

The soil microorganisms beneficial for production and protection and maintenance of soil health and fertility are known for centuries. However, in the present day intensive agricultural practices the contributions of these microorganisms have been overlooked, since their effects are more pronounced under sustainable conditions as compared to nutrient sufficient soil environment. Use of excessive inorganic fertilizers and chemical pesticides in intensive cropping has destroyed the microbial equilibrium in soil where microorganisms harmful to plant growth have predominated over the beneficial ones. However the Soil Biologists continued their efforts to highlight the role of the beneficial forms and have understood the physiology, biochemistry and genetics of these organisms which directly or indirectly benefit the plants in nutrient supply, crop production and protection and maintenance of soil fertility. The Book "Biotechnological Approaches in Soil Microorganisms for Sustainable Crop Production" is a compilation of information on such beneficial soil microorganisms which have been recognised as plant nutrient suppliers, nutrient solubilizers or mobilizers, plant growth promoters, plant health promoters, pathogen suppressors, entomopathogens, rhizosphere colonizers, pesticide degraders, pollutant detoxifiers and organic matter decomposers.

The biochemical and physiological aspects of plant nutrient supply system through microorganisms (mainly nitrogen and phosphorus) have been discussed extensively with molecular approaches attempted by scientists to modify the microorganism or the plant system to get maximum benefits from the plant-microbe association or interaction. Similarly, molecular studies carried out with soil microorganisms involved in plant growth promotion, disease suppression, pest control, pesticide degradation and pollutant detoxification have been discussed in taming and enhancing the effectivity of the process in which they are involved. The production technologies for large scale production of the organism or the end product of metabolism involved in benefiting the plant, with their limitations involved in the processes, are also elaborated.

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Role of Free Living and Associative Diazotrophs in Non-legumes

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1. INTRODUCTION

The quantum jumps in cereal crop yields during the era of "green revolution" in agriculture in the 1960's were largely through the adoption of highly N-fertilizer responsive plant genotypes. In spite of an unlimited supply of N₂ in the air, manufacturing of 1 kg of fertilizer N requires 18.5 MCal, six times more energy than that needed to produce either P or K fertilizer (1). Manufacturing the

fertilizer for today's needs requires 544×10^9 MJ of fossil fuel energy (2, 3) which is equivalent to about 13 million tonnes of oil - a non-renewable resource. In India we have to reach the estimated target of 230-240 million tonnes of foodgrains by 2000 AD. On the other hand the demand for fertilizer nitrogen produced by using non-renewable fossil fuels cannot be met through domestic production. In such a scenario, help of microbes which do not need fossil energy is of immense value for increasing soil productivity in India where most of the agriculture is low-input subsistence farming through biological nitrogen fixation (BNF) or increased efficiency of the fertilizers applied. The association between non-legumes and N_2 -fixing bacteria as shown by increased N_2 ase activity is now well established (4-7). The list of N_2 -fixing bacteria associated with non-legumes includes species of *Achromobacter*, *Acetobacter*, *Alcaligenes*, *Arthrobacter*, *Azotobacter*, *Azotomonas*, *Bacillus*, *Beijerinckia*, *Clostridium*, *Campylobacter*, *Corynebacterium*, *Derxia*, *Desulfovibrio*, *Enterobacter*, *Erwinia*, *Herbaspirillum*, *Klebsiella*, *Lignobacter*, *Mycobacterium*, *Methylosinus*, *Pseudomonas*, *Rhodospirillum*, *Rhodopseudomonas*, and *Xanthobacter* (8).

Diazotrophs like *Herbaspirillum* spp. grow endophytically in the stems and leaves of sugarcane. There is evidence to show that *Acetobacter diazotrophicus* is the main contributor of endophytic BNF, which according to N-balance studies was found to be as high as $150 \text{ kg N ha}^{-1} \text{ y}^{-1}$ in sugarcane (9). Another N_2 -fixing endophyte which is of considerable interest is *Azoarcus*. This diazotroph inhabits the roots of Kallar grass (*Leptochloa fusca*) which yields 20-40 t of hay $\text{ha}^{-1} \text{ y}^{-1}$ without the addition of any N fertilizer in saline-sodic, alkaline soils having low fertility (10). Recent studies have demonstrated the endophytic colonization of rice roots by other N_2 -fixing bacteria *Alcaligenes faecalis* (11, 12) and *Herbaspirillum* spp. (13). *Herbaspirillum* spp. have also been isolated from stems and leaves of rice (13). Hurek et al. (14) showed that the N_2 -fixing endophyte *Azoarcus* BH72 has the ability to invade and colonize rice roots.

Although many genera and species of N_2 -fixing bacteria are isolated from the rhizosphere of various cereals, mainly members of *Azotobacter* and *Azospirillum* genera have been widely tested to increase yields of cereals under field conditions. These bacteria are stimulated in the rhizosphere of cereal crops and a selection for particular type of bacteria also occur in the root zone. Azospirilla and azotobacters are active N_2 fixers under laboratory conditions, generally found wherever these are sought and can use a variety of carbon and energy sources for their growth on combined N or N_2 (8).

A survey of 200 fields in the traditional sorghum and millet-growing areas in North-Western India showed the most probable number (MPN) of N_2 fixers varying from $10^2 - 10^5 \text{ g}^{-1}$ soil. Out of 3760 isolates obtained from these soils following MPN and dilution plate count technique, 42% isolates showed N_2 ase activity *in vitro* (6). In another study out of 546 different isolates obtained from the rhizosphere of pearl millet grown at ICRISAT Center, only 17% isolates showed N_2 ase activity *in vitro*. Pearl millet rhizosphere was dominated by azospirilla, constituting 72% of total N_2 -fixing isolates followed by enterobacters (12%), azotobacters (11%) and pseudomonads (5%) (15).

It is commonly thought that these bacteria could be exploited to increase crop yields through increased BNF. To increase crop yields, the route of artificial inoculation of plants with N_2 -fixing bacteria has been tried. Many experiments have been performed in several countries to investigate the effects of inoculation of various strains of *Azotobacter chroococcum* and *Azospirillum* spp. on cereals and grasses. Several field experiments in Belgium, Brazil, Czechoslovakia, Egypt, Israel, India, Germany, Poland, USA and erstwhile USSR with different crops, inoculated with different N_2 -fixing bacteria, showed increased yields and / or increased N accumulation by plants, and sometimes resulted in decreased yields because of inoculation.

In this article, the results of several field inoculation trials with azospirilla and azotobacters, mechanisms of increasing crop yields and the extent of BNF's contribution to sustainable agriculture are reviewed. Recent developments in the area of non-legume N_2 -fixation are also discussed.

2. CROP RESPONSES TO INOCULATION

Plant responses to inoculation with azotobacters and azospirilla in cereals and non-cereals are often reported in terms of increased grain yield, plant biomass yield, nutrient uptake, grain and tissue N contents, nitrogenase activity, early flowering, tiller numbers, greater plant height, leaf size, increased enzyme levels in plant parts, increased number of spikes and grains per spike, thousand grain weight, increased root length and incidences of reduced insect and disease infestation (8,16). Recent reviews (8,17) have evaluated the worldwide crop responses to inoculation with azotobacters and azospirilla. The results indicated that in many cases inoculations increased plant yields but such increases were variable (statistically

significant increases and sometime negative). The responses varied with crops, cultivars, locations, seasons, agronomic practices, bacterial strains, level of soil fertility, and interaction with native soil microflora.

Wani (8) reviewed the comprehensive data obtained from field experiments conducted in erstwhile USSR which showed that out of 1095 experiments, 890 (81%) experiments showed increases in yields of cereals and vegetables and the increases amounted to 10% in only 514 (47%) experiments. Similarly, several subsequent field experiments showed increased crop yields due to *Azotobacter* inoculation (Table 1). In the Estonian S.S.R. region, results of 117 field experiments on the use of *Azotobacter* demonstrated that *Azotobacter* is effective only in soil with a native *Azotobacter* population. This observation looks strange since it is generally thought that inoculation is successful in soils that have very low or no population of the inoculant bacteria. Further, it was suggested that instead of *Azotobacter* inoculation it would be more convenient to enhance the growth of the native *Azotobacter* population in the soil by treating seeds with trace elements and other growth factors (18). In Australia, out of 71 field trials with *Azotobacter* inoculation of wheat, in 28 trials grain yields increased by 5%, in 4 trials negative results were observed and in 39 trials no effect on grain yields were observed (19).

Table 1. Summary of cereal crop responses to *Azotobacter* inoculation in different regions of the USSR (8).

Crop	No. of experiments	Av. % increase in yield
Spring wheat	66	15.9
Winter wheat	34	12.7
Barley	56	8.9
Foxtail millet	2	39.0
Oats	73	12.3
Rye	7	19.1
Millet	9	5.6
Corn	27	10.0

Of late, attention has been shifted from *Azotobacter* to *Azospirillum* as an inoculant as it has widespread distribution in soil, is easy to culture and identify because of its curved form and type of motility, and is relatively efficient in utilization of carbon to support N₂-fixation. In an evaluation of the reported world wide success of *Azotobacter* and *Azospirillum* inoculation, it was concluded that

statistically significant yield increases were obtained in approximately 60% of the trials in erstwhile USSR, Israel and India (8). Mean grain yield of pearl millet increased significantly (up to 33%) due to inoculation with N₂-fixing bacteria over the respective noninoculated controls in 14 out of 24 field experiments (Table 2). In one experiment with *A. lipoferum* and two experiments with *A. chroococcum* no response was observed. In two other experiments grain yields decreased by 2.7% after inoculation with *A. lipoferum* and by 4.5% after inoculation with *A. chroococcum*. Field experiments at ICRISAT Asia Center (IAC) with sorghum showed that inoculation with *Azospirillum* and *Azotobacter* increased the grain yields marginally over the uninoculated control. In a field trial on an Alfisol with three sorghum hybrids CSH 1, CSH 5, and CSH 9, inoculated with *Azospirillum lipoferum* and *A. chroococcum* grain yield was marginally increased by 6% over the control because of inoculation (5). Another trial with three sorghum cvs CSH 5, CSH 9, and SPV 351 and 10 inoculation treatments showed only marginal increase (2 - 10%) in grain and plant dry matter yield across the cvs because of inoculation with N₂-fixing bacteria over the uninoculated control (6). Several field trials with different crops inoculated with azotobacters and azospirilla reviewed by Wani (8) indicated that pearl millet and sorghum which are grown as dryland crops showed 11 - 12% increased yields due to inoculations. Maize, wheat and rice, which receive better management and inputs than pearl millet and sorghum showed 15 - 20% increased yields due to inoculation.

In Israel, field inoculation experiments with *Azospirillum* were carried out using different cereal crops, varieties, and different fertilization levels (23). These experiments were conducted on large plots (200 - 1000 m²) with 4 - 6 replications and the agronomic practices used were identical to those used for commercial production. Thirty-one such field experiments were conducted and in most cases, the effect of *Azospirillum* varied with the seasons, years, and the crop (Table 3). In general, inoculation of the C-4 plants corn, sorghum, *Panicum*, and *Setaria* showed greater yield increases than the inoculated spring wheat, a C-3 plant. With the summer crops, 75% of the experiments showed significant increases and 90% of the experiments showed increases >5%. The optimum temperature for *Azospirillum* growth is 32 - 35°C and it is possible that bacterial activity, including BNF was greater in the summer, particularly in irrigated crops. During vegetative phase of wheat growth, the soil temperatures in Israel are 10 - 15°C; nevertheless, inoculation of wheat with *Azospirillum* also showed significant increases in foliage and grain yield with lower increases than the summer crops.

increased N assimilation by plants (26-29). In pearl millet inoculation with *Azospirillum* or *Azotobacter* generally increased total plant N assimilation, and such increases were higher at sub-optimal levels of applied N with inoculation (8). Based on several field trial analysis, the average increase in N assimilation by inoculated pearl millet was found to be about 5 kg ha⁻¹ (8). Pearl millet inoculation experiments were conducted for three consecutive years in the same plot. Following three years of inoculation with N₂-fixing bacteria, N uptake by a pearl millet cv. ICMV1 was studied at 0, 20 and 100 kg N application under field conditions. In case of 20 kg N ha⁻¹ treatment, apparent fertilizer recovery by plants in uninoculated treatment was 45% whereas the apparent fertilizer recovery in case of *Azosp. lipoferum* inoculated treatment was 86% and with *Azob. chroococcum* it was 113%. In case of 100 kg N ha⁻¹ treatment inoculation with N₂-fixing bacteria increased apparent fertilizer N recovery marginally by 7% over the uninoculated treatment (55 vs. 48%). Similarly, maximum increased N assimilation (21 kg N ha⁻¹) due to inoculation with *Azosp. brasilense* was observed in 20 kg N ha⁻¹ treatment over the 20 kg N ha⁻¹ alone showing 105% apparent plant N recovery of applied N (22).

4. EFFECT OF SOIL NUTRIENTS

Soil and fertilizer N affect the response to inoculation. Largest differences in yields are obtained when the soil is adequately but not excessively fertilized. In a multi-location experiments with pearl millet, higher increases in grain, plant biomass, and total N uptake were observed with zero N + inoculation and the extent of response declined with the increasing levels of applied N (Table 4). In another set of experiments conducted at four locations in India with pearl millet over five years it was observed that the maximum benefits of inoculation was seen either at 0 or 10 kg N ha⁻¹ application than with 20 or 40 kg N ha⁻¹ application (30). Grain yields obtained from zero N treatments inoculated with N₂-fixing bacteria were similar to the yields from the non-inoculated plots receiving 20 kg N ha⁻¹. It is therefore not uncommon to observe yield increases equivalent to 20 kg N ha⁻¹ depending on locations, soil fertility and other factors (8).

Table 4. Mean grain, total plant biomass yield and total plant N uptake by pearl millet inoculated with N₂-fixing bacteria with different N levels (20).

levels (kg ha ⁻¹)	Bacterial culture		uninoculated control	Mean	SE ±
	<i>A. lipoferum</i>	<i>A. chroococcum</i>			
Grain yield (t ha ⁻¹)*, mean of 7 locations					
	1.8(16)	1.8(16)	1.5	1.7	
	2.0(10)	1.9(4)	1.8	1.9	0.059 NS
	2.0(6)	2.0(3)	1.9	2.0	
Mean	1.93	1.88	1.76		0.033*
±		0.036*			
√		20			
Total plant N uptake (kg ha ⁻¹), mean of 2 locations					
0	32.2(27)	29.9(18)	25.3	29.1	
20	37.0(13)	36.6(12)	32.6	35.4	
40	39.2(8)	37.3(3)	36.2	37.6	
Mean	36.1	34.6	31.4		

P = 0.01, NS = Nonsignificant.

Figures in parentheses indicate % increase over controls.

5. ORGANIC MANURES AND INOCULATIONS

Nitrogen-fixing bacteria associated with non-legumes require carbon for their growth and activity in soil. Addition of organic substances introduced into the low organic matter containing tropical soils serve as carbon source for N₂-fixing bacteria and also help the bacteria to overcome the antagonistic effect of soil microflora. Increased nitrogenase activity was observed in the soil when straw was incorporated and the activity enhanced further under warm and moist conditions (31). Similarly, addition of 3% w/w farmyard manure to sand considerably enhanced nitrogenase activity associated with sorghum and millet roots (32). Incorporation of straw (5% w/w) into Nile Delta Soil together with *Azospirillum* inoculation increased dry matter, nitrogen content, and plant height of 12-week old maize plants. Nitrogenase activity associated with corn roots was also increased (33). The inoculation experiment

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