. The test plants placed on top of the glass plates grow with part of their roots in the sand and with the other part in the soil. Different amounts of the trace element are added to either the sand or the soil of each jar in the series. Large differences between the amounts of the trace element required in the soil and in the sand indicate either fixation in the soil or the presence of substances interfering with its uptake.

BIOLOGY OF MANGANESE IN RELATION TO SOIL FERTILITY

INTRODUCTION

Although most cultivated soils contain large amounts of manganese, the availability of this trace element to the plant roots under certain soil conditions may be very poor. Therefore, manganese deficiency of plants is sometimes the cause of a serious reduction in yield of agricultural crops. This may even happen on fertile soils having excellent physical and chemical properties and able to produce maximum yields if one succeeds in introducing small amounts of the trace element into the plants.

The reason for this abnormal behaviour of manganese in the soil is its relatively easy change in valency. Since the higher-valent ions generally have a much greater tendency to form insoluble compounds, conditions favouring oxidation curtail availability and thus promote the appearence of deficiency symptoms in the higher plants. There is no absolute rule for this, however, and there are many secondary factors affecting the availability of this micronutrient.

Manganese exists in the soil in various states of oxidation, depending mainly on pH and oxygen supply in relation to organic matter content and microbial processes. In acid soils it is chiefly present in the bivalent state, either in the soil solution or in an exchangeable form bound by the soil colloids. Additional amounts may be fixed in certain minerals and in organic compounds. Manganous ions are readily taken up by the plants, at pH values below 5 sometimes in excessive amounts so that toxicity symptoms may occcur which give rise to serious losses of crop yields (Löhnis, 1951).

In neutral and alkaline soils manganese is much less available to the higher plants and as a result deficiency symptoms may occur. According to many authors, under these conditions manganese exists mainly in the tetravalent and presumably partly in the trivalent state (Dion and Mann, 1946). These higher-valent manganese ions occur in the form of manganic oxides (MnO_2 and Mn_2O_3) which may represent various types of minerals (Groot, 1962-63; Jones and Leeper, 1951*a*, 1951*b*). Complexes of higher-valent manganese ions and organic matter may presumably also be found.

The conversion of manganous to manganic ions upon neutralization of acid soils may be either chemical or biochemical (microbial). The reverse, the reduction of manganic oxides to manganous ions, can also be carried out by micro-organisms when the soil is kept under anaerobic conditions.

Although the availability of the manganese in manganic oxides is much less than that of bivalent manganese, there are large differences between different oxides in their ability to supply manganese to plants. This was shown by Jones and Leeper (1951*a*) by the addition of various synthetic manganic oxides to a number of neutral and alkaline soils on which oats showed severe symptoms of manganese deficiency. Some of the manganic oxides tested gave healthy plants, others were completely inactive in supplying manganese to the oat plants. Particle size was found to be one of the factors affecting activity, small particles being much more active than large ones owing to their higher specific surface which makes them more liable to reduction by plant roots or micro-organisms. A further factor is crystallinity, highly crystallized oxides being more inert than those of low crystallinity.

Bromfield (1958) studied the properties of a manganic oxide (presumably MnO_2) formed by a manganese-oxidizing *Corynebacterium*. It was not crystallized and it was easily reduced to manganous ions by an aqueous quinol solution (0.05 per cent w/v). When added to a neutral soil, on which oat plants were showing symptoms of serious manganese deficiency, only a slight response was observed, but in sand (pH 6.7-7.0) the MnO_2 was available as readily as $MnSO_4$. The reduction of MnO_2, at least in this particular experiment, was found to be due to the root system itself and not to contaminating bacteria. The roots excreted certain compounds which, upon collection and addition to MnO_2 at pH values below 6.5, could reduce and dissolve the oxide specifically. Only one oxide was tested by Bromfield. It is highly probable that the large spherical globules of manganic oxide formed outside fungi and other micro-organisms owing to their relatively small surface area are more resistant to this dissolving action.

Soils containing a certain amount of organic matter (humus) are generally more prone to manganese deficiency than those low in organic matter when neutralized or made slightly alkaline. According to some authors (Groot, 1962-63; Heintze, 1957; Page, 1962) this is due to the soil organic matter itself fixing the manganous ions as complexes so that they become unavailable to the plants.

TRANSFORMATION OF MANGANOUS TO MANGANIC IONS BY MICRO-ORGANISMS

The oxidation of the bivalent to the higher-valent manganese ions may proceed in different ways depending on the pH of the medium. In the absence of micro-organisms and organic compounds the reaction does not take place to any extent, unless the pH exceeds a value of 9.

However, in the presence of hydroxyacids chemical oxidation of manganous ions proceeds at a considerably lower pH (from approximately 8 upward). Such acids, e.g., citric, malic or lactic acid, are common products of the carbohydrate metabolism of micro-organisms. This type of chemical reaction which apparently leads to the formation of trivalent manganese is affected by the pH, the concentration of manganous salt and the concentration of hydroxyacid. At high concentrations of both these compounds the reaction proceeds at considerably lower pH values (approximately 7) than at low concentrations (van Veen, Laboratory of Microbiology, Wageningen, unpublished results).

This effect of hydroxyacids on microbial manganese oxidation was studied for the first time by Söhngen (1914). Small pieces of sodium bicarbonate placed on agar plates containing 1 per cent $MnSO_4$ and 2 per cent Ca citrate or gluconate caused the formation of dark brown higher oxides of manganese, presumably MnO_2, in an area surrounding the bicarbonate. The formation of MnO_2 from the trivalent manganese in an area surrounding the $NaHCO_3$ in Söhngen's experiments was presumably due to the high concentration of the bicarbonate in this zone. No manganese oxidation took place when 2 per cent Ca butyrate instead of the citrate or gluconate was present. A number of pure cultures of different bacteria, including *Azotobacter chroococcum*, *Pseudomonas fluorescens*, *Ps. trifolii* (*Bact. herbicola*) and *Aerobacter aerogenes* when streaked on the hydroxy-acid-containing agar plates also became surrounded by a greyish brown zone of an oxidized Mn compound. The growing organisms, after utilization of the organic acid, apparently succeeded in bringing the pH of the medium to a value comparable to that obtained with $NaHCO_3$, namely to approximately 8. According to Söhngen this would result in the formation of manganic oxides.

In addition to micro-organisms of the above-mentioned type which presumably include the majority of soil organisms, namely those which can utilize hydroxyacids, a second group of non-specific micro-organisms—those forming and excreting hydroxyacids—is thought to oxidize Mn^{++} in alkaline media.

Specific manganese-oxidizing micro-organisms are able to oxidize Mn^{++}, presumably to MnO_2, in the absence of added hydroxyacids at pH values as low as 6 and sometimes even below pH 6. These specific manganese oxidizers, bacteria as well as fungi, were isolated for the first time by Beijerinck (1913) from soil. Bromfield (1956) studied a manganese-oxidizing corynebacterium obtained from a manganese-deficient Australian soil. Sand filters of waterworks and slowly running iron and manganese-containing brooklets are also well known habitats of this type of manganese-oxidizing micro-organisms (Mulder, 1964; Mulder *et al.*, 1963; Wolzogen Kühr, 1927).

Although such organisms are thought by some authors to derive energy from the oxidation of manganous to manganic ions, so far no convincing evidence of this hypothesis has been provided (Mulder, 1964). Oxidation of manganese by these micro-organisms in general proceeds most readily in the presence of low concentrations of organic nutrients; higher concentrations of several nutrients, particularly N-containing organic compounds, although promoting growth, often retard or even entirely depress the formation of MnO_2. The manganese concentration at which the specific manganese-oxidizing micro-organisms give optimal formation of manganic oxide is much lower than that at which manganese oxidation proceeds in the presence of hydroxyacids as a result of the activity of non-specific bacteria. Bromfield found manganese oxidation by his corynebacterium only under starvation conditions (50-100 mg of both $MnSO_4$ and yeast extract per litre of medium were optimal). Similar results have been obtained in the authors' laboratory with a number of *Leptothrix* strains (van Veen, unpublished results). In a basal medium containing 0.02 per cent glucose and 0.02 per cent peptone MnO_2 formation was much more pronounced than with a ten-times higher concentration of these organic nutrients; often it was entirely suppressed under the latter conditions.

ACTIVITY OF MANGANESE-OXIDIZING MICRO-ORGANISMS IN THE SOIL

To demonstrate that micro-organisms in the soil are able to oxidize manganous compounds, Gerretsen (1936, 1937) used the modified Söhngen technique. Soil was mixed with 2 per cent agar cooled to 40°C, and poured into Petri dishes. When solidified, a hole of 2.5 cm diameter was made in the centre of each plate and filled with an agar suspension containing 1 per cent $MnSO_4$. The manganous ions diffused into the soil-agar medium where they were converted to manganic oxide when the conditions for oxidation were favourable (pH between 6.8 and 7.8, optimum pH being 7.0). In that case a brown ring was formed, surrounding the central core of $MnSO_4$ agar and consisting of numerous brown colonies of fungi and bacteria. Mixing the soil-agar suspension with chloroform or keeping the plate at 0°C prevented the formation of the brown ring, showing that the formation of the manganic oxides was caused by microbial activity.

Leeper and Swaby (1940), repeating Gerretsen's soil plaque technique, found a somewhat wider range of pH values at which manganese oxidation occurred but activity was maximal between pH 6.0 and 7.5.

When growing on agar plates with mineral salts, 0.5 per cent $MnSO_4$ and 2 per cent Ca citrate, a large proportion of the bacteria isolated by Gerretsen from the brown ring of the soil plaques formed dark-brown colonies with a brown halo apparently containing trivalent manganese-citrate complex. Although this was not tested by Gerretsen, it may be assumed that the majority of his isolates belonged to the above-mentioned non-specific manganese oxidizers, i.e., required hydroxyacids for manganese oxidation. This is concluded from similar experiments of Bromfield and Skerman (1950) who from the brown ring of soil agar-$MnSO_4$ plates isolated a number of bacteria of the genera *Pseudomonas*, *Aerobacter*, *Proteus*, *Corynebacterium* and *Bacillus* which oxidized Mn^{++} on Gerretsen's citrate medium but not on sterilized-soil-agar-$MnSO_4$ plates. However, on the original soil-agar-$MnSO_4$ plates, which contained large numbers of different micro-organisms, the brown ring of oxidized manganese was formed without added hydroxyacids. This means that the manganese oxidation was probably carried out by specific manganese oxidizers which, owing to the presence of large numbers of contaminating organisms, were difficult to isolate. A second explanation is that the formation of MnO_2 depended on the activity of mixed cultures of two different organisms, which when grown separately were unable to oxidize Mn^{++}. Examples of MnO_2 formation by mixed cultures of two micro-organisms have been recorded by Bromfield and Skerman (1950, see also Bromfield, 1956) and by Zavarzin (1962). The former obtained manganese oxidation by a *Corynebacterium* when this organism was growing in association with a *Flavobacterium* or a *Chromobacterium*. No

MnO_2 was formed when filtrates or dead cells of these cultures were added to the *Corynebacterium* culture or when the bacteria grew in the same media as the *Corynebacterium* but separated by a collodion membrane. Zavarzin found manganese oxidation in mixed cultures of two *Pseudomonas* strains. So far no explanation has been given of the effect of associated bacterial growth on manganese oxidation. Neither is it known whether this phenomenon is common among manganese-oxidizing micro-organisms from soil agar plaques.

In a subsequent paper Zavarzin (1964) dezcribed a manganese-oxidizing bacterium (*Metallogenium*) requiring the presence of living fungi for growth and manganese-oxidation.

In addition to the brown colonies of specific manganese-oxidizing bacteria (coryneform bacteria,[1] *Hyphomicrobium* spp.[1]) and yeasts (*Cryptococcus albidus*[1]) the brown rings of soil-agar-$MnSO_4$ plaques upon microscopical observation (low magnification) always show brown fungal colonies. Upon transfer to similar soil-agar-$MnSO_4$ plates these fungi maintain their capacity to form MnO_2. This means that fungi belong to the specific manganese oxidizers in soil.

ROLE OF MICRO-ORGANISMS IN RENDERING SOIL MANGANESE UNAVAILABLE TO PLANTS

There is disagreement among research workers as to the effect of micro-organisms in rendering soil manganese unavailable to plants. Microbiologists usually believe that the above-mentioned microbial formation of manganic oxides is the main cause of the decreased availability of the manganese when added in the bivalent form to neutral or slightly alkaline soils containing a certain amount of organic material.

Some authors minimize this effect of micro-organisms, particularly in view of the rapidity with which the exchangeable manganese of acid soils disappears upon liming. Fixation of Mn^{++} by soil organic matter as a result of the formation of complexes (chelates) is thought by these authors to be the main reason for its unavailability (Heintze, 1957; Page, 1962).

To decide which part of the Mn fixation is due to microbial processes and which part to chelate formation, a comparison of the fate of Mn^{++} added to sterilized and non-sterilized soil should be made. Furthermore, samples of sterilized soil should be inoculated with various types of manganese-oxidizing bacteria and the effect of these bacteria on the availability of manganese to higher plants should be studied. Such experiments have only rarely been made. Gerretsen (1936, 1937) grew oat plants in manganese-deficient soil sterilized by formalin treatment. Healthy plants were obtained, in contrast with the untreated soil which gave plants with severe symptoms of manganese deficiency. The favourable influence of the formalin treatment on the manganese supply of the plants may have been due to the solubilizing effect of formalin on the unavailable soil manganese. Treatment of the sterilized soil with 5 per cent of unsterilized soil caused manganese deficiency of the oat plants, apparently owing to microbial manganese oxidation.

In separate experiments, Gerretsen cultivated oat plants in sterile sand and in water cultures low in manganese. No symptoms of manganese deficiency were seen unless the cultures had been infected either spontaneously or by adding a small amount of a manganese-deficient soil.

Strong evidence of the contribution of micro-organisms to Mn^{++} fixation in neutral or slightly alkaline soil was obtained by Quastel, employing the soil perfusion technique (Quastel, 1955). When $MnSO_4$ was perfused through such a soil, the rate of oxidation of the Mn^{++} increased sharply after a lag period. Sterilization of the soil or the addition of cell poisons stopped manganese fixation.

A further indication of the importance of micro-organisms in causing manganese deficiency in plants may be derived from the authors' observation that acid sandy and peaty soils may continue to produce healthy plants for a considerable length of time after neutralization. However, once manganese deficiency has appeared, its elimination by adding manganese salts to the soil is very difficult as the added manganese is readily rendered unavailable.

So far it is unknown which type of manganese-oxidizing micro-organism is responsible for the fixation of manganese under natural conditions. The fact that the non-specific organisms, which require hydroxyacids for oxidizing Mn^{++}, were unable to bring about the oxidation on soil-agar-$MnSO_4$ plates, indicates that this type of organism presumably is not involved in manganese fixation in soil. Manganese oxidation by this type of organism may be expected to occur in those cases where hydroxyacids are formed or added (rhizosphere, application of straw and other plant residues, stable manure, etc.). It is not yet known whether, under these conditions, manganese may be rendered unavailable by the non-specific type of organism.

Timonin (1946), comparing numbers of manganese-oxidizing bacteria in the rhizosphere of oat varieties resistant to grey-speck disease (manganese deficiency) and of plants suffering from this disease, found far larger numbers in the latter. However, counts were made on Gerretsen's citrate agar so that it is highly probable that many manganese oxidizers of the non-specific type were involved. The fungi isolated by Timonin (1950) from manganese-deficient soil on the calcium citrate-manganese sulphate medium proved to be able to oxidize Mn^{++} in soil agar plaques.

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[1] Isolated in the authors' laboratory.

REDUCTION OF MANGANIC TO MANGANOUS COMPOUNDS

Reduction of manganic oxide in soil is a biological process. Several micro-organisms which decompose organic matter can apparently use MnO_2 instead of oxygen as the hydrogen acceptor, reducing it to Mn^{++} (Mann and Quastel, 1946).

MnO_2 can furthermore be reduced to manganous ions by certain compounds formed by microbial processes (hydrogen sulphide, sulphydryl compounds and polyhydric phenols (Quastel, 1963). Both phenomena will occur in the soil when the supply of oxygen is inadequate, i.e., when the soil structure is poor or when the soil is water-soaked.

The biological reduction of manganic oxides to Mn^{++} is strongly favoured by a drop in the soil pH to a value below 5.5.

Roots of living plants may promote the reduction of manganic oxides to Mn^{++} in three different ways:

1. By the excretion of unknown organic compounds which dissolve MnO_2. This was shown for oat plants by Bromfield (1958). The reducing power of the latter compounds, which were fairly resistant to microbial decomposition, increased with the decrease in pH. Above pH 6.5 their dissolving activity was very small.
2. More readily decomposable root excretions bring about a pronounced increase of microbial activity in the rhizosphere and thus an increased demand for oxygen. When the oxygen supply becomes inadequate, manganic oxides will be used as the hydrogen acceptor.
3. The demand for oxygen in the rhizosphere is much increased as a result of root respiration.

METHODS OF CURING MANGANESE DEFICIENCY OF PLANTS

When manganese deficiency is the result of specific soil conditions favouring the oxidation of Mn^{++} (neutral or slightly alkaline reaction, presence of a certain amount of organic matter), the cultivation of healthy plants is often no easy task. The addition of a moderate amount of manganese sulphate (e.g., 50 kg per hectare) which contains many times the amount of manganese required by a crop of cereals or sugar-beet, is often inadequate to give optimal yields, most of the added manganese being rendered unavailable during the first part of the growing period. Since manganese is transported poorly from the older parts of the plants to the growing tissues (Henkens and Jongman, 1965), deficiency symptoms may occur in the higher leaves in spite of a relatively high manganese content of the lower parts of the plants.

Spraying plants with a dilute solution of $MnSO_4$ eliminates the effect of the soil on the response to the added manganese; however, the poor translocation of this micronutrient from one leaf to the others also applies to this treatment, so that the spraying has to be repeated in the course of the growing period (Henkens, 1962).

If the pH of the soil is relatively high, owing to the application in previous years of fertilizers which have shifted the reaction towards alkalinity, lowering the pH to values below 6 may have a beneficial effect on the manganese supply on sandy or peaty soils. In fact, this method of eliminating the grey-speck disease of oats, occurring on the reclaimed peaty soils in the north of Holland, was applied successfully by Hudig (1911) even before the cause of this disease was known. For lowering the pH, ammonium sulphate may be used as the nitrogenous fertilizer. Owing to its physiologically acid reaction, this compound brings about a considerable shift in pH in the vicinity of the roots, thus improving the availability of manganese in this region. Conversion of the NH_4 ions to nitric acid by nitrifying bacteria may even enhance this effect.

Lowering the oxidation-reduction potential of manganese-deficient soils, as may be achieved by the flooding of low land or of rice fields, in many instances raises the availability of manganese to such a level that, after drainage, normal plants can be grown.

BIOLOGY OF COPPER IN RELATION TO SOIL FERTILITY

INTRODUCTION

Copper deficiency of higher plants is often associated with the presence in the soil of black organic matter derived from the peaty material underlying the turf of heathland. When the latter is brought into cultivation, severe symptoms of copper deficiency may appear when sensitive crops like cereals are grown. High-moor peat derived from *Sphagnum* is also copper-deficient.

Although the copper requirement of higher plants is low (approximately 25 g of copper for 1 hectare of a cereal crop), an amount almost a thousand times higher of this trace element in the form of a soluble copper salt has to be supplied to soils of the above-mentioned types to obtain healthy plants. This is due to the tight binding of the copper ions by the soil organic matter, as can be shown easily by the application of the divided-root-system technique mentioned above.

In contrast to soils liable to manganese deficiency where large amounts of added manganous ions are made unavailable to plants within a few months after dressing, copper-fixing soils may continue to give healthy plants for several years after the application of a large amount of a soluble copper salt. This shows that the mechanism of Mn fixation is entirely different from that of copper fixation. Further evidence of this difference may be derived from the following observations:

1. In general, there is no parallelism between manganese and copper fixation of soil types.
2. Copper fixation is much less pH-dependent than manganese fixation; it often occurs on moderately acid peat soils.
3. Eliminating Cu-deficiency by the addition of a soluble copper salt sometimes brings about manganese deficiency of the plants (Mulder, 1938). Since manganese oxidation by pure culture of fungi is catalysed by copper ions (Mulder, 1938), this may be the explanation of the observed effect.

EFFECT OF TYPE OF ORGANIC MATTER ON COPPER FIXATION IN SOIL

To demonstrate that the organic matter of copper-deficient soils is responsible for the fixation of copper, two types of experiments have been carried out (Mulder, 1938, 1939, 1940) one by a microbiological technique and the other by a soil percolation method. The former depends on the requirement of the fungus *Aspergillus niger* for small amounts of available copper in its nutrient medium. Copper compounds unavailable to higher plants growing in soil are generally also unavailable to the fungus. This test may therefore be used to study the availability of copper in soil to plants.

Copper fixation by black organic matter from heathland was studied by adding increasing amounts of a copper sulphate solution to small amounts of the organic matter, incubating the mixture at room temperature, sterilizing it at 110°C and subsequently adding the suspension to *Aspergillus* cultures. Considerable amounts of copper were found to be fixed by this soil type in a form unavailable to the fungus.

In the percolation experiments, dilute copper sulphate solutions were percolated through soils contained in glass tubes. When saturated with copper, the soils were washed with distilled water until the wash water was nearly free from copper, and subsequently with a 1 per cent calcium nitrate solution until practically no more exchangeable copper was released. Non-exchangeable ('fixed') copper was determined by estimating total residual copper. A soil on which the plants were severely copper-deficient was found to bind 73 mg Cu per gramme of organic matter, 34 per cent of which was in the non-exchangeable form. A soil with healthy plants bound 42.9 mg of copper per gramme of organic matter; only 12 per cent of this amount was in the non-exchangeable form. In both cases the retained copper was released upon treatment with 0.05 N HCl.

The high value for copper retention by the organic matter of the above-mentioned copper-deficient soil is in agreement with those Schlichting (1955) found for the binding of copper by humus isolated from such soils (205-274 mval/100 g of organic matter). Although part of this copper may have been available to higher plants, culture solution experiments with oats (Schlichting, 1955, 1962) clearly showed that copper supplied as Cu bentonite was more readily available to plants than that supplied in combination with humus compounds from copper-deficient soil.

That copper may be retained by soil organic matter in different ways, corresponding with a different tenacity of retention, may also be concluded from the experiments of Broadbent (1957) and Ennis (1961-62*a*). These authors saturated organic matter preparations from different soils with copper sulphate and then released the copper ions by treating the preparations with hydrochloric acid solutions of different strengths. Several types of copper retention were found to exist in the same preparation, namely those resisting release by H_2O only, and by 0.01, 0.05, 0.1 and 1 N HCl. Complexes of copper and peat resistant to 1 N HCl treatment were fund by Ennis and Brogan (1961-62) to be unavailable to oat plants when present in copper-deficient sand cultures; those resisting 0.1 N HCl were slightly available and those surviving 0.01 N HCl treatment were somewhat better available. However, availability of the latter was much lower than that of copper sulphate.

It is not known whether these types of copper retention correspond to different copper-binding compounds within the soil organic matter, or represent different copper-binding groupings within one compound. An extensive study by Ennis (1961-62*b*) indicated that carboxyl and hydroxyl groups were responsible for the binding of 90 per cent of the copper contained in his Cu-saturated peat compounds. The more tenaciously retained copper ions were thought to have formed complexes with thiol and NH_2 groups.

Addition of nitrogenous fertilizers to soils poor in available copper, which is known to bring about a serious aggravation of copper deficiency in plants (Fleming and Delaney, 1961-62; Mulder, 1950*b*) did not affect the copper-fixing properties of the soil organic matter (Fleming and Delaney, 1961-62). This suggests an interfering effect of the N compounds on the uptake of copper ions by the plant roots or on their biochemical activity in the plant, rather than an effect in the soil.

The soil or plant factors responsible for the formation of soil organic matter with a high retention capacity for copper ions have not yet been identified. Since black humus from heathland was found in experiments with cereals and in those with *Aspergillus niger*, as well as in percolation experiments, to be one of the strongest copper-fixing organic materials, heathland vegetation and the conditions causing its decomposition in the natural soil may be considered to be important factors in forming soil organic matter with a high retention capacity.

There are some indications that certain types of organisms may aggravate copper deficiency in cereals. This was shown by Sorteberg (1962) studying copper deficiency in peat soils in Norway. Inoculation of uncultivated slightly humified peat rich in *Sphagnum*, on which no or slight symptoms of copper deficiency in higher plants occurred, with 0.5 to 1 per cent of copper-deficient soil, caused severe symptoms of copper deficiency which were absent when the added soil had been sterilized by heating or with formaldehyde. More details of the effect of micro-organisms on copper transformations in soil will be given in the next section.

TRANSFORMATION OF COPPER COMPOUNDS BY MICRO-ORGANISMS

Micro-organisms affect the copper supply of higher plants growing in soil in more than one way. Hydrogen-sulphide-producing bacteria may precipitate soluble copper compounds as the sulphide or other insoluble S compounds. Organisms of the *Thiobacillus* and *Ferrobacillus* groups oxidize the sulphide and thus liberate the copper ions.

Micro-organisms decomposing plant and animal residues liberate copper locked up in organic compounds. On the other hand, they may form the soil organic matter which fixes the soluble copper.

Copper precipitation by hydrogen-sulphide-producing organisms

Two types of H_2S-producing micro-organisms may be involved in copper precipitation (Mulder, 1938, 1939). The first type includes bacteria producing under anaerobic conditions H_2S from sulphur-containing amino acids and peptides (cystine, methionine, glutathione, etc.). *Clostridium lentoputrescens* and other proteolytic clostridia as well as *Proteus vulgaris*, *Escherichia coli*, etc., belong to this group. The second type includes the sulphate-reducing bacteria of which *Desulfovibrio desulfuricans* is the most important representative.

That H_2S-producing bacteria may precipitate considerable amounts of copper ions may be shown easily when organisms of the coli group are streaked on agar plates containing: meat extract, approximately 1 per cent; glucose, 0.25 per cent; $CaCO_3$, 0.25 per cent; and $CuSO_4.5\,H_2O$, 0.005 per cent. Colonies of these bacteria grow readily on this medium and turn brown as a result of accumulated copper which is presumably present as CuS. When the plates are covered with a solution of a copper reagent no reaction takes place in a wide area surrounding the colonies, showing that the metal has been concentrated in the colony. A more pronounced copper precipitation takes place when the above nutrient medium is enriched with 1 per cent peptone and 0.1 per cent cystine, while glucose is omitted.

To test the availability of copper precipitated by H_2S-forming bacteria, experiments were carried out with *Aspergillus niger* and with barley and oats in culture solution (Mulder, 1938). For the *Aspergillus* test, *E. coli* and *Clostridium lentoputrescens* were cultivated in a meat extract-cystine medium with small amounts of copper sulphate. When the bacteria had grown, the cultures were heated for 0.5 h at 65°C and then added to the culture media for *Aspergillus niger*. Available copper was estimated after five days' incubation of the *Aspergillus* cultures at 30°C by comparing the colour of the fungal spores with those of a standard series cultivated with increasing amounts of copper. The results of this experiment showed that a large part of the copper had been rendered unavailable in the bacterial cultures, presumably by the accumulation of CuS or some other S compounds of copper within the cells. That formation of CuS in the culture solution was not the cause of the copper precipitation may be concluded from the observations that CuS, formed chemically in the culture solution of *Aspergillus niger*, was readily taken up by the fungus, presumably after oxidation of the sulphide to the sulphate.

In the experiments with barley and oats, copper precipitated by H_2S-forming *Desulfovibrio desulfuricans* and *E. coli* was added to copper-deficient plant cultures. No recovery of these plants was observed during the two weeks' period following the addition of the bacterial cultures. This was in contrast to the plants which had received sterile bacterial medium enriched with copper sulphate. Within a few days these plants recovered from the Cu-deficiency symptoms.

The results of the above experiments clearly show that copper rendered insoluble by H_2S-forming bacteria is unavailable to *Aspergillus niger* as well as to cereal plants growing in culture solution. Since microbial H_2S formation may occur in natural soils and peats under anaerobic conditions, i.e., at a high water content of the soil, this microbial process under certain conditions is thought to be responsible for the poor copper supply of the plants. Since sulphate-reducing bacteria for their development require a neutral or alkaline reaction, copper fixation due to the activity of such micro-organisms would be expected to occur in low-moor peats rather than in high-moor peats which are generally much more acid.

Solubilization of copper sulphide by micro-organisms

Bacteria of the genera *Thiobacillus* and *Ferrobacillus* are able to oxidize copper sulphide to copper sulphate (Bryner and Jameson, 1958). These bacteria can resist very high concentrations of copper ions. Booth and Merces (1963) give the following limiting Cu^{++} concentrations for a number of bacteria involved in the oxidation of copper ores and for a number of H_2S-producing bacteria: *Thiobacillus concretivorus*, 10,000 ppm; *Th. thiooxydans*, 20,000 ppm; *Ferrobacillus ferrooxydans*, 10,000 ppm; *Desulfovibrio desulfuricans*, 50 ppm; *D. orientis*, 30 ppm; *Clostridium nigrificans*, 30 ppm. Although the values for the representatives of the *Thiobacillus* and *Ferrobacillus* groups are much higher than those given for the H_2S-producing bacteria, the values of the latter are considerably higher than those of organisms not involved in S-transformations. In experiments with *Acetobacter aceti*, for instance, an amount of copper as low as 0.5 ppm was found to be toxic (Mulder, 1938, 1939).

Mobilization of fixed soil copper by increased biological activity

To investigate whether the availability of fixed soil copper can be improved by an increased microbial activity in the soil, two types of experiments have been carried out by the senior author (Mulder, 1938) with a copper-fixing soil containing approximately 10 per cent black organic matter derived from a heathland vegetation. Cereal plants growing in this soil, on the field as well as in pots, had symptoms of very serious copper deficiency.

In the first type of experiment, yellow lupins were cultivated on an experimental field. These plants showed no symptoms of copper deficiency, and a heavy crop of plant material was obtained both in the absence and presence of added copper sulphate. The lupins were ploughed under in the spring of the subsequent year and shortly afterwards oats were sown. A few months later symptoms of serious copper deficiency appeared on the plots which had received no copper treatment. There was no difference in appearance between the plants growing on control plots (no lupins) and on those with the residues of the lupin crop.

In the pot experiment, mycelia of the fungus *Aspergillus niger*, grown in a copper-deficient nutrient medium, were used as the organic substrate. The mycelia were crushed and mixed with the copper-deficient soil. After an incubation period of some months at 20°C barley was sown. As in the field experiment, no effect of the organic matter treatment on the copper supply of the plants was observed.

Soil biology and soil structure

INTRODUCTION

Soil structure is one of the fundamentals of soil fertility. This is partly due to its direct effect on soil fertility factors like aeration, water movement and water content, partly to its effect on microbial processes.

The most suitable soil structure for plant growth is the crumb structure, i.e., the presence in the soil of moderately stable aggregates built up from the smaller particles. Such a structure may be obtained in different ways. In clay soils saturated with calcium and magnesium ions, colloidal clay may be the most important cementing material; in sandy soils and presumably also in leached clay soils organic matter functions in this respect.

In addition to humus (the more or less stable product of degraded plant and microbe residues) less stable products like polysaccharides, polyuronides and other gums, produced by soil micro-organisms, are thought to contribute to crumb formation.

EFFECT OF ROOTS AND ROOT HAIRS ON SOIL STRUCTURE

The effect of roots and root hairs on crumb formation can be seen easily when plants growing in structureless sand are carefully lifted. A quite large amount of aggregated sand particles is held by the roots and root hairs. Evidently, this is the result of mechanical and chemical forces, the latter depending on root exudates and the activity of rhizosphere micro-organisms.

A further effect of root systems, particularly in the case of deep-rooted plants, is the formation of holes which, after decay of the roots, may serve in the discharge of excessive amounts of water.

EFFECT OF PLANT RESIDUES ON SOIL STRUCTURE

It is a well-known fact that the fertility of an arable soil is at its best in the first years following the ploughing of a pasture. This is partly due to the liberation of nitrogen from decomposing organic matter, partly to its much improved soil structure. Agricultural practice often takes advantage of this dual effect by introducing short-term grass-legume associations (leys) into the crop rotation. It must be stressed, however, that the beneficial effect of such leys is less than that of old pastures.

To demonstrate the effect of pastures on soil structure, Low (1950, 1955) compared the percentages of water-stable aggregates > 2 mm in size of old arable soils and nearby pastures on a large number of farms in different parts of England. Pasture soil was found to have a considerably higher content of such aggregates than arable soil. To restore the good structure of pasture, arable soil had to be under grass, grass-clover associations or clover for a considerable time. For clay soils this was longer (25-50 years) than that for coarse sandy soils (5-10 years).

Structural improvement of pasture soil was found by Low to be often linked with earthworm activity.

The beneficial effect of pasture on soil structure presumably depends on several factors, namely root and root-hair development, decomposing plant residues, fungal and bacterial growth, humus formation, earthworm activity and perhaps others. These different activities partly proceed simultaneously, partly in succession.

EFFECT OF MICRO-ORGANISMS AND THEIR REACTION PRODUCTS ON CRUMB FORMATION

Micro-organisms are thought to play an important part in the formation of soil aggregates (Geoghegan and Brian, 1946, 1948; Gilmour *et al.*, 1948; Greenland *et al.*, 1962; Martin, 1946; Martin and Waksman, 1940; Mishustin and Gromyko, 1945; Quastel, 1952). In the case of fungi this can be a mechanical effect owing to the presence of filaments which may bind the soil particles together. Of more importance is the physicochemical effect, depending on: (a) products of microbial degradation of plant and animal residues; (b) products synthesized by micro-organisms during decomposition of the organic matter; or (c) humus compounds formed in the course of the microbial degradation of added plant residues, stable manure, etc. Although humus is able to form water-stable soil aggregates, thus contributing materially to soil fertility, readily decomposable plant residues exert their strongest effect on crumb formation during the period of microbial degradation. This indicates that the beneficial effect on soil structure of added plant and animal residues which is often observed shortly after their application, depends on compounds belonging to (a) or (b) rather than to (c). The after-effect of such a treatment, which lasts much longer, is supposed to depend on the humus compounds which are much more resistant to microbial breakdown than the compounds of (a) and (b).

Several authors (Geoghegan and Brian, 1946, 1948; Martin, 1946; Martin and Waksman, 1940; Quastel, 1952) are of the opinion that polysaccharides and polyuronides which may be formed by certain micro-organisms play the most important part in microbial crumb formation. Martin and Waksman (1940) obtained an excellent formation of water-stable aggregates in mixtures of 97.5 per cent white river sand and 2.5 per cent bentonite, and of 80 (or 60 per) cent sand and 20 (or 40) per cent clay, supplied with a basal nutrient medium and saccharose as the carbon and energy source, and inoculated with pure cultures of slime-forming micro-organisms (*Azotobacter indicum* = *Beijerinckia*; *Rhizopus nigricans*, *Aspergillus niger*). A similar aggregate formation was obtained by Martin (1946) with a fructosan-forming *B. subtilis* introduced into a soil supplied with saccharose.

These results led to the conclusion that microbial slimes, mostly consisting of polysaccharides and polyuronides, are common crumb-forming compounds in soil. This hypothesis was strengthened by the observation of the pronounced aggregate-forming properties of long-chain polysaccharides when added in quite low concentrations to soil (see Quastel and Webley's (1947) experiments with alginic acid).

It should be stressed, however, that the experiments with slime-forming bacteria have been carried out in media containing large amounts of carbohydrate and relatively low amounts of nitrogen. In the experiments of Martin and Waksman, for instance, 20 g saccharose and 0.28 g nitrogen were added per kilogramme of soil. Griffiths and Jones (1965) added 40 g of various carbohydrates to 1 kg of soil without adding nitrogen. Under such conditions, polysaccharide formation by micro-organisms is often stimulated as a result of nitrogen deficiency (see Mulder *et al.*, 1962; Zevenhuizen, 1966). Furthermore, the amounts of carbohydrate added to the soils in these experiments are much higher than those occurring under natural conditions. On the other hand there is no indication that, during the microbial breakdown of plant and animal residues, when aggregate formation is at its best, excessive amounts of polysaccharides are formed.

Since Swaby (1949-50*a*) has shown that, in addition to polysaccharides and polyuronides, several other compounds (fats, some types of protein, lignin-containing substances) have crumb-forming properties, it may be assumed that the beneficial effect of decomposing plant and animal residues on soil aggregation is brought about by various compounds.

Since the soil-aggregating compounds of the types mentioned above are liable to microbial attack, synthetic polyelectrolytes (Krilium soil conditioners) have been prepared which have a similar or even stronger aggregating effect on soil particles but which are highly resistant to microbial degradation. These compounds, which are used in amounts of approximately 0.1 per cent by weight of dry soil, are particularly effective on sticky clay soils (Allison, 1952; Hedrick and Mowry, 1952; Martin *et al.*, 1952).

THE INFLUENCE OF HUMUS ON SOIL AGGREGATION

To demonstrate the influence of humus on crumb formation, Swaby (1949-50*a*) separated the humus fraction from a number of widely different soil types and mixed it in amounts of 1 per cent with particles smaller than 1 mm of a clay soil. This resulted in a clear crumb formation. To determine the type of substance responsible for the aggregation, Swaby subjected the humates to various chemical treatments. The aggregate-forming properties of the treated humate were compared with those of the unaltered control. These experiments provided indirect evidence that proteins, polysaccharides, amino-polyuronides and lignin-like material might all contribute to the aggregating action of soil humus.

A somewhat deviating result as to the occurrence of polysaccharides as a crumb-forming component in humus was obtained by Mehta, Streuli, Müller and Deuel (1960). These authors isolated the polysaccharide from soil aggregates of a 'Braunerde' which contained 3.2. per cent C and 30 per cent clay, and had a pH of 6.8. The isolated polysaccharide was added in an amount of 1 per cent to particles < 0.2 mm in size of the same soil, and crumb formation was easily obtained. The aggregates were destroyed by treatment with a dilute solution of sodium periodate, followed by treatment with a buffer of pH 10, which oxidizes the polysaccharides. Natural aggregates, however, were not affected by even a much longer exposure to the periodate. Since similar results were obtained with various other Swiss soils, it was concluded that soil polysaccharides are not responsible for crumb formation under natural conditions. This was also concluded from the fact that ClO_2 treatment, which oxidizes lignin and humic compounds with practically no degradation effect on polysaccharides, destroyed the natural aggregates in less than 24 h, whereas synthetic aggregates (0.3 per cent guar gum treatment) were stable.

EFFECT OF EARTHWORMS ON AGGREGATE FORMATION

It is rather generally accepted that earthworms play a more or less important part in the formation of soil crumbs. They ingest relatively large amounts of soil particles and plant material which are submitted to bacterial activity in the gut and subsequently excreted as structural soil. Swaby (1949-50*b*) found a large difference between the stability of worm casts from grassland and those from arable land. This was presumably due to the larger amounts of root residues in the grassland soil which resulted in much larger numbers of intestinal bacteria than was the case with the earthworms of the much poorer arable soil. Part of these bacteria produced gums which were thought to be responsible for the stable aggregates. Owing to the restricted aeration inside the earthworm, fungi occurred in very low quantities. This was in contrast to both arable and grassland soil supplied with ground grass roots where, in the absence of earthworms, stable aggregates were formed by soil fungi.

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