Development of Co-aggregated Cells as Bioinoculants Using Plant Seed Powders – A Novel Delivery System for Rice Grown under Lowland Condition

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Summary

Co-aggregation was attempted in *Azorhizobium caulinodans* ORS-571 with other agriculturally important microorganisms such as *Azospirillum brasilense* MTCC-125, *Azotobacter chroococcum* MTCC-446, *Bacillus megatherium* MTCC-3353, and *Pseudomonas fluorescens* MTCC-4828 to develop coaggregates with multiple benefits using seed powders of different plants *viz*., *Moringa oleifera, Strychnos potatorum* and *Sappindus emaignatus*. Among the different treatments evaluated, the combination of *Azorhizobium caulinodans* ORS-571 and *Azospirillum brasilense* MTCC-125 with the plant seed powder of *Moringa oleifera* recorded the maximum co-aggregation of cells to the tune of 96.8%. The co-aggregates were also studied for their phyto-stimulatory effect such as seed vigour, plant height, plant dry weight, plant N content and endophytic colonization of *A. caulinodans* ORS-571 in rice var. ADT 43 grown under *in vitro* conditions. The co-aggregates of *A. caulinodans* and *A. brasilense* were found to be superior in positively augmenting the characters studied above.

Key words

coaggregation, *Azorhizobium caulinodans,* plant seed powder, rice, endophytic colonization

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Introduction

The sole dependence on chemical inputs based agriculture is not sustainable in the long run and only integrated plant nutrient systems involving a combination of fertilizers, organic and or green manures and biofertilizers are essential to sustain crop production and soil biodiversity. The associative diazotrophs colonize the surface of roots and other plant parts and remain vulnerable to competition from other rhizosphere microorganisms (Ladha et al., 1986). Recently, evidence has been obtained that naturally occurring rhizobia isolated from nodules of non-legume *Parasponia* sp were able to colonize roots through lateral root colonization (LRC) by crack entry. Reproductive invasion of LRCs and subsequent intercellular colonization of *Azorhizobium caulinodans* ORS-571 (a stem nodulating isolate of *Sesbania rostrata*) in low land rice ecosystem could provide opportunities for endophytic nitrogen fixation (Webster et al., 1997). Moreover inoculant formulation has a critical effect on the inoculation process since it determines the potential success of the inoculant (Bashan et al., 1984). Van Veen et al. (1997) critically reviewed the reasons for the poor performance of agricultural bioinocula in natural environments and in rhizosphere of host plants and suggested using multiple microbial consortia for multiple benefits that can also thrive together in unique ecological niches in ideal proportions, instead of using a single strain, for a single trait.

Co-aggregation is a bacteria-bacteria interaction and the interactions are highly specific, that only certain cell types are partners (Kolenbrander, 1988). Co-aggregation was first reported by Gibbons and Nygard (1970) who called it inter-bacterial aggregation and it was readily observed with naked eye (Cisar et al., 1979; Kelstrup and Funder-Neilsel, 1974). It is found to be strongest when equal numbers of partners are present and a genetic stability for co-aggregation is mediated by surface components that recognize a carbohydrate on the cell of the partner (Bougeu and Mc Bride, 1976; Kolenbrander and Phucus, 1984; Mc Intre et al., 1975). The present study was aimed to develop co-aggregated cells as inoculum with the following objectives: (1) To study the co-aggregation of *A. caulinodans* with other agriculturally important microorganisms (AIM) using different plant seed powders (2) Effect of co-aggregation cells of *A. caulinodans* with other AIM on seed vigour index, plant height, plant dry weight, nitrogen content and endophytic colonization in rice roots.

Materials and methods

Azorhizobium caulinodans ORS-571, *Azospirillum brasilense* MTCC-125, *Azotobacter chroococcum* MTCC-446, *Bacillus megatherium* MTCC-3353, and *Pseudomonas fluorescens* MTCC-4828 were obtained from IMTECH, Chandigarh, India and maintained in respective agar slants at 35°C with monthly transfer to fresh agar slants.

Preparation of inoculum for co-aggregation

The bacteria were grown in a minimal medium, and they were maintained in an orbital shaker (100 × g) at 30 ± 2°C for 24 hr. Then the broth was centrifuged at 5000 × g for 10 min to harvest the log phase cells and the pellets were washed three times with 0.1 M phosphate buffer (pH 6.8). Finally the cells were resuspended in the same buffer to a cell concentration of 1×10^7 cfu/ml by measuring the absorbance at 420 nm, which was used as an inoculum source for the co-aggregation assay.

Co-aggregation assay

The effect on plant seed powders on augmentation of co-aggregation was carried out with seed powders of *M. olifera*, *S. emaginatus* and *S. potatorum* at one per cent concentration. The seeds of *Moringa oleifera, Strychnos potatorum* and *Sappindus emaignatus* were collected crushed and sieved (0.8 mm mesh) separately. The sieved powder of each plant species was mixed with sterile water to form a paste and then diluted to required strength and added to the Co-AG buffer. Control was also maintained to check natural co-aggregation in Co-AG buffer without seed powder.

The 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, were adjusted finally to a pH of 7.8 with the dilute HCl or NaOH. The buffer is then stored at 10°C. After the incubation period the aggregates settled at the bottom of the tube, while some of the free cells remained in the suspension. The supernatant was sampled and its turbidity measured in spectronic-20 colorimeter at 420nm. The co-aggregation percentage was determined according to the procedure of Madi and Henis (1989) in which the aggregates were mechanically dispersed by treatments in a tissue homogenizer for 1 min, the total OD was measured and percent aggregation was calculated as follows:

$$Percentage\ Aggregation = \frac{OD_t - OD_s \times 100}{ODt}$$

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

Effect of co-aggregated cells on the plant growth characters and endophytic occurrence of *A. caulinodans* in rice roots

The effect of co-aggregated cells of *A. caulinodans* and other AIM on the growth of rice variety ADT 43 and seedling vigour index was carried out according to Abdul Baki and Anderson (1973). The seeds of rice variety ADT

Table 1. Effect of plant seed powders on augmentation of co-aggregation between *A. caulinodans* ORS 571 and other Agriculturally Important Microorganisms (AIM)

Bacterial cultures	Co-aggregation percentage in five minutes with seed powders of			
	M. olifera	*S. emaginatus*	*S. potatorum*	Control
A. caulinodans + *A. brasilense*	96.80±1.4[a]	88.42±1.7[a]	78.75±1.6[a]	56.40±1.4[a]
A. caulinodans + *A. chroococcum*	85.60±1.9[b]	78.15±1.7[b]	70.40±1.4[b]	44.25±1.75[b]
A. caulinodans + *B. megatherium*	65.10±1.3[d]	58.70±1.2[d]	52.35±1.2[d]	32.75±1.25[d]
A. caulinodans + *P. fluorescens*	80.75±1.8[c]	76.80±1.5[c]	65.20±1.4[c]	39.42±1.62[c]

The experiments were performed five times, and similar results were obtained each time. The values are a mean of five replications ± SD for five replicates per treatment. 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

43 were surface sterilized with 0.1 per cent mercuric chloride, soaked in sterile water for overnight and germinated in sterile Petri plates containing sterile filter paper moistened with sterile water for 48 hrs period. The germinated seeds were then transferred to Semisolid Weaver's medium (Weaver et al., 1975) in a test tube at the rate of one seed per tube. The co-aggregated cells (Inoculation load of 1 x 10^7 cfu/ml) were inoculated close to the seeds on the surface of the Weaver's medium (Weaver et al., 1975). The inoculated tubes were maintained for about 30 days at 30±2°C in a Plant Growth Chamber with 10 h/ 14 h light and dark cycle. Five replications were maintained for each treatment.

The plant dry weight, plant N content and seedling vigour index were determined as per the methods given below and endophytic colonization of *A. caulinodans* was studied following the method of Yanni et al. (1997).

Plant height

The height of the plants from each treatment was measured on 15th day after sowing (DAS). The mean value of the plants from five replications was recorded.

Plant dry wt

The dry wt. of the entire plant was recorded on 15th day after sowing (DAS). Five plant samples from each treatment were drawn, washed, dried and finally dried to a constant weight in an oven at 50°C. The oven dry wt. of the plant sample was noted.

'N' content of the plant

The plant samples were collected on the 15th day after sowing (DAS), washed in water, air dried and later dried to a constant wt. in an oven at 50°C. Then they were powdered, sieved and 100 mg of sample was taken for analysis. The total nitrogen content was estimated by Microkjeldahl method (Bremner, 1960).

Statistical analysis

The experimental results were statistically analyzed in Duncan's multiple range test (DMRT) as per the procedure described by Gomez and Gomez (1984).

Results and discussion

In order to augment the artificial induction of co-aggregation, the seed powders of *M.olifera, S.emaginatus* and *S.potatorum* were tried (Table 1). Seed powders of all the three plant species tested were able to augment the co-aggregation process although the seed powder of *M.olifera* augmented co-aggregation percentage better than other seed powders. Heller et al. (2000) emphasized the role of *M.olifera* seeds as water clarifier and explained that the water-soluble proteins released from the crushed seeds kernels functioned as natural flocculating agents. Okada et al. (2000) reported that the plant seed powder of *Moringa oleifera* have coagulation properties, which has been positively utilized in the treatment of water and wastewater. In addition, he reported the role of *Moringa oleifera* as a potential coagulant especially for waters with very high turbidity. However, reports regarding the use of plant products for the development of coaggregates that could be used as potential source of agricultural inoculum are scarce. On the other hand, coaggregation has been reported earlier among certain bacterial species. Bougeu and Mc Bride (1976), Kolenbrander and Phucus (1984) and Mc Intre et al. (1975) reported that *Actinomyces viscosus* T14V and *Streptococcus sanguis* 34 co-aggregated by a mechanism not inhibited by 1 M NaCl and dextran independent, requires calcium, pH dependent with an optimum pH of 8.0 to 8.5. The interactions require the interaction of a protein or glycoprotein on *A. viscosus* with a carbohydrate on *S. sanguis*. The inoculation effect of *A. caulinodans* co-aggregated cells with other AIM on plant height, plant dry wt, plant N content, vigour index and endophytic occurrence of *A. caulinodans* in rice roots were also studied (Table 2), with better response of rice to inoculation of *A. caulinodans* + *A. brasilense* co-aggregated cells. The observations indirectly envisaged the synergistic effect of *Azospirillum* in augmenting the endophytic colonization in rice roots by *A. caulinodans* (Day and Dobereiner, 1976). Endophytic diazotrophs such as *Azorhizobium caulinodans, Gluconacetobacter diazotrophicus, Azoarcus spp, Herbaspirillum spp.* and some strains of *Azospirillum* are

Table 2. Inoculation effect of co-aggregated cells of *A. caulinodans* ORS 571 with other AIM on plant height, seedling vigour index plant dry weight, N content and endophytic colonization of *A. caulinodans* in rice roots under *in vitro* conditions

Treatments	Plant height (cm)	Plant dry wt. (mg/plant)	Plant N content (mg/g plant dry wt.)	Endophytic colonization of *A. caulinodans* $\log_{10}$ CFU/g fresh wt.	Seedling vigour index
Control	9.8±0.4^{e}	1.8±0.02^{e}	0.13±0.01^{c}	-	6050±50^{f}
A. caulinodans alone	14.4±0.7^{b}	4.2±0.84^{b}	0.32±0.04^{b}	4.92±0.84^{c}	7560±65^{e}
A. brasilense alone	14.8±0.7^{b}	4.4±0.40^{b}	0.34±0.04^{b}	ND	8100±50^{e}
A. chroococcum alone	13.0±0.4^{b}	3.8±0.50c	0.26±0.02^{c}	ND	7800±70^{e}
B. megatherium alone	10.2±0.6^{e}	3.1±0.53^{c}	0.18±0.02^{c}	ND	6650±65^{f}
P. fluorescens alone	11.4±0.4^{d}	3.6±0.70^{c}	0.20±0.02^{c}	ND	7150±85^{e}
A. caulinodans + *A. brasilense*	16.2±0.4^{a}	5.2±0.90^{a}	0.40±0.05^{a}	6.34±0.84^{a}	18410±50^{a}
A. caulinodans + *A. chroococcum*	13.8±0.8^{b}	4.8±0.54^{b}	0.34±1.04^{b}	5.18±0.72^{b}	13200±95^{b}
A. caulinodans + *B. megatherium*	11.2±0.6^{d}	4.0±0.40^{b}	0.21±0.03^{c}	4.32±0.94^{c}	9800±70^{d}
A. caulinodans + *P. fluorescens*	12.0±0.6^{c}	4.5±0.50^{b}	0.24±0.04^{c}	4.76±1.02^{c}	12500±120^{c}

*Initial inoculation load 1x 10^{9} cfu/ml. ND - not determined.The experiments were performed five times, and similar results were obtained each time. The values are a mean of five replications ± SD for five replicates per treatment. 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

able to colonize the root cortex (Cocking et al., 1994). This intracellular colonization to the adjacent cells of the epidermis is probably due to their ability to secrete cellulases and *pectinases* (Cocking, 2003).

The seed bacterization and its augmentation effect of AIM on rice were reported by many workers (Bashan et al.,1984; Okon, 1985; Webster et al., 1997).

Hence, the present study has opened the possibilities of using co-aggregates as inoculants for obtaining multiple benefits of the different agents for field crops. It has also been found that plant seed could augument co-aggregation to a larger extent and it is also one among the cheaply available raw material. The findings of the present study open up the possibilities for further investigation of genetic basis of effective co-aggregation and also the nature of cellular adhesion.

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