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Long term survivability of *Azospirillum* co-aggregates: Bioinoculation effect on the growth and yield of sunflower

*Manoharan Melvin JOE, Palanivel Karpagavinayaga SIVAKUMAAR

Department of Agricultural Microbiology, Faculty of Agriculture, Annamalai University, Annamalainagar-608002, India

Azospirillum brasilense AZP-18 was co-aggregated with other Plant Growth Promoting Rhizobacteria (PGPR), such as *Azotobacter chroococcum* MTCC-2805, *Azorhizobium caulinodans* ORS-571, *Bacillus megatherium* MTCC-3353 and *Pseudomonas fluorescens* MTCC-4828. These different combinations of *Azospirillum* co-aggregates were studied for their long-term survival efficiency in vermiculite. Among the different combinations the combination of *Azospirillum* with *Azotobacter* showed the highest survival efficiency. The different combinations of *Azospirillum* co-aggregates were found to have a positive influence on the total bacterial and *Azospirillum* population on the rhizosphere of sunflower. It has been found that the combination of *Azospirillum brasilense* with *Azotobacter chroococcum* to be superior in positively augmenting the growth (plant height, capitullum diameter and dry matter production) and yield (no of seeds capitullum^{-1}, stalk yield and seed yield) parameters of sunflower crop.

Key words: *Azospirillum brasilense*, Co-aggregation, Plant Growth Promoting Rhizobacteria, sunflower

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are soil bacteria that have the ability to colonize roots and to stimulate plant growth through the production of phytohormones. This plant growth promotion activity has been reported for strains belonging to many different genera such as *Azoarcus, Azospirillum, Azotobacter, Arthrobacter, Bacillus, Clostridium, Enterobacter, Gluconoacetobacter, Pseudomonas* and *Serratia* (Somers et al. 2004).

Among these different PGPR strains, *Azospirillum, Azotobacter, Azorhizobium, Bacillus* and *Psuedomonas* are widely used as bioinoculants. *Azospirillum brasilense*, a free living, PGPR can fix nitrogen under microaerophilllic conditions, and are frequently associated with the rhizosphere of a large number of agriculturally important crops and cereals (Bashan and Levanony 1990, Bashan and Holguin 1997). *Azotobacter chroococcum*, a cyst forming and free-living PGPR is found to promote plant growth due to its ability to fix dinitrogen (Tchan 1984). Later yield improvements observed in this case are attributed more to the ability of *Azotobacter* to produce plant growth promoting substances such as phytohormone IAA and siderophore azotobactin, rather than diazotrophic activity (Saikia and Brezbaruah 1995). *Azorhizobium* is particularly interesting since it has a unique capacity among rhizobia to fix N2 in the free living state and in plants (Dreyfus et al. 1988). Moreover, its role as helper bacterium was also reported earlier (Yanni et al. 1997). The most efficient phosphate solubilizing microorganism (PSM) include genera *Bacillus* and *Pseudomonas* (Tilak et al. 2005). The bacilli include *Bacillus megatherium* isolated from the rhizosphere of legumes and cereals (Sundara-Rao and Sinha 1963) and *Pseudomonas fluorescens* isolated from chick pea, maize, soybean and other crops (Bardiya and Gaur 1974). Besides its phosphate solubilizing ability *Pseudomonas fluorescence* is also known for its biocontrol efficiency (Leeman et al. 1995, Mayer and Hofte 1997, Sivakumaar and Joe 2007).

Though *Azospirillum*, one among these strains is well known for its ability to enhance plant growth and yield under a variety of environmental conditions (Blaha and Schrank 2003) its growth promotion activity under field conditions is not consistent (Öğüt et al. 2005). Moreover, Okon and Laberandera-Gonzalez (1994) reported that only 60–70% of the field inoculations over a 20-year period produced significant increase in yield. This inconsistency in the field reports of *Azospirillum* has led to the emergence of a new research subfield namely co-inoculation of *Azospirillum* with other *microorganisms* (Bashan and Holguin 1997).

Co-inoculation with *Azospirillum* is based on mixed inoculants or combinations of microorganisms that interact synergistically, where *Azospirillum* function as a "helper" bacteria, which enhance the performance of other beneficial microorganisms. It has been found that these microorganisms interact synergistically by providing nutrients, removing some inhibitory products, or stimulating each other through physical or biochemical mechanisms (Khammas and Kaiser 1992).

However, the major limitation in this approach is competition; an indirect interaction that has negative effect on both. In competition, each population competes for the same substrate or for required nutrients, when grown together (Shuler and Kargi 2006).

*Correspondence to:
Tel.: +919894095443
E-mail: micromelvin@gmail.com

This competition, which the organisms encounter either when grown together in a growth media or when stored in inoculant carrier, has eventually led to the search for other alternatives. One among these alternatives is co-aggregation; a bacteria-bacteria interaction and the interactions are highly specific and that only certain cell types are partners. This phenomenon can be defined as clumping when different cell types are mixed (Kolenbrander et al. 1993). Co-aggregation is prevalent among bacteria isolated from human oral cavity and was reported by Gibbons and Nygaard (1970).

Earlier we advanced this concept to the field of agriculture and developed co-aggregated cells as bioinoculants for rice crop using A. *caulinodans*-ORS-571 (Sivakumaar and Joe 2008). Further, we in our earlier studies (Joe et al. 2009) investigated the effects of various physical and chemical factors influencing the co-aggregation and also evaluated the stability of *Azospirillum* co-aggregates.

Though, crop response to *Azospirillum* was well demonstrated in many cereal and forage crops (Okon 1985, Hegazi and Saleh 1985, Sharma 1997, Bashan and Holguin 1997). Work done on biofertilization of oil crops and in particular sunflower under the context of southern Indian conditions is less despite its well acceptance among the farming community in these regions. This well acceptance is due to its adaptability to a wide range of soil and climatic conditions and shorter duration. Moreover in Tamil Nadu, South India sunflower is an important oil crop next to groundnut and grown over area of 17,000 ha with a production of 7000 tonnes.

Hence the present work was undertaken with the following objectives:

1. Evaluation of the long-term survivability of different *Azospirillum* co-aggregates.
2. Studies on the effect of these co-aggregated cells on the total bacterial and *Azospirillum* population, followed by a critical evaluation of its influence on the growth and yield of sunflower variety sunbeam.

MATERIALS AND METHODS

Culture and growth conditions

Azotobacter chroococcum - MTCC - 446, *Azorhizobium caulinodans* ORS-571, *Bacillus megatherium* MTCC-3353, *Pseudomonas fluorescens* MTCC-4828 were obtained from IMTECH(Institute of Microbial Technology), Chandigarh, India, the *Azospirillum brasilense* isolate AZP-18 was isolated, purified and characterized in the Department of Microbiology, Faculty of Agriculture, Annamalai university. The bacterial strains were maintained at -20°C in nutrient broth containing 20% (v/v) glycerol and, before being used, they were grown overnight at 30°C at 120 X g in Nutrient broth (Himedia) or on Nutrient agar medium (Himedia) at 30°C for 24h.

Preparation of different combination of Azospirillum co-aggregates

The bacterial inoculum was made as follows: all the PGPR strains namely, *Azospirillum*-AZP-18, *Azotobacter* MTCC-446, *Azorhizobium* ORS-571, *Pseudomonas* MTCC-4828 and *Bacillus* MTCC-3353 were inoculated separately on M 9 salts minimal media with a slight modification as described by Sambrook et al. (1989) in a shaking bath at 30 ± 2°C for 5 days. For the induction of aggregation a slight modification was made to the minimal salt medium in which the carbon and nitrogen sources were replaced by fructose (6.67 g/L) and NH_4Cl (0.214 g/L) in the ratio of 30:1. Then the medium was centrifuged at 5000 x g for 10 min to harvest the stationary phase cells and the pellets were washed three times with 0.1 M-phosphate buffer (pH 6.8). Finally, the cells were re-suspended in the same buffer to a final concentration of 1 x 10^9 CFU/ mL by measuring the absorbency at 650 nm and used as inoculums (OD value of 0.6).

Co-aggregation assay

One ml aliquot of *Azospirillum* AZP-18 with other any of the other PGPR strain was mixed together in 10 ml Co-Ag buffer as described by (Grimaudo and Nesbitt 1997) consisting of 20mM Tris -HCl buffer (pH 7.8), 0.01mM $CaCl_2$, 0.01mM $MgCl_2$, 0.15 M NaCl, 0.02% NaN_3. Uninoculated buffer served as control. The mixture was vortexed for 10s, shaken on a rotary platform shaker for 3 min, and left undistributed at room temperature for 24 h. All Co-Ag reactions were performed in triplicate. Finally, the cells were resuspended in the same buffer. The final concentration is adjusted to 1 x 10^9 CFU /mL for each strain.

Long term survivability in vermiculite as an inoculant carrier

Vermiculite, the most suitable carrier based on previous studies (Joe and Sivakumaar 2008; Joe et al. 2009) was selected for survival studies. Standard procedures for carrier preparation were followed (Somasegaran and Hoben 1994). Ten gram of vermiculite as a carrier material was aseptically injected with buffer containing the *Azospirillum* co-aggregates (minimum 10^9 CFU/mL for each strains). *Azospirillum* strains containing specific antibiotic markers (ampicillin at a concentration of 100 mg/L) were used in this study. The buffer: carrier ratio was chosen according to the water-holding capacity of substrate as per the procedures of Nieuwenhove et al. (2000). The treatments simulated realistic conditions of storage: room temperature (28 ± 2°C). Sampling was done in three replicate bags per treatment. The total survival population, every two months up to a period 12 months after inoculation (MAI) was estimated by plating decimal dilution series in Phosphate buffer of 1 g stored material on Nutrient agar medium.

The individual *Azospirillum* population was determined by Most Probable Number (MPN) method (Cochran 1950) by plating in NFB semisolid agar supplemented with the antibiotic.

Effect of co-aggregated cells on the plant growth and yield of sunflower

Pot culture experiment

The experiment was conducted in the Department of Microbiology, Faculty of Agriculture, Annamalai University, Tamil Nadu, India in the period of October-December- 2008. The soil was sieved through a 20-mesh sieve thoroughly mixed and placed in clay pots. Clay pots were filled with clay loam soil having a pH 7.4 and EC of 0.93dSm^{-1}. The total available nitrogen of soil was 86.24 kg ha^{-1} and the organic carbon (%) was 0.34. Each pot including the control treatment was given a basal dose of triple super phosphate (37.5mg P_20_5), murate of potash (25mg K_2O) and ammonium molybdate (0.625mg). Five replications were maintained for each treatment.

Seed bacterization

For seed treatment, the seeds of sunflower variety sunbeam were treated with cell suspensions containing the co-aggregates at the rate of 10ml per pot (minimum inoculation load of 1x10^9, individual population of cells) mixed with lignite and 5ml of gum arabinose to enhance the adhesiveness. Sunflower seeds without seed bacterization were maintained as control. The treated seeds were grown under pot culture condition.

Enumeration of total bacterial population in the rhizosphere of sunflower

The total bacterial population in the rhizosphere of sunflower was enumerated by serial dilution and plating in glucose agar medium (Allen 1974).

Enumeration of total Azospirillum population in the rhizosphere of sunflower

The population of *Azospirillum* in the rhizosphere of sunflower soil was enumerated by following the most probable number (MPN) technique (Cochran 1950).

Bioinoculation effect of different combination of Azospirillum co-aggregates germination percentage and vigour index

The treatment effects of different combination of *Azospirillum* co-aggregated cells were studied for their influence on the germination percentage and vigour index of sunflower.

One-hundread seeds were taken in a sterile petriplate and treated with ten ml of *Azospirillum* co-aggregates (with an initial population 10^9; individual population of strains). Control treatment was maintained without any seed treatment. The seeds were then shade dried for 30 min. Then, these inoculated seeds were tested for the germination rate using paper towel method (ISTA 1976). The germination percentage was calculated from eight days after sowing (DAS) to 12 DAS. The morphological characters like shoot and root length was measured on 20 DAS. The vigour index (*VI*) of the seedlings was estimated as suggested by Abdul-Baki and Anderson (1973):

$VI=RL+SL\times GP$, where *RL* is root length (cm), *SL* is shoot length (cm) and *GP* is germination percentage.

Plant height

The height of the plants from each treatment was measured on 60th day after sowing (DAS). The mean value of the plants from three replications was recorded.

Dry matter production (DMP)

Three plants randomly selected from each treatment was washed and dried in an oven at 80°C till constant weight was observed. The plants were weighed and DMP was expressed in kg ha^{-1} on 60 DAS.

Nitrogen content of the plant

The plant samples were washed in water, air dried and later dried to a constant weight in an oven at 50°C. Then they were ground, sieved and 100mg of sample was taken for analysis. The total nitrogen content was determined by microkjeldahl method (Yoshida et al. 1972).

Capitullum diameter

Capitullum diameters of three representative plants from each treatment pots are measured at harvest and their mean values were recorded.

Total number of seeds per capitullum

Total number of seeds in the three representative samples was counted and the mean value per plant was recorded.

Seed yield

The seeds of three representative samples were collected and weighed. The mean value plant^{-1} was expressed in g plant^{-1}.

Stalk yield

The straw yield was determined at the time of harvest and expressed in g plant^{-1}.

Grain yield

Grain yield of the crop g plant^{-1} was determined at the time of the harvest. Mean values of three plants were recorded.

Oil content

The oil content of the seed was estimated using diethyl ether as extractant using soxhelet extractor and expressed in percentage.

Protein content

Crude protein content of seed was calculated by multiplying the nitrogen content of the kernel with 6.25 (Humphries 1956).

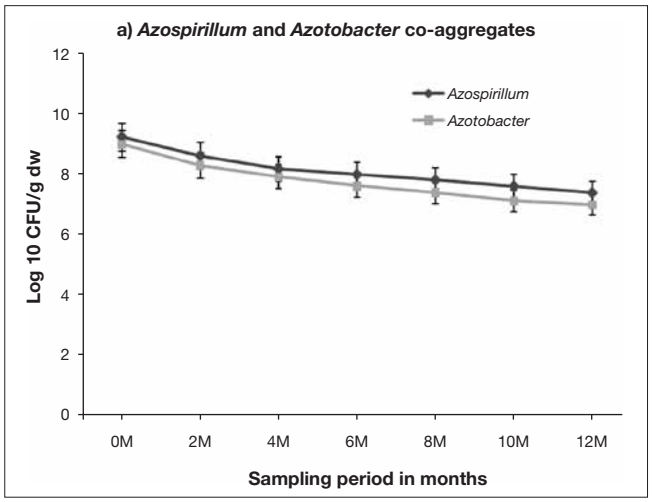
Statistical analysis

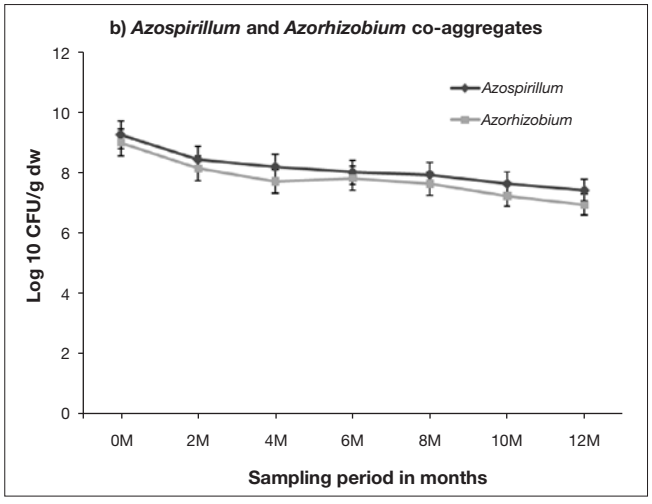
Experimental results were statistically analyzed by analysis of variance (ANOVA) and the treatment means were compared relative to control following Duncan's Multiple Range Test (DMRT) or least significant difference (LSD) test unless indicated otherwise, differences were only considered when significant at $p<0.05$ as per procedure described by Gomez and Gomez (1984).

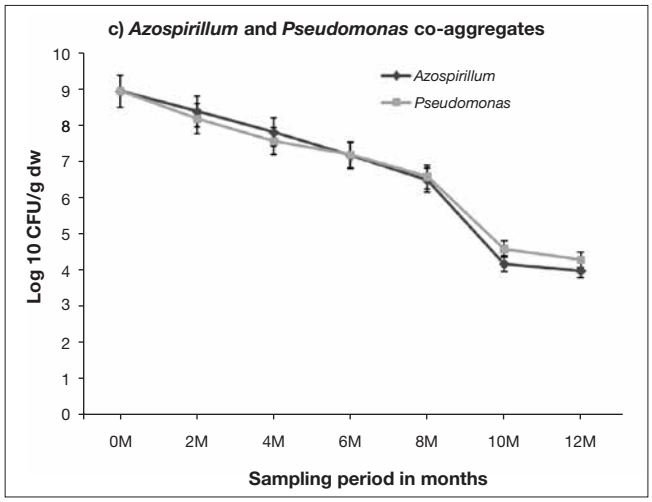
RESULTS AND DISCUSSION

The phenomenon of bacterial aggregation is of great interest in the production, storage and survival of bacterial inoculants for agriculture application (Bahat-Samet et al. 2004).

In the present study, the long-term survivability of different *Azospirillum* co-aggregates was evaluated in vermiculite as an inoculant carrier (Fig 1). Among the different combinations, the combination of *Azospirillum* and *Azotobacter* showed good compatibility among them. Further, they showed higher survival rates in vermiculite, this was closely followed by the other PGPR combination of *Azospirillum* with *Azorhizobium*.







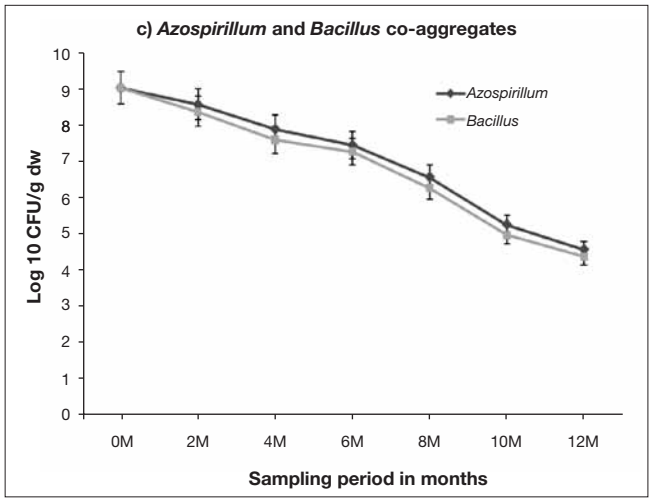


Figure 1. Long term survival of *Azospirillum* co-aggregates

(log CFU g^{-1} dry wt of carrier; consisting of Azospirillum and other PGPR combination) on seven sampling dates, in vermiculite stored at 28 ± 2°C. Error bars indicate the minimum significant difference (5%) for comparing treatments on each sampling date)

The results of this study are in line with our previous studies (Sivakumaar and Joe (2008), Joe et al. (2009)) that the co-aggregation percentage and compatibility was high among different combination of diazotrophic bacteria, when compared with other combination of PGPR's. Previous studies by Bashan and Holguin (1998) reported that this co-culture could be considered as a metabolic association where the sugar degrading bacteria produce degradation and fermentation products that can be used effectively by *Azospirillum*.

However, the combinations of *Azospirillum* with *Pseudomonas* and *Bacillus* did not go well and showed a significant reduction in the survival population. The results of our study is in conformity with the reports of Felici et al. (2008) that *A. brasilense* strain Sp245 failed to co-work well in association with other the other PGPR strain B. *subtilis* 101.

We suppose that this negative effect is due to the production of toxin or other inhibitors by these strains against *Azospirillum*.

The influence of different *Azospirillum* co-aggregates on the total bacterial and *Azospirillum* population was studied and the results are presented in Table 1. Among the different combinations studied, the combination of *Azospirillum* with *Azotobacter* showed a greater influence on the total bacterial and *Azospirillum* population. This is clearly evidence from the Table 1 that the combination of *Azospirillum* and *Azotobacter* sustained the highest total bacterial (8.42±0.12 Log 10 CFU/g dw) and *Azospirillum* population (6.70±0.09 Log 10 CFU/g dw) (Table 1).

Table 1. Influence of different combination of *Azospirillum* co-aggregates on the total bacterial and *Azospirillum* population on the rhizosphere of sunflower

Treatment	Total bacterial population*	*Azospirillum* population*
	Log 10 CFU/g dw	
Azospirillum + Azotobacter	8.42±0.1[a]	6.70±0.1[a]
Azospirillum + Azorhizobium	8.04±0.1[b]	6.46±0.1[b]
Azospirillum + Pseudomonas	7.76±0.1[c]	6.20±0.1[c]
Azospirillum + Bacillus	7.84±0.1[c]	6.14±0.1[c]
Azospirillum	7.56±0.1[d]	5.70±0.2[d]
Control	6.79±0.1[e]	5.35±0.1[e]

* Observation on 21 DAS. Values are a mean of three determinants ± S.D. Within a column different letters after values indicate that there is a significant difference at a p value of 0.05 as determined by a post hoc test

However, little is known about the interaction of *Azospirillum spp.* with specific soil microorganisms besides its being parasitized by *Bdellovibrio spp*. and bacteriophages, while having a synergistic association with *Bradyrhizobium* (Bashan 1999).

He further suggested that the application of PGPB, particularly under wet conditions, increases the population of nearby microorganisms, which "prey" on the applied PGPB until they are extinct.

The different combination of *Azospirillum* co-aggregates were studied for their bioinoculation effect on various growth and yield parameters of sunflower crop. Among the different combinations tried the combination of *Azospirillum* and *Azotobacter* positively augmented the growth and yield of sunflower crop, followed by the combination of *Azospirillum* with *Azorhizobium* (Table 2, 3).

Table 2. Bioinoculation effects of different *Azospirillum* co-aggregates on germination percentage (%), vigour index, plant ht, plant dry wt and 'N' uptake of sunflower

Treatment	Germination percentage	Vigour index	Plant ht* in cm	Dry matter production (kg ha^{-1})	'N' uptake*A (Kg ha^{-1})
Azospirillum + Azotobacter	94.5[a]	1264.9[a]	118.4[a]	2403.7[a]	235.6[a]
Azospirillum + Azorhizobium	90.6[b]	1234.6[b]	110.2[b]	2378.6[b]	220.9[b]
Azospirillum + Pseudomonas	86.4[c]	1204.6[c]	105.7[c]	2336.6[c]	207.1[c]
Azospirillum + Bacillus	84.8[c]	1182.6[d]	106.4[c]	2324.4[c]	205.4[c]
Azospirillum	80.4[d]	1154.6[e]	101.4[d]	2300.1[d]	194.2[d]
Control	70.5[e]	730.64[f]	80.6[e]	1794.6[e]	152.6[e]
LSD	2.8	18.6	3.1	12.8	5.1

*Observations at 60 DAS. A "N" uptake assayed according to Microkheldhel assay. Values are a mean of six replications. Mean values followed by different letters are differed significantly according to least significant difference test (p<0.05)

Table 3. Bioinoculation effect of *Azospirillum* co-aggregates on flower head diameter, number of seed capitullum^{-1}, stalk yield, seed yield, oil content and 'N' uptake of sunflower

Treatment	Capitullm Diameter (cm)	Number of seed capitullum^{-1}	Stalk yield g plant^{-1}	Seed yield g plant^{-1}	Oil content (%)	Protein content (%)
Azospirillum + Azotobacter	13.6[a]	720.4[a]	3040.7[a]	1212.4[a]	38.4[a]	11.9[a]
Azospirillum + Azorhizobium	13.4[a]	700.6[b]	2914.3[b]	1140.6[b]	38.2[a]	10.7[a,b]
Azospirillum + Pseudomonas	13.2[a]	690.2[c]	2892.7[c]	1194.5[c]	38.1[a]	10.4[b]
Azospirillum + Bacillus	13.1[a]	687.2[c]	2842.7[d]	1180.4d	38.1[a]	10.3[b]
Azospirillum	13.1[a]	660.8[d]	2564.7[e]	1112.4[e]	38.0[a]	10.1[b,c]
Control	10.3[b]	573.2[e]	2040.1[f]	966.4[f]	36.4[b]	9.1[c]
LSD	1.4	10.8	1.2	2.2	1.2	1.2

Values are a mean of six replications. Mean values followed by different letters are differed significantly according to least significant difference test (p<0.05)

Elshanshoury (1995) reported that dual inoculation of *Azospirillum brasilense* with *Azotobacter chroococcum*, in sterilized soil resulted in significant stimulation of their populations in the rhizosphere of wheat seedlings. Furthermore, he suggested that dual inoculations significantly increased the plant growth, concentrations of indole acetic acid (IAA), P, Mg, N and total soluble sugars in wheat shoots. Further studies by Hegazi et al. (1998) reported an increase in total 'N' content of rice due to the co-inoculation of *Azospirillum* with *Azotobacter*.

Numerous reports (Acharya et al. 1999, Selvakumari et al. 2000, de-Freitas 2000) also suggest the positive influence of coinoculation of *Azotobacter* and *Azospirillum* in augmenting the growth and yield of various crops.

Results of our present study showed that the other diazotrophic combination of *Azospirillum* with *Azorhizobium* has increased the growth and yield of sunflower crop followed by the successful combination of *Azospirillum* with *Azotobacter*.

Neyra et al. (1997) reported an increase in the growth and yield parameters of common bean due to application of co-flocs of *Azospirillum* and *Rhizobium*. Moreover, the performance of *Rhizobium* sp, as helper bacterium, in the rhizosphere of rice has been reported by Yanni et al. (1997).

The combination of *Azospirillum* with *Pseudomonas* and *Bacillus* also found to increase the growth and yield of sunflower, when compared with single inoculation of *Azospirillum*.

The results obtained in our present study are in conformity with the earlier reports of Algawadi and Gaur (1992) that combined inoculation of *Azospirillum brasilense* and the phosphate-solubilizing bacteria *Pseudomonas striata* or *Bacillus polymyxa* on field-grown sorghum significantly increased grain and dry matter yield, and N and P uptake as compared with single inoculation of individual organisms.

The results of our present study are encouraging and suggest that co-aggregation of *Azospirillum* with other microorganisms as one among the major frontiers in biofertilizer technology.

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