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Co-aggregation in *Azospirillum brasilensense* MTCC-125 with other PGPR strains: Effect of physical and chemical factors and stress endurance ability

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ABSTRACT

This work aims at exploring the co-aggregation of *Azospirillum brasilense* MTCC-125 with other plant growth promoting rhizobacterial (PGPR) strains. The PGPR strains include *Azotobacter chroococcum* MTCC-446, *Azorhizobium caulinodans* ORS-571, *Bacillus megatherium* MTCC-3353 and *Pseudomonas fluorescens* MTCC-4828. The influence of different physical and chemical factors involved in this phenomenon was investigated. Co-aggregation was found to be dependent on various factors: pH, temperature, inoculation level and growth phase. Treatment with urea, protease and chelating agents such as EDTA (ethylene diamine, tetra acetic acid) and EGTA (ethyl glycol-bis(β-amino ethyl ether) N-N′-tetra acetic acid) was found to inhibit co-aggregation. Sonication was found to increase co-aggregation, while heat treatment has a detrimental effect. The ability of *Azospirillum* co-aggregates to endure desiccation, heat, osmotic tolerance and osmotic shock and for its survivability in different carrier materials were also evaluated. Among the different combinations, the co-inoculation of *Azospirillum* MTCC-125 and *Azotobacter* MTCC-446 exhibited a higher degree of tolerance as compared to the other combinations tested.

1. Introduction

Plant growth promoting rhizobacteria (PGPR) may promote growth directly either by fixation of atmospheric nitrogen or by solubilization of minerals such as phosphorus (Karthikeyan *et al.*, 2007, 2008a,b). This can promote growth indirectly by the production of plant growth regulators (Jaleel *et al.*, 2007; Klopper and Schroth, 1978). This PGPR activity has been reported for strains belonging to different genera such as *Azoarcus*, *Azospirillum*, *Azotobacter*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Enterobacter*, *Gluconoacetobacter*, *Pseudomonas*, and *Serratia* (Jaleel *et al.*, 2009; Somers *et al.*, 2004).

Among these different PGPR strains: *Azospirillum*, *Azotobacter*, *Azorhizobium*, *Bacillus* and *Psuedomonas* are widely used as bioinoculants in the field of agriculture. *Azospirillum brasilense* a free living, PGPR can fix nitrogen under microaerophilic conditions, and are frequently associated with the root and in the rhizosphere of a large number of agriculturally important crops and cereals (Bashan and Levanony, 1990; Bashan and Holguin, 1997). Inoculation with *Azospirillum* improves root growth, increases root hair biomass production, which in turn increases the adsorption of water and minerals that eventually exerts beneficial effects on plants (Dobbelaree *et al.*, 2001; Russo *et al.*, 2008).

Azotobacter chroococcum, a cyst forming and free-living PGPR is found to promote plant growth due to its ability to fix dinitrogen (Tchan and Family, 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 (Kennedy, 1998; Saikia and Brezbaruah, 1995). The rhizobium of *Sesbania rostrata* has been classified as *Azorhizobium caulinodans* (Dreyfus and Dommergues, 1981). *Azorhizobium* is particularly interesting since it has a unique capacity among rhizobia to fix N_2 in the free-living state and in plants (Dreyfus *et al.*, 1988).

The most efficient phosphate solubilizing microorganism (PSM) includes 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

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Pseudomonas fluorescens isolated from chick pea, maize, soybean and other crops (Bardiya and Gaur, 1974). Besides its phosphate solubilizing ability *P. fluorescens* is also known for its biocontrol efficiency (Leeman *et al.*, 1995; Meyer and Hofte, 1997; Sivakumar and Joe, 2007). Though PGPR activity has been successfully demonstrated and reported in green house experiments, its commercial application has not been a great success. The main obstacle in achieving field success is the large inconsistency of field results (Okon and Laberandera-Gonzalez, 1994).

This inconsistency has been effectively overcome by the emergence of a new research sub-field namely co-inoculation of *Azospirillum* with other microorganisms (Bashan and Holguin, 1997). Further, Russo *et al.* (2005) reported an indirect effect of *Azospirillum* on mycorrhization as a consequence of the positive effect on root growth. Based on the field and greenhouse experimental results they concluded that the *Azospirillum*–AM fungus co-inoculation as a suitable methodology for sustainable agriculture practices. Co-inoculation is based on mixed inoculants, combinations of microorganisms that interact synergistically. The bacteria are found to interact synergistically, by providing nutrients, removing some inhibitory products, or stimulating each other through physical or biochemical mechanisms (Bashan *et al.*, 2004; Khammas and Kaiser, 1992).

VanVeen *et al.* (1997) also 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. Adding impetus to this approach Neyra *et al.* (1995, 1997) proposed the concept of "multigeneric microbial co-aggregates" for the production of multifunction agricultural inoculants with multiple benefits.

Macroscopically the phenomenon of co-aggregation can be defined as clumping when the different cell types are mixed (Cisar *et al.*, 1979; Gibbons and Nygaard, 1970). This inter-bacterial aggregation was readily observed with naked eye (Kolenbrander, 1989). The recognition may be intrageneric, intergeneric or multigeneric in nature (Kolenbrander *et al.*, 1993). In the recent past the authors of this work, have developed and reported co-aggregated cells as bioinoculants for rice crop using *A. caulinodans*-ORS-571 (Sivakumar and Joe, 2008).

Azospirillum cells aggregate and flocculate under diverse stress conditions and in the presence of various carbon and nitrogen sources, this phenomenon being generally accompanied by an accumulation of poly-β-hydroxy butyrate (PHB) granules (Burdman *et al.*, 1998; Sadasivan and Neyra, 1985). Co-aggregation in *Azospirillum* with other agriculturally important microorganisms has not been extensively studied. In the present work the authors extensively bring out the effectiveness of co-aggregation of *Azospirillum* MTCC-125 with other PGPR strains.

In this work, we have studied the effects of various physical and chemical factors influencing the co-aggregation and also the stability of *Azospirillum* co-aggregates. The ability of *Azospirillum* co-aggregates to endure different stress conditions and its survivability in different inoculant carrier materials were also investigated.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A. brasilense MTCC-125, *A. chroococcum* MTCC-446, *A. caulinodans* ORS-571, *B. megatherium* MTCC-3353, and *P. fluorescens* MTCC-4828 were obtained from IMTECH, Chandigarh, India. The bacterial strains were maintained at −20 °C in NA broth containing 20% (v/v) glycerol and, before being used, they were grown overnight at 30 °C and 120 rpm in Nutrient broth medium (Himedia) or on Nutrient agar medium (Himedia) at 30 °C for 24 h.

The bacterial inoculum was made as follows: all the PGPR strains namely, *Azotobacter* MTCC-446, *Azorhizobium* ORS-571, *Pseudomonas* MTCC-4828 and *Bacillus* MTCC-3353 were inoculated separately on M 9 salts minimal media as described by Sambrook *et al.* (1989) in a shaking bath at 30 ± 2 °C for 5 days. However 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 × *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 1×10^9 CFU/mL by measuring the absorbency at 650 nm and used as inoculums (OD value of 0.6).

2.2. Co-aggregation assay

One mL aliquot of *Azospirillum* MTCC-125 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 20 mM Tris–HCl buffer (pH 7.8), 0.01 mM $CaCl_2$, 0.01 mM $MgCl_2$, 0.15 M NaCl, 0.02% NaN_3. Uninoculated buffer served as control. The mixtures were vortexed for 10 s, 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.

2.3. Estimation of co-aggregation

The degree of co-aggregation was monitored by a visual assay (Cisar *et al.*, 1979). The degree of resultant co-aggregation was assigned a score ranging from 0 to 3+ by the following criteria—0: no visible aggregates in the cell suspension; 1+: small uniform co-aggregates in suspension; 2+: definite co-aggregates seen but the suspension remained turbid; 3+: large co-aggregates that settled rapidly, leaving a clear supernatant. Tubes containing each cell suspension alone (0.1 mL) plus 0.1 mL of buffer were always included as controls.

However the exact percentage of co-aggregation was estimated by a spectrophometric assay. After degree of co-aggregation was scored visually, the reaction mixture was diluted with 0.5 mL of buffer, mixed gently with a Vortex mixer, allowed to stand for 30 min at room temperature and centrifuged at 7000 × *g* for 2 min. The supernatants were analyzed spectrophotometrically at 650 nm to estimate the co-aggregation percentage. The percentage of co-aggregation was calculated using following mathematical formulae as described by McIntire *et al.* (1978)

$$\text{Percent co-aggregation} = \frac{A650(A) + A650(S) - A650(A+S)}{A650(A) + A650(S)} \times 100$$

where *A* is the control containing *Azospirillum* cells alone, *S* is the control containing the other PGPR cells alone, and *A* + *S* is the reaction mixture containing both the cells. The co-aggregation assay was usually performed in triplicate to confirm the reproducibility of the data.

2.4. Compatibility of Azospirillum MTCC-125 with other PGPR strains

The compatibility of *Azospirillum* MTCC-125 with other PGPR strains was studied by co-inoculating *Azospirillum* strain containing specific antibiotic markers (ampicillin at a concentration of 100 mg/L was added) with either one of the other PGPR strain such as *Azorhizobium* ORS-571, *Azotobacter* MTCC-446, *Pseudomonas* MTCC-4828 and *Bacillus* MTCC-3353 in M9 salts minimal medium and incubated at 28 ± 2 °C for 72 h. After 72 h of incubation, 1 mL of the culture was serially diluted and the total bacterial population of the co-inoculated strains was determined by plating in nutrient agar