NITROGEN FIXATION ASSOCIATED WITH MAIZE PLANTS (*Zea mays* L.) IN A TEMPERATE AND A TROPICAL SOIL.

BY

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DECLARATION

I, Francis B. Mwaura, hereby declare that this thesis is my original work and has not been presented for a degree in any other University. All sources of information have been acknowledged by means of references.

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This thesis has been submitted for examination with my approval as University supervisor.

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ABSTRACT

In the temperate soil, low rates of acetylene reduction activity were detected in excised roots of field grown maize without preincubation. A higher acetylene reduction activity (20 nmol C_2H_4/ g d.wt./ 24 h) was recorded under an atmosphere containing 5% oxygen than when the roots were incubated under 0% and 20% oxygen. Most probable number (MPN) counts of N_2-fixing bacteria associated with field grown maize in the temperate soil were estimated at 7.0×10^6 cells/ g d.wt. roots. The acetylene reduction activity in rhizosphere soil was very low (1.7 - 5.8 nmol C_2H_4/ 10 g d.wt. soil/ 24h) but higher than that recorded in soil sampled between the maize plant rows (0.8 - 2.8 nmol C_2H_4/ 10 g d.wt. soil/ 24 h). Soil amendment with glucose stimulated the nitrogenase activity three-hundred fold or more over a 72 h period. The reduction of acetylene to ethylene by intact maize plants grown in the temperate soil was immediate without significant lag periods. Higher rates of nitrogenase activity were observed in intact maize plants incubated under aerobic than under anaerobic conditions.

In the temperate soil appreciable increases in

shoot length, dry matter and N-yields of up to 10%, 27% and 35% respectively were obtained in three-week-old inoculated maize plants under greenhouse conditions. Maize plants inoculated with various bacterial strains had significantly higher mean nitrogenase activities (27 - 239 nmol C_2H_4/ plant/ h) than uninoculated plants (19 nmol C_2H_4/ plant/ h). Experimental results indicated that there was enrichment of rhizosphere diazotrophs on the roots of inoculated maize plants. A time course study of acetylene reduction by intact maize plants showed that the nitrogenase activity dropped under dark conditions but increased rapidly on reillumination of the plants. Cultural studies of a rhizosphere diazotroph and its maize plant reisolate showed a "transient" increase in growth vigour and nitrogenase activity of the reisolate bacterium.

Tropical soil incubated at 28°C had a significantly higher acetylene reduction activity (17.1 nmol C_2H_4/ 10 g d.wt. soil) than soil incubated at 20°C (9.9 nmol C_2H_4/ 10 g d.wt. soil) during a 72 h period. Whereas no acetylene reduction activity was detected in air-dried soil, the activity increased with increase in soil moisture and was highest in water logged soil (12.5 nmol C_2H_4/ 10 g d.wt. soil/ 24 h). Heterotrophic nitrogenase activity in the tropical soil was energy limited. Soil amendment with maize straw stimulated the

activity by nearly two thousand times during a 72 h period. Glucose stimulated the activity to a lesser extent but no stimulation in N_2-ase activity was observed in soil amended with malate. Higher acetylene reduction activities were detected in intact maize plants under aerobic than under anaerobic conditions.

In the tropical soil, inoculation of maize plants with diazotrophic rhizosphere bacteria promoted growth of the plants. Inoculation with the bacteria improved the vegetative growth of maize plants and increased their plant dry matter yields by up to 67.9 %. In addition the dry weight of maize seeds harvested from inoculated plants increased by up to 49.4% over that of uninoculated control plants. Substantial increases in ear dry weight (31.9 %) were also observed in inoculated maize plants compared to the uninoculated plants. However inoculation of maize plants in the tropical soil with the N_2-fixing bacteria failed to significantly increase plant associated acetylene reduction activity as well as the percentage N content of the maize plants. The crude protein content in maize grain from inoculated and uninoculated plants were also quite comparable. Unlike maize plants, wheat and oat plants showed no significant response to inoculation with the N_2-fixing bacteria previously isolated from maize plant roots.

Amendment of the tropical soil with maize straw increased the soil acetylene reduction activity significantly during a six-week study period. Very high rates of nitrogenase activity were detected in intact maize-plant-soil-straw systems (0.6 - 34 µmol C_2H_4/ plant/ 4h). Soil amendment with straw increased the formation of root biomass by 35.7 - 63 % in three-week old maize plants. Maize plants grown in soil amended with maize straw demonstrated improved water status under water stress conditions compared to plants grown in unamended soil.

Several N_2-fixing bacterial strains were isolated from the temperate and the tropical soils. Although various bacterial strains were isolated from maize plant roots using malate medium, the dominant diazotrophic rhizosphere bacteria in both soils were acid-producing glucose-fermenting types. The optimum temperature for N_2-dependent growth of the temperate soil isolates was around 28°C. On the other hand the optimum temperature for growth of the tropical soil isolates in a N-deficient medium was around 37°C. It was however noticed that the optimum pH range for N_2-dependent growth for both the tropical and the temperate soil isolates was between pH 6.0 and 7.5. From the results of various physical and biochemical tests carried out on the isolates *Enterobacter cloacae*,

Pseudomonas sp. and a *Klebsiella* sp. were identified among the temperate soil isolates. *Klebsiella* spp. and other members of the family *Enterobacteriaceae* were identified among the tropical soil isolates.

INTRODUCTION

In Kenya and many other developing countries, the demands placed upon land to produce food for the rapidly expanding human population are considerable and are proving to be difficult to meet. In tropical Africa this has led to impoverishment of cultivated soils and a decline in crop yields (Keya, 1977). One of the factors contributing to the decline in food production is the unavailability of chemical fertilizers to maintain high soil productivity. When fertilizers are available they are often expensive and beyond the reach of the ordinary farmer. Thus the increasing demand for food, coupled with increasing fertilizer costs have emphasized the need to fully exploit biological nitrogen fixation in farming systems (Day, 1977; Albrecht, Okon, Lonnquist and Burris, 1981). In the tropics, the enhancement of the capacity for biological nitrogen fixation is of major significance since N is usually a limiting nutrient for production of food crops (Kang, Nangju and Ayanaba, 1977). Nitrogen fertilizer is also known to represent one of the largest energy inputs involved in cropping (Pohlman and McColl, 1982).

Biofertilizers such as *Rhizobium* inoculants for legumes and *Azolla* and algal inoculants for rice paddies have emerged as suitable alternatives to

chemical nitrogen fertilizers. Studies on the enhancement of biological nitrogen fixation have mainly emphasized the *Rhizobium*-legume symbiotic system (Gibson, Scowcroft and Pagan, 1977; Parker, 1985). However with the discovery that certain free-living nitrogen-fixing bacteria form associative symbiosis with graminaceous plants (Raju, Evans and Seidler, 1972; Dobereiner and Day, 1976) interest has been focused on the possibilities of inducing these bacteria to live in association with roots of cereals and forage grasses. This is important since maize and other cereals constitute an important part of the human diet and require lavish use of nitrogen fertilizers to produce high protein grains (Parker, 1985). Rice has been cited as the staple food for 60% of the world's population (Singh, 1981). In addition to savings in terms of agricultural inputs for the farmer, the limited use of chemical nitrogen fertilizers would also help to curb water pollution due to leaching and runoff from fertilized fields.

Rhizospheric nitrogen fixation has been demonstrated in various cereals and forage grasses in temperate environments (Nelson, Barber, Tjepkema, Russel, Powelson, Evans and Seidler, 1976; Larson and Neal, 1978; Pedersen, Chakrabarty, Klucas and Vidaver, 1978; Albrecht, Okon, Lonnquist and Burris, 1981;

Wright and Weaver, 1982). In Scandinavian countries, few studies on rhizospheric nitrogen fixation have been reported (Idris, Memon and Vinther, 1981; Haahtela, Wartiovaara, Sundman and Skujins, 1981; Lindberg and Granhall, 1984a). In Sweden, the only research carried out has largely been on wheat and has only been published recently (Lindberg and Granhall, 1984a, 1984b and Linberg, Granhall and Tomenius, 1985). In the present study, nitrogen fixation associated with the roots of field-grown maize was investigated during the summer of 1984, in Uppsala, Sweden. Plant response to inoculation with nitrogen-fixing bacteria was studied in young maize plants under green house conditions during the following winter period. Although maize is of little importance as human food in Sweden and many other temperate countries, forage corn is grown in summer and processed into silage which is fed to cattle and other animals over the winter months.

Maize is the staple food for millions of people in East and Central Africa. The crop residues are also commonly used as animal feed and for cooking and heating purposes in rural homesteads where the majority of people live. In Africa, non-symbiotic nitrogen fixation by free-living bacteria and by bacteria associated with the roots of various grasses in natural ecosystems has been reported (Meiklejohn and Weir,

1968; Abd-el-Malek, 1971; Spiff and Odu, 1972; Okafor, 1977; Mwatha, 1981; Mwaura, 1981). However, few studies have been made on the potential for enhancement of rhizosphere nitrogen fixation in agronomically important cereals and forage grasses. Most of the studies on associative nitrogen fixation have been carried out on rice in West Africa (Balandreau, Rinaudo, Hamad-Fares and Dommergues, 1975; Rinaudo, Hamad-Fares and Dommergues, 1977) and more recently on maize (Hegazi, Monib, Amer and Shokr, 1983) and barley (Fayez and Vlassak, 1984) in Egypt. In this study, nitrogen fixation associated with maize grown in a Kenyan soil was investigated. The response of maize to inoculation with N_2-fixing bacteria and the possible use of maize straw to enhance nitrogen fixation in the soil-plant system was also examined. In addition, the response of different maize cultivars to inoculation with nitrogen-fixing bacteria was evaluated.

LITERATURE REVIEW

Survey of free-living nitrogen-fixing bacteria.

The capacity to fix atmospheric elemental nitrogen is confined to prokaryotic micro-organisms. These are bacteria and the cyanobacteria. Among bacteria this property is restricted to a limited number of families

or genera (Mulder, 1975). They include:a)*Azotobacter* (aerobic) ; b) *Klebsiella* and *Bacillus* species (facultatively anaerobic); c) *Clostridium* and the sulphate reducing bacteria in the genera *Desulphovibrio* and *Desulphotomaculum* (anaerobic) and d) some anaerobic photosynthetic bacteria. A number of detailed reviews on the physiology and ecology of free-living nitrogen-fixing bacteria have been published (Mulder and Brotonegoro, 1974; Mulder, 1975; Sprent, 1979; Thompson and Skerman, 1979; Dalton, 1980). More recently, a great deal of interest has been shown in facultatively anaerobic or microaerophilic nitrogen -fixing bacteria such as *Azospirillum* (previously known as *Spirillum*) species (Dobereiner and Day, 1976; Klingmuller, 1982 ; Krieg and Dobereiner, 1984).

Diazotrophic rhizocoenoses.

Nitrogen fixation by heterotrophic bacteria in the rhizosphere of grasses has been referred to as associative symbiosis (Burns and Hardy, 1975) and in more recent times as diazotrophic rhizocoenoses (Dobereiner and De-polli, 1980). Optimization of N_2 fixation in grass-bacteria associations for economic gains has been described as one of the remaining major challenges in agriculture and bacteriology (Dobereiner and De-polli, 1980). Many graminaceous plants have

been shown to support measurable levels of nitrogen fixation activity in their rhizospheres. Among these plants are economically important crops such as maize (von Bulow and Dobereiner, 1975; Tjepkema and van Berkum, 1977); wheat (Klucas and Pedersen ,1980); barley (Pohlman and McColl, 1982); sugarcane (Ruschel, 1981); rice (Watanabe, Osamu and Barraquio, 1981) and forage grasses (Thompson, Gemell, Roughley, Evans and Nicolls , 1984). In these and other grass crops, the absence of structures comparable to root nodules observed in legumes has been cited as a major drawback in the plant-bacteria association (Patriquin, Dobereiner and Jain, 1983). Such stuctures would protect the nitrogen-fixing bacteria against oxygen and other environmental effects which are thought to be responsible for the large site to site variations in associative symbioses (Dobereiner and De-polli, 1980).

Nitrogen fixation in C-4 and C-3 grasses.

Many tropical grasses which have been shown to support significant nitrogenase activity have the C-4 type of photosynthetic pathway (Dobereiner and Day, 1975; Day, Harris, Dart and van Berkum, 1975). Unlike C-3 types, such plants are able to attain greater photosynthetic rates and minimal loses of carbon due to photorespiration; which may result in greater

availability of photosynthate for plant growth and nitrogen fixation by bacteria associated with the plant roots (Neyra, 1978). The *Paspalum notatum-Azotobacter paspali* association was the first tropical C-4 grass-bacteria association to be studied in detail (Neyra and Dobereiner, 1977). Dobereiner, Day and Dart (1972) suggested that in the *P. notatum-A. paspali* association, the bacteria were located in the mucigel layer on the root surface since only a small loss of nitrogenase activity was observed even after vigorous washing of the roots under a water tap.

Sugarcane, a C-4 grass, has been grown in monoculture for many years in various parts of the world without the need for nitrogen fertilizers (Neyra and Dobereiner, 1977). Dobereiner (1961) demonstrated selective stimulation of the nitrogen-fixing bacteria ,*Beijerinckia* by roots of sugarcane plants. In this crop, most of the nitrogen is reportedly fixed in the rhizosphere soil and only a minor part of it is fixed in or on the roots themselves (Ruschel, 1981). Maize and sorghum are important grain crops which also have the C-4 type of photosynthetic pathway. These cereals and C-4 forage grasses such as *Digitaria decumbens*, *Panicum maximum* and *Andropogon* sp. are known to fix nitrogen in association with *Azospirillum* spp. and other soil bacteria (Dobereiner and Day, 1976; Barber

and Evans, 1976; Jagnow, 1986). The nitrogen content of a soil (0-20 cm depth) planted to three cultivars of *D. decumbens* in Brazil was reported to increase by 216 - 468 g N /ha /day (Neyra and Dobereiner, 1977).

Rice and wheat are among C-3 grasses which have been reported to fix atmospheric dinitrogen. Rinaudo, Balandreau and Dommergues (1971) demonstrated bacterial nitrogen fixation in intact rice cultures grown in test tubes. This was later confirmed by Yoshida and Ancajas (1973) using excised roots of field-grown rice. Acetylene reduction activities ranging from 2 - 6 μ mol C_2H_4 /h /g d. wt. roots were detected in rice seedlings grown under controlled conditions (Dommergues, Balandreau, Rinaudo and Weinhard, 1973). Nitrogen fixation by bacteria associated with roots of wheat plants has also been demonstrated (Day, Harris, Dart and van Berkum, 1975; Lethbridge, Davidson and Sparling, 1982; Lindberg and Granhall, 1984a).

Colonization of the rhizosphere and rhizoplane.

It has generally been observed that there is enrichment of diazotrophs in rhizosphere soil compared with nonrhizosphere soil (Balandreau, Ducerf, Hamad -Fares, Weinhard, Rinaudo, Millier and Dommergues, 1978). These workers calculated R/S values (ratio of

diazotroph numbers in rhizosphere soil to the number in nonrhizosphere soil) from various sources and found that they were mostly in the range of 1 - 20. Watanabe and Barraquio (1979) reported that as high as 81% of the bacteria isolated from the inner rhizosphere of rice were nitrogen fixers. Many of these were later found to be *Achromobacter*-like microaerophiles (Barraquio, de Guzman, Barrion and Watanabe, 1982). More recently, Reihold, Hurek, Nieman and Fendric (1986) have reported diazotrophic population densities of up to 85% of the total aerobic heterotrophs in the endorhizosphere of Kallar grass, *Leptochloa fusca*. However, lower percentages of N_2-fixing microaerophiles (1 - 10%) have been observed on *Spartina* roots (Boyle and Patriquin, 1980).

Various factors have been attributed to the enrichment of diazotrophs in plant rhizospheres. These are : 1) competitive advantage in a carbon-rich, N-deficient environment which is typical of rhizospheres (Patriquin, Dobereiner and Jain, 1983); 2) provision by the plant of essential vitamins such as biotin (Martin and Glatzle, 1982) or 3) aerotactic attraction of microaerophilic bacteria to reduced oxygen tension in the root zone (Okon, Cakmacki, Nur and Chet, 1980). Colonization of roots by bacteria is normally very patchy except when the plants are grown in nutrient

solutions or in semisolid media (Rovira, 1981). This observation may explain the high variability of acetylene reduction activities of individual root pieces in *Digitaria decumbens* (Dobereiner and Day, 1976). It has also been shown that diazotrophs make up a higher proportion of the total bacteria numbers associated with plant roots in wetland when compared with plants in dryland environments (Barraquio and Watanabe, 1981). This may be due to a more favourable Po_2 for nitrogen fixation near roots in wet soils than in dry soils (Patriquin *et al.*, 1983).

Legume plants such as *Vicia* contain proteins which bind selectively to rhizobia and can promote adherence of these bacteria to the legume roots (Dazzo, Napoli and Hubbell, 1976). A study carried out by Umali-Garcia, Hubbell, Gaskins and Dazzo (1980) demonstrated that pearl millet roots released protease-sensitive nondialyzable substances which bound to azospirilla and promoted their selective adherence to root hairs. These results were in agreement with the lectin-recognition model proposed by Solheim (1975) to explain the mechanism of bacterial adherence in the *Rhizobium*-legume symbiosis (Umali-Garcia *et al.*, 1980). According to the model, a glycoprotein lectin excreted from the plant roots binds to the bacteria and promotes their attachment to the

root surface. In adsorption studies, grass roots were found to display selectivity in the binding of bacteria to discreet regions of the root (Umali-Garcia *et al.*, 1980). Azospirilla were firmly attached to root hairs of grasses grown without combined nitrogen, while no bacteria attached to root hairs of plants supplied with fixed nitrogen. The presence of NO_3^- and NH_4^+ ions in the rooting medium has similarly been found to reduce the selective adherence of *Rhizobium trifolii* to clover root hairs (Umali-Garcia *et al.*, 1980). This corresponded with an observed reduction in the level of a clover lectin, trifoliin, on the root hair surface (Dazzo and Brill, 1978).

Colonization of the root interior.

The presence of bacteria in intercellular spaces between the epidermis and the cortex and in the outer cortical layers has previously been reported in axenically grown grasses inoculated with *Azospirillum* (Umali-Garcia *et al.*, 1978; Patriquin and Dobereiner, 1978). In their studies on wheat plants inoculated with N_2-fixing *Bacillus* cultures under sterile conditions, Larson and Neal (1978) found that the bacteria invaded the intercellular spaces between the cortical cells and that such spaces were much larger than the intercellular spaces of uninoculated wheat

plants. Considerable nitrogenase activity was detected in the roots of the inoculated plants even after surface sterilization (Larson and Neal, 1978).

In a study carried out by Patriquin and Dobereiner (1978) to determine the location of *Azospirillum lipoferum* in roots of maize grown under tropical conditions, the bacteria were demonstrated to be at various depths in the cortex from just below the epidermis to adjacent to the endodermis. Stele and inner cortex infections were reportedly dense in sections of maize roots which included lateral branches. From their observations, these workers suggested that infections of inner cortex and stele tissues occurred initially in branches, possibly near their sites of emergence from main roots and then spread longitudinally into the main roots.

In a recent study (Lindberg *et al.*, 1985) the invasion of roots of axenically grown wheat seedlings by various strains of diazotrophic rhizosphere bacteria was investigated. Observations made under an electron microscope showed that two unidentified but taxonomically related bacterial strains did not penetrate the wheat plant roots. A strain of *Azospirillum brasilense* was found to occur mainly in the root mucigel outside epidermal cells and sporadically between and within the outermost

epidermal root cells. The authors further reported the presence of a nitrogen-fixing *Bacillus* strain both intercellularly and intracellularly in the epidermis. Plant cells containing bacterial endospores at various stages of maturity were clearly identified. A strain of *Enterobacter agglomerans* was detected between and within epidermal cells, in cortex cells close to the endodermis and even within the stele. Kleeberger, Castorph and Klingmuller (1983) had earlier reported that *E. agglomerans* was a dominant bacterial species in surface-sterilised roots of wheat. These findings revealed that there was a large variation in the degree of root penetration by the different diazotrophs (Lindberg *et al.*, 1985).

The presence of pectin lyase and endopolygalacturonase enzyme activities has been demonstrated in cultures of *Azospirillum brasilense* (Umali-Garcia *et al.*, 1978). As a result, these workers suggested that these pectin-modifying enzymes may contribute to the limited invasive properties of azospirilla on grass roots. *Azospirillum* spp. may also enter the plant roots through lysed root hairs and void spaces of epidermis created by epithelia desquamation and lateral root emergence (Umali-Garcia *et al.*, 1978). A suggestion has also been made that mechanical injury of the root, as a result of contact

with the solid support medium, might also provide other portals of entry for microorganisms under natural conditions (Umali-Garcia *et al.*, 1980). However, Patriquin *et al.* (1983) have argued that invasion of cortical tissues apparently remains intercellular as long as host cells are healthy. Infection of stems of field-grown maize by *Azospirillum* sp. has also been reported (Magalhaes, Patriquin and Dobereiner, 1979). It has been suggested that the low Po_2 and the presence of malate in the xylem (Burtz and Long, 1979) may provide conditions very conducive to nitrogen fixation in plant roots (Patriquin *et al.*, 1983). These workers have suggested that owing to the low pH (about 5.3) of the xylem fluid, only acid-tolerant microaerophilic bacteria would be able to colonize this environment. More recently Boddey and Dobereiner (1984) have shown that *Azospirillum* isolates from surface-sterilised maize and wheat roots can grow at pH 5.5 or even lower.

Interactions between nitrogen-fixing bacteria and other soil microorganisms.

Growth of *Azotobacter* in plant rhizospheres is slow and depressed partly due to microbial antagonism by actinomycetes and other soil bacteria (Strzelczyck ,1961). In their study on the possible role of

actinomycetes in inhibiting growth of introduced *Azotobacter*, Patel and Brown (1969) reported that with increase in plant age, the number of antagonists in the rhizosphere grew while the population of *Azotobacter* declined.

Mutualistic and other analogous relationships between nitrogen-fixing bacteria and other microorganisms have been desribed where the associative microorganism breaks down an otherwise unavailable substrate thereby making it available to the nitrogen fixer (Jensen and Holm, 1975). Lind and Wilson (1942) showed that a *Bacillus* sp. could improve the growth of *Azotobacter* in the soil by decomposing iron humates so that iron was made available. Similarly, other essential mineral elements such as molybdenum which occur in suboptimal concentrations may be released by the decomposition of organic substances (Jensen and Holm, 1975). Production of organic growth factors by associative organisms which result in stimulation of nitrogen fixation has also been suggested (Hervey and Greaves, 1941; Jensen and Holm, 1975; Lindberg and Granhall, 1984b). Lindberg and Granhall (1984b) found that two rhizospheric bacterial isolates reduced acetylene vigorously when grown together but failed to do so as separate cultures. They suggested that one of the isolates was a non nitrogen-fixing bacterium which

produced certain growth factors that stimulated growth and nitrogen fixation by the other bacterium.

It has also been suggested that associative microorganisms may improve the growth of a nitrogen-fixing bacterium by removing toxic substances from the growth medium (Line and Loutit, 1973). These workers demonstrated that a mixed culture of a nitrogen-fixing *Clostridium* sp. and an aerobic organism, *Pseudomonas azotogensis*, was capable of nitrogen fixation in an aerobic environment. *Clostridium* when grown alone failed to fix nitrogen in the presence of oxygen. It was therefore suggested that in the mixed culture, the aerobe detoxified the medium by using up the oxygen thus making it possible for *Clostridium* to grow and fix nitrogen. Diem and Dommergues (1980) have therefore proposed that it may be more suitable to use synergistic associations of microorganisms rather than pure cultures for inoculating plant rhizospheres.

Exchanges of energy and metabolites between bacteria and host plants.

Reciprocal exchanges of energy and metabolites between the host plant and a diazotroph is an important feature of diazotrophic rhizocoenoses (Dommergues, 1978). Nitrogen-fixing bacteria in plant rhizospheres are heterotrophs and depend upon the

plant for their supply of energy and carbon. In the plant rhizosphere, energy-yielding compounds come from root exudates, root lysates and root litter. Root exudates have been described as materials which are released from healthy and intact roots (Diem and Dommergues, 1980). On the other hand, Martin (1977) has defined root lysates as compounds which are released from the autolysis of root hairs and sloughed off root cap cells as well as epidermal and cortical cells of still functional roots. In young wheat plants, Martin (1977) found that a major loss of root carbon (14 - 47%) was due to autolysis of the root cortex. The third source of energy and carbon, root litter, includes moribund or dead and decaying roots in the soil as the roots and root-lets of the plant are renewed. Root litter may provide rhizosphere microorganisms with a large extra supply of energy-yielding compounds in more mature plants (Diem and Dommergues, 1980).

Free-living nitrogen-fixing bacteria are known to be relatively inefficient in terms of nitrogen fixed for every unit weight of carbon compound consumed. The amounts of nitrogen fixed for every gram of carbohydrate consumed ranges from 10 - 20 mg for *Azotobacter*; about 5 mg for *Klebsiella* and 5 - 10 mg for *Clostridium* species (Daesch and Mortenson, 1972; Mulder, 1975). These figures are very low compared with those obtained

in the *Rhizobium*-legume symbiotic systems where figures in excess of 270 mg N_2 fixed for every gram of carbon consumed have been recorded (Minchin and Pate, 1973).

Fate of fixed nitrogen.

The fate of nitrogen fixed in associative symbiosis is a matter which has largely remained unresolved. It has been demonstrated by $^{15}N_2$ studies that the nitrogen fixed in the rhizospheres of sugarcane (Ruschel, Henis and Salati, 1975) and flooded rice (Watanabe, Osamu and Barraquio, 1981) was at least partially absorbed by the plants during their growth cycle. However the quantity of $^{15}N_2$ which is incorporated into plant parts is very small which suggests that there is no direct bacteria to plant transport of fixed nitrogen (Okon, 1984). The proportion of fixed nitrogen which is excreted by bacteria and absorbed directly by the roots and the length of time that fixed nitrogen is immobilised in the diazotroph are basic questions which have yet to be answered. It has however been argued that if the ammonification rate of the proteins synthesized by the nitrogen-fixing bacteria is high, the host plant can itself benefit from the ammonium-nitrogen which is subsequently released (Diem and Dommergues, 1980). On the other hand if the rate of ammonification is low, nitrogen fixed in

the cells of diazotrophs is incorporated into the soil nitrogen pool and can only be available to plants after mineralization (Diem and Dommergues, 1980; Lethbridge and Davidson, 1983; Okon, 1984).

Factors affecting nitrogen fixation in grasses.

Diurnal and seasonal variations

Diurnal changes in nitrogenase activity have been observed in several grasses. *Paspalum notatum*, sorghum and other graminaceous plants are known to have one peak of nitrogenase activity around midday and a lower second peak of activity at night (Dobereiner and Day, 1975). However rice and other graminaceous plants have only one peak of nitrogenase activity during daytime (Balandreau *et al.*, 1975). In general most of the nitrogenase activity associated with grasses occurs during the light period and reflects the dependence of nitrogenase activity upon available photosynthate as is the case in symbiotic systems (Neyra and Dobereiner, 1977). A suggestion has been made that enhanced production of photosynthate could increase root exudation and the exudate in turn would support increased bacterial growth and higher nitrogenase activity in the plant rhizosphere (Dobereiner, 1974). Light may also act upon nitrogen fixation in the

rhizosphere by affecting stomatal opening, hence the diffusion of gases especially nitrogen, through the plant (Balandreau *et al.*, 1975).

Nitrogenase activity has been observed to change during the growth cycle of plants. Highest activities have generally been recorded during reproductive growth (Neyra and Dobereiner, 1977). In field-grown maize maximal nitrogenase activities have been found to occur during silk emergence and at the onset of grain filling (von Bulow and Dobereiner, 1975). Conversely little nitrogenase activity is observed before tasseling of the plants and after grain filling (Neyra and Dobereiner, 1977). Maximal activities in field grown sorghum have been reported to occur during flowering and the activity declines with the beginning of grain filling (van Berkum and Neyra, 1976). Competition for available photosynthates by the grain has been cited as the possible cause for the observed decline of nitrogenase activity during the grain filling period (Neyra and Dobereiner, 1977). In legumes nitrogenase activity has similarly been reported to decline with ontogeny of the plants (Harper and Hageman, 1972).

Temperature.

Nitrogenase activities in intact maize plants have been shown to have a positive correlation with

temperature changes (Albrecht *et al.*, 1977). In rice, insignificant nitrogenase activity was recorded at 10 - 15°C but maximal activity was observed at 35°C (van Berkum and Sloger, 1982). Results obtained from *in situ* assays of nitrogenase activity in maize plants also showed an increase in activity with rise in temperature from 18 - 35°C (Balandreau *et al.*, 1978). Nitrogenase activity in roots of *Digitaria decumbens* and in *Azospirillum lipoferum* cultures isolated from *Digitaria* roots is reportedly inhibited by temperatures below 25°C (Neyra and Dobereiner, 1977). Due to the relatively high temperature requirement for this bacterium it has been suggested that tropical areas would be more favourable for its development (Dobereiner, Marriel and Nery, 1976).

Moisture.

Studies on nitrogenase activity of soil cores from various grasslands have shown highly significant correlations with soil moisture (Vlassak, Paul and Harris, 1973; Day, Harris, Dart and van Berkum, 1975; Tjepkema and Burris, 1976). This has also been demonstrated in maize plant-soil cores and similar cores of other cereal plants in Brazil and elsewhere (Tjepkema and van Berkum, 1977; Balandreau *et al.*, 1978; Boddey and Dobereiner, 1984). It has been

suggested that at high moisture levels, roots have more microsites in which diazotrophs can be active (Boddey and Dobereiner ,1984). In addition high moisture may increase the availability of nutrients for the bacteria (Lethbridge *et al.*, 1982).

Oxygen.

Studies on the *Paspalum notatum-Azotobacter paspali* association revealed that nitrogenase activity is extremely sensitive to changes in oxygen tension (Dobereiner *et al.*, 1972). These workers found that nitrogenase activity was almost completely inhibited in air or in the absence of oxygen but was highest at Po_2 0.004 atm. Various aerobic, facultative and anaerobic bacteria are able to fix nitrogen in the soil or in association with the roots of grasses. Aerobic N_2-fixing organisms have received most attention largely because aerobic metabolism is more efficient and most agricultural soils are well aerated (Neyra and Dobereiner, 1977). Among the most studied aerobic N_2-fixing bacteria is *A. lipoferum*. However this organism cannot grow in air using N_2 as the nitrogen source due to its sensitivity to high oxygen tension (Day and Dobereiner, 1976; Volpon, De-Polli and Dobereiner, 1981). Okon, Albrecht and Burris (1977) found that nitrogen fixation and growth of *A. lipoferum*

was most rapid at a Po_2 of 0.005 - 0.007 atm. It has been suggested that this preference for low Po_2 may explain the oxygen sensitivity of nitrogenase activity, on the roots of various tropical grasses, in association with this bacterium (Neyra and Dobereiner, 1977).

Inorganic nitrogen.

A high level of combined nitrogen in soil or the application of high doses of nitrogen fertilizer reduces the potential for associative N_2 fixation in grasses (Balandreau and Dommergues, 1973; Neyra and Dobereiner, 1977). The addition of 10 µg /ml of urea to rice-water-culture systems has been shown to greatly reduce the nitrogenase activity of excised roots (Trolldenier, 1977) and intact plants (Watanabe and Cabrera, 1979). A similar inhibition of nitrogenase activity has been demonstrated in excised sorghum roots after addition of ammonium or nitrate N (Neyra and van Berkum, 1976). In another study, Cohen, Okon, Kigel, Nur and Henis (1980) observed acetylene reduction activity in maize plants supplied with 0 - 0.08 g of NH_4NO_3 per plant but not with plants supplied with 0.16g of the ammonium salt. Due to similar results in other studies, it has been suggested that a reasonable starter dose of nitrogen fertilizer may not reduce the

nitrogen fixed in association with grasses (Subba Rao, 1980; Okon, 1984; Boddey and Dobereiner, 1984).

Plant genotype.

Acetylene reduction assays of excised roots have revealed a wide range of activities in different maize genotypes (Neyra and Dobereiner, 1977). Von Bulow and Dobereiner (1975) reported that mean nitrogenase activities of some maize lines were 10 - 20 times higher than the original cultivars. Larson and Neal (1978) showed that there was selective colonization of a genetically defined line of wheat among various genotypes by a facultative nitrogen-fixing *Bacillus*. More recently it has been demonstrated that certain lines of spring wheat respond better than other lines to inoculation with nitrogen-fixing bacteria (Rennie, 1981). Ela, Anderson and Brill (1982) have also shown that the ability in maize to support bacterial nitrogen fixation in or on its roots can be increased through screening and selection. These and other findings have increasingly shown the importance of plant genotypes in plant-bacteria associations with high potential for nitrogen fixation.

The measurement of nitrogen fixation.

The acetylene reduction technique

The acetylene reduction method has been used extensively in the last fifteen or so years as an indicator of nitrogen fixation in biological systems. This popularity has largely been due to the high sensitivity and simplicity of the method. The technique was developed after the discovery by Michael Dilworth in 1966 that acetylene was reduced to ethylene by the nitrogen-fixing enzyme complex (Burris, 1975). The ethylene produced could then be measured in a gas chromatograph.

The acetylene reduction technique as a tool for the measurement of biological nitrogen fixation has been reviewed by several workers (Burris, 1972; Hardy, Burns and Holsten, 1973). A common criticism of the method is that it is an indirect measurement of nitrogen fixation since it assumes that the quantity of acetylene reduced is directly related to the amount of nitrogen that could have been reduced. However difficulties have been encountered in attempts to quantify the nitrogen fixed from acetylene reduction assays. The ratio of C_2H_2 : N_2 has been shown to vary from the theoretical ratio of 3 : 1 in a number of living systems (Bergersen, 1970; Burris, 1974). Additional difficulties arise during the

extrapolation of results obtained under controlled laboratory conditions to the field situation due to environmental variations (Dommergues *et al.*, 1973).

Various merits and demerits of the acetylene reduction method in the study of nitrogen fixation in grass systems have been cited (Patriquin and Denike, 1978; van Berkum and Bohlool, 1980). It has been suggested that the technique may be least prone to error when used on undisturbed monoxenic plants inoculated with nitrogen-fixing bacteria provided short assay periods are used and ethylene production is linear (Lethbridge *et al.*, 1982). In the study of grass-bacteria associations the acetylene reduction method may be used with excised roots or with intact plants (Burris, 1974).

The excised root assay.

The excised root assay has been used to localize nitrogen fixation in the roots of grasses (Dobereiner *et al.*, 1972; Dobereiner and Day, 1975). The technique has also been employed to estimate the rates of nitrogen fixation in maize genotypes (von Bullow and Dobereiner, 1975); Halondule wrightii and other grasses (Smith and Hayasaka, 1982). This method of assay has however attracted a lot of criticism. Various reports indicate that a lag period of 8 - 18 h is

normally observed before the excised root samples start to reduce acetylene (Dobereiner *et al.*, 1972; Zuberer and Siver 1978; van Berkum, 1980; Mwaura, 1981). To overcome this period of inactivity, the roots are preincubated overnight at low Po_2 without acetylene addition (Dobereiner and Day, 1976). Due to this requirement, the validity of estimating nitrogen fixation in grasses using excised roots has been questioned (Barber, Tjepkema, Russel and Evans, 1976; Tjepkema and van Berkum, 1977; van Berkum, 1980). Proliferation of nitrogen-fixing bacteria is known to occur during the preincubation period (Barber *et al.* , 1976 ; Okon *et al.*, 1977) and may account for the very high rates of nitrogenase activity in preincubated excised roots compared to the rates of activity observed in corresponding soil cores (Tjepkema and van Berkum, 1977). Van Berkum (1980) observed increases in the nitrogen-fixing microflora ranging from 15 to 1800 fold during the preincubation period of excised roots of five tropical grasses in Brazil. The excised root assay involving prolonged incubations may therefore overestimate N_2 fixation associated with grasses in the field (Barber *et al.*, 1976; Okon *et al.*, 1977; Tjepkema and van Berkum, 1977).

However other studies have shown that immediate acetylene reduction by excised roots of maize (Sloger,

1976) and *Spartina* sp. (van Berkum and Sloger, 1979) can occur without preincubation of the roots. Van Berkum (1980) has suggested that the excised root assay should be considered valid for measuring nitrogen fixation only when immediate reduction of acetylene is observed without prior incubation under low Po_2. Despite the difficulties, the technique is however useful in the screening of nitrogenase activity in large numbers of root samples from the field (Neyra and Dobereiner, 1977).

Non-destructive acetylene reduction assay.

This method of assay involves enclosing a whole plant or its intact root system alone under acetylene. Since the plants are intact, the observed acetylene reduction activities are taken to represent their *in situ* nitrogenase activities. One version of this method involves the placing of a large perspex cylinder over the plants and driving it several centimeters into the soil to enclose the soil-plant root system (Patriquin and Denike, 1978; O'Hara, Davey and Lucas, 1981). The top end of the cylinder and the barrel are fitted with rubber closures through which gasses can be added or withdrawn. In potted plant assays the whole plant may be enclosed in a gas-tight plastic bag which is then sealed by heat (Lindberg and Granhall, 1984a). Tips of

disposable syringes fitted in the wall of the plastic bag are stoppered with suba-seals through which gasses can be injected into or removed from the bag.

Other acetylene reduction studies using potted plants have involved placing the pot in a rigid container and forming a gas-tight junction between the plant stem and the lid which carries rubber septa used as the gassing ports (Nur, Okon and Henis, 1980; Lethbridge *et al.*, 1982). This system allows normal photosynthesis of intact plants which are being assayed for acetylene reduction activity associated with their roots. It is however difficult to obtain a completely leak-proof system when assaying potted plants. Small amounts of propane have been added to such systems for exact volumetric determinations and to correct for gas leakage (Balandreau and Dommergues, 1973; Lindberg and Granhall, 1984a).

Nitrogenase activity associated with intact plants has also been studied in plant-soil cores which are similar to potted plants (Tjepkema and van Berkum , 1977; van Berkum and Day 1980; Weaver, Wright, Varanaka, Smith and Holt, 1980; Weir, 1980). Other studies of associative N_2 fixation in intact plants have involved the culturing of such plants in test tubes over limited periods (Balandreau *et al.*, 1975; Lindberg *et al.*, 1985). A common problem with the

intact plant assay is the slow diffusion of gases which is partly dependent on the soil type and moisture content of the soil (van Berkum and Day, 1980; Lethbridge *et al.*, 1982). The assay may therefore mean prolonged incubations under acetylene which are undesirable because of possible adverse effects on plant metabolism and microbial processes other than nitrogen fixation (Patriquin and Denike, 1978). Some workers have used sand-soil mixtures in potted-plant nitrogenase activity studies in a bid to improve the diffusion of gases in the system (Lindberg and Granhall, 1984a).

N difference method.

The classical difference method of estimating N_2 fixation is based on total Kjeldahl N determinations. Due to the poor sensitivity of this method, it is often necessary that the plants are grown for prolonged periods so that the differences in total N are reasonably large. Difficulties may however be encountered if only a small absolute increase in N occurs during the exposure period since the increase may be obscured by sampling errors and by errors inherent in the Kjeldahl method (Burris, 1974). The errors may therefore invalidate conclusions based upon total N analysis which are interpreted in terms of

absolute nitrogen gains (Burris, 1974).

^{15}N Isotope techniques.

Measurement of $^{15}N_2$ fixation has been described as the only completely unequivocal means of demonstrating nitrogen fixation (Patriquin, 1982). The application of the method has however been limited by the high cost of the necessary equipment and isotopes. It is only recently that ^{15}N has been extensively used to quantify nitrogen fixation both in the green house and in the field (Rennie, 1981). Two techniques which are commonly applied in nitrogen fixation studies are: 1) $^{15}N_2$ reduction and 2) ^{15}N-isotope dilution. The main use of $^{15}N_2$ reduction is to prove that nitrogen fixation is taking place. This is achieved by incubating an intact plant in an atmosphere enriched with $^{15}N_2$ and finding ^{15}N-labelled ammonia or other derivatives in the plant. This method is comparable to the nondestructive acetylene reduction test since an incubation chamber is used and therefore only short term assays are suitable (Rennie, 1981).

In the ^{15}N-isotope dilution method, the soil or growth medium is labelled with ^{15}N. The principle is that if a plant is growing in a medium with a higher ^{15}N / ^{14}N ratio than the atmosphere then the isotopic composition of plant nitrogen will reflect the

proportions of nitrogen derived from the medium and from the atmosphere (Patriquin, 1982). The technique provides an integrated estimate of N_2 fixed directly in the field (Danso, 1986). The method however requires reference to control plants which do not fix nitrogen and also assumes uniform labelling of the soil or growth medium with the ^{15}N fertilizer. After determination of the ^{15}N : ^{14}N ratios on the Kjeldahl distillate of the plants, the percentage of fixed nitrogen is then calculated using the equation below (Rennie, 1981).

$$\%N \text{ fixed} = 1 - \frac{\%^{15}N \text{ atom excess (fs)}}{\%^{15}N \text{ atom excess (nfs)}} \times 100$$

where : fs = N_2 fixing system and
nfs = nonfixing system.

Improvement of associative N_2 fixation in grasses.

Although nitrogen fixation is known to occur commonly in the rhizospheres of many graminaceous plants, the amounts of nitrogen fixed are generally low and have not been unequivocally shown to exceed 5-10 Kg N fixed/ ha/ year (Diem and Dommergues, 1980). This view is supported by many other published reports which also indicate nitrogen fixation rates of the same order (Day, Harris, Dart and van Berkum, 1975; Tjepkema and Burris, 1976; Pedersen, Chakrabarty, Klucas and Vidaver, 1978; Lethbridge *et al.*, 1982). Two major

avenues have been suggested for the improvement of nitrogen fixation in grass-bacteria associations (Okon, 1984). These are : 1) the manipulation of rhizospheric microflora in plants and 2) plant breeding and selection.

Manipulation of bacteria in plant rhizospheres.

Direct manipulation of rhizospheric microflora has largely been attempted by inoculating young seedlings or seed material before or immediately after planting, with high doses of N_2-fixing bacteria. The inoculum is commonly applied in the form of a bacterial suspension (Smith, Schank, Bouton and Quesenberry, 1978; Bouton and Zuberer 1979; Gaskins, Hubbell and Albrecht, 1981; Reynders and Vlassak, 1982) or in a peat-based form (Kapulnik, Sarig, Nur, Okon, Kigel and Henis, 1981b; Okon, 1984). The assumption is that the large numbers of the introduced bacteria should give the inoculant strain a competitive advantage over the indigenous soil microflora in colonization of the roots. The introduced diazotroph would then become dominant in the rhizosphere and subsequently more N_2 would be fixed in association with the host plant. In this study attempts were made to improve nitrogen fixation associated with maize and other grasses using this method.

MATERIALS AND METHODS

2.1 Experimental materials and common methods.

2.1.1 Soil, seed materials and composition of the isolation medium.

a) Soil: The temperate soil used in this study was collected from a maize field at Saby in Uppsala, Sweden. The tropical soil was from a weeded plot at Chiromo, in the Nairobi area.

b) Seed materials: Seeds of maize cultivar LG 11 were obtained from the Department of Plant Husbandry, Swedish University of Agricultural Sciences, Uppsala, Sweden. Seeds of maize cultivars Katumani and Makueni were obtained from Katumani Dryland Research Station at Machakos. Wheat and oat seeds used in this study were supplied by a farmer in the Nakuru area; the cultivars were unknown.

c) Composition of the isolation NFb-medium (Dobereiner *et al.*, 1976):

4g KOH; 0.5g K_2HPO_4; 0.2g $MgSO_4.7H_2O$; 0.1g NaCl; 0.02g $CaCl_2$; 0.5g $FeSO_4.7H_2O$; 0.002g $NaMoO_4.2H_2O$; 0.01g $MnSO_4.H_2O$; 0.1g yeast extract; 2 ml. alcoholic solution bromothymol blue (0.5 % w:v). The volume was made up to 1 l with distilled water and the pH was adjusted to 6.8. Except where otherwise stated, 1.5g agar (Difco Bacto) was added to make 1 l semisolid medium.

In the NFb-glucose medium, KOH and K_2HPO_4 were omitted and water was replaced with 0.05M phosphate buffer pH 7.0 (Lindberg and Granhall, 1984b). Glucose (10 g) was autoclaved separately in a small volume of distilled water and then added to the autoclaved medium to make up 1000ml of the NFb-glucose medium. For the NFb-malate medium, 0.5g malate was added to every 100 ml of the basal medium prior to autoclaving.

Sterile 0.05M phosphate buffer pH 7.0 was used to wash bacterial cells or make bacterial suspensions for use as inoculants.

2.1.2 Seed preparation.

Seeds were washed under running water for 2 h to remove the fungicide. The washed seeds were then placed in petri dishes, on a bed of filter paper moistened with distilled water, and germinated in the dark at room temperature. When surface sterilisation of seeds was required, the seeds were washed free of fungicide, rinsed in 95 % ethanol and immersed in 0.2 % acidified mercuric chloride for three minutes (Vincent, 1970). The seeds were then washed in several changes of sterile distilled water and aseptically germinated on water-agar plates in the dark at room temperature.

2.1.3 Inoculant preparation.

The inoculant bacterial strains were grown in a liquid N-deficient glucose medium (0.05 % agar w:v) in 150 ml. Erhlenmeyer flasks. After incubation for four to five days at 28°C without shaking, the cultures were centrifuged (Wifug Centrifuges, Bradford, England) and the pellets washed twice with sterile 0.05 M phosphate buffer pH 7.0 (Berg, Tyler, Novick and Vasil, 1980). The cells were finally resuspended in sterile phosphate buffer using a mixer (Vortex Genie, New York, U.S.A.). Where necessary, optical density measurements of the bacterial suspensions were read at 560 nm using a spectrophotometer (Shimadzu UV-120-01, Japan).

2.1.4 Greenhouse experiments.

The seeds were sown in large glass tubes (25 - 30mm x 300 - 320mm). At the bottom of each glass tube, a 2 cm thick layer of washed and sterile vermiculite was placed and 5 - 8 ml of distilled water was added. The vermiculite was then covered with a 3 cm thick layer of soil moistened with distilled water. Germinated seeds were placed in the glass tubes and covered with a thin layer of soil. One maize plant was sown in each glass tube but three wheat or oat plants were sown per tube. Finally the soil was covered with a

thin layer of sterile vermiculite to minimise algal growth on the soil surface. The tubes were then sealed with parafilm to minimise contamination and water loss (Scher, Ziegle and Kloepper, 1984) and their lower parts were covered with dark paper to shield the soil and plant roots from light.

The greenhouse was illuminated with 400 W greenhouse lamps (Phillips, Holland) positioned 1.3 m above the benches on which the plants were grown in glass tubes. A light/dark cycle of 16/8 h respectively was maintained during the experimental period. The greenhouse temperature was regulated between 20° and 25°C as necessary.

2.1.5 Acetylene reduction assays.

a) Bacterial cultures.

Bacterial cultures were assayed for nitrogenase activity under aerobic conditions. After a satisfactory growth period, the cotton wool plugs or screw caps of the culture vials were replaced with sterile gas-tight Suba-seal rubber closures (W. Freeman and Co. Ltd., Barnsley, Yorkshire). Using a sterile hypodermic syringe and needle (Gillete, Scimitar, Great Britain), 10 % of the air in the head space of the culture vials was withdrawn and replaced with acetylene. Sterile culture medium was included in each assay as a control.

After suitable incubation periods, gas samples were withdrawn and analysed for ethylene content as detailed in methods section 2.1.5d.

b) **Excised roots and soil samples.**

The materials were assayed under gas phases containing 0 %, 5 % and 20 % oxygen. Root or soil samples assayed under 0 % and 5 % oxygen were first flushed with pressurised nitrogen gas for three minutes and the exposure vessels sealed immediately with gas-tight Suba-seal caps. Materials to be assayed under 20 % oxygen were not flushed with nitrogen gas. For samples to be assayed under 5 % oxygen, an appropriate volume of nitrogen gas in the exposure vessel was replaced with air to achieve the required oxygen level in the gas phase. No air was added to the root or soil samples to be assayed under 0 % oxygen. Finally 10 % of gas in the exposure vessels was replaced with acetylene. In all cases gases were removed or added to the exposure vessels through the rubber closures using a hypodermic syringe and needle. Gas aliquots were removed and analysed for ethylene after suitable incubation periods.

c) **Intact plants.**

Intact plants grown in large glass tubes in a greenhouse were assayed for acetylene reduction activity. The parafilm seal used to cover the mouth of each glass tube was replaced with a gas-tight rubber bung modified to carry a Suba-seal rubber cap. Plants to be assayed for nitrogenase activity under anaerobic conditions were first flushed with nitrogen gas for three minutes and the rubber bung replaced immediately in each case. Intact plants to be assayed under aerobic conditions were not flushed with nitrogen gas. Using a sterile hypodermic syringe and needle, 10 % of the gas phase in the glass tubes was replaced with acetylene through the rubber closures. Except where otherwise stated gas samples were removed from the glass tubes and analysed for ethylene content after a 4 h incubation period. In experiments where the plants were assayed weekly, the glass tubes were flushed with air, sealed with parafilm and returned to the green house.

d) **Analysis.**

A Packard model 428 gas chromatograph equipped with a hydrogen flame ionisation detector was used to analyse the ethylene content in gas samples withdrawn from the exposure vessels. A stainless steel column, 2 m long and 3.2 mm internal diameter, packed with Porapack N (80 - 100 mesh) and operated at 60°C was

used to separate the gases. Nitrogen was used as the carrier gas and the detector temperature was set at 100°C. For analysis of gases in the exposure vessels, 0.5 ml aliquots were removed through the Suba-seal rubber closures using a syringe and needle (Precision Sampling Corp., Baton Rouge, Louisiana, U.S.A.) and injected directly into the GC column. After injection of each sample, the syringe was flushed several times with air to avoid cross-contamination between samples. The instrument was calibrated using ethylene standards just before and immediately after each batch of assays. Acetylene standards and gas samples from control exposure vessels incubated without acetylene were tested for ethylene contamination during each assay.

2.1.6 Determination of plant dry weight and N-content.

The plant materials were dried to constant weight in a forced-air oven (Electrolux, Wascator, Alingsas, Sweden) at 80°C. The materials were then milled and the nitrogen content was determined by a semimicro Kjeldahl method (Nelson and Sommers, 1973) in a Kjeltec Autoanalyzer (Tecator, Hoganas, Sweden).

2.2 Temperate Soil

2.2.1 Excised root assay.

Root stumps of mature maize plants (*Z. mays* L.) cultivar LG 11 were dug up from an experimental plot at Saby, near Uppsala. The maize crop had been sown in the summer of 1984 and the root samples were taken late in the autumn. Nitrogen fertilizer had been added at the rate of 75 Kg N/ha at the time of sowing. The root stumps together with the adhering soil were put in polythene bags and taken to the laboratory. About 10 g batches of the roots were immediately excised, washed free of the sticky clay soil, and put in 60 ml gas-tight screw-capped bottles with about 2 ml of water to keep them from drying. Four replicate root samples were assayed under 0 %, 5 % and 20 % oxygen as detailed previously in methods section 2.1.5b. In an additional experiment excised maize roots were thoroughly washed in tap water and surface sterilised in 1 % sodium hypochlorite (Okon *et al.*, 1977). The roots were rinsed in several changes of sterile water and incubated under 5% oxygen. All root samples, under the various gas phases, were incubated in the dark at 24°C and assayed for acetylene reduction activity at 24 h intervals as detailed in methods section 2.1.5b. Gas samples from control root samples incubated without acetylene were also analysed for ethylene.

2.2.2 Enumeration of the root-associated N_2-fixing bacteria.

The MPN (Most Probable Number) method was used to estimate the numbers of N_2-fixing bacteria associated with the roots of field-grown maize at Saby. Bacteria on the root surface were removed by shaking weighed roots in sterile 0.05M phosphate buffer pH 7 for 1h (Okon *et al.*, 1977). Serial dilutions of the root washings were then made in sterile buffer. To determine the total number of N_2-fixing bacteria associated with the roots, weighed root samples were crushed in sterile phosphate buffer using a sterilised mortar and pestle. Serial dilutions of the homogenate were then prepared. The number of bacteria inside the roots were estimated by first sterilising the root surface with 1 % sodium hypochlorite (Okon *et al.*, 1977). The roots were then washed in several changes of sterile water, crushed in sterile phosphate buffer and serial dilutions made as before. For each of the prepared dilutions, five replicate vials containing 4 ml of a semi-solid N-deficient glucose medium (Lindberg and Granhall, 1984b) were inoculated. Sets of five replicate vials containing 4 ml of a semi-solid N-deficient malate medium were similarly inoculated with the prepared dilutions. All vials were plugged

with sterile cotton wool and incubated at 28°C for 48 h. The cultures were incubated under 10 % acetylene for 1 h and then assayed for nitrogenase activity as detailed in methods section 2.1.5d. Vials with visible growth and detectable nitrogenase activity were considered positive. MPN's were calculated using McGrady's probability tables (Postgate, 1969).

2.2.3 Acetylene reduction activity in soil.

Soil was collected from the maize field at Saby. Mud closely associated with maize plant root stumps was labelled rhizosphere soil and was put separately from that collected from between the plant rows. For each type of soil 10 g (dry wt.) samples were put in four replicate 30 ml bottles. The samples were incubated under 0, 5 and 20 % oxygen and assayed for acetylene reduction activity at 24 h intervals as detailed in methods section 2.1.5b. All bottles were incubated in the dark at 24°C. Mud samples incubated without acetylene were used as controls.

2.2.4 Effect of soil amendment with carbon substrates on the nitrogenase activity.

Soil from maize plant rhizosphere and between plant rows was separately collected as detailed above. The mud samples (about 10 g dry wt.) were transferred into

30 ml bottles. Four replicate samples were amended with 0.5 g glucose and a similar number of soil samples was amended with 0.5 g sodium malate. Unamended mud samples were used as controls. All soil samples were incubated under 5 % oxygen in the dark at 24°C. The acetylene reduction assays were carried out at 24 h intervals as detailed in methods section 2.1.5b. Mud samples incubated without acetylene were also tested for endogenous production of ethylene.

2.2.5 Acetylene reduction activity associated with intact maize plants.

Seeds of maize cultivar LG11 were washed, surface sterilised and aseptically germinated on water agar plates as detailed in methods section 2.1.2. Two days after germination the seedlings were sown in large glass tubes (25mm x 300mm) containing a Swedish clay soil as detailed previously (methods section 2.1.4). One seedling was sown in each glass tube. Twelve replicate tubes were set up, sealed with parafilm and placed in a green house. The green house temperature was maintained at 21°C throughout the experiment. The rest of the greenhouse conditions were as detailed in methods section 2.1.4.

After two weeks in the greenhouse the plants were assayed for acetylene reduction activity under aerobic

conditions as detailed previously (methods section 2.1.5c). Ethylene formation was monitored over a 6 h period at 2 h intervals. The rubber bungs were then removed and the gas phase was allowed to return to normal over a period of two days. The glass tubes were then flushed with pressurised nitrogen gas and the plants were assayed for acetylene reduction activity under anaerobic conditions at 2 h intervals over a 6 h period (methods section 2.1.5c). In both the aerobic and the anaerobic assays, control plants were incubated without acetylene to check for endogenous formation of ethylene.

2.2.6 Nitrogenase activity in initial enrichment cultures of maize plant roots.

Six acetylene-reducing intact maize plants were selected from the experiment described above. The plants were pulled out of the tubes and several root pieces each 0.5 cm long were cut from each plant and kept separately. Two root pieces from each plant were randomly taken and transferred into separate vials containing 4ml of the sterile semisolid N-deficient medium with malate or glucose as the carbon source (methods section 2.1.1c). For each plant three replicate vials were set up with each of the two carbon sources. The vials were immediately sealed with sterile

cotton wool plugs and incubated at 28°C for three days without shaking. The plugs were replaced with sterile rubber closures and the enrichment cultures were assayed for nitrogenase activity after a 4 h incubation period as detailed previously (methods section 2.1.5a).

2.2.7 Isolation of N_2-fixing bacteria from roots of field-grown maize.

The composition of the isolation medium was similar to that of the NFb-medium employed by Dobereiner *et al.* (1976) in *Azospirillum* isolation studies. However yeast extract was also added to promote growth of the bacteria (Okon *et al.*, 1977; Watanabe and Barraquio, 1979; Lindberg and Granhall, 1984b). The composition of the NFb-medium used in this study is given in methods section 2.1.1c.

Enrichment cultures.

Maize roots with many lateral branches were sampled from the field-grown maize at Saby. The roots were cut into short pieces 0.5-1.0 cm long. Some of the root pieces were surface-sterilised in 1% sodium hypochlorite as previously described in methods section 2.2.2. A few surface-sterilised and unsterilised root pieces were transferred into separate vials containing 4ml of sterile semisolid N-deficient

NFb-malate and NFb-glucose media. The vials containing media and root pieces were stoppered with sterile cotton wool plugs and incubated at 28°C for two days. The cultures were then tested for nitrogenase activity after incubation for 1 h under acetylene as detailed in methods section 2.1.5a. Enrichment cultures incubated without acetylene addition were used as controls.

Purification of the cultures.

Enrichment cultures with high nitrogenase activities were selected and transfers were made into fresh semisolid media using a wire loop under aseptic conditions. The cultures were incubated for three days at 28°C. After two other tranfers into fresh media, followed by 2-3 day incubation periods after each transfer, the cultures were again tested for acetylene reduction activity. Nitrogenase positive cultures were then selected and streaked on NFb-malate and NFb-glucose agar plates. After incubation for 5-6 days dominant colonies were stabbed with a straight wire and tranferred into sterile semisolid media. Cultures with substantial acetylene reduction activities after two days of incubation were purified by repeated streaking until pure cultures of putative N_2-fixing bacteria were obtained.

2.2.8 Response of maize plants to inoculation with N_2-fixing bacteria.

Inoculant preparation.

Three isolate strains A,B and C were selected from among the acetylene-reducing bacteria isolated from the roots of field-grown maize in methods section 2.2.7 for use as inoculants. These bacteria were aerobic rods and were selected because they grew fast and reduced acetylene vigorously in the semisolid N-deficient glucose medium. Their possible identities will be discussed later. The bacteria were grown on glucose medium since they had been isolated using the same medium. After incubation for five days at 28°C, the cultures were centrifuged and inoculants prepared as detailed in methods section 2.1.3. The three inoculants were diluted with sterile buffer so that each had an optical density of 0.8 units at 560nm.

Inoculation and growth of the plants.

Seeds of maize cultivar LG11 were surface sterilised and germinated in the dark on water-agar plates as detailed in methods section 2.1.2. Seed sterility was confirmed by the absence of microbial growth on nutrient agar plates containing a sample of the sterilised seeds (O'Hara *et al.*, 1981). Two-day old maize seedlings of uniform size were sown in large

glass tubes (25mm x 300mm) containing some sterile vermiculite covered with moist soil from the maize field at Saby (methods section 2.1.1a). Another experiment was similarly set up using autoclaved soil collected from the same site. One seedling was sown per tube and inoculated by dripping 0.1ml of the bacterial suspension on it. Control plants were treated with sterile buffer. The glass tubes were sealed with parafilm and the plants were grown in a green house as detailed previously (methods section 2.1.4). The greenhouse temperature was maintained at 21^{o}C during the experiment. Once every week the plants were assayed for acetylene reduction activity under aerobic conditions over a 4 h period (methods section 2.1.5c). After three weeks of growth, the plant tops were harvested and shoot lengths, dry weights and nitrogen contents were determined as detailed in methods section 2.1.6. Some of the maize plant roots were used immediately for the enrichment cultures study detailed below.

2.2.9 Acetylene reduction activity in preliminary enrichment cultures of inoculated maize plant roots.

Maize plants inoculated with bacterial strains A, B, C and control plants with the highest nitrogenase

activities in each treatment were selected from both the unsterile and the autoclaved soils in the experiment described immediately above. An uninoculated plant in the sterile soil treatment was randomly chosen since no control plant in the treatment had detectable acetylene reduction activity. For each selected plant two root pieces, 0.5cm long, were cut and transferred aseptically into each of four replicate bottles containing 4ml of sterile semisolid NFb-glucose medium. Other root pieces were cut from the same plants and similarly transferred into sterile semisolid NFb-malate medium. Extreme care was taken in all cases to avoid cross contamination between the root materials. All bottles were stoppered with sterile cotton wool and incubated at 28°C for three days. The cotton wool plugs were then replaced with gas-tight rubber closures and the nitrogenase activities in the enrichment cultures were determined after a 4 h incubation period as detailed in methods section 2.1.5a.

2.2.10 Effect of light on nitrogenase activity associated with inoculated maize plants.

Seeds of maize cultivar LG11 were surface sterilised, germinated in the dark and sown in large glass tubes (25mm x 300mm) containing soil from a maize field as detailed in methods sections 2.1.2 and 2.1.4.

The seedlings were inoculated with suspensions of acetylene-reducing bacterial strains A and C previously isolated from the roots of field grown maize (methods section 2.2.7). Six replicate seedlings were inoculated with isolate A and a similar number of plants were inoculated with isolate C. Uninoculated control plants were treated with sterile phosphate buffer. Care was taken to avoid cross-contamination between the glass tubes. The plants were grown in a greenhouse for 18 days and then assayed for acetylene reduction activity under aerobic conditions as detailed in methods sections 2.1.5c and 2.1.5d respectively. Alternate light/dark/light conditions were imposed on the plants during a 35 h assay period. The plants were covered with thick light proof cardboard boxes during the dark period. Gas samples from tubes incubated without acetylene addition were also analysed for ethylene.

2.2.11 Maize-diazotroph association and its influence on bacterial growth and nitrogenase activity in a culture medium.

Establishment of plant-bacteria associations.

Seeds of maize cultivar LG11 were surface sterilised and germinated aseptically as described earlier in methods section 2.1.2. A few seeds were

germinated on nutrient agar to confirm sterility. The seedlings were aseptically sown in large glass tubes (30mm x 320mm) containing some autoclaved soil as detailed in methods section 2.1.4. One maize seedling was sown in each tube and inoculated with 0.1 ml suspension of the bacterium C previously isolated from the roots of field-grown maize. The seedlings were covered with a thin layer of sterile soil and the glass tubes sealed with parafilm. The plants were grown in a greenhouse as detailed previously (methods section 2.1.4). The greenhouse temperature was maintained at 24°C. After a period of three weeks the plant-bacteria systems were assayed for nitrogenase activity (methods section 2.1.5c) to identify the plants which had established effective associations with the bacterium strain C.

Reisolation of the bacterium from the plant.

A maize plant showing good vegetative growth and substantial nitrogenase activity (112.8 nmol C_2H_4 /h) was chosen. The plant was pulled out of the tube and a few short root pieces (0.5 - 1.0cm) were cut with a sterile knife and aseptically transferred into sterile semisolid NFb-glucose medium. The enrichment cultures were incubated at 28°C for 36 h and then streaked on NFb-glucose plates. After three days of incubation,

colonies of a single type were observed on the plates. Bacteria were then taken with a sterile inoculating wire and aseptically transferred to a small volume of sterile phosphate buffer. This was labelled the reisolate strain.

Preparation of bacterial inoculants

Stock strain colonies of the same age as the reisolate strain described above were similarly suspended in sterile buffer. Each bacterial suspension was thoroughly homogenised with a mixer (methods section 2.1.3) and then diluted with sterile phosphate buffer so that both the reisolate and the stock strain suspensions had the same optical density of 0.072 units at 560nm. Microscopic examinations showed that the two bacteria were morphologically identical. The bacterial suspensions were used for the growth and nitrogenase activity studies described below.

Growth studies

Twenty one vials, each containing 4ml of sterile semisolid NFb-glucose medium were prepared for each of the two bacterial strains; the stock strain and the reisolate strain. Agar concentration was lowered to 0.05% to make it possible for the optical density measurements to be read (Berg *et al.*, 1980). Each

bottle was inoculated with four loopfuls of the bacterial suspension, stoppered with a sterile cotton wool plug and incubated at 28°C without shaking. At suitable intervals three bottles of each culture strain were randomly removed and their optical densities measured as detailed in methods section 2.1.3.

Nitrogenase activity studies.

The experimental set up was the same as that of growth studies described immediately above but the agar concentration was raised to 0.175% (Dobereiner *et al.*, 1976). At suitable intervals, triplicate vials of each culture strain were randomly taken and assayed for nitrogenase activity after incubation under 10% acetylene for 1 h (methods section 2.1.5a). The last batch of triplicate cultures to be assayed were pooled together and the pH determined using a pH meter (Radiometer pH meter, Copenhagen, Denmark).

2.3 Tropical Soil

2.3.1 Effect of various environmental factors on soil acetylene reduction activity.

Temperature.

Soil samples (10 g) were put in each of twelve 30 ml McCartney bottles and moistened well with distilled

water. The samples were incubated aerobically under 10 % acetylene as detailed in methods section 2.1.5b. Control soil samples were incubated without acetylene. Four replicate soil samples were assayed for acetylene reduction activity at each of the following temperatures: 20°, 24° and 28°C. The activities were monitored at 24 h intervals over a 72 h period.

Moisture.

The acetylene reduction activity in soil with four different moisture levels was measured. These were air-dried soil; soil at 40 and 60 % WHC (water holding capacity) and water logged soil. Soil (10 g d.wt.) was put in each of four replicate 30ml McCartney bottles and sterile distilled water was added to achieve the required moisture content. The bottles were stoppered with gas tight rubber closures and 10 % acetylene was added. No acetylene was added to the control soil samples. All bottles were incubated at 24°C and gas samples were withdrawn at 24 h intervals and analysed for ethylene content (methods section 2.1.5d).

Carbon substrate.

Approximately 10 g of soil was put in each of several 30 ml McCartney bottles. Four replicate soil samples were amended with one of three carbon

substrates (5 % w:w): glucose, sodium malate and macerated pith from dry maize stalks. The soil samples were moistened well with distilled water and all bottles were incubated at 24°C. The samples were then assayed for acetylene reduction activity at 24 h intervals as detailed in the previous experiment. Gas samples from control soil samples set up without substrate amendment or acetylene addition were also analysed for ethylene content.

2.3.2 Acetylene reduction activity associated with intact maize plants.

Seeds of maize cultivar LG11 were washed, surface sterilised and aseptically germinated on water agar plates as detailed in methods section 2.1.2. Seedlings with straight radicles 1 - 2 cm long were sown in large glass tubes (25mm x 300 mm) containing moist soil as detailed previously (methods section 2.1.4). Twelve replicate tubes were prepared each containing one maize seedling. The tubes were immediately sealed with parafilm and the plants were grown in a green house as detailed in methods section 2.1.4. When the maize plants were two weeks old, they were assayed for acetylene reduction activity under aerobic and anaerobic conditions as detailed in methods section 2.2.5. Gas samples from glass tubes with plants

incubated without acetylene were also tested for ethylene.

2.3.3 Effect of repeated subculturing of enrichment cultures on nitrogenase activity.

Several intact plants from the experiment detailed above in methods section 2.3.2 were taken. The plants were pulled out of the tubes and a few 0.5 cm long root pieces were excised from each plant and pooled together. Twelve vials containing 4 ml of sterile semisolid NFb-glucose medium and a similar number of vials containing 4 ml of sterile semisolid NFb-malate medium were prepared. Two root pieces were transferred into each vial and the bottles were plugged with sterile cotton wool and incubated at 28°C for 72 h. All the enrichment cultures were then assayed for nitrogenase activity over a 4 h period as previously described in methods section 2.1.5a.

Three glucose and three malate enrichment cultures with the highest acetylene reduction activities were selected. Each culture was mixed well and three loopfuls were aseptically transferred into fresh media. Four replicate vials were inoculated for each culture. The cultures were grown at 28°C for 72 h and then assayed for nitrogenase activity. Three cultures with the highest acetylene reduction activities in glucose

and in malate media were again selected and transfers made into fresh media as before. The cultures were then grown and assayed for nitrogenase activity as described previously.

2.3.4 Isolation of N_2-fixing bacteria associated with maize plant roots.

Plants with substantial acetylene reduction activities in the experiment described above (methods section 2.3.2) were selected. They were pulled out of the glass tubes and shaken free of soil.

Enrichment cultures.

Root pieces (0.5 - 1 cm long) were cut from the plants and immediately transferred into 14 ml vials each containing 4 ml of sterile N-deficient semisolid NFb-glucose or NFb-malate media (methods section 2.1.1c). All vials were sealed with sterile cotton wool plugs and incubated at 24°C for 4 days. The enrichment cultures were then assayed aerobically for nitrogenase activity as detailed in methods section 2.2.2.

Purification of the cultures.

Cultures showing vigorous nitrogenase activity were selected. Further enrichment of these cultures was carried out by repeated transfers into fresh semisolid

media after every two days of incubation at 28°C. After three such transfers, the enriched cultures were then streaked on NFB-glucose and NFb-malate agar plates and the dominant colonies selected and tested for nitrogenase activity as before. Purification was achieved by alternately growing selected colonies on agar plates and in semisolid media until pure cultures were obtained. The purity of the cultures was further verified by observations under a light microscope.

2.3.5 Effect of straw amendment and inoculation with N_2-fixing bacteria on maize under green house conditions.

Soil preparation.

Moist soil amended with dried macerated maize stalks (5 % w:w) was transferred into large glass tubes (30 mm - 320 mm) containing washed and sterile vermiculite as detailed in methods section 2.1.4. A second batch of tubes was similarly prepared using soil which had been fumigated with methyl bromide at the rate of 100 g /m^3 for 24 h (Anticimex Co., Stockholm, Sweden). Glass tubes with unamended raw or fumigated soil were also prepared.

Preparation of the inoculants

Two vigorous acetylene-reducing bacterial strains J and L were chosen from amongst the bacteria isolated from the roots of maize plants grown in the tropical soil (methods section 2.3.4). The cultures were grown at 28°C and the inoculants were prepared as detailed in methods section 2.1.3. The optical density of each inoculant bacterial suspension in sterile phosphate buffer was adjusted to 0.5 units at 560 nm.

Seed preparation, inoculation and plant growth.

Seeds of maize cultivar LG11 were washed, surface sterilised and aseptically germinated on water agar as previously described (methods section 2.1.2). Seedlings of uniform size were then selected and one seedling was sown in each of the prepared glass tubes. Some of the seedlings in both the amended and the unamended soil were inoculated with 0.2 ml bacteria suspension of strain J. Other seedlings were similarly inoculated with the bacterium strain L. Uninoculated control seedlings were drenched with 0.2 ml of the sterile buffer. Each treatment was replicated six times and all seedlings were covered with soil and sterile vermiculite as detailed in methods section 2.1.4. The glass tubes were finally sealed with parafilm and the plants were grown in a green house as detailed in

methods section 2.1.4. The temperature was maintained at 25°C throughout the experiment. The experiment was terminated after three weeks when the plants attained the maximum size possible in the glass tubes. The plant shoot lengths were measured and the plants were harvested and their dry weights determined as detailed in methods section 2.1.6.

Acetylene reduction assays of the plants.

At weekly intervals the plants were incubated aerobically under 10% acetylene for 4 h and assayed for acetylene reduction activity as detailed previously in methods section 2.1.5c. Ethylene production was also tested for in control plants incubated without acetylene. After each assay the rubber bungs were removed and the glass tubes were flushed with air before resealing them with parafilm.

2.3.6 Effect of straw amendment on nitrogenase activity in soil over a prolonged period.

The experimental soil was mixed with dried macerated maize stalks (5 % w:w) and 10 g samples were transferred into large glass tubes (25mm x 300mm) as detailed in methods section 2.1.4. Other tubes were similarly prepared using unamended soil. Five replicate tubes were set up with each soil. The glass tubes were

then sealed with parafilm and transferred to a green house in which the temperature was maintained at 25°C. The acetylene reduction activity under aerobic conditions was measured weekly for six weeks as described in methods section 2.1.5c. The soil samples were incubated under acetylene for 4 h during each assay. Endogenous production of ethylene was always checked for in soil samples incubated without acetylene.

2.3.7 Effect of straw amendment and inoculation on the growth of maize plants under water limiting conditions.

Seed preparation, inoculation and planting.

Seeds of three maize cultivars, LG11, Katumani and Makueni were washed for 2 h under running water and then in several changes of sterile water to remove the fungicides. The seeds were germinated on a bed of moistened filter paper as detailed in methods section 2.1.2. Three-day-old maize seedlings were inoculated by immersing them in suspensions of N_2-fixing bacterial strains C and L in sterile phosphate buffer as detailed in the preceeding experiment. Uninoculated seeds were immersed in sterile buffer. For each maize cultivar five maize seedlings were sown in each 20 l plastic pot

containing straw-amended or unamended soil. In the amended soil dried chopped maize straw was applied in the top 3 cm soil layer. Seeds inoculated with bacteria strains C and L and the uninoculated controls were sown in separate pots; care being taken in each case to avoid cross contamination between them. Each treatment was replicated four times and the pots were kept in an open shed with a clear plastic roof to keep out rain.

Growth of the plants.

All pots were watered every other day as necessary. When the plants were four weeks old, half of the pots in each treatment were watered normally but no water was added to the others. Leaf water potentials of both the watered and water-stressed plants were determined at suitable intervals using a pressure chamber (PMS Instruments, corvallis, Oregon, U.S.A.). All measurements were taken during day time between 11.00 a.m. and 1.00 p.m. The experiment was terminated after two weeks when some of the water-stressed plants started to wither. All plants were then harvested and the root and shoot dry weights were determined as detailed in methods section 2.1.6.

2.3.8 Growth and nitrogenase activity of maize seedlings inoculated with reisolates of a N_2-fixing bacterium.

Preliminary investigations were conducted to test whether a bacterium, originally isolated from surface sterilised maize roots and which had been passaged through another maize plant would be more effective in promoting growth and nitrogen fixation in other maize plants. Apart from the original isolate C, two other strains of the N_2-fixing, plant-growth-promoting bacterium were obtained as follows.

Plant reisolate (PR)

The stock culture of the original isolate C was used to prepare an inoculum as detailed in methods section 2.1.3. Gnotobiotic maize seedlings were inoculated and grown in autoclaved soil for three weeks under aseptic conditions. The bacterium was then reisolated from nitrogenase positive maize plants by incubating the root pieces in the isolation medium (methods section 2.2.7) and plating the cultures. Repeated subculturing of the isolate in artificial medium was avoided since it might lead to some loss of nitrogenase activity (Berg *et al.*, 1980).

Soil reisolate (SR)

Inoculum prepared from the stock culture of the original isolate C was added to autoclaved moist soil in glass tubes without plants. The glass tubes were sealed with parafilm and kept in a green house for three weeks. A little of the soil was then aseptically transferred into the isolation medium and the bacterium reisolated as for the plant reisolate above.

Inoculation and plant growth.

The original isolate or stock culture (SC); the plant reisolate (PR) and the soil reisolate (SR) were grown in liquid NFb-glucose medium and inoculants were prepared as detailed earlier in methods section 2.1.3. The optical densities of each of the three bacterial suspensions was adjusted to 0.8 units at 560 nm with sterile phosphate buffer. Care was taken to avoid cross contamination between the cultures.

Gnotobiotic seedlings of maize cultivar LG11 were aseptically sown in glass tubes (25mm x 300mm) containing raw or fumigated soil as previously described in methods section 2.1.4. A third batch of seedlings was transferred into tubes containing 7ml of a semi solid water agar medium (0.25 % agar w:v). The only additions to this medium were: 0.5g K_2HPO_4; 0.2g $MgSO_4.7H_2O$; 0.02g $CaCl_2$; 0.002g $NaMoO_4.2H_2O$; 0.01g

$MnSO_4.H_2O$ per liter of the medium. The pH was adjusted to 7.0. The semisolid nature of the medium enabled the seedlings to remain on the surface and their roots could grow easily into the medium.

For each of the three bacterial cultures (SC, PR, and SR) six replicate seedlings in raw soil, fumigated soil and in the water-agar medium were inoculated. The inoculum applied to each seedling was 0.2ml. Uninoculated plants were treated with sterile phosphate buffer. Seedlings sown in soil were covered with a little soil and a thin layer of vermiculite to keep out light. Glass tubes containing the water agar medium were plugged with sterile cotton wool while those containing soil were sealed with parafilm. All seedlings were grown in a green house as detailed in methods section 2.1.4 and the greenhouse temperature was maintained at 24°C. At weekly intervals the maize seedlings were assayed for nitrogenase activity under aerobic conditions, after incubation for 24 h under acetylene, as detailed in methods section 2.1.5c. After two weeks the plants were removed from the glass tubes and their shoot lengths and dry weights were determined as previously described in methods section 2.1.6. Plate counts of total and N_2-fixing bacteria associated with the plant roots were made on nutrient agar and NFb-glucose plates respectively. This was

achieved by crushing root samples in sterile 0.05 M phosphate buffer pH 7.0 using a sterile mortar and pestle. Serial dilutions of the homogenates were made in buffer and 0.1 ml aliquots were spread on the plates. Colony counts were made after incubating the plates for four days at 28°C.

2.3.9 Effect of seed bacterization on growth and yield of three maize cultivars.

Inoculant preparation

Cultures of three N_2-fixing bacterial strains C, J and L previously isolated from maize roots (methods sections 2.2.7 and 2.3.4) were used to prepare inoculants as detailed in methods section 2.1.3. The bacteria were suspended in sterile phosphate buffer and transferred into sterile petri dishes.

Seed preparation, inoculation and growth of the plants.

Seeds of three maize cultivars LG11, Makueni and Katumani were washed under running water for 2 h and then in several changes of sterile distilled water to remove the fungicides. The seeds were placed on moistened filter paper in petri dishes and germinated in the dark at room temperature. Seedlings of uniform size were selected for each cultivar and each seedling lot was divided into four batches. For every cultivar, one

batch of seeds was used as a control while the other three batches were inoculated with bacterial strains C, J and L respectively.

The germinated seeds were inoculated by placing them in petri dishes containing the bacterial suspensions for 30 minutes. Control seeds were immersed in sterile phosphate buffer. Three maize seedlings were sown in 20 l plastic buckets while four seedlings were sown in 30 l plastic troughs also containing well moistened soil from a weeded plot (methods section 2.1.1a). Great care was taken to avoid cross contamination between the containers. Each treatment was replicated four times and all containers were randomly arranged in an open space but under a roof of clear plastic sheeting to prevent flooding of the containers by rain water. All plants were watered every two to three days as necessary. The soil in each container was covered with washed vermiculite to minimise growth of algae. When the plants were ten days old, they were thinned to two in the buckets and to three in the larger plastic containers. The plants were grown for one hundred and ten days before harvesting. The plant heights were measured with a ruler and the shoot, ear and 1000-seed dry weight was determined in every treatment. The nitrogen contents of the shoot and the maize seeds were then determined as detailed previously (methods section

2.1.6). The crude protein content of the maize grain was estimated by multiplying the N-content with a factor of 6.25 (AOAC, 1960).

2.3.10 Response of wheat and oat plants to inoculation with N_2-fixing bacteria.

Inoculant preparation

Three acetylene-reducing bacterial strains C, J and L previously isolated from the roots of maize (methods sections 2.2.7 and 2.3.4) were used to prepare inoculants as detailed in methods section 2.1.3. Each inoculant bacterial suspension in sterile phosphate buffer had an optical density of 0.5 units at 560 nm.

Inoculation and plant growth

Wheat and oats seeds were washed in running water and germinated on moistened filter paper in the dark at room temperature. Three days after germination, the seedlings were sown in large glass tubes (30mm x 320mm) containing soil as detailed in methods section 2.1.4. Three wheat or oat seedlings were sown in each tube and each seedling was inoculated with 0.1 ml of the bacterial suspension. For each inoculant, four replicate glass tubes were set up. This was repeated for both wheat and oat plants. Control plants were treated with sterile phosphate buffer. The glass tubes

were finally sealed with parafilm and the plants were grown in a green house as detailed in methods section 2.1.4. At weekly intervals, the plants were aerobically incubated under 10% acetylene for 22 h and assayed for nitrogenase activity as detailed previously (methods section 2.1.5c). Gas samples from glass tubes incubated without acetylene addition were also tested for ethylene. After three weeks, the plants were pulled out of the tubes and shoot lengths and plant dry weights were determined as detailed earlier in methods section 2.1.6.

2.4 Studies of the N_2-fixing temperate and tropical soil isolates.

2.4.1 Physiological studies.

These studies were restricted to the rhizosphere bacteria which had been used in various inoculation experiments described above.

a) Effect of temperature on growth and nitrogenase activity.

Stock cultures of the nitrogen-fixing bacterial isolates A and C from the temperate soil and isolate L from the tropical soil were grown on NFb-glucose agar plates for three to four days at 28°C. Colonies

were then transferred into small volumes of sterile phosphate buffer using a sterile inoculating wire. Each bacterial suspension was thoroughly homogenised with a mixer (methods section 2.1.3) and four loopfuls were used to inoculate each vial containing 4 ml of sterile semisolid NFb-glucose medium (0.05 % agar w:v) (Berg *et al.*, 1980). For each isolate, twenty five replicate vials were inoculated, plugged with sterile cotton wool and incubated without shaking at each of the following temperatures: 24°, 28° and 37°C. Triplicate cultures were randomly picked and assayed for nitrogenase activity at suitable intervals over a 72 h period as previously described (methods section 2.1.5a). Immediately after each assay, the cultures were homogenised by gentle shaking and their optical densities measured at 560 nm as detailed in methods section 2.1.3.

b) Effect of pH on growth and nitrogenase activity.

Three-day-old colonies of bacterial isolates A and C from the temperate soil and isolate L from the tropical soil were suspended in sterile phosphate buffer and homogenised as detailed in the preceeding experiment (a) above. For each isolate, three loopfuls of the bacterial suspension were transferred to each vial containing 4 ml of sterile semisolid (0.05% agar)

NFb-malate medium. Four replicate vials were set up at each of the pH values 5, 5.5, 6, 6.5, 7, 7.5 and 8.0. Glucose medium was not used because rapid changes in pH had previously been observed when glucose was the carbon source. All vials were stoppered with sterile cotton wool and incubated for 19 h at 28°C as stagnant cultures. At the end of this period, the cultures were incubated under acetylene for 1 h and assayed for nitrogenase activity as detailed in methods section 2.1.5a above. The cultures were then homogenised by gentle shaking and their optical densities determined as detailed previously (methods section 2.1.3).

2.4.2 Characterisation and identification of the isolates.

Attempts were made to characterise and identify the N_2-fixing bacterial isolates from the temperate and the tropical soils using standard texts (Bergey's Manual Of Determinative Bacteriology, 1974; Cowan, 1974; Collins and Lyne, 1984) and literature accompanying the test reagent kits. However due to the large number of tests required in such an excercise, only three isolates were selected from each of the two soils. The selected bacteria were amongst the dominant N_2-fixing isolates and had also been used in inoculation experiments elsewhere in this study. The various physical and

biochemical tests were carried out on pure cultures of the isolates. Microscopic observations of the cultures were made under a phase contrast microscope (Olympus MKC-720, Olympus Optical Company Ltd., Japan).

a) **Colony morphology.**

The bacterial isolates were grown on N-deficient NFb-glucose agar plates (methods section 2.1.1c) for 4 - 5 days at 28°C. Colony characteristics such as texture, elevation and margin were then noted.

b) **Cell shape and size.**

Pure cultures of the isolates were grown on NFb-glucose agar plates at 28°C. Bacteria from 30 h old colonies were suspended in a drop of water and the wet mounts were observed without staining under a phase contrast microscope. The cell shape of each isolate was noted. Stage and ocular micrometers were used to determine the breadth and length of four or more cells in the wet mounts. The average cell size for each isolate was then found.

c) **Cell motility.**

A sterile semisolid NFb-glucose medium (0.25 % w:v agar) in 14 ml culture vials was inoculated with the bacterial isolates using a straight inoculating wire.

All cultures were incubated at 28°C. Cultures showing diffuse growth were scored as motile while those showing growth along the point of inoculation even after 40 h of incubation were considered as non-motile. The results were compared with those obtained from microscopic observations of the wet mounts.

d) **Gram reaction.**

Bacterial smears of 30 h old colonies grown on NFb-glucose plates at 28°C were prepared on clean microscope slides. The smears were fixed and Gram stained following standard microbiological procedures (Collins and Lyne, 1984). The stained bacterial smears were then examined under oil immersion using a light microscope.

e) **Endospore formation.**

The isolates were grown on NFb-glucose agar plates for 4 days at 28°C. Bacterial suspensions were then made in sterile water and heated in a water bath at 80°C for 10 min. The suspensions were streaked on glucose agar plates and incubated at 28°C for 48 h. Growth indicated the presence of spores in the culture.

f) **Growth on MacConkey agar.**

The isolates were streaked on MacConkey agar plates

and incubated at 30°C for 24 h. Growth and colony colour were then noted.

g) **Catalase test.**

A loopful of a 20 h old culture was placed in a drop of diluted hydrogen peroxide. Observation of effervescence indicated the presence of catalase.

h) **Cytochrome oxidase test.**

A drop of an Oxidase Test Reagent (Marion Scientific Corp., Kansas City, U.S.A.) was placed on 30h old colonies of the pure culture. Reactions were recorded as positive when a violet to purple colour was observed immediately after addition of the reagent.

i) **Indole formation.**

The isolates were grown on peptone broth for 48 h at 30°C. The development of a red colour after addition of Kovac's reagent (Marion Scientific Corp., Kansas City, U. S. A.) indicated indole formation.

j) **Citrate utilisation.**

The isolates were inoculated on Simmon's citrate agar medium (Collins and Lyne, 1984). The plates were incubated for 48 h at 30°C. Production of an alkaline reaction indicated citrate utilization.

k) **Carbohydrate fermentation tests.**

The ability of the isolates to ferment various carbohydrates was also tested (Cowan, 1974). The carbohydrates included glucose, sucrose, lactose, mannitol, maltose, dulcitol and inositol. The isolates were inoculated in nutrient broth containing 1 % (w:v) of the carbohydrate and 0.5 % (w:v) bromothymol blue indicator. The cultures were incubated at 30°C for 1 - 2 days. In the glucose fermentation test Durham's tubes were inverted in the medium to test for gas formation (Collins and Lyne, 1984).

l) **Aesculin hydrolysis.**

Aesculin agar medium (Cowan, 1974) was inoculated with the isolates and incubated for 1 - 2 days at 30°C. Blackening of the medium indicated hydrolysis of aesculin.

RESULTS

3.1 Temperate soil.

3.1.1 Acetylene reduction activity of excised roots.

Low rates of acetylene reduction activity were detected in washed and excised maize roots. The activity decreased after 24 h of incubation (Table 1). Root-associated nitrogenase activity was higher with an initial atmosphere of 5% oxygen than when under ambient and anaerobic conditions. Surface sterilised roots had no detectable acetylene reduction activity during the first 24 h . However after this period, the activity increased rapidly and was slightly higher than that of the unsterilised roots under 5% initial oxygen concentration after 72 h of incubation (Table 1).

3.1.2 MPN counts of putative N_2-fixing rhizosphere bacteria.

Bacterial growth was visually recorded in the vials after 48 h of incubation at 28°C. In nearly all cases , growth in the N-deficient glucose medium was accompanied by a decrease in pH. In some of the cultures grown in N-deficient malate medium the pH became alkaline. These observations were made from the change in colour of the bromothymol blue indicator incorporated in the culture media. Occasionally, a

Table 1. Acetylene reduction activity of washed excised roots of field-grown maize.

Initial O_2%	nmol C_2H_4 / g d.wt. roots		
	24h	48h	72h
0	5.0 ± 1.0	6.2 ± 0.5	6.3 ± 0.5
5	20.0 ± 3.8	21.9 ± 3.7	24.4 ± 5.9
20	9.0 ± 2.0	11.5 ± 0.9	11.6 ± 1.6
* 5	0	21.0 ± 0.1	25.5 ± 3.2

* Surface-sterilised roots.

Roots were sampled from field-grown maize. They were washed in water to remove mud and incubated under 0, 5 and 20 % oxygen as detailed in methods section. Surface-sterilised maize roots were incubated under 5% oxygen. Acetylene (10 %) was added to each exposure vessel and ethylene content in the gas phase was determined every 24h using a gas chromatograph. The results are means of four replicate root samples ± S.E.

Table 2. MPN counts of putative N_2-fixing bacteria associated with roots of field-grown maize.

Treatment	cells / g d.wt. roots	
	GLU medium	MAL medium
washed	5.0×10^6	0.7×10^6
washed and crushed	7.0×10^6	2.0×10^6
washed, surface-sterilised and crushed.	2.5×10^6	0.5×10^6

GLU = glucose ; MAL = malate.

Fresh root samples from field-grown mature maize plants were washed in water to remove mud. The Most Probable Numbers of N_2-fixing bacteria on the root surface (washed); on and inside roots (washed and crushed); and in the endorhizosphere alone (surface-sterilised and crushed) were then determined as detailed in methods section.

thick surface pellicle due to aerobic growth was observed, while microaerophilic growth was evident in most of the other cultures. In the latter cultures, the bacteria formed a band 1 - 2 mm below the surface or grew in the subsurface without forming a distinct pellicle. Pellicle formation was observed in vials inoculated with the high dilutions and only when malate was the carbon source. Such cultures were found to have no detectable acetylene reduction activity and were disregarded. The highest numbers of putative nitrogen-fixing bacteria were observed in washed and crushed roots while the lowest numbers were present in crushed and surface sterilised roots (Table 2). Higher numbers of bacteria were detected using glucose medium than with the malate medium (Table 2).

3.1.3 Nitrogenase activity in soil associated with maize plants.

Generally low rates of acetylene reduction activity were detected in soil collected from the maize field. In all cases the activity was particularly low under anaerobic and ambient conditions during the first 24 - 48 h of incubation after which substantial increases were recorded (Table 3). Higher nitrogenase activity was found in rhizosphere soil than in soil sampled between the plant rows. In the rhizosphere soil,

Table 3. Nitrogenase activity in soil associated with field -grown maize.

		nmol C_2H_4 /*bottle.		
	% O_2	24h	48h	72h
Rhizosphere soil	0	2.6 ± 0.6	4.3 ± 0.7	10.5 ± 3.7
	5	2.6 ± 0.4	11.6 ± 3.6	15.5 ± 4.5
	20	1.7 ± 0	4.6 ± 0.6	9.8 ± 4.8
Soil between plant rows	0	2.0 ± 0.6	2.6 ± 1.1	8.3 ± 2.3
	5	1.9 ± 1.0	2.9 ± 1.0	3.8 ± 1.4
	20	1.6 ± 0.1	1.7 ± 0.4	2.3 ± 0.3

*each bottle contained 10 g d.wt. soil collected from the maize field at Saby.

The mud samples were put in 30 ml bottles and incubated under 0, 5 and 20 % oxygen as detailed in methods section. Acetylene (10 %) was finally added and the soil samples were incubated in the dark at 24°C. Results are means of four replicates ± S.E.

optimum activity was detected between 24 and 48 h when the initial oxygen level was 5%. Soil sampled between the plant rows had its highest acetylene reduction activity under anaerobic conditions after 72 h of incubation (Table 3).

3.1.4 Effect of carbon substrate addition on soil acetylene reduction activity.

Large increases in acetylene reduction activity were observed in glucose-amended soil samples during the first 24 h of incubation (Table 4). Glucose stimulated the activity more in the rhizosphere soil than in soil sampled between the plant rows. In both soils, malate stimulation of nitrogenase activity was very much lower than that effected by glucose. It was noted however that substantially greater stimulation of acetylene reduction activity occurred in soil collected between plant rows than in rhizosphere soil after amendment with malate (Table 4).

3.1.5 Nitrogenase activity in intact maize plant-soil systems.

In the temperate soil the reduction of acetylene to ethylene by intact maize plant soil systems was immediate without a significant lag period under both aerobic and anaerobic conditions. In these intact

Table 4. Effect of soil amendment with carbon substrates on the acetylene reduction activity.

		nmol C_2H_4/* bottle		
	substrate	24h	48h	72h
Rhizosphere soil.	none	2.1 ± 0.7	6.5 ± 2.8	9.4 ± 2.2
	glucose	451.5 ± 118	1760 ± 450	2726.1 ± 295
	malate	2.9 ± 0.4	8.6 ± 3.4	12.4 ± 3.9
Soil between plant rows	none	1.8 ± 0.6	3.3 ± 0.8	5.0 ± 1.9
	glucose	297.2 ± 88.5	1498.7 ± 378	2263.7 ± 642
	malate	30.9 ± 12.3	56.1 ± 22.2	73.4 ± 30.5

*each bottle contained mud (10 g d.wt. soil) from the maize field at Saby.

Mud samples (10 g)in 30 ml bottles were amended with 1% glucose or 1% sodium malate (w / w) and incubated under 5% oxygen at 24°C. Acetylene reduction activities were determined over a 72 h period. Results are means of four replicate soil samples ± S.E.

Fig. 1 Nitrogenase activity associated with intact maize plants in the temperate soil.

Maize seedlings cultivar LG11 were sown in large glass tubes containing the temperate soil. One seedling was sown in each tube. The plants were grown for two weeks in a greenhouse with a dark/light cycle of 16/8 h respectively. The temperature was maintained at $24^{o} \pm 2^{o}C$. The plants were then assayed for acetylene reduction activity under aerobic (O) and anaerobic (●) conditions as detailed in methods section. Each point represents the mean nitrogenase activity of ten replicate maize plants.

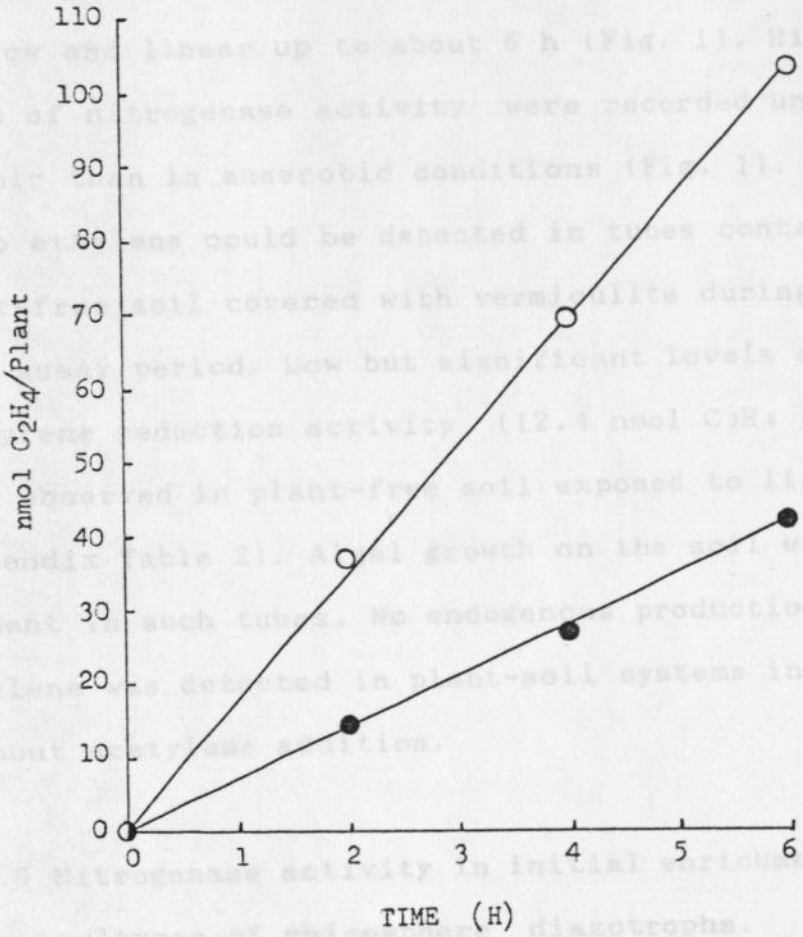

110
100
90
80
70
60
50
40
30
20
10
0
nmol C_2H_4/Plant
0
1
2
3
4
5
6
TIME (H)

Fig.1

plant-soil systems, large variations in nitrogenase activity were observed among the plants (Appendix Table 1). In general the mean acetylene reduction activity was low and linear up to about 6 h (Fig. 1). Higher rates of nitrogenase activity were recorded under aerobic than in anaerobic conditions (Fig. 1). Little or no ethylene could be detected in tubes containing plant-free soil covered with vermiculite during the six hour assay period. Low but significant levels of acetylene reduction activity (12.4 nmol C_2H_4 /tube /h) were observed in plant-free soil exposed to light (Appendix Table 2). Algal growth on the soil was evident in such tubes. No endogenous production of ethylene was detected in plant-soil systems incubated without acetylene addition.

3.1.6 Nitrogenase activity in initial enrichment cultures of rhizosphere diazotrophs.

Very varied acetylene reduction activities were detected in the initial enrichment cultures of roots excised from six different maize plants (Fig. 2). Though some variation was also observed between nitrogenase activities in replicate enrichment cultures, the activities were largely comparable. In nearly all cases however, enrichment cultures in glucose medium had much higher acetylene reduction

Fig. 2 Nitrogenase activity in initial enrichment cultures of maize plant rhizosphere diazotrophs.

Six acetylene-reducing two-week-old intact maize plants were selected. Two root pieces (each 0.5 cm. long) were cut from each plant and transfered into separate vials containing semisolid N-deficient glucose (▨) or malate (□) media as detailed in methods section. The vials were incubated at 28°C for three days and the enrichment cultures were assayed for N_2-ase activity. Results are mean activities in three replicate cultures. Bars represent standard error.

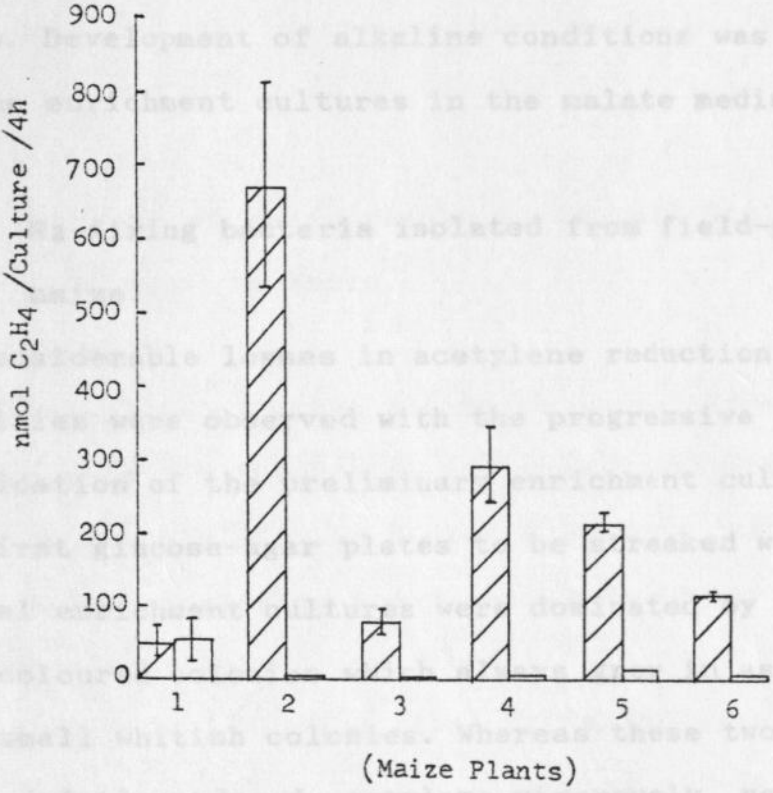

900
800
700
600
500
400
300
200
100
0
nmol C_2H_4 /Culture /4h
1
2
3
4
5
6
(Maize Plants)

Fig.2

activities than cultures grown in malate medium. Acid was evidently produced in all enrichments in glucose medium since the colour of bromothymol blue incorporated in the medium changed from green to yellow. Development of alkaline conditions was observed in some enrichment cultures in the malate medium.

3.1.7 N_2-fixing bacteria isolated from field-grown maize.

Considerable losses in acetylene reduction activities were observed with the progressive purification of the preliminary enrichment cultures. The first glucose-agar plates to be streaked with the initial enrichment cultures were dominated by large buff coloured colonies which always grew in association with small whitish colonies. Whereas these two colony types jointly reduced acetylene vigorously, no nitrogenase activity was detectable in either culture when the two were grown separately. A total of seven acetylene-reducing bacterial isolates were obtained from the maize roots. These bacteria were considered different owing to differences in colony characteristics such as form, size and colour. Only two of these isolates were obtained from the malate enrichment cultures whilst the rest were isolated in the glucose medium. The malate isolates had very low acetylene

reduction activities. Further subculturing of the glucose isolates showed that they were stable and reduced acetylene vigorously in the nitrogen -deficient glucose medium. Various properties of some of the isolates and their possible identities are shown in Table 20.

3.1.8 Response of maize plants to inoculation with N_2-fixing bacteria.

a) **Plant growth.**

The response of maize plants to inoculation with the temperate soil bacterial isolates was investigated. Among the three N_2-fixing bacteria tested, inoculation with strain C increased the plant shoot length by over 10% while inoculants A and B had little or no effect in this regard (Table 5).The shoot dry matter yields of maize plants inoculated with bacterial strains A, B and C increased by 1.9%, 19.6% and 27.4 % respectively. The inoculated plants also had a slightly higher nitrogen content in their shoots than the control plants (Table 5). Thus, shoot nitrogen yields in the inoculated treatments increased by 6.3 to 35.2 % over that of the uninoculated plants. Maize plants inoculated with the bacteria also showed increases in plant shoot/root ratios from 0.51 in uninoculated plants to between 0.57 and 0.89 in the inoculated

Table 5. Effect of bacteria inoculation on growth, N-content and shoot / root ratio of young maize plants.

Inoculants	shoot length (cm)	shoot d.w. (mg)	% N in shoots	Shoot N-yield (mg)	shoot/root ratio
A	26.0 ± 0.7	104 ± 7	2.62	2.72	0.57
B	25.7 ± 0.6	122 ± 9	2.68	3.27	0.65
C	28.4 ± 0.9	130 ± 12	2.66	3.46	0.89
control	25.7 ± 1.2	102 ± 7	2.51	2.56	0.51

Maize seedlings were inoculated with N_2-fixing bacteria strains A, B and C as detailed in methods section. The plants were grown at 21 °C in a green house for three weeks. Plant shoot lengths, dry weights and N-content were then determined. Results are means of nine replicate plants ± S.E.

treatments.

b) **Acetylene reduction by inoculated intact plants.**

The effect of inoculation on the acetylene reduction activity associated with intact maize plants was investigated. Acetylene reduction activity was detectable in seven-day-old intact soil-maize plant systems without significant lag periods (Table 6). The activity varied widely between the treatments and also within the treatments. After growing for two weeks, plants inoculated with bacterial strains A and C had significantly higher mean nitrogenase activities than the uninoculated control plants as well as those inoculated with the B strain (Table 6). When the plants were three weeks old, those inoculated with bacterial strain A had still high acetylene reduction activities but those inoculated with strain C showed a drop in activity. Whereas only 22% of uninoculated maize plants had detectable acetylene reduction activity after one week of growth, over 78% of the inoculated maize plants had measurable levels of activity during the same period (Table 6). In both the inoculated and the uninoculated plants, the acetylene reduction activity generally increased with plant age.

It was noted that some maize plants had detectable nitrogenase activity throughout the three week study

Table 6. Nitrogenase activity associated with intact maize plants inoculated with three strains of N_2-fixing bacteria.

Inoculant	plant age (days)	ARA +ve plants	nmol C_2H_4 /plant /h ARA range	mean ARA	mean ARA nitrogenase +ve plants ± S.E.
A	7	9	3 - 49	12	12 ± 5
	14	9	3 - 638	183	183 ± 76
	21	8	0 - 413	194	218 ± 63
B	7	7	0 - 28	5.7	7.3 ± 2.8
	14	7	0 - 90	21	27 ± 10
	21	5	0 - 119	29	52 ± 14
C	7	7	0 - 41	11	14 ± 5
	14	7	0 - 963	186	239 ± 102
	21	9	8 - 133	81	81 ± 14
Control	7	2	0 - 9	1.3	5.9 ± 1.0
	14	9	3 - 78	19	19 ± 8
	21	7	0 - 194	67	86 ± 23

ARA = Acetylene Reduction Activity.

Maize seedlings cultivar LG11 were sown in soil in large test tubes and inoculated with N_2-fixing bacteria strains A,B or C as detailed in methods section. One plant was sown in each test tube. The plants were grown in a green house for three weeks and assayed weekly for nitrogenase activity. Results are means of nine replicate plants. S.E. is standard error.

period. However other plants had no detectable activity during the second or third week though some nitrogenase activity had been recorded in the previous assay. This phenomenon was not restricted to particular maize plants but was found to occur randomly among plants in all treatments. In their third week of growth, most maize plants in the inoculated treatments had reached their largest possible size in relation to the size of the culture vessels. The leaves of these plants were bundled together in the tubes and rapidly turned yellow. Such plants invariably showed decreased acetylene reduction activity which was reflected in the lower mean and range of activities for some treatments (Table 6). In comparison the uninoculated maize plants were green and smaller and showed a progressive increase in their mean nitrogenase activities during the three-week study period.

3.1.9 Nitrogenase activity in inoculated maize plant root enrichment cultures.

Higher acetylene reduction activity was generally recorded in glucose than in malate enrichment cultures for the same root samples (Fig. 3). The only exceptions were enrichments from plants inoculated with the bacterium strain B in the sterile soil where malate cultures had higher nitrogenase activities than the

Fig. 3 Nitrogenase activity in preliminary enrichment cultures of inoculated maize plant roots.

Several root pieces (0.5 cm long) were aseptically sampled from inoculated maize plants grown on sterilised and unsterile soil as detailed in methods section. A, B and C were the inoculant bacteria. Two root pieces were transfered into vials containing N-deficient glucose or malate media. The vials were incubated at 28°C for three days and the cultures were assayed for N_2-ase activity. Results are mean activities of four replicates. Bars represent standard error.

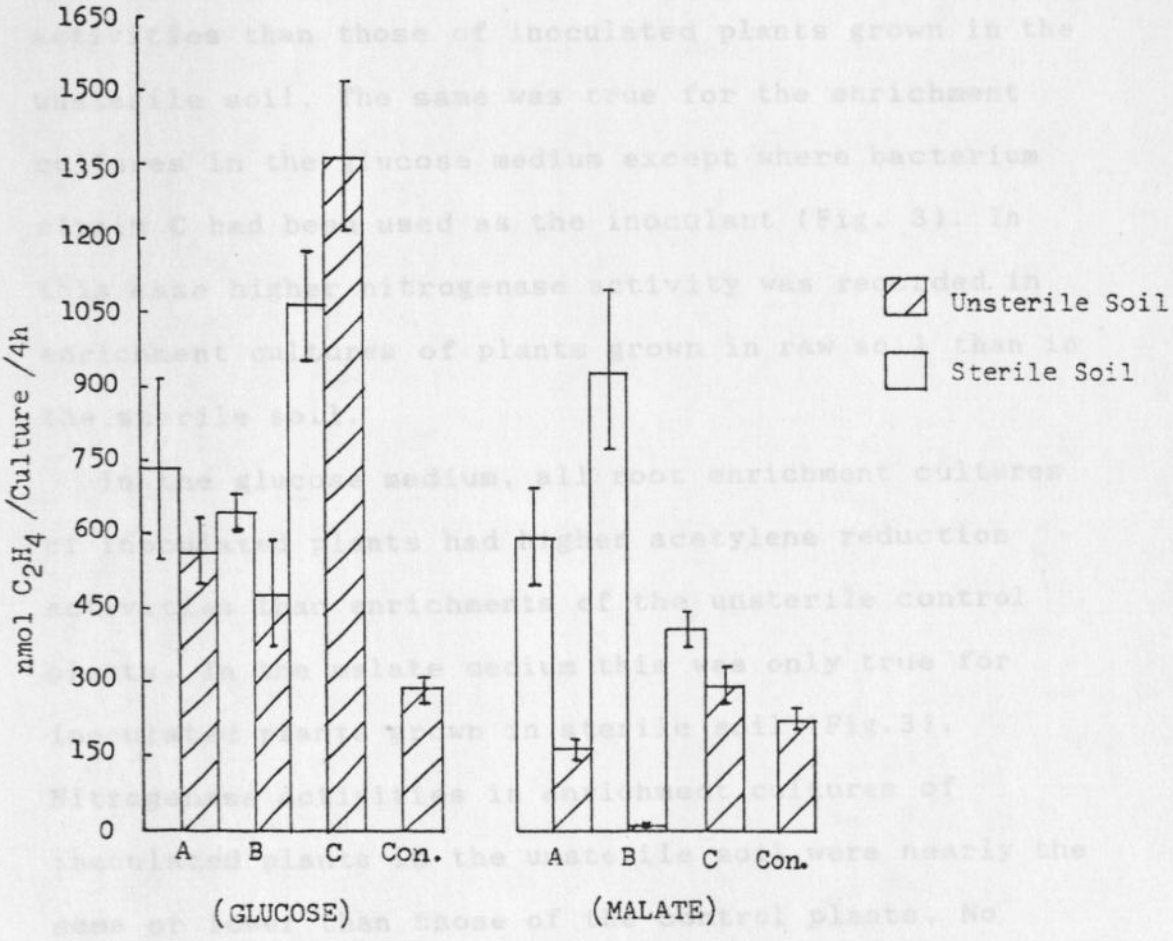

1650
1500
1350
1200
1050
900
750
600
450
300
150
0
nmol C_2H_4 /Culture /4h
A
B
C
Con.
(GLUCOSE)
A
B
C
Con.
(MALATE)
Unsterile Soil
Sterile Soil

Fig.3

glucose root enrichment cultures. In the malate medium all enrichment cultures of inoculated plants grown in sterile soil recorded higher acetylene reduction activities than those of inoculated plants grown in the unsterile soil. The same was true for the enrichment cultures in the glucose medium except where bacterium strain C had been used as the inoculant (Fig. 3). In this case higher nitrogenase activity was recorded in enrichment cultures of plants grown in raw soil than in the sterile soil.

In the glucose medium, all root enrichment cultures of inoculated plants had higher acetylene reduction activities than enrichments of the unsterile control plants. In the malate medium this was only true for inoculated plants grown in sterile soil (Fig.3). Nitrogenase activities in enrichment cultures of inoculated plants in the unsterile soil were nearly the same or lower than those of the control plants. No acetylene reduction activity was detected in enrichment cultures of uninoculated plants grown in the sterile soil.

3.1.10 Influence of light on acetylene reduction activity associated with intact maize plants.

The time course of acetylene reduction by intact maize plants was determined over a 35 h period during

Fig. 4 Influence of light on acetylene reduction activity associated with intact maize plants.

Seedlings of maize cultivar LG11 were grown in large glass tubes containing soil sampled from the maize plot at Saby. Some of the seedlings were inoculated with bacteria strains A (O) or C (▽) and others were left uninoculated (□). One plant was grown in each tube. A light/dark cycle of 16/8h respectively and a temperature of 24 °C were maintained in the greenhouse. When the plants were 18 days old, they were assayed for acetylene reduction activity over a 35 h period during which light/dark/light conditions were imposed as detailed in methods section. Each point represents the mean acetylene reduction activity of five replicate plants.

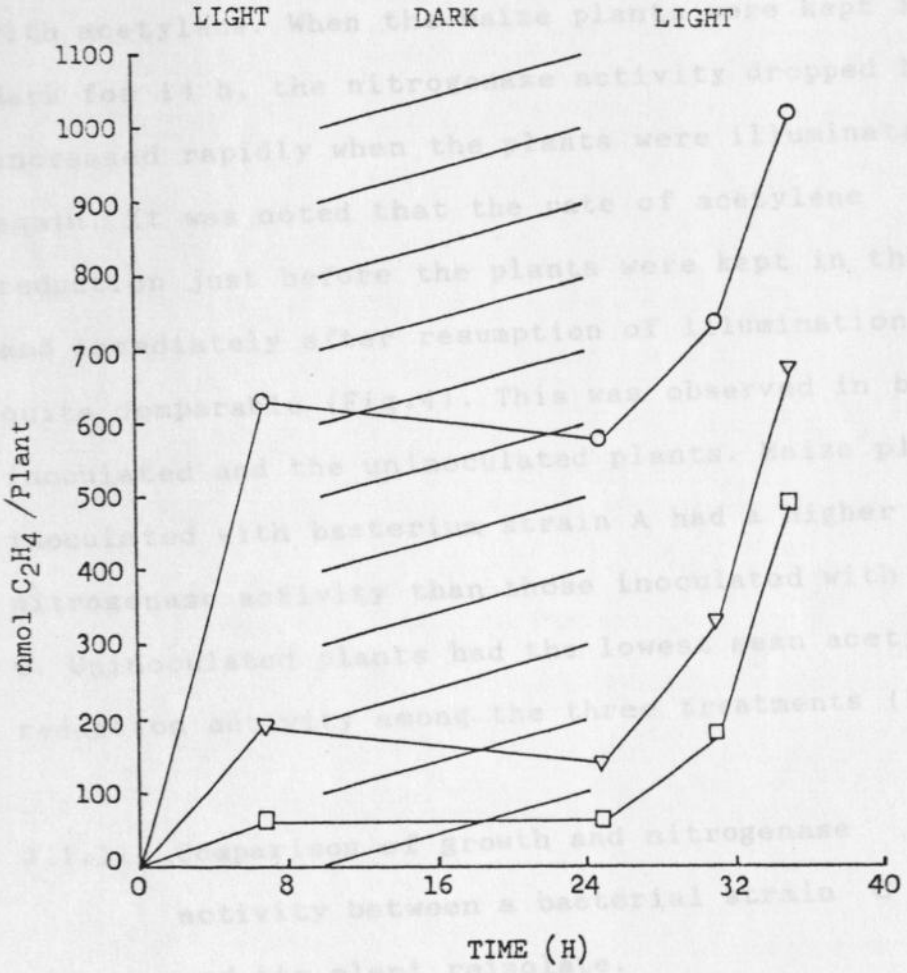

LIGHT
DARK
LIGHT
1100
1000
900
800
700
600
500
400
300
200
100
0
nmol C_2H_4 /Plant
0
8
16
24
32
40
TIME (H)

Fig.4

which light/dark/light conditions were imposed on the plants (Fig.4). Acetylene-dependent ethylene production was detectable within 2 h of commencement of incubation with acetylene. When the maize plants were kept in the dark for 14 h, the nitrogenase activity dropped but increased rapidly when the plants were illuminated again. It was noted that the rate of acetylene reduction just before the plants were kept in the dark and immediately after resumption of illumination were quite comparable (Fig.4). This was observed in both the inoculated and the uninoculated plants. Maize plants inoculated with bacterium strain A had a higher mean nitrogenase activity than those inoculated with strain C. Uninoculated plants had the lowest mean acetylene reduction activity among the three treatments (Fig.4).

3.1.11 Comparison of growth and nitrogenase activity between a bacterial strain C and its plant reisolate.

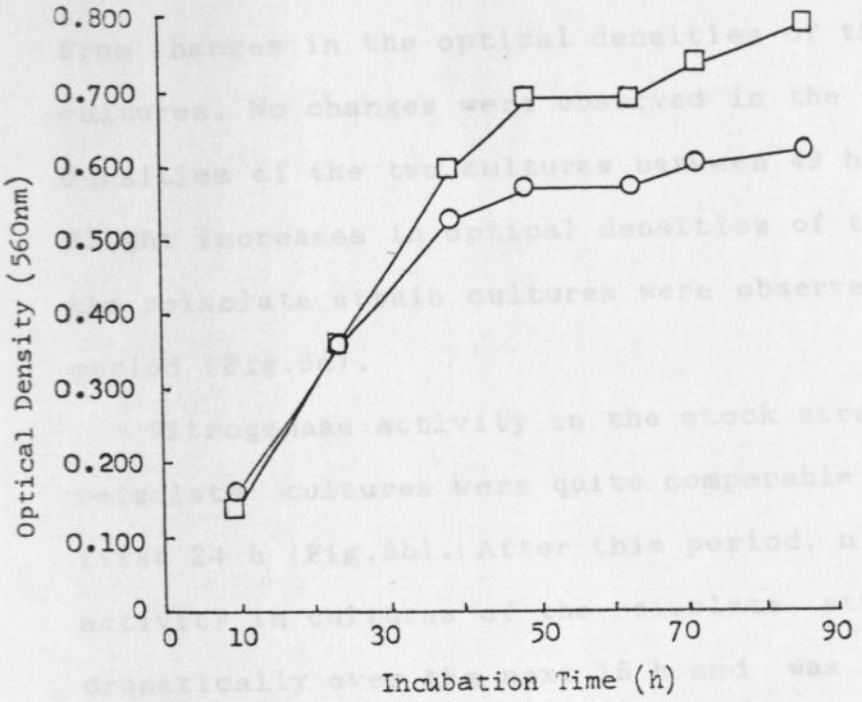
A study was carried out to examine the influence of a host maize plant on the growth and nitrogenase activity of a reisolate bacterial strain in a culture medium. During the first 24 h after inoculation, the stock strain and the plant reisolate had a similar growth rate (Fig.5a). However after this period, the reisolate grew faster than the stock strain as observed

Fig. 5 Comparison of growth and nitrogenase activity between a bacterial strain C and its plant reisolate.

a) Growth of strain C (O) and its plant reisolate (□) in a semisolid N-deficient glucose medium (0.05% agar) were compared by measuring optical densities of the two cultures at 560mm over a period of 86h. Results are means of three replicates.

b) Nitrogenase activities of strain C (O) and its plant reisolate (□) in a semisolid N-deficient glucose medium (0.175% agar) were determined under aerobic conditions as detailed in methods section. Results are means of three replicate cultures. All cultures were incubated at 28°C.

a) Growth



b) Nitrogenase activity

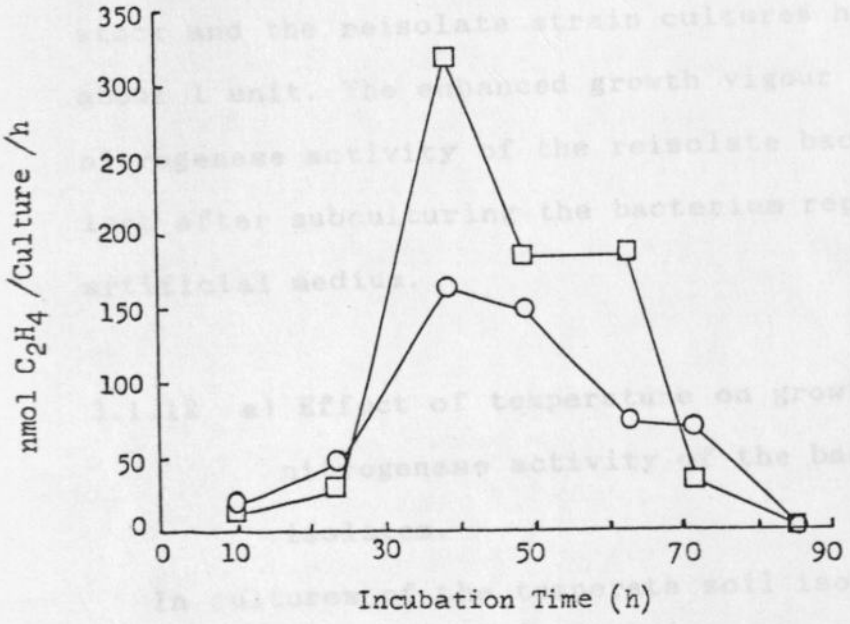


Fig.5

from changes in the optical densities of the two cultures. No changes were observed in the optical densities of the two cultures between 49 h and 62 h. Slight increases in optical densities of the stock and the reisolate strain cultures were observed after this period (Fig.5a).

Nitrogenase activity in the stock strain and the reisolate cultures were quite comparable during the first 24 h (Fig.5b). After this period, nitrogenase activity in cultures of the reisolate strain increased dramatically over the next 15 h and was nearly twice that detected in the stock culture. The acetylene reduction activities of the two cultures then declined gradually to undetectable levels. At the end of the experiment, the pH of the growth medium in both the stock and the reisolate strain cultures had dropped by about 1 unit. The enhanced growth vigour and nitrogenase activity of the reisolate bacterium was lost after subculturing the bacterium repeatedly in artificial medium.

3.1.12 a) Effect of temperature on growth and nitrogenase activity of the bacterial isolates.

In cultures of the temperate soil isolates, the highest rates of acetylene reduction activity were

Fig. 6 Effect of temperature on nitrogenase activity and growth of the temperate soil isolate A.

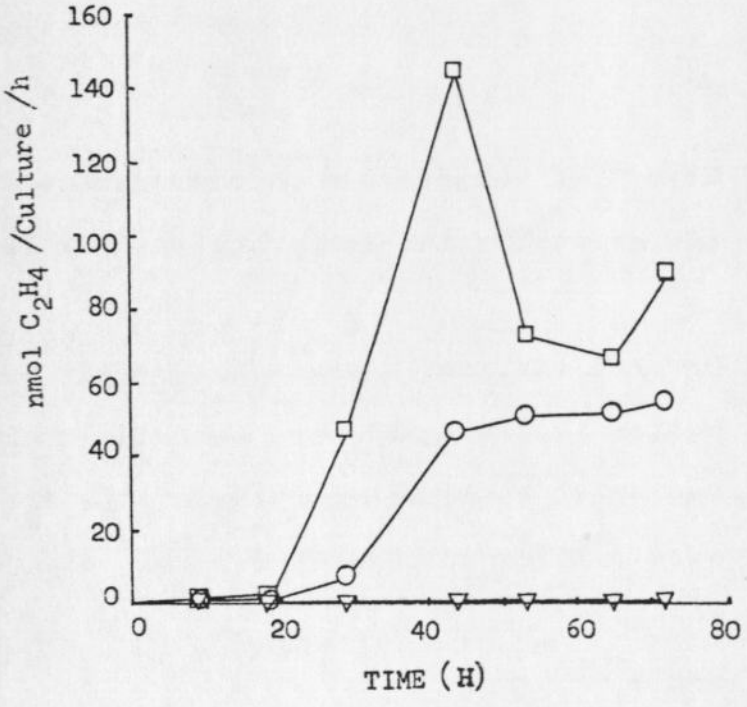
Several vials of a semisolid N-deficient glucose medium (0.05% agar) were inoculated with a bacterial suspension of isolate A. The cultures were divided into three batches which were incubated aerobically at 24°, (○), 28° (□) and 37°C (▽).

a) Nitrogenase activity:

At suitable intervals, triplicate cultures were randomly selected at each incubation temperature and assayed for acetylene reduction activity.

b) Growth: Immediately after each nitrogenase activity assay, the optical densities of the cultures were determined at 560nm using a spectrophotometer.

a) N_2 -ase activity



b) Growth

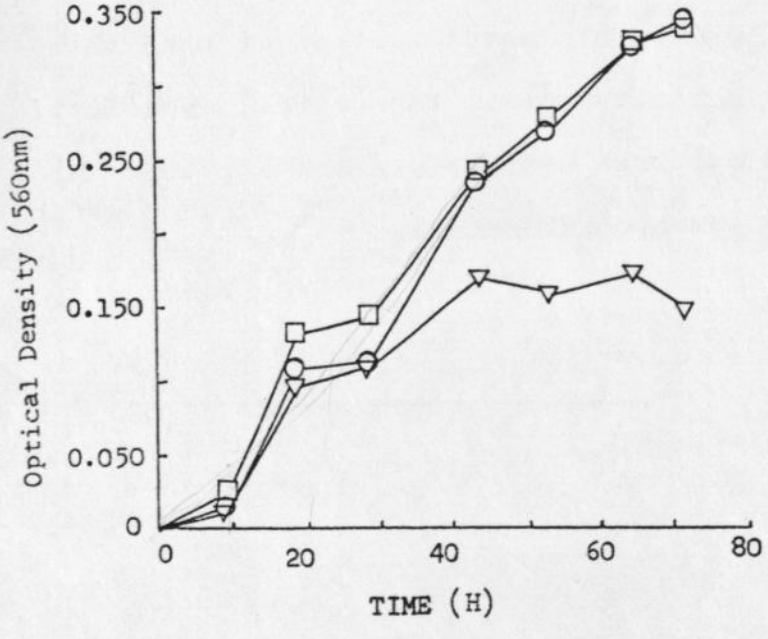


Fig.6

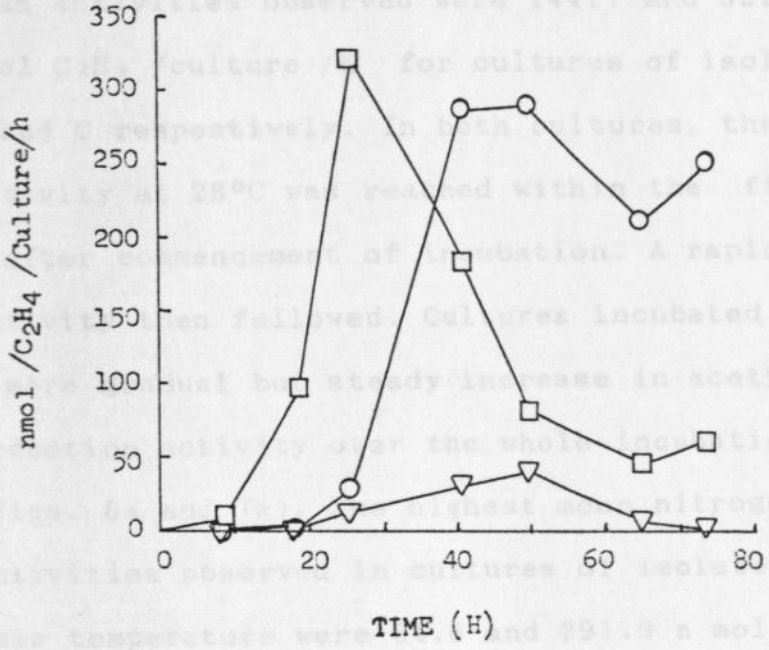
Fig. 7 Effect of temperature on nitrogenase activity and growth of the temperate soil isolate C.

Several vials of a semisolid N-deficient glucose medium (0.05% agar) were inoculated with a bacterial suspension of isolate C. The cultures were divided into three batches which were incubated aerobically at 24°, (○) 28° (□) and 37°C (▽).

a) Nitrogenase activity: At suitable intervals triplicate cultures were randomly selected at each incubation temperature and assayed for acetylene reduction activity.

b)Growth: Immediately after each nitrogenase activity assay, the optical densities of the cultures were determined at 560nm using a spectrophotometer.

a) N_2-ase activity



b) Growth

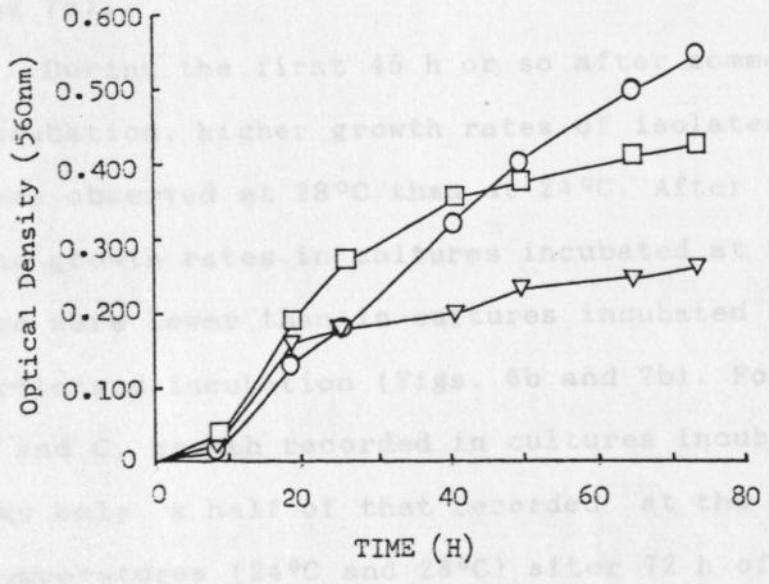


Fig.7

recorded at 28°C (Figs. 6a and 7a). The highest mean activities observed were 144.7 and 321.3 nmol C_2H_4 /culture /h for cultures of isolates A and C respectively. In both cultures, the peak activity at 28°C was reached within the first 53 h after commencement of incubation. A rapid decline in activity then followed. Cultures incubated at 24°C had a more gradual but steady increase in acetylene reduction activity over the whole incubation period (Figs. 6a and 7a). The highest mean nitrogenase activities observed in cultures of isolates A and C at this temperature were 54.6 and 291.9 n mol C_2H_4/ culture /h respectively. For both isolates, cultures incubated at 37°C had very low acetylene reduction activities throughout the experimental period (Figs. 6a and 7a).

During the first 45 h or so after commencement of incubation, higher growth rates of isolates A and C were observed at 28°C than at 24°C. After this period, the growth rates in cultures incubated at 28°C declined and were lower than in cultures incubated at 24°C after prolonged incubation (Figs. 6b and 7b). For both isolates A and C, growth recorded in cultures incubated at 37°C was only a half of that recorded at the lower temperatures (24°C and 28°C) after 72 h of incubation (Figs. 6b and 7b).

Fig. 8 Effect of pH on nitrogenase activity and growth of the temperate soil isolate A.

Several vials of a semisolid N-deficient malate medium (0.05% agar) with a pH range of 5 - 8 were inoculated with a bacterial suspension of isolate A. Four replicate vials were set up with the medium at each of the pH values : 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. All vials were incubated aerobically for 19 h at 28°C. The cultures were then assayed for nitrogenase activity (O) and their optical densities (□) determined at 560nm as detailed in methods section.

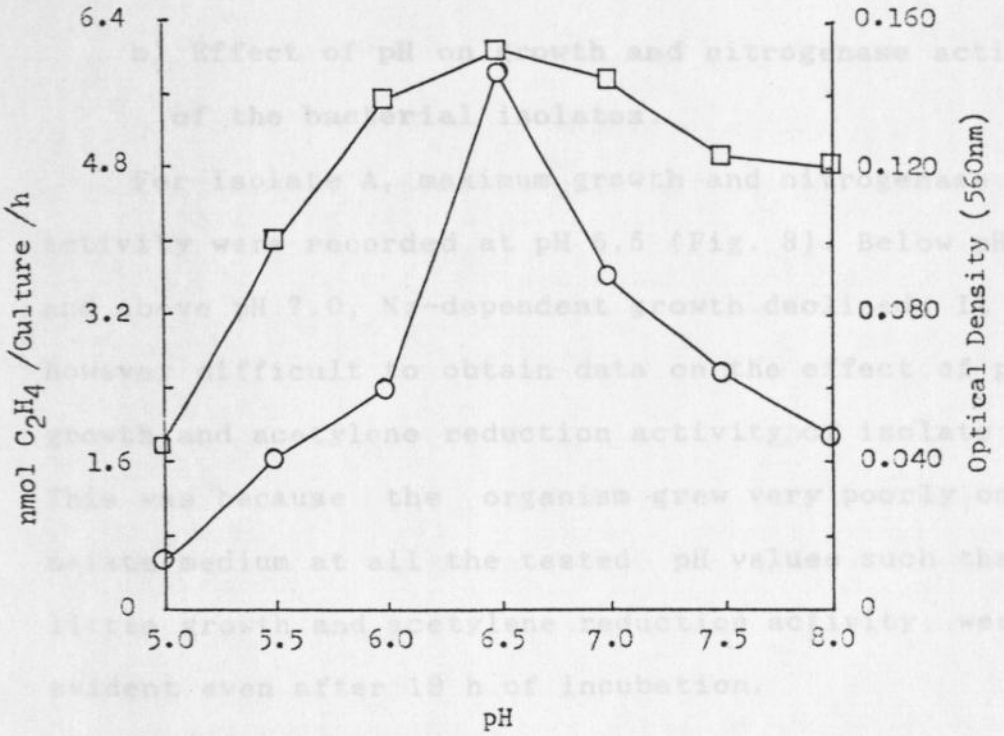

6.4
4.8
3.2
1.6
0
nmol C_2H_4 /Culture /h
0.160
0.120
0.080
0.040
0
Optical Density (560nm)
5.0
5.5
6.0
6.5
7.0
7.5
8.0
pH

Fig.8

b) **Effect of pH on growth and nitrogenase activity of the bacterial isolates.**

For isolate A, maximum growth and nitrogenase activity were recorded at pH 6.5 (Fig. 8). Below pH 6.0 and above pH 7.0, N_2-dependent growth declined. It was however difficult to obtain data on the effect of pH on growth and acetylene reduction activity of isolate C . This was because the organism grew very poorly on the malate medium at all the tested pH values such that little growth and acetylene reduction activity were evident even after 19 h of incubation.

3.2 Tropical Soil.

3.2.1 Effect of various environmental factors on acetylene reduction activity in the soil.

Temperature

Low rates of nitrogenase activity were generally detected in the aerobically incubated soil at 20°, 24° and 28°C (Table 7). Soil samples incubated at 24°C showed only a slight increase in activity over that recorded in samples incubated at 20°C. However when the incubation temperature was increased to 28°C, acetylene reduction activity in the soil increased substantially and was nearly double that recorded at 20°C after 72 h of incubation (Table 7).

Table 7. Effect of temperature on nitrogenase activity in a tropical soil.

Temperature (°C)	nmol C_2H_4 /* bottle		
	24h	48h	72h
20	6.8 ± 0.5	7.4 ± 0.0	7.5 ± 0.1
24	7.5 ± 0.1	7.5 ± 0.1	7.9 ± 0.1
28	9.5 ± 1.1	10.0 ± 0.8	13.7 ± 1.1

* each bottle contained approximately 7.5 g d.wt. soil.

Soil samples were put in 30 ml. bottles and moistened to about 50% water holding capacity with distilled water. The samples were then aerobically incubated under 10% acetylene as detailed in methods section. Some of the soil samples were incubated at 20°C, others at 24°C and another batch at 28°C. Results are means of acetylene reduction activity ± S.E. in four replicate samples.

Moisture

In the air-dried soil, no acetylene reduction activity was detected even after 72 h incubation under acetylene. Soil moistened to 40% WHC (water holding capacity) showed a substantial increase in activity compared to the air-dried soil. Additional wetting of the soil up to 60% WHC had no observable effect on the nitrogenase activity compared to that detected at 40% WHC (Table 8). A significant increase in acetylene reduction activity was however detected in the water-logged soil samples (Table 8).

Organic carbon

Soil amendment with a plant residue or glucose greatly stimulated the soil acetylene reduction activity. The plant residue consisting of macerated pith from dry maize stalks stimulated nitrogenase activity in the soil by about twenty times within the first 24h. Prolonged incubation of the amended soil samples for 72h resulted in increased activity by nearly two thousand times over that of unamended soil (Table 9). Glucose stimulated the acetylene reduction activity to a lesser extent than the plant residue. Addition of sodium malate to the soil had little or no effect on the activity even after prolonged incubation under acetylene.

Table 8. Influence of moisture on the acetylene reduction activity in a tropical soil.

moisture level (WHC)	nmol C_2H_4 /*bottle 24h	48h	72h
air dried	0	0	0
40%	7.9 ± 1.3	8.9 ± 0.8	10 ± 0.8
60%	7.2 ± 1.7	8.3 ± 1.5	11.2 ± 1.1
water logged	10 ± 1.1	13.7 ± 2.6	16.4 ± 3.2

* each bottle contained approximately 7.5 g d.wt. soil.

WHC is the water holding capacity of the soil.

Soil samples in 30 ml bottles were moistened to various degrees using distilled water. All samples were aerobically incubated under 10% acetylene at 28°C. Results are means of acetylene reduction activity of four replicate samples ± S.E.

Table 9. Effect of soil amendment with carbon substrates on the acetylene reduction activity.

	nmol C_2H_4 /*bottle		
substrate	24h	48h	72h
glucose	12.9	1517.3	8568
malate	8.8	11.2	14.0
maize straw	189	7500.2	22539.8
control	9.1	10.6	12.9

* Each bottle contained approximately 7.5 g d.wt. soil.

The soil was well moistened with distilled water and put in 30ml bottles. The samples were then amended with 5% (w:w) glucose, sodium malate or macerated maize stalks. Nothing was added to the control soil samples. Acetylene (10%) was added to each sample and the bottles were incubated at 28°C. Results are means of nitrogenase activity in four replicate samples.

3.2.2 Acetylene reduction activity associated with intact maize plants.

In the tropical soil, acetylene reduction by the plant-soil system was immediate without a significant lag period (Fig. 9). Ethylene production was evident within the first hour of incubation under acetylene and the activity was linear for 6h. Considerable variations in acetylene reduction activities were observed amongst the plants (Appendix Table 3). Higher activities in the maize plant-soil systems were detected under aerobic than under anaerobic conditions (Fig. 9). The mean acetylene reduction activities after 6h of incubation were 44.6 and 29.6 nmol C_2H_4 /plant /h (cumulative values) under aerobic and anaerobic conditions respectively. Plant-free soil exposed to light had nitrogenase activities of up to 7.3 nmol C_2H_4 /tube /h while that covered with vermiculite had only trace levels of activity over the incubation period (Appendix Table 4). Maize plants incubated aerobically or anaerobically without acetylene addition had no detectable ethylene production activity.

Fig. 9 Acetylene reduction activity associated with intact maize plants in the tropical soil.

Maize seedlings cultivar LG 11 were sown in large glass tubes containing the tropical soil. One seedling was sown in each tube. The plants were grown for two weeks in a greenhouse with a dark / light cycle of 16 / 8 h respectively. The temperature was maintained at $21^{0} \pm 2^{0}C$. The plants were then assayed for acetylene reduction activity under aerobic (O) and anaerobic (●) conditions as detailed in methods section. Each point represents the mean acetylene reduction activity of ten replicate maize plants.

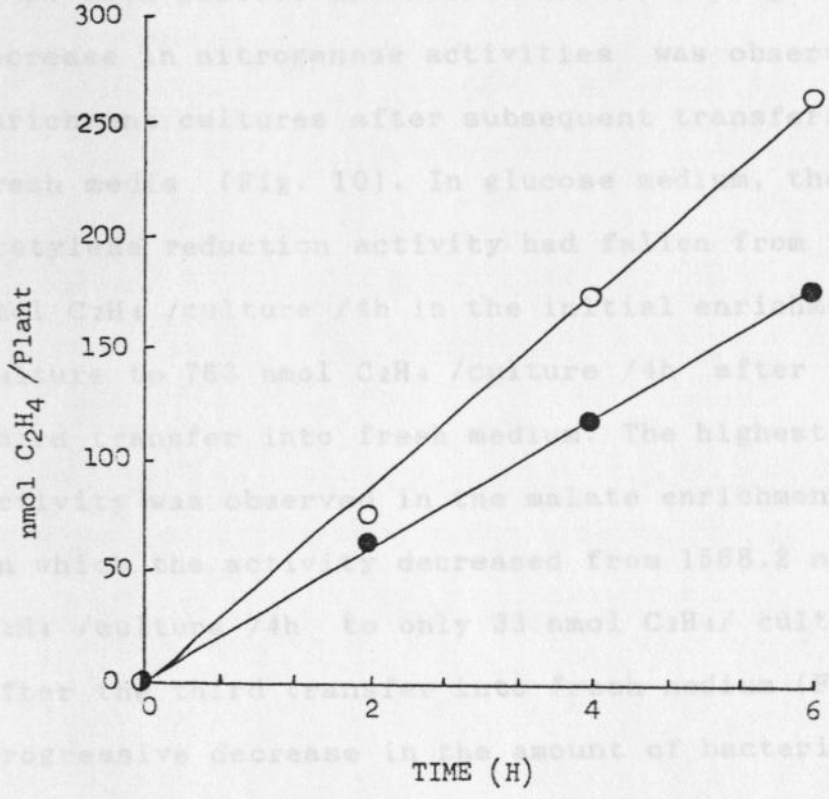


Fig.9

3.2.3 Nitrogenase activity in subcultured maize root enrichment cultures.

In both glucose and malate media, a progressive decrease in nitrogenase activities was observed in the enrichment cultures after subsequent transfers into fresh media (Fig. 10). In glucose medium, the acetylene reduction activity had fallen from 1586.6 nmol C_2H_4 /culture /4h in the initial enrichment culture to 763 nmol C_2H_4 /culture /4h after the third transfer into fresh medium. The highest loss of activity was observed in the malate enrichment cultures in which the activity decreased from 1568.2 nmol C_2H_4 /culture /4h to only 33 nmol C_2H_4/ culture/ 4h after the third transfer into fresh medium (Fig. 10). A progressive decrease in the amount of bacterial growth, with subsequent transfers into fresh media, was also visually detectable.

3.2.4 Isolation of putative N_2-fixing bacteria associated with maize plants.

High nitrogenase activities were observed in the initial enrichment cultures. Considerable losses in activity were observed after subsequent transfers into fresh medium during the purification process. Although a total of fourteen different types of colonies were observed in the first glucose and malate media plates

Fig. 10 Nitrogenase activity in subcultured maize root enrichment cultures.

Root pieces (each 0.5 cm long) from several acetylene-reducing maize plants were pooled together. Two root pieces were placed in each of twelve replicate vials containing semisolid N-deficient glucose medium. Other root pieces were similarly placed in vials containing semisolid N-deficient malate medium. All cultures were incubated at 28°C for 72 h after which three cultures with the highest nitrogenase activities in each medium were selected. Each selected culture was homogenised well and inoculum transfered into four vials of fresh medium. Two other cycles of incubation, selection and transfer into fresh media were repeated as before. Results are mean N_2-ase activities of twelve replicate cultures for the three successive enrichments. Bars represent standard error.

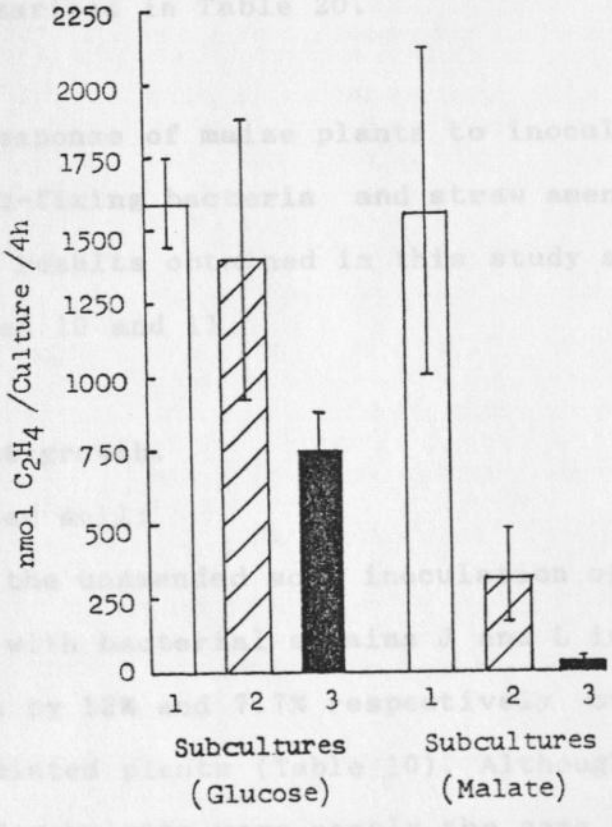

2250
2000
1750
1500
1250
1000
750
500
250
0
nmol C_2H_4 /Culture /4h
1
2
3
1
2
3
Subcultures
(Glucose)
Subcultures
(Malate)

Fig.10

to be streaked, only three types of colonies on glucose plates and one type of colony on malate plates had stable nitrogenase activities after repeated subculturing. In glucose medium, the isolates were largely dominated by acid-producing types of bacteria. Characteristics of the stable N_2-fixing isolates are summarised in Table 20.

3.2.5 Response of maize plants to inoculation with N_2-fixing bacteria and straw amendment.

The results obtained in this study are summarised in Tables 10 and 11.

Plant growth.

Fumigated soil:

In the unamended soil inoculation of the maize plants with bacterial strains J and L increased shoot lengths by 12% and 7.7% respectively over the uninoculated plants (Table 10). Although the total plant dry weights were nearly the same in both the inoculated and the uninoculated treatments, inoculation promoted shoot dry weights by 7.4 - 13.3%. Soil amendment with organic matter depressed the shoot/root ratio in all treatments and clearly favoured the formation of root biomass during the three week period. Inoculation with bacterial strains J and L raised the shoot dry

Table 10. Response of maize plants to inoculation with N2-fixing bacteria and straw amendment in a fumigated and an unsterile soil.

	Inoculant	shoot legth(cm)	shoot d.wt.(mg)	root d.wt.(mg)	S / R ratio
Fumigated soil	control	23.4 ± 1.7	143.9 ± 9.5	130.2 ± 7.2	1.105
	J	26.2 ± 1.2	154.6 ± 6.8	121.5 ± 14.7	1.272
	L	25.2 ± 0.9	163.0 ± 12.1	117.6 ± 10.9	1.386
Fumigated soil + straw	control	17.5 ± 1.7	135.5 ± 11.3	135.2 ± 9.0	1.002
	J	21.3 ± 1.5	152.8 ± 8.9	141.3 ± 15.9	1.081
	L	20.8 ± 0.8	141.5 ± 11.4	144.8 ± 6.3	0.977
Unsterile soil	control	23.8 ± 0.8	142.4 ± 6.3	96.9 ± 3.3	1.470
	J	25.9 ± 1.9	160.6 ± 12.3	111.5 ± 9.6	1.440
	L	24.2 ± 1.6	164.4 ± 17.8	116.3 ± 8.8	1.413
Unsterile soil + straw	control	18.7 ± 0.6	148.5 ± 8.5	158.3 ± 14.2	0.938
	J	19.9 ± 0.6	141.3 ± 11.5	151.3 ± 15.7	0.934
	L	19.5 ± 0.6	154.0 ± 7.6	160.1 ± 7.1	0.962

Seedlings of maize cultivar LG11 were inoculated with N2-fixing bacterial strains J and L and then sown in a fumigated soil and an unsterile soil with and without straw amendment. After growing in a greenhouse for a period of three weeks, the maize plants were harvested and their shoot lengths and shoot and root dry weights determined. Results are means of five replicate plants ± S.E.

weights by 12.8% and 4.4% respectively over control plants in the straw-incorporated soil (Table 10). Substantial increases in plant shoot lengths (18.9 - 21.7%) in the straw-amended soil were observed in inoculated plants (Table 10). It was however noted that soil amendment with organic matter depressed shoot elongation by between 21.2% and 33.7% during the experimental period.

Unsterile soil:

In unamended soil, bacterial strains Jand L raised the shoot dry matter yields of the inoculated maize plants by 12.8% and 15.4 % respectively over the uninoculated plants. It was however noted that the shoot/root ratios between the inoculated and the control plants were quite comparable (Table 10). Inoculation of maize plants with the bacteria also promoted root development.

Soil amendment with straw affected shoot dry matter yields negatively during the experimental period (Table 10). Converseley, addition of straw to the soil increased the formation of root biomass by 35.7 - 63.4% compared to the unamended soil. These large increases in root biomass in the straw-incorporated soil were also reflected in the depressed shoot/root ratios (Table 10). Little or no changes were observed in

total plant dry weights of inoculated plants compared to those of the controls.

Acetylene reduction activity.

In the unamended fumigated soil, inoculation with bacteria strains J and L failed to increase the number of nitrogenase positive plants and also acetylene reduction activity in individual plants. The highest acetylene reduction activity in the fumigated soil (951.5 nmol C_2H_4/plant /4h) was recorded among uninoculated maize plants after two weeks of growth (Table 11). In straw-amended soil, inoculation with the acetylene -reducing bacteria strains did not increase the incidence of nitrogenase activity among the plants. Heterotrophic nitrogen fixation activity in this soil was not stimulated by straw addition (Table 11).

In unamended unsterile soil, maize plants inoculated with the bacterium strain L had higher acetylene reduction activities than the control plants after two weeks of growth. However higher plant -associated nitrogenase activities (up to 3124 nmol C_2H_4 /plant /4h) were detected in uninoculated treatments in three-week-old maize plants (Table 11). Very high rates of acetylene reduction activity were observed in all plant-soil systems where the soil had been amended with maize straw (Table 11). Large

Table 11. Acetylene reduction activity associated with inoculated maize plants in a fumigated and an unsterile soil with and without straw amendment.

		nmol C2H4 /*tube /4h					
		Week 1		Week 2		Week 3	
	Inoculant	mean ARA	ARA range	mean ARA	ARA range	mean ARA	ARA range
Fumigated soil	control	38.7	0 - 79.2	211.2	0 - 951.5	53.1	0 - 260.7
	J	88.9	0 - 220	50.2	0 - 88	2.2	0 - 11
	L	15.8	0 - 79.2	20.2	0 - 88	1.3	0 - 6.6
Fumigated soil + straw	control	22.9	trace- 44	34.9	5.5 - 72.6	40.9	6.6 - 72.6
	J	40.5	22 - 105.6	55.8	5.5 - 104.5	0	0 - trace
	L	55.9	0 - 132	42.8	0 - 99	4.4	0 - 22
Unsterile soil	control	74.3	0 - 264	115.2	13.2 - 211.2	852.5	72.6 - 3124
	J	20.7	0 - 92.4	31.5	0 - 151.8	20.9	0 - 71.5
	L	8.8	0 - 33	133.3	16.5 - 495	17.6	trace- 59.4
Unsterile soil + straw	control	429	110 - 1293.6	8595.9	616 - 34215	666.4	264 - 1540
	J	123.2	13.2 - 275	187	33 - 324.5	541.4	6.6 - 1298
	L	121.4	22 - 356.4	221.5	27.5 - 534.6	102.3	33 - 242

* one maize plant was sown in each glass tube.

ARA = Acetylene Reduction Activity.

Seeds of maize cultivar LG11 were inoculated with bacteria strains J and L and sown in a fumigated and an unsterile soil with and without straw incorporation (5% w:w). Each treatment was replicated five times. The plants were grown in a green house for three weeks and assayed weekly for acetylene reduction activity.

variations in nitrogenase activity were noted within treatments. In the uninoculated treatment, the range of activity was 0.6 - 34 µmol C_2H_4 /plant /4h after two weeks. Inoculation with the bacteria did not increase the acetylene reduction activity associated with the plant-soil systems.

3.2.6 Nitrogenase activity in straw-amended soil over a prolonged period.

Very low acetylene reduction activity was detected in the unamended tropical soil during the six-week study period. During the first three weeks, only trace amounts of ethylene could be detected in the exposure vessels 4h after commencement of incubation under acetylene. Slightly higher rates of acetylene reduction activity were evident after the fourth week (Fig. 11). The activity however remained far below that detected in the straw amended soil throughout the study.

Soil amendment with organic matter greatly stimulated the acetylene reduction activity during the first two weeks (Fig. 11). A mean nitrogenase activity in excess of 6 µmol C_2H_4 /tube /4h was detected in straw-amended soil within this period. The level of activity remained generally high during the six-week study period. A gradual decline in the nitrogenase activity was however observed after the second week

Fig. 11 Nitrogenase activity in straw-amended soil over a prolonged period.

The tropical soil was mixed with macerated maize stalks (5% w:w) and 10g samples were transfered into large glass tubes. Other glass tubes were similarly prepared using unamended soil. All soil samples were moistened with sterile water and were incubated at 24°C for six weeks. At weekly intervals, both the amended (O) and unamended (●) soil samples were assayed for acetylene reduction activity as detailed in methods section. Bars represent standard error.

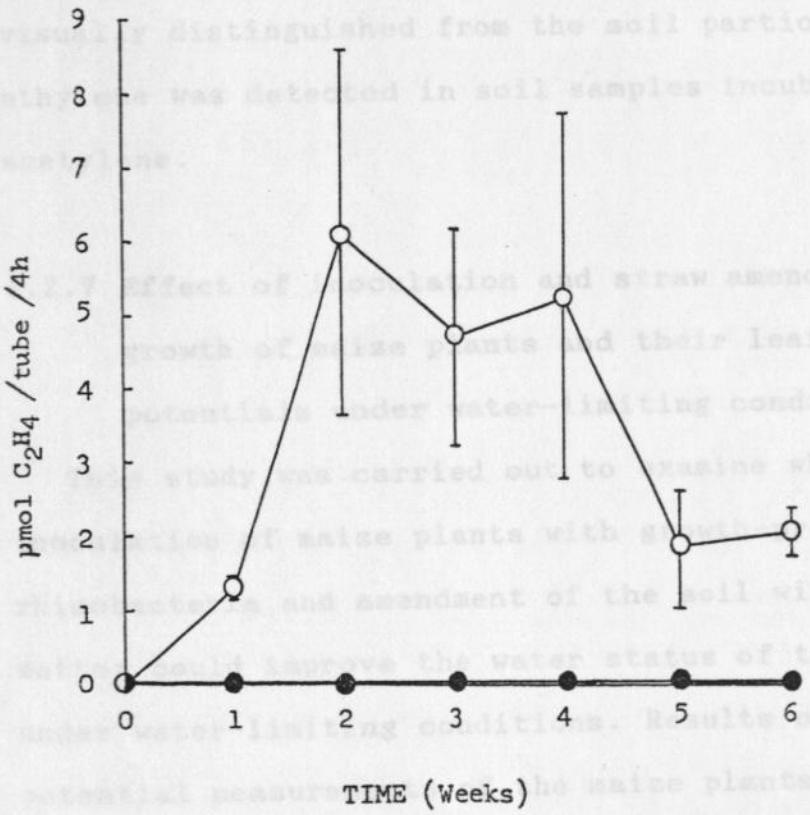


Fig.11

though small peaks were noted during the fourth and sixth weeks of incubation (Fig. 11). By the fifth week, the organic matter which had been added could not be visually distinguished from the soil particles. No ethylene was detected in soil samples incubated without acetylene.

3.2.7 Effect of inoculation and straw amendment on the growth of maize plants and their leaf water potentials under water-limiting conditions.

This study was carried out to examine whether inoculation of maize plants with growth-promoting rhizobacteria and amendment of the soil with organic matter could improve the water status of the plants under water-limiting conditions. Results of leaf water potential measurements of the maize plants during the experimental period are shown in Figs. 12a, b and c. For each cultivar, mean leaf water potentials of all watered plants were the same on any particular day regardless of the treatment. The leaf water potentials of all watered plants were also fairly constant during this period. Among the water-stressed treatments, maize plants grown in unamended soil started rolling their leaves by the seventh day and many had wilted by the thirteenth day from the day they were last watered. Rapid decreases in plant leaf water potentials were

Fig. 12 Effect of inoculation and straw-amendment on leaf water potentials of maize plants grown under water-limiting conditions.

Inoculated seedlings of maize cultivars a) LG 11, b) Katumani and c) Makueni were grown in unamended and straw-amended soil. C and L were the inoculant bacterial strains. Leaf water potentials were measured at suitable intervals for two weeks as detailed in methods section. Each point represents the mean leaf water potential of five replicate maize plants.

i) All watered treatments in unamended and amended soil: uninoculated plants; plants inoculated with C or L (○).

ii) Water stressed plants in unamended soil: uninoculated (⊙); inoculated with C (□); inoculated with L (▽).

iii) Water stressed plants in amended soil: uninoculated (●); inoculated with C (■); inoculated with L (▼).

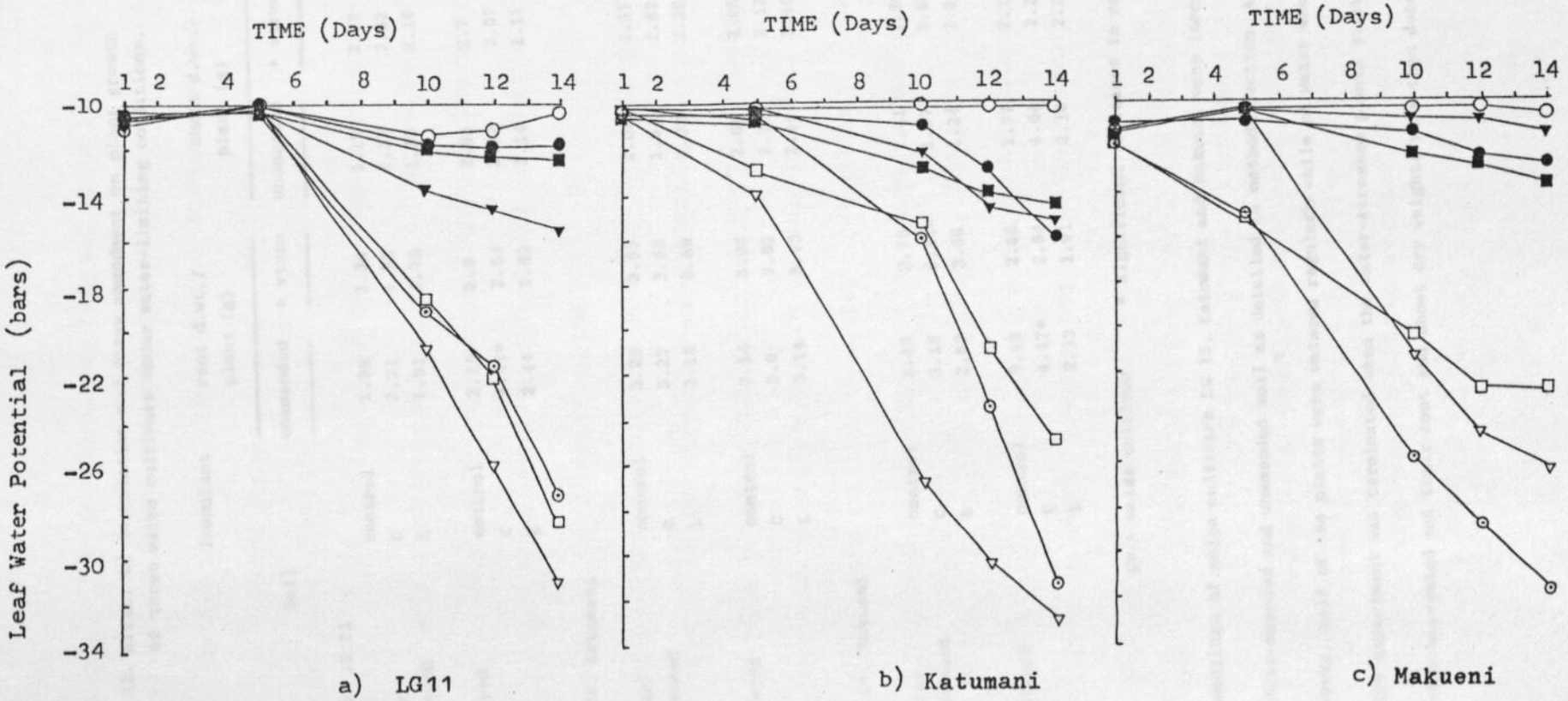

TIME (Days)
1 2 4 6 8 10 12 14
-10
-14
-18
-22
-26
-30
-34
Leaf Water Potential (bars)
a) LG11
TIME (Days)
b) Katumani
TIME (Days)
c) Makueni

Fig.12

Table 12. Effect of inoculation and straw amendment on plant growth of three maize cultivars under water-limiting conditions.

Soil	Inoculant	root d.wt./plant (g)		shoot d.wt./plant (g)	
		unamended	+ straw	unamended	+ straw
a) Cv. LG 11					
water stressed	control	1.96	3.34	1.15	2.28
	C	2.21	4.78	1.37	2.62
	L	1.91	3.78	1.38	2.16
watered	control	2.28	2.9	2.85	2.7
	C	4.41*	2.84	2.92	2.07
	L	2.44	2.82	3.14	2.11
b) Cv. Katumani					
water stressed	control	3.28	3.63	2.04	2.57
	C	3.27	3.83	1.94	2.82
	L	3.18	3.89	2.02	2.26
watered	control	3.54	3.08	2.05	2.05
	C	3.0	3.02	2.11	2.12
	L	3.14	3.73	2.46	3.40
c) Cv. Makueni					
water stressed	control	3.48	2.30	1.41	1.96
	C	3.16	5.84*	1.57	3.60
	L	2.60	3.66	1.24	2.61
watered	control	2.49	2.08	1.77	2.12
	C	4.17*	2.64	4.04	2.25
	L	2.33	1.91	2.10	2.24

Cv.= maize cultivar * significant increases in root d.wt. (P = 0.05).

Seedlings of maize cultivars LG 11, Katumani and Makueni were inoculated and sown in a straw-amended and unamended soil as detailed in methods section. After growing for three weeks, half of the plants were watered regularly while no water was added to the other half. The experiment was terminated when the water-stressed plants started wilting. The plants were harvested and their root and shoot dry weights were then determined.

observed in the three cultivars starting from the fifth day after watering was stopped (Figs. 12a, b and c).

Unlike plants grown in the unamended soil, most maize plants grown in soil amended with straw did not show leaf-rolling or any other signs of water stress until twelve or so days after watering had been stopped. The decrease in plant leaf water potential was relatively small and gradual during the experimental period (Figs. 12a, b and c). No significant differences in plant leaf water potentials were however detected between the inoculated and the corresponding control treatments in straw-amended soil.

Significant increases in root dry weights were observed in maize plants of cultivars LG11 and Makueni inoculated with the bacterium strain C (Table 12). Inoculation effects on root biomass and total plant dry weights were most pronounced in plants grown in unamended, watered soil and in straw-amended soil under water-limiting conditions. The increases in total plant dry matter (shoot and root) were particularly high for the Makueni cultivar for which increases in excess of 100% over the uninoculated treatment were observed (Table 12). For the Katumani cultivar, substantial increases in plant dry weight were recorded in watered plants inoculated with bacterium strain L in the straw amended soil.

3.2.8 Response of maize plants to inoculation with reisolates of a N_2-fixing bacterium.

Plant growth:

A study was carried out to test whether a maize plant bacterial reisolate could be more effective than the original isolate in promoting the growth and nitrogenase activity of inoculated maize plants. The plant reisolate (PR), the soil reisolate (SR) and the stock culture promoted growth of the inoculated maize plants (Table 13). These observations were made on plants grown on the semisolid water-agar medium, the fumigated and the unsterile soil. However, no significant differences in plant growth were observed between plants inoculated with the three cultures.

Inoculation effects were best seen in maize plants grown in the semisolid water-agar medium where shoot length increases of 17.5 - 32.5% and plant dry weight increases of 31.1 - 53.4% were observed compared to the uninoculated plants (Table 13). In the water-agar medium, inoculation visibly stimulated formation of root biomass. This was also reflected in decreased shoot/root ratios of the inoculated plants compared to those of uninoculated control plants. Proliferation of bacteria within the immediate vicinity of the roots was

Table 13. Response of maize plants to inoculation with reisolate strains of a N_2-fixing bacterium.

Growth medium	Inoculant	shoot length (cm)	shoot d.wt. (mg)	root d.wt. (mg)	%plant d.wt. increase	S / R ratio
semisolid agar	control	12	57.8	52.1	-	1.109
	SS	14.1	70.4	73.7	31.1	0.955
	PR	15.4	81.9	86.7	53.4	0.945
	SR	15.9	74.8	75.0	36.3	0.997
Fumigated soil	control	19	88.7	89.3	-	0.993
	SS	20.2	118.4	76.8	10.9	1.542
	PR	19.8	122.9	74.9	11.1	1.641
	SR	20.1	135.1	78.8	20.2	1.714
unsterile soil	control	16	85.7	66.9	-	1.281
	SS	19.6	115.6	73.1	23.7	1.581
	PR	19.4	117.1	77.1	27.3	1.519
	SR	19.6	113.1	73.9	22.5	1.530

No significant differences between SS, PR and SR inoculants in a particular medium ($P = 0.05$).

SS = stock strain; PR = plant reisolate; SR = soil reisolate.

Maize seedlings cultivar LG 11 were sown in test tubes with various media as detailed in methods section. The seedlings were inoculated with the stock strain (SS) and the two reisolates (PR and SS) of the original N_2-fixing isolate C. The plants were grown in a green house for two weeks. Plant shoot lengths, shoot and root dry weights were then determined. The results are means of six replicate plants.

also clearly visible in the semisolid water-agar medium.

Plant associated acetylene reduction activity:

Low acetylene reduction activities were detected in inoculated maize plants grown in the semisolid water-agar medium (Table 14). However no nitrogenase activity was detectable in uninoculated plants. Similarly, inoculated plant-free water-agar medium had no detectable activity. In fumigated and unsterile soils, inoculated plants had substantially higher acetylene reduction activities than uninoculated plants (Table 14). However, the plant reisolate was not more effective than the other cultures in increasing plant-associated acetylene reduction activity.

Bacteria numbers in the rhizosphere:

From the plate counts, it was evident that even uninoculated plants grown in the fumigated soil had high total bacteria numbers (1.8×10^8 CFU /g d. wt. root) in the rhizosphere. Higher total bacteria counts were however recorded in the rhizosphere of uninoculated plants grown in the unsterile soil (3.6×10^8 CFU /g d. wt. root) (Table 15). Inoculation with the three acetylene-reducing bacteria clearly increased the ratio of N_2-fixing bacteria to the

Table 14. Nitrogenase activity associated with maize plants inoculated with reisolates of a N_2-fixing bacterium isolate C.

Growth medium	Inoculant	nmol C_2H_4 /*tube /24h week 1	week 2
semisolid agar	control	0	0
	SS	1.5	11.4
	PR	0.9	7.9
	SR	0.5	9.4
Fumigated soil	control	11.8	40
	SS	237.7	306.4
	PR	20.8	132.9
	SR	12.9	100.9
Unsterile soil	control	14.6	19.4
	SS	0.7	50.1
	PR	168.6	182.8
	SR	23.6	367.3

* one maize plant was grown in each tube.

Maize seedlings cultivar LG 11 were sown in test tubes with various media as detailed in methods section. The seedlings were inoculated with the stock strain (SS) and the two reisolates (PR and SR) of the original N_2-fixing isolate C. The plants were grown in a green house for two weeks and assayed weekly for acetylene reduction activity. Results are means of six replicate plants.

Table 15. Counts of total and diazotrophic bacteria associated with maize plant roots inoculated with reisolates of a N_2-fixing bacterium.

Growth medium	Inoculant	CFU / g d.wt. roots: Total bacteria	CFU / g d.wt. roots: N_2-fixing bacteria
		x 10^8	x 10^7
semisolid agar	control SS,PR,SR	N D	N D
fumigated soil	control	1.8	0.6
	SS	2.9	1.7
	PR	2.2	2.4
	SR	2.6	1.9
unsterile soil	control	3.6	2.1
	SS	2.7	5.6
	PR	2.9	4.4
	SR	2.7	4.1

CFU = colony forming units; N D = not determined.
SS = stock strain; PR = plant reisolate; SR = soil reisolate.

Gnotobiotic maize seedlings cultivar LG 11 were sown in glass tubes with various media as detailed in methods section. The seedlings were inoculated with the stock strain (SS) or one of the two reisolates (PR and SR) of the original N_2-fixing isolate C. The plants were grown in a green house for two weeks. Total and diazotrophic bacteria associated with the plant roots were counted on nutrient agar and NFb-GLU medium respectively as detailed in methods section.

total bacteria numbers in the plant rhizosphere (Table 15). The number of nitrogen-fixing bacteria in the rhizosphere of plants inoculated with the two reisolates and the stock cultures were however quite comparable for each soil. The plant reisolate strain had no consistent competitive advantage in terms of root colonisation over the soil reisolate and the stock cultures.

3.2.9 Response of three maize cultivars to inoculation with N_2-fixing bacteria strains.

A preliminary study was carried out to examine the response to inoculation by various maize cultivars. The results are summarised in Tables 16 and 17. Improved vegetative growth was observed in maize cultivars LG11 and Katumani after inoculation with the bacteria. Substantial increases in plant height and shoot dry weights (3.1 - 34.3%) were recorded (Table 16). However little or no response to inoculation was observed in plants of the Makueni cultivar. Among the three inoculants tested, bacterial strain C was the most effective in increasing the shoot dry matter yield. With the exception of cultivar LG11 maize plants inoculated with bacterial strain C, inoculation did not affect the nitrogen content of maize plant shoots.

On yield parameters, the 1000-seed dry weight of

Table 16. Effect of inoculation on the vegetative growth and N-yield of three maize cultivars.

Cultivar	Inoculant	Plant height (cm)	Shoot d.wt. /plant (g)	% N	N-yield /plant (mg)
LG 11	control	130.2 ± 12.1	39.1 ± 4.9	0.35	136.9
	L	152.1 ± 5.4	51.1 ± 2.8*	0.34	173.7
	J	186.0 ± 5.6	47.7 ± 2.5	0.37	176.5
	C	180.3 ± 9.1	52.5 ± 4.4*	0.54	283.5
Katumani	control	184.7 ± 14.3	77.6 ± 7.0	0.65	504.4
	L	206.2 ± 6.6	80.0 ± 8.1	0.64	512.0
	J	225.5 ± 15.4	74.5 ± 6.4	0.61	454.5
	C	244.9 ± 9.2	97.2 ± 9.2*	0.57	554.0
Makueni	control	175.1 ± 8.9	75.4 ± 6.6	0.41	309.1
	L	196.8 ± 10	76.1 ± 7.3	0.39	296.8
	J	187.7 ± 11.9	80.0 ± 8.1	0.38	304.0
	C	162.7 ± 11.5	74.2 ± 6.0	0.39	289.4

*significant increases (P = 0.05).

Maize seedlings cultivars LG 11, Katumani and Makueni were inoculated with bacterial strains L, J and C as detailed in methods section. The seedlings were sown and the plants were grown to maturity. The plant height, shoot dry weight and N-content were then determined. Results are means of six replicate plants ± S.E.

Table 17. Effect of inoculation on yield parameters of three maize cultivars.

Cultivar	Inoculant	ear d.wt. /plant(g)	1000-seed d.wt.(g)	mean seed d.wt.(mg)	% crude protein
LG 11	control	57.3 ± 5.2	129.8	129.8	13.1
	L	56.7 ± 4.8	130.8	130.8	12.8
	J	54.0 ± 0.5	135.2	135.5	9.5
	C	67.7 ± 13.2	141.7	141.7	11.2
Katumani	control	32.9 ± 4.3	74.1	74.1	13.2
	L	38.4 ± 3.7	96.0	96.0	10.5
	J	43.4 ± 7.2	110.8	110.8*	11.6
	C	39.2 ± 4.1	76.5	76.5	14.1
Makueni	control	44.2 ± 1.9	81.6	81.6	9.7
	L	46.4 ± 10.7	112.9	112.9*	10.5
	J	49.8 ± 6.6	100.8	100.8	10.5
	C	42.8 ± 2.5	79.5	79.5	11.4

* significant increases (P = 0.05).

Maize seedlings cultivars LG 11, Katumani and Makueni were inoculated with bacterial strains L, J and C as detailed in methods section. The seedlings were sown and the plants were grown until mature. The plants were harvested and ear dry weight, 1000-seed dry weight and % crude protein in the grain were then determined. Results are means of six replicate plants ± S.E.

inoculated plants increased by up to 9.1%, 49.4% and 38.4% for cultivars LG11 , Katumani and Makueni respectively (Table 17). The most significant increases in seed dry weight were observed in maize plants inoculated with bacterial strains J and L. The crude protein content in the grain was however unaffected by inoculation except for the Makueni cultivar in which higher crude protein levels were recorded in grain from inoculated plants than in grain from the uninoculated control plants (Table 17). It was also noted that the inoculated maize plants generally matured slightly earlier than the control plants.

3.2.10 Response of wheat and oat plants to inoculation with the maize root isolates.

Wheat

Inoculation with the acetylene-reducing bacteria strains J, L and C increased plant shoot lengths by 16.7%, 20.7% and 8.3% respectively over the uninoculated plants during a three-week period. Inoculation of the wheat plants with strains L and C had little or no effect on the plant dry weights (Table 18). However, plants inoculated with bacterium strain J showed a 15.3% increase in plant dry weight compared to

Table 18. Response of wheat and oat plants to inoculation with maize-root N_2-fixing isolates.

Plant	Inoculant	shoot length (cm)	*plant d.wt.(mg)	% increase plant d.wt.
wheat	control	22.7 ± 0.5	125.4	-
	J	26.5 ± 16.7	144.6	15.3
	L	27.4 ± 0.4	125.1	0
	C	24.6 ± 0.7	126.1	0.5
oats	control	17.2 ± 0.8	151.5	-
	J	20.4 ± 0.9	164.4	8.5
	L	19.3 ± 1.2	158.6	4.6
	C	19.2 ± 0.6	155.7	2.7

Plant d.wt. increases not significant (P = 0.05).

* three plants grown in each tube.

Three pregerminated wheat and oat seeds were sown in each glass tube and inoculated with N_2-fixing maize-root isolates J, L and C as detailed in methods section. The plants were grown in a green house and were harvested after three weeks. Plant shoot lengths and dry weights in each tube were then determined. Results are means of four replicate tubes ± S.E.

the control plants.

Inoculation with the three bacterial strains J, L and C failed to increase plant-associated acetylene reduction activity in inoculated plants. Uninoculated wheat plants had a higher mean nitrogenase activity (326 nmol C_2H_4 /tube /22h) than the inoculated plants (54.5 - 244.5 nmol C_2H_4 /tube /22h) at the age of three weeks. In both inoculated and uninoculated plants the activity was observed to increase with plant age (Table 19).

Oats.

Plants inoculated with the bacterial strains J, L and C recorded shoot length increases of 18.6%, 12.2% and 11.6% respectively compared to the control plants. Inoculation with the three acetylene-reducing bacterial strains however did not increase the plant dry weights significantly during the three-week experimental period (Table 18).

Plant-associated acetylene reduction activity increased with plant age. When the plants were three weeks old, control plants had a mean acetylene reduction activity of 110 nmol C_2H_4 /tube /22h compared to 153 and 341.3 nmol C_2H_4 /tube /22h which were detected in plants inoculated with bacterial strains J and C respectively (Table 19). No increases

Table 19. Acetylene reduction activity associated with intact wheat (*T.aestivum*) and oat (*A.sativa*) plants inoculated with N_2-fixing bacteria.

Plant	Inoculant	n mol C_2H_4 /*tube /22h Week 1	Week 2	Week 3
Wheat	control	4.3	53.8	326.0
	J	90.0	117.5	243.0
	L	1.3	2.8	54.5
	C	3.0	6.0	244.5
Oats	control	trace	15.0	110.0
	J	40.0	83.8	153.0
	L	52.6	61.3	81.3
	C	171.0	216.0	341.3

* three plants were grown in each glass tube.

Three pregerminated wheat or oat seeds were sown in each tube and inoculated with N_2-fixing bacteria strains J,L and C. The plants were grown in a greenhouse and their nitrogenase activities were determined at weekly intervals for three weeks. Results are means of four replicate tubes.

in activity were noted after three weeks in oat plants inoculated with bacterium strain L compared to the uninoculated control plants.

3.2.11 Effect of temperature and pH on growth and nitrogenase activity of a tropical soil isolate.

Temperature

Among the three incubation temperatures (24°,28° and 37°C) the highest nitrogenase activity in cultures of the tropical soil isolate L was recorded at 37°C during the first 20h of incubation (Fig. 13a). Peak activity in cultures incubated at 28°C (698.5 nmol C_2H_4 /culture /h) and 24°C (432.6 nmol C_2H_4 /culture /h) were observed only after prolonged incubation periods. It was however noted that the acetylene reduction activity decreased rapidly at each of the three incubation temperatures and was barely detectable 60h after commencement of incubation.

Cultures incubated at 37°C recorded the highest growth rate for the first 20h of incubation. After this period, the growth rate declined to a level below that recorded at the lower incubation temperatures (24° and 28°C) (Fig. 13b).

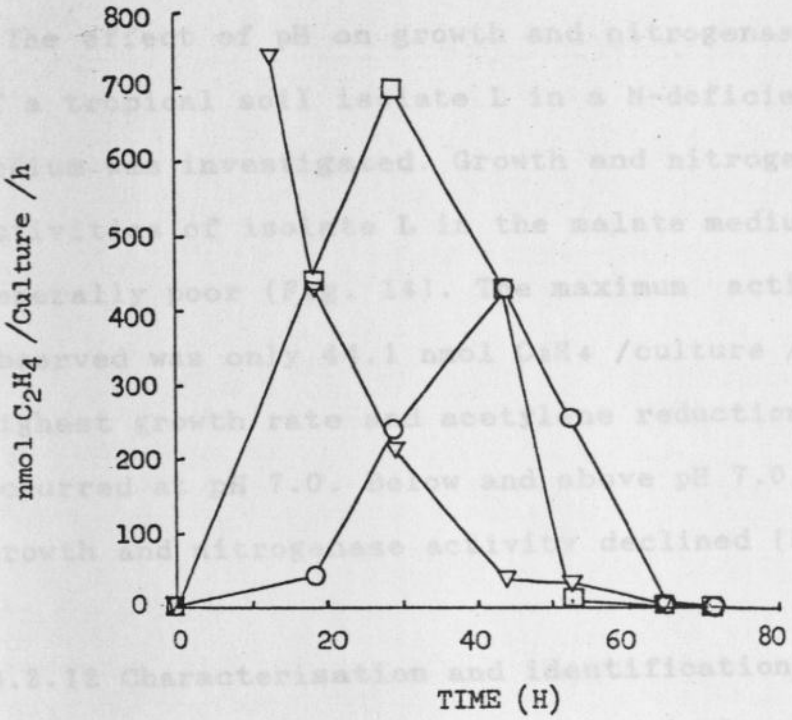
Fig. 13 Effect of temperature on nitrogenase activity and growth of a tropical soil isolate.

Several vials of a semisolid N-deficient glucose medium (0.05% agar) were inoculated with a bacterial suspension of isolate L. The cultures were divided into three batches which were incubated aerobically at 24° (○); 28° (□) and 37°C (▽).

a) Nitrogenase activity: At suitable intervals, triplicate cultures were randomly selected at each incubation temperature and assayed for acetylene reduction activity.

b) Growth: Immediately after each nitrogenase activity assay, the optical densities of the cultures were determined at 560nm using a spectrophotometer.

a) N_2-ase activity



b) Growth

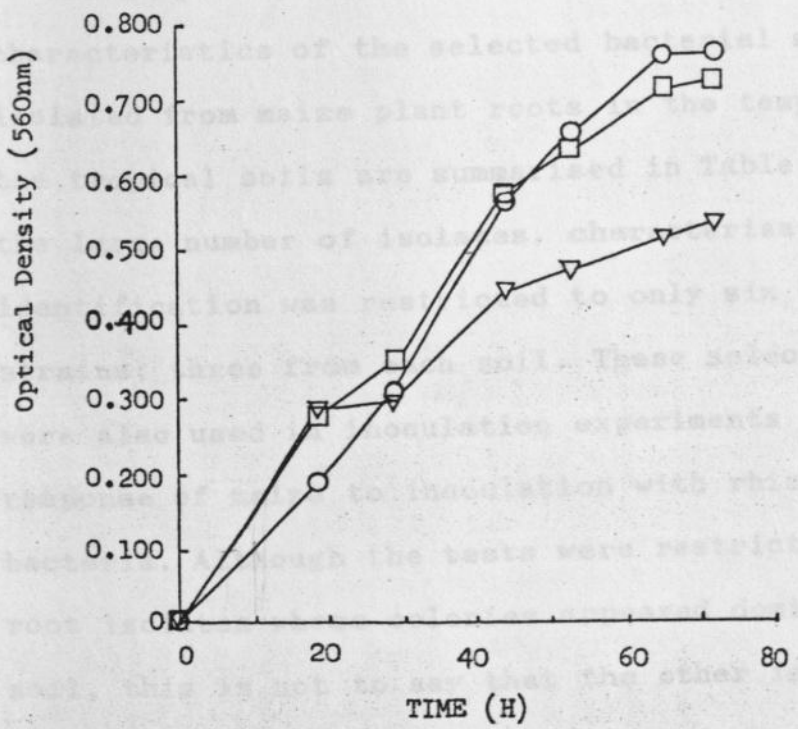


Fig.13

pH

The effect of pH on growth and nitrogenase activity of a tropical soil isolate L in a N-deficient malate medium was investigated. Growth and nitrogenase activities of isolate L in the malate medium were generally poor (Fig. 14). The maximum activity observed was only 44.1 nmol C_2H_4 /culture /h. The highest growth rate and acetylene reduction activities occurred at pH 7.0. Below and above pH 7.0, N_2-dependent growth and nitrogenase activity declined (Fig. 14).

3.2.12 Characterisation and identification of the N_2-fixing temperate and tropical soil isolates.

The morphological, cultural and biochemical characteristics of the selected bacterial strains isolated from maize plant roots in the temperate and the tropical soils are summarised in Table 20. Among the large number of isolates, characterisation and identification was restricted to only six bacterial strains; three from each soil. These selected strains were also used in inoculation experiments to study the response of maize to inoculation with rhizosphere bacteria. Although the tests were restricted to three root isolates whose colonies appeared dominant in each soil, this is not to say that the other isolates were

Fig. 14 Effect of pH on nitrogenase activity and growth of a tropical soil isolate.

Several vials of a semisolid N-deficient malate medium (0.05% agar) with a pH range of 5 - 8 were inoculated with a bacterial suspension of isolate L. Four replicate vials were inoculated at each of the pH values: 5.0, 6.0, 6.5, 7.0, 7.5 and 8.0. All vials were incubated aerobically for 19h at 28°C. The cultures were then assayed for nitrogenase activity (○) and their mean optical densities (□) determined at 560nm as detailed in methods section.

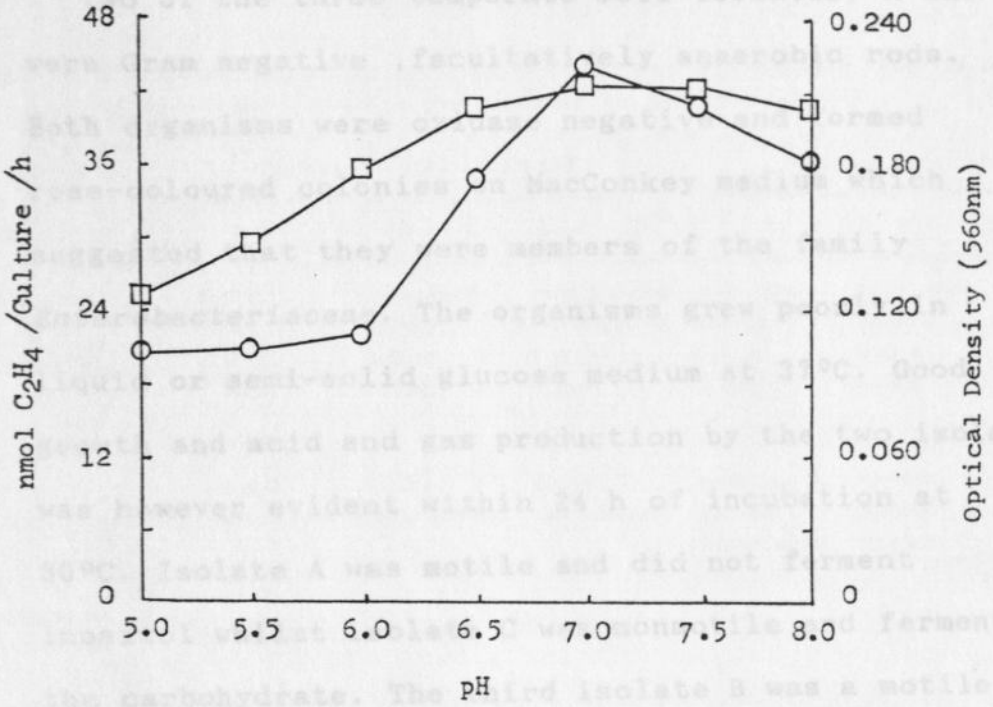

48
36
24
12
0
0.240
0.180
0.120
0.060
0
5.0
5.5
6.0
6.5
7.0
7.5
8.0
pH
nmol C_2H_4 /Culture /h
Optical Density (560nm)

Fig.14

of lesser significance to the maize plants.

Two of the three temperate soil isolates, A and C, were Gram negative ,facultatively anaerobic rods. Both organisms were oxidase negative and formed rose-coloured colonies on MacConkey medium which suggested that they were members of the family *Enterobacteriaceae*. The organisms grew poorly in liquid or semi-solid glucose medium at 37°C. Good growth and acid and gas production by the two isolates was however evident within 24 h of incubation at 30°C. Isolate A was motile and did not ferment inositol whilst isolate C was nonmotile and fermented the carbohydrate. The third isolate B was a motile, Gram negative aerobic rod which was oxidase positive. Neither acid formation nor gas production by the isolate were observed even after prolonged incubation at 30°C in semi-solid glucose medium. According to Cowan and Steel's Manual for the Identification of Medical Bacteria (Cowan, 1974), isolates A, B and C fall in the genera *Enterobacter, Pseudomonas* and *Klebsiella* respectively. Failure of isolate A to hydrolyse esculin suggests that isolate A is *E. cloacae*. The *Pseudomonas* and *Klebsiella* strains have yet to be identified to the species level.

The three selected tropical soil maize root isolates J, K and L were all facultatively anaerobic

Table 20. Characteristics of the putative N2-fixing bacteria isolated from the roots of maize plants grown in the temperate and the tropical soil.

Character or test	Temperate soil isolates			Tropical soil isolates		
	A	B	C	J	K	L
Cell shape	short rods	rods,some curved	rods,single /shortchain	rods,short chains	rods,some paired	short rods
Cell size	0.8 x 1.4µm	0.8 x 2.5µm	1.0 x 3.0µm	0.8 x 2.6µm	1.0 x 2.8µm	1.0 x 1.7µm
Motility	+	+	-	-	-	-
Grams test	-	-	-	-	-	-
Colony characteristics}	raised, translucent	flat, transparent	domed, translucent	smooth, raised, translucent	smooth, raised, opaque	domed, opaque.
Endospores	-	-	-	-	-	-
Growth on MacConkey agar}	+	ND	+	+	+	+
Catalase	(+)	+	+	+	(+)	+
Oxidase	-	+	-	-	-	-
Indole	-	ND	-	+	+	-
Citrate utilizition}	+	ND	-	+	+	-
Fermentation of:						
Glucose	+	-	+	+	+	+
Sucrose	+	-	+	+	+	+
Lactose	+	-	+	+	+	+
Mannitol	+	-	+	+	+	+
Maltose	+	-	+	+	+	+
Dulcitol	-	-	-	+	+	-
Inositol	-	ND	+	+	-	+
Esculin	-	-	+	+	+	+
Tentative identification.	*Enterobacter cloacae*	*Pseudomonas* sp.	*Klebsiella* sp.	*Klebsiella oxytoca*	*Citrobacter* sp?	*Klebsiella* sp.

All tests were carried out in accordance with Bergey's Manual of Determinative Bacteriology (1974) as detailed by Cowan (1974) and Collin's and Lyne (1984).

+ = positive; - = negative ; (+) = weak reaction; ND = not determined.

Gram negative rods. All three bacterial strains produced acid and gas when cultured on semi-solid or liquid glucose medium at 37°C. The isolates formed rose-coloured or brownish colonies on MacConkey medium which indicated that all were members of the family *Enterobacteriaceae*. Isolate K was motile but J and L were not. Isolate K was identified as a *Citrobacter* sp. rather than *Eschericia* sp. due to its ability to grow on citrate as the sole carbon source; while the fermentation of dulcitol by the isolate ruled out *Enterobacter* species. Bacterial strains J and L were identified as *Klebsiella* species from their biochemical and cultural characteristics. Isolate J was further identified as a strain of *K. oxytoca* due to indole formation (Cowan, 1974; Cakmacki *et al.*, 1981). The bacterial strain L has yet to be identified to the species level.

DISCUSSION

Maize roots excised from field-grown plants in temperate environments have been reported to reduce acetylene without preincubation under reduced oxygen tension (De-Polli *et al.*, 1982; Van Verkum and Sloger , 1981; Ela *et al.*, 1982). In a temperate soil of North America De-Polli *et al.* (1982) found that at the ear-development stage, excised maize roots assayed under air had nitrogenase activities in the order of 40 - 60 nmol C_2H_4/ g d.wt./ 24h. In this study, comparatively lower acetylene reduction activities were detected in excised roots of field - grown maize in a Swedish soil. Roots assayed under 5% and 20% oxygen without preincubation had activities of 20 and 9 nmol C_2H_4/ g d.wt. root respectively for the first 24h. It is however possible that some nitrogenase activity was lost, as has previously been reported (Dobereiner *et al.*, 1972), when the roots were washed under running water to remove the adhering mud prior to incubation under acetylene. Besides, the roots were assayed at harvest when root-associated nitrogenase activity in maize is reportedly on the decline (Neyra and Dobereiner, 1977 ; Albrecht *et al.*, 1981). In field-grown maize, it has been demonstrated that maximal nitrogenase activities occur during silk emergence and the onset of grain

-filling (Von Bullow and Dobereiner, 1975). Excised maize roots incubated under 0% oxygen had a lower acetylene reduction activity than roots incubated under 5% and 20% oxygen concentrations. Since maize plant roots incubated under 5% oxygen had the highest activity it is possible that the dominant N_2-fixing bacteria associated with the roots were microaerophiles or facultative anaerobes. In a similar study (De-Polli et al., 1982) excised maize roots incubated under 1% oxygen had a lower acetylene reduction activity than roots incubated under air.

In surface-sterilised maize roots, no acetylene reduction was detectable during the first 24h of incubation with acetylene. This may have been due to the elimination of the majority of N_2-fixing bacteria resident in the rhizoplane. O'Hara *et al.* (1981) found that washing maize roots did not significantly decrease the total number of bacteria or the number of N_2-fixing *Azospirillum* spp. associated with the roots. However surface sterilization of the roots greatly reduced the bacteria numbers indicating that endorhizosphere populations were at least one order of a magnitude lower than the total number of diazotrophs associated with the roots. In grasses, no specialized root structures harbouring associative N_2-fixing bacteria have yet been found

(Patriquin and Jain, 1983). Various reports indicate that proliferation of N_2-fixing bacteria occurs in excised roots of grasses during prolonged incubations (Barber *et al.*, 1976; Okon *et al.*, 1977; van Berkum, 1980). The observed increase in nitrogenase activity after prolonged incubation of the roots may have similarly been as a result of an increase in numbers of N_2-fixing bacteria present in the endorhizosphere; which had escaped the deleterious effects of the sterilising agent.

Figures available in the literature (Scott *et al.*, 1978; Diem and Dommergues, 1980; Magalhaes, Patriquin and Dobereiner, 1979; O'Hara *et al.*, 1981) indicate that the most probable number (MPN) putative nitrogen -fixing rhizosphere bacteria associated with maize roots range from 1.5×10^3 to 10^7 /g d.wt. root. Wright and Weaver (1981) also reported diazotrophic population densities in the range of 1×10^4 to 1×10^7/ g d.wt. root on the roots of various grasses, indicating the ubiquitous nature of such associations. Others (Barber *et al.*, 1976; Okon *et al.*, 1977; Watanabe *et al.*, 1979) have reported similar ranges of bacteria numbers. In comparison with other reports (Diem and Dommergues, 1980; O'Hara *et al.*, 1981) the endorhizosphere N_2-fixing population was relatively high (2.5×10^6

cells /g d.wt. root) in this study. This may be attributed to the senescent nature of the roots which may have made them more amenable to penetration by bacteria as has been suggested by Foster and Rovira (1976). Recently however, even higher endorhizosphere populations of N_2-fixing bacteria (7.3×10^7 cells/g d.wt. root) have been reported in Kallar grass, *Leptochloa fusca* (Reinhold, Hurek, Nieman and Fendrick, 1986).

Very low rates of nitrogenase activity were detected in both the temperate and the tropical soils. The results obtained indicate that heterotrophic nitrogen fixation in these soils is energy limited (Tables 4 and 9). This has previously been demonstrated in many soils particularly those with no readily available carbon source and generally those with a low organic matter content (Okafor and MacRae, 1972; Spiff and Odu, 1972; Chang and Knowles, 1965; Bristow, 1974; Mwatha, 1981). Amendment of such soils with a readily available carbon source greatly enhances non-symbiotic nitrogen fixation (Okafor,1977). In this study, malate had little or no effect on the nitrogenase activity in the tropical soil but stimulated the activity several fold in the temperate soil. Glucose however stimulated the acetylene redution activity much more in the tropical soil than in the temperate soil. These differences in

response to soil amendment with substrates may have been due to the difference in the type of diazotrophs dominant in the two types of soil. Failure of malate to stimulate N_2-ase activity in certain soils has previously been reported (Dobereiner, 1978). It has been suggested that glucose and other sugars increase soil acetylene reduction activity most effectively while malate is most effective in increasing the activity in excised roots (Boyle and Patriquin, 1980). Certain factors present in the plant material may have stimulated the proliferation of diazotrophs in the tropical soil. This could have resulted in the very high acetylene reduction activities which were detected in tropical soil amended with maize straw compared to that amended with glucose. The relatively high heterotrophic nitrogenase activity observed over a period of several weeks in straw amended soil could result in substantial nitrogen gains by the soil. This has previously been suggested in some parts of Australia where high levels of nitrogenase activity in the soil have been demonstrated after repeated incorporation of wheat straw (Roper, 1983).

Whereas air-dried tropical soil had no detectable nitrogenase activity, increase in moisture content of the soil stimulated the activity. It has previously been reported that there is a high correlation between

moisture content and nitrogenase activity in soils (Koch and Oya, 1974; Barber *et al.*, 1978; Balandreau and Ducerf, 1980; Lethbridge *et al.*, 1982). Increase in the soil moisture is known to increase total microbial numbers (Koch and Oya, 1974) and a suggestion has also been made that high soil moisture increases the availability of nutrients for soil bacteria (Lethbridge *et al.*, 1982). These factors together with the restriction of oxygen diffusion into the soil by water (Knowles, 1975), may account for the increased nitrogenase activities at the higher moisture levels.

A substantially higher nitrogenase activity, particularly after prolonged incubation periods, was detected in the tropical soil incubated at 28°C than at 20°C. This may be significant since soil temperatures below 25°C have been reported to be a major limiting factor to N_2-ase activity of field-grown *Digitaria decumbens* (Neyra and Dobereiner, 1977). Kapulnik *et al.* (1981a) have also shown that higher temperatures of up to 32°C enhance acetylene reduction in *Setaria-A.brasilense* associations. Kenya has mean temperatures of around 20°C in some of its main grain growing areas (Lusigi, 1978). Thus despite the fact that the country lies in the tropics, low temperatures may be a limiting factor in the fixation of atmospheric nitrogen by grass-bacteria associations.

This contention is supported by the fact that the optimum temperature for growth and nitrogenase activity of the Kenyan soil isolates was between 28°C and 37°C.

Immediate acetylene reduction activity by intact maize plants in nondestructive assays has previously been demonstrated (Albrecht *et al.*, 1977; Cohen *et al.*, 1980; Ela *et al.*, 1982). In the present study, acetylene reduction activity by intact maize plants in both the temperate and the tropical soil was immediate and linear for up to 6h. This was possibly due to the small size of the exposure vessels as well as use of small amounts of loosely packed soil which may have facilitated rapid equilibration of the gases in the air-soil-plant system. Lag periods in intact plant assays have been ascribed to the slow diffusion of acetylene to and ethylene from the active sites on or in the roots (Ela *et al.*, 1982). Similarly O'Hara *et al.* (1981) attributed their failure to observe *in situ* nitrogenase activity in maize plants to their large exposure vessels and to poor gaseous exchange in the soil. In the temperate soil, the mean nitrogenase activity recorded in three-week-old maize plants was 86 nmol C_2H_4 /plant /h. This was considerably lower than the mean activity (213.1 nmol C_2H_4 /plant /h) detected in same age maize

plants grown in the tropical soil at a similar temperature.

On a root dry weight basis, the mean nitrogenase activities of intact plants in the two soils were 430 and 2131 nmol C_2H_4 g d.wt. root/ h in the temperate and the tropical soil respectively. The latter figure compares well with acetylene reduction activities of maize seedlings grown in West African soils (Dommergues *et al.*, 1973). The mean acetylene reduction activity associated with intact maize plants in the Swedish soil was within the range of activity (77 nmol C_2H_4 /core /h) reported by Tjepkema and van Berkum (1977) in maize plant-soil cores in Brazil. Thus in this study, it was observed that the influence of soil type on nitrogenase activity associated with intact plants may be considerable. This has similarly been observed in some West African soils where nitrogenase activity associated with *Eleusine coracana* was a hundred times greater in one soil than in another (Dommergues *et al.*, 1973).

In general, acetylene reduction activities associated with two to three-week-old plants were low in both the tropical and the temperate soil. This was however not surprising since nitrogenase activity in maize has been shown to increase with plant age and to reach its peak during silking and the onset of grain filling (Von bullow and Dobereiner, 1975). Due to the

small amounts of loosely packed soil used in this study, it is likely that the soil-root system was well aerated. This could have resulted in aerobic and microaerophilic N_2-fixing bacteria becoming dominant over anaerobic N_2 fixers in the soil-root system. This would explain the higher nitrogenase activities observed under ambient than under anaerobic conditions. In biological nitrogen fixation, interest in aerobic organisms has generally been greater since aerobic metabolism is more efficient and because agricultural soils, with the exception of a few such as paddy soils, are well aerated (Neyra and Dobereiner, 1977).

Results obtained in this work indicate that plant function and rhizospheric nitrogen fixation may be closely related (Fig. 4). Whereas little or no plant -associated nitrogenase activity was detectable when the plants were placed in the dark, the activity was restored when lighting was resumed. De-Polli *et al.*(1982) reported that intact maize plants and excised maize roots had two different time course profiles of acetylene reduction activity over a 24h diurnal period. The nitrogenase activity associated with the intact plant-soil system practically stopped during the night while that of excised roots and detopped maize plants did not change. In maize *-Azospirillum* associations, it has been shown that nitrogenase activities are positively corelated with light

intensity in intact plants (Albrecht *et al.*, 1977). Recently, tight coupling of root-associated nitrogen fixation and plant photosynthesis has been demonstrated in the salt marsh grass *Spartina alterniflora* (Whiting, Gandy and Yoch, 1986). Dobereiner (1974) suggested that higher light intensities may lead to enhanced production of photosynthate which could increase root exudation and ultimately support increased nitrogen fixation by the rhizosphere diazotrophs.

In general inoculation of maize plants grown in the temperate soil, with three N_2-fixing bacterial strains previously isolated from the same soil, improved growth of the plants. The observed increases in shoot length (10%); dry matter yield (27%) and shoot nitrogen yield (35%) were appreciable, considering the short duration of the experiment. Reports on maize crop response to inoculation with N_2-fixing bacteria are very varied. Various instances have been reported where little or no increases in plant dry weight and N-content of inoculated maize plants has been observed (Albrecht *et al.*, 1977; 1981). On the other hand, significant increases in plant dry weight have been recorded after inoculating maize with diazotrophic bacteria (Cohen *et al.*, 1980; O'Hara *et al.*, 1981; Lin *et al.*, 1983). Cohen *et al.* (1980) reported increases of up to 55% in plant dry matter yield and

30% in shoot nitrogen content of maize plants inoculated with *Azospirillum* sp. Nur *et al.* (1980) similarly reported significant increases in the dry weight and the nitrogen content of inoculated maize plants compared to the uninoculated controls. In temperate environments, increases in the protein yield of maize plants have also been recorded after inoculation with diazotrophic bacteria (Vlassak and Reynders, 1981).

In the tropical soil, substantial increases in plant height and shoot dry weight were observed in maize plants inoculated with the nitrogen-fixing bacteria isolated from the temperate and the tropical soil. In the short green house experiments positive inoculation effects, including shoot dry weight increases of up to 15.4 %, were obtained. In large glass tubes containing soil amended with maize straw, inoculation effects were masked by soil toxicity apparently due to the decomposing straw.

It has previously been reported that microbial decomposition of straw may retard seedling establishment due to toxic effects and is one of the reasons why straw is burned before sowing in temperate countries (Lynch *et al.*, 1984). However this might not pose a serious problem in the tropics where decomposition rates are normally high. In Nigeria,

Jenkinson and Ayanaba (1977) reported that decomposition of a ^{14}C labelled plant material was four times higher than in England. The observed toxicity of straw-amended soil in the glass tubes may have been aggravated by poor aeration conditions since such effects were not observed in similar pot experiments.

Inoculation effects in the tropical soil were best seen in maize plants grown to maturity in large containers under uncontrolled conditions. In addition to the inoculated plants maturing slightly earlier than the controls, inoculation increased plant height and dry matter yields by up to 42.9% and 67.9% respectively. Earlier heading and flowering has similarly been observed in wheat, *Setaria italica* ,sorghum and *Panicum* sp.in the green house and in the field after inoculation with N_2-fixing bacteria (Kapulnik *et al.*, 1983; Yahalom *et al.*, 1984; Kapulnik *et al.*, 1981b; Lin *et al.*, 1983; Sarig *et al.*, 1984). Inoculation of the Makueni cultivar did not promote vegetative growth of the maize plants but the seed dry weight of inoculated plants increased by up to 38.5%. Large increases in ear dry weight (31.9%) and seed dry weight (49.5%) were also recorded for the Katumani maize cultivar after inoculation with the test bacterial strains. The observed differences in maize cultivar response to inoculation with the N_2-fixing

bacteria were remarkable in that the third cultivar LG11 responded best in terms of plant dry matter yield increases but recorded the lowest increase in seed dry weight among the three cultivars. Similar genotype effects have been established for maize (Hegazi *et al.*, 1983; Kapulnik *et al.*, 1981b) and other cereals (Millet, Avivi and Feldman, 1984). In Israel, Kapulnik *et al.* (1981b) reported seed and cob dry weight increases of 28.2% and 34.4% respectively in inoculated maize grown under field conditions. Few published reports on inoculation effects in maize and other cereals in Africa are presently available. In Egypt, Hegazi *et al.* (1983) reported increases of 150% - 170%, 180% - 270% , 120% - 130% for straw yield, grain yield and total-N yield respectively in maize inoculated with *Azospirillum* sp. under field conditions.

In the temperate soil, bacteria strains A and C were the most effective in increasing plant associated nitrogenase activity, while strains B and C were most effective in promoting shoot biomass. The increase in plant top nitrogen by strain B was noteworthy since plant-associated acetylene reduction activity was not increased above that of the control plants. Various published reports indicate that plant-associated nitrogenase activity in maize may be enhanced by

inoculation with diazotrophic bacteria (Kapulnik *et al.* , 1981b; Albrecht *et al.*, 1981; Hegazi *et al.*, 1983). In this study, the acetylene reduction activity *per se* associated with the maize plants was enhanced by inoculation with bacterial strains A and C. Maize plants inoculated with these two strains recorded up to 638 and 963 n mol C_2H_4 /plant /h respectively compared to the uninoculated plants which had nitrogenase activities of up to 194 n mol C_2H_4/ plant /h. The activities were however higher than those detected by Ela *et al.*(1982) (39 n mol C_2H_4 /plant /h) and Cohen *et al.*(1980) (410 n mol C_2H_4 /plant /h) in sterile vermiculite-maize plant or sand-maize plant systems inoculated with *Azospirillum* spp.

Unlike in the temperate soil, inoculation of maize plants in the tropical soil failed to increase plant-associated acetylene reduction activity above that observed in uninoculated control plants (Table 11). This was so even in the fumigated straw-amended soil where heterotrophic nitrogen fixation was not stimulated and could not therefore mask plant-associated nitrogenase activity. In a maize inoculation experiment, O'Hara *et al.* (1981) similarly reported significant increases in the dry weight and nitrogen yield of shoots but detected no enhancement of root-associated acetylene reduction activity above the control plants.

Gaskins and Hubbel (1979) observed increases in nitrogenase activity of inoculated plants but only when competing bacteria had been excluded from the system.

It is known that certain cultural practices such as the addition of straw to soil improves soil structure and increases soil biomass which together with organic residues constitute the main source of nutrients in the soil (Mulongoy, 1986). In this study, young maize plants grown in straw-amended soil had generally higher root biomass than plants grown in unamended soil (Tables 10 and 12). This enhancement of root development in straw-amended soil may have resulted in higher shoot dry matter yields observed in older maize plants (Table 12) possibly due to improved nutrient uptake by the plants (Lin *et al.*, 1983). Straw incorporation in the soil could also have promoted growth of the plants by improving the soil structure; availability of plant nutrients and the enhancement of biological nitrogen fixation as has previously been suggested (McCalla, 1945).

The improvement in water status of maize plants grown in straw-amended soil compared to plants grown in unamended soil was significant. Leaf water potentials of plants grown under water-limiting conditions in straw-amended soil remained relatively high compared to those observed in plants grown in unamended soil

(Fig.12). Lignin, resins and other similar molecules, all products of microbial degradation of organic matter, are known to stabilize soil and increase its capacity for water uptake (McCalla, 1945; Tisdall and Oades, 1982). Thus decomposition of maize straw in this study may have resulted in improvement of the water holding capacity of the soil compared to the unamended soil. This factor together with the mulch effect of the undecomposed organic matter (Mulongoy, 1986) in minimising evaporation from the soil surface , may have extended the period of water availability to the plants in the straw-amended soil.

Among inoculated plants grown in unamended soil under water-limiting conditions, plants inoculated with bacterial strain C had a somewhat better water status than the control plants (Fig.12). This may have been due to enhanced root development in the inoculated plants which may have improved water and nutrient uptake by the plants (Tien *et al.*, 1979). This inturn could have contributed to the improvement in growth of the plants as seen from the increased shoot dry weights compared to the uninoculated control plants. Okon (1984) has suggested that the water status of field-grown plants may similarly be favoured by inoculating the plants with *Azospirillum*.

Inoculation of wheat and oat plants with the

diazotrophic rhizosphere bacteria, originally isolated from maize plant roots, promoted increases in plant height in all cases. However little or no increase in plant dry matter yield and plant-associated nitrogenase activity were detected in inoculated treatments compared to the controls. Previously failure of wheat plants to increase their dry weight and nitrogen content even after inoculation with a N_2-fixing *Klebsiella oxytoca* has similarly been reported (Cakmacki *et al.*, 1981). However substantial yield increases in wheat and fodder oats due to inoculation with *Azospirillum* spp. and other N_2-fixing bacteria have been reported in India (Subba Rao, 1980; 1981; Rai and Gaur, 1982) and elsewhere (Reynders and Vlassak, 1982; Mertens and Hess, 1984; Millet and Feldman, 1984; Millet, Avivi and Feldman, 1984). Baldani and Dobereiner (1980) reported that maize and wheat were predominantly infected by labelled streptomycin-resistant *Azospirillum* species. They however noted that maize was preferentially infected by *A. lipoferum* while wheat selected for *A. brasilense* strains. Specificity of infection by *Azospirillum* species in plants with the C4-photosynthetic pathway has also been demonstrated (Rocha, Baldani and Dobereiner, 1981). Such specific plant -bacteria affinities may account for the poor responses to inoculation by wheat and fodder oats

inoculated with the maize-root isolates in the present study. It is possible that use of more specific bacteria isolated from the roots of these crops, as proposed by Subba Rao (1981), may be more effective in promoting growth of wheat and oat plants.

The increases in dry matter yield, nitrogen yield , ear dry weight and 1000-seed dry weight observed in various inoculation experiments in this study were substantial in many cases. Experimental evidence shows that inoculation of the maize plants with the selected bacteria strains clearly enriched the plant rhizosphere with diazotrophs (Table 15 and Fig. 3). Relative enrichment of diazotrophs has similarly been demonstrated in the endorhizosphere of plants inoculated with N_2-fixing bacteria (O'Hara et al., 1981; Patriquin *et al.*, 1979).

Beneficial effects of cereal inoculation have been ascribed to various factors. It has been suggested that the observed increase in growth and total nitrogen content of inoculated plants may result from the utilisation of the nitrogen fixed by the bacteria (Nur *et al.*, 1980; Kapulnik *et al.*, 1981b; Tien *et al.*, 1979). Experimental evidence has also shown that addition of a bacterial inoculum may cause yield increases if the plant assimilates nitrogen contained in the inoculum (Lethbridge and Davidson, 1983). Using

the ^{15}N isotope dilution method, Rennie (1980) estimated that up to 12.6% of maize plant nitrogen could be derived from associative nitrogen fixation by *A. brasilense* using plant exudates as the sole carbon source. However since only very small amounts of fixed nitrogen are incorporated into plant parts, it has been suggested that there is no direct transfer of N from the bacterium to the plant and that the bacteria have to die and be mineralised first before N can become available to the plants (Okon, 1984; Diem and Dommergues, 1980). This may explain the poor correlation between acetylene reduction activity and yield increases reported in some inoculation experiments (Smith *et al.*, 1978; Wright and Weaver, 1982).

Bacterial production of plant growth regulators (Tien *et al.*, 1979; Brown, 1982; Hartman *et al.*, 1983) has been proposed as the major mechanism through which inoculation promotes crop yields. Lin *et al.* (1983) observed production of more lateral roots and root hairs in pearl millet seedlings in response to inoculation with *A. brasilense*. In the present study, enhancement of root branching and root hair fomation were particularly evident in inoculated plants grown in semisolid water agar medium. Stimulation of root development and root hair formation

in inoculated plants has also been demonstrated in other graminacious plants (Tien *et al.*, 1979; Yahalom *et al.*, 1984; Lindberg *et al.*, 1985). A suggestion has been made that inoculated plants may react to bacterial colonization by producing plant-growth promoting factors (Kapulnik *et al.*, 1983; Okon, 1984).

It is noteworthy that inoculation effects are most pronounced in soils with low nitrogen status (Schank *et al.* 1981; Dobereiner and Baldani, 1981; Mertens and Hess, 1984; Fayez and Vlassak, 1984). Sarig *et al.* (1984) observed significant increases in N, P and K in *Sorghum bicolor* plants inoculated with *Azospirillum* compared to the control plants. The enhancement of root development due to inoculation may enable the plant to scavenge limiting nutrients and therefore improve the efficiency of applied mineral fertilizers (Rai and Gaur, 1982; Lin *et al.*, 1983). This was demonstrated by Kapulnik *et al.* (1981b) who obtained higher maize yields at intermediate levels of nitrogen fertilization in inoculated treatments than in the fully fertilized uninoculated plots. It is possibly due to the enhanced mineral uptake by plants that inoculation can partly replace valuable nitrogen fertilizer as has been demonstrated by various workers (Kapulnik *et al.*, 1981b; Smith *et al.*, 1976; Taylor,

1979; Rai and Gaur, 1982). Apart from enhanced mineral uptake by plants, other demonstrated inoculation benefits include increased plant tillering (Reynders and Vlassak, 1982) and favoured water status of inoculated plants (Okon, 1984). Inoculation may also benefit plants by competition and antagonism in the rhizosphere by the introduced bacteria against pathogens that inhibit root development (Suslow, 1982).

Nitrogenase activities in N_2-fixing bacteria enrichment cultures were invariably higher in glucose medium than in the malate medium. Similar observations have been reported in enrichment cultures of diazotrophic bacteria associated with various temperate cereals and forage grasses (Lindberg and Granhall, 1984b). This would appear logical since microorganisms in maize plant rhizospheres have previously been shown to prefer glucose rather than organic acids present in the plant root exudates as the carbon source (Kraffczyk *et al.* 1984). The observed loss of nitrogenase activity by some of the dominant colonies after axenic cultures were obtained is difficult to explain. However, it is noteworthy that similar losses of activity by diazotrophic rhizosphere bacteria, after purification of the cultures, has hitherto been reported (Lethbridge *et al.*, 1982; Lovett and Sagar, 1978; Lindberg and Granhall, 1984b). A

suggestion has been made that some of the N_2-fixing bacteria are lost during purification of the cultures leaving only associated non-fixing heterotrophs (Lindberg and Granhall, 1984b). In addition to this, *n i f* genes in these diazotrophs are bound to plasmids which may be lost if the bacteria are cultured under non-selective conditions (Guierinot and Patriquin, 1981). Various mutualistic and other analogous relationships between N_2-fixing bacteria and other microorganisms have also been described where the association creates conditions favourable for nitrogen fixation (Lind and Wilson, 1942; Hervey and Greaves, 1941; Line and Loutit, 1973; O'campo *et al.*, 1975; Jensen and Holm, 1975; Whitehead *et al.*, 1978; Lindberg and Granhall, 1984b). It is possible that some of these factors were responsible for the observed loss of nitrogenase activity during purification of the cultures.

The enhanced growth and nitrogenase activity of the N_2-fixing bacterium C after association with gnotobiotic plants was transient since it was lost after repeated subculturing of the bacterium in artificial medium. A similarly transient enhancement of nitrogenase activity has been demonstrated in a strain of *A. brasilense* cultured in association with sugarcane callus (Berg *et al.*, 1980). It is unclear

how association of the bacterium with the plant tissue improved the growth and nitrogenase activity of the bacterium. However in bacteria-callus tissue associations, it has been suggested that the callus may provide nutritional conditions which also enhance nitrogenase activity (Berg *et al.*, 1980). Similar effects have been proposed for *Rhizobium* grown on soybean cultures (Bednarski and Reporter, 1978) and for *Azospirillum* grown on several callus tissues (Child and Kurz, 1978). From the results obtained in the present study, it is apparent that repeated infection of the host plant with a rhizosphere bacterium does not confer a permanent advantage on the bacterium in terms of infectivity or improve its growth promoting properties in subsequent inoculations.

The optimum temperature for N_2-dependent growth of the temperate soil isolates was around 28°C. Although some growth occured at 37°C during the first 20h little growth was evident for the rest of the incubation period. The little growth observed during the initial incubation period may have been largely due to the small amounts of combined nitrogen due to the addition of 0.01% yeast extract and chemical contaminants in the growth medium. Very low levels of nitrogenase activity were detectable throughout the incubation period. The optimum temperature for growth

and nitrogenase activity of these temperate soil isolates compares well with that reported for N_2-fixing bacteria isolated from the roots of *Digitaria sanguinalis* (Barber and Evans, 1976) and wheat (Larson and Neal, 1978) in temperate environments (30°C). In contrast with the temperate soil isolates, the optimum temperature for growth and nitrogenase activity of the tropical soil isolates was around 37°C. This temperature was in the same range with the optimum temperature (32° - 40°C) of *Azospirillum* species responsible for nitrogen fixation in the roots of a tropical grass *Digitaria decumbens* cv. Transvala (Day and Dobereiner, 1976). It has been suggested that such temperature optima differences between temperate and tropical soil isolates may be of ecological significance (Day and Dobereiner, 1976). In temperate climates, soil temperatures are generally low for most of the year while in tropical soils they are relatively high all the year round.

It was interesting to find that the optimum pH range for N_2-dependent growth in both the temperate and the tropical soil isolates was between pH 6.0 and 7.5. Day and Dobereiner (1976) observed that nitrogenase activity in *Azospirillum lipoferum* was very sensitive to pH. These workers reported that

N_2- dependent growth occurred only between pH 5.5 and 9.0 even when the bacterium had been isolated from soil whose pH was seldom above 5.0. A similar range of pH requirement (5.5 - 8.0) has been reported for N_2-dependent growth of a *D. sanguinalis* root isolate (Barber and Evans, 1976); *Klebsiella oxytoca* (Cakmacki *et al.*, 1981) and *Bacillus azotofixans* (Seldin *et al.*, 1984). These observations render support to the suggestion by Day and Dobereiner (1976) that good growth and active N_2 fixation by diazotrophic bacteria does not occur in the soil but at the root surface or within the root where these specific pH requirements can be easily met.

The acetylene-reducing rhizosphere organisms isolated from maize plants grown in the temperate soil indicate that *Pseudomonas* species and members of the family *Enterobacteriaceae* may constitute an important part of the maize rhizosphere diazotroph population in this soil. Nitrogen fixation in the rhizosphere of some grasses in Northern Europe by *Pseudomonas* species has previously been reported (Haahtela, Kari and Sundman, 1983). Root-associated nitrogen fixation by this organism has also been demonstrated in flooded rice (Barraquio, Ladha and Watanabe, 1983). In their studies on the rhizosphere microflora of wheat and barley in a temperate

enviromnment, Kleeberger *et al.* (1983) reported that pseudomonads were prominent in both untreated and surface-sterilised roots. It is therefore not surprising that this organism appearsto have been one of the dominant organisms in the rhizosphere of field-grown maize in the temperate soil.

Apart from *Pseudomonas* species, certain members of the family *Enterobacteriaceae* have been shown to fix nitrogen in association with grass plants. In a Northern American subtropical enviroment, Wright and Weaver (1981) reported that the majority of the nitrogen-fixing bacteria isolated from the roots of various forage grasses were *Enterobacter cloacae* and *Klebsiella pneumoniae*. Nitrogen-fixing *Klebsiella pneumoniae* have been found in the rhizosphere of grasses in Finland (Haahtela *et al.*, 1981) and strains of N_2-fixing *E. cloacae, K. pneumoniae* and *Erwinia herbicola* have been isolated from the roots of wheat in temperate regions of North America (Nelson, Barber, Tjepkema, Russel, Powelson, Evans and Seidler, 1976; Pedersen *et al.*, 1978). Previously, nitrogen fixation by *E. cloacae* associated with the roots of maize has also been reported (Raju *et al.*, 1972). The isolation of *E. cloacae* and *Klebsiella* sp. from the roots of field-grown maize in the temperate soil, further

suggests that *Enterobacteriaceae* may be important rhizosphere diazotrophs for grasses in such environments. The low incidence of N_2-fixing *Azospirillum* species in temperate soils compared to warm tropical environments has previously been noted (Dobereiner *et al.*, 1976; Tyler *et al.*, 1979; Lindberg and Granhall, 1984b).

Klebsiella species and a *Citrobacter*-like organism appear to have been among the dominant N_2-fixing *Enterobacteriaceae* isolated from the maize plant roots in the tropical soil. Nitrogen-fixing *Klebsiella* species have previously been isolated from several sources including pulp mill effluents (Neilson and Sparell, 1976) and living wood (Aho, Seidler, Evans and Raju, 1974). A nitrogenase positive strain of *Klebsiella oxytoca* has also been isolated from wheat plant roots in Turkey (Cakmakci, Evans and Seidler, 1981) and more recently, N_2-fixing *E. cloacae* and *K. planticola* have been isolated from rice roots and leaf sheaths in the Philippines (Ladha, Barraquio and Watanabe, 1983). *Citrobacter* species have previously been isolated from roots of cereal crops using low nitrogen medium (Kleeberger *et al.*, 1983) and have been included among N_2-fixing members of the *Enterobacteriacea* in various reviews of asymbiotic nitrogen fixation (Sprent, 1979; Dalton,

1980). Reports on their capacity to fix atmospheric dinitrogen are however rare.

It is noteworthy that *Azospirillum* was not isolated from the roots of maize plants grown in the tropical soil. A vibrio-shaped isolate which formed a pellicle comparable to that formed by *Azospirillum* species in semi-solid malate medium had no detectable acetylene reduction activity. No further work was therefore carried out on this isolate. In their studies on the ecological distribution of *Azospirillum* species, Dobereiner *et al.*(1976), noted the rare occurrence of this organism in temperate regions and in soil and grass root samples collected from Nairobi area in Kenya. These workers also noted that Nairobi had an altitude of 1700 m and low mean night temperatures (13°C) to which they attributed the low incidence of *Azospirillum* in this area compared to warmer tropical environments. The tropical soil used in this study was collected in the Nairobi area which may explain why typical acetylene-reducing *Azospirillum* species were not isolated from the maize roots.

It has also been observed that the composition of the isolation and enumeration medium is an important factor when determining the species and numbers of N_2-fixing bacteria in soil or grass roots (Watanabe and Barraquio, 1979; Thomas-Bauzon, Weinhard,

Villecourt and Balandreau, 1982; Jagnow, 1986). Mwatha (1981) reported that *Beijerinckia, Derxia* and *Azotobacter* species were the dominant free-living N_2-fixing bacteria in soils sampled in a Nairobi area. However this worker used sucrose as the carbon source in the isolation medium which has previously been recommended for isolating N_2-fixing members of the *Azotobacteriacea* (Dobereiner, 1980). It is therefore possible that the isolation of *Enterobacteriaceae* as the dominant N_2-fixing microflora may partly be due to the composition of the isolation medium employed in the present study. Further biochemical tests as well as base composition and parallel serological studies with reference cultures, would be necessary for the complete identification and authentication of these N_2-fixing bacterial isolates.

CONCLUSION

The results obtained provide further proof that plants stimulate N_2 fixation in the soil and that plant associated nitrogenase activity in maize may be closely linked to the plants photosynthetic processes. Inoculation experiments showed that it was possible to enrich the maize plant rhizosphere with diazotrophic bacteria through inoculation. This suggests that the inoculant bacterial strains survived and were able to compete favourably with the normal soil microflora in the maize plant rhizosphere. It would be interesting to find out how long this enrichment of diazotrophs in the maize plant rhizosphere is likely to persist during the plants growth period.

In the Swedish soil, inoculation of maize plants with suitable N_2-fixing rhizobacteria could increase forage production appreciably without addition of chemical nitrogen fertilizers. A good selection of bacteria would however be necessary since plant associated nitrogenase activity was promoted by inoculation of the plants with some but not all N_2-fixing rhizobacteria. Although inoculation with the putative N_2-fixing bacteria increased the nitrogen yield of the maize plants considerably, it was mainly due to the increased dry matter yied of the inoculated plants rather than

their nitrogen content. Even in cases where inoculation increased plant associated nitrogenase activity, other factors such as phytohormone production by the bacteria and increased nutrient uptake by the plants may have also contributed to the increase in shoot dry weights of the maize plants.

Considering the significant increases in plant dry matter, grain and nitrogen yields obtained in the tropical soil, it is possible that the potential for increasing forage and grain production by maize through inoculation with suitable bacteria may be economically useful. This has already been demonstrated in Israel where extensive inoculation studies have been successfully carried out with maize (Kapulnik *et al.* 1981b; Kapulnik *et al.*, 1982) and sorghum (Sarig *et al.* 1984) on a commercial scale. The faster rate of growth observed in inoculated compared to uninoculated maize plants could be useful in many of Kenya's maize growing areas where rainfall periods are normally short. Inoculation in such cases could improve the chances of a maize crop maturing before the dry season sets in. The enhancement of root development due to inoculation with selected rhizobacteria may also minimise the need for high fertilizer dosage due to improvement in nutrient uptake by the plants. Further studies should be carried out to examine the response of maize plants

to the application of suitable bacterial inoculants and various levels of chemical nitrogen fertilizers.

The addition of straw to the tropical soil could be highly beneficial to maize growing. The observed improvement in water status of maize plants grown in straw-amended soil under water-limiting conditions may prove particularly useful for maize growing in marginal areas where rainfall is rarely adequate. In addition, significant savings in nitrogen fertilizer may be realised due to stimulation of biological nitrogen fixation by straw incorporation in the soil. Mineral constituents left behind in available form during decomposition processes could also be used by the plants. Additional investigations need to be carried out with a view to utilising maize straw as a substrate for cooperative nitrogen fixation. In Australia, the potential for using cellulose degrading bacteria and their interaction with *Azospirillum brasilense* in the degradation of wheat straw and associated nitrogen fixation has shown considerable promise (Halsall and Goodchild, 1986; Halsall and Gibson, 1986).

It is generally acknowledged that lack of consistent inoculation response is the major obstacle to the widespread use of bacterial inoculants for grass crops (Gaskins *et al.*, 1981; Smith *et al.*,

1984; Millet *et al.*, 1984). Further studies need to be carried out to better understand these plant-bacteria associations. The development of suitable plant cultivars and selection of highly effective bacterial strains to make inoculation effects reproducible under varied environmental conditions has previously been suggested (Rennie, 1981; Dobereiner and De-Polli, 1980). Substantial savings due to partial replacement of chemical fertilizers could be easily realised without compromising the quality and yield of the grain produced. This would be particularly useful in tropical countries where food production is low partly due to the unavailability of chemical fertilizers.

REFERENCES

ABD-EL-MALEK, Y. 1971. Free-living nitrogen-fixing bacteria in Egyptian soils and their possible contribution to soil fertility. *Plant Soil*, Special volume 423 - 442.

AHO, E.P., SEIDLER, R.J., EVANS, H.J. AND RAJU, P.N. 1974. Distribution, enumeration and identification of nitrogen-fixing bacteria associated with decay in living white fir trees. *Phytopathology* 64 :1413 - 1420.

ALBRECHT, S.L., OKON, Y. AND BURRIS, R.H. 1977. Effects of light and temperature on the association between *Zea mays* and *Spirillum lipoferum*. *Plant Physiol*. 60 :528 - 531.

ALBRECHT, S.L. ,OKON, Y., LONNQUIST, J. AND BURRIS, R.H. 1981. Nitrogen Fixation by corn -*Azospirillum* associations in a temperate climate. *Crop Sc*. 21 :301 - 306.

AOAC (Association of Official Agricultural Chemists) 1960. Methods of Analysis. Washington D.C.

BALANDREAU, J. AND DOMMERGUES, Y. 1973. Assaying nitrogenase (C_2H_2) activity in the field. In: Modern methods in the study of microbial ecology (ed. T. Rosswall), *Bull Ecol. Res. Comm*. (Stockholm), 17 :247-254. Quoted by

Lindberg and Granhall (1984a).

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), 243-268. Elsevier : Amsterdam.

BALANDREAU, J., DUCERF, P., HAMAD-FARES, I., WEINHARD, P., RINAUDO, G., MILLIER, C. AND DOMMERGUES, Y. 1978. Limiting factors in grass nitrogen fixation. *In*: Limitations and potentials for biological nitrogen fixation in the tropics (eds. J. Dobereiner, R.H. Burris and A. Hollaender) 275-302. Plenum Press : New York.

BALANDREAU, J., RINAUDO, G., HAMAD-FARES, I. AND DOMMERGUES, Y.1975. Nitrogen fixation in the rhizosphere of rice plants. *In*: Biological Nitrogen Fixation by free-living microorganisms (ed. W.D.P. Stewart) , 57 - 70. Cambridge University Press : Cambridge.

BALANDREAU, J. AND DUCERF, P.1980. Analysis of factors limiting nitrogenase (C_2H_2) activity in the field. *In*: Nitrogen fixation, vol. 11 (eds. W.E. Newton and W.H. Orme-Johnson), 229 - 242. University Park Press : Baltimore.

BALDANI, V.L. AND DOBEREINER, J. 1980. Host-plant specificity in the infection of cereals with *Azospirillum* spp. *Soil Biol. Biochem.* 12: 433 - 439.

BARBER L.E. AND EVANS, H.J. 1976. Characterization of a nitrogen-fixing bacterial strain from the roots of *Digitaria sanguinalis*. *Can. J. Microbiol.* 22 :254 - 260.

BARBER, L.E., TJEPKEMA, J.D., RUSSEL, S.A. AND EVANS, R.J. 1976. Acetylene reduction (nitrogen fixation) associated with corn inoculated with *Spirillum*. *Appl. Environm. Microbiol.* 32 :108 - 113.

BARBER, L.E., TJEPKEMA, J.D. AND EVANS, H.J. 1978. Acetylene reduction in the root environment of some grasses and other plants in Oregon. In: Environmental Role of Nitrogen-fixing Blue-green Algae and Asymbiotic Bacteria (ed. U. Granhall) *Ecol. Bull.* (Stockholm) 26 :366 - 372.

BARRAQUIO, W.L., DE-GUZMAN, M.R., BARRION, M. AND WATANABE, I. 1982. Population of aerobic heterotrophic nitrogen-fixing bacteria associated with wetland and dryland rice. *Appl. Environm. Microbiol.* 43 :124 - 128.

BARRAQUIO, W.L., LADHA, J.K. AND WATANABE, I. 1983. Isolation and Identification of N_2-fixing *Pseudomonas* associated with Wetland rice. *Can. J. Microbiol.* 29 :867 - 873.

BARRAQUIO, W.L. AND WATANABE, I. 1981. Occurence of aerobic Nitrogen-fixing bacteria in Wetland and dryland plants. *Soil Sc. Plant Nutr.*, 27 :121 - 125.

BEDNARSKI, M.A. AND REPORTER, M. 1978. Expression of rhizobial nitrogenase : influence of plant cell-conditioned medium. *Appl. Environm. Microbiol.* 36 :115 - 120.

BERG, R.H., TYLER, M.E., NOVICK, N.J., VASIL, V. AND VASIL, I.K. 1980. Biology of *Azospirillum* -sugarcane association : Enhancement of nitrogenase activity. *Appl. Environm. Microbiol.* 39 :642 - 649.

BERGERSEN, F.J. 1970. The quantitative relationship between nitrogen fixation and the acetylene - reduction assay. *Aust. J. biol. Sci.* 23,1015 - 1026. Quoted by Dommergues, Balandreau, Rinaudo and Weinhard (1973).

BERGEY'S MANUAL OF DETERMINATIVE BACTERIOLOGY 8TH EDN. 1974. Eds. R.E. Buchanan and N.E. Gibbons. The William and Wilkins Company : Baltimore.

BODDEY, R.M. AND DOBEREINER, J. 1984. Nitrogen fixation associated with grasses and cereals. *In*: Current developments in Biological Nitrogen fixation (ed. N.S. Subba Rao), 277 - 313. Edward Arnold : London.

BOUTON, J.H. AND ZUBERER, D.A. 1979. Response of *Panicum maximum* Jacq. to inoculation with *Azospirillum brasilense*. *Plant Soil* 52 :585 - 590.

BOYLE, C.D. AND PATRIQUIN, D.G. 1980. Endorhizal and exorhizal acetylene reduction activity in a grass *Spartina alterniflora* (Loisel) - diazotroph association. *Plant Physiol.* 66 :276 - 280.

BRISTOW, J.M.1974. Nitrogen fixation in the rhizosphere of fresh water angiosperms. *Can. J. Bot.* 52 :217 - 221.

BROWN, M.E. 1982. Nitrogen fixation by free-living bacteria associated with plants - fact or fiction ? *In*: Bacteria and plants (ed. M.E. Rhodes and F.A. Skinner), 25 - 41. Academic Press : London.

BURNS, R.C. AND HARDY, R.W.F. 1975. Nitrogen fixation in bacteria and higher plants. Springer Verlag : Berlin.

BURRIS, R.H. 1972. Measurements of Biological N_2 fixation with $^{15}N_2$ and acetylene. *In*: Techniques for the Assessment of Microbial Production and Decomposition in fresh waters (ed. Y.I. Sorokin and H. Kadota), 3-14. Blackwell Scientific Publications : Oxford.

BURRIS, R.H. 1974. Methodology. *In*: The biology of Nitrogen fixation (ed. A. Quispel), 9-33. North Holland: Amsterdam.

BURRIS, R.H. 1975. The acetylene reduction technique. *In*: Nitrogen fixation by free-living microorganisms (ed. W.D.P. Stewart), 249 - 257. Cambridge University Press : Cambridge.

BUTZ, R.G. AND LONG, R.C.1979. L-malate as an essential component of the xylem fluid of corn seedling roots. *Plant Physiol.* 64 :684 - 689.

CAKMACKI, M.L., EVANS, H.J. AND SEIDLER, R.J. 1981. Characteristics of nitrogen-fixing *Klebsiella oxytoca* isolated from wheat roots. *Plant Soil.* 61 :53 - 63.

CHANG, P.C. AND KNOWLES, R. 1965. Non-symbiotic nitrogen fixation in some Quebec soil. *Can. J. Microbiol.* 11 :29 - 33.

CHILD, J.J. AND KURZ, W.G.W. 1978. Inducing effect of plant cells on nitrogenase activity by *Spirillum* and *Rhizobium* in vitro. *Can. J.*

Microbiol. 24 :143 - 148.

COHEN, E., OKON, Y., KIGEL, J., NUR, I. AND HENIS, Y. 1980. Increase in dry weight and total nitrogen content in *Zea mays* and *Setaria italica* associated with nitrogen-fixing *Azospirillum* spp. *Plant Physiol.* 66 :746 - 749.

COLLINS C.H. AND LYNE P.M. 1984. Microbiological Methods, 5th Edn. Butterworths : London.

COWAN, S.T. 1974. Cowan and Steel's Manual for the Identification of Medical bacteria. 2nd ed. Cambridge University Press : Cambridge.

DAESCH, G. AND MORTENSON, L.E. 1972. Effect of ammonia on the synthesis and function of the N_2-fixing enzyme system in *Clostridium pasteurianum*. *J. Bacteriol.* 110 :103 - 109.

DALTON, H. 1980. The cultivation of Diazotrophic Microorganisms. *In*: Methods for Evaluating Biological Nitrogen Fixation (ed. F.J. Bergersen), 13 - 64. John Wiley and Sons : Chichester.

DANSO, S. 1986. Quantification of nitrogen fixation. *In* : Proceedings of the IFS workshop on "Biological Improvement of soil fertility". March 17-26, Dakar, Senegal.

DAY, J.M. 1977. Nitrogen-fixing Associations between Bacteria and Tropical grass roots. *In*:

Biological Nitrogen Fixation in Farming Systems of the Tropics (ed. A. Ayanaba and P.J. Dart), 273 - 288. John Wiley and Sons : Chichester.

DAY, J.M. AND DOBEREINER, J. 1976. Physiological aspects of N_2 fixation by a *Spirillum* from *Digitaria* roots. *Soil Biol. Biochem.* 8 :45 - 50.

DAY, J.M., HARRIS, D., DART, P.J, AND VAN BERKUM, P. 1975. The Broadbalk experiment. An investigation of nitrogen gains from nonsymbiotic nitrogen fixation. *In*: Nitrogen fixation by free-living microorganisms (ed. W.D.P. Stewart), 71 - 84. Cambridge University Press : Cambridge.

DAZZO, F.B. AND BRILL, W.J. 1978. Regulation by fixed nitrogen of host-symbiont recognition in the *Rhizobium* - clover symbiosis. *Plant Physiol.*62 :18 - 21.

DAZZO, F.B. AND MILAM, J.R. 1976. Serological studies of *Spirillum lipoferum*. *Soil Crop Sci. Soc. Fla. Proc.* 35 :122 - 126.

DAZZO, F.B. , NAPOLI, C. AND HUBBELL, D.H. 1976. Absorption of bacteria to roots as related to host specificity in the *Rhizobium* - clover symbiosis. *Appl. Environm. Microbiol.* 32 :166 - 171.

DE-POLLI, H., BOYER, C.D. AND NEYRA, C.A. 1982. Nitrogenase activity associated with roots and stems of field-grown corn (*Zea mays* L.) plants. *Plant Physiol.* 70 :1609 - 1613.

DIEM, H.G. AND DOMMERGUES, Y.R. 1980. Significance and improvement of rhizosphere N_2 fixation. *In*: Recent Advances in Biological Nitrogen Fixation (ed. N.S. Subba Rao), 190 - 226. Edward Arnold : London.

DOBEREINER, J. 1961. Nitrogen-fixing bacteria of the genus *Beijerinckia* Derx in the rhizosphere of sugar cane. *Plant Soil* 14 :211 - 217.

DOBEREINER, J. 1974. Nitrogen-fixing bacteria in the rhizosphere. *In*: The Biology of Nitrogen Fixation (ed. A. Quispel), 86 - 117. North-Holland : Amsterdam.

DOBEREINER, J. 1978. Influence of environmental factors on the occurrence of *Spirillum lipoferum* in soils and roots. *In*: Environmental Role of Nitrogen-fixing Blue-green Algae and Asymbiotic Bacteria (ed. U.Granhall) *Ecol. Bull.* (Stockholm) 26 :343 - 352.

DOBEREINER, J. 1980. Forage Grasses and Grain Crops. *In*: Methods for Evaluating Biological Nitrogen Fixation (ed. F.J. Bergersen), 535 - 555. John Wiley and Sons : Chichester.

DOBEREINER, J. AND BALDANI, V.L.D. 1981. Prospects for inoculation of grasses with *Azospirillum* spp. *In*: Associative nitrogen fixation vol. II (eds. P.B. Vose and A.P. Ruschel), 1 - 9. CRC Press Inc. : Florida.

DOBEREINER, J. AND DAY, J.M. 1975. Nitrogen fixation in the rhizosphere of tropical grasses. *In*: Nitrogen fixation by free-living microorganisms (ed. W.D.P. Stewart), 39 - 56. Cambridge University Press : Cambridge.

DOBEREINER, J. AND DAY, J.M. 1976. Associative symbiosis and free-living systems. *In*: Proceedings of the First International Symposium on Nitrogen Fixation (eds. W.E. Newton and C.J. Nyman), 518 - 538. Washington State University Press : Washington.

DOBEREINER, 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.

DOBEREINER, J. AND DE-POLLI, H. 1980. Diazotrophic rhizocoenosis. *In*: Nitrogen fixation (eds. W.D.P. Stewart and J.R. Gallon), 301 - 333. Academic Press : London.

DOBEREINER, J., MARRIEL, I.E. AND NERY, M. 1976. Ecological distribution of *Spirillum lipoferum*

Beijerinck. *Can. J. Microbiol.* 22 :1464 - 1473.

DOMMERGUES, Y.R. 1978. The plant - microorganism system. *In*: Interactions between non-pathogenic soil microorganisms and plants (eds. Y.R. Dommergues and S.V. Kruppa), 1 - 36. Elsevier : Amsterdam.

DOMMERGUES, Y., BALANDREAU, J., RINAUDO, G. AND WEINHARD, P. 1973. Non-symbiotic nitrogen fixation in the rhizosphere of rice, maize and different tropical grasses. *Soil Biol. Biochem.* 5 :83 - 89.

ELA, S.W., ANDERSON, M.A. AND BRILL, W.J. 1982. Screening and selection of maize to enhance associative bacterial nitrogen fixation. *Plant Physiol* .70 :1564 - 1567.

FAYEZ, M. AND VLASSAK, V. 1984. Effect of inoculation with *Azospirillum brasilense* on barley grown under semi-arid conditions. *Zbl. Mikrobiol.* 139 :359 - 365.

FOSTER, R.C. AND ROVIRA, A.D. 1976. Ultrastructure of wheat rhizosphere. *New Phytol.* 76 :343 - 352.

GASKINS, M.H. AND HUBBEL, D.H. 1979. Response of nonleguminous plants to root inoculation with free-living dizotrophic bacteria. *In*: The soil root interface (eds. J.L. Harley and R.S. Russel), 175 - 182. Academic Press : London.

GASKINS, M.H., HUBBELL, D.H. AND ALBRECHT, S.L. 1981. Interactions between grasses and rhizosphere nitrogen-fixing bacteria. *In*: Proceedings of the XIV International Grassland Congress, Kentucky, U.S.A. (eds. J.A. Smith and V.W. Hays), 327 - 329. Westview Press : Colorado.

GIBSON, A.H., SCOWCROFT, W.R. AND PAGAN, J.D. 1977. Nitrogen fixation in plants : An expanding horizon ? *In*: Recent Developments in Nitrogen fixation (eds. W. Newton, J.R. Postgate and C. Rodriguez-Barrueco) 387 - 417. Academic Press : London.

GUIERINOT, M.L. AND PATRIQUIN, D.G. 1981. N_2-fixing vibrios isolated from the gastrointestinal tract of sea urchins. *Can. J. Microbiol.* 27 :311 - 317.

HAAHTELA, K., KARI, K. AND SUNDMAN, V. 1983. Nitrogenase activity (acetylene reduction) of root-associated, cold climate *Azospirillum, Enterobacter, Klebsiella and Pseudomonas* species during growth on various carbon sources and at various partial pressures of oxygen. *Appl. Environm. Microbiol.* 45 :563 - 570.

HAAHTELA, K., WARTIOVAARA, T., SUNDMAN, V. AND SKUJINS, J. 1981. Root-associated N_2 fixation (acetylene reduction) by *Enterobacteriaceae* and

HALSALL, D.M. AND GIBSON, A.H. 1986. Comparison of two *Cellulomonas* strains and their interaction with *Azospirillum brasilense* in degradation of wheat straw and associated nitrogen fixation. *Appl. Environm. Microbiol.* 51 :855 - 861.

HALSALL, D.M. AND GOODCHILD, D.J. 1986. Nitrogen fixation associated with development and localization of mixed populations of *Cellulomonas* sp. and *Azospirillum brasilense* grown on cellulose or wheat straw. *Appl. Environm. Microbiol.* 51 :849 - 854.

Azospirillum strains in cold climate spodosols. *Appl. Environm. Microbiol.* 41 :203 - 206.

HARDY, R.W.F., BURNS, R.C. AND HOLSTEN, R.D. 1973 Application of the acetylene-ethylene assay for measurement of nitrogen fixation. *Soil Biol. Biochem* 5 :47-81.

HARPER, J.E. AND HAGEMAN, R. H. 1972. Canopy and seasonal Profiles of Nitrate Reductase in Soybeans (*Glycine max* L. Merr.). *Plant Physiol.* 49: 146 - 154.

HARTMAN, A., SINGH, M. AND KLINGMULLER, W. 1983. Isolation and characterisation of *Azospirillum* mutants excreting high amounts of indoleacetic acid. *Can. J. Microbiol.* 29 :916 - 923.

HEGAZI, N.A., MONIB, M., AMER, H.A. AND SHOKR, E. 1983. Response of maize plants to inoculation with azospirilla & (or) straw amendment in Egypt. *Can. J. Microbiol.* 29 :888 - 894.

HEGAZI, N.A., MONIB, M. AND VLASSAK, K. 1979. Effect of Inoculation with N_2-fixing Spirilla and *Azotobacter* on Nitrogenase activity on roots of Maize grown under Subtropical conditions. *Appl. Env. Microbiol.* 38: 621-625.

HERVEY, R.J. AND GREAVES, J.E. 1941. Nitrogen fixation by *Azotobacter chroococcum* in the presence of soil protozoa. *Soil Sc.* 51 :85 - 100.

IDRIS, M., MEMON, G.H. AND VINTHER, F.P. 1981. Occurrence of *Azospirillum* and *Azotobacter* and Potential Nitrogenase activity in Danish agricultural soil under continuous barley cultivation. *Acta Agric. Scand.* 31 :433 - 437.

JAGNOW, G. 1986. Nitrogen-fixing bacteria in the rhizosphere. *In*: Plant Research and Development, 23 :7 - 20. Institute for Scientific Cooperation : Tubingen.

JENKINSON, D.S. 1973. Organic matter and nitrogen in soils of the Rothamsted classical experiments. *J. Sci. Food Agric.* 24 :1149 - 1150.

JENKINSON, D.S. AND AYANABA, A. 1977. Decomposition of C-14 labeled plant material under tropical conditions. *Soil Sc. Soc. Amer. J.* 41 :912 - 915.

JENSEN, V. AND HOLM, E. 1975. Associative growth of nitrogen-fixing bacteria with other microorganisms. *In*: Nitrogen fixation by Free-living Microorganisms (ed. W.D.P. Stewart), 101 - 119. Cambridge University Press : Cambridge.

KANG, B.T., NANGJU, D. AND AYANABA, A. 1977. Effects of Fertilizer use on Cowpea and Soybean Nodulation and Nitrogen Fixation in the Lowland Tropics. *In*: Biological Nitrogen Fixation in Farming

Systems of the Tropics (ed. A. Ayanaba and P.J. Dart), 205 - 216. John Wiley and Sons : Chichester.

KAPULNIK, Y., OKON, Y., KIGEL, J., NUR, I. AND HENIS, Y. 1981a. Effects of temperature, nitrogen fertilisation and plant age on nitrogen fixation by *Setaria italica* inoculated with *Azospirillum brasilense* (strain cd). *Plant Physiol.* 68 :340 - 343.

KAPULNIK, Y., SARIG, S., NUR, I., OKON, Y., 1983. Effect of *Azospirillum* inoculation on yield of field-grown wheat. *Can. J. Microbiol.* 29 :895 - 944.

KAPULNIK, Y., SARIG, S., NUR, I. OKON, Y., KIGEL, J. AND HENIS, Y. 1981b. Yield increases in summer cereal crops of Israeli fields inoculated with *Azospirillum. Exp. Agric.* 17 :179 - 187.

KAPULNIK, Y., SARIG, S., NUR, I., OKON, Y. AND HENIS, Y. 1982. The effect of *Azospirillum* inoculation on growth and yield of corn. *Isr. J. Bot.* 31 :247 - 255.

KEYA, S.O. 1977. Nodulation and Nitrogen Fixation in Legumes in East Africa. *In*: Biological Nitrogen Fixation in Farming Systems of the Tropics (ed. A. Ayanaba and P.J. Dart), 233 - 243. John Wiley and Sons : Chichester.

KLEEBERGER, A., CASTORPH, H. AND KLINGMULLER, W. 1983. The rhizosphere microflora of wheat and barley with special reference to gram-negative bacteria. *Arch. Microbiol.* 136 :306 - 311.

KLUCAS, R.V. AND PEDERSEN, W. 1980. Nitrogen fixation associated with roots of sorghum and wheat. *In*: Nitrogen fixation Vol. II (eds. W.E. Newton and W.H. Orme-Johnson), 243 - 255. University Park Press : Baltimore.

KLINGMULLER, W. 1982. *Azospirillum*: Genetics, Physiology and Ecology Workshop, University of Bayreuth, Germany, 16 to 17 July, 1981. Birkhauser Verlag : Basel.

KNOWLES, R. 1975. The significance of asymbiotic dinitrogen fixation by bacteria. *In*: Dinitrogen (N_2) fixation Vol. II (ed. R.W.F. Hardy). Wiley-Interscience : New York.

KOCH, B.L. AND OYA, J. 1974. Non-symbiotic nitrogen fixation in some Hawaaian pasture soils. *Soil Biol. Biochem.* 6 :363 - 367.

KRAFFCZYK, I., TROLLDENIER, G. AND BERINGER, H. 1984. Soluble root exudates of maize: Influence of potassium supply and rhizosphere microorganism. *Soil Biol. Biochem.* 16 :315 - 322.

KRIEG, W.R. AND DOBEREINER, J. 1984. Genus *Azospirillum* Tarrand, Krieg and Dobereiner 1979. *In*: Bergeys

Manual of Systematic Bacteriology Vol.I (eds. N.R. Krieg and J.G. Holt), 94 - 104. Williams and Wilkins : Baltimore.

LADHA, J.K., BARRAQUIO, W.L. AND WATANABE, I. 1983. Isolation and identification of nitrogen-fixing *Enterobacter cloacae* and *Klebsiella planticola* associated with rice plants. *Can. J. Microbiol.* 29 :1301 - 1308.

LARSON, R.I. AND NEAL, J.L. 1978. Selective colonisation of the rhizosphere of wheat by nitrogen-fixing bacteria . *In*: Environmental Role of Nitrogen-fixing Blue-green algae and Asymbiotic bacteria (ed. U. Granhall) *Ecol. Bull.* (Stockholm) 26 :331 - 342.

LETHBRIDGE, D. AND DAVIDSON, M.S. 1983. Root-associated nitrogen-fixing bacteria and their role in the nitrogen nutrition of wheat estimated by N-15 isotope dilution. *Soil Biol. Biochem.* 15 :365 - 374.

LETHBRIDGE, G., DAVIDSON, M.S. AND SPARLING, G.P. 1982. Critical evaluation of the acetylene reduction test for estimating the activity of nitrogen-fixing bacteria associated with the roots of wheat and barley. *Soil Biol. Biochem.* 14 :27 - 35.

LIN, W., OKON, Y. AND HARDY, R.W.F. 1983. Enhanced

mineral uptake by *Zea mays* and *Sorghum bicolor* roots inoculated with *Azospirillum brasilense*. *Appl. Environm. Microbiol.* 45 :1775 - 1779.

LIND, C.J. AND WILSON, P.W. 1942. Nitrogen fixation by *Azotobacter* in association with other bacteria. *Soil Sc.* 54 :105 - 111.

LINDBERG, T. AND GRANHALL, U. 1984a. Nitrogenase activity in Different Cereals and forage grasses. *Swedish J. agric. Res.* 14 :77 - 84.

LINDBERG, T. AND GRANHALL, U. 1984b. Isolation and characterisation of dinitrogen-fixing bacteria from the rhizosphere of temperate cereals and forage grasses. *Appl. Environm. Microbiol.* 48 :683 - 689.

LINDBERG, T., GRANHALL, U. AND TOMENIUS, K. 1985. Infectivity and acetylene reduction of diazotrophic rhizosphere bacteria in wheat (*Triticum aestivum*) seedlings under gnotobiotic conditions. *Biol. Fer. Soils* 1 :123 - 129.

LINE, M.A. AND LOUTIT, M.W. 1973. Nitrogen fixation by mixed cultures of aerobic and anaerobic miro-organisms in an aerobic environment. *J. gen. Microbiol.* 74 :179 - 180.

LOVETT, J.V. AND SAGAR, G.R. 1978. Influence of bacteria in the phyllosphere of *Camelina sativa*

L. Crantz on germination of *Linum usitalissimum* L. *New Phytol.* 81 :617 - 625.

LUSIGI, W.J. 1978. Planning human activities on protected natural ecosystems. Strauss and Cramer GmbH : West Germany.

LYNCH, J.M., HARPER, S.H.T., CHAPMAN, S.J. AND VEAL, D.A. 1984. Approaches to the controlled production of novel agricultural composts. *In*: Microbiological Methods for Environmental Biotechnology, Techn. Series No.19 (eds. J.M. Grainger and J.M. Lynch),69 - 78. Academic Press Inc.: London.

MAGALHAES, F.M.M., PATRIQUIN, D.G. AND DOBEREINER, J. 1979. Infection of field-grown maize with *Azospirillum* sp. *Rev. Brasil. Biol.* 39 :587 - 596.

MARTIN, J.K. 1977. Factors influencing the loss of organic carbon from wheat roots. *Soil Biol. Biochem.* 9 :1-7

MARTIN, P. AND GLATZLE, A. 1982. Mutual influences of *Azospirillum* spp. and grass seedlings. *In*: *Azospirillum*: genetics, physiology and ecology (ed. W. Klingmuller), 108 - 220. Berkhauser Verlag : Basel.

McCALLA, T.M. 1945. Influence of microorganisms and

some organic substances on soil structure. *Soil Sci.* 59 :287 - 297.

MEIKLEJOHN, J. AND WEIR, J.B. 1968. Nitrogen fixers - *Pseudomonas* and other aerobic bacteria from Rhodesian soils. *J. gen. Microbiol.* 50 :487 - 496.

MERTENS, T. AND HESS, D. 1984. Yield increases in spring wheat (*Triticum aestivum* L.) inoculated with *Azospirillum lipoferum* under green house and field conditions of a temperate region. *Plant Soil.* 82 :87 - 99.

MILLET E. AND FELDMAN, M. 1984. Yield response of a common spring wheat cultivar to inoculation with *Azospirillum brasilense* at various levels of nitrogen fertilization. *Plant Soil.* 80 :255 - 259.

MILLET, E., AVIVI, Y. AND FELDMAN, M. 1984. Yield responses of various wheat genotypes to inoculation with *Azospirillum brasilense*. *Plant Soil* 80 :261 - 266.

MINCHIN, F.R. AND PATE, J.S. 1973. The carbon balance of a legume and the functional economy of its nodules. *J. exp. Bot.* 24 :259 - 271.

MULDER, E.G. 1975. Physiology and ecology of free-living nitrogen-fixing bacteria. *In*: Nitrogen fixation by free-living microorganisms

(ed. W.D.P. Stewart), 3 - 28. Cambridge University Press : Cambridge.

MULDER, E.G. AND BROTONEGORO, S. 1974. Free-living heterotrophic nitrogen- fixing bacteria. *In*: The Biology of Nitrogen fixation (ed. A. Quispel), 37 - 85. North-Holland : Amsterdam.

MULONGOY, K. 1986. Influence of biological processes on soil fertility: A research priority for sustained crop production in the tropics. *In*: Proc. of IFS Workshop on "Biological Improvement of Soil Fertility". March 17 - 26, Dakar, Senegal.

MWATHA, E.W. 1981. A study of non-symbiotic nitrogen-fixing bacteria in some Kenyan soils. M Sc. Thesis, University of Nairobi, Nairobi, Kenya.

MWAURA, F.B. 1981. Nitrogen fixation in the Papyrus swamps of Lake Naivasha. M Sc. Thesis, University of Nairobi, Nairobi, Kenya.

NEILSON, A.H. AND SPARREL, L. 1976. Acetylene reduction (Nitrogen fixation) by *Enterobacteriaceae* isolated from paper mill process waters. *Appl. Environm. Microbiol.* 32 :197 - 205.

NELSON, A.D., BARBER, L.E., TJEPKEMA, J.,RUSSEL, S.A., POWELSON, R., EVANS, H.J. AND SEIDLER, R.J. 1976. Nitrogen fixation associated with grasses

in Oregon. *Can. J. Microbiol.* 22 :523 - 530.

NELSON, D.W. AND SOMMERS, L.E. 1973. Determination of total nitrogen in plant material. *Agron. J.* 65: 109 - 112.

NEYRA, C. 1978. Interactions of plant photosynthesis with dinitrogen fixation and nitrate assimilation. *In*: Limitations and Potentials for Biological Nitrogen Fixation in the Tropics (eds. J. Dobereiner, R.H. Burris, and A. Hollaender), 111 - 120. Plenum Press : New York.

NEYRA, C. AND VAN BERKUM, P. 1976. Nitrogenase activity in isolated sorghum roots. Effect of bicarbonate and inorganic nitrogen. *Plant Physiol.* 57, suppl. abs. 533. Quoted by Boddey and Dobereiner (1984).

NEYRA, C.A. AND DOBEREINER, J. 1977. Nitrogen fixation in grasses. *Adv. Agron.* 29 :1 - 38.

NORTHCOTTE, D.H. AND PICKETT-HEAPS, J.D. 1966. A function of Golgi apparatus in polysaccharide synthesis and transport in the root cap cells of wheat. *Biochem. J.* 89 :159 - 167.

NUR, I., OKON, Y. AND HENIS, Y. 1980. An increase in nitrogen content of *Setaria italica* and *Zea mays* inoculated with *Azospirillum*. *Can. J. Microbiol.* 26 :482 - 485.

O'CAMPO, 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.

ODU, C.T.I. 1977. Contributions of free-living bacteria to the nitrogen status of humid tropical soils. *In*: Biological Nitrogen fixation in Farming Systems of the Tropics (eds. A. Ayanaba and P.J. Dart), 257 - 266. John Wiley and Sons : Chichester.

O'HARA, G.W., DAVEY, M.R. AND LUCAS, J.A. 1981. Effect of inoculation of *Zea mays* with *Azospirillum brasilense* strains under temperate conditions. *Can. J. Microbiol.* 27 :871 - 877.

OKAFOR, N. 1977. Non-symbiotic nitrogen fixation in tropical humid soils with particular reference to Nigeria. *In*: Biological Nitrogen Fixation in Farming Systems of the Tropics (eds. A. Ayanaba and P.J. Dart) , 267 - 272. John Wiley and Sons : New York.

OKAFOR, N. AND MACRAE, I.C. 1972. The influence of moisture level, light and glucose upon acetylene reduction by a black earth soil. *Soil Biol. Biochem.* 5 :181 - 186.

OKON, Y. 1984. Response of cereal and forage grasses to

inoculation with N_2-fixing bacteria. *In*: Advances in nitrogen fixation research . Proc. 5th Int. Symp. Nitrogen fixation, Noordwijkehout, The Netherlands (eds. C. Veeger and W.E. Newton),303 - 309. Martinus Nijhoff / Dr. W. Junk : The Hague.

OKON, Y., ALBRECHT, S. L. AND BURRIS, R.H. 1977. Methods for growing *Spirillum lioferum* and for counting it in pure culture and in association with plants. *Appl. Environm. Microbiol.* 33 :85 - 87.

OKON, Y., CAKMACKI, L., NUR, I. & CHET, I. 1980. Aerotaxis and Chemotaxis of *Azospirillum brasilense* : a note. *Microb. Ecol.* 6 :277 - 280.

PARKER, C.A. 1985. Nitrogen fixation for developing countries with special reference to Africa. *In*: Proc. of the First Conference of the AABNF,Nairobi Kenya 23 to 27 July 1984 (eds. H. Ssali and S.O. Keya),10 - 29, *Rhizobium* MIRCEN Centre : Nairobi.

PATEL, J.J. AND BROWN, M.E. 1969. Interactions of Azotobacter with rhizosphere and root-surface microflora. *Plant Soil* 31 :273 - 281.

PATRIQUIN, D.G. 1982. New developments in grass-bacteria associations. *In* : Advances in

agricultural microbiology (ed. N.S. Subba Rao), 139 - 190. Butterworth Scientific : London.

PATRIQUIN, D.G. AND DENIKE, D. 1978. In situ acetylene reduction assays of nitrogenase activity associated with the emergent halophyte *Spartina alterniflora* Loisel: Methodological problems. *Aquat. Bot.* 4 :211-226.

PATRIQUIN, D.G. AND DOBEREINER, J. 1978. Light microscopy observations of tetrazolium-reducing bacteria in the endorhizosphere of maize and other grasses in Brazil. *Can. J. Microbiol.* 24 :734 - 742.

PATRIQUIN, D.G., BOYLE, C.D., LIVINGSTONE, D.C. AND McCLUNG, C.R. 1979. Physiology of the associative symbiosis in salt marsh, *Spartina alterniflora* Loisel. *In*: Proceedings of the international Workshop of Associative N_2 Fixation, Piracicaba, Brazil. CRC Press Inc. : Florida.

PATRIQUIN, D.G., DOBEREINER, J. AND JAIN, D.K. 1983. Sites and processes of association between diazotrophs and grasses. *Can. J. Microbiol.* 29 :900 - 915.

PEDERSEN, W.L., CHAKRABARTY, K., KLUCAS, R.V. AND VIDAVER, 1978. Nitrogen fixation (acetylene reduction) associated with roots of winter

POSTGATE, J.R. 1969. Viable Counts and Viability. *In*: Methods in Microbiology, Vol. I (eds. J.R. Norris and D.W. Ribbons), 611 - 628. Academic Press Inc. : New York.

wheat and sorghum in Nebraska. *Appl. Environm. Microbiol.* 35 :129 - 135.

POHLMAN, A.A. AND McCOLL, J.G. 1982. Nitrogen fixation in the rhizosphere and rhizoplane of barley. *Plant Soil* 69 :341 - 352.

RAI, S.N. AND GAUR, A.C. 1982. Nitrogen fixation by *Azospirillum* spp. and effect of *Azospirillum lipoferum* on the yield and N-uptake of wheat crop. *Plant Soil.* 69 :233 - 238.

RAJU, P.N., EVANS, H.J. AND SEIDLER, R.J. 1972. An asymbiotic nitrogen-fixing bacterium from the root environment of corn. *Proc. Natl. Acad. Sci.* U.S.A. 69 :3474 - 3478.

REINHOLD, B., HUREK, T., NIEMAN, E. AND FENDRIK, I. 1986. Close association of *Azospirillum* and diazotrophic rods with different root zones of Kalar grass. *Appl. Environm. Microbiol.* 52 :520 - 526.

RENNIE, R.J. 1980. N-15 isotope dilution as a measure of dinitrogen fixation by *Azospirillum brasilense* associated with maize. *Can. J. Bot.* 58 :21 - 24.

RENNIE, R.J. 1981. Potential use of induced mutations to improve symbiosis of crop plants with N_2-fixing bacteria. *In*: Proceedings of an International Symposium on induced mutations as a tool for crop plant improvement, 293 - 321.

IAEA, Vienna.

REYNDERS,L. AND VLASSAK, K. 1982. Use of *Azospirillum brasilense* as biofertilizer in intensive wheat cropping. *Plant Soil.* 66 :217 - 223.

RINAUDO, G., BALANDREAU, J. AND DOMMERGUES, Y. 1971. Algal and bacterial nonsymbiotic nitrogen fixation in paddy soil. *Plant Soil*, Special volume 471 - 479.

RINAUDO, G., HAMAD-FARES, I. AND DOMMERGUES, Y. R. 1977. Nitrogen fixation in the rice rhizosphere : methods of measurement and practices suggested to enhance the process. *In*: Biological Nitrogen fixation in Farming Systems of the Tropics (eds. A. Ayanaba and P.J. Dart), 313 - 322. John Wiley and Sons : Chichester.

ROCHA, R.E.M., BALDANI, J.I. AND DOBEREINER, J. 1981. Specifity of infection by *Azospirillum* spp. in plants with C-4 photosynthetic pathway. *In*: Associative N_2 fixation (eds. P.B. Vose and A.P. Ruschel), 67 - 69. CRC Press Inc. : Florida.

ROPER, M.M. 1983. Field measurements of nitrogenase activity in soils amended with wheat straw. *Austr. J. Agric. Res.* 34 :725 - 739.

ROVIRA, A.D. 1981. The microbiology and biochemistry of

the rhizoplane in relation to root disease of wheat and direct drilling in Brazil. *In*: The Soil/Root system in relation to Brazilian agriculture. (eds. R.S. Russell, K. Igue and Y.R. Mehta), 259-277. Fundaca Instituto. Agronomica do Parana: Brazil. Quoted by Patriquin, Dobereiner and Jain (1983).

ROVIRA, A.D. AND DAVEY, C.B. 1971. Biology of the rhizosphere . *In*: The plant root and its environment (ed. E.W. Carson), 153 - 204. university Press : Virginia, Charlottesville.

RUSCHEL, A.P. 1981. Associative N_2 fixation by sugar cane. *In*: Associative N_2-Fixation (eds. P.B.F Vose and A.P. Ruschel), 81 - 89. CRC Press Inc. : Florida.

RUSCHEL, 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 .

SARIG, S., KAPULNIK, Y., NUR, I. AND OKON, Y. 1984. Response of non-irrigated *Sorghum bicolor* to *Azospirillum* inoculation. *Exp. Agric.* 20 :59 - 66.

SCHANK, S.C., WEIR, K.L. AND MACRAE, I.C. 1981. Plant yield and nitrogen content of a digit grass in response to *Azospirillum* inoculation. *Appl.*

Environm. Microbiol. 41 :342 - 345.

SCHER, F.M., ZIEGLE, J.S. AND KLOEPPER, J.W. 1984. A method for assessing the root-colonizing capacity of bacteria on maize. *Can. J. Microbiol.* 30 :151-157.

SCOTT, C.A., MAGALHAES, F.M.M., DIVAN, V.L.S. 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. Dobereiner, R.H. Burris and A. Hollaender), 371 - 372. Plenum Press : New York.

SELDIN, L., VAN ELSAS, J.D. AND PENIDO, E.G.C. 1984. *Bacillus azotofixans* sp. nov., a nitrogen-fixing species from Brazilian soils and grass roots. *Int. J. Syt. Bact.* 34 :451 - 456.

SHIMSHICK, E.J. AND HEBERT, R.R. 1978. Adsorption of Rhizobia to cereal roots. *Biochem. Biophys. Res. Commun.* 84 :736.

SINGH, P.K. 1981. Use of *Azolla* and blue-green algae in rice cultivation in India. *In*: Associtive N_2 fixation , Vol. II (eds. P.B. Vose and A.P. Ruschel) , 183 - 196. CRC Press : Florida.

SLOGER, C. 1976. Associated nitrogen fixation in corn. *Plant Physiol.* 57 : Suppl. Abstr. 532. Quoted by Van Berkum (1980).

SMITH, G.W. AND HAYASAKA, S.S. 1982. Nitrogenase activity associated with *Halodule Wrightii* roots. *Appl. Environm. Microbiol.* 43 :1244-1248.

SMITH, R.L., BOUTON, J.H., SCHANK, S.C. QUESENBERRY, R.H., TYLER, M.E., GASKINS, 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*. In: Environmental Role of Nitrogen-fixing Blue-green Algae and Asymbiotic Bacteria (ed. U. Granhall), *Ecol. Bull.* (Stockholm), 26 :380 - 385.

SMITH, R.L., SCHANK, S.C. AND LITTELL, R.C. 1984. The influence of shading on associative N_2 fixation. *Plant Soil* 80 :43 - 52.

SOLHEIM, B. 1975. Possible role of lectins in the infection of legumes by rhizobia. *In*: North Atlantic Treaty Organization Conference on Specificity in Plant Diseases. Advanced Study Institute, Sardinia. Quoted by Umali-Garcia, Hebbell, Gaskins, and Dazzo (1980).

SPIFF, E.D. AND ODU, C.T.I. 1972. An assessment of non-symbiotic nitrogen fixation in some Nigerian soils by the acetylene reduction

technique. *Soil Biol. Biochem.* 4 :71 - 77.

SPRENT, J.I. 1979. The Biology of Nitrogen-fixing Organisms. McGraw Hill Book Co. (U.K.) Ltd. : London.

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

SUBA RAO, N.S. 1980. Crop response to microbial inoculation. *In*: Recent Advances in Biological Nitrogen Fixation (ed. N.S. Subba Rao), 406 - 420. Edward Arnold : London.

SUBBA RAO, N.S. 1981. Response of crops to *Azospirillum* inoculation in India. *In*: Associative N_2-fixation Vol. 1 (eds. P.B. Vose and A.P. Ruschel), 137 - 144. CRC Press Inc. : Florida.

SUSLOW, T.V. 1982. Role of root-colonizing bacteria in plant growth. *In*: Phytopathogenic procaryotes, Vol. I (eds. M.S. Mount and G.H. Lacy), 187 - 223. Academic Press : New York.

TARRAND, J.J., KRIEG, N.R. AND DOBEREINER, J. 1978. Taxonomic Study of *Spirillum lipoferum* group, with description of a new genus, *Azopirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) Comb. nov. and *Azospirillum brasilense* sp. nov. *Can. J.*

Microbiol. 24 :967 - 980.

TAYLOR, R.W. 1979. Response of two grasses to inoculation with *Azospirillum* spp. in Bahamian soil. *Tropical agric.*, Trinidad 56 :361 - 365.

THOMAS-BAUZON, D., WEINHARD, P. VILLECOURT, P. AND BALANDREAU, J. 1982. The Spermosphere model: Its use in growing, counting and isolating N_2-fixing bacteria from the rhizosphere of rice. *Can. J. Microbiol.* 28 :922 - 928.

THOMPSON, J.P. AND SKERMAN, V.B.D. 1979. *Azotobacteriaceae* : The taxonomy and ecology of the aerobic nitrogen-fixing bacteria. Academic Press : London.

THOMPSON, J.A., GEMELL, L.G., ROUGHLEY, R.J., EVANS,J. AND NICHOLS, D.J. 1984. Nitrogenase activity associated with pasture grasses in Northern New South Wales. *Soil Biol. Biochem.* 16 :217 - 222.

TIEN, T.M., GASKINS, M.H.AND HUBBEL, D.H. 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). *Appl. Environm. Microbiol.* 37 :1016 - 1024.

TISDALL, J.M. AND OADES, J.M. 1982. Organic matter and water stable aggregates in soils. *J. Soil Sci.* 33 :141 - 163.

TJEPKEMA, J.D. AND BURRIS, R.H. 1976. Nitrogenase

activity associated with some Wisconsin prairie grasses. *Plant Soil*, 45: 81-94.

TJEPKEMA, L.D. AND VAN BERKUM, P. 1977. Acetylene reduction by soil cores of maize and sorghum in Brazil. *Appl. Environm. Microbiol.* 33 :626 - 629.

TROLLDENIER, G. 1977. Influence of some enviromental factors on nitrogen fixation in the rhizosphere of rice. *Plant Soil* 47 :203 - 217.

TYLER, M.E., MILAM, J.R. AND ZUBERER, D.A. 1978. Taxonomy of the diazotroph of tropical grasses called *Spirillum lipoferum*. *In*: Limitations and potentials for Biological Nitrogen Fixation in the Tropics (eds. J. Dobereiner, R.H. Burris and A. Hollaender), 77. Plenum Press : New York.

TYLER, M.E., MILAM, J.R., SMITH, R.L., SCHANK, S.C. AND ZUBERER, D.A. 1979. Isolation of *Azospirillum* from diverse geographic regions. *Can. J. Microbiol.* 25 :693 - 697.

UMALI-GARCIA, M., HUBBELL, D.H. AND GASKINS, M.H. 1978. Process of infection of *Panicum maximum* by *Spirillum lipoferum*. In: Environmental Role of nitrogen-fixing blue-green Algae and Asymbiotic bacteria (ed. U. Granhall), *Ecol. Bull.* (Stockholm) 26 :373 - 379.

UMALI-GARCIA, M., HUBBELL, D.H., GASKINS, M.H. AND DAZZO, F.B. 1980. Association of *Azospirillum*

with Grass roots. *Appl. Environm. Microbiol.* 39 :219 - 226.

VAN BERKUM, P. 1980. Evaluation of acetylene reduction by excised roots for the determination of nitrogen fixation in grasses. *Soil Biol. Biochem.* 12 :141 - 145.

VAN BERKUM, P. AND BOHLOOL, B.B. 1980. Evaluation of nitrogen fixation by bacteria in association with roots of tropical grasses. *Microbiol. Rev.* 44 :491 - 517.

VAN BERKUM, P. AND DAY, J.M. 1980. Nitrogenase activity associated with soil cores of grasses in Brazil. *Soil Biol. Biochem.* 12 :137 - 140.

VAN BERKUM, P. AND NEYRA, C. 1976. Nitrogenase activity in isolated sorghum roots : The effect of bicarbonate and inorganic nitrogen. *Plant Physiol.* 57 : Suppl. Abstr. No.533. Quoted by Neyra and Dobereiner (1977).

VAN BERKUM, P. AND SLOGER, C. 1979. Immediate acetylene reduction by excised grass roots not previously preincubated at low oxygen tensions. *Plant Physiol.* 64: 739-745.

VAN BERKUM, P. AND SLOGER, C. 1981. Comparing time course profiles of immediate acetylene reduction by grasses and legumes. *Appl. Environ. Microbiol.* 41 :184 - 189 .

VAN BERKUM P. AND SLOGER, C. 1982. Physiology of root -associated nitrogenase activity in *Oryza sativa*. *Plant Physiol*. 69 :1161 - 1164.

VINCENT. J.M. 1970. A Manual for the practical study of Root Nodule bacteria. Blackwell Scientific Publication : Oxford.

VLASSAK, K. AND REYNDERS, L. 1981. Agronomic aspects of biological dinitrogen fixation by *Azospirillum* spp. in temperate region . *In*: Associative N_2 fixation Vol.1 (ed. P.B. Vose and A.P. Ruschel), 93 - 101 CRC Press Inc. : Florida.

VLASSAK,K., PAUL, E.A. AND HARRIS, R.E. 1973. Assessment of biological nitrogen fixation in grassland and associated sites. *Plant Soil* 38 :637 - 649.

VOLPON, A.G.T., DE-POLLI, H. AND DOBEREINER, J. 1981. Physiology of nitrogen fixation in *Azospirillum lipoferum*. Br 17 (ATCC29 709). *Arch. Microbiol.* 128 :371-375.

VON BULOW , J.F.W. AND DOBEREINER, J. 1975. Potential for nitrogen fixation in maize genotypes in Brazil . *Proc. Natl. Acad.* Sc. U.S.A. 72 :2389 - 2393.

WATANABE, I. AND BARRAQUIO, W.L. 1979. Low levels of fixed nitrogen required for isolation of free-living N_2-fixing organisms from rice

roots. *Nature* (London) 277 :565 - 566.

WATANABE, I. AND CABRERA, D.A. 1979. Nitrogen fixation associated with the rice plant grown in water culture. *Appl. Environm. Microbiol.* 37 :373 - 378.

WATANABE, I., BARRAQUIO, W.L., DE GUZMAN, M.R.AND CABRERA, D.A.1979. Nitrogen-fixing (acetylene reduction) activity and population of aerobic heterotrophic nitrogen-fixing bacteria associated with wetland rice. *Appl. environ. Microbiol.* 37 :813 - 819.

WATANABE, I., OSAMU, I. AND BARRAQUIO, W. 1981. Nitrogen-fixing population and activity associated with wetland rice. *In*: Associative N_2-Fixation (eds. P.B. Vose and A.P. Ruschel), 177 - 182. CRC Press Inc.: Florida.

WEAVER, R.W., WRIGHT, S.F., VARANAKA, M.W., SMITH, O.E. AND HOLT, E.C. 1980. Dinitrogen fixation (C_2H_2) by established forage grasses in Texas. *Agron. J.* 72 :965 - 968. Quoted by Patriquin (1982).

WEIR, K.L. 1980. Nitrogenase activity associated with three tropical grasses growing in undisturbed soil cores. *Soil Biol. Biochem.* 12 :131 - 136.

WELSH, J.R. 1981. Fundamentals of Plant Genetics and Breeding. John Wiley and Sons : Chichester.

WHITEHEAD, H.C.M., ELLIOT, D., GILES, K.L., RONGAL, S.

AND PATEL, J.J. 1978. A naturally occurring nitrogen-fixing rhizosphere association in the Manawatu. *In*: Microbial Ecology (eds. M.W. Loutit and J.A.R. Miles), 144 - 146. Springer-Verlag : Berlin.

WHITING, G.J., GANDY, E.L. AND YORK, D.C. 1986. Tight coupling of root-associated nitrogen fixation and plant photosynthesis in the salt marsh grass *Spartina alterniflora* and carbon dioxide enhancement of nitrogenase activity. *Appl. Environm. Microbiol.* 52 :108 - 113.

WRIGHT, S.F. AND WEAVER, R.W. 1982. Inoculation of forage grasses with N_2-fixing *Enterobacteriaceae*. *Plant Soil.* 65 :415 - 419.

YAHALOM, E., KAPULNIK, Y. AND OKON, Y.1984. Response of *Setaria italica* to inoculation with *Azospirillum brasilense* as compared to *Azotobacter chrooccoccum*. *Plant Soil.* 82 :77 - 85.

YOSHIDA, T. AND ANCAJAS, R.R. 1973. The fixation of atmospheric nitrogen in the rice rhizosphere. *Soil Biol. Biochem.* 5 :153 - 155.

ZUBERER, D.A. AND SILVER, W.S. 1978. Biological Dinitrogen fixation (acetylene reduction) associated with Florida mangroves. *Appl. Environ. Microbiol.* 35 :567-575.

APPENDIX

Table 1. Acetylene reduction activity associated with intact maize plants in the temperate soil.

	nmol C_2H_4/ plant			
	Aerobic		Anaerobic	
Time(h)	range	mean	range	mean
0	0 - 0	0	0 - 0	0
2	1.5 - 259.1	50.1	5.3 - 36.8	17.3
4	1.9 - 455.4	89.5	3.2 - 89.3	22.3
6	3.9 - 486.9	92.7	5.3 - 210	42.3

Table 2. Acetylene reduction activity in plant-free temperate soil.

		n mol C_2H_4/ *tube		
		2h	4h	6h
Aerobic	Soil (covered)	0	trace	trace
	Soil (illuminated)	24.7	28.1	31.9
Anaerobic	Soil (covered)	0	0	trace
	Soil (illuminated)	10.2	15.2	15.2

* each tube contained 10g d.wt. soil.

Table 3. Acetylene reduction activity associated with intact maize plants in the tropical soil.

	n mol C_2H_4/ plant			
	Aerobic		Anaerobic	
Time(h)	range	mean	range	mean
0	0 - 0	0	0 - 0	0
2	5.2 - 481.7	75.9	5.3 - 105.3	63.2
4	5.6 - 1216.7	175.2	5.3 - 908.4	117.0
6	9.2 - 1846.7	267.3	5.3 - 1333.5	177.4

Table 4. Acetylene reduction activity in plant-free tropical soil.

		n mol C_2H_4/ *tube		
		2h	4h	6h
Aerobic	Soil(covered)	0	trace	trace
	Soil(illuminated)	14.5	17.5	19.6
Anaerobic	Soil(covered)	0	trace	trace
	Soil(illuminated)	9.9	11.6	11.8

* each tube contained 10g d.wt. soil.