the general trend. To achieve this we would have to carry out research on some of the grey areas that still exist, like infection of cultures, maintenance of quality, and packaging and transportation systems. Each of these problems is important. For instance the last mentioned must be carefully considered since the products are highly perishable. Contamination of cultures, on the other hand, can threaten the very survival of the industry. Product price is another important criterion to consider in making the marketed product acceptable. This means that, between the development of a laboratory-scale process and its utilization for commercial-scale production, intense research and development is essential to make the process economically viable.

New methods of automation and production of synthetic seeds can be considered for production of cost-effective propagules in the future. The big role of micropropagation in India is mainly in the case of crops and trees where the quality of the product, rotation cycle and yield are of relevance. This would include fruit and vegetable crops, ornamentals, forest trees, and horticultural, medicinal and plantation crops. Improvements in greenhouse technology provide the advantage of exploiting year-round production, unaffected by seasonal effects. Other biotechnological approaches like genetic engineering, somaclonal variation, etc. should be exploited to generate a new range of superior genotypes.

We are on the threshold of a biotechnology revolution. India can exploit the situation with its vast pool of both talented manpower in tissue culture and diverse genetic resources of plants growing in a variety of climatic and soil conditions. The initiative and bold stand taken by DBT in funding advanced research in key areas and the entry of commercial companies could help India be a leader in this field.

Biological nitrogen fixation in the context of Indian agriculture

H. K. Das

Plants obtain their nitrogen as inorganic nitrates from the soil or from added fertilizer, and synthesize organic nitrogen compounds. Leguminous plants, however, can form associations with nitrogen-fixing bacteria (Rhizobium) and obtain organic nitrogen directly. The advantages of inoculation of Rhizobium for pulse crops, which are leguminous plants, are obvious. Success in increasing yield depends on proper selection of Rhizobium strains and large-scale production of culture. Recombinant-DNA technology helps in strain improvement, increase of efficiency of nitrogen fixation by the bacteria, and introduction of other traits into Rhizobium.

Biological nitrogen fixation is the reduction of atmospheric nitrogen to ammonia by a metabolic process. The process of biological nitrogen fixation is carried out by many species of microorganisms, mostly unicellular ones. These microorganisms can be classified broadly into two main groups, the free-living and the symbiotic (Figure 1). The former can fix nitrogen by themselves; they do not require the help of any other microorganism or a plant. The symbiotic microorganisms require association with plants; they fix nitrogen when they are in close contact with plants, and the two together create a situation where nitrogen is fixed. Of the symbiotic microorganisms, the most important by far are those belonging to the genus *Rhizobium*. These microorganisms form associations with leguminous plants, and also a few non-leguminous ones. They are very specific with regard to the kind of plant they associate with. For example, *Rhizobium meliloti* will only associate with plants like lucerne (alfalfa, *Medicago sativa*) or fenugreek (methi, *Trigonella foenum-graecum*) but never with French bean (*Phaseolus vulgaris*) or soybean (*Glycine max*). Table 1 lists examples of such specificity. All *Rhizobium* species are unicellular. They form nodular structures in the roots of leguminous plants and it is in these nodules that nitrogen is fixed. In agricultural practice, seeds are coated with the appropriate *Rhizobium* culture before

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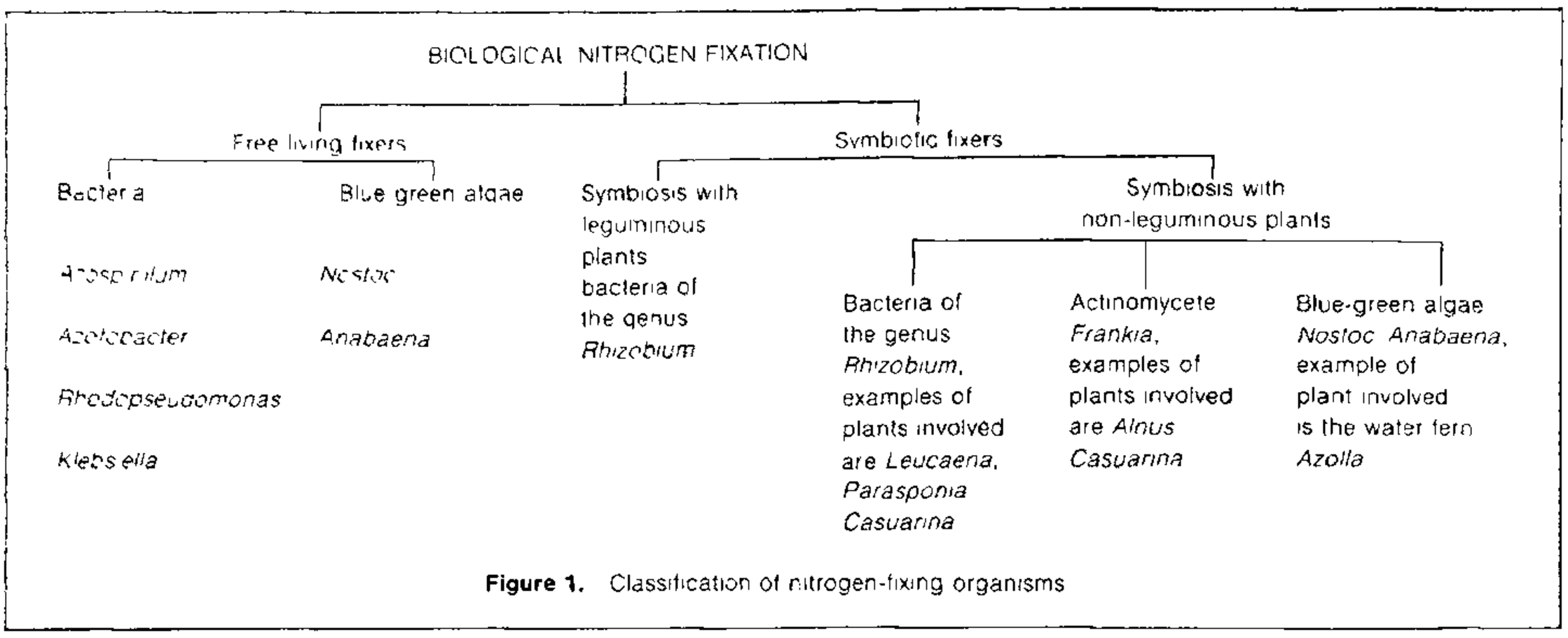

Figure 1. Classification of nitrogen-fixing organisms

sowing. Sometimes the soil is also inoculated with the culture. Once the microorganisms are put in the soil, they start multiplying and colonize the roots of the plants, form nodules, and start fixing nitrogen. As a result, grain yield goes up. The efficacy of *Rhizobium* inoculation has been established in our country beyond any doubt by the results of coordinated trials conducted by the Indian Council of Agricultural Research[1] (ICAR) (Table 2). These trails have been done in the fields of farmers, not in experimental fields. There is large variation in yield increase, which reflects the unpredictability of the extent of enhancement of crop yield. Nevertheless, in terms of nitrogen fixed, in some areas it could be as high as 170 kg nitrogen per hectare.

Commercial exploitation

The commercial aspects of *Rhizobium* inoculum are presented in Table 3 (ref. 2). A thorn in the rose is that, while the production capacity of the units making *Rhizobium* inoculum now is of the order of two million kg, actual production is only about half the capacity. Why? An important reason is that there is not enough actual demand for the inoculum. Quite often it does not give the expected result and farmers are either not convinced about its efficacy or easily disenchanted. The key points that deserve attention are listed in Table 4. Survival of the inoculum during storage in distribution centres is a point to be considered seriously. Quite often the inoculum is not kept in a cold store and, as a result, when it is applied to the soil, very few *Rhizobium* cells are viable. The Indian Standards Institution has laid down detailed specifications for *Rhizobium* inocula[3]. Proper storage is essential for meeting these specifications. Proper selection of strains is seldom done by the commercial concerns. This has to be based on soil characteristics—pH, salinity and moisture—and the temperature of the area Many different strains of each species are available in the culture collections of various research and teaching

Table 1. Specificity of different *Rhizobium* species for association with particular leguminous plants

Rhizobium species	Associative plant
R meliloti	Lucerne (alfalfa, *Medicago sativa*) Fenugreek (methi, *Trigonella foenum-graecum*)
R trifolii	Clover (berseem, *Trifolium* spp)
R leguminosarum	Pea (*Pisum sativum*) Lentil (*Lens culinaris*)
R japonicum	Soybean (*Glycine max*)
R phaseoli	Phaseolus beans (French bean *Phaseolus vulgaris*)
R cowpea miscellany	Green gram (mung *Phaseolus aureus*) Black gram (urad *Phaseolus mungo*) Groundnut (*Arachis hypogea*) Chickpea (chana *Cicer arietinum*) Pigeon pea (*Cajanus cajan*)

Table 2. Efficacy of *Rhizobium* inocula [Results of ICAR coordinated trials]

Pulse	Increase in grain yield due to inoculation
Chickpea or gram (*Cicer arietinum*)	9–76%
Pigeon pea or arhar (*Cajanus cajan*)	10–46%
Mungbean or mung (*Vigna mungo*)	9–55%

Table 3. Commercial aspects of *Rhizobium* inoculum

Area under legume cultivation in India	30 million hectares
Requirement of *Rhizobium* inoculum (carrier-based) on the basis of 1/2 kg per hectare	15 million kg
Present production	1 million kg
Production cost	Rs 4 5 per kg
Business potential	Over Rs 60 million per year

Table 4. Biotechnology of *Rhizobium*, Phase I — key points that deserve attention

1	Survival of the inoculum during storage in distribution centres
2	Selection of proper strains for survival in the field after application, based on (i) soil characteristics — pH, salinity, moisture, temperature (ii) competition with other microorganisms already present in the soil
3	Large-scale production of culture

Table 5. Biotechnology of *Rhizobium*, Phase II — key points to be considered

1	Enhancement of competitive ability (i) antibiotic resistance (ii) production of antibiotics or toxins
2	Enhancement of tolerance of adverse pH, salinity or temperature
3	Counteracting ammonia repression
4	Increasing efficiency of nitrogen fixation
5	Conferring ability to produce plant-growth substances

institutes. These have all been characterized with respect to survival, growth parameters and nitrogen-fixation ability in different kinds of soils. It is important to choose the right strain, using the available information, before multiplying it and giving it to the farmer.

How can one go about doing it? A well-organized industry that would like to get into this business must have small laboratories in the distribution centres. The farmer should bring a sample of the soil and the soil should be tested for its chemical characteristics before a particular strain is suggested for a particular field. This is necessary for consistent success and is not at all difficult to do. One point that has also to be considered very seriously is competition with other microorganisms already present in the soil. The microbial content of the soil is also relevant to the survival and multiplication of the inoculum. Quite often the microorganism that is put in the soil fails to multiply because of competition from other microorganisms. Survival of an inoculum in a particular soil can be checked easily in the laboratory before a particular strain is recommended. All these can be done only if the production is on a large scale. A small producer cannot afford to have a laboratory at every distribution centre, and this is one reason why large-scale production must come in. Large-scale production is also necessary for maintaining the standard and the quality of the inoculum. Today if random samples of the available commercial inoculum are taken, one would find that they are mostly contaminated, and the other microorganisms present may affect the growth of the desired culture. This is because these cultures are prepared on a small scale under unscientific conditions. Production must be done in a scientific manner in fermenters under aseptic and defined conditions to ensure good quality and uniformity from batch to batch. The points mentioned in Table 4 can be taken care of immediately. All the information required is available. It is a matter of an organized industry taking over.

Recombinant-DNA technology

The phase-II biotechnology (Table 5) would have to involve recombinant-DNA technology. When I stated that several institutions have worked on the characteristics of different strains, I meant that whatever was available in nature have been characterized. Quite often it is found that a particular strain, which, say, is resistant to high temperature or high-alkali soil, does not fix nitrogen efficiently, while a strain that fixes nitrogen efficiently is sensitive to these environmental conditions. This is where the phase-II biotechnology would be useful. Let us first consider enhancement of competitive ability. The soil contains many kinds of microorganisms. Some of them may have the ability to produce antibiotics. If the inoculum that has been put in the soil is sensitive to these antibiotics, it has no chance of survival. In this case genes would have to be introduced into the inoculum strains that would make them resistant to the antibiotics. This is easy to do using recombinant-DNA technology. Similarly, production of antibiotics or toxins by the *Rhizobium* culture could also be envisaged. Strains of the free-living nitrogen fixer *Azotobacter* that elaborate toxins that eliminate pathogenic fungi and bacteria are already known[4,5]. These fungi and bacteria are harmful to plants. So, if the inoculum is capable of producing such toxins, not only would it be able to survive, but also the plants would be protected from the action of the pathogenic organisms. It is possible to transfer genes producing such toxins into the *Rhizobium* strains of interest. The genes involved in tolerance of salinity have been well worked out in *Escherichia coli*[6]. These again could be transferred into *Rhizobium* strains. Similarly, it should be possible to transfer genes that contribute to resistance to adverse pH and high temperature into strains that are commercially important. Some of the species that fix nitrogen have been found to produce plant-growth substances[7]. These microorganisms thus help the plant not just by supplying fixed nitrogen, but also by making available plant hormones. Some of the genes responsible for the production of plant hormones have been characterized and cloned[8,9]. These genes could be introduced into *Rhizobium* strains so that plant hormones are produced by these strains.

The other two points mentioned in Table 5, viz. counteracting ammonia repression and increasing efficiency of nitrogen fixation, require understanding of the basics of nitrogen fixation. It is common knowledge among farmers that the nitrogen-fixing microorganisms fail to do their job if chemical fertilizers are added to the soil. This is because there is a regulatory mechanism

that operates inside all nitrogen-fixing microorganisms that would shut off nitrogen fixation if sufficient fixed nitrogen is already present[10]. It is the enzyme nitrogenase that reduces atmospheric nitrogen to ammonia. This enzyme is composed of two components, component I and component II (Figure 2). The component I has two α-subunits and two β-subunits,

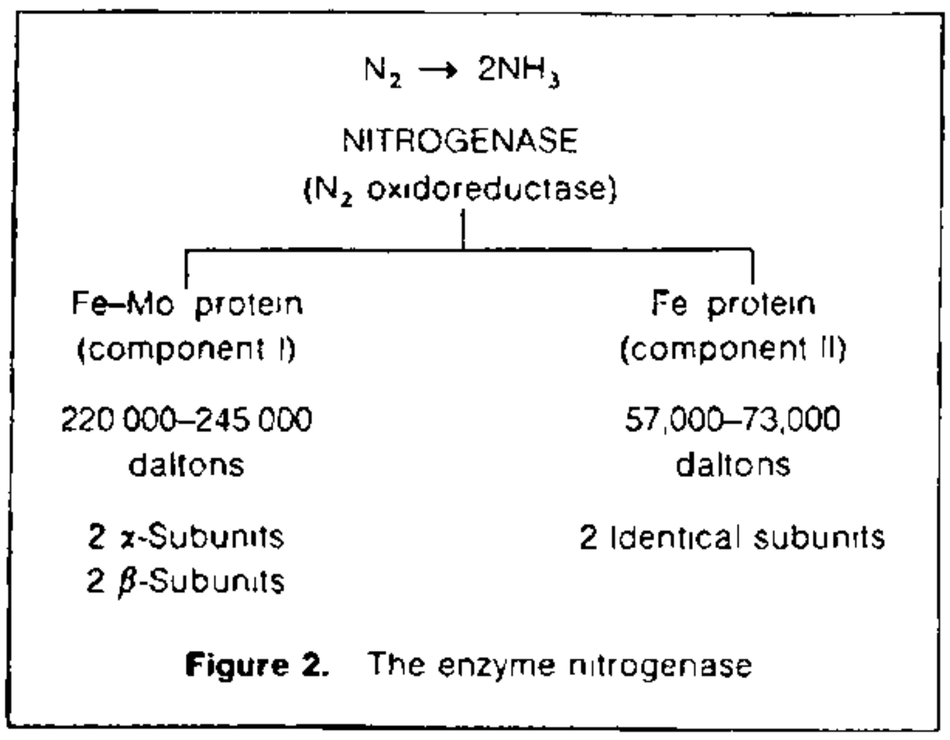

Figure 2. The enzyme nitrogenase

while the component II is composed of two chains of a third subunit[11]. The three different polypeptides of nitrogenase are the products of the genes *nifH*, *nifD* and *nifK* (ref. 12). Figure 3, which shows the cluster of genes involved in nitrogen fixation in the free-living nitrogen fixer *Klebsiella pneumoniae*, shows other genes that are ancillary genes and are all necessary for nitrogen fixation[12]. Some are involved in electron transport, while some others are responsible for processing of the translation products of the genes *nifH*, *nifD* and *nifK*. The gene *nifA* codes for a regulatory protein[13] which exerts positive control over all the other genes. Thus the presence of the nifA gene product is essential for expression of all the other *nif* genes. The expression of the *nifA* is in turn regulated by two other genes, *ntrC* and *ntrB*. The combined product of *ntrC* and *ntrB*, which are normally activators of *nifA* expression, is converted into a repressor in the presence of fixed nitrogen. This is the mechanism by which fixed nitrogen shuts off nitrogen fixation. It has been possible to isolate mutants[14], which, even in the presence of fixed nitrogen, continue to fix nitrogen. This should be possible in the commercially important *Rhizobium* strains also. The efficiency of nitrogen fixation in a particular strain is controlled by the level of the nifA gene product, and this efficiency can also be improved by manipulating the regulatory system using recombinant-DNA technology. In *Rhizobium* a similar organization of *nif* genes is seen, and in addition, there are other genes that are necessary for the symbiotic process[15]. A signal from the plant activates the expression of *nifA*, and once *nifA* is expressed, its product activates the other genes, as in *K. pneumoniae.*

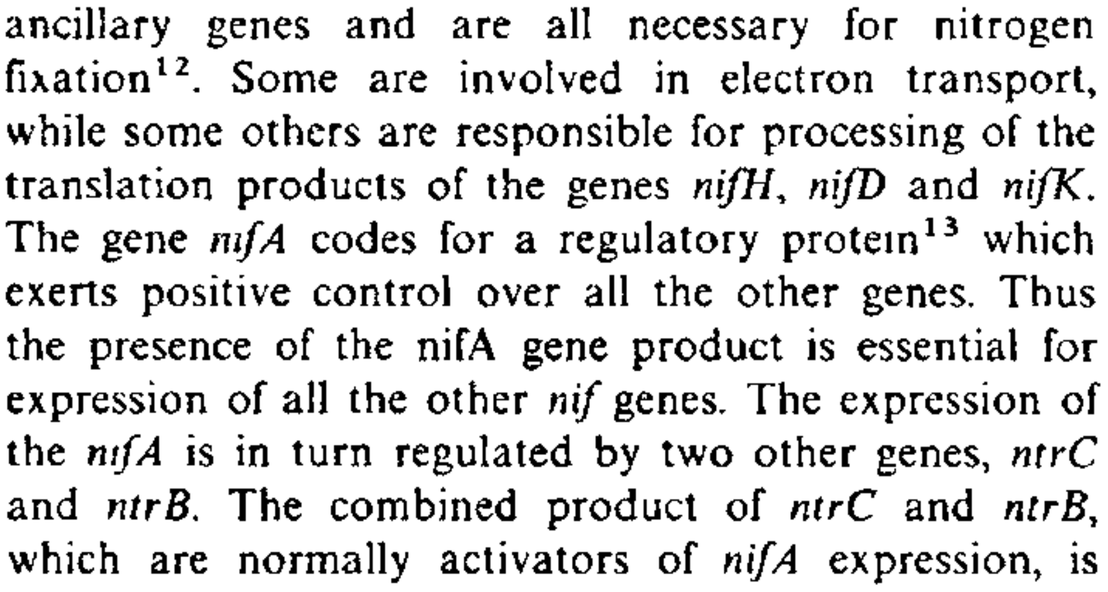

Figure 3. The *nif* genes of *Klebsiella pneumoniae*

Free-living nitrogen fixers

Rhizobium is effective only with leguminous plants, but in India cereal crops, which are grasses, are more important commercially. The area under rice cultivation is much more than that under all the legumes combined. Table 6 gives an idea of the areas under different crops[16]. The free-living nitrogen fixers *Azospirillum* and *Azotobacter* are useful for these crops. The effect of *Azospirillum* application on the yield of crop can be seen from Table 7. Rice, barley, oat, sorghum and pearl millet show considerable increase in grain yield[17–19]. Really substantial yield increase can be seen in the case of forage and fodder crops like Transval digit grass and Guinea grass[20]. Similarly *Azotobacter* application has been found to produce beneficial effect in yield of sorghum and vegetables like potato, carrot, cauliflower and tomato (Table 8)[4,5,21–25]. *Azotobacter* is now being recommended for application to cotton plants. Sugarcane responds to both *Azospirillum* and *Azotobacter* applications. The points mentioned under biotechnology phase-I and phase-II (Tables 4 and 5) are all relevant to *Azospirillum* and *Azotobacter* also.

Another important class of free-living microorganisms with members that fix atmospheric nitrogen are the blue-green algae or cyanobacteria. Two members of this class, *Nostoc* and *Anabaena*, are very useful in submerged paddy fields. *Nostoc* is a unicellular organism while *Anabaena* is a filamentous one. An actinomycete, *Frankia*, has been found to be involved in nitrogen fixation in the case of trees like *Alnus* and *Casuarina.*

Table 6. Area under various crops in India

Crop	Area (million hectares)
Rice	41
Sorghum	16
Millets	14
Sugarcane	3
Barley	1
Maize	6
Cotton	2

Table 7. Yield increase due to *Azospirillum* application

Crop	Increase in yield (%)
Food grains	
Wheat	6
Rice	15
Maize	8
Barley	18–27
Oat	17
Sorghum	23–64
Pearl millet	3–30
Forage/fodder	
Transval digit grass	160
Guinea grass	150

Table 8. Yield increase due to *Azotobacter* application

Crop	Increase in yield (%)
Food grains	
Wheat	8–10
Rice	5
Maize	8
Sorghum	15–20
Vegetables	
Potato	13
Carrot	16
Cauliflower	40
Tomato	2–24
Fibre	
Cotton	7–27
Sugarcane	9–24

This microorganism is therefore important for forest trees.

Conclusion

In summary, it may be emphasized that nitrogen-fixing microorganisms have been demonstrated, beyond any doubt, to enhance production of leguminous crops, cereal crops, forage and fodder crops, some vegetables, and cotton and sugarcane. The inconsistent reports on yield are not because of any deficiency in the science or technology involved; it is because of wrong practice, inadequate quality control, and lack of involvement of organized industry in it. If the strains are chosen properly, results are bound to be reproducible. If the production of inocula is done scientifically, product quality would be ensured and grain yield would continue to show the expected enhancement year after year.

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