Introduction
Some comments about a better use of biological nitrogen fixation in rice cultivation

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Abstract

Research on biological nitrogen fixation began in Western Europe during the nineteenth century, under conditions where a mere increase in nitrogen fertilization inevitably increased yields: it was the beginning of the triumphal era of fertilizers. In the thirties began the era of legume inoculation: and this again was due to a very simplistic situation in Western countries: the absence of bacterial symbionts adapted to crops such as soybeans. Biological nitrogen fixation (BNF) appeared as an extension of nitrogen fertilization, with the same effects on farmers' incomes. The amount of nitrogen available was the limiting factor of the farmer's income, whatever its origin: mineral nitrogen from soil or fertilizers as well as nitrogen derived from biological fixation. In a way, this very clear-cut situation allowed for the rapid development of our knowledge about BNF, and its use by farmers. Nevertheless, when the time came to extrapolate to tropical countries, some difficulties arose. Some were due to a lack of knowledge about BNF systems in warm countries. Other difficulties were due to the interference of many yield-limiting factors other than nitrogen. But the main difficulty resulted from a misunderstanding about the objectives: the goal of developing BNF is not to achieve the maximum nitrogen input, it is really to achieve the maximum income (money and/or food) for farmers. In many tropical countries, the farmer's income is not directly proportional to nitrogen availability. We, as scientists, are confined to scientific objectives (maximum nitrogenase activity) whereas countries, such as Bangladesh, must aim at a maximum farming efficiency, biological science being largely secondary to other disciplines such as sociology or economics.

Introduction

In Western Europe, the second part of the nineteenth century was the beginning of the triumphal era of chemical fertilizers. Vast areas of low-fertility soils opened to modern agriculture. This was permitted by the fact that fertilizer elements (N and P, essentially) were the main yield-limiting factors in most instances: conditions were such that a mere increase in nitrogen fertilization almost inevitably increased yields. Awareness about this role of nitrogen in soils also favoured the inclusion of a legume in rotations. The breeding of new high-yielding cultivars in most crops

M. Rahman (ed): Biological Nitrogen Fixation Associated with Rice Production, 1–12.

followed shortly, so that within a century, yields and acreage of many crops increased in such a way that the term 'green revolution' could be coined. Farmers' incomes followed the same trend, at the beginning.

In the early thirties, the success of legume inoculation began. This again was due to a very simplistic situation in Western countries: the absence (e.g. soybean) or scarcity (alfalfa in acidic soils) of bacterial symbionts adapted to some leguminous crops. Biological nitrogen fixation (BNF) appeared as a simple extension of nitrogen fertilization, with the same positive effect on yield.

In consequence, the amount of nitrogen available was perceived as the main limiting factor for farmers' incomes as well as for plant growth, whatever its origin: mineral nitrogen from soil, the previous crop or applied fertilizers, as well as nitrogen derived from biological fixation.

New sources of nitrogen fixation had been found as early as the end of the last century but their actual importance was ignored until the sensitive acetylene assay became available, in 1968. This method is sensitive and cheap, and it boosted research on nitrogen fixation, in non-legumes as well as legumes. When research on BNF boomed (in the seventies), it was tempting, in tropical countries, to extrapolate research conducted under temperate conditions, with a view to increasing yields, through a better use of biological nitrogen fixation, in the same proportion as in Europe and the USA.

Regarding rice-growing countries, in the early period, some methodologies could be exported with success. In these countries also, new N-fixing systems were demonstrated and used, such as the N-fixing *Azolla-Anabaena* symbiosis, or cyanobacterial blooms. Nevertheless, recent decades have led to disillusionment and made highly questionable the rationale of simply applying the knowledge and know-how acquired under temperate conditions to rice-based agrosystems. Obviously, a reappraisal of BNF interest is necessary.

1. Extrapolating to rice-growing countries

Rice agrosystems are a privileged niche for biological nitrogen fixation, as they possess a variety of N-fixing systems (Figure 1), ranging from symbiotic (*Azolla*, legumes), to associative (heterotrophic rhizosphere N fixers), and free living (photosynthetic bacteria, including cyanobacteria). Traditional rice management already provides a large N input through BNF: the persistence of rice cultivation in the same area for thousands of years, in the Banaue region of the Philippines cannot be explained without a large contribution of BNF to this agrosystem. This fixation compensates for N exports at harvest.

1.1. Cropping sequence

The simplest way to increase BNF-derived soil N, is the inclusion of a leguminous crop in the cropping sequence. In Egypt, for instance, the sequence includes a winter Berseem crop (*Trifolium alexandrinum*), whereas rice can grow only as a summer crop. Soybean is traditionally grown alternating with rice in some parts of

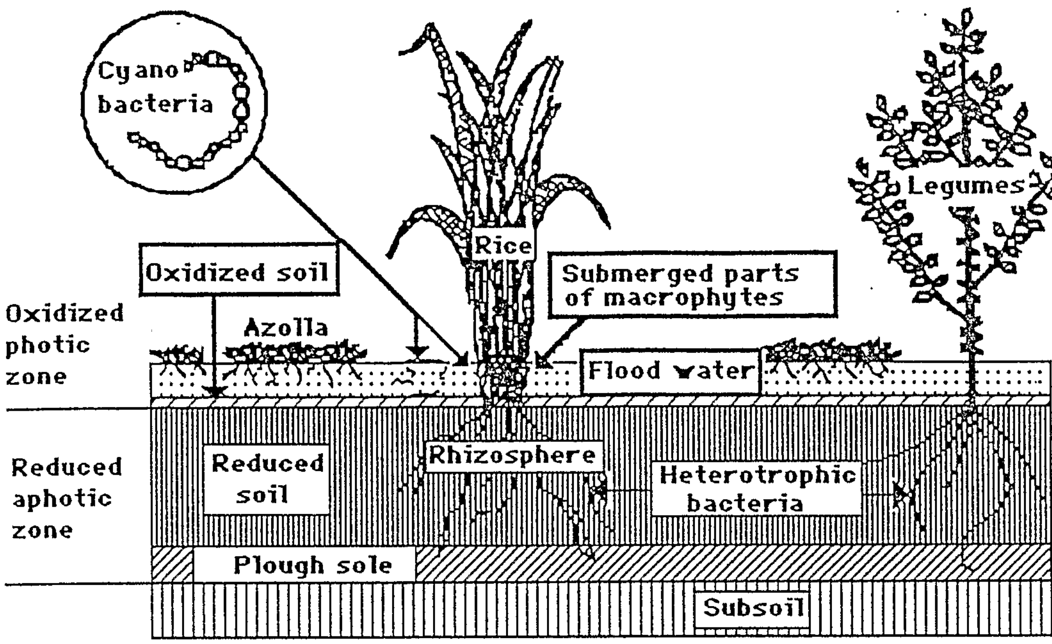

Figure 1. Schematic representation of a rice field and its several ecological niches.

Vietnam. In most countries of the developing world, this is feasible only with legumes usable as fodder or for man; in many instances, the priority need for more rice excludes this practice.

1.2. Azolla *management*

Azolla has been a part of rice cultivation in Vietnam and China for centuries and has been applied or tested more recently in other rice-growing countries (Roger and Watanabe, 1986). In addition to N supply, the benefits of *Azolla* as a green manure include provision of other mineral nutrients and organic matter to the soil. When established in rice fields, *Azolla* also reduces water evaporation and NH_3 volatilization (Rains and Talley, 1979). However, the realizable potential of *Azolla* as a green manure is restricted by climatic factors (air temperature), water availability and quality, soil factors (pH, salt) and mineral nutrition (P). When all these conditions are optimized, and biological interactions such as grazing or competition from other vascular plants and algae are controlled, *Azolla* can double its fresh weight in 2 days (Peters *et al.*, 1980). Main limiting factors for the agronomic use of *Azolla* are: (1) competition with rice for light and acreage, (2) overwintering and (3) labour involved in ploughing it in.

1.3. Inoculation

Inoculation was attempted as a promising way to increase BNF of different systems:

1. **Legumes**: soybean inoculation in Vietnam can increase nodulation and yield in some regions, thus contributing to an increase in soil N. The inoculation of

Sesbania rostrata, the stem nodulating legume, is not very widespread because it is difficult to convince a farmer that he should grow a shrub at a place and a time when he could grow rice. Moreover soil incorporation of chopped *Sesbania* residues implies an extra load of work for the farmer at a difficult time of the year.

2. In the case of **cyanobacteria** (BGA: blue-green algae), many inoculation trials have been performed. A recent survey of published results (Roger, 1991) shows that:
   a. Without inoculation, the contribution of BGA to the N balance of rice fields is significant, average values being 13 kg N ha^{-1} (ARA measurements) or 15 kg N ha^{-1} (average bloom N content).
   b. After inoculation (634 experiments analysed), yield increases have been reported, up to 450 kg grain ha^{-1}. Nevertheless, their significance is highly questionable, mainly due to poor quality of experimental designs: in most instances, 4 × 4 m plots are used, in four replicates, which usually gives a coefficient of variation higher than 10% and a minimum detectable difference of 14.5% (Gomez, 1972).
   c. Many unsuccessful results are not published.
   d. N-fixing BGA are present in rice fields at a much higher rate than was previously thought, making questionable the need for inoculation.
   e. Foreign strains rarely establish.

3. In the case of **rhizosphere heterotrophic N fixation**, research has suffered most from what could be called 'the magic bug concept'. Field inoculation trials have been conducted using strains assumed to be 'good' candidates to improve rice yield. The best instance is strain Sp7 of *Azospirillum brasilense*, which has been inoculated into a great diversity of plant species, including rice. When one tries to understand the reason for such a choice, it appears that this strain was chosen simply because it was available, and its physiology, genetics and molecular biology studied in several laboratories. There is no reason to think that this strain is likely to reproducibly establish in the rhizosphere of rice and positively interact with a non-host plant, such as rice. As a matter of fact, when inoculated into rice in the field, in Egypt (Figure 2), this strain proved to have a significant negative effect on rice yield (Tran *et al.*, 1994). Beside Sp7, many bacteria have been used in inoculation trials, mainly *Azospirillum* and *Azotobacter*. In a literature survey of 210 sets of data, Roger *et al.* (1993) concluded that:
   a. The average effect of inoculation is a 19.8% increase in yield. The average increase is 27.6% in pot experiments (known to overestimate N fixation). It is only 14.4% in field experiments; this is close to the minimum detectable difference (14.5%) that can be expected (Gomez, 1972) from the experimental design most commonly used in field experiments (4 × 4 m plots, four replicates).
   b. Many unsuccessful results are not published.
   c. Establishment of strains has rarely been studied.

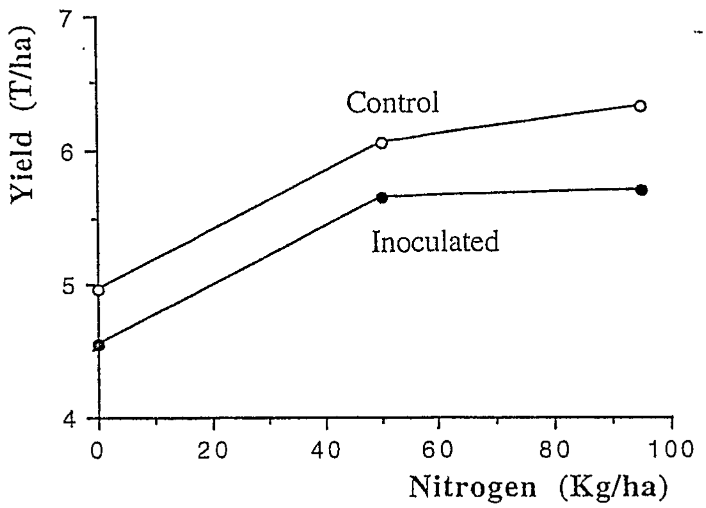

Figure 2. Sakha Experimental Station (Egypt), inoculation of rice cv Giza 172 by strain Sp7 of *Azospirillum brasilense*. This *Digitaria decumbens* strain has a detrimental significant effect (p<0.1) on rice yield.

Thus, only the introduction of a legume in the cropping sequence, some instances of legume inoculation, and the use of *Azolla* in low-labour-cost countries, are clear successes towards a better use of BNF in rice. On a statistical basis, the overall effect of practices, such as field BGA inoculation or seed inoculation by heterotrophic N-fixing bacteria, is close to zero.

2. New prospects

The above state of the art is rather disheartening. Fortunately, recent discoveries and a more ecological way of thinking are able to cast some light of hope on this grim prospect. To adopt a more positive language, it must be said that BNF is far more diverse and complex in the tropics than under temperate conditions: we must increase our knowledge, we must think more about limiting factors and modify our simplistic ideas about inoculation.

2.1. There is much more to discover

2.1.1. New BNF systems and species

Tropical countries are still able to surprise us with impressive discoveries in the area of BNF. When Trinick discovered nodules on *Parasponia* roots (Akkermans *et al.*, 1978), it was astonishing: it was the first instance of a rhizobiaceae symbiosis with a non-legume. Surprisingly also, *Acetobacter diazotrophicus* (Gillis *et al.*, 1989) was found only recently to be an essential N-fixing symbiont of sugar cane.

More relevant in rice cultivation is the description of the *Azoarcus* genus, a rhizosphere N-fixing associate of Kallar grass (*Leptochloa fusca*). The ecology of this grass is not very different from rice ecology, and *Azoarcus* has been shown to colonize rice roots in vitro. Nevertheless, the presence of this bacterium on or in rice roots, in the field, has never been reported.

A N-fixing *Pseudomonas* sp. has been described as an abundant rice root colonizer in the Philippines by Barraquio and Watanabe in 1981, but it still awaits a precise taxonomic status.

It is probable that other *α-Proteobacteria* of major importance are still to be discovered: many of the already known taxa of this superfamily are able to colonize rice roots: *Azospirillum*, *Rhodopseudomonas*, *Xanthobacter*, *Beijerinckia* and *Sphingomonas*. It seems important to re-evaluate their importance.

Last but not least, Tran *et al.* (1993), in a study of the rice rhizosphere on acid sulphate soils of Vietnam, discovered that the most abundant and efficient N-fixing bacterium was a new species of *Burkholderia*, a *β-Proteobacterium* (Gillis *et al.*, 1995). This species offers a very good prospect for rice seed inoculation (Figure 3).

2.1.2. Specificity

It has often been assumed that, in non-symbiotic plant–bacteria associations, there was no specificity. This contention has no grounds other than facility: it explains the proliferation of heterologous inoculation experiments, i.e. inoculation of a plant with a strain isolated from another plant, another soil or another country. As a matter of fact, evidence is accumulating that plant–bacteria rhizosphere associations can be highly specific. Of special importance, in that respect, is the study of wheat-associated *Bacillus polymyxa* populations performed by Mavingui *et al.* (1992). Diversity among 130 isolates was studied; bacteria were isolated by immuno-

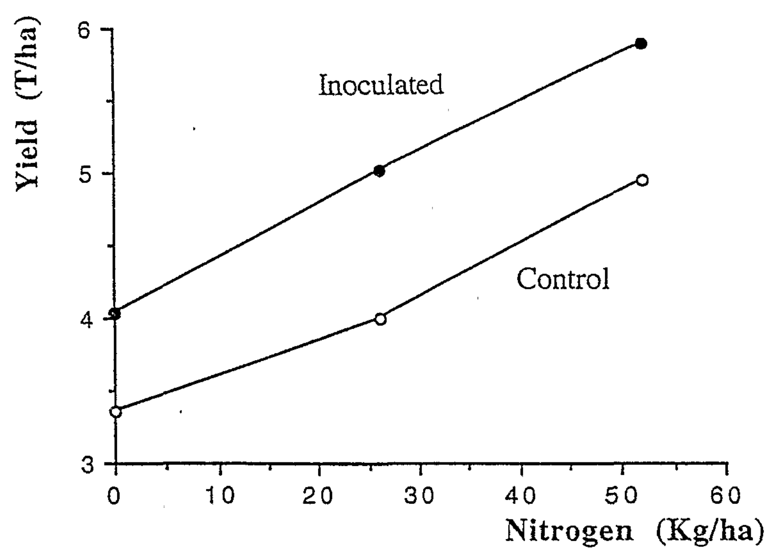

Figure 3. Nha Bè experiment (Vietnam), inoculation of rice cv Nang Huong by strain TVV75 of *Burkholderia cepacia*. N is limiting the yield: yield increases when N fertilization is increased in control uninoculated plots. There is a significant effect of inoculation.

trapping from non-rhizosphere soil (32 strains), rhizosphere soil (38 strains) and the rhizoplane (60 strains) of wheat plantlets growing in a growth chamber. Strains were characterized phenotypically by 63 auxanographic (API) and morphological features, serologically (ELISA), and genetically by restriction fragment length polymorphism (RFLP) profiles of total DNA in combination with hybridization patterns obtained with an rRNA gene probe. Similarity analysis of phenotypic characters by the unweighted pair group method with averages indicated four groups at a similarity level of 93%. Clustering of *B. polymyxa* strains from the various fractions showed that the strains isolated from non-rhizosphere soil fell into two groups (I and II), while the third group (III) mainly comprised strains isolated from rhizosphere soil. The last group (IV) included strains isolated exclusively from the rhizoplane. Strains belonging to a particular group exhibited a similarity level of 96%. RFLP patterns also revealed a high degree of homogeneity in rhizoplane strains as opposed to a greater genetic diversity among strains isolated from non-rhizosphere and rhizosphere soil which, therefore, could not be clearly grouped. It thus appears that wheat roots select a specific subpopulation from the diverse soil *B. polymyxa* population. In an in-vitro study of root colonization by a symplasmata-forming strain of *Enterobacter agglomerans*, Achouak *et al.* (1994) showed that the bacterium could colonize wheat as well as its host plant, rice, but only the latter was colonized by symplasmata, which again shows an unexpectedly high level of specific interaction. This ignored specificity could explain the failure of many inoculation experiments.

2.1.3. Colonization

Very little work has been done on colonization by inoculated bacteria under natural conditions. Most studies relate inoculation at sowing with yield characteristics at harvest, that is a long time later. What is happening, in between, to the inoculated bacteria remains unclear. The difficulty arises from several causes: (1) most strains have no natural markers allowing for a phenotypic typing, (2) serology is of no help with *Azospirillum*, (3) specific phages or bacteriocins have not been studied enough to be used. The only available method is to use genetically labelled bacteria, which restricts experiments to laboratory or controlled greenhouse conditions (Nayak *et al.*, 1986) and incurs the risk of modifying the microbial behaviour relative to wild-type strains. Using DNA probes of very variable regions of the chromosome could offer an interesting alternative to be developed.

2.2. *What are the actual limiting factors?*

Most studies aimed at improving BNF in western temperate countries have been done under conditions where N is the limiting factor of yield. It is so commonplace that it is rarely assessed. As far as tropical countries are concerned, especially in rice-growing agrosystems, it is important to verify that N is actually limiting before improving BNF. What would be the rationale of improving BNF if N had no effect on yield?

2.2.1. When is N the limiting factor?

N is the limiting factor when the level of available N determines the level of yield. It is extremely important, especially for non-agronomists, to realize that this has nothing to do with the level of available N. Nitrogen can be limiting even under very high rates of N fertilization: it simply means that if the fertilizer is further increased, yield will further increase. Nitrogen can also be limiting in poor soils: if N fertilizer is applied, an increase in yield will follow. Nevertheless, it must be stressed that, in poor soils, other elements can become limiting shortly after N: a moderate increase in available N (through fertilizers or BNF) could result in a situation where P is limiting. To be safe, experiments designed to assess a way of increasing BNF should bring the proof that N is limiting under the conditions employed. The simplest way is to include treatments in which available N is increased (N fertilizer): if this does not affect the yield, it means that a factor other than N has been limiting. Knowing whether N is the actual limiting factor or not is a great help to interpret results. As an illustration, Table 1 shows the work of Omar *et al.* (1992) on the inoculation of rice by a strain of *Azospirillum brasilense* in Egypt. Out of five field inoculation trials, only three showed a significant effect of N fertilizers on the yield of control non-inoculated plots. If a statistical approach is adopted, i.e. if the five experiments are treated together, the overall effect of inoculation is not significant, whereas, if only the three N-limited experiments are taken into account, a significant effect (p = 5.6%) of inoculation on yield can be seen. This example is interesting also because the two experiments in which N was non-limiting are very different: in 1988, the yield level was very low, whereas in 1989, it was very high and in both cases the yield did not respond to N fertilizers (Figure 4). This illustrates the fact that the availability of N does not indicate whether N is limiting or not. In the experiment shown in Figure 5, N availability is probably very good (the yield is extremely high) but still limiting, and inoculation has a significant effect (Omar *et al.*, 1992).

Table 1. Analysis of five years of inoculation trials of rice cv Giza 172 by strain NO40 of *Azospirillum brasilense* at Sakha Experimental Station (Egypt).

Pooled trials	Median yield (t/ha)	Effect of the factors[a]		Median grain yield[b] (t/ha)				
		N dose	Inoculation	N dose			Inoculation	
				0	Half dose	Full dose	Control	NO40
5 years	7.3	H=6.5 (p<5%)	H=1.0 (NS[c])	6.5	7.2	8.1	7.03	7.49
1985, 1987 and 1990	7.6	H=16.5 (p<5%)	H=3.6 (p=5.6%)	6.5	7.5	8.8	7.16	8.04

No statistical effect of inoculation is seen when all five trials are pooled. If only trials conducted under N-limiting conditions are considered, a significant effect of inoculation can then be demonstrated. Plot size: 14 m^2; number of replicates: 5 (1987), 6 (1988, 1989, 1990), 8 (1985).
[a]Kruskall–Wallis' H statistics; [b]Median of replicates; [c]Non-significant (p>5%).

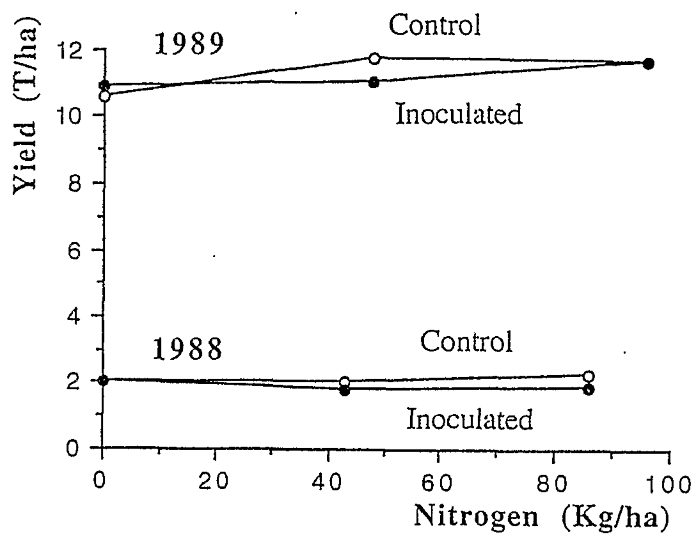

Figure 4. Sakha Experimental Station (Egypt), inoculation of rice cv Giza 172 by the local strain NO40 of *Azospirillum lipoferum*. In these two experiments, N is not the yield-limiting factor: yield does not increase when N fertilization is increased in control uninoculated plots. In both cases, inoculation has no effect on yield. It must be stressed that N is not the limiting factor, whatever the yield level: very high in 1989 (suggesting a high N availability), very low in 1988.

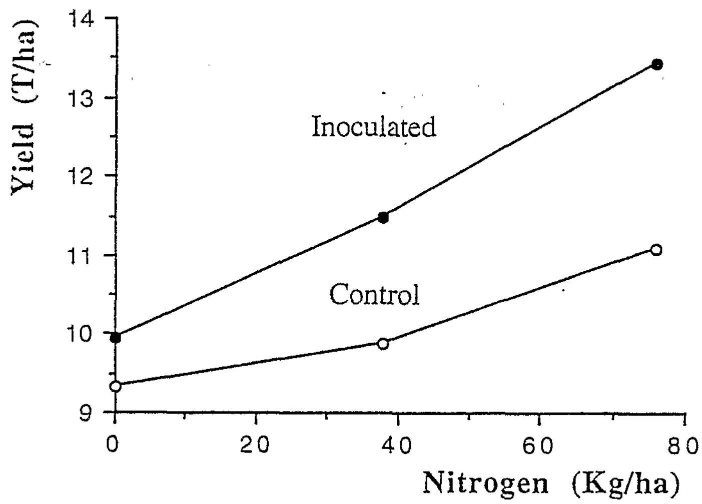

Figure 5. Gemmeiza Experimental Station (Egypt), inoculation of rice cv Giza 172 by the local strain NO40 of *Azospirillum lipoferum*. N is limiting the yield: yield increases when N fertilization increases in control uninoculated plots. There is a significant effect of inoculation, even at this very high level of fertility.

2.2.2. Other biological limiting factors

In cultivated soils, other elements than N can limit plant growth: frequent ones are phosphorus and iron, especially in neutral to alkaline soils. Situations exist where alleviating the limiting character of N is not really useful because another element becomes limiting shortly after. For instance *Azolla* can be used to increase N but the system is very rapidly P limited and needs phosphate fertilization as well.

Diseases are another frequent limiting factor to take into account and many field studies conducted by microbiologists do not take enough account of the possibility of diseases simply because plant pathology is another discipline.

Depredators (birds, rats, diverse insects) can also strongly interfere with experimental design and erase the statistical differences.

2.2.3. Non-biological limiting factors

Other factors are not biological in nature but constitute severe obstacles in a strategy of BNF improvement. They are mainly cultural or sociological by nature. For instance, practices generating an extra work load at the time of sowing are not likely to be adopted by farmers: sowing is often a time of reduced food and maximum labour investment. As already mentioned above, cultivated areas are a very frequent limiting factor: how could a farmer devote some plots to *Sesbania* or *Azolla* cultivation when he is trying to sow as much rice as he can?

2.3. Changing our understanding of inoculation

As stated above, it is difficult to introduce foreign bacteria into a soil and the same holds true for cyanobacteria. Does this make inoculation useless?

In the inoculation work performed in Egypt, an in-vitro-selected strain was used: *Azospirillum brasilense* NO40. This strain was isolated at a rice-growing experimental station called Moshtoor, near Cairo. As mentioned above, its inoculation greatly stimulated yield in all inoculation trials where N was limiting. It has also been used with success in an inoculation experiment in Moshtoor (Omar *et al.*, 1987), i.e. in a soil which already contained this strain: a 20% significant ($p = 0.05$) yield increase has been obtained. In the absence of a method to follow the fate of introduced bacteria, this result can only be interpreted with caution. Nevertheless, the success of inoculation was not due to the introduction of a bacterium absent from this soil: the situation is obviously very different from the inoculation of a legume. At this stage, it is possible to risk a few tentative explanations:

1. Inoculation forces the plant to associate preferably with one among many possible partners. As a matter of fact, it is well known that the rhizosphere of rice can harbour a large variety of bacterial taxa, differing by:

   a. The efficiency of exudate C utilization to generate the large amounts of ATP (fermenters less efficient than aerobes) necessary to fix nitrogen.
   b. The efficiency of nitrogenase activity, which can differ greatly between strains and even within a single species (Heulin *et al.*, 1989).

   Inoculation would diminish biodiversity by replacing a mixture of efficient and inefficient C users and N fixers by a population of selected efficient aerobic N fixers.

2. Inoculation could have a yet simpler role: most successfully inoculated bacteria are K strategists (De Leij *et al.*, 1994), i.e. slow growers, specialized and well-adapted bacteria. This type of bacterium usually colonizes the rhizosphere after a first bloom of r type bacteria (fast growers, abundant in new niches, but susceptible to competition). Inoculation would thus shorten or skip the phase of colonization by r bacteria (presumably enterobacteria) and decrease the delay necessary for the establishment of efficient specialized K-type colonizers.

If this is confirmed, then inoculation is advisable with local strains. This type of strategy increases the likelihood of dealing with bacteria adapted to the plant and to the local edaphic and climatic conditions.

And this could also be the case with BGAs: introduced BGAs are difficult to produce in large quantities and rarely establish. Moreover, observed blooms are mainly from local strains in inoculated plots. "Available data are not sufficient to draw definite conclusions, but they clearly suggest that use of an inoculum produced from the soil to be inoculated should be tested whenever experiments are conducted" (Roger *et al.*, 1993). A sensible effect of BGA inoculation would then be to shorten the time necessary for local adapted strains to multiply and colonize the plot efficiently. In this case, the limiting factor addressed would simply be time.

This strategy of local bacteria inoculation seems especially advisable under conditions where there is a period of time between harvest and the following rice crop when soils are left to dry out, which often results in partial sterilization in very warm climates. In this case, the real limiting factor is the total microflora level.

Conclusion

After a period of simply extrapolating from temperate countries, it appears that use of BNF in warm rice-growing countries must explore new avenues, and overcome specific difficulties. Some are due to a lack of knowledge about BNF systems in warm countries: in that respect, we are far from knowing enough about the N-fixing microbial components of the agrosystems to be managed. Other difficulties are due to the frequent interference of many yield-limiting factors other than nitrogen, which is not the case in western temperate countries. Some difficulties, also, result from the division of the relevant knowledge into different disciplines: microbiology, soil science, agronomy and plant pathology.

The main difficulty results from a misunderstanding about the objectives: the goal of developing BNF is not to achieve the maximum nitrogen input, it is really to achieve the maximum income (money and/or food) for farmers. In many tropical countries, the farmer's income is not directly proportional to nitrogen availability: it has other limiting factors. We, as scientists, are confined to scientific objectives (maximum nitrogenase activity) whereas many countries must aim primarily at a maximum farming efficiency, biological science being largely secondary to other disciplines, such as sociology and economics.

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