H.G. Diem and Y.R. Dommergues

CNRS/ORSTOM. Dakar, Sénégal

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Significance and Improvement of Rhizospheric N_2 Fixation

Introduction

N_2 fixation in the rhizosphere of grasses and non-leguminous crops was indicated some time ago (Parker 1957). The first undisputable report on this process was that of Hassouna & Weireing (1964) who found active N_2 fixation in the rhizosphere of *Ammophila arenaria*, which they attributed to *Azotobacter* associated with roots. The introduction of the acetylene reduction technique by the end of the sixties, facilitated investigations of what is known as loose type of symbiosis, termed "associative symbiosis" by Burns & Hardy (1975). The present review is an attempt to summarize the enormous volume of research carried out in this field during the last decade.

Populations of N_2 fixing bacteria in the rhizosphere

Methodology

Enumeration techniques: Until recent years, the most common method used to estimate populations of N_2 fixing bacteria (Syn. diazotrophs) in soil was the spreading a soil suspension on a nitrogen-free silica gel medium and counting the colonies. Plates were incubated under aerobic conditions for counting *Azotobacter* or *Beijerinckia* and under anaerobic conditions for counting *Clostridium* and other anaerobic bacteria. Alternatively, the usual pour plate technique was also used by mixing soil suspensions with an agar medium cooled to 40–45°C. These techniques were then modified by Fenglerova (1965) who, instead of silica gel plates, used a thin layer of soft agar to enhance the development of *Azotobacter*. Other authors used extinction

dilution technique by which the most probable number (MPN) of N_2 fixing bacteria could be estimated. This procedure was considered by Abd-el-Malek & Ishac (1968) as more sensitive than the plating method.

The MPN method has also been used for estimating *Clostridium* population. In this case, the reading of positive tubes is facilitated by the addition of phenosafranine to the medium (Brouzes *et al.*, 1971; Balandreau *et al.*, 1975).

The extinction dilution technique combined with the acetylene reduction test is now used to estimate the total N_2 fixing microflora (Villemin *et al.*, 1975; Diem *et al.*, 1978a) or *Spirillum* populations colonizing peanut roots (Okon *et al.*, 1977). This technique may be combined with the use of Pankhurst tubes (Campbell & Evans, 1969) to assess the number of facultative and anaerobic nitrogen fixing bacteria.

Spirillum lipoferum and other microaerophilic bacteria may now be easily isolated by introducing soil suspensions or root pieces in a soft agar medium where micro-aerophilic bacteria grow at depths characterized by PO_2 most suitable for individual species. This original method first devised by Dobereiner & Day (1976) was based on the fact that most N_2 fixing bacteria are sensitive to oxygen (Dobereiner *et al.*, 1976). In order to separate different N_2 fixing bacteria from mixed population, Rinaudo (personal communication) incubated streaked plates in desiccator under anaerobic or microaerophilic conditions.

The advent of the fluorescent antibody (FA) technique as a tool for ecological studies allowed De Ville & Tchan (1970) to estimate *Azotobacter* populations in natural soil. Using this method, Diem *et al.* (1978b) were able to count *Beijerinckia* cells (cells count technique) in the midst of spermosphere or rhizosphere microflora. When the plating procedure was used, colonies of a given bacteria were assessed by Unger & Wagner's method (1965) which consisted of staining impressed enumeration plates with fluorescent labelled antiserum.

Immunofluorescence techniques (Schmidt 1973, 1974, 1978) especially the FA-membrane filter counting method allows quantification of a specific microorganism *Nitrobacter* at densities as low as 10^2–10^3 per gram of soil in the midst of a complete mixed microbial population (Rennie & Schmidt, 1977). Enumeration of colonies grown on a membrane filter previously placed on the surface of an appropriate medium was also recommended by Hegazi & Niemela (1976) for routine quantitative estimation of *Azotobacter*.

Working with an isolate of *Beijerinckia* artificially introduced into nonsterile soil, Diem (not published) was also able to calculate the MPN of the bacteria at a level 10^4 cell/g by using an enrichment culture technique coupled with FA staining (Fig. 1).

Microscopic observations: Many modern methods, including transmission and scanning electron microscopy, are now widely used to observe the establishment and the behaviour of microorganisms in the root region. As far as

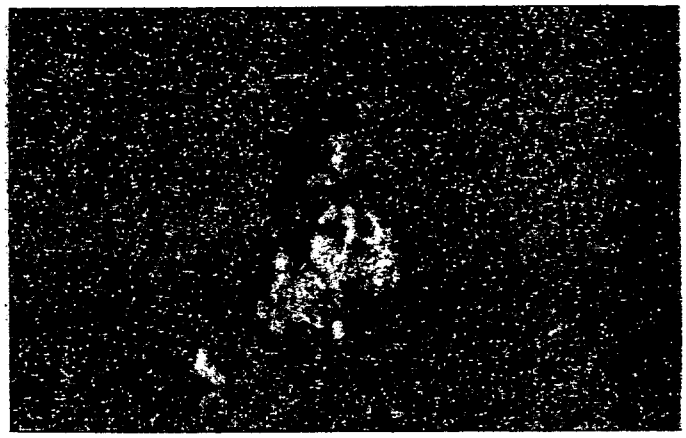

Fig. 1. Fluorescing colonies of *Beijerinckia* sp. mixed with non fluorescing cells of unidentified soil bacteria (arrow) after staining with fluorescent antibodies against *Beijerinckia* sp.

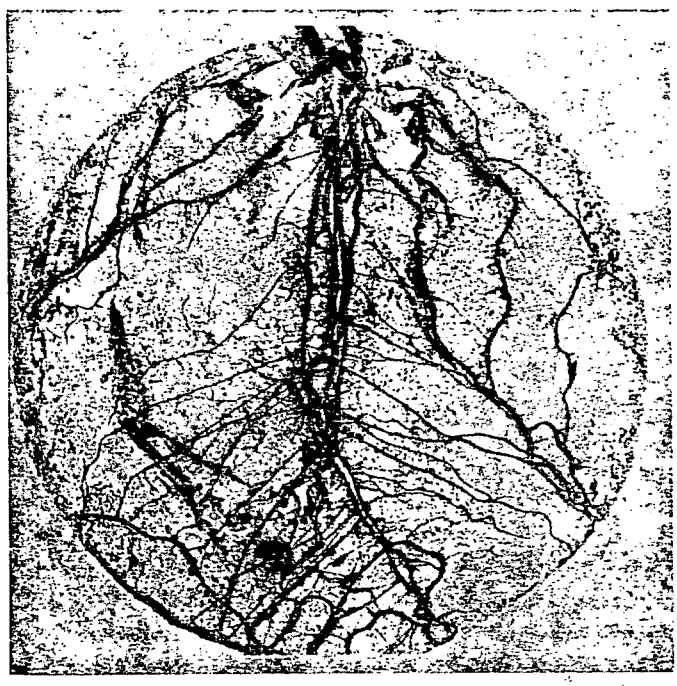

Fig. 2. Root system of rice seedlings embedded in dried agar pellicle after removal from the plastic plate.

we know, Jackson & Brown (1966) first used the FA technique to follow the germination of *Azotobacter* encysted cells and the growth of the bacterium in natural rhizosphere conditions. Using an electron microscope, Hamad-Fares (1976) showed extensive colonization of the root surface of axenic rice seedlings by some N_2 fixing bacteria. Diem *et al.* (1978b) proposed a method in which developing roots grew on an agar layer poured on a plastic plate. The plate containing the soil was placed at an angle of 45°. At the end of the experiment and after drying, the agar pellicle on which the plant roots were embedded could be easily removed (Fig. 2) and stained for microscopic observation. By using this method in conjunction with FA staining, these authors were able to observe the behaviour of *Beijerinckia* sp. in different compartments of the rice seedling rhizosphere.

Other methods to be explored: General methods devised for studying soil saprophytes can also be used to investigate the ecology of N_2 fixing bacteria. Modern techniques have been developed recently (Rosswall, 1973). To examine microbial competition and estimate the number of soil microorganisms besides the use of such methods as electron microscopy (Foster & Rovira, 1976), staining with a fluorescent brightener (Trolldenier, 1973; Soderstrom 1977) or fluorescent antibodies (Schmidt, 1973) or radio-labelled antibodies (Benbough & Martin, 1976), there are a number of attractive techniques as exemplified by auto-radiography to detect active microbial cells in natural habitats (Waid *et al.*, 1973; Fliermans & Schmidt, 1975) and the use of antibiotic resistant mutants (Obaton, 1971; Schwinghamer & Dudman, 1973) or auxotrophic markers (Johnston & Beringer, 1975).

Survey of the microorganisms

Qualitative distribution: From the data regularly reported, it may be presumed that N_2 fixing bacteria are widely distributed taxonomically, but high potentials of N_2 fixation seem to belong to species of Azotobacteriaceae, Spirillaceae and, to some extent, Enterobacteriaceae and Bacillaceae.

Bacteria of widespread families such as Pseudomonadaceae and Achromobacteriaceae are also sporadically recorded to fix N_2 (Paul & Newton, 1961; Koch & Oya, 1974). Further details on the taxonomy of N_2 fixing bacteria have already been reviewed (Postgate, 1971; Knowles, 1977; Stewart, 1977).

There are some main considerations which emerge from a review of literature on N_2 fixing bacteria: Some of the N_2 fixers recorded are not true root inhabitants. The best known example is that of some *Azotobacter* species which cannot be definitively considered as rhizosphere microorganisms. The ability to fix N_2 by the so-called N_2 fixing microorganisms has not always been conclusively demonstrated. Some *Arthrobacter* were claimed to be able to assimilate N_2 (Cacciari & Lippi, 1973). However, a number of *Arthrobacter* species have been tested by Mulder & Brotonegoro (1974) and found not

to possess any N_2 fixing ability. Recently, interest has been devoted to facultative anaerobic or microaerophilic N_2 fixers such as *Spirillum* (= *Azospirillum*) *lipoferum*, *Bacillus polymyxa*, *B. macerans*, *Enterobacter cloacae*, *E. aerogenes* and some species of *Klebsiella*. Surprisingly, little attention has been paid in the past to obligate anaerobic N_2 fixing bacteria in the rhizosphere although they may be abundant in soil and well-adapted to a wide variety of edaphic conditions (Katznelson *et al.*, 1948). Rinaudo (1970) suggested that N_2 fixation in rice soils was partly due to *Clostridium*. This is plausible since waterlogging in paddy soils increased the number of anaerobic sites at least in the outer rhizosphere where O_2 excreted by roots cannot diffuse. Anaerobic sulfate-reducing bacteria (*Desulfovibrio*) are also known to fix N_2 (Riederson-Henderson & Wilson, 1970) but their significance in natural conditions is not yet known.

Quantitative assessment: A complete survey of *Azotobacter* and *Beijerinckia* populations in soil was given by Mishustin & Shilnikova (1971), Abd-el-Malek (1971) and Dobereiner (1974). Under field conditions, populations of *Azotobacter* amounted to about a few hundred cells per gram of soil and this is generally recognized as negligible. Even in Polish soils characterized by a relative abundance of the bacterium, Golebiowska & Pedziwilk (1975) reported 5×10^3 cells per g of rhizosphere soil. In the rhizosphere of *Paspalum notatum*, the number of *A. paspali* was only between 5 and 10×10^3 colonies per g of soil (Dobereiner, 1974). Only Middle East regions with poor alkaline soils appear to be favourable to the development of *Azotobacter*. According to Vancura *et al.* (1965) and Abd-el-Malek (1971), these bacteria are particularly abundant in Egyptian soils ranging from 9×10^5 to 3×10^7 cells per g of rhizosphere soils of wheat, *Vicia* or clover, although inconsistent.

Bacteria of the genus *Beijerinckia* occur mostly in tropical or subtropical conditions but they are never abundant even in the root area and are generally not more than a thousand microcolonies per g of rhizosphere soil (Dobereiner, 1974). Information on rhizosphere populations of other groups of N_2 fixing bacteria is still very scanty. As observed by Scott *et al.* (1978), numbers of *Azospirillum* associated with field-grown maize may reach 6×10^5 cells per g of wet weight of root.

Unfortunately, most work on *Spirillum lipoferum*, which may be responsible for nitrogenase activity in many plant rhizospheres, are of a qualitative nature and quantitative estimates are lacking.

Specificity: It has been suggested that C_4 tropical plants having an efficient photosynthetic pathway are capable of selectively enhancing the growth of N_2 fixing bacteria at the expense of other groups of the rhizosphere microflora. Certain forage grasses, rice and sugar cane stimulated *Beijerinckia* multiplication and depressed the numbers of amino-acid requiring bacteria, molds and actinomycetes (Dobereiner & Campelo, 1971). The rhizosphere of grasses was found to be more favourable to *Spirillum lipoferum* than that of legumes

(Dobereiner *et al.*, 1976), the latter appeared to be favourable to *Klebsiella* (Evans *et al.*, 1972). The variability of *Spirillum* populations in different rhizospheres, as observed by Lakshmi *et al.* (1976), may also be another example of loose specificity in plant-N_2 fixers associations.

A certain specificity was hypothesized in the following cases: (1) a spontaneous and abundant presence of *A. paspali* was found in the rhizosphere of some varieties or ecotypes of *Paspalum notatum* (Dobereiner, 1974). (2) an association between a N_2 fixing *Bacillus* and a chromosome substitution line of wheat was obtained, whereas none of the bacilli were isolated from either of the two parent cultivars (Neal & Larson, 1976). However, the authors did not indicate whether the bacilli were the sole N_2 fixers colonizing their host.

Sites of colonization

Colonization by *Azotobacter*, when it occurred, was reportedly limited to the rhizosphere soil, while very few *Azotobacter* cells were found on the root surface, probably due to acidity of the concentrated exudates of many plant species (Dobereiner, 1974). By contrast, acid-tolerant *Beijerinckia* grow better on the root surface than in the surrounding soil. In their experiment conducted by Diem *et al.* (1978b) with non-sterile soil using the FA technique, root colonization by *Beijerinckia* was considered to be poor, although a slight increase in its population occurred in some areas. In the seedling stage of rice, few colonies of *Beijerinckia* were observed on the root surface or in the root hair regions (Figs. 3, 4), while the root tip was not at all colonized. They

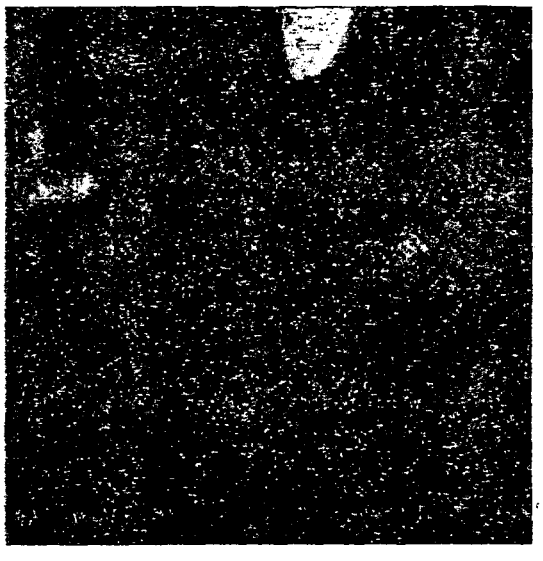

Fig. 3. Growth of *Beijerinckia* sp. as observed by immunofluorescent technique in the root hair region of 2-week old rice seedlings.

Fig. 4. Fluorescing *Beijerinckia* cells on the surface of root hairs of 2-week old rice seedlings.

partly attributed the reduced growth of *Beijerinckia* to an antagonism exerted by actinomycetes abundant in the used soil and also to a low exudation rate of rice at the seedling stage in the experimental conditions.

Colonization of living root tissues: As early as 1948, Katznelson *et al.* discussed the invasion of plant roots by soil bacteria resulting in a so-called "bacteriorrhiza" and noticed that this may involve true symbiosis (legume symbiosis) or may result in an entirely passive association.

The plant-*Spirillum lipoferum* association, as described by Dobereiner & Day (1976), would be an intermediate system if the activity of the bacteria within root tissues is confirmed to be intracellular or intercellular.

Intracellular colonization

A number of papers reported the occurrence of *S. lipoferum* as a symbiotic associate with the roots of many plants (Dobereiner *et al.*, 1976; Lakshmi *et al.*, 1976; Hernandes 1978; Sarro Da Silva & Dobereiner, 1978). The occurrence of *S. lipoferum* within living root cells was initially asserted because (a) the bacterium was often retrieved from roots after surface sterilization; (b) its presence in these habitats was presumed to be related to N_2 fixation as revealed by the reduction of tetrazolium salt.

Neither of these two arguments appear to be valuable or convincing evidence for advocating such a hypothesis. Firstly, when a microorganism is isolated from surface sterilized roots, it may only be embedded with soil

debris in moribund or dead tissues of a root cortex and, thus, escape the deleterious action of the sterilization agent (Fig. 5; Diem *et al.*, 1978a). Mosse (1975) wrote that the crevices and deeply embedded pockets on the root surface provided protected niches for microorganisms and this may explain the difficulty of sterilizing roots by washing. Secondly, caution should be exercised when relating the presence of N_2 fixing bacteria to the reduction of tetrazolium salt because this reaction is not specific to N_2 fixing bacteria.

Fig. 5. Soil bacteria (*b*) embedded with soil debris (*s*) between remnants of cortical cell walls (*w*) of mature, field-grown rice (× 32000). (Microphotograph due to the courtesy of M. Rougier).

More recently inoculation experiments under gnotobiotic conditions were performed in different laboratories. Umali-Garcia *et al.* (1976) reported the colonization of epidermal walls that were beginning to slough off. But Vasil *et al.* (1978) were not able to ascertain that *S. lipoferum* remained biologically viable within plant protoplasts. Lakshmi *et al.* (1977) observed many bacterial cells within root hairs and sometimes within cells of xylem (rice). In the case of gnotobiotic wheat and sorghum systems, Patriquin & Dobereiner (1978b) observed three patterns of colonization: "(i) dispersed individual bacteria or discrete colonies on epidermal cells and root hairs; (ii) longitudinal distributions between epidermal cells or outer cortex cells, and (iii) intracellular distribution in root hairs and in epidermal cells of outer cortex. Intracellular localization was observed only in the sorghum plants".

These experiments show that intracellular colonization may occur but it is generally discrete. Moreover, effective N_2 fixation by the bacteria infecting plant cells has not yet been demonstrated unequivocally.

Intercellular colonization: The colonization of root cell walls by soil

bacteria is often reported, but it generally occurs in the old regions of roots and especially with the onset of cortex decomposition (Old & Nicolson, 1975; Foster & Rovira, 1976). According to Umali-Garcia *et al.* (1978) and Vasil *et al.* (1978), many *S. lipoferum* cells can be observed within the middle lamella of the walls of living cell cultures; but whether this colonization can also occur under natural conditions is not known.

Colonization of dead or moribund root cortex: As reported by Umali-Garcia *et al.* (1978), infection of *Panicum maximum* by *S. lipoferum* occurred only on older parts of roots and bacterial colonization was closely associated with sloughing off of epidermal cells. Diem *et al.* (1978a) indicated that the colonization of endorhizospheric tissues of still functional roots by N_2 fixing bacteria in soil would be especially restricted to the basal zone where these tissues (old epidermis and cortex) are generally damaged. Such a colonization by soil bacteria is now considered to be a common phenomenon in nature (Waid, 1974; Martin, 1977). Holden (1975) reports that 70% of the cortical root cells are dead even in samples of 3 to 4 week old roots. According to the author, the degeneration and sloughing off of root cells has been noted in many plants, especially grasses and cereals, and in mature regions of such roots only the stele remains functional. Foster & Rovira (1976) described that at the end of the growing season, the root surface of wheat is

Table 1. Most probable number of N_2 fixing bacteria estimated from rice and maize roots after surface sterilization or successive washings (Fetiarison, Diem & Villemin, unpublished data)

Plant	Root zone	Number of remaining bacteria (10^3 per g wet weight of root)	
		After surface sterilization (endorhizosphere bacteria)	After 20 washings (rhizoplane + endorhizosphere bacteria)
Rice cv. SE 302G	A	2.2	120.7
	B	0	10.6
	C	0	40.3
Maize cv. F16	A	8.1	469.5
	B	8.3	28 248.5
	C	2.4	125.2

Root zones: A: 0–5 cm from root crown; B: intermediate, 5–15 cm from crown; C: apical and sub-apical zone, more than 15 cm from crown.

Bacteria remaining after surface sterilization were assumed to be endorhizosphere bacteria; becteria remaining after 20 washings with water were assumed to be rhizoplane + endorhizosphere bacteria.

separated from living host cells by a sheath of collapsed, cortical cells occupied by bacteria. This observation is not consistent with that of Patriquin & Dobereiner (1978a) who found that soil bacteria occurred in the inner cortex and in xylem without being accompanied by a collapse of the outerlying tissues. Diem *et al.* (1978a) observed that most N_2 fixers are situated on the rhizoplane, only a low population is inside the root that is in the endorhizosphere (see also Table 1). In this case, bacteria would only be associated with the outer root tissues and are probably enclosed with soil debris between remnants of dead cortical cell walls. The finding that most N_2 fixing bacteria are located "outside" the roots is thoroughly consistent with that of Okon *et al.* (1977) and Scott *et al.* (1978).

Colonization of root mucigel: In studies on detection of preferential sites of bacterial colonization, root mucigel has attracted much attention because this substance is not only situated in the soil-root interface, but it may also serve as a substrate for microorganisms (Darbyshire & Greaves, 1971; Balandreau & Knowles, 1978). Since the development of free living N_2 fixing bacteria is greatly favoured by a high C/N ratio in soil, it is reasonable to ask whether polysaccharides and pectic polymers of root mucigel may be assimilated by these bacteria or decomposed by other soil microorganisms to liberate carbon compounds available to the growth of N_2 fixers. Many electron microscopic studies (Bowen & Rovira, 1976) have shown that microcolonies of bacteria or individual bacterial cells can be seen within the mucigel layer creating electron-transparent zones around them. However, the occurrence of bacteria within the mucigel could supposedly be due to active colonization and/or to passive embedding phenomenon that takes place when the root surface comes in contact with soil bacteria. The electron-transparent zones could be due either to the lysis of root mucilage or, as stated by Mosse (1975), to the synthesis of bacterial polysaccharides.

While, at present, one can only speculate about the ecological significance of the root mucigel, some investigations suggest that its utilization by microorganisms, as hypothesized by Brown (1975), may be limited.

Electron microscopic studies of Old & Nicolson (1975), Rovira & Campbell (1975) and Dayan *et al.* (1977) showed that the surface of young roots, well covered with mucigel, is sparsely colonized by bacteria. By contrast, when mucigel disappeared from old roots (Old & Nicolson, 1975) or from roots infected by *Gaeumannomyces graminis* (Rovira & Campbell 1975), the number of bacteria on the surface increased.

Colonization of the root cap: Mota *et al.* (1975) have observed many bacteria in the intercellular spaces of some root caps of *Luzula purpurea*. The rice root cap area was found to be extensively colonized by *Beijerinckia* and other N_2 fixing bacteria when inoculation experiments were carried out with axenic rice seedlings grown on agar plates (Hamad-Fares, 1976). However, extrapolation from results obtained from such artificial conditions, should be

made with utmost caution. An analogous example was described by Bowen and Rovira (1976). Studies on plants grown in solution or in sand culture have always suggested that almost the entire surface of a root in soil was covered by microorganisms forming a continuous sheath between soil and root but, in fact, direct observation revealed that bacteria only covered 4–10% of the root surface of plants grown in natural soil. The low occupancy of root surface by soil microorganisms is well described by Bowen & Rovira (1976) who were, themselves, surprised by the relative paucity of root colonization, especially in segments 1–2 days old.

Using the device described above Diem (unpublished) also sometimes observed bacterial microcolonies within the mucigel adjacent to the root cap, but as similar colonies can also occur in soil areas devoid of roots, he concluded that observed microcolonies might be formed in soil prior to being embedded within the root cap mucigel.

The inability of soil microorganisms to colonize the root cap is suggested by the fact that they are sparse or even absent in this zone (Samtsevich, 1968; Rovira, 1973; Bowen & Rovira, 1976; Diem *et al.*, 1978b; Dayan *et al.*, 1977). This absence may be explained by the thickness of the root cap mucigel (Brown, 1975) or by the anti-microbial properties of root exudates in this region (Samtsevich, 1968). Another explanation was suggested by Diem *et al.* (1978b) that the root apex and its newly formed mucigel cannot be promptly colonized because, under natural conditions, the growth rate of bacteria is depressed by soil bacteriostasis and, hence, is much lower than that of the plant root itself.

Interactions of N_2 fixing bacteria with other soil microorganisms

Strzelczyk (1961) suggested that the abundance of antagonistic actinomycetes and bacteria may be partly responsible for the reduced growth of *Azotobacter* in the rhizosphere due to microbial antagonism. The author also discussed the hypothesis that conditions in the root zone are favourable for the production of minute but effective amounts of inhibitory substances. The observed inhibition cannot obviously be ascribed to microbial competition and according to Strzelczyk, *Azotobacter*, which is a relatively slow-growing organism, cannot compete successfully with other rhizospheric microorganisms.

Patel & Brown (1969) studied the possible role of inhibitory actinomycetes against introduced *Azotobacter*. In their experiments, *Azotobacter* initially had a competitive advantage over other soil microorganisms because it was introduced in large numbers, but the population of antagonists increased considerably with plant age and induced a reduction in numbers of *Azotobacter* in the rhizosphere. Brown (1974) suggested that an actively growing population of *Azotobacter* at first directly supplied metabolites or altered the composition of root exudates rendering the root environment more favour-

able to activity of actinomycetes. Thus, for a brief period, the rhizospheric *Azotobacter* population created an environment leading to its self-destruction.

Associative interactions between N_2 fixing bacteria and other microorganisms were recently reviewed by Jensen & Holm (1975). This aspect of microbial interactions is worth exploring because it has been claimed that larger amounts of N_2 can be fixed when nitrogen fixers were associated with other microorganisms. Dommergues & Mutaftschiev (1965) observed that N_2 fixation in cultures of both *Beijerinckia indica* and *B. fluminensis* were strongly stimulated by the presence of a soil yeast, *Lipomyces starkeyi*. Line & Loutit (1973) reported N_2 fixation by mixed cultures of aerobic and anaerobic microorganisms in an aerobic environment.

Other interesting data are provided by Fedorov & Kalininskaya (1959) who noticed that mixed cultures of oligonitrophilic soil bacteria could fix N_2 even though none of them is able to fix nitrogen in pure culture.

Other types of associations especially with cellulose decomposers were recorded in the review of Jensen & Holm (1975). However, most of the works reported were carried out to study the role of microbial association on the N_2 fixing process *in vitro* and not on the growth of N_2 fixers in the rhizosphere (*in vivo*).

According to Remacle & Rouatt (1968), in the early stages of growth of barley, pectinolytic bacteria contribute to the decomposition of root and seed reserves and liberate available carbohydrates, thus stimulating the multiplication of *A. chroococcum*. A recent paper of Ocampo *et al.* (1975) reported that inoculation with phosphate-solubilizing bacteria stimulated the natural population of *Azotobacter* in the rhizosphere and, conversely, the presence of *Azotobacter* was also favourable to the multiplication of phosphate-solubilizing-bacteria. These results, in addition to others, support the suggestion that it is necessary to use synergic associations of microorganisms rather than pure cultures for inoculating plant rhizospheres.

Reciprocal exchanges of energy and metabolites

Like any plant-microorganism system (Dommergues, 1978a), the plant-rhizosphere N_2 fixing system involves exchanges of energy and metabolites.

Energy flow from the plant to rhizosphere N_2 fixing bacteria

Rhizosphere N_2 fixing bacteria are heterotrophic microorganisms; they depend upon the host-plant for their supply of energy and carbon. In the rhizosphere (*sensu lato*), energy yielding compounds come from three different sources:

1) *root exudates*, i.e. materials released from healthy, intact roots, which have been grouped according to their mobility through the soil as diffusible-volatile, diffusible-water soluble and non-diffusible compounds, the latter

category comprising the mucigel (Rovira & Davey 1971; Balandreau & Knowles, 1978).

2) *root lysates*, i.e. "compounds resulting from the autolysis of root hairs and sloughed off root cap cells, as well as epidermal and cortical cells" of still functional roots (Martin, 1977).

3) *root litter*, i.e. moribund or dead, decaying roots in the soil (process termed as rhizo-deposition by Waid (1974) as the roots and rootlets are renewed (process termed as root turnover), without noticeable variation in the total biomass in natural prairie formations (Warembourg & Morrall, 1978).

There is increasing agreement that root lysates provide a much more important source of energy than it was thought a few years ago. By using ^{14}C labelling, Martin (1977) showed that a major loss of root carbon (14–47%) resulted from autolysis of the root cortex. However, Martin's experiments are based on young plants (3–8 week-old). It is reasonable to assume that in older plants root-deposition provides rhizosphere microorganisms with a large extra supply of energy-yielding compounds. In the rhizosphere of flooded rice, there is some indication that N_2 fixers make up a large percentage of the total rhizosphere bacterial population (Trolldenier, 1977; Watanabe & Cholitkul, 1978). If bacteria are dominant in the rhizosphere of flooded rice, N_2 fixing bacteria ought to benefit from an important part of the energy flux.

Experimental evidence shows that N_2 fixing bacteria are relatively inefficient when viewed in terms of mg N_2 fixed per g of carbohydrate consumed whose values range from 2 to 25 (Dalton & Mortenson, 1972). Thus, according to Jensen (1953) *Azotobacter* sp. fixes only 15–10 mg N_2 per g of sugar consumed; this is a low figure, indeed, when compared with 80 mg of NH_4-N per g of glucose incorporated by a coliform bacteria. However, higher efficiency figures have been reported—as high as 45 mg N_2 fixed per g of mannitol consumed (Dalton & Postgate, 1969, a, b). In some cases, an increase of efficiency was attributed to the environment such as especially low pO_2 resulting, for example, from the association of the N_2 fixing bacteria with other organisms exhibiting high O_2 requirements (La Rue, 1977).

Fate of fixed N_2

At the present stage of our knowledge, we have very little information about the fate of fixed N_2. By placing sugarcane (L. 61.41) seedlings in an atmosphere enriched with $^{15}N_2$, Ruschel *et al.* (1975) found that a high amount of ^{15}N accumulated in the leaves, "suggesting rapid translocation of the fixed N_2 from the roots without its prior incorporation into microbial proteins as an intermediate step". Similar results were recently obtained by Eaglesham (personal communication) who found that N_2 fixed in the rhizosphere of flooded rice was at least partially absorbed by the plant during its growth cycle. Until these preliminary conclusions are quantified, the following questions remain: (1) What is the relative proportion of fixed N_2 which is excreted

and absorbed directly by the roots? (2) How long is fixed N_2 immobilised in the cells of N_2 fixing bacteria?

If the ammonification rate of the proteins synthesized by the rhizosphere N_2 fixing bacteria is high, the host-plant itself can benefit from the NH_4^+-N which is thus made available; but if the breakdown rate of the proteins is low, N_2 fixed in the cells of N_2 fixing bacteria is incorporated into the soil organic nitrogen pool and, thus, stocked until it is mineralized and absorbed by another crop. In the former case, one could expect that the nitrogen requirements of the host-plant will be partially met during its growth cycle, although this would not be true in the latter case.

Production of growth-regulating substances and antibiotics by rhizosphere N_2 fixing bacteria

Any rhizosphere microorganism may stimulate or depress the root growth of its host-plant. Among the different hypotheses that are invoked to explain such an effect, the one concerning the production of growth-regulating substances by microorganisms should not be overlooked.

It has been shown that several N_2 fixing bacteria produce growth-regulating substances. Thus, Brown & Burlingham (1968) reported that *Azotobacter chroococcum* produces an average of 0.05 μg gibberellic acid GA3 equivalents per ml of culture in 14 days. Production of cytokinin-like substances by *A. chroococcum* was reported by Coppola *et al.* (1971). More recently, Barea & Brown (1974) treated seedlings of several plant species with 14 day-old cultures of *A. paspali*, which is known to be a typical inhabitant of the rhizosphere of *Paspalum notatum* and *P. plicatum* (Dobereiner, 1970; Brown, 1976). Roots of germinated seedlings were dipped into a culture of *A. paspali* so that each received 7×10^5 cells and they were then transplanted into two different soils: (1) light yellow latosol from the Cerrado, Brazil, (2) standard J.I. compost. The treatment did not affect the development of plants grown in the Cerrado soil (presumably because of the occurrence of an unknown limiting factor) but affected significantly that of plants grown in the compost. The number of *A. paspali* in the rhizosphere of treated plants declined rapidly, even around the roots of the *Paspalum notatum*. The decline was more rapid in the compost (Table 2). The decline of *A. paspali* populations in different rhizospheres suggests that improvements in plant growth was mainly due to growth-regulating substances elaborated by *A. paspali* such as IAA, at least 3 gibberellins and 2 cytokinins.

Mishustin (1966) had proposed earlier that *Azotobacter* inoculants acted not by stimulating N_2 fixation, but by affecting plant growth through gibberellin or cytotokinin-like substances. However, if Barea and Brown's experiments show that young seedlings can absorb such growth regulators produced by *A. paspali*, it does not, however, exclude the possibility that old, mature roots can fix N_2 or that a wide range of other substances of bacterial origin

Table 2. Change in numbers of *A. paspali* in rhizospheres of different plant species over a period of 2–8 weeks (Barea & Brown, 1974)

Species	No/g of dry rhizosphere soil							
	J.I. Compost				Brazilian soil			
	2	4	6	8	2	4	6	8
L. perenne	182	66	0	58	17600	1300	290	0
Wheat	40	12	22	48	14300	0	0	0
Lettuce	1970	0	0	55	1600	13	155	7
Tomato	—	—	—	—	27600	20500	7200	1800
C. pubescens	58	7	0	7	14300	4600	450	180
P. notatum	4823	219	2079	1325	180	63	63	6

might affect plant growth (Lynch & White, 1977).

Since antibiotics were reportedly isolated from cultures of *Azotobacter*, it was also suggested that *Azotobacter* inoculants could improve yields by controlling diseases, especially those affecting seedlings when poor quality seeds are used (Mishustin, 1966).

The agronomic significance of rhizospheric N_2 fixation

Total non-symbiotic N_2 fixation

In ecosystems where leguminous plants are absent or too low to account for much of the fixation, N_2 fixation, as measured by any method, can be attributed not only to rhizosphere N_2 fixing bacteria but also to N_2 fixing systems such as blue-green algae (Cyanobacteria), lichens and *Azolla* (in paddy fields). Since the contribution of each group cannot be specified, the nitrogen input as it is measured is termed here as "total non-symbiotic N_2 fixation".

An example of a high rate of total N_2 fixation has already been given. Other examples of high rates are given in the reviews of Moore (1966) and Dart & Day (1975) who report nitrogen gains up to 50 kg and even higher than 70 kg ha^{-1} $year^{-1}$. But one should be aware of the fact that high figures of total non-symbiotic N_2 fixation are not the rule and that total N_2 fixation varies considerably with environmental parameters and in many cases its rate is very low, or even nil.

Contribution of N_2 fixing bacteria to nitrogen gains in the rhizosphere

Field studies based either on the balance approach, or on any other methodology, cannot provide information on the respective contribution of different N_2 fixing systems. In order to get this kind of information, one has to use experimental devices specifically set up to eliminate the growth of N_2 fixing microorganisms other than N_2 fixing rhizosphere bacteria, especially blue-

green algae which are the most active fixers. Elimination of blue-green algae was usually obtained by covering the soil with a black cloth to prevent light from striking the surface; algicides could also be used, but rates of application should be low enough to avoid a reduction of plant growth.

Recently the International Rice Research Institute group (Watanabe *et al.*, 1978), devised a simple method to eliminate the bulk of the N_2 fixing activity by blue-green algae that reside in the flood water on the surface of paddy fields. Before the acetylene assay was performed in the plastic chamber enclosing the plant, flood water and a small layer of the surface soil was removed followed by addition of distilled water.

Direct assessment by the balance approach and by the ARA method: From a greenhouse pot, containing Philippino soil (blue-green algae, *Azolla*, rhizosphere N_2-fixing bacteria) planted with rice grown under continuously flooded conditions, the IRRI group established a nitrogen balance sheet (Table 3). It was found that a quantity of nitrogen equal to 50% of the

Table 3. N balance sheet for four crops of rice (International Rice Research Institute, 1978)

Treatment	Debit			Credit		Balance	
	Decrease soil N (mg/pot)	Misc. N input (mg/pot)	Total N (mg/pot)	N in four crops (mg/pot)	Increase soil N (mg/pot)	Unacc't N (mg/pot)	Unacc't N / Crop N %
Standard	469	25	494	967	—	473* a	49%
Covered	895	25	920	915	—	−5 (N.S.) b	—
+algae (+P, Fe)	252	70	322	1,093	—	771* a c	71
+Azolla (+P, Fe)	260	78	338	1,316	—	978* c	74
Fallow	—	22	22	—	177	149 (N.S.) b	—

*Significant at 1% level.

Note: Values followed by a common letter are not significantly different at the 5% level.

nitrogen removed by the crop was fixed in the standard pot, where blue-green algae were spontaneously growing. But if soil in pots were covered by a black cloth leaving the plant free, no significant amount of N_2 was fixed. The flooded fallow control pots without plants did not fix significant amount of nitrogen. This suggests that the rice plant must be present growing in soil to stimulate N_2 fixation. Inoculation with either *Azolla*, or with blue-green algae (with phosphorus and iron fertilization) stimulated N_2 fixation. Inoculant was taken from the field, and depending on the crop, contained *Nostoc*, *Gloeotrichia*, *Anabaena* and *Azolla pinnata*. All the net N_2 fixation detected in this experiment was due to phototrophic microorganisms".

Similar results were obtained by Alimagno and Yoshida (1977) using the ARA method. Soil samples covered with black cloth did not exhibit any significant N_2 fixing activity, whereas 18–33 kg N_2 ha^{-1} and 2–6 kg ha^{-1} were fixed in farmer's fields in Albay province in Philippines. Thus, in Philippino rice fields studied by these authors, N_2 fixation was attributed mostly to blue-green algae and, eventually, *Azolla*.

Field assays performed at the IRRI using the water replacement technique as described above, showed that removing the bulk of algal community and replacing the flood water with algal free water resulted in a dramatic decrease of ARA (Fig. 6), suggesting greater contribution by free living algae than by other organisms associated with rice (Watanabe & Cholitkul, 1978). According to these authors, the microflora responsible for the residual ARA (Fig. 6 A) was predominantly made up of algae attached on the rice stems, the contribution of rhizosphere bacteria being trivial.

However, the possibility of the contribution of rhizosphere N_2 fixing bacteria in rice fields other than Philippines cannot be excluded. Thus Yoshida (personal communication) in Japan, Rinaudo (personal communication) in Senegal and (Camargue) in France found that the rhizosphere acetyiene reducing activity (ARA) of 3 week-old rice seedlings growing in the absence of blue-green algae could reach 5,000 nanomoles C_2H_4 ha^{-1} g (dry weight) $root^{-1}$. Such values might be of agronomic significance.

The interference of blue-green algae in normally drained agrosystems (including upland rice field) may be negligible. ARA's (expressed as nanomoles C_2H_4 h^{-1} g dry $root^{-1}$) observed in such agrosystems are generally low (100 or less) and values higher than 1,000 are the exception (Table 4). However, figures higher than 2,000 nanomoles C_2H_4 g dry $root^{-1}$ h^{-1} have been exceptionally reported (Dart, 1977).

Assuming (1) that the daily rhizosphere ARA is ca. 12 times the ARA expressed on a one-hour basis, (2) that the rhizosphere ARA remains constant during half of the growth cycle (ca. 50 days) and is nil during the other half, (3) that the mean root weight of a rice crop is ca. 1,000 kg ha^{-1} i.e. 10^6 g ha^{-1} (Charreau et Nicou 1971), (4) that the molar C_2H_4: fixed N_2 conversion ratio is 3.0, extrapolation of the reported figure for flooded rice at 5,000 nanomoles C_2H_4 h^{-1} g $root^{-1}$ gives:

$$5{,}000 \times 12 \times 50 \times 10^6 \times \frac{1}{3} = 1 \times 10^{12} \text{ nanomoles } N_2 \text{ ha}^{-1} \text{ per growth cycle}$$

i.e. 28,000 g or 28 kg N_2 fixed ha^{-1} per growth cycle, which is a significant nitrogen input.

Such extrapolation, however, cannot be accepted without caution since assumption (1) and (2) still have to be checked and the C_2H_4:N_2 conversion ratio may be largely affected by different factors (Knowles, 1976). Moreover, making comparisons of ARA on a root weight basis is questionable because of the heterogeneity of the ARA distribution in the root systems.

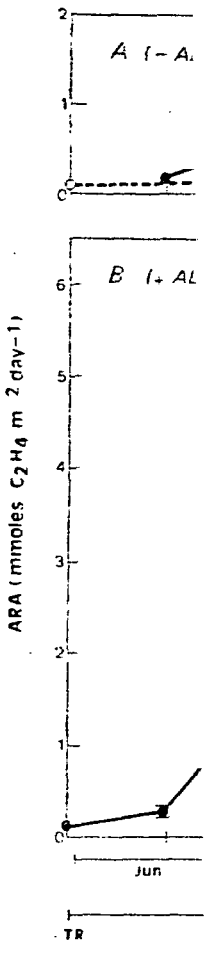

Fig. 6. AR
blue-green

Indirect assess
pounds: We have
bacteria expressed
(or 0.2–2.5 if resul
of carbohydrate a
mate N_2 fixation,
ing bacteria efficie
the quantity of car
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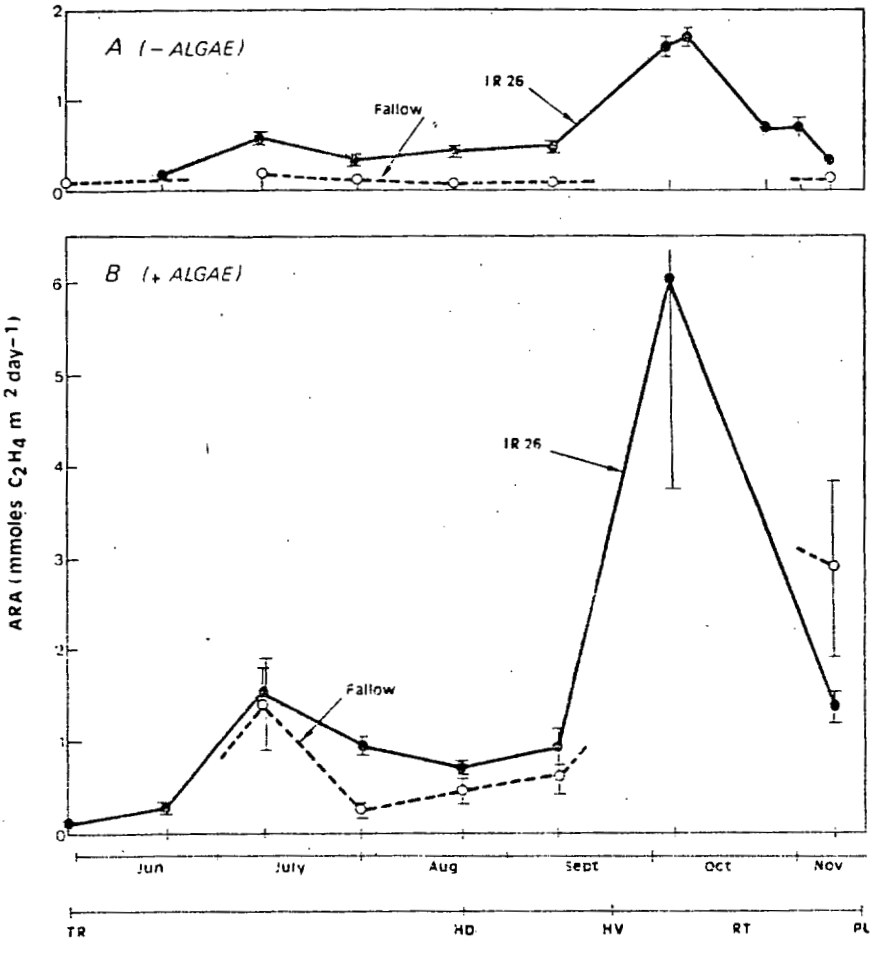

Fig. 6. ARA as measured in the rice field in presence or in absence of blue-green algae. TR = transplanting, HD = heading, HV = harvesting, PL = ploughing (Watanabe & Cholitkul, 1978).

Indirect assessment based on amount of available energy yielding compounds: We have already reported that the efficiency of free-living N_2 fixing bacteria expressed as mg N_2 fixed per g of carbohydrate consumed was 2–25 (or 0.2–2.5 if results are expressed on a percentage basis). Thus, if the amount of carbohydrate available in the rhizosphere is known, it should be easy to estimate N_2 fixation, using the ratio: N_2 fixed = carbohydrates available × N_2 fixing bacteria efficiency. Unfortunately, as already stressed, our knowledge of the quantity of carbohydrate supplied to the rhizosphere is very poor and restricted to young plants. However, two examples of calculations will be given here, with the assumption that non N_2 fixing heterotrophs do not compete with N_2 fixing bacteria.

Table 4. Acetylene reducing activity of non-legume roots (Dart & Day, 1975)

Plant	Origin	N_2ase activity (expressed as nmoles C_2H_4 g dry root^{-1} h^{-1})
Brachiaria mutica	Brazil	150–750
B. rugulosa		5–150
Cymbopogon citratus		2
Cynodon dactylon		20–270
Cyperus rotundus		10–30
Digitaria decumbens		20–400
Hyparrhenia rufa		20–30
Melinis minutiflora		15–40
Panicum maximum		20–300
Paspalum notatum		2–300
Pennisetum purpureum		5–1000
Saccharum officinarum (sugar cane)		5–20
Sorghum vulgare seedlings		10–100
Andropogon gayanus	Nigeria	15–270
Cenchrus ciliaris		16
Cymbopogon giganteus		60–85
Cynodon dactylon		10–50
Cyperus spp.		2
Hyparrhenia rufa		30–140
Hypothelia dissoluta		10–15
Panicum maximum		75
Paspalum commersonii		25–30
P. virgatum		3
Pennisetum purpureum		60
P. coloratum		13
Setaria anceps		1–120
Pennisetum typhoides (millet)		3–195
Sorghum		22–83
Andropogon spp.	Ivory Coast	50–380
Brachiaria brachylopha		100–140
Bulbostylis aphyllanthoides		74
Cyperus obtusiflorus		30–620
C. zollingeri		50–160
Cyperus sp.		150–1900
Fimbristylis sp.		80–190
Hyparrhenia dissoluta		2–4
Loudetia simplex		54
Maize seedlings	France	100–3000
	USA	14–16
Rice	Philippines	8–80
Rice seedlings (in test-tubes)	Ivory Coast	1040–2360
	France	10–3000
Setaria anceps	Australia	68
Pennisetum clandestinum		21–140

·y, 1975)
ivity (expressed ioles C_2H_4 g root^{-1} h^{-1})
50–750
5–150
2
20–270
10–30
20–400
20–30
15–40
20–300
2–300
5–1000
5–20
10–100
15–270
16
60–85
10–50
2
30–140
10–15
75
25–30
3
60
13
1–120
3–195
22–83
50–380
100–140
74
30–620
50–160
150–1900
80–190
2–4
54
100–3000
14–16
3–80
1040–2360
10–3000
68
21–140

The first example is based on exudate estimation made by Barber & Lynch (1977). According to these authors, the total exudation of carbohydrates by barley is 0.2 mg per mg plant dry weight. Assuming the efficiency of involved N_2 fixers is 1.5%, N_2 fixation would be at most 2–3 μg N_2 fixed per mg plant (dry weight), that is about 15 per cent of the N content of the plant.

The second example is based on Martin's experiments (1977), indicating, that an average 10% of the total photosynthate is released as exudates or lysates into the soil. Given a wheat crop yielding 2T of grain, 2T of straw and 1T of roots ha^{-1}, that is 5T of dry matter ha^{-1}, 500 kg of material would be released into the soil. Assuming the same efficiency as in the previous example, estimated N_2 fixation would be 7.5 kg ha^{-1} year^{-1} (Rovira, personal communication). Such calculations may overestimate or underestimate the actual N_2 fixation. Overestimation results mainly from the fact that the assumption of no competition by non-N_2 fixing heterotrophs is unrealistic. Presently, we have no idea of the amount of energy which is bypassed by these organisms; but one could presume that, even under the most favorable conditions, at least 50–90% of the carbohydrates are not available for N_2 fixing bacteria.

Underestimations may be as important as overestimations. Thus in the above mentioned examples, amounts of released carbohydrates were estimated without taking into account the input of carbohydrates through root deposition, which is mainly known to occur after the heading stage. Root deposition could presumably be 5–10 times higher in terms of carbohydrates added to the soil than root exudates or lysates.

N_2 fixation is now well known to occur spontaneously in the rhizosphere of many plants. Latest estimations suggest that its amount is not negligible but is lower than anticipated amounts and agronomic significance of fixed nitrogen is questionable in many agrosystems since amounts higher than 5–10 kg N_2 fixed ha^{-1} have not yet been demonstrated unequivocally.

Improvement of rhizospheric N_2 fixation

Improvement in N_2 fixation could be achieved by several ways as outlined below:

Plant breeding and selection

Genetic variability in legumes responding to association with *Rhizobium* is well-known (Nutman 1969, 1975). Many reports suggest that certain species, e.g. rice, sugar cane, sorghum, maize and some forage grasses, preferentially stimulate N_2 fixing bacteria in their rhizosphere (e.g. Dommergues *et al.*, 1972; Dobereiner, 1974). Improving these species by plant breeding and selection appears to be promising and realistic since intervarietal and intra-varietal differences have been observed by some investigators working with rice (Hamad-Fares, 1976; Lee & Yoshida, 1977), sorghum (Dart, 1977), maize

(Bulow and Dobereiner, 1975), wheat (Neal & Larson, 1976) and forage grasses such as *Paspalum notatum* (Dobereiner *et al.*, 1972). Table 5 illustrates the intra-varietal variability of rice cv. Cesariot observed in a phytotron experiment (Marie *et al.* in Dommergues, 1978b). It is interesting to compare the last data, concerning rice, with data published by Bulow & Dobereiner (1975) concerning maize. These authors found that the original cultivar of maize studied showed a mean ARA of 313 nanomoles, whereas the best lines showed mean ARAs of 2,026, 2,315 and 7,124 nanomoles per g dry root per h. By screening sixty lines and land race cultivars of pearl millet grown in a red soil at an ICRISAT station, Dart (1977) found that the line ICI 1,530 showed a high ARA (2,290 nanomoles C_2H_4 g dry root^{-1} h^{-1}). On the other hand, "New variability may be produced through mutation breeding, hybridization and genetic manipulation" (Bulow, 1978). However, there are limitations in breeding programmes for maximizing N_2 fixation mainly due to variability in genetic lines. Thus when testing 70 clones of cultivated and wild rice for ARA in the field and greenhouse, Watanabe & Cholitkul (1978) could not find any significant difference between the nitrogen fixing activity of the different clones, possibly because large fluctuations occurred during the growth stage.

Table. 5. Acetylene-reducing activity (ARA in the rhizosphere of different mutants of rice cv. Césariot grown in an alluvial soil (3-week old seedlings) (Marie *et al.* quoted by Dommergues, 1978b)

Mutants of rice cv. Césariot	ARA in the rhizosphere (nmoles C_2H_4 g dry root^{-1} h^{-1})
Rampant 2	5,320 ± 5,010
Criblé à glumelles bicolores	4,890 ± 2,770
Grain très long non perlé	3,310 ± 2,190
Rampant 1	2,890 ± 1,670
Grain long	2,870 ± 2,730
Original Cesariot cv.	2,120 ± 1,470
Petit pois	2,020 ± 1,800
Tardif à grain long très perlé	2,010 ± 1,370
Raide tardif à grain fin	1,820 ± 1,600
Raide 1	1,560 ± 1,250
Criblé	1,550 ± 860
Glumelles chamois	1,270 ± 580
Grain très long perlé	950 ± 380
Rachis noir	860 ± 470
Piment	690 ± 580
Glumelles bicolores	630 ± 190
Glumelles chocolat	500 ± 310
Court à grain grossi	80 ± 40

Manipulation of rhizosphere N_2 fixing bacteria

Effect of inoculation upon plant growth and yields (also see chapter 14)

Azotobacter: According to Rubenchik (1963), the first attempts at using *Azotobacter* to inoculate plants were made as early as 1902. Then many experiments were performed, especially in the Soviet Union, Eastern Europe and India and the practice of inoculation was often recommended in these countries. However, Mishustin & Shilnikova (1971) drew attention to the fact that significant, positive effects were obtained only in about one third of the field trials. Increases ranged from 10% to 39%, an increase of 10% being almost within the limits of experimental error. When they occurred, such increases were attributed to different processes: production of growth-regulators, protection against root pathogens, stimulation of rhizosphere microorganisms beneficial for the plant, modification of nutrient uptake by the plant and enhancement of N_2 fixation (Brown, 1975).

Beijerinckia: Few experiments have been performed with this bacterium. We quote here the results of field trials at 5 places in acidic soils of Maharashtra, India where at 3 out of 5 studied sites, inoculation of *Beijerinckia* alone was significant at the 5% level, but inoculation of *Beijerinckia* with the application of molybdenum was significant at 1% level, in increasing the yield of rice (Sulaiman, 1971).

Azospirillum (*Spirillum*): Out of four inoculation experiments with maize in Wisconsin where practically no *Spirillum* were present in the soils before inoculation, only two showed significant, positive results. (Albrecht *et al.* quoted by Dobereiner, 1978). Inoculation of field-grown wheat (Neyra and Dobereiner, 1977) did not increase crop yields. By contrast, Smith *et al.* (1976) reported that field-grown pearl millet (*Pennisetum americanus*) and Guinea grass (*Panicum maximum*) lightly fertilized (ca. 40 kg N ha^{-1}) and inoculated with *Spirillum lipoferum* produced significantly higher yields of dry matter than non-inoculated controls. Generally, nitrogen content (%) was not changed but greater amounts of nitrogen were harvested per unit area. However, these experiments do not show unequivocally the ability of *S. lipoferum* to fix N_2 and thus enhance plant growth (Smith *et al.*, 1978).

Effect of inoculation on N_2 fixation and root colonization: Reports on the effect of inoculation upon N_2 fixation generally measured by the ARA method—are much less numerous than reports relating to the effects upon crop yields.

Inoculation with pure strains: (1) Gnotobiotic systems. It is tempting to set up experimental models consisting of a sterile plant inoculated with a given strain of N_2 fixing bacterium, and to estimate the ARA of this simple system. Such an approach was adopted by Watanabe (International Rice Research Institute, 1976) and by Hamad-Fares (1976). Non-inoculated seedlings did not exhibit any ARA. Inoculated seedlings exhibited some ARA, but it was always low. Maximum ARA was only 21 nanomoles C_2H_4 per $hour^{-1}$ for

3-week old rice seedlings (0.2 μg N_2 fixed plant^{-1} d^{-1}) inoculated with isolates from rice roots or *Azotobacter chroococcum* (International Rice Research Institute, 1976). The mean ARA was 56 nanomoles C_2H_4 hour^{-1} per 10 seedlings, that is $15 \times 12 = 180$ nanomoles C_2H_4 per day (12 hours of active fixation) per seedling (1, 6 μg N_2 fixed plant^{-1} d^{-1}), in the system made up by rice inoculated with a *Beijerinckia* sp. strain isolated from Camargue soil (Hamad-Fares, 1976). In both experiments no improvement of plant growth and nitrogen uptake could be detected "probably because the amount of N_2 fixed was too low to meet the plant's nitrogen requirements (400 μg N for 3 weeks)" (International Rice Research Institute, 1976). The low ARA observed in simple gnotobiotic systems may be attributed to a deficiency of photosynthesis due to CO_2 deficiency or/and relatively low light intensity (ca. 10 klux) usually obtained in growth cabinets. Another explanation is that young seedlings do not provide rhizosphere bacteria with as much energy-yielding material as older plants would do. Moreover, inoculated N_2 fixing bacteria might lack the enzymes necessary for making use of some of the substrates provided by the host-plant (cell lysates, decaying roots).

2) Inoculation of non-sterile soil-plant systems. From a series of pot and field experiments performed in Oregon, Barber *et al.* (1976) concluded that inoculating maize with *Spirillum* increased moderately the ARA of intact plant systems but that the increase of ARA was two-fold higher when plants inoculated with *Spirillum* were grown in "used" soil (previously inoculated and used for growth of corn in an earlier experiment), indicating that continual application of *Spirillum* might lead to the establishment of the desired N_2 fixing bacteria.

Laboratory experiments performed by Hamad-Fares (1976) using rice grown in an alluvial soil from Camargue, France, inoculated with *Beijerinckia* sp. and simultaneously by Rinaudo (personal communication) using rice grown in different Senegalese alluvial soils and inoculated with *Spirillum*, showed that the odds for an increase of N_2fixation through inoculation were higher when the non sterile soil was diluted, suggesting that the dilution of the soil decreased the competition of the native N_2 fixing microfolia and/or the antagonism of non-fixing heterotrophs. Thus the failure of inoculation might be attributed to antagonism processes which tend to eliminate the introduced strain. In the case of the alluvial Camargue soil, actinomycetes were thought to be responsible for the limited development of *Beijerinckia* sp. (Diem *et al.*, 1977).

Mixed inocula: Instead of inoculating plants with pure cultures some authors have used mixed inocula made up of different strains. Two examples will be given here.

The first one is that of an experiment set up by Ocampo *et al.* (1975) who inoculated lavender plants grown in soils with or without organic matter. Two kinds of inocula were compared: (1) inoculum A, consisting of a mixture of

three *Azotobacter* strains (*A. chroococcum*, *A. beijerinckia*, *A. vinelandii* (2) inoculum A+P, consisting of a mixture of inoculum A and inoculum P, which is itself a mixture of three phosphate-solubilizing bacteria (*Pseudomonas* sp.; *Agrobacterium* sp.; and *Bacillus* sp.). In all cases, the number of *Azotobacter* still living after inoculation declined but there were always more *Azotobacter* in the rhizosphere of plants inoculated with A+P than in that of plants inoculated with A alone. In one case (soil n°1 with 2% organic matter), *Azotobacter* population was still large after 16 weeks, when inoculum A+P was used (Table 6).

Table 6. Numbers of *Azotobacter* in lavender rhizospheres (Ocampo *et al.*, 1975)

Soil No.	Organic matter added	Inoculation treatment	Nos. ($\times 10^3$)/g dry rhizosphere soil, weeks after inoculation				
			6	8	10	12	16
1	2%	A	1020	292	157	65	9
		A+P	2099†	801‡	480†	352‡	283‡
1	0	A	236	153	18	8	3
		A+P	1160‡	250*	44*	27‡	7*
8	2%	A	1600	211	143	56	27
		A+P	1500	272	186	120*	55*
8	0	A	901	180	80	77	29
		A+P	1900†	760‡	212†	159*	58*

Note: A=*Azotobacter*-inoculated pots; P=phosphobacteria-inoculated pots. Significance at 0.1% level (‡), 1% level (†), and 5% level (*). A+P vs. A treatments have been compared for each experimental soil.

Questions to be answered

What are the N_2 fixing strains/or associations of strains which are active in the rhizosphere? What are the criteria which could be used in the selection and genetic improvement of rhizosphere N_2 fixing bacteria? How can selected strains or associations of strains be established in the rhizosphere?

Efficient N_2 fixing bacteria involved in rhizosphere N_2 fixation: We have already indicated that spontaneous plant-rhizosphere systems can fix N_2. But, until now, nobody has been able to relate the observed N_2 fixing activity to the growth or activity of a given strain or association of strains among the different N_2 fixing bacteria which have been isolated from the rhizosphere: *Azotobacter*, *Beijerinckia*, *Azospirillum*, *Enterobacter*, *Klebsiella*, *Clostridium*. "Measurement of the N_2 fixing activity alone will not be adequate because other N_2 fixers may be impredictably present and go unrecognized while a real issue must be the way in which changes [in a given N_2 fixing population] are related to N_2 fixed" (Dommergues *et al.*, 1978). On the other hand it should be stressed that N_2 fixing bacteria do not fix N_2 in the field as they

would do in a pure culture, since they may be associated with other N_2 fixing or non-fixing microorganisms which increase (or decrease) their efficiency. More attention should be given to associations of N_2 fixing bacteria with other microorganisms such as those already reported by Rubenchick (1963), Dommergues & Mutaftschiev (1965), Florenzano *et al.* (1968), Kobayashi *et al.* (1965), Line & Loutit (1973), and Babeva *et al.* (1977) including associations of N_2 fixing bacteria and mycorrhizae (Silvester and Bennett, 1973).

Criteria used in the selection and genetic improvement of rhizospheric N_2 fixing bacteria: The main properties which are prerequisite for active N_2 fixation in the rhizosphere are not yet clearly defined. However, taking into account most recent experiments, it can be assumed that the most desirable properties would be the following:

1) Ability to survive in soil;

2) Ability to compete successfully with other rhizosphere microorganisms and to actively colonize the root system;

3) Ability to fix N_2 in the presence of combined nitrogen and to provide the host-plant directly with easily absorbed forms of nitrogen;

4) High nitrogenase activity;

5) Protection against oxygen.

Characteristics (1) and (2) will be dealt with later. As already mentioned, rhizosphere N_2 fixation cannot provide all the nitrogen required by the plant; consequently, nitrogen fertilizers must be added to the soil to meet the plant's nitrogen requirements. But, such additions generally result in the inhibition of N_2 fixation, unless additions are low, or compatible forms of fertilizers are used. Otherwise, the solution to the problem lies in the discovery of N_2 fixing strains which are able to fix N_2 in the presence of combined nitrogen. Actually, mutants have already been obtained in which the *nif* genes are constitutive or de-repressed, which excrete large amounts of fixed nitrogen (Shanmugam & Valentine, 1975). Recently, Gauthier & Elmerich (1977) obtained spontaneous revertants from *Spirillum lipoferum* mutants which exhibited levels of nitrogenase activity up to 50% higher than that of the wild strain, suggesting that nitrogenase activity of N_2 fixers could be improved by genetic manipulations. With the exception of *Azotobacter*, most aerobic N_2 fixers are relatively oxygen-sensitive, and are, thus, ill-adapted to aerobic soil micro-habitats. It could be of interest to select strains possessing good oxygen protection mechanisms for their nitrogenase. But, to date, no investigation has been initiated in that direction.

Establishment of a given strain in the rhizosphere: Introducing a given strain or a given association into a non-sterile environment such as the rhizosphere soil is difficult and most observations indicate that sooner or later there is generally a marked decline of the inoculated strain (Clark, 1948; Alexander, 1961; Barea & Brown, 1974; Ocampo *et al.*, 1975). How can the

competition be overcome? Up to now, we are unable to answer that question properly. However, some preliminary results suggest that it might be promising to use associations of microorganisms and/or massive inoculants improved with inoculum carriers.

Soil management

At our present state of knowledge, soil management is still one of the most practical ways to improve rhizosphere N_2 fixation. Practices that could be recommended are aimed at eliminating or, at least, reducing the effects of edaphic limiting factors, especially an excess or a deficiency of different elements. Control of the soil water regime may also be desirable.

Excess or deficiency of different elements: The most important aspect, from the agronomic point of view, is that of the effect of mineral nitrogen fertilizers on N_2 fixation in the rhizosphere. Laboratory experiments performed by Rinaudo (Balandreau *et al.*, 1975) showed that addition of ammonium sulphate of up to 40 ppm, to rice seedlings, stimulated ARA, but higher application induced a marked decrease. Field experiments confirm these laboratory results. A low application of mineral N (20 kg N/ha) every two weeks did not alter rhizosphere ARA in roots of *Pennisetum purpureum* or *Digitaria decumbens* (Day *et al.*, 1975). By contrast, higher applications reduced (Balandreau and Dommergues, 1972) or inhibited rhizosphere ARA (Dobereiner, 1978). In order to avoid the inhibition of symbiotic N_2 fixation, Hardy *et al.* (1973) suggested the use of other forms of nitrogen fertilizers which do not inhibit N_2 fixation, while they provide the plant with the complementary nitrogen required for their growth. Such new forms of chemical fertilizers, designed as compatible fertilizers by Hardy *et al.* could also be recommended in the case of rhizosphere N_2 fixation. This possibility has not yet been explored seriously, but it could be a promising approach (Dommergues, 1978c).

Taking into account the information we have about legume symbiosis, we can assume that soil acidity, which is prevalent in tropical situations, and which is usually associated with low Ca, P and Mo contents, drastically limits rhizosphere N_2 fixation. Applying required fertilizers should probably stimulate the activity of rhizosphere N_2 fixing bacteria either directly, or by promoting the development of the root system, thus increasing the amount of energy yielding substrates in the soil.

Control of the soil water regime: By controlling the soil water regime, it should be possible to assist rhizosphere N_2 fixation. Thus, increasing soil water content, during the most favorable period, can electively favor N_2 fixing bacteria (most of which are known for their sensitivity to oxygen) and reduce the competition of aerobic microorganisms for energy-yielding substrates in the rhizosphere. Obviously the practice of monitoring the water regime would be restricted to sites allowing irrigation or water conservation in the soil.

Stimulation of N_2 fixation through herbicides: Unexpectedly, Marriel and Cruz (1978) found that two herbicides (atrazin and alachlor[1]) used in maize culture, increased the number of *Azospirillum lipoferum* in soil and enhanced rhizosphere ARA of field-grown maize. How herbicides affect rhizosphere ARA is still unknown, but the possibility of increasing rhizosphere N_2 fixation through herbicide application should be explored further.

Concluding remarks

Our knowledge of rhizospheric N_2 fixation has obviously progressed during the last ten years. But in spite of this vast research effort, four basic questions have still not been satisfactorily answered:

1) What is the structure of the plant-rhizosphere N_2 fixers association and how does it function?

2) What is the actual agronomic significance of rhizosphere N_2 fixation?

3) Even if the spontaneous rhizosphere N_2 fixation is not agronomically significant, can it be increased sufficiently to be economically exploited?

4) Beside improvement of the N_2 fixing system as described above, other approaches have been proposed. Which are the most promising?

1) The question of the structure of the association is still debated: are the associated N_2 fixing bacteria simply embedded in the root mucilage, are they mainly situated on the root cortex or amidst sloughing off cortical cells, or are they inside the cortex cells? The functioning of the associative symbiosis is still poorly understood, especially as far as the energy flux is concerned.

2) Estimation of actual rhizosphere N_2 fixation cannot be accepted without caution, since the present field techniques of measurement are not yet totally reliable.

3) Improvement of the associative symbiosis seems to be an attainable objective. Different approaches have been suggested in this chapter: they are related on one hand to the microflora involved and on the other, to the plant itself.

4) In addition to conventional microbiological and plant breeding methods, other exciting possibilities should be considered, especially introducing *nif* genes into the plant. The transfer of *nif* genes from N_2 fixing bacteria to higher plant cells has been considered for several years (Streicher *et al.*, 1972). But, it must be known that besides the problem of introducing *nif*, other difficulties can already be foreseen. These concern the phenotypic expression of *nif* which depends upon the protection of nitrogenase against destruction by oxygen, the regulation of nitrogenase and the functioning of the proper electron transport systems. In order to overcome such considerable difficulties, geneticists are trying to incorporate a plasmid containing the *nif*

[1]Atrazin: 2 chloro-4-ethylamine-6-isopropylamine-S triazine; Alachlor: 2 chloro-2′-6′-diethyl-N-mexomethyl-acetanilide.

genes connected with regulation and electron transport into plant protoplasts (Shanmugam & Valentine, 1975). The possibility of incorporating the *nif* genes into the DNA molecule which can be transcribed into plant cells has been theorized. Other approaches have also been proposed. Gibson *et al.* (1967) suggested attempting protoplast fusion of cells of a legume (or a nodulated non-legume) and a non-nodulating plant, hoping that "all the genes required for the establishment and function of a symbiotic association will persist through to the plant developed from the resulting callus". Postgate (1974) proposed to incorporate the *nif* genes either into a benign endocellular pathogen which would grow harmlessly in the plant tissue, or into mycorrhizae. Recently, Giles & Whitehead (1975, 1977) reported a successful transfer of acetylene reduction activity to *Rhizopogon* sp., a mycorrhizal fungus, and the subsequent occurrence of acetylene reduction activity in *Pinus radiata* roots reassociated with the modified *Rhizopogon* strain. These claims are obviously encouraging, but more experiments are necessary in order to check the stability and effectiveness of such systems.

References

Abd-el-Malek, Y. (1971). Free-living nitrogen-fixing bacteria in Egyptian soils and their possible contribution to soil fertility. *Plant and Soil.* Special Volume. 423–442.

Abd-el-Malek, Y. and Ishac, Y.Z. (1968). Evaluation of methods used in counting Azotobacters. *J. appl. Bacteriol.*, **31**, 267–275.

Alexander, M. (1961). Introduction to Soil Microbiology. John Wiley, New York.

Alimagno, B.V. and Yoshida, T. (1977). *In situ* acetylene assay of biological nitrogen fixation in lowland rice soils. *Plant and Soil*, **47**, 239–244.

Bab'eva, I.P., Moavad, K.H. and Marchenko, A.L. (1977). Nitrogen fixation in mixed cultures of *Lipomyces* and bacteria. *Mikrobiologiya*, **46**, 270–272.

Balandreau, J. and Dommergues, Y.R. (1972). Assaying nitrogenase C_2H_2 activity in the field. *Bull. Ecol. Res. Comm.* (Stockholm), **17**, 247–254.

Balandreau, J. and Knowles, R. (1978). The rhizosphere. *In:* Interactions between Non-Pathogenic soil Microorganisms and Plants (eds. Y.R. Dommergues and S.V. Krupa), pp. 243–268. Elsevier, Amsterdam.

Balandreau, J. Rinaudo, G., Hamad-Farès, I. and Dommergues, Y.R. (1975). Nitrogen fixation in the rhizosphere of rice plants. *In:* Nitrogen Fixation by Free-Living Micro-Organisms (ed. W.D.P. Stewart), pp. 57–70. Cambridge University Press.

Barber, D.A. and Lynch, J.M. (1977). Microbial growth in the rhizosphere. *Soil Biol. Biochem.*, **9**, 305–308.

Barber, L.E., Tjepkema, J.D., Russell, S.A. and Evans, H.J. (1976). Acetylene reduction (Nitrogen fixation) associated with corn inoculated with

Spirillum. *Appl. Environ. Microbiol.*, **32**, 108–113.

Barea, J.M. and Brown, M.E. (1974). Effects on plant growth produced by *Azotobacter paspali* related to synthesis of plant growth regulating substances. *J. appl. Bact.*, **37**, 583–593.

Benbough, J.E. and Martin, K.L. (1976). An indirect radiolabelled antibody staining technique for the rapid detection and identification of bacteria. *J. appl. Bact.*, **41**, 47–58.

Bowen, G.D. and Rovira, A.D. (1976). Microbial colonization of plant roots. *Ann. Rev. Phytopathol.*, **14**, 121–142.

Brouzes, R., Mayfield, C.I. and Knowles, R. (1971). Effect of oxygen partial pressure on nitrogen fixation and acetylene reduction in a sandy loam soil amended with glucose. *Plant and Soil*, Special Volume, 481–494.

Brown, M.E. (1974). Seed and root bacterization. *Ann. Rev. Phytopathol.*, **12**, 181–197.

Brown, M.E. (1975). Rhizosphere microorganisms—Opportunists, bandits or benefactors. *In:* Soil Microbiology. (ed. N. Walker). pp. 21–38. Butterworths, London.

Brown, M.E. (1976). Role of *Azotobacter paspali* in association with *Paspalum notatum*. *J. appl. Bact.*, **40**, 341–348.

Brown, M.E. and Burlingham, S.K. (1968). Production of plant growth substances by *Azotobacter chroococcum*. *J. gen. Microbiol.*, **53**, 135–144.

Bülow, J.F.W. von. (1978). Plant influence in symbiotic nitrogen fixation. *In:* Limitations and Potentials for Biological Nitrogen Fixation in the Tropics. (eds. J. Döbereiner, R.H. Burris and A. Hollaender). pp. 75–94. Plenum Press, New York and London.

Bülow, J.F.W. von and Döbereiner, J. (1975). Potential for nitrogen fixation in maize genotypes in Brazil. *Proc. Nat. Acad. Sci.* U.S.A., **72**, 2389–2393.

Burns, R.C. and Hardy, R.W.F. (1975). Nitrogen Fixation in Bacteria and Higher Plants. Springer-Verlag, Berlin.

Cacciari, I. and Lippi, D. (1973). Nitrogen fixation by *Arthrobacter* sp. II. Ability to fix nitrogen by some *Arthrobacter* sp. isolated from soil. *Ann. Microbiol.*, **23**, 69–73.

Campbell, N.E.R. and Evans, H.J. (1969). Use of Pankhurst tubes to assay acetylene reduction by facultative and anaerobic nitrogen-fixing bacteria. *Can. J. Microbiol.*, **15**, 1342–1343.

Charreau, C. and Nicou, R. (1971). L'amélioration du profil cultural dans les sols sableux et sablo-argileux de la zone tropicale sèche Ouest-Africaine et ses incidences agronomiques. *Agronomie Tropicale*, 209–255, 565–631, 1184–1247.

Clark, F.E. (1948). *Azotobacter* inoculation of crops. III. Recovery of *Azotobacter* from the rhizosphere. *Soil Sci.*, **65**, 193–202.

Coppola, S., Percuoco, G., Zoina, A. and Picci, G. (1971). Citochinine in germi terricoli e relative significato nei rapporti plante-microorganismi.

Annali di M

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Annali di Microbiologia ed Enzimologia, **21**, 45–53.

Dalton, H. and Mortenson, L.E. (1972). Dinitrogen (N_2) fixation (with a biochemical emphasis). *Bact. Rev.*, **36**, 231–260.

Dalton, H. and Postgate, J.R. (1969a). Effect of oxygen on growth of *Azotobacter chroococcum* in batch and continuous cultures. *J. gen. Microbiol.*, **54**, 463.

Dalton, H. and Postgate, J.R. (1969b). Growth and physiology of *Azotobacter chroococcum* in continuous cultures. *J. gen. Microbiol.*, **56**, 307.

Darbyshire, J.F. and Greaves, M.P. (1971). The invasion of pea roots *Pisum sativum* by soil microorganisms. *Acanthamoeba palestinensis* and *Pseudomonas* sp. *Soil Biol. Biochem.*, **3**, 151–155.

Dart, P.J. (1977). The ICRISAT microbiology program. *In:* Isotopes in Biological Dinitrogen Fixation. I.A.E.A., Vienna. In press.

Dart, P.J. and Day, J.M. (1975). Non-symbiotic nitrogen fixation in soil. *In:* Soil Microbiology (ed. N. Walker), pp. 225–252. Butterworths, London and Boston.

Day, J.M., Neves, M.C.P. and Döbereiner, J. (1975). Nitrogenase activity on the roots of tropical forage grasses. *Soil Biol. Biochem.*, **7**, 107–112.

Dayan, E., Banin, A. and Henis, Y. (1977). Studies on the mucilaginous layer of barley (*Hordeum vulgare*) roots. *Plant and Soil*. **47**, 171–191.

De-Ville, R.R. and Tchan, Y.T. (1970). Etude quantitative de la population azotobactérienne du sol par la méthode d'immunofluorescence. *Ann. Inst. Pasteur*, **119**, 492–497.

Diem, H.G., Godbillon, G. and Schmidt, E.L. (1977). Application of the fluorescent antibody technique to the study of an isolate of *Beijerinckia* in soil. *Can. J. Microbiol.*, **23**, 161–165.

Diem, H.G., Rougier, M., Hamad-Farès, I., Balandreau, J. and Dommergues, Y.R. (1978a). Colonization of rice roots by diazotroph bacteria. *Bull. Ecol. Res., Comm.*, **26**. In press.

Diem, H.G., Schmidt, E.L. and Dommergues, Y.R. (1978b). The use of the fluorescent antibody technique to study the behaviour of a *Beijerinckia* isolate in the rhizosphere and spermosphere of rice. *Bull. Ecol. Res. Comm.*, (Stockholm), **26**. In press.

Döbereiner, J. (1970). Further research on *Azotobacter paspali* and its variety specific occurrence in the rhizosphere of *Paspalum notatum*. *Zentralbl. Bact. Parasitenk.* Abt. II., **124**, 224–230.

Döbereiner, J. (1974). Nitrogen fixing bacteria in the rhizosphere. *In:* The Biology of Nitrogen Fixation (ed. A. Quispel). pp. 86–120. North Holland, Amsterdam.

Döbereiner, J. (1978). Nitrogen fixation in grass-bacteria associations in the tropics. *In:* Isotopes in Biological Dinitrogen Fixation. I.A.E.A., Vienna. In press.

Döbereiner, J. and Campelo, A.B. (1971). Non-symbiotic nitrogen fixing

bacteria in tropical soils. *Plant and Soil*, Special Volume, 457–470.

Döbereiner, J. and Day, J.M. (1976). Associative symbioses and free-living systems. *In:* Proc. 1st International Symposium on Nitrogen Fixation (eds. W.E. Newton and C.J. Nyman), pp. 518–538. Washington State University Press.

Döbereiner, J., Day, J.M. and Dart, P.J. (1972). Nitrogenase activity and oxygen sensitivity of the *Paspalum notatum—Azotobacter paspali* association. *J. gen. Microbiol.*, **71**, 103–116.

Döbereiner, J., Marriel, I.E. and Nery, M. (1976). Ecological distribution of *Spirillum lipoferum* Beijerinck. *Can. J. Microbiol.*, **22**, 1464–1473.

Dommergues, Y.R. (1978a). The plant microorganism system. *In:* Interactions between Non-Pathogenic Soil Microorganisms and Plants (eds. Y.R. Dommergues and S.V. Krupa), pp. 1–36. Elsevier, Amsterdam.

Dommergues, Y.R. (1978b). Impact on soil management and plant growth. *In:* Interactions between Non-Pathogenic Soil Microorganisms and Plants (eds. Y.R. Dommergues and S.V. Krupa), pp. 443–458. Elsevier, Amsterdam.

Dommergues, Y.R. (1978c). The effect of edaphic factors on nitrogen fixation with special emphasis on organic matter in soils. *In:* Proc. FAO/SIDA Regional Workshop in Africa on Organic Recycling in Agriculture. Buea Cameroun, December, 1977. In press.

Dommergues, Y.R., Balandreau, J., Rinaudo, G. and Weinhard, P. (1972). Nonsymbiotic nitrogen fixation in the rhizospheres of rice, maize and different tropical grasses. *Soil Biol. Biochem.*, **5**, 83–89.

Dommergues, Y.R., Belser, L.W. and Schmidt, E.L. (1978). Limiting factors for microbial growth and activity in soil. *Adv. in microbial. Ecol.*, **2**, 49–104.

Dommergues, Y.R. and Mutaftschief, S. (1965). Fixation synergique de l'azote atmosphérique dans les sols tropicaux. *Ann. Inst. Pasteur*, **109**, 112–120.

Evans, H.J., Campbell, N.E.R. and Hill, S. (1972). Asymbiotic nitrogen-fixing bacteria from the surface of nodules and roots of legumes. *Can. J. Microbiol.*, **18**, 13–21.

Fedorov, M.V. and Kalininskaya, T.A. (1959). Nitrogen fixing activity of mixed cultures of oligonitrophile microorganisms. *Mikrobiologiya*, **28**, 323–330.

Fenglerova, W. (1965). Simple method for counting *Azotobacter* in soil samples. *Acta Microbiol. Polonica*, **14**, 203–206.

Fliermans, C.B. and Schmidt, E.L. (1975). Autoradiography and immunofluorescence combined for autecological study of single cell activity with *Nitrobacter* as a model system. *Appl. Microbiol.*, **30**, 676–684.

Florenzano, G., Materassi, R. and Favilli, F. (1968). Indagini sperimentali sul sinergismo *Beijerinckia Lipomyces* nell'azotofissazione. *Ric. Sci.*, **38**, 1273–1276.

Foster, R.C. a
New Phytol

Gauthier, D. a
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Jackson, R.M.

Foster, R.C. and Rovira, A.D. (1976). Ultrastructure of wheat rhizosphere. *New Phytol.*, **76**, 343–352.

Gauthier, D. and Elmerich, C. (1977). Relationship between glutamine synthetase and nitrogenase in *Spirillum lipoferum*. *FEMS Microbiology letters*, **2**, 101–104.

Gibson, A.L., Scowcroft, W.R. and Pagan, J.D. (1977). Nitrogen fixation in plants: an expanding horizon? *In:* Recent Developments in Nitrogen Fixation. (eds. W.E. Newton, J.R. Postgate and C. Rodriguez-Barrueco), pp. 387–417, Academic Press, London.

Giles, K.L. and Whitehead, H.C.M. (1975). The transfer of nitrogen fixing ability to a eukaryote cell. *Cytobios*, **14**, 49–61.

Giles, K.L. and Whitehead, H.C.M. (1977). Reassociation of a modified mycorrhiza with the host plant roots (*Pinus radiata*) and the transfer of acetylene reduction activity. *Plant and Soil*, **48**, 143–152.

Golebiowska, J. and Pedziwilk, Z. (1975). Variation in the *Azotobacter* population from several habitats in the botanical garden in Poznan. *Acta Microbiol. Polonica*, B, **7**, 219–222.

Hamad-Farès, I. (1976). La fixation de l'azote dans la rhizosphère du riz. Thèse, Université de Nancy I, France.

Hardy, R.W.F., Burns, R.C. and Holsten, R.S. (1973). Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. *Soil Biol. Biochem.*, **5**, 47–81.

Hassouna, M.G. and Wareing, P.F. (1964). Possible role of rhizosphere bacteria in the nitrogen nutrition of *Ammophila arenaria*. *Nature*, London, **202**, 467–469.

Hegazi, N.A. and Niemelä, S.I. (1976). A note on the estimation of *Azotobacter* densities by membrane filter technique. *J. appl. Bacteriol.*, **41**, 311–313.

Hernandes, G.R. (1978). Experimental evidence on the presence of symbiotic bacteria in *Coffea*, with the capacity of triphenyl tetrazolium chloride reduction. *In:* Limitations and Potentials for Biological Nitrogen Fixation in the tropics (eds. J. Döbereiner, R.H. Burris and A. Hollaender), p. 364. Plenum Press, New York and London.

Holden, J. (1975). Use of nuclear staining to assess rates of cell death in cortices of cereal roots. *Soil Biol. Biochem.*, **7**, 333–334.

International Rice Research Institute (1976). Annual Report for 1975. Los Baños, Philippines.

International Rice Research Institute (1977). Annual Report for 1976. Los Baños, Philippines.

International Rice Research Institute (1978). Annual Report for 1977. Los Baños, Philippines.

Jackson, R.M. and Brown, M.E. (1966). Behaviour of *Azotobacter chro-*

ococcum introduced into the plant rhizosphere. *Annls. Inst. Pasteur.*, **111**, 103–112.

Jensen, H.L. (1953). *Azotobacter* as a crop inoculant. *In:* Proc. International Congr. Microbiol., Rome, **6**, 245.

Jensen, V. and Holm, E. (1975). Associative growth of nitrogen fixing bacteria with other microorganisms. *In:* Nitrogen Fixation by Free-Living Micro-Organisms (ed. W.D.P. Stewart), pp. 101–119. Cambridge Uni. Press.

Johnston, A.W.B. and Beringer, J.E. (1975). Identification of the *Rhizobium* strains in pea root nodules using genetic markers. *J. gen. Microbiol.*, **87**, 343–350.

Katznelson, H., Lochhead, A.G. and Timonin, M.I. (1948). Soil micro-organisms and the rhizosphere. *Bot. Rev.*, **14**, 543–587.

Knowles, R. (1976). Factors affecting dinitrogen fixation by bacteria in natural and agricultural systems. *In:* Proc. 1st International Symposium on Nitrogen Fixation (eds. W.E. Newton and C.J. Nyman), pp. 539–555. Washington State University Press.

Knowles, R. (1977). The significance of asymbiotic dinitrogen fixation by bacteria. *In:* A Treatise on Dinitrogen Fixation, Sect. 4: Agronomy (eds. R.W.F. Hardy and A.H. Gibson), pp. 33–83. Wiley and Sons, New York.

Kobayashi, M., Katayama, T. and Okuda, A. (1965). Nitrogen fixing micro-organisms in paddy soils. 15. Nitrogen fixation in a mixed culture of photosynthetic bacteria (*Rhodopseudomonas capsulatus*) heterotrophic bacteria. 5. *Azotobacter agilis*. *Soil Sci. Pl. Nutr.*, 11, **3**, 1–5.

Koch, B.L. and Oya, J. (1974). Non-symbiotic nitrogen fixation in some Hawaiian pasture soils. *Soil Biol. Biochem.*, **6**, 363–367.

Lakshmi Kumari, M., Kavimandan, S.K. and Subba Rao, N.S. (1976). Occurrence of nitrogen fixing *Spirillum* in roots of rice, sorghum, maize and other plants. *Ind. J. exp. Biol.*, **14**, 638–639.

Lakshmi, V., Satyanarayana Rao, A., Vijayalakshmi, K., Lakshmi-Kumari, M., Tilak, K.V.B.R. and Subba Rao, N.S. (1977). Establishment and survival of *Spirillum lipoferum*. *Proc. Indian Acad. Sci.*, **86**, 397–404.

La Rue, T.A. (1977). The bacteria. *In:* A Treatise on Dinitrogen Fixation Sect. 3. Biology (eds. R.W.F. Hardy and W.S. Silver), pp. 19–62. Wiley, New York.

Lee, K.K. and Yoshida, T. (1977). An assay technique of measurement of nitrogen activity in root zone rice for varietal screening by the acetylene reduction method. *Plant and Soil*, **46**, 127–134.

Line, M.A. and Loutit, M.W. (1973). Nitrogen fixation by mixed cultures of aerobic and anaerobic microorganisms in an aerobic environment. *J. gen. Microbiol.*, **74**, 179–180.

Lynch, J.M. and White, N. (1977). Effects of some non-pathogenic micro-organisms on the growth of gnotobiotic barley plants. *Plant and Soil*, **47**, 161–170.

Marriel, I.E.
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Marriel, I.E. and Cruz, J.C. (1978). Increased nitrogen fixation (C_2H_2) in field grown maize by herbicide treatments. *In:* Limitations and Potentials for Biological Nitrogen Fixation in the Tropics (eds. J. Döbereiner, R.H. Burris and A. Hollaender), p. 340. Plenum Press, New York and London.

Martin, J.K. (1977). Factors influencing the loss of organic carbon from wheat roots. *Soil Biol. Biochem.*, **9**, 1–7.

Mishustin, E.N. (1966). Action d'*Azotobacter* sur les végétaux supérieurs. *Ann. Inst. Pasteur*, **111**, 121–135.

Mishustin, E.N. and Shil'Nikova, V.K. (1971). Biological Fixation of Atmospheric Nitrogen. MacMillan, London.

Moore, A.W. (1966). Non-symbiotic nitrogen fixation in soil and plant systems. *Soil Fertilizers*, **29**, 113–128.

Mosse, B. (1975). A microbiologist's view of root anatomy. *In:* Soil Microbiology, (ed. N. Walker), pp. 39–66. Butterworths, London and Boston.

Mota, M., Silva, M.T. and Salema, R. (1975). Electron microscopic study of bacteria in the intercellular spaces of the root cap of *Luzula purpurea*. *J. Submicr. Cytol.*, **7**, 373–378.

Mulder, E.G. and Brotonegoro, S. (1974). Free-living heterotrophic nitrogen fixing bacteria. *In:* The Biology of Nitrogen Fixation (ed. A. Quispel), pp. 37–85. North Holland, Amsterdam.

Neal, J.L. (jr.) and Larson, R.I. (1976). Acetylene reduction by bacteria isolated from the rhizosphere of wheat. *Soil Biol. Biochem.*, **8**, 151–155.

Neyra, C.A. and Dobereiner, J. (1977). Nitrogen fixation in grasses. *In:* Advances in Agronomy (ed. N.C. Brady), **29**, pp. 1–38. Academic Press, New York and London.

Nutman, P.S. (1969). Genetics of symbiosis and nitrogen fixation in legumes. *Proc. Roy. Soc., B.*, **172**, 417–437.

Nutman, P.S. (1975). Symbiotic Nitrogen Fixation in Plants. Cambridge University Press, London.

Obaton, M. (1971). Utilisation de mutants spontanés résistant aux antibiotiques pour l'étude écologique des *Rhizobium*. *C.R. Acad. Sci. Paris*, **272**, 2630–2633.

Ocampo, J.A., Barea, J.M. and Montoya, E. (1975). Interactions between *Azotobacter* and "phosphobacteria" and their establishment in the rhizosphere as affected by soil fertility. *Can. J. Microbiol.*, **21**, 1160–1165.

Okon, Y., Houchins, J. P., Albrecht, S.L. and Burris, R.H. (1977). Growth of *Spirillum lipoferum* at constant partial pressures of oxygen, and the properties of its nitrogenase in cell-free extracts. *J. gen. Microbiol.*, **98**, 87–93.

Old, K.M. and Nicolson, T.H. (1975). Electron microscopy of the microbial colonization of roots of sand dune grasses. *In:* Biodegradation et Humification (ed. G. Kilbertus, O. Reisinger, A. Mourey and J.A. Cancela da Fonseca), pp. 208–215. Pierron, Sarreguemines, France.

Parker, C.A. (1957). Non-symbiotic nitrogen fixing bacteria in soil. III. Total nitrogen changes in a field soil. *J. Soil Sci.*, **8**, 48–59.

Patel, J.J. and Brown, M.E. (1969). Interactions of *Azotobacter* with rhizosphere and root-surface microflora. *Plant and Soil*, **31**, 273–281.

Patriquin, D.G. and Döbereiner, J. (1978a). Bacteria in the endorhizosphere of maize in Brazil. *In:* Limitations and Potentials for Biological Nitrogen Fixation in the Tropics (eds. J. Döbereiner, R.H. Burris and A. Hollaender), p. 349. Plenum Press, New York and London.

Patriquin, D.G. and Döbereiner, J. (1978 b). Light microscopy observations of tetrazolium-reducing bacteria in the endorhizosphere of maize and other grasses in Brazil. *Can. J. Microbiol.*, **24**, 734–742.

Paul, E.A. and Newton, J.D. (1961). Studies of aerobic non-symbiotic nitrogen fixing bacteria. *Can. J. Microbiol.*, **7**, 7–13.

Postgate, J.R. (1971). Relevant aspects of the physiological chemistry of nitrogen fixation. *In:* Symposia of the Society for General Microbiology. Microbes and biological productivity, **21**, 287–307.

Postgate, J.R. (1974). New advances and future potential in biological nitrogen fixation. *J. Appl. Bact.*, 37, 185–202.

Remacle, J. and Rouatt, J.W. (1968). Culture mixte de *Azotobacter chroococcum* et de germes pectinolytiques dans la rhizosphere de l'orge. *Ann. Inst. Pasteur*, **115**, 745–754.

Rennie, R.J. and Schmidt, E.L. (1977). Immunofluorescence studies of *Nitrobacter* populations in soils. *Can. J. Microbiol.*, **23**, 1011–1017.

Riederer-Henderson, M.A. and Wilson, P.W. (1970). Nitrogen fixation by sulphate reducing bacteria. *J. gen. Microbiol.*, **61**, 27–31.

Rinaudo, G. (1970). Fixation biologique de l'azote dans trois types de sols de riziére de Côte d'Ivoire. Thèse Université de Montpellier, France.

Rosswall, T. (1973). Modern methods in the study of microbial ecology. *Bull. Ecol. Res. Comm.*, Stockholm, **17**, 1–508.

Rovira, A.D. (1973). Zones of exudation along plant roots and spatial distribution of microorganisms in the rhizosphere. *Pestic. Sci.*, **4**, 361–366.

Rovira, A.D. and Campell, R. (1975). A scanning electron microscope study of interactions between microorganisms and *Gaeumannomyces graminis* (Syn. *Ophiobolus graminis*) on wheat roots. *Microbial Ecology*, **2**, 177–185.

Rovira, A.D. and Davey, C.B. (1971). Biology of the rhizosphere. *In:* The Plant Root and its Environment (ed. E.W. Carson), pp. 153–204. University Press, Virginia, Charlottesville.

Rubenchik, L.I. (1963). *Azotobacter and its use in agriculture.* Israel Program for Scientific Translations, Jerusalem. Oldbourne Press, London.

Rushel, A.P., Henis, Y. and Salati, E. (1975). Nitrogen-15 tracing of N-Fixation with soil-grown sugar cane seedlings. *Soil Biol. Biochem.*, **7**, 181–182.

Samtsevich, S.A. (1968). Gel-like excretion of plant roots and their influence

upon soil and rhizosphere microflora. *In:* Methods of Productivity Studies in Root Systems and Rhizosphere Organisms (ed. M.S. Ghilarov, V.A. Kovda, L.N. Novichkova-Ivanova, L.E. Rodin and V.M. Sveshnikova), pp. 200–204. Publ. House, Nauka, Leningrad.

Sarro da Silva, M.F. and Döbereiner, J. (1978). Occurrence of *Azospirillum* spp. In soils and roots. *In:* Limitations and Potentials for Biological Nitrogen Fixation in the Tropics. (eds. J. Döbereiner, R.H. Burris and A. Hollaender), pp. 372. Plenum Press, New York and London.

Schmidt, E.L. (1973). Fluorescent antibody techniques for the study of microbial ecology. *In:* Modern Methods in the Study of Microbial Ecology (ed. T. Rosswall). *Bull. Ecol. Res. Comm.*, Stockholm, **17**, 67–76.

Schmidt, E.L. (1974). Quantitative autecological study of microorganisms in soil by immunofluorescence. *Soil Sci.*, **118**, 141–149.

Schmidt, E.L. (1978). Ecology of the legume root nodule bacteria. *In:* Interactions between Non-Pathogenic Soil Microorganisms and Plants (ed. Y.R. Dommergues and S.V. Krupa), pp. 269–303. Elsevier, Amsterdam.

Schwinghamer, E.A. and Dudman, W.F. (1973). Evaluation of streptomycin resistance as a marker for ecological studies with *Rhizobium* spp. *J. appl. Bact.*, **36**, 263–272.

Scott, C.A., Magalhaes, F.M.M., Dos Santos Divar, V.L. and Scott, D.B. (1978). Numbers of *Azospirillum* spp. associated with the roots of field grown maize. *In:* Limitations and Potentials for Biological Nitrogen Fixation in the Tropics (eds. J. Döbereiner, R.H. Burris and A. Hollaender), pp. 371–372. Plenum Press, New York and London.

Shanmugam, K.T. and Valentine, R.C. (1975). Molecular biology of nitrogen fixation. *Science.*, **187**, 919–924.

Silvester, W.B. and Bennett, K.J. (1973). Acetylene reduction by roots and associated soil of New Zealand conifers. *Soil Biol. Biochem.*, **5**, 171–179.

Smith, R.L., Bouton, J.H., Schank, S.C., Quesenberry, K.H., Tyler, M.E., Milan, J.R., Gaskin, M.H. and Littel, R.C. (1976). Nitrogen fixation in grasses inoculated with *Spirillum lipoferum*. *Science*, **193**, 1003–1005.

Smith, R.L., Schank, S.C., Bouton, J.H. and Quesenberry, K.H. (1978). Yield increases of tropical grasses after inoculation with *Spirillum lipoferum*. *Ecol. Bull.* Stockholm, **26**. In press.

Söderström, B.E. (1977). Vital staining of fungi in pure culture and in soil with fluorescein diacetate. *Soil Biol. Biochem.*, **9**, 59–63.

Stewart, W.D.P. (1977). Present-day nitrogen-fixing plants. *Ambio.* **6**, 166–173.

Streicher, S.L., Gurney, E.G. and Valentine, R.C. (1972). The nitrogen fixation genes. *Nature*, London, **239**, 495–499.

Strzelczyk, E. (1961). Studies on the interaction of plant and free-living nitrogen fixing microorganisms. *Can. J. Microbiol.*, **7**, 507–513.

Sulaiman, M. (1971). Non-symbiotic nitrogen fixation in rice plants. *Beitr. Trop. Subtrop. Landwirt, Tropenveterinärmed.* **9**, 139–146.

Trolldenier, G. (1973). The use of fluorescence microscopy for counting soil microorganisms. *In:* Modern Methods in the Study of Microbial Ecology (ed. T. Rosswall), pp. 53–59. *Bull. Ecol. Res. Comm.*, **17**, 53–59.

Trolldenier, G. (1977). Influence of some environmental factors on nitrogen fixation in the rhizosphere of rice. *Plant and Soil*, **47**, 203–217.

Umali-Garcia, M., Hubbell, D.H. and Gaskins, M.H. (1978). Process of infection of *Panicum maximum* by *Spirillum lipoferum*. *Bull. Ecol. Res. Comm.*, Stockholm, **26**. In press.

Unger, H. and Wagner, M. (1965). A new method for quantitative exploitation of immunofluorescence and the possibilities for its utilization in rhizosphere research. *In:* Plant Microbes Relationships (eds. J. Macura and V. Vancura), pp. 311–315. Academy Sciences, Prague, Czechoslovakia.

Vancura, V., Abd-El-Malek, Y. and Zayed, M.N. (1965). *Azotobacter* and *Beijerinckia* in the soils and rhizosphere of plants in Egypt. *Folia Microbiol.*, **10**, 224–229.

Vasil, V., Vasil, I.K., Zuberer, D.A. and Hubbell, D.V. (1978). Forced association of *Spirillum lipoferum* with tissue cultures of some tropical grasses. *In:* Limitations and Potentials for Biological Nitrogen Fixation in the Tropics (eds. J. Döbereiner, R.H. Burris and A. Hollaender), pp. 351–352. Plenum Press, New York and London.

Villemin, G., Balandreau, J. and Dommergues, Y.R. (1975). Utilisation du test de réduction de l'acétyléne pour la numération des bactéries libres fixatrices d'azote. *Ann. Microbiol.*, **24**, 87–94.

Waid, J.S. (1974). Decomposition of roots. *In:* Biology of Plant Litter Decomposition (ed. C.H. Dickinson and G.J.F. Pugh), **1**, pp. 175–211. Academic Press, London.

Waid, J.S., Preston, K.J. and Harris, P.J. (1973). Autoradiographic techniques to detect active microbial cells in natural habitats. *In:* Modern Methods in the Study of Microbial Ecology (ed. T. Rosswall), *Bull. Ecol. Res. Comm.*, Stockholm, **17**, 317–322.

Warembourg, F.R. and Morrall, R.A.A. (1978). Energy flow in the plant microorganisms system. *In:* Interactions between Non-Pathogenic Microorganisms and Plants (eds. Y.R. Dommergues and V.S. Krupa), pp. 205–242. Elsevier, Amsterdam.

Watanabe, I. and Cholitkul, W. (1978). Field studies on nitrogen fixation in paddy soils "Nitrogen and Rice" Symposium, International Rice Research Institute, Los Banos. In press.

Watanabe, I., Lee, K.K. and Alimagno, B.V. (1978). Seasonal change of N_2 fixing rate in rice field assayed by *in situ* acetylene reduction technique. *Soil Sci. Plant Nutr.*, **24**, 1–13.

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Edited by

N.S. SUBBA RAO

Microbiology Division
Indian Agricultural Research Institute
New Delhi, India

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