DEVELOPMENT AND USE OF *AZOSPIRILLUM* CO-AGGREGATES USING CERTAIN CATIONIC IONS AND ITS BIOINOCULATION EFFECT ON RICE GROWTH AND YIELD

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ABSTRACT

A study was conducted in the Department of Agricultural Microbiology, Faculty of Agriculture, University of Aannamalai, India during 2006. *Azospirillum brasilense* MTCC (microbial type cultural collection)-125, obtained from Institute of Microbial Technology, Chandigarh, India were studied for co-aggregation with other plant growth promoting rhizobacteria, such as *Azotobacter chroococcum* MTCC-2805, *Azorhizobium caulinodans* ORS (collection de O.R.S.T.O.M, Senegal) - 571, *Bacillus megaterium* MTCC-3353 and *Pseudomonas fluorescens* MTCC-4828. The co-aggregation efficiency was found to be enhanced by adding certain cationic ions, such as calcium chloride, aluminium sulphate, magnesium sulphate, ferric chloride and sodium sulphate. Combination of *Azospirillum brasilense* with *Azotobacter chroococcum* using calcium chloride at a concentration of 1.6 mM augmented the highest co-aggregation percentage and floc yield. *Azospirillum* co-aggregates exhibited higher survival in seed surface and spermosphere as compared to log phase PGPR cells. different combinations of *Azospirillum* co-aggregates were also studied for phyto-stimulatory effect, such as plant height, plant dry weight, plant nitrogen content, number of panicles, number of productive tillers and grain yield in rice (cv. ADT-43) grown under pot culture condition. It was found that combination of *Azospirillum brasilense* and *Azotobacter chroococcum* proved superior in positively augmenting growth and yield of rice crop.

KEYWORDS: ***Oryza sativa; Azospirillum brasilense; Rhizobium;* plant growth substances; India.**

INTRODUCTION

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Plant growth promoting rhizobacteria (PGPR) are soil bacteria that have the ability to colonize roots and stimulate plant growth. 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* (36). It is generally believed that main mechanism, by which *Azospirillum* enhances plant growth, is the production of certain plant hormones (37). These growth-promoting substances stimulate the density and length of root hairs and root surface area and improve utilization of water and mineral nutrients. As an outcome of inconsistency in the field of *Azospirillum*, a new research subfield has emerged, namely co-inoculation of *Azospirillum* with other microorganisms.

Co-inoculation with *Azospirillum* is based on mixed inoculants, combinations of microorganisms that interact synergistically, where *Azospirillum* function as a "helper" bacteria, which enhance the performance of other beneficial microorganisms. On the other hand it has been found that these bacteria would also interact synergistically by providing nutrients, removing some inhibitory products, or stimulating each other through physical or biochemical mechanisms (20).

Inoculant formulation has a critical effect on the inoculation process since it determines the potential success of any agricultural bio-inoculant (6). Van Veen *et al.* (39) critically reviewed the reasons for poor performance of agricultural bioinocula in natural environments and in the rhizosphere of host plants and suggested that instead of using a single strain, for a single trait, use of multiple microbial consortia for multiple benefits, can also thrive together in unique ecological niches in ideal proportions.

Co-aggregation is a bacteria-bacteria interaction and 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 (26). Co-aggregation is prevalent among bacteria isolated from human oral cavity and was reported by Gibbons and Nygaard (15). This inter-bacterial aggregation was readily observed with naked eye (11, 23). Co-aggregation is found to be strongest when equal numbers of partners are present (8). This recognition may be intrageneric, intergeneric or multigeneric in nature (24). In all three kinds of coaggregations, cells appear to interact independently of other cells in the population (22)

As reports regarding interbacterial interaction in other ecosystem have been scarce (25), this fascinated us to undertake research which could positively to

develop *Azospirillum* co-aggregated cells as bioinoculants in the field of agriculture. The research was conducted to study co-aggregation of *Azospirillum brasilense* with other PGPR cells using different cations and to evaluate bio-inoculation effect of *Azospirillum* co-aggregates on the plant height, grain yield, number of panicles, productive tillers (%), plant dry weight and nitrogen content of rice.

MATERIALS AND METHODS

The study was conducted in the Department of Agricultural Microbiology, Faculty of Agriculture, University of Aannamalai, India during 2006. *Azospirillum brasilense* MTCC-125 (Microbial type cultural collection), *Azotobacter. Chroococcum, Azorhizobium caulinodans* ORS-571, *Bacillus megatherium* MTCC-3353, *Pseudomonas fluorescens* MTCC-4828 were obtained from Institute of Microbial Technology, Chandigarh, India and were maintained in respective agar slants at 35°C with monthly transfer.

Preparation of inoculum for co-aggregation

The bacteria were grown in M 9 salts minimal media as described by Sambrook *et al.* (32) and these were maintained in an orbital shaker, at 100 rpm, 30 ± 2°C for 24 hours. The broth was centrifuged at 5000 rpm, 10 min to harvest the log phase cells. The pellets were washed three times with 0.1 M phosphate buffer (pH 6.8). Finally the cells were resuspended in same buffer to a cell concentration of 1×10^7 Cfu/ml which confined to an optical density(OD) value 0.4 by measuring absorbency at 420 nm, which was used as an inoculum source for co-aggregation assay.

Co-aggregation assay

Cationic effect on Co-aggregation activity: Co-aggregation assay was done as per methods described by Grimado and Nesbit (17) and the additions of cationic ions to buffer were made following Toeda and Kurane (38), initially with calcium chloride solution (0.05ml; 1.6mM) which was replaced later by various bivalent (magnesium sulphate, ferric chloride and sodium sulphate) and trivalent (aluminium sulphate) cations(0.05ml; 1.6mM) and their co-aggregation activity was measured.

The co-aggregation buffer was prepared according to Grimaduo and Nesbitt (17), which consisted of 20µg $MgCl_2$, 0.15µg NaCl and 0.02% $NaNO_3$; the contents were finally adjusted to a final pH of 7.8 with dilute HCl or NaOH and stored at 10°C.

Equal volumes (2 ml) of each cell suspension were mixed together in pairs by vortexing for 10 s. Control tubes were set up at the same time, containing bacterial suspension without the addition of cations. After incubation period the aggregates settled at the bottom of tube, while some of free cells remained in suspension. The supernatant was sampled and its turbidity was measured in spectronic-20 colorimeter at 420nm. The co-aggregation percentage was determined according to procedure of Madi and Henis (27) in which aggregates were mechanically dispersed by treatments in a tissue homogenizer for 1 min and total OD (optical density) was measured and percent aggregation was calculated as follows:

$$\text{Percentage aggregation} = \frac{OD_t - OD_s \times 100}{OD_t}$$

where OD_t = Total optical density after mechanical dispersion and
OD_s = OD of aggregate after aggregate had settled.

Quantification of co-aggregates (33): Net weight of co-aggregates was obtained by subtracting the weight of dried filter mg/L. and was expressed as mg/L. For dry weight determination filter papers with co-aggregates were placed in a desiccator oven at 60°C for 2 hours.

Survival of *Azospirillum* co-aggregates on seed surface and spermosphere.

Seed bacterization: Seeds were surface-disinfected for 3 minutes in 1 percent (w/v) NaOCl, rinsed in 70 percent (v/v) ethanol for 3 minutes before finally rinsing three times in sterile distilled water (13). Efficacy of disinfection was tested by placing samples of treated seeds on potato dextrose agar (PDA) and nutrient agar (Difco) plates for any microbial growth. One ml of *Azospirillum* co-aggregates or Log phase cells of different PGPR isolates (control) was mixed with one gram of surface sterilized rice seed of variety ADT-43.

Seed surface survival: Bacterized seeds were placed in paper towels and air-dried in a laminar flow chamber overnight. Seed samples were placed in 30x 15 cm^2 brown paper bags and then stored at room temperature. After one week, a sub sample of each seed lot was removed from storage and microbiological analysis of coated seeds and controls were conducted to determine microbial load. For enumeration of attached cells, 1 g of each seed

lot was added to potassium phosphate buffer (0.06M, pH 6.8) and stirred at 200 x g for 2 hours at 30˚C.Bacterial viability of PGPR isolates was determined by plating on selective media.

Spermosphere survival: *Azospirillum* co-aggregates were compared for ability to colonize the spermosphere of rice in tube assays in growth chamber. Plastic tubes (4.0 cm diameter × 20.5 cm long) were plugged in the bottom with cotton and filled with 50 mg of sterile vermiculite followed by 50 g of clay loam from Annamalai University experimental farm. Soil was collected from top 10 to 15 cm of soil profile and sieved through a 0.5-cm-mesh screen before use. Soils were prepared 1 week prior to each experiment and air-dried at room temperature on a bench in the greenhouse. Soil was treated at 60°C for 30 minutes using air mixed with steam. One-gram seed, coated with cells of *Azospirillum* co-aggregates or log phase cells was sown on soil surface in each tube and then covered with 20 mg of sterile vermiculite. Each tube received 30 ml of sterile water at planting and was incubated in the growth chamber under a 12-h photoperiod at 15°C. After one week, individual seedlings were removed from tubes by pushing column of soil and vermiculite containing the plants upward from the bottom and then gently shaking the seedling to remove excess soil from roots. Spermosphere populations were estimated by dissolving one mg of soil in close vicinity with roots in 10 ml phosphate buffer. The suspension was vortexed for 30 min at maximum speed for 30 seconds, serially diluted and plated on selective medium to estimate surviving spermosphere population.

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

Pot culture experiment: Soil was sieved through a 20-mesh sieve thoroughly mixed and placed in clay pots Each pot 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.

Bioinoculation effect of co-aggregates: Seeds of rice variety ADT-43 were treated with cell suspensions containing co-aggregates @ 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. The treated seeds were grown under pot culture condition.

Plant height: Height of plants from each treatment was measured on 60th day after sowing (DAS). Mean values of plants from five replications were recorded.

Plant dry weight: Dry weight of entire plant was taken on 60th DAS. Five plant samples were drawn, washed and later dried to a constant weight in an oven at 50°C. The oven dry weight of plant samples was recorded.

Nitrogen content of plant: The plant samples were washed in water, air dried and later dried to a constant weight in an oven at 50°C. Then these were ground, sieved and 100mg of sample was taken for analysis. Total nitrogen content was determined by micro Kjeldahl method (41).

Number of panicles and productive tillers: The panicles and number of productive tillers of plants from each treatment were measured on 60th DAS. Mean values of five plants were recorded.

Grain yield: Grain yield of crop (g/plant) was determined at time of harvest. Mean values of five plants were recorded.

Statistical analysis

Experimental results were statistically analyzed by analysis of variance(ANOVA) and 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 (16).

RESULTS AND DISCUSSION

In present investigation co-aggregation was attempted in *Azospirillum* with other PGPR isolates. It was observed that co-aggregation exhibited among different PGPR isolates with *Azospirillum*. This aggregation was enhanced by adding different cationic ions. Among different cationic ions studied for aggregation, it was found that Ca^{2+} ions at a concentration of 1.6mM augmented maximum co-aggregation (80.00 %), followed by Al^{3+} ion (71%) (Table-1).

Table 1. Effect of different cations on co-aggregation of *A. brasilense* with other PGPR.

Combination	Co-aggregation percentage in 30 minutes					
	Calcium chloride	Aluminium sulphate	Magnesium sulphate	Ferric chloride	Sodium sulphate	Control
A. brasilense + *A. hroococcum*	72±1.5[a]	80±2.0[a]	68.6±1.4[a]	54.6±1.2[a]	51.4±1.2[a]	44±2.0[a]
A. brasilense + *A. caulinodans*	69.75±1.25[b]	74.20±1.20[b]	64.5±1.25[b]	50.5±1.5[b]	48.40±1.20[b]	40±1.5[b]
A. brasilense + *P. fluorescens*	67.5±1.5[c]	71.8±1.20[c]	62.40±1.20[c]	48.24±1.24[c]	46.5±1.5[c]	36.5±1.25[c]
A. brasilense + *B. megatherium*	66.40±1.60[d]	68.5±1.5[d]	60.5±1.5[d]	46.5±1.25[d]	45.40±1.20[d]	30.5±1.5[d]

The experiments were performed five times, and similar results were obtained each time. The values are a mean of five replications ± SD. Within a column different letters after values indicate a significant difference (P = 0.05) as determined by DMRT.

It was found that Al^{3+} ions were also able to induce co-aggregation to a considerable extent followed by Ca^{2+} ions. Toeda and Kurane (38) reported that aggregation activity was synergistically stimulated by the addition of bivalent/trivalent cations such as Ca^{2+} and Al^{3+}. They also reported that synergistic effects of trivalent cations were stronger than bivalent cations, Al^{3+} being the most effective cation. Ca^{2+} ion was also found considerably increase co-aggregation. Calcium is known to induce encystment in *Phytopthora cinnamomi* at lower concentration (10). Smith *et al.* (35) reported that no aggregation occurred in absence of Ca^{2+} in *Saccharomyces cerevisiae*.

Table 2. Effect of different cations on yield of *Azospirillum* co-aggregates.

Combination	Calcium chloride	Aluminium sulphate	Magnesium sulphate	Ferric chloride	Sodium sulphate	Control
A. brasilense + *A. chroococcum*	1.84±0.24[a]	1.92±0.04[a]	1.65±0.15[a]	1.42±0.04[a]	1.32±0.12[a]	1.02±0.04[a]
A. brasilense + *A. caulinodans*	1.64±0.08[b]	1.75±0.05[b]	1.39±0.03[b]	1.31±0.110[b]	1.22±0.08[b]	0.94±0.06[b]
A. brasilense + *P. fluorescens*	1.42±0.12[c]	1.50±0.08[c]	1.21±0.07[c]	1.12±0.80[c]	1.04±0.04[c]	0.84±0.04[c]
A. brasilense + *B. megatherium*	1.26±0.04[d]	1.34±0.10[d]	1.12±0.04[d]	0.94±0.04[d]	0.71±0.110[d]	0.68±0.02[d]

The experiments were performed five times, and similar results were obtained each time. The values are a mean of five replications ± SD. Within a column different letters after values indicate that there is a significant difference at a P value of 0.05, as determined by DMRT

Among different combinations tried, *A. brasilense* with *A.chroococcum* exhibited the highest co-aggregation efficiency both in terms of co-aggregation percentage and a co-aggregate yield (1.84 g/L). This was closely followed by other diazotrophic combination of *Azospirillum* with *Azorhizobium*, (1.64 g/L) (Table-2).

Reports regarding co-aggregation between *Azospirillum* and other PGPR isolates in the field of agriculture, were scarce, but it is believed that *Azospirillum* can be associated with a wide variety of sugar or polysaccharide degrading bacteria. The co-culture can be considered as a metabolic association where sugar degrading bacteria produce degradation and fermentation products that can be used effectively by *Azospirillum* (5).

Azospirillum co-aggregates exhibited a higher degree of survival in seed surface and spermosphere as compared to log phase PGPR cells (Table-3)

Table 3. Percentage survival of growth promoting rhizobacteria in seed surface and spermosphere of rice variety ADT-43.

PGPR combinations*	Percentage survival**			
	Seed surface survival		Spermosphere survival	
	Co-aggregated cells	Log phase cells	Co-aggregated cells	Log phase cells
A. brasilense + *A. chroococcum*	81.47±1.13[a]	74.72 ±1.42[a]	92.43±1.23[a]	84.12 ±1.42[a]
A. brasilense + *A. caulinodans*	74.23±1.33[b]	67.62 ±2.32[b]	85.43±1.33[b]	77.32 ±2.32[b]
A. brasilense + *P. fluorescens*	66.32 ±2.24[d]	61.22± 2.34[d]	73.12 ±2.24[d]	71.42± 2.34[d]
A. brasilense + *B. megatherium*	77.42 ±1.22[c]	69.12 ±1.18[c]	80.12 ±1.22[c]	65.12 ±1.12[c]

*Initial inoculation load 1 × 10^9 CFU/mL **Survival percentage in seed surface/ spermosphere of other PGPR used with *Azospirillum* in co-aggregation assay after one week. Values are a mean of three determinants ± S.D. Within a column different letters after values indicate that there is a significant difference at P = 0.05 as determined by DMRT.

Present results are in line with findings of Neyra *et al.* (30) where *Enterobacter* and *Pseudomonas* sp. showed good survival rates as co-flocs of *Azospirillum*, when compared to non-flocculated *Pseudomonas* or *Enterobacter.* Previous studies (7,19) have also showed that flocculated cells of *Azospirillum* are rich in PHB granules with a high degree of resistance to desiccation. This phenomenon of flocculation has increased resistance to environmental stress, as they remain metabolically dormant as long as stress conditions persist (28) Flocculation has also provided a micro-environment, which is highly protective against physical and chemical stress and provides a safe niche for survival and cell release upon seed sowing in favorable surroundings, which enhances its survivability (21)

Different *Azospirillum* co-aggregates were studied for their bioinoculation effect on various growth and yield parameters namely plant height, plant dry

weight, plant N content, number of panicles, number of productive tillers and grain yield in rice crop. Among different combinations combination of *Azotobacter* and *Azotobacter* positively augmented rice growth and yield followed by combination of *Azospirillum* and *Azorhizobium* (Table 4).

Table 4. Inoculation effect of *Azospirillum* co-aggregates on different parameters of rice variety ADT-43 (pot culture experiment).

Treatment	Plant height(cm)	Grain yield* (g/plant)	Panicles (no/plant)	Productive tillers (%)	Total dry weight (g/plant)	Total "N" uptake (mg/plant)
Control	72.40±2.04^{e}	4.80±0.40^{e}	7.02±01.14^{e}	70.00±2.00^{e}	17.60±2.02^{e}	16.0±2.0^{e}
A. brasilense + *Azotobacter*	91.34±2.14^{a}	16.2±1.02^{a}	17.01±0.71^{a}	94.00±2.40^{a}	46.40±1.40^{a}	38.50±2.50^{a}
A. brasilense + *A. caulinodans*	85.12±1.12^{b}	11.10±1.10^{b}	13.10±1.10$_{b}$	80.00±2.00^{b}	37.40±1.04^{b}	29.10±1.03^{b}
A. brasilense + *P. fluorescens*	81.40±2.44^{c}	9.04±0.14^{c}	11.00±1.00^{c}	78.50±2.25^{c}	30.40±2.40^{c}	26.06±2.04^{c}
A. brasilense + *B. megatherium*	76.40±2.74^{d}	7.02±0.64^{d}	9.24±0.44^{d}	73.50±2.50^{d}	25.40±1.40^{d}	22.04±2.20^{d}
LSD(<0.05)	2.80	1.20	1.24	2.24	2.20	2.20

Values are a mean of five replications ± SD. Means differed significantly according to least significant difference test (LSD) at P< 0.05. *Grain yield recorded at the time of harvest.

The coinoculation effect of *Azotobacter* and *Azospirillurn*, *Azotobacter* + *Rhizobium* and *Azospirillum* + *Rhizobium* in augmenting the growth and yield of cereal crops has also been reported earlier (1, 31, 34, 12)

Hegazi *et al.* (18) reported an increase in total 'N' content of rice due to inoculation of *Azospirillum* and *Azotobacter*. Elshanshoury (14) reported that dual inoculation of *Azospirillum brasilense* with *Azotobacter chroococcum,* in sterilized soil resulted in significant stimulation of their populations in rhizosphere of wheat seedlings. These dual inoculations also significantly increased the plant growth, concentrations of indole acetic acid (IAA), P, Mg, N and total soluble sugars in wheat shoots.

Performance of *Rhizobium* sp, as helper bacterium, in the rhizosphere of rice has been reported by Yanni *et al. (*40). Neyra *et al.* (29) reported that co-cultivation of *Azospirillum brasilense* Cd and *Rhizobium leguminosarum* bv *phaseoli* in culture medium resulted in co-aggregation of the two bacteria and production of large flocs. Neyra *et al.* (30) reported an increase in growth and yield parameters of common bean due to application of coflocs of *Azospirillum* and *Rhizobium*.

Combined inoculation of *Azospirillum brasilense* and 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 (2).

CONCLUSION

In summary, co-aggregation with *Azospirillum* and other microorganisms is one of major frontiers of biofertilizer technology and perhaps main area for future application. The findings of present study opened up the possibilities for further investigation of genetic basis of effective co-aggregation and also the nature of cellular adhesion.

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