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Biological nitrogen fixation in non-leguminous field crops: Recent advances

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Key words: *Azospirillum*, bacteria-plant association, non-legumes, *para*-nodules, *Rhizobium*, root nodules

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Abstract

There is strong evidence that non-leguminous field crops sometimes benefit from associations with diazotrophs. Significantly, the potential benefit from N_2 fixation is usually gained from spontaneous associations that can rarely be managed as part of agricultural practice. Particularly for dryland systems, these associations appear to be very unreliable as a means of raising the nitrogen status of plants. However, recent technical advances involving the induction of nodular structures on the roots of cereal crops, such as wheat and rice, offer the prospect that dependable symbioses with free-living diazotrophs, such as the azospirilla, or with rhizobia may eventually be achieved.

Introduction

Past and future

It is now well over 100 years since the existence of microorganisms capable of biological fixation of atmospheric N_2 was experimentally proven (Tchan, 1988). Barely 100 years ago the N_2-fixing capacity of the legume-rhizobial symbiosis was firmly established. Since then, this symbiotic system has become well understood and exploited as an effective means of raising the nitrogen status of soils, providing nitrogen for crops and pastures (Vincent, 1984). Optimism that non-leguminous crops could be similarly benefited was fueled in the 1970s and 1980s by the discovery of several N_2-fixing organisms (diazotrophs) forming apparently specific associations with non-legumes. On the whole, however, this optimism has not be rewarded.

Recently, several approaches using techniques developed in the area of biotechnology have raised new hopes that success in this secondary objective may yet be realized. It is the authors' opinion that there are now sound reasons to anticipate that at least some non-leguminous

To avoid a too voluminous bibliography, where possible the authors have used review papers to provide background information. Earlier important papers were not overlooked, but are covered in the relevant, more recent, reviews.

field crops may also become independent of soil nitrogen. We intend to explain the reasons for this renewed optimism, against the background of knowledge accumulated in the past century that will be relevant to any ultimate success in exploiting these new approaches.

Nitrogen fixation and sustainable agriculture

A theme of this book is the relationship between biological N_2 fixation and sustainable agriculture. Almost by definition, biological N_2 fixation is synonymous with sustainability. Systems capable of fixing their own nitrogen exploit their own environment less and may even provide a positive contribution.

Nitrogen applied in fertilizers usually provides a benefit to plants. But, if applied inefficiently, it can also have serious disadvantages in causing pollution. It is difficult to match nitrogen supply to actual requirements of a crop at a given ecosite and any excess may damage this or other ecosites. Excess reduced nitrogen (ammonium) in agricultural or forest ecosystems may lead to their acidification through the process of nitrification, if significant leaching of nitrate occurs (Kennedy, 1986a; 1991). In agricultural ecosystems, however, biological N_2 fixation may usually be expected not to exceed the actual nitrogen requirements of an ecosystem, thus being less likely than fertilizers to cause pollution. This conclusion is based on the active repression of the N_2-fixing apparatus by a condition of ample fixed nitrogen and the poorer competitive ability of N_2-fixing organisms when nitrogen supply is adequate.

Key features in achieving N_2-fixing activity

The nitrogenase reaction

Chemically, biological N_2 fixation is essentially the conversion of dinitrogen (N_2) to ammonia, catalyzed by the enzyme nitrogenase according to the reaction (Fd = ferredoxin):

$$N_2 + 8H^+ + 8Fd^- + 16MgATP^{2-} + 18H_2O \rightarrow 2NH_4^+ + 2OH^- + H_2 + 8Fd + 16MgADP^- + 16H_2PO_4^-$$

Of course, the process of N_2 fixation is much more complex than this, but the biochemical reaction contains the key ingredients and points to the major requirements. It indicates the dual needs for reducing potential and the substantial energy requirement in the form of ATP. It also suggests the need for a mechanism for ammonia utilization that will simultaneously neutralize the alkalinity generated (Kennedy, 1986a, 1991). In order to fully appreciate the challenges of extension of the range of N_2-fixing systems, we must examine these requirements in greater detail.

The energy requirement

Estimates of the energy requirement (Schubert, 1982) have been based on theoretical as well as many experimental results. However, the energy requirement for N_2 fixation is actually almost identical to that required for nitrate assimilation (Gibson, 1966; Kennedy, 1988), the other main source of nitrogen for most field crops, excepting rice.

A number of studies have attempted to estimate energy requirements for biological N_2 fixation by comparing the rate of growth of legumes growing on nitrate with their growth on dinitrogen. Measurement of dry matter accumulation has generally indicated little or no difference in growth between the two nitrogen sources provided in ample quantity (except for the initial period, when nodules are being established), suggesting little difference in the need for photosynthate, in agreement with the theoretical predictions based on metabolic pathways.

The other main experimental approach has been the use of efflux of carbon dioxide from legumes growing with either nitrogen source (e.g. Silsbury, 1977). In general, a greater carbon dioxide efflux has been observed on plants growing with dinitrogen than with nitrate (see Schubert, 1982), suggesting an increased energy demand as extra carbohydrate required. Unfortunately, none of these studies considered the fact that nitrate-grown plants produce bicarbonate rather than carbon dioxide in assimilating nitrate (Kennedy, 1986b). When this difference in the metabolism of the two nitrogen sources is taken into account, the apparent difference in energy consumption could disappear.

Nevertheless, for diazotrophs with only indirect access to energy compounds, the criticism regarding adequacy of energy supply is probably valid. Calculations using models of the diffusive flux of 'energy' compounds from the roots of plants indicate that adequate growth of associative organisms in the rhizosphere with biological N_2 fixation significant to the plant is not possible (Whipps and Lynch, 1983). Competition from non-diazotrophs would make this benefit even less likely.

On the other hand, if the diazotrophic organism in an engineered system could have direct access to an ample energy supply from the plant, such negative opinions about proposals to engineer cereals to fix dinitrogen based on energy requirement could be questioned. Substantial infection of the root cortex by organisms like Azospirillum would be a case in point. For such pessimism to be sustained, one would need to dismiss the positive role of leguminous nodules in fixing N_2, which is absurd. To be effective, however, not only penetration but also adequate colonization with the diazotrophs and some kind of symbiosis would be required.

Sensitivity to oxygen

Another potentially negative factor in extending the range of N_2-fixing systems is the well-known sensitivity to molecular oxygen. This sensitivity results from the great instability of nitrogenase when exposed to oxygen. Most diazotrophs regulate this sensitivity by using one of several protective mechanisms (Kennedy, 1979; Postgate, 1974). These protective mechanisms include excessive respiratory consumption of oxygen, the cellular location of nitrogenase, the presence of protective proteins associated with nitrogenase, oxygen buffering as by leghaemoglobin in legume nodules and the less direct means of hydrogenase, catalase and superoxide dismutase activities (Clara and Knowles, 1985). For example, without the protection of leghaemoglobin, isolated Rhizobium bacteroids will fix N_2 only when external oxygen concentration is reduced to about 0.01 atm (Bergersen and Turner, 1967). At the same time, excepting obligately anaerobic clostridia or some facultative organisms, molecular oxygen is an absolute requirement for significant N_2 fixation, since it is the terminal electron acceptor and it is needed to allow formation of ATP by oxidative phosphorylation.

The actual pO_2 at the root surface in soil has rarely been determined. It might seem that, in a well-aerated soil, the oxygen pressure would be too high for sustained N_2 fixation by many diazotrophs. On the other hand, in a waterlogged soil, oxygen pressure would probably be too low. The bulk concentration of oxygen in the soil atmosphere is commonly about 10% of gas content, half that of the free atmosphere and oxygen pressure in soil obviously varies in the range 0–0.20 atm (Nye and Tinker, 1977). Root growth has been observed to be retarded below 0.10 atm, although root metabolism is not seriously affected until oxygen pressure is reduced to 0.02–0.05 atm. Furthermore, an idealized model suggests that, as a result of oxygen consumption by microbes and healthy plant roots, a pO_2 of 0.02 atm at the root surface 0.5 metres below the surface is anticipated for a wheat crop (Kirkham, 1990) – one-tenth that of the free atmosphere, even in an aerated soil at field moisture capacity. Obviously, more experimental data on this point is essential. (However, it can be noted now that 0.02 atm oxygen coincides with the value that we use in our standard assay system for N_2 fixation by *para*-nodulated wheat, to be discussed at length in the second half of this chapter).

Non-leguminous N_2-fixing systems

Nitrogen fixation in non-leguminous symbioses

A number of non-leguminous plants are recognized to be well-endowed with N_2-fixing capacity. Those capable of forming specialized symbioses with Frankia provide many such examples (Millet et al., 1989; Quispel, 1974; Rodriquez-Barrueco et al., 1989). The symbioses between cyanobacteria and various plants (Millbank, 1974) including Azolla (see Skinner et al, 1989) are also significant N_2-fixers. It is not intended to discuss these highly developed symbioses in this chapter, except rarely where comparisons are to be made. These functional non-leguminous symbioses are well described elsewhere.

Free-living N_2-fixing organisms

The free-living diazotrophs were the first to be recognized. It is now clearly understood that a diverse range of microbial species are capable of biological N_2 fixation (Mulder and Brotonegoro, 1974; Postgate, 1972). At the very least, this capacity allows these organisms to pioneer the colonization of nitrogen-poor niches in ecosystems because of the selective advantage they enjoy. It is also recognized that these organisms provide a positive contribution to ecosystems in the nitrogen they fix, perhaps indirectly when they decompose.

As pointed out by Döbereiner (1989), the use of selective media that simulate the natural environment of diazotrophs is still leading to the discovery of previously undescribed N_2-fixing bacteria. This expands the genetic diversity available in the known library of diazotrophs and should be helpful in new technological developments. In this paper, the possible value of these organisms is viewed less from the effect of their growth in situ, but for their potential to be exploited in new technology.

Azotobacter

The hope of increased crop yields from soil inoculation with N_2 fixing bacteria can be traced back almost a century to Caron in Germany. Azotobacter was one of the organisms involved in early research, with favourable results reported from Russia by Kostychev et al. (1926), though the findings in Russia and elsewhere were generally inconsistent and variable (Mishustin and Shil'Nikova, 1971). The problem of inconsistency was not overcome by using well authenticated strains of inoculum. One of the problems with Azotobacter is its poor performance in colonizing the rhizosphere with the exception of the *Azotobacter paspali – Paspalum notatum* association (Döbereiner, 1974). Obviously, *A. paspali* forms a very close and specialized association with a cultivar of paspalum and can be considered as an exceptional associative diazotroph although it does not penetrate the root as does Azospirillum.

Much of the early work with Azotobacter inoculation was carried out in Russia, Eastern Europe and India. Only about a third of field trials showed any positive effects, which ranged up to 39%. Several processes other than N_2 fixation could account for these positive effects, including production of growth regulators, protection from root pathogens and modification of nutrient uptake by the plant (Tchan, 1988).

A report that Azotobacter had been inserted into a fungal mycorrhiza associated with a woody plant (Pinus) (Giles and Whitehead, 1977) – thus providing the first case of a biologically engineered diazotrophic symbiosis – is in serious doubt. Apart from a controversy about a potential threat to pine plantations from associated disease, the validity of the introduction has been strongly disputed (Terzachi and Christensen, 1986).

Beijerinckia

In contrast to the acid-sensitive Azotobacter, Beijerinckia is indifferent to soil pH. This bacterium is adapted to sub-tropical or tropical soils (which are often acid). However, it is not regarded as a rhizosphere organism.

Derxia

Derxia, discussed by Postgate (1972), is unable to commence N_2 fixation and most colonies fail to develop unless oxygen pressure is reduced (Ravishankar et al., 1986). Apparently, Derxia has a relatively inefficient respiratory protection compared to Azotobacter.

Clostridium

The clostridial species such as *Clostridium pasteurianum* and *Cl. butyricum* are strict anaerobes, and therefore fix N_2 only in the condition of the absence of oxygen. Provided sufficient soluble energy substrates are available they grow readily and are quite easy to isolate in nitrogen-deficient media using soil as inoculum.

Photosynthetic N_2-fixing bacteria and cyanobacteria

These organisms grow well in rice fields well supplied with water and could contribute to the supply of nitrogen to rice crops.

Associative diazotrophic systems

Attempts to discover beneficial associations be-

tween plants and N_2-fixing bacteria have a long history. Obviously, a large proportion of the total microbial activity in soil is likely to be associated with the growth of plants, or their subsequent breakdown, simply because this is where growth substrates occur in greatest abundance. For N_2-fixers, Döbereiner (1989) has stressed that too much may be assumed about the degree of an associative relationship simply because bacteria are located in the rhizosphere near the root surface. It may also be remembered that as long as the N_2 fixation carried out by such diazotrophs remains bound to the growth of the bacterium, the possibility of a significant effect on the plant remains slight. Nevertheless, certain bacterial species (in particular the genus Azospirillum, are thought to have a close association with plants, to a point bordering on symbiosis. Dart (1986) has prepared a comprehensive review of N_2-fixing organisms associated with nonlegumes (other than rice) in agriculture.

Azospirillum

The genus Azospirillum is extensively discussed in the book edited by Skinner et al. (1989). This organism is known to penetrate the root of gramineous plant species and to grow intercellularly to a degree, as well as growing in the rhizosphere (Reinhold and Hurek, 1989).

Physiological aspects of the growth and N_2 fixation of various *Azospirillum* species and related bacteria were discussed by Hartmann (1989). The nitrogenase activity of Azospirillum is strongly influenced by both ammonium (Gallani and Bazzicapulo, 1985; Hartmann et al., 1986) and oxygen. When ammonium is added, or anaerobic conditions are imposed, the nitrogenase of *A. brasilense* and *A. lipoferum* is 'switched-off' by ADP-ribosylation (Hartmann et al., 1985, 1986; Hartmann and Burris 1987; Kanemoto and Ludden, 1984) of nitrogenase reductase (iron-sulphur protein). High oxygen levels, inhibitory to nitrogenase activity, do not cause this covalent modification to occur (Hartmann and Burris, 1987).

The release of nitrogen compounds by Azospirillum has been examined on only a few occasions. Release of nitrogen compounds in batch cultures has been observed for *A. lipoferum* (Volpon et al., 1981), *A. amazonense* and *A. halopraeferens* (Hurek et al., 1987). Energy-starved cultures of Azospirillum (Hartmann et al., 1985) and at low levels of glutamate or aspartate (Hartmann et al., 1986) release low amounts of ammonium. Glutamine auxotrophic mutants of *A. brasilense*, defective in glutamine synthetase (Gauthier and Elmerich, 1977) or glutamate synthase (Bani et al., 1980) release ammonium under some conditions (Hartmann, 1989). Of course, the rapid and prolonged release of ammonia is a prominent feature of Rhizobium bacteroids in legume nodules, but the potential for efficient release of nitrogen compounds to the host plant by Azospirillum is poorly understood at this stage. No clear evidence that azospirilla actively excrete newly-fixed nitrogen compounds into plant tissues is available. If it is true that N_2 fixation by azospirilla is bound to growing cells of the microorganism in plant associations, this would be a possible reason for poor performance of the system.

The oxygen sensitivity of the nitrogenase activity of Azospirillum has been discussed in detail (see Fig. 1). In the figure redrawn from Hartmann (1989), the complete abolition of nitrogenase at 1.0 kPa oxygen concentration is shown, with a strain of *A. amazonense* being more tolerant of oxygen.

The natural occurrence, distribution and survival of *Azospirillum* spp as a crop inoculant in Belgian agricultural soils was discussed by De Coninck et al. (1989). They point out that the three modes of entry by N_2-fixing bacteria into plant roots are through wounds, especially at the junction with lateral roots, through root hairs and between undamaged epidermal cells; rhizobia are known to employ all three methods, and Frankia the second two. But the mode of entry of Azospirillum remains unknown.

Acetobacter

Acetobacter diazotrophicus, an associative diazotroph, provides an outstanding example of the bacterial ecological requirement. This organism needs high sugar concentration in the medium to grow (Döbereiner, 1989), which need is apparently satisfied in association with sugarcane (Li and MacRae, 1991).

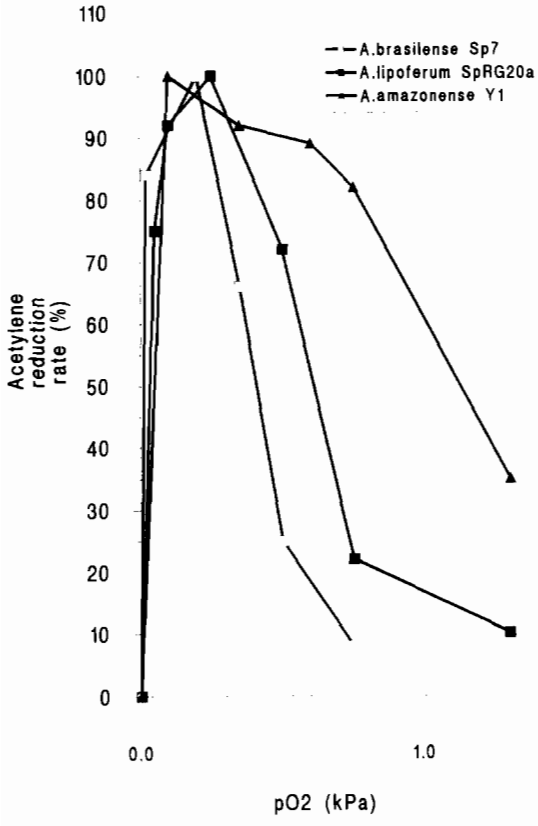

Fig. 1. Oxygen tolerance of nitrogen fixation (C_2H_2 reduction) in azospirilla. Redrawn from Hartmann (1989).

Pseudomonas

Wetland rice may have large populations of N_2-fixing *pseudomonas diazotrophicus*, sometimes amounting to about 80% of the total bacterial population associated with rice roots. The ability of this organism to grow autotrophically with H_2 and CO_2 may be of great significance under flooded rice conditions where H_2 is often evolved (Barraquio et al., 1983; Watanabe et al., 1987a). Pseudomonads have also been observed in the root systems of sugarcane (Li and MacRae, 1991).

Alcaligenes

The diazotrophic *Alcaligenes faecalis* is capable of entering rice roots, as shown by You and Zhou (1989).

Klebsiella

Klebsiella pneumoniae has been identified as a prominent N_2-fixing inhabitant of the root system of sugarcane (Li and MacRae, 1991).

Miscellaneous species of significance

Azorhizobium

The diazotrophic organism, *Azorhizobium caulinodans*, capable of free-living N_2 fixation, is the microsymbiont in the stem nodules of Sesbania (discussed by Ladha et al., 1990). Stem nodules are regarded as an adaptation to flooding to achieve adequate oxygenation. The rates of nitrogen fixation achieved in such nodules can be as high as 17 kg N per ha per day. The symbiosis between *A. caulinodans* and *S. rostrata* is highly specific and the microsymbiont is unable to produce stem or root nodules in any of the stem-nodulating *Aeschynomene* species. *A. caulinodans* is also exceptional in being able to grow on N_2 under free-living conditions without added fixed nitrogen (Dreyfus et al., 1983). Recently, Ladha et al (1989) reported the presence of *A. caulinodans* in the rhizosphere of rice. Further, rice seedlings when inoculated with this organism showed significant nitrogenase activity. There are also recent reports (Ladha et al., 1990) that this organism can contain bacteriochlorophyll and photosynthetic reaction centres. If the organism can carry out photosynthesis growing at up to 0.03 atm O_2, this is most exceptional for a photosynthetic bacterium and this proposal requires careful examination. Such a capability would be of obvious benefit to a system biologically fixing nitrogen.

Effectiveness of Azospirillum and other plant associations/symbioses

Since the excitement of the initial findings about Azospirillum by Döbereiner and her colleagues (Döbereiner, 1983), expectations regarding the practical application of this organism in agriculture have been largely unfulfilled. No significant agricultural systems have been established that involve extensive inoculation by farmers of field crops with organisms such as Azospirillum, a fact that highlights the lack of its successful application. Despite this, a considerable amount of

information exists with several field crops that needs to be considered.

Associative organisms and wheat

Experimental work in countries other than Australia has shown that inoculation of wheat with *A. brasilense* and *A. lipoferum* can increase significantly yields of foliage and grain (Hegazi and Saleh, 1985; Kapulnik et al., 1983; Mertens and Hess, 1984; Rai and Gaur, 1982). The cause of such increases has not been determined, but improved nitrogen nutrition from N_2 fixation by azospirilla cannot be excluded, although this has been questioned (Jagnow, 1990). Positive results from work in Israel (Millet and Feldman, 1984) has even led to some field inoculations as an agronomic practice (Okon et al., 1988), although its success under farming conditions still needs verification.

From a survey of wheat plants grown in the field throughout the eastern Australian wheat belt (New and Kennedy, 1989) a culture collection of more than 20 strains of Azospirillum isolated from the root systems has been established. A pattern of distribution has emerged in which only neutral soils contain a significant population of these diazotrophs (New and Kennedy, 1989). Soils below pH 5.0 contained few if any native azospirilla.

Although our greenhouse trials have failed to show significant effects from inoculation of wheat with azospirilla, apart from some stimulation of early vegetative yield, tests in laboratory model systems have shown that particular strains of azospirilla are more effective than others in giving significant C_2H_2 reduction with particular cultivars of wheat. The best of these was a *A. brasilense* (Sp 107)/Sunelg, University of Sydney) combination (Kennedy et al., 1990), where 3-week-old wheat plants produced 48 nmoles of ethylene per h per plant (30°C). Assuming that this nitrogenase activity could be sustained and would increase proportional to root weight, a substantial contribution to the nitrogen needs of the wheat plant would be made. More typically, C_2H_2 reduction activity in these model associative systems lies between just above zero activity in more than half our tests to about 10 nmoles ethylene produced per hr per plant at this plant age. No obvious reason for this variability with wheat seedlings, documented extensively by Jagnow (1990), could be found. Furthermore, even those plants with a high rate of C_2H_2 reduction did not necessarily contain the greatest number of viable azospirilla and the two parameters – number of Azospirillum and rate of N_2 fixation- were poorly correlated.

A major problem in achieving significant and reliable N_2 fixation in such associations could result from the poor internal colonization of the roots of wheat by the azospirilla. For example, a factor of 10^4 fewer azospirilla (*A. brasilense* Sp7) were found in the root interior (endorhizosphere) compared to the root exterior (New et al., 1991). Even if the endorhizosphere population is underestimated by a factor of 100 because of the procedure employed to establish most probable numbers of bacteria in this location, it would appear that only a low proportion of the azospirilla associated with wheat roots has direct access to energy substrates. Whether the occasional seedlings that display strong C_2H_2 reduction have more internal colonization, or show strong excretion of energy substrates or are affected otherwise, is not known.

In view of the difficulty in satisfying all the demands for a satisfactory symbiosis, it is not surprising that positive effects are inconsistently observed. One must question whether sufficient numbers of azospirilla with sufficient carbon substrates are present in most cases to make a significant contribution to the nitrogen needs of the plant.

Rice

Published evidence on experiments with rice present an overall picture that is, on the whole, more positive for associative N_2 fixation than for wheat.

Several reports of N_2 fixation associated with the rhizosphere of rice have been made. These are summarised by Yoo et al. (1986), who indicate a number of associated organisms (Azospirillum, Clostridium, Enterobacter, Alcaligenes, Pseudomonas) in a report in which they described the contribution of *Klebsiella oxytoca* to N_2 fixation benefiting rice plants. A 6% increase in plant + soil nitrogen content and a significant incorporation of $^{15}N_2$ was observed. Up to 30–40 kg nitrogen ha^{-1} $crop^{-1}$ are attribu-

ted to association with unidentified diazotrophs (App et al., 1984). Fujii et al. (1987) have described the benefits of use of strains of *Klebsiella oxytoca* and *Enterobacter cloacae*, able to fix N_2 in the presence of ammonium, for inoculation of rice.

A substantial number of studies have been conducted at the International Rice Research Institute in the Philippines (Ladha et al., 1986; 1987; 1989; Roger and Ladha, 1990) using rice. On the whole, these results suggest that significant nutrition of rice supplying perhaps 20–25% of the total needs of rice from associative fixation is occurring (App et al., 1980; Watanabe et al., 1987b; Zhu, 1989). Critical evidence of the likelihood of actual yield increases by inoculation of rice is lacking.

Maize

Diem and Dommergues (1979) reviewed results from some field trials using inoculation with Azospirillum in Wisconsin, where the soils were practically devoid of the organism before treatment. Of four experiments with maize only two showed significant positive results.

An evaluation of the effect of oxygen pressure and available carbon substrates (Alexander and Zuberer, 1989) on C_2H_2 reduction by maize showed that both these factors were significant. C_2H_2 reduction activity was greatest with a pO_2 between 1.3–2.1 kPa and amendment with several organic substrates greatly increased activity if it was initially low. Inorganic nitrogen (4–20 mg L^{-1}) abolished C_2H_2 reduction within about 2 h. Rates of activity with individual plants varied greatly but the maximum rates observed were of the order of 300–800 nmoles C_2H_4 per h per g dry weight of roots. There was no effect of inoculation with azospirilla on dry weight of plants grown in soil in a series of 15 experiments, and C_2H_2 reduction was depressed compared to uninoculated controls with an indigenous population of bacteria. Thus, either the indigenous bacteria are more effective or inoculation is counterproductive.

Millet

Smith et al. (1976) found that pearl millet showed a significant increase in dry matter, associated with increased harvest of nitrogen per unit area, but subsequent work did not prove that these gains occurred from N_2 fixation by the plant (Smith et al., 1978, 1984). Instead, as Tien et al. (1979) and Harari et al. (1989) have indicated, growth substances produced by Azospirillum may be the source of these gains by plants.

Occasional trials conducted at ICRISAT with pearl millet have produced increased grain yield up to 33% over uninoculated controls. More typically, no significant increase was observed (Wani et al., 1989) from inoculation with *A. lipoferum*. A similarly variable result was obtained from inoculation with *Azotobacter chroococcum*. Although an increased nitrogen uptake by plants was measured in this series of experiments, equivalent to about 3–19 kg per ha over three seasons, no evidence was obtained that this was the result of N_2 fixation.

Sugarcane

A substantial input of nitrogen from associative fixation is indicated in trials using ^{15}N (Urquiaga et al., 1989), with from 20–55% of the plant nitrogen indicated from this source. The range of organisms involved under Australian conditions includes pseudomonads, enterobacters, Acetobacter, Beijerinckia and *Klebsiella pneumoniae*, but did not include Azospirillum or Azotobacter (Li and MacRae, 1991).

Sorghum

Recent studies on the effect of inoculation with azospirilla (Pacovsky, 1989; Pereira et al., 1989) do not provide convincing evidence that grain yield can be increased from the association with this diazotroph.

New nodulation technology for non-leguminous field crops

The discovery of the first known rhizobial symbiosis with a non-legume (Parasponia-Rhizobium) by Trinick (1973, 1988) provided an important object lesson for scientists with interests in biological N_2 fixation. Sprent and de Faira (1989) have recently emphasised that many of the widely accepted dogmas for 'normal' symbioses, for example root hair infection and the

necessity for bacteria to be released from infection threads before they differentiate into N_2-fixing forms, are not universal. They suggest that a comparative study of a range of systems may help exploit the possibilities for increasing the range of N_2-fixing systems. Infection through wounds is now well established as a normal part of nodule initiation in some legumes (Sprent and de Faria, 1989), so that the idea of a fixed, obligatory procedure cannot be sustained although such schemes provide helpful conceptual models for study.

The recognition that Bradyrhizobium cells initially enter the roots of Parasponia at localized sites of plant cell division having disrupted the epidermis (Bender et al., 1987) also emphasises that various developmental paths may lead to a functional nodule. Only subsequently do infection threads develop from within the cortex and participate in the nodule development. The absence of infection threads in the formation of lupin nodules contrasts with their occurrence in nodule formation in serradella roots, even though *Rhizobium lupini* is the microsymbiont in each case (Haack, 1961). Despite genetical studies lasting more than ten years, no evidence has been found that the legume-Rhizobium symbiosis involves unique plant gene products that are necessarily restricted to the plant species that so far successfully nodulate and fix N_2. Neither do any of the proposed steps in Rhizobium-legume symbiosis (e.g. coded as *hac* (hair curling), *noi* (nodule initiation), etc.) infer a uniqueness that would absolutely exclude different plant species from also allowing establishment of persistent symbioses with diazotrophs.

The concept of establishment of artificially induced symbioses between plants and so-called L-forms of bacteria was experimentally explored by Aloysius and Paton (1984). Protoplasts of bacteria (L-forms) were suggested to have the ability to penetrate the cell wall and membrane structures of living plant cells and to colonize plant tissue. L-forms of Azotobacter, *Pseudomonas syringae*, *Bacillus polymyxa* and *Beijerinckia indica* were all considered as capable of penetration of plant tissue and some evidence of expression of bacterial metabolic activity in plant tissue was obtained. No tests for nitrogenase activity were performed. However, approaches such as this offer possible means of allowing non-legumes such as cereals to fix their own N_2. Further investigations are needed.

Here, we will review recent developments suggesting that the occurrence of N_2-fixing symbioses may eventually comprise a much more diverse range of systems than has previously been imagined.

Induction with hydrolytic enzymes

Experiments performed at Nottingham in the U.K. (Cocking et al., 1990) have developed new associations between *Rhizobium* spp. and a range of non-leguminous field crops. One of the areas of investigation has been the removal of barriers in the plant to entry by the bacterium, using enzymatic degradation of the plant cell walls and of the root hairs. Some progress has been made with a range of legumes and non-legumes, including cereals. Their technique involves immersion of rapidly growing roots in cell wall-degrading enzymes in hormone-free culture medium with sucrose. Rapid degradation of the plant cell wall at the apices of root hairs has been observed. The use of a cellulase-pectolyase enzyme solution has been extended from white clover seedlings (where the specificity of rhizobial infection was broken down) to rice. Up to ten per cent of rice seedlings developed nodular structures after treatment with enzymes and inoculation with various rhizobia, both singly and in combination. Omission of the enzyme treatment resulted in no such structures being formed.

Al-Mallah et al. (1989) investigated the formation of nodular structures by rhizobia on rice seedlings. They suggested that nodulation of non-legumes may be promoted by enzymatic cell wall degradation coupled with polyethylene glycol treatment. This apparently assists the entry of rhizobia, though nitrogenase activity in the resulting nodules was barely detectable. Bacteria in the structures were mostly located between cell layers, with some infection of cells that usually appear to lack well-defined cytoplasm.

Trials with some varieties of wheat (*Triticum aestivum*) also produced nodules on the roots, following enzyme treatment and inoculation with

Rhizobium in the presence of polyethylene glycol (PEG). Signs of N_2 fixation were barely detectable, possibly due to the limited extent of infection of host cells.

Some varieties of Rhizobium induce nodules on the roots of oilseed rape, inoculated subsequent to an enzyme treatment of the seedlings. A synergistic interaction between *R. loti* and *Bradyrhizobium* produced the highest rate of nodulation on all plant varieties tested. Invasion occurred both within and between the cells of the nodules. Nodules which developed on oilseed rape reduced C_2H_2 per unit fresh weight very feebly, at less than 0.1% of that seen typically in legume nodules.

Nodulation of oilseed rape by Parasponia-infecting rhizobia

Bradyrhizobium parasponium is capable of infecting the roots of oilseed rape (*Brassica napus*) without need of enzyme treatment (Cocking et al., 1990). A variety of nodule types have been observed after infection with *B. parasponium*; some contain red/pink pigmentation, suggesting but not proving the production of leghaemoglobin. Rhizobia are present both within and between the cells of the host tissue, and some were also associated with the surface of the nodules.

Non-genetical approaches to induced nodulation of non-legumes

From the early 1980s, Nie and his colleagues at Shandong University in China have published a series of papers in Chinese journals, reporting the nodule-inducing effect of 2,4-dichlorophenoxyacetate (2,4-D) on the roots of a large number of plant species, including wheat (see Tchan and Kennedy, 1989, for review). This approach resulted from an initial observation by Nie, made when using a plant tissue culture medium containing 2,4-D.

Even earlier observations were made of the development of such structures in both legumes (Allen et al., 1953; Vincent, pers. commun.; Wilde, 1951) and non-legumes (Nutman, 1944, pers. commun.) on treating plants with hormone herbicides. However, no inference was made in earlier work that these structures might contain bacteria or that the structures might be beneficial. To its credit, Nie's group recognized this, although it was also understood that the structures were primarily a response to 2,4-D treatment, since they were formed irrespective of whether the roots were inoculated with a *Rhizobium* species or not. The Chinese researchers have continued this work in the hope of eventually emulating leguminous N_2-fixing nodules, but without obtaining acceptable evidence of positive N_2 fixation (see Tchan and Kennedy, 1989).

Other research centres in China have subsequently carried out work on inducing nodular structures on non-legumes not normally nodulated. Chen and his colleagues in Beijing confirmed the results of Nie's group. They have also reported stimulation in the growth of wheat when treated with 2,4-D, but this effect was not dependent on inoculation with rhizobia. Chen (personal communication, 1990) has also reported positive but low rates of N_2 fixation in 2,4-D-induced nodulated roots of wheat using *Azorhizobium caulinodans* as microsymbiont.

Jing et al. (1990a) also contributed in China to this activity regarding an extended potential for diazotrophs. Their findings, published in a European journal, suggest that magnetic effects are capable in some unexplained way of inducing nodular structures on barley roots. These structures apparently contain inoculated bacteria (*Rhizobium astragali*) and formation of the 'pseudonodules' was accompanied by the presence of infection threads and formation of bacteroids enclosed within a peribacteroid membrane. No C_2H_2-reducing activity was observed. Unfortunately, it is impossible to assess their published results critically, since no quantitative data were given on the relative rates of nodulation in test plants versus control plants. The paper does not explicitly state that the controls were also negative. These experiments will need to be supported by suitable quantitative data before their relevance can be assessed.

Genetical approaches to induced nodulation of non-legumes

One approach to forming nodules on plants not normally nodulated is the purely genetical one.

For example, a successful transfer of genes associated with root hair curling (*hac*) was made from *Rhizobium trifolii* into a derivative *Rhizobium trifolii* on the plasmids pKT230 and pRK290 (Plazinski et al., 1985). The root hair-curling character could then be expressed on infection of rice seedlings. A similar transfer of the genes from *Rhizobium meliloti* into *Azospirillum brasilense* on the plasmid pVK100 was successfully achieved. Subsequent inoculation of maize seedlings also resulted in root hair curling (Piana et al., 1988).

Rolfe and Bender (1990) recently described further developments in their attempts to evolve rhizobia for non-legume nodulation. A strain of Rhizobium reconstructed from one capable of nodulating Parasponia with a *nodD* allele inserted into its genome inoculated onto rice seedlings occasionally resulted in nodule-like structures on the roots (see Fig. 2). These contained vascular bundles and membrane-encapsulated bacteria, though there was no sign of an infection thread and no detectable N_2 fixation.

In other work with rice, Jing et al. (1990b) have described formation of root nodules using a mutant of *Rhizobium sesbania* (*Azorhizobium caulinodans*). The structure of these nodules and their formation was claimed to be similar to those of legume nodules such as soybean, including the presence of leghaemoglobin. A rate of C_2H_2 reduction per g of nodule tissue about 1.5% of that shown by legume nodules was observed, although it is not clear that adequate controls were performed.

Recent studies on 2,4-D-induced para-*nodules*

Tchan and Kennedy (1989) reviewed work on 2,4-D induction from original Chinese sources (supplied by Nie in late 1987) and reported their own confirmation of the effect of 2,4-D in inducing nodular structures on wheat. Deliberately seeking an alternative term for these structures that emphasises their 'apart-ness' from legume nodules, Tchan and Kennedy have named them *para*-nodules (Kennedy et al., 1990; e.g. *para* = beyond). Since our review and confirmation of the results obtained by Nie in China from 1980 (Tchan and Kennedy, 1989), we have consistently regarded it as counterproductive to consider *para*-nodules simply as models of legume nodules. We have the firm opinion that progress is more likely if the dogma of the process of legume nodulation is not used in their future development, although the reasons for success of legume nodules obviously need to be considered.

Bender et al. (1990) recently also examined the effect of 2,4-D on wheat seedlings. Within a narrow concentration range, some lateral roots became modified to form structures resembling the nodules formed on legumes, while other lateral roots and top growth were normal. Plants exposed to higher concentrations (5×10^{-6} *M* 2,4-D and above) had few or no lateral roots, while the top growth was yellow and reduced in size. As previously observed (see also Tchan and Kennedy, 1989) all inoculated wheat plants formed nodules (unlike nodules on rice as referred to above), but these were not observed to be highly organized internally. No evidence of N_2 fixation has been reported for these nodules, using rhizobia as the infective bacteria.

Experiments with wheat plants

Inoculation with rhizobia

Under aseptical conditions modelled on an approach pioneered by Nie and his colleagues at Shandong University, para-nodules are readily formed on wheat (see Fig. 3) using 2,4-D (0.5–1 ppm in the nitrogen-free hydroponic medium); these may occasionally be infected with inoculated rhizobia. In experiments conducted in Sydney (Kennedy et al., 1991), there is no clear-cut evidence of intra-cellular infections with bacteroids surrounded by peribacteroid membranes as in legume nodules (although some earlier electron micrographs obtained by Nie in China are suggestive of these). Significant infection by rhizobia occurs in only about 10–20% of cases and the *para*-nodules are disorganized internally with most bacteria being located distant from rudimentary central vascular tissue in extracellular pockets (Fig. 4) or in cells without normal cytoplasmic contents. Similar results were obtained by Yu (1988).

Furthermore, no N_2 fixation (C_2H_2 reduction) is observed when rhizobia are used for inoculation (Kennedy et al., 1990). In one recent experiment using 15 diverse strains of rhizobia

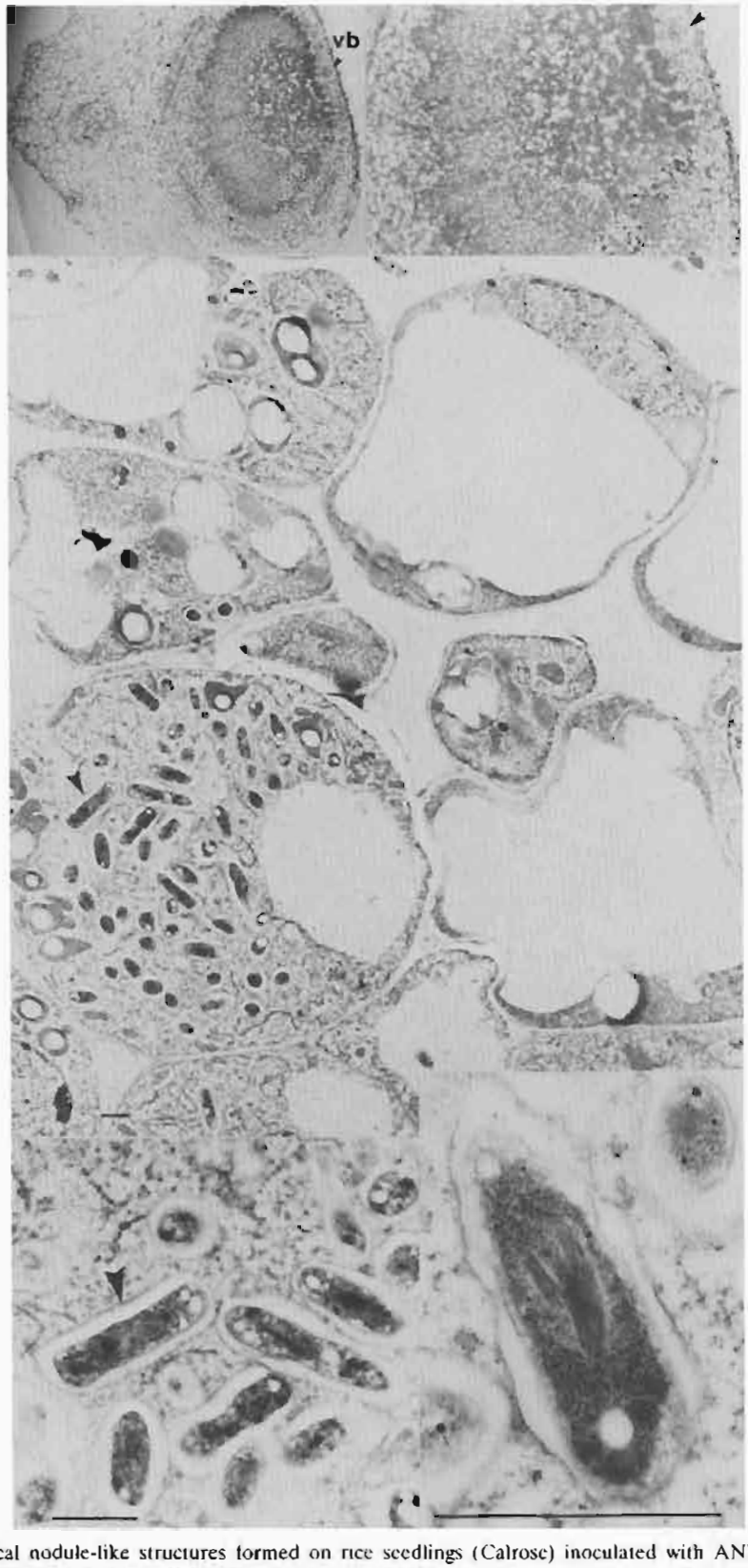

Fig. 2. Small, spherical nodule-like structures formed on rice seedlings (Calrose) inoculated with ANU536. (a) and (b) light micrograph sections showing infected cells and vascular bundles (vb); (c), (d) and (e) electron micrographs of infected plant cells showing membrane encapsulated bacteria. *Bar* = 1 μm. Reproduced from Rolfe and Bender (1990). These micrographs were kindly provided by Professor B. Rolfe.

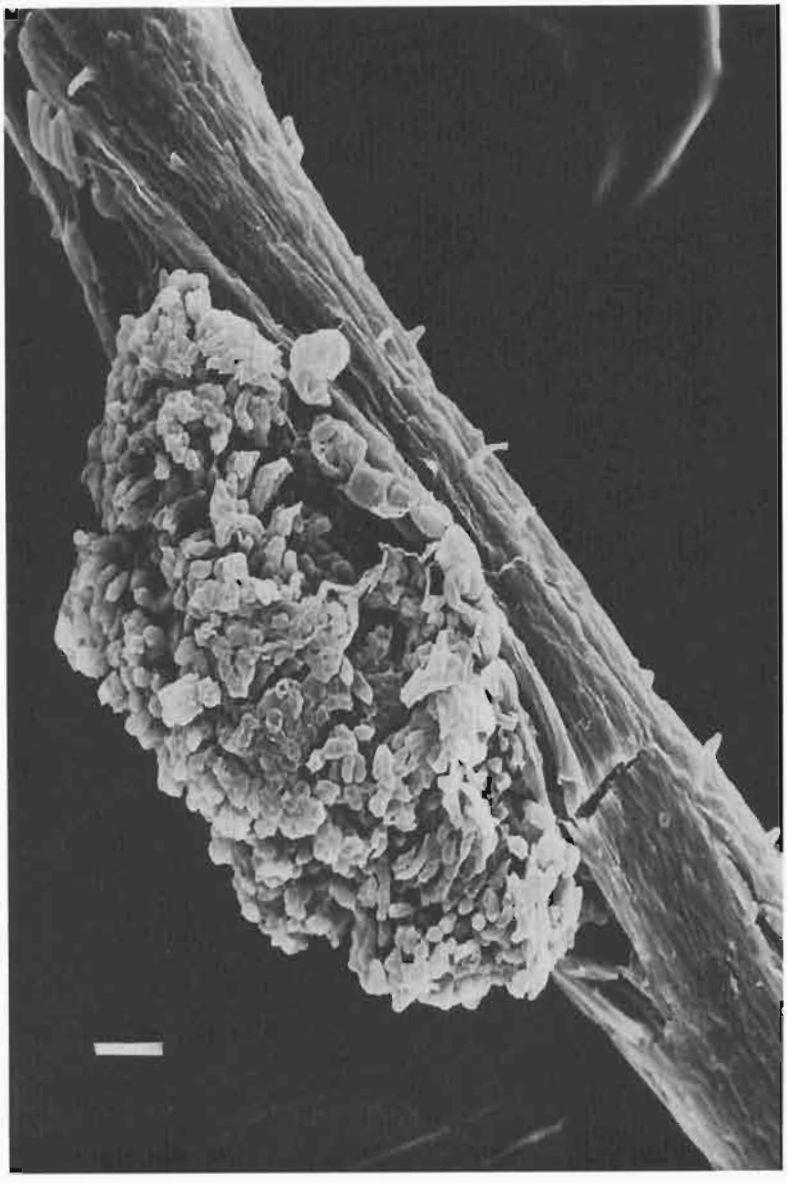

Fig. 3. Scanning electron micrograph showing a *para*-nodule of wheat, inoculated with *Rhizobium leguminosarum*. *Bar* = 100 μm.

representing the range of commercial strains used in Australia (Roughley, 1988), provided by the Australian Inoculants Research and Control Service, no C_2H_2-reducing activity was observed for each association. In these experiments, oxygen pressure was varied during a three-day exposure to C_2H_2 over the range 0.02 atm to 0.21 atm and still no ethylene was formed.

Inoculation with azospirilla

Our philosophical viewpoint that *para*-nodules should not be regarded as models of the legume-Rhizobium symbiosis immediately led to a proposal to employ a free-living diazotroph such as Azospirillum as a potential N_2-fixing microsymbiont. This decision was also based on the well-known fact that azospirilla are associative diazo-

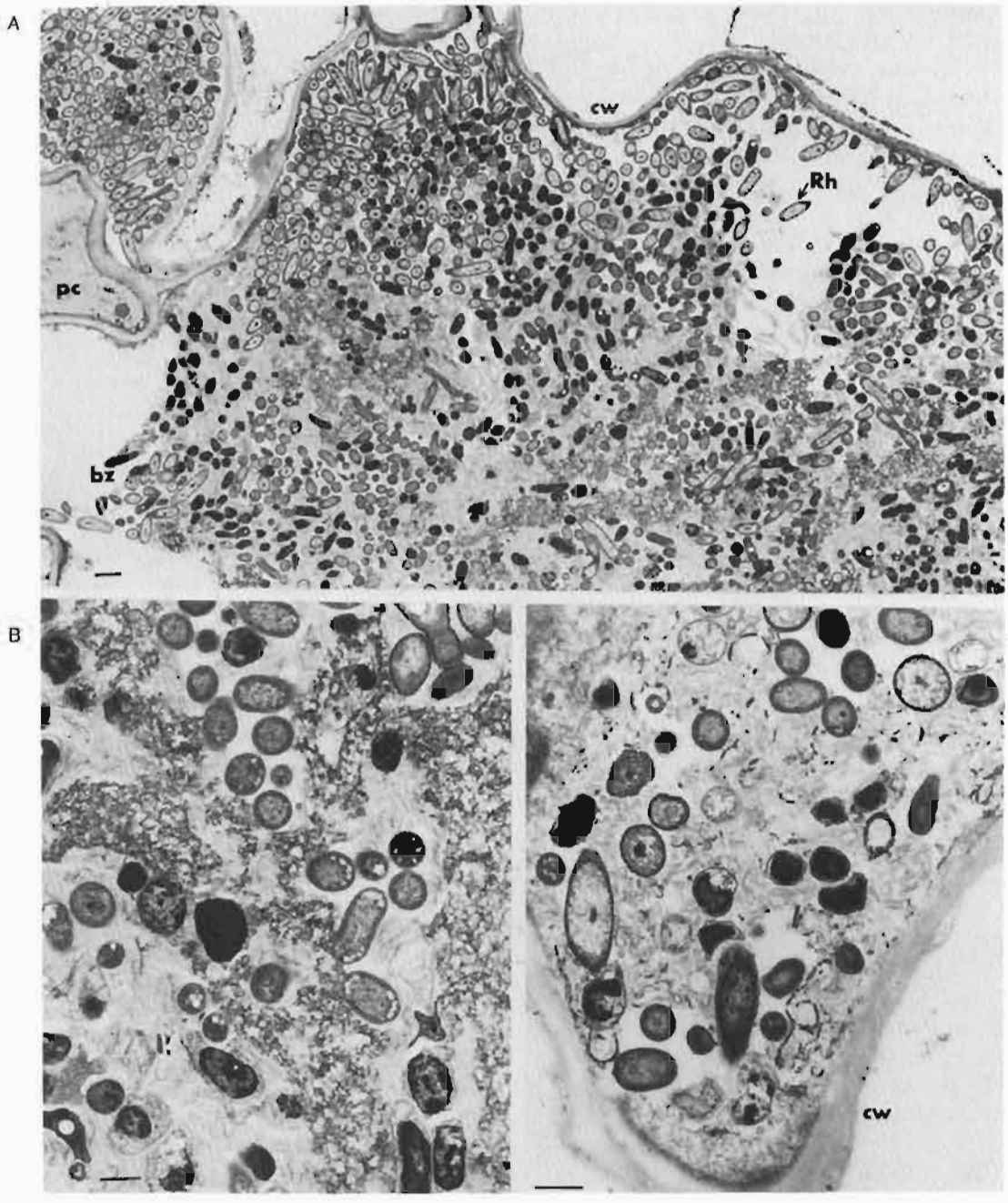

Fig. 4. Electron micrograph of wheat *para*-nodule infected with *Rhizobium astragali*. The cell wall (cw) and rhizobia (Rh) are shown, without evidence of infection of plant cell cytoplasm (pc). **A**, *Bar* = 1 μm; **B**, **C**, *Bar* = 0.5 μm.

trophs of grasses, such as wheat. Encouraging results have been obtained since adopting this viewpoint, in testing the hypothesis that these *para*-nodules based on Azospirillum as the infective agent provide a new model of a N_2-fixing symbiosis in non-legumes (Zeman et al., 1991).

In contrast to experiments with rhizobia, we now consistently observe substantial rates of

ethylene production when *para*-nodulated wheat seedlings have been inoculated with Azospirillum. The ethylene production under a range of treatments is dependent on both inoculation with azospirilla and on the presence of C_2H_2. There is no spontaneous measurable ethylene production by the plant seedlings treated with 2,4-D unless both C_2H_2 and azospirilla are present.

Seedlings inoculated with Azospirillum, but with no 2,4-D treatment, often reduce C_2H_2 as well, but only in the range of 0–20% the rate of the para-nodulated plants within individual experiments. Another characteristic of 2,4-D-influenced C_2H_2 reduction by azospirilla is a much higher degree of reproducibility with replicated plants compared to seedlings inoculated with Azospirillum alone.

In order to be certain that the nitrogenase activity is not that of free-living azospirilla, we routinely flood the roots with Winogradsky's mineral nitrogen-free medium and shake the flasks using an atmosphere of 0.02 atm O_2 (Kennedy et al., 1990; Tchan et al., 1990). We have verified (Tchan et al., 1991) that, under these conditions, the nitrogenase activity of free-living bacteria and of azospirilla added to sterile wheat seedlings is abolished by oxygen inhibition at 0.02 atm oxygen, as would be predicted from the known sensitivity of Azospirillum shown in Figure 1. Apparently, the agitation in Winogradsky's medium is sufficient to disperse microbes that develop in colonies on the root surface. Consequently, if exposures are not too prolonged, the activity measured should be of azospirilla infecting the para-nodules or colonizing the interior of root tissues. However, this situation is complex and more critical tests will be needed before strong assertions about the exact location of the 2,4-D-dependent activity can be made.

The rate of C_2H_2 reduction by *Azospirillum brasilense* Sp7 in associative and *para*-nodulated wheat seedlings in the range 0–0.08 atm pO_2 is shown in Figure 5. Note that in the standard assay system with Winogradsky's medium added, no C_2H_2 reduction occurs in the associative system with pO_2 higher than 0.01 atm, a result similar to that obtained when Azospirillum in liquid culture is added to uninoculated wheat seedlings at the commencement of the nitrogenase assay. This suggests that nearly all active azospirilla in the associative system without added 2,4-D reside near the surface of the roots, within easy reach of external oxygen.

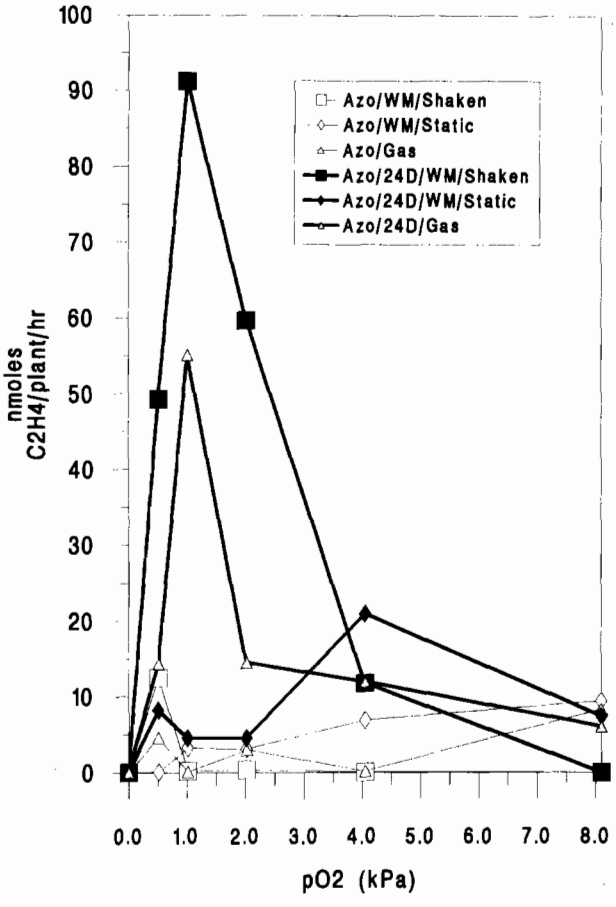

Fig. 5. Effect of pO_2 on the rate of C_2H_2 reduction by associative azospirilla and in *para*-nodules. Seedlings with and without 2,4-D treatment were inoculated with *A. brasilense* Sp7 at one week of age and grown under continuous light for two more weeks. C_2H_2-reducing activity was measured at 30°C, with Winogradsky's N-free medium (WM) sometimes immersing the roots.

Inspection by phase microscopy of individual plants previously tested as positive for N_2-fixing activity suggests that the azospirilla colonize the *para*-nodules and their vicinity strongly (Kennedy et al., 1991a). INT-stained root systems from the most active plants invariably show large numbers of stained bacteria, particularly near the base of the *para*-nodules. While not direct proof that these bacteria in *para*-nodules are reducing C_2H_2, this qualitative association is suggestive of this.

The findings of dependence on inoculation with azospirilla, the presence of C_2H_2, the inhibition by oxygen and a positive correlation between high rates of activity and the obvious presence of azospirilla in the basal *para*-nodule cells suggest strongly that the C_2H_4 production observed results from nitrogenase activity. Furthermore, or preliminary experiments with $^{15}N_2$ indicate significant enrichment of total Kjeldahl nitrogen (0.040 atom per cent excess ^{15}N) in *para*-nodulated root systems. In view of these results, it is unlikely that the ethylene production observed is other than nitrogenase-related.

Effects of plant growth regulators and colchicine
We have tested the effects of indoleacetic acid (IAA), naphthalene acetic acid (NAA) and colchicine on the rate of development and C_2H_2-reducing activity of *para*-nodules (Kennedy et al., 1991a). For *para*-nodules to play a significant role in the nutrition of field crops such as wheat, they will need to remain in function throughout the vegetative growth phase of the plants or be continuously initiated on the root system. Therefore, information on the effects of growth regulators or other substances on their rate of initiation and development should be of value.

Initially, the effect of colchicine was tested in case, by increasing the frequency of polyploid cells, it might increase the frequency of para-nodulation, an argument based on the observation that the initiation of legume nodules involves production of polyploid cells (Vincent, 1980). It was found that no such increase occurred but that a marked increase in the size of *para*-nodules was obtained with a treatment for 24 h of 0.005% colchicine (see Fig. 6). This occurred below the polyploidy threshold of 0.007% for wheat seedlings (Darvey, 1972) and the effect was abolished by more than 0.010% colchicine. With *Rhizobium meliloti* as inoculant, no C_2H_2 reduction was observed with these colchicine-affected *para*-nodules.

In contrast, with *Azospirillum brasilense* as inoculant, strong C_2H_2 reduction is obtained with 2,4-D-treated seedlings and in a limited number of experiments a substantial increase in the rate of C_2H_2 reduction with colchicine treatment as well has been observed, although this result is rather erratic. Colchicine did not reliably increase the rate of C_2H_2 reduction of wheat seedlings inoculated with azospirilla alone without the formation of *para*-nodules (Kennedy et al., 1991a). Colchicine has been reported to affect the initiation of lateral root primordia (Foard et al., 1965).

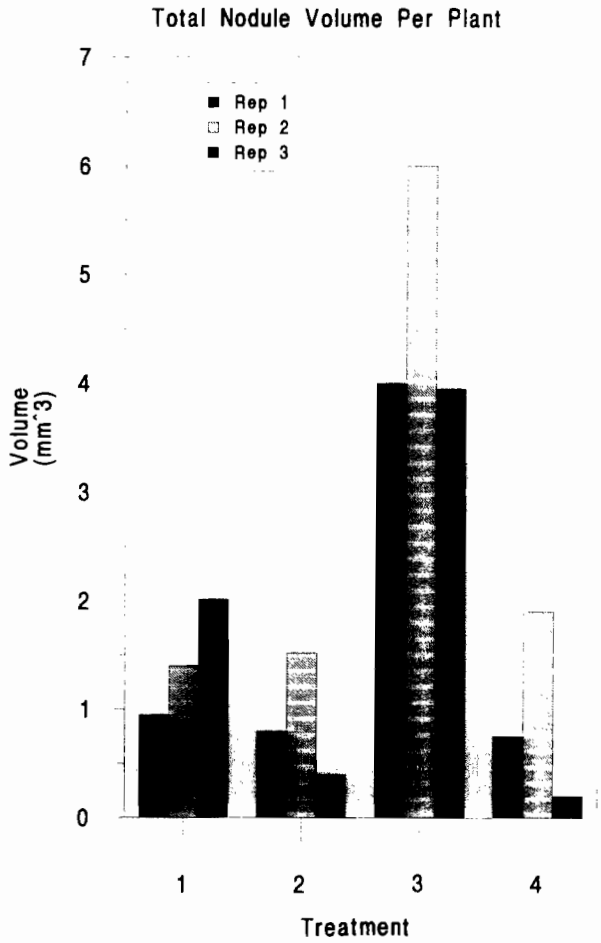

Fig. 6. Effect of colchicine treatment on the volume of *para*-nodules of wheat infected with *R. meliloti* Wheat (*Triticum aestivum* L.) seedlings were germinated on agar and grown hydroponically until one week old before pre-treatment with colchicine for 24 h just prior to *para*-nodulation (Kennedy et al., 1991). Nodule dimensions were determined two weeks later using root systems stained with iodonitrotetrazolium (0.2% in water), using a micrometer eyepiece on an Olympus dissecting microscope. Treatments: 1 = 2,4-D; 2 = 2,4-D + *R. meliloti*; 3 = 2 + 0.005% colch.; 4 = 3 with continuous colchicine.

With NAA *plus* 2,4-D, a strong stimulation of C_2H_2 reduction was obtained (Table 1) accompanied by some modification of the development of the *para*-nodules. Perhaps significantly, wheat seedlings without 2,4-D treatment but with IAA

Table 1. C_2H_2 reduction in *para*-nodules and associative systems

Para-nodules	nmoles h^{-1} $plant^{-1}$ (ca. 100 mg f.w. roots)			
	Azos.[c]	2,4-D + *Azos.*	NAA + *Azos.*	NAA/2,4-D/*Azos*
Exp. 1 (n = 5)[a]	0.4(0.4)[b]	34.1(35.0)	2.7(3.1)	79.2(86.8)
Exp. 2 (n = 9)	0.0(0)	7.0(5.4)	7.9(19.1)	38.3(21.8)
Exp. 3 (n = 5)	0.2(0.4)	18.9(18.3)	–	–

×40 converts these values to per g dry weight roots (approx.)

	nmoles h^{-1} g^{-1} f.w. nodules	ref.
Oil-seed rape nodules	10	Cocking et al. (1990)
Rice nodules	204	Jing et al. (1990b)
Para-nodulated seedlings	24,750	Mean value, data above[d]

[a]n = number of replicate seedlings.
[b]The standard error is shown in parentheses.
[c]Azos = *Azospirillum brasilense* Sp7.
[d]not neccessarily restricted to *para*-nodules.

(5 ppm) and NAA (1 ppm) treatment also sometimes reduced C_2H_2 much more rapidly than did control plants without any hormone treatment (Kennedy et al., 1991a). However, both NAA and IAA also induced strong modification of lateral roots, which were thickened and prolific in root hairs. Inspection by microscopy indicated that azospirilla colonized in a similar manner as with 2,4-D-treated plants, at the basal region close to the root stele of the main root. Although these auxin-affected structures lack the well-rounded appearance of 2,4-D induced *para*-nodules, this process may be akin to *para*-nodulation. Indeed, when the NAA concentration was raised to 10 ppm, the well-rounded shape of 2,4-D-induced nodules was also obtained.

The rate of N_2 fixation in para*-nodules, other induced nodules and associative systems*

In Table 1 are shown the approximate C_2H_4-forming activities in several of our experiments. A control of wheat seedlings inoculated with Azospirillum, but without 2,4-D added, is routinely included. Under the conditions of assay, azospirilla in the rhizosphere are considered as limited in their ability to express nitrogenase activity because of oxygen toxicity (see Fig. 5).

It is difficult to compare these rates with those of associative systems, since data for young seedlings are rarely provided. However, between 100–800 nmoles of C_2H_2 reduction per g dry weight roots per hour have been reported for systems not necessarily involving Azospirillum (Alexander and Zuberer; Diem and Dommergues, 1979; Ladha et al. 1986). A pure culture of *Azospirillum brasilense* Sp7 is capable of producing 240 nmoles C_2H_4 per h per mL of culture containing about 10^8 cells in the log phase of growth (Hartmann and Burris, 1987).

The apparent rate of C_2H_2 reduction in 2–3-week-old *para*-nodules is already clearly significant. Although the variation in nitrogenase activity is high, with standard deviations of the same order as the value of the mean, this is not unexpected. Observations have shown that activities are qualitatively correlated with the extent of colonization of *para*-nodules – a process that is likely to follow the Poisson distribution rather than a normal distribution.

However, now that an effective model system (viz. the *para*-nodule, Zeman et al., 1991) is available for testing, further improvements in the consistency of the rate of C_2H_2 reduction can be anticipated. We are currently testing about 20 collected strains of azospirilla, isolated (New and Kennedy, 1989) from the roots of wheat plants growing in the eastern Australian wheat zone.

Comparisons of legume nodules with para*-nodules and other induced nodules on non-legumes*

The concept of *coupling mechanisms* has been advanced to allow a more systematic analysis of

the ecological success of different entitites in ecosystems (Kennedy 1983, 1986a; Tchan and Kennedy, 1988). A coupling mechanism is a physical, chemical or biological apparatus catalysing a thermodynamic work process (an action). Such an action process (e.g. the growth to maturity of a legume such as soybean) occurs with greatest efficiency when factors affecting the coupling mechanism(s) are optimised. In the case of N_2 fixation by legumes, each of the actions, viz.

i) infection and colonization of root nodules by rhizobia (Dart, 1988)
ii) the delivery of an energy supply (as photosynthate)
iii) the supply of oxygen (*via* leghaemoglobin)
iv) an efficient assimilation of ammonia (Schubert, 1986), as favoured by a high carbon-to-nitrogen ratio is optimised.

All the coupling mechanisms involved are subject to a complex *genotype(s)* × *environment* that determines the actual success of each. In legumes, these mechanisms have been refined through evolution to the point where the operation of each appears to integrate almost perfectly with the others. Indeed, the specificity between particular bacterial strains and legume species may be seen as an evolutionary refinement to ensure that greatest optimisation is achieved by excluding less efficient symbioses.

Para-nodules

Even by comparisons with legume nodules, the *para*-nodulated wheat roots have creditable rates of C_2H_2 reduction. If all the C_2H_2 reduction was taking place in the *para*-nodules, these would have an activity several times greater than that of legume nodules (over 1000 nmoles min^{-1} g^{-1} f.w. versus ca. 200 nmoles min^{-1} g^{-1} f.w.). It should be pointed out that the weight of the wheat nodules (ca. 1–4 mg) as a proportion of the root system (ca. 100 mg f.w.) is several times lower than in the case of legume nodules. Also, it has not been established that the C_2H_2 reduction is restricted to azospirilla located in the *para*-nodules.

Further, it must be emphasised that these *para*-nodulated seedlings have only been tested when around 2–3 weeks of age. Experiments to test the persistence of the *para*-nodules and the capacity to continue these rates of C_2H_2 reduction and to contribute to the nitrogen nutrition of the wheat plants are now in progress.

We have also found a possible direct link between photosynthesis and C_2H_2 reduction in *para*-nodules on wheat, similar to that known for legume nodules. When seedlings had exhausted their seed reserves of carbohydrates, the C_2H_2 reduction continued provided the seedlings were exposed to light (Tchan et al., 1991). In preliminary experiments, the application of diuron (DCMU), an inhibitor of photosystem II in chloroplasts, abolished C_2H_2 reduction (Zeman et al., 1991). Thus, one of the major functional characteristics expected in a true symbiosis will be satisfied by *para*-nodules, if this effect of diuron can be confirmed as direct result of preventing supply of fresh photosynthate to Azospirillum and photosynthate is needed to allow nitrogenase activity to proceed. This would then indicate that the elevated activity of Azospirillum in *para*-nodules compared to that in associative systems (Table 1) is not a result of extensive parasitism of wheat seedlings imposed by a degenerative condition caused by 2,4-D treatment, as suggested by some scientists. On the contrary, their N_2 fixation may be viably linked to the transport of photosynthate from the leaves.

However, the other key characteristic of symbiosis, the ability of a microsymbiont to benefit the plant, has not been established. Thus, it is too early to say whether *para*-nodules possess a sufficient number of features described above allowing legume nodules to be so successful. Some of these features of legume nodules are compared below to those of *para*-nodules, possibly throwing some light on the prospects for eventual success of the new technology.

Nodules on rice

Nodules induced on rice, by the use of genetically modified rhizobia (Rolfe and Bender, 1990), are characterized by internal structures rather similar to legume nodules, with up to 20% of the plant cells infected and containing bacteroids encapsulated by peribacteroid membranes. Furthermore, these nodules contain vascular bundles surrounding the infected zone, similar to legume nodules, although only about one in 400–1000 rice plants forms nodules. Better re-

sults have been claimed by Jing, using a mutagenized strain of *Rhizobium sesbania* (*A caulinodans*), with a much greater frequency of nodulation. If the possibility of successful N_2 fixation depends on achieving a legume nodule-like structure, then this is clearly a significant result. In neither case is significant nitrogenase activity found in these nodules.

Rice nodules formed by the use of enzymes resemble legume nodules less. No evidence of peribacteroid membranes has been reported (Cocking et al., 1990) and infected plant cells appear to lack normal cell cytoplasm (Al-Mallah et al., 1989). Neither do these nodules display nitrogenase activity in the C_2H_2 reduction test.

Nodules on oilseed rape

Nodules formed on oilseed rape roots (Cocking et al., 1990) have an internal structure similar to legume nodules. They also show slight rates of C_2H_2 reduction, although rates reported so far would be too low to significantly benefit the plant.

General

Legume symbioses are thought to have evolved from loose associations between free-living bacteria (Rhizobium's ancestors) and the Leguminoseae family (Dilworth and Parker, 1969). The question of why (with the exception of Parasponia) this development was limited to the legumes has not been answered. To what extent *para*-nodules already satisfy the requirements for success outlined above for legume nodules must still be evaluated. Whether a process of evolution possibly (but perhaps, not necessarily) taking an extremely lengthy period to optimise can be achieved artificially in a few years is also a controversial question that will only be answered in the future.

On the basis of their relative frequencies on each root, *para*-nodules on wheat appear to be modified lateral roots. Their frequency closely matches that of actual laterals, although the depressive effect of 2,4-D on root extension usually shortens their separation. With 2,4-D, most lateral roots that might have developed during the treatment become *para*-nodules.

According to Nutman's 'focus hypothesis', legume nodules are considered to be modified lateral roots (Libbenga and Bogers, 1974). These authors appraised the evidence for and against the focus hypothesis suggested by Nutman. This hypothesis states that sites of latteral root initiation are the focal points for infection and initiation of nodules. Libbenga and Bogers concluded that the lateral root are so fundamentally different from nodules in structure and development that the idea of a common origin cannot be sustained. They also suggested that one of the most persuasive pieces of evidence in favour of the focus hypothesis, viz. the approximately similar numbers of laterals plus nodules produced with effective and ineffective inoculation, might be mere coincidence. The phenomenon of 'supernodulation' in soybeans also seems to refute this idea. Despite the controversy concerning the focus hypothesis, the possible link between lateral root initiation and nodule development in legumes adds credence to the possibility that these new nodular structures in non-leguminous crop plants may also eventually be functional.

Another possible viewpoint would be that vascularization of legume nodules should be a key factor in deciding whether or not they are valid nodules. On this basis, the tendency of *para*-nodules to possess a central vascular bundle with infecting bacteria occurring in surrounding cortical tissue would rule them invalid. However, the nodules of Parasponia also have this arrangement (Trinick, 1988), with a central vascular bundle and they are fully functional, providing rates of N_2 fixation per hectare exceeding most legumes including soybean. Also, *para*-nodules infected with Azospirillum are clearly quite different in character from legume nodules and a criterion such as this need not be considered as significant.

Abolition of non-nodulating genetical character by 2,4-D

Very recently, Akao et al. (1991) have demonstrated that a non-nodulating line of soybean (T201) could be effectively nodulated by treatment of soybean roots with 2,4-D at the same time as Bradyrhizobium was added. The shape of these 2,4-D-induced nodules (formed irrespective of whether rhizobia were added or not, as in

the case of *para*-nodules on wheat) was quite different from the normal nodules that appear on nodulating soybean lines only when Bradyrhizobium was present. The 2,4-D-induced nodules were usually gourd-shaped or in a doublet ('bar-bell') form, shown in Figure 7, and these were inducible on a nodulating cultivar (T202) as well. When infected with *Bradyrhizobium japonicum*, these induced nodules were effective in fixing N_2, as shown by the greening of the soybean leaves and by the C_2H_2 reduction test.

A result such as this indicates that the effect of 2,4-D observed in wheat seedlings can also be compared to the normal nodulation process in leguminous plants, such as soybean. Obviously, in both non-nodulating and nodulating soybean (lacking rhizobia), 2,4-D initiates a process quite similar to normal nodule formation induced by rhizobia. The fact that the soybean nodules initiated under the influence of 2,4-D have unusual morphology is not surprising. In the normal process, any auxin production associated with the penetration and colonization by rhizobial bacteria is spatially directed (i.e. vectorial), whereas 2,4-D in solution is not.

We have observed a similar 2,4-D-induced nodulation of lucerne plants (*Medicago sativa*) in the absence of *Rhizobium meliloti* (Yu, pers. comm.). As mentioned above, Allen et al. (1953) have also described pseudo-nodulation of legumes induced with hormone herbicides, although they failed to report microbial infection of these structures.

The future

Areas of inadequate knowledge

Although we have successfully attempted to construct an in vitro cellulo-biochemical model of N_2-fixing symbiosis between wheat seedlings and Azospirillum, there are many challenges to be negotiated before this model system can be considered as a true symbiosis. At this stage the main questions that need to be asked about the model are:

i) is there direct transfer of fixed nitrogen to the host plant, or is any N_2 fixation simply bound to the growth of Azospirillum? If the latter is true, it is unlikely that sufficient

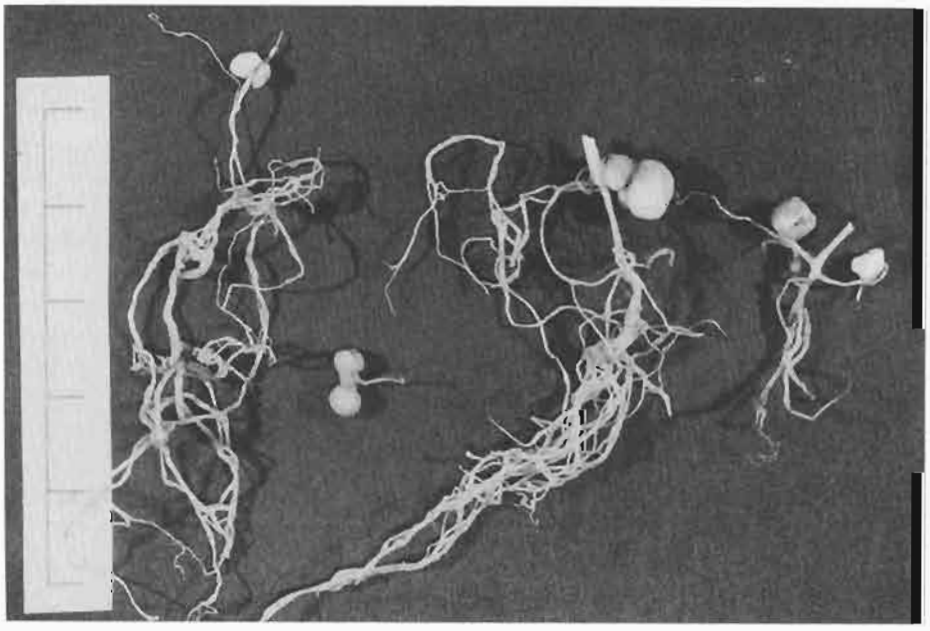

Fig. 7. Barbell-shaped nodules formed on non-nodulating soybean (cv. T201), treated with 2,4-D. *Scale divisions* = 1 cm. We are grateful to Dr. S. Akao for providing this photograph.

nitrogen could be transferred to the host plant. It is surprising, considering the potential importance that has been attributed to Azospirillum, that little is known of any mechanism of transfer of fixed nitrogen to a host plant. Indeed, no evidence exists that a direct transfer occurs at all (Zimmer and Bothe, 1989).

ii) how long a period are the *para*-nodules likely to remain as N_2-fixing organs? It is possible that the infected tissue might be unstable, or that nodulation might be restricted to an insufficient number of sites and not be able to provide sufficient N_2-fixing tissue to satisfy the needs of mature plants. The effect of 2,4-D or NAA is to induce many lateral root initials simultaneously, possibly stressing the plant by causing excessive demand for photosynthate (C. Atkins, pers. commun.). However, Nie (see Tchan and Kennedy, 1989) has pointed out that a nodular structure, when 2,4-D is removed, may revert to a lateral root, so that the process of *para*-nodulation may be reversible.

iii) is the oxygen requirement for N_2 fixation likely to be satisfied in the *para*-nodules? Without an effective means of regulating oxygen activity around the bacteria, the rate of fixation may be far from maximal.

The reasons for the success of legumes discussed above are far from verified in this model system in wheat. Quite plainly, there is a need to determine the actual effect of *para*-nodulation with diazotrophs on the growth of wheat. The negative effect of 2,4-D on root growth may increase the difficulty of demonstrating a positive effect on wheat growth, and alternatives to 2,4-D may need to be found. There is also a potential environmental objection to the use of 2,4-D for field crops, even for localized applications on roots. At this stage, our attitude is to continue the use of 2,4-D as an experimental tool, but to also seek better alternatives so that these will be available in case they are required. It should be remembered, however, that 2,4-D is still extensively used as a herbicide for broad-leaved plants and it is rapidly broken down in soil (Jackson, 1985). Fears of environmental damage may be exaggerated when balanced against the potential environment benefits of wheat fixing its own N_2.

The problem of the longevity of effective *para*-nodules might prove even more intractable, but it is too early to predict how difficult this will be, or to predict that other serious problems will not emerge.

Genetical techniques

It is significant to observe that the degree of success achieved with *para*-nodules so far owes nothing to genetic engineering, but it should also be clear that further progress may depend on genetic manipulation of either microsymbiont (Elmerich, 1984), host plant or both. For example, the problem of nitrogen transfer might need to be overcome by producing ammonia-'leaky' mutants. Indeed, mutants of Azospirillum that might enhance the process have been described (Pedrosa et al., 1989), capable of excreting ammonia to an external concentration of at least 5.1 m*M*. A recent report that *Azospirillum brasilense* Sp7 possesses plasmid genes homologous to nodulation genes of *Rhizobium meliloti* (Vieille and Elmerich, 1990) may also be of relevance, although we would emphasize the uniqueness of the *para*-nodule.

Applications in agriculture

There is no doubt that if this in vitro model can be shown to be a working system in the field, this would lead to widespread demand for the application of *para*-nodulation in agriculture. Even then, many challenging tasks would need to be faced in providing a viable system for the use of farmers. The process of *para*-nodulation under field conditions could require extensive redesign before a reliable procedure could be available. The effective delivery of azospirilla to seedlings would also represent a challenge, although extensive experience with legume nodulation must provide valuable lessons here. Possibly, initial applications of the *para*-nodule model will need to be conducted for horticultural crops that can be grown hydroponically.

The various attempts to achieve effective nodulation of non-leguminous field crops using Rhizobium may also yet prove beneficial. This will require suitable measures to be taken to allow expression of nitrogenase activity. Similar

problems for persistence of nodules and continued activity would still remain.

Despite these severe challenges, we are convinced that the eventual application of this developing technology in one form or other is now more likely than not. By her brilliant examples represented in the legume-Rhizobium and the plant-Frankia symbioses, 'Nature' has provided the models that must be matched, at least in overall function. However, these new technologies with very different species of plants may not necessarily mimic these naturally evolved symbioses. The next ten years will tell whether this confidence is justified. It is also conceivable that the technique of *para*-nodulation with desirable bacteria may find new applications outside the N_2-fixing area.

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