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Original Research Paper

Co-aggregation of *Pseudomonas fluorescens* and *Bacillus subtilis* in culture and co-colonization in black gram (*Vigna mungo* L.) roots

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ABSTRACT

Many plant growth promoting rhizobacteria (PGPR), biocontrol agents and biofertilizer bacteria are used in agriculture. They are applied to crops by seed or soil application which colonizes in the rhizosphere. Although there are extensive reports on the output (growth, yield, disease resistance, phenotypes, etc) of the combined inoculations of these bacteria the companionship among these bacteria and the relative contribution to overall output is largely unknown. The starting point to unravel this would be to study the bacterial companionship in terms of ability to co-aggregate, stability of co-aggregation and developing molecular evidences for co-colonization in crop roots. Our work mainly aimed at understanding the extent of co-aggregation between *Pseudomonas fluorescens* and *Bacillus subtilis* and their co-colonization abilities in plant roots. This was verified by conducting several experiments of co-aggregation and effect of various factors like pH and growth phase, effect of various treatments like urea, protease K, EDTA, heat, thermal stability, sonication and desiccation on the extent of the co-aggregation. Co-aggregation between these two bacteria was found to be pH and growth phase dependent. Protease and EDTA treatment reduced the co-aggregation whereas sonication treatment improved the same. Talc based formulation of the bacteria was prepared separately and used for seed treatment in black gram. The seeds were germinated under laboratory conditions and growth parameters were observed. DNA from roots of germinated seedlings was used as template to amplify bacterial DNA with species specific primers. PCR analysis results indicated that *P. fluorescens* and *B. subtilis* are compatible in colonizing black gram.

1. Introduction

Many bacterial species including *Azospirillum*, *Azotobacter*, *Azorhizobium*, *Pseudomonas fluorescens*, *Bacillus subtilis* are used in agriculture for different purposes and applied as formulation either in the form of seed treatment or soil application. Though plant growth promotion and biocontrol were observed in laboratory, green house and field experiments, application in the farmers field suffers from inconsistency (Okon and Laberandera-Gonzalez, 1994). To overcome this problem, application of more than one bacteria as mixed inoculants was suggested (Bashan and Holguin, 1997). VanVeen et al. (1997) suggested mixing many microbes in the form of consortia towards varied benefits to the plants. Multigeneric microbial coaggregates were proposed by Neyra et al. (1997) for application as microbial inoculants in agriculture.

Still growing discovery of beneficial effects of *Pseudomonas fluorescens* include i) plant growth promotion ii) antifungal compounds and enzymes iii) induction of systemic resistance against pests and pathogens iv) imparting tolerance to abiotic stresses and v) interaction with other PGPR species. *Bacillus* species exist naturally in the plant surface (Arias et al., 1999) and rhizosphere (Mohammadipour et al., 2009). The metabolites and proteins produced by *Bacillus* are known to play important role in plant growth as well as control of pest and diseases (Priest, 1993).

The first step towards developing compatible consortia of beneficial bacteria for the purpose of application to crop plants would be to study their compatibility in culture and laboratory plant inoculation experiments. Cultural compatibility is often measured by the ability of bacteria to co-aggregate with the partner bacteria to which they are mixed with. Co-aggregation is a phenomenon of clumping of cells of different bacteria (Gibbons and Nygaard, 1970; Cisar et al., 1979), and when it is an inter-bacterial co-aggregation, it can even be viewed with naked eye (Kolenbrander, 1989).

In our current work, we studied the effect of different chemical and physical parameters affecting the co-aggregation of *P. fluorescens* and *B. subtilis* and its stability. Very less research has been carried out on the cellular and molecular level interaction between

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these two bacteria either in the culture, in the soil or root vicinity. It is very important to determine the physical interaction at cultural co-aggregation and plant root co-colonization level, to understand the beneficial effects of mixed formulations and the relative contribution of each of the member bacteria in the consortia. This will ultimately lead to selection of efficient and compatible species and also to design a better formulation to improve the performance under field conditions.

2. Materials and methods

2.1. Bacterial cultures

Pseudomonas fluorescens (strain PF1) and *B. subtilis* (strain TNAU G-1) cultures used in the study were obtained from Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India. GenBank accession numbers for 16S rRNA gene sequence are: AY818674 (*P. fluorescens*) and KP419701.1 (*B. subtilis*). These two cultures were characterized and commercially produced in Tamil Nadu Agricultural University and widely used by farmers of India.

2.2. Co-aggregation assay

One ml aliquots *of B. subtilis* and *P. fluorescens* overnight cultures (OD $A_{600}=1.0$) were mixed together in 10 ml co-aggregate buffer (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) and vortexed for 10 s. The mixture was incubated in a rotary shaker for three min and left undisturbed for 24 h (Grimaudo and Nesbitt, 1997).

2.3. Estimation of co- aggregation

The degree of co-aggregation was calculated using the scores 0: no visible aggregates, 1+: small uniform co-aggregates, 2+: definite co-aggregate but with turbidity suspension, 3+: large co-aggregates that separates rapidly with clear supernatant. The mixture was diluted with 0.5 ml of buffer and was gently vortexed for few s. The diluted mixture was allowed to stand for 30 min at room temperature and centrifuged at $7000\times g$ for two min. The supernatant was collected and absorbance was read at 650 nm (Cisar et al., 1979). The percentage of co-aggregation was calculated using the formula by McIntire et al. (1978) as follows:

$$\text{Percentage co}-\text{aggregation}=\frac{A650(B)+A650(P)-A650(B+P)}{A650(B)+A650(P)}\times 100$$

where B is the control containing *B. subtilis*, P is the control containing *P. fluorescens* cells and $B+P$ is the mixture containing both bacteria. The assay was carried out in triplicates to check the reproducibility of observed results.

2.4. Quantification of flocs

The flocs were filtered through Whatman no. 1 filter paper. The filter paper along with flocs was dried in the oven at 60 °C for two hours. Based on weight of empty filter paper, floc weight was determined and expressed in mg/l (Sadasivan and Neyra, 1985).

2.5. Effect of pH on the co-aggregation

The strains were grown in M9 salt minimal media (Sambrook et al., 1989) maintained at pH ranging 4–8 in a rotatory shaker at 30 °C. After incubation of 120 h, the cultures were centrifuged and co-aggregation was estimated as described.

2.6. Effect of different growth phase

The strains were grown in M 9 minimal media for five days and the bacterial cells were separated at different phases of growth (lag, log and stationary phase). Co-aggregation was estimated after transferring the cells to co-aggregation buffer.

2.7. Effect of protease and urea

The co-aggregates used were treated with proteinase K at 1 mg ml^{-1} for three h at 37 °C. The co-aggregation percentage was estimated. Co-aggregates in the phosphate buffer were supplemented with 2 M urea. Aggregates were sonicated for a minute and incubated at 50 °C for three hours. After transferring to co-aggregation buffer, percentage of co-aggregation was estimated.

2.8. Effect of sonication and heat

The co-aggregates were centrifuged twice at $5000\times g$ for 10 min, and resuspended in 10 mM of phosphate buffer. After five min of sonication, the cells were centrifuged at $5000\times g$ for five min. Pellets were collected and suspended in the co-aggregate buffer.The percentage of co-aggregation was estimated. Sonication was done for one minute and the suspension was incubated at 50 °C for three hours. The cells were transferred to co-aggregate buffer and the stability was determined as described earlier.

2.9. Effect of chelating agent

EDTA was added at a concentration of 1 mM (final concentration) to phosphate buffer containing co-aggregates. The cells were then sonicated for one min and harvested by centrifugation at $5000\times g$ for 15 min. The percentage of co-aggregation was estimated.

2.10. Observation on thermal tolerance

The co-aggregates were mixed with five ml of phosphate buffer (10 mM) in test tubes and maintained in water bath at 50 °C for 20 min followed by rapid cooling. One ml of sample was serially diluted and 10^{-5} and 10^{-6} diltuion cultures were used for nutrient agar plating. After 24 h of incubation, the growth of colonies was observed.

2.11. Effect of desiccation on co-aggregates

The co-aggregates were transferred to 1.5 ml tubes and kept open in sterile Petri plate in 37 °C incubator for one week. The dried cells were washed with 1 ml of sterile distilled water and plated in nutrient agar to check the viability of cells.

2.12. Preparation of talc based formulation

B. subtilis and *P. fluorescens* were grown in nutrient broth in a rotary shaker at 200 rpm until the cell population reached 10^{10}–10^{11} CFU ml^{-1}. Talc based carrier material was prepared as described by Vidhyasekaran et al. (1997) with 20 g kg^{-1} carboxy methyl cellulose and 15 g kg^{-1} calcium carbonate. About 200 ml of the bacterial culture was used per kg of the carrier material in the case of single culture. For the mixed culture, 100 ml each of the bacterial cultures were mixed. The talc based formulation thus prepared was air dried in culture room and packed aseptically in sterile polythene bags. The formulation for uninoculated control contained talc, carboxy methyl cellulose and calcium carbonate without any bacteria.