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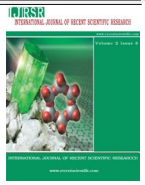

CO-AGGREGATES OF PGPR CELLS: POSITIVE EFFECT OF CERTAIN PHYSICAL AND CHEMICAL FACTORS ON THE ENHANCEMENT OF CO- AGGREGATION AMONG *PSEUDOMONAS FLUORESCENS* AND *PAENIBACILLUS POLYMYXA*

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ABSTRACT

The positive effect of certain physical and chemical factors *viz.*, inoculum level and growth phase, cultural condition, temperature, pH, presence of divalent cation and chelating agent, on the enhancement of coaggregation among the PGPR isolates *viz., Pseudomonas fluorescens* (PFR-3) and *Paenibacillus polymyxa* (PBR-3) was studied under *in vitro* condition. It was observed that 10^7: 10^7 inoculum level of PGPR partners was found to be optimum for achieving the maximum coaggregation percentage while any increase or decrease to this inoculum level could reduce the coaggregation percentage of PGPR partners. The use of PGPR partners at stationary growth phase augmented the " Intergeneric coaggregation" to a higher level when compared to lag and log growth phase. The cells of PGPR partners, harvested from N-deficient media, yielded more coaggregation when compared to the cells harvested from N- rich media. Further, the PGPR cells cultured at 35^{o}c at a pH level of 7.5 recorded higher coaggregation percentage than other levels. Moreover, the addition of Ca^{2+}, as divalent cation, recorded higher coaggregation percentage of PGPR cells whereas the addition of EDTA, as chelating agent, derastically reduced the stability of PGPR coaggregates. It was concluded that the use of PGPR cells cultured in N-deficient media, at a growth temperature of 35^{o}c and at a pH level of 7.5 during the stationary growth phase yielded more coaggregation percentage among PGPR partners. Moreover, the use of 10^7:10^7 inoculum level of PGPR partners and addition of Ca^{2+}, as divalent cation, was found to maximise the coaggregation percentage among PGPR partners.

Key words: Co-aggregation, PGPR partners, inoculum level and growth phase, temperature, divalent cation.

1. INTRODUCTION

Beneficial, freeliving bacterial isolates have been shown to improve the plant health or increase yield and are usually referred as "plant growth promotiong rhizobacteria" (PGPR) or by one group of workers in china as "yield increasing bacteria" (YIB) (Tang, 1994). PGPR may increase plant growth by different mechanisms *viz.*, nitrogen fixation (Hong *et al.*, 1999), regulating ethylene production in roots (Glick, 1995) releasing phytohormones (Frankenberger and Arshad, 1995), synthesizing siderophores (Budzikiewicz,1997) and indirectly either by the suppression of well known diseases caused by major pathogens or by reducing the deleterious effect of minor pathogens (Shishido and Chanway, 1999). PGPR include bacteria belonging to the genera, namely, *Pseudomonas fluorescens* and *Paenibacillus polymyxa*.

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The beneficial effects of *Pseudomonas fluorescens* and *Paenibacillus polymyxa*, as PGPR, have been reported by many authors (Suslow and Schroth, 1982; Glandorf *et al.*, 1994 Hofte *et al.*, 1991; Timmusk, 2003; Yao *et al.*, 2006; Raza *et al.*, 2008) and the same are used as agricultural bioinoculant, worldwide, for the enhancement of growth and yield in field crops, including, lowland rice. However, the use of the agricultural bioinoculant recorded a poor performance in natural environment and in the rhizosphere of host plants.vanVeen *et al*. (1997) critically reviewed the reasons for the same and suggested that instead of trying single microbial strain with single trait, as agricultural bioinoculant, trying to use microbial consortia for multiple benefits.

Higher degree of stress tolerance, longer shelf life, enhanced survivability in soils and on seeds and consistent plant response to inoculation are the important characteristics of any agricultural bioinoculant (Neyra *et al.*, 1997). Okon and Labaendra-Gonzalez (1994)

suggested the importance of the physiological status of the microorganisms in agricultural bioinoculant rather than their cell numbers to ensure more survival in carriers, survival in soil and on seed, colonisation in the rhizosphere and positive plant response to bioinoculation. Some of the most promising research topics on novel agricultural bioinoculant technology, include, the use of flocculated cell forms of microorganisms, as delivery system (Olubayi *et al.*, 1998). Cell aggregation/ flocculation is a widespread phenomenon in the microbial world and occurring under certain physiological conditions (Callega, 1984). Neyra *et al*.(1999) proposed the use of "Intergeneric microbial coflocs", as a novel delivery system and a new generation of agricultural bioinoculant. Co-flocculation involves the inoculation of a bacterial culture with additional microorganisms prior to flocculation such that the additional microbes are entrapped in the biopolymer network produced by the flocculant bacteria (Neyra *et al.*, 1997). Further, the microbial coflocs contained high cell titre, increased adhesiveness to plant roots, enriched in encysted cells with thick capsules surrounded by the EPS rich network which provided higher stress tolerance and longer shelf life to bioinocula.

In the present investigation, the role of various physical and chemical factors *viz.*, Inoculum level, growth phase, culture media, growth temperature, pH, divalent cations and chelating agents for the maximisation of co-aggregation among the PGPR cells *viz., Pseudomonas fluorescens* and *Paenibacillus polymyxa* was studied.

2. MATERIALS AND METHODS

2.1. Preparation of inoculum

The PGPR strains *viz.*, *Pseudomonas fluorescens* (PFR-3) and *Paenibacillus polymyxa* (PBR-3) were grown in King´s B and Nutrient glucose broth, respectively, maintained in a shaking bath at 30±2°C for 5 days to get stationary phase cultures. Then, each medium was centrifuged, separately, at 5000×g for 10 min to harvest the stationary phase cells and the pellets washed three times with 0.1 M phosphate buffer (pH 6.8). Finally, the PGPR cells were resuspended, separately, in the same buffer to get a cell concentration of 1×10^7 CFU ml^{-1} by measuring the absorbance at 420 nm and used as inoculum.

2.2. Preparation of co-aggregation buffer (Grimaudo and Nesbitt, 1997)

The co-aggregation buffer was prepared with the following composition: 20 mM Tris- HCI buffer; 0.01 m M $CaCl_2$; 0.15 M NaCl and 0.02% NaN_3.

2.3. Co-aggregation assay (Jabra-Rizk et al., 1999)

One ml aliquot of each bacterial culture *viz.*, *Pseudomonas fluorescens* (PBR-3) and *Paenibacillus polymyxa* (PBR-3) was mixed together in 10 ml Co-Ag buffer. The mixture was vortexed for 10s, shaken on a rotary platform shaker for 3 min. and left undisturbed for 24h. Uninoculated buffer served as control. All Co-Ag reaction were performed in triplicate.

2.4. Estimation of co-aggregation percentage (Madi and Henis, 1989)

After the incubation period, the aggregates settled at the bottom of the tube while some of the free cells remained in suspension. The supernatant was sampled and the turbidity measured in spectronic-20 colorimeter at 420nm. Then the flocs were mechanically dispersed by treating in a tissue homogenizer for 1 min and the total OD was measured and the percent of coaggregation was calculated as follows:

$$\%\,\text{Co - aggregation} = \frac{(OD_t - OD_s) \times 1OO}{OD_t}$$

where,

OD_t = total optical density after mechanical dispersion and

OD_s = OD of supernatant after aggregate had settled

2.5. Factors affecting the coaggregation of PGPR cells

2.6. Effect of cell age on co-aggregation of PGPR isolates

The PGPR isolates *viz*., *Pseudomonas fluorescens* (PFR-3) and *Paenibacillus polymyxa* (PBR- 3) were grown in King's B and Nutrient glucose broth, respectively, in a shaking broth at 30 ± 2°C.The lag, log and stationary phase cultures of the PGPR cells were harvested at 0, 24 and 72 hours, respectively, and the co-aggregation percentage was estimated.

2.7. Effect of temperature on co-aggregation of PGPR cells

The PGPR isolates, namely, PFR- 3 and PBR- 3 were grown separately for 5 days as per the conditions mentined above but the temperature was maintained at different levels, namely 25, 30, 35, 40 and 45°C for the growth of the isolates. After 72 h incubation, the co-aggregation percentage of the PGPR isolates, maintained at different levels of temperature, was estimated.

2.8. Effect of pH on co-aggregation of PGPR cells

The PGPR isolates, namely, PFR- 3 and PBR- 3 were grown for 72h as per the conditions mentined above. After 72h of incubation, the cells of each bacterial isolates were harvested and the co-aggregation percentage was estimated according to Madi and Henis (1989) in co-aggregation buffer (Grimaudo and Nesbitt, 1997) maintained at different pH levels, namely, 6.0, 6.5, 7.0 and 7.5.

2.9. Effect of culture media on co-aggregation of PGPR cells

The PGPR isolates, namely, PFR- 3 and PBR- 3 were grown in King's B and Nutrient glucose broth, respectively, broth under N free and N supplemented condition in a shaking bath at 35°C for 72h. After the incubation period, the cells of each PGPR cells were harvested separately and the co-aggregation percentage was estimated as stated earlier in co-aggregation buffer (Grimaudo and Nesbitt,1997) maintained at a pH level of 7.5.

2.10. Effect of cell number on co-aggregation of PGPR cells

The PGPR isolates, namely, PFR- 3 and PBR- 3 were grown for 72h in King's B broth and Nutrient glucose broth, respectively as per the condition mentined above. After the incubation period, the cells of each PGPR isolates were harvested separately and the co-aggregation percentage was estimated at different levels of cell number, namely, 10^4:10^4, 10^5:10^5 and 10^6: 10^6, 10^7: $10^7$and 10^8:10^8.

2.11. Effect of divalent cations on co-aggregation of PGPR cells

The PGPR isolates, namely, PFR-3 and PFR-3 were grown in Kings'B broth, and nutrient glucose broth respectively, at 35°C for 72h. Then, the cells were harvested and the co-aggregation percentage was estimated as stated elsewhere in the text in the coaggregation buffer (Grimaudo and Nesbitt, 1997) supplemented with different divalent cations *viz*., Ca^{2+}, Mg^{2+} and Ba^{2+}, with a view to test their efficacy on the induction of co-aggregation, at 0.1mM level.

2.12. Effect of chelating agents on co-aggregation of PGPR cells

The PGPR isolates, namely, PFR-3 and PBR-3 were grown in N-free Kings'B and Nutrient glucose broth, respectively and the coaggregation percentage was estimated in coaggregation buffer (Grimaudo and Nesbitt, 1997) maintained at pH 7.5 together with the addition of EDTA (Ethylene diamine tetra acetic acid) or EGTA (Ethylene glycol-bis-(β-amino ethyl ether) N-N'tetracetic acid) at 1mM level.

3. RESULT AND DISCUSSION

In the present study, the PGPR genera *viz*., *Pseudomonas* and *Paenibacillus*, recorded the highest intergeneric co-aggregation percentage at a inoculum level of 10^7:10^7 and any increasing or decreasing values to this level of inoculum caused a reduction in their coaggregation ability (Fig-1). The importance of inoculum level of coaggregating partners in intergeneric coaggregation has been reported by Gibbons and Nygaard (1970), Kolenbrander and Andersen, (1986) and Kolenbrander, (1988).

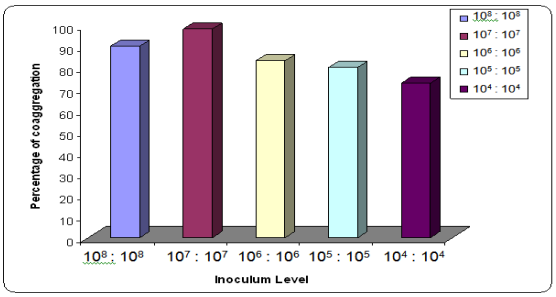

Fig 1 Effect of different inoculum levels on co-aggregation of *pseudomonas fluorescens* and *paenibacillus polymyxa*

Gibbons and Nygaard (1970) reported the importance of equal number of coaggregating partners to obtain a visibly stronger coaggregation. The higher inoculum levels leads to the formation of rosette type colonies that are visible only under microscope (Kolenbrander and Andersen, 1982). The results of the present study clearly revealed the importance of inoculum level of coaggregating PGPR partners to achieve the maximum coaggregation percentage. The effect of different growth phases of the coaggregating partner's *viz.*, *Pseudomonas* and *Paenibacillus* on the coaggregation percentage revealed that the growth phase of the coaggregating partners played a critical role on the coaggregation percentage of the same. Among the different growth phases tested, the stationary growth phase of coaggregating PGPR partners recorded the highest coaggregation percentage followed by lag and log growth phases (Fig-2).

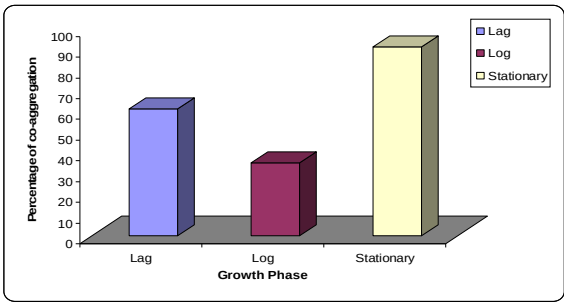

Fig 2 Effect of different growth phases on co aggregation of *Pseudomonas fluorescens* and *paenibacillus polymyxa*

The lowest coaggregation percentage recorded with the log growth phase of the coaggregating partners revealed the fact that active metabolic state of the microbial cell was not conducive for coaggregation. Kolenbrander *et al.* (1983) and Kolenbrander and Williams (1983) reported the effect of growth phases on the coaggregation of *Streptococcus* with other human oral bacterial isolates, collected from the same site. The effect of the culture age on the composition of the cell surface of bacteria has been reported by many workers (Burdman *et al., 1998*;

Burdman *et al.*, 2000). Nikitina *et al.* (2000) reported that the coaggregation of *Azospirillum brasilense* SP7 (S) and SP.7.2.3 changed with the culture age. The results of the present study clearly revealed the determining role of growth phase of PGPR cells on coaggregation percentage of the same. The effect of cultural conditions *viz.*, N-free and N- supplmentation conditions of *Pseudomonas* and *Paenibacillus* cells on the coaggregation percentage revealed that the cultural conditions of the coaggregating partners played a key role in determining the coaggregation percentage of PGPR cells. Between the two cultural conditions tested, namely, PGPR cells grown in N-free medium and PGPR cells grown in N-supplemented medium, the PGPR cells collected from N-free medium recorded more coaggregation percentage (Fig-3).

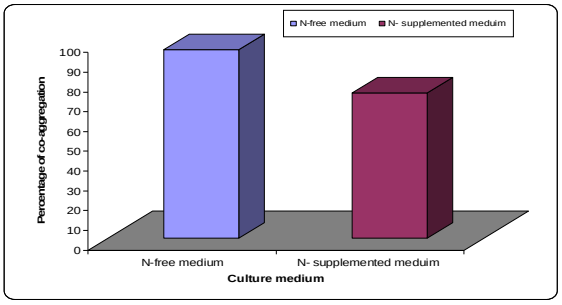

Fig 3 Effect of different culture media on coaggregation of *Pseudomonas fluorescens* and *paenibacillus polymyxa*

Sadasivan and Neyra (1985) reported the effect of high C:N ratio on the coaggregation of *Azospirillum brasilense* Cd. strains. Kolenbrander, (1988) summarized the effect of culture medium on the coaggregation of *Streptococci* and *Actinomyces* suspensions, collected from human oral ecosystem. Burdman *et al.* (1998) reported that when *Azospirillum brasilense* strains FAJ0204 grown under high C:N ratio medium accumulated high amount of poly-β-hydroxybutyrate with a change in cell surface properties, namely, a well-defined electron-dense layer outside the outer membrane. It has been previously reported that the cells of *Azospirillum brasilense g*rowing in culture medium at high C: N ratio tends to aggregate and it was also shown that the amount of arabinose present in the EPS correlated with the extent of cell aggregation of different *Azospirillum* strains (Burdman *et al*., 2000). The results of present study also revealed the highest coaggregation percentage of PGPR cells, *viz.*, *Pseudomonas* and *Paenibacillus* collected from N-deficient medium which affected the cell surface characteristics of PGPR cells and resulted in more coaggregation percentage of the same.

The effect of different growth temperature levels *viz.*, 25, 30, 35, 40 and 45 on the coaggregation percentage of PGPR cells *viz*., *Pseudomonas* (PFR-3) and *Paenibacillus* (PBR-3), revealed that the growth temperature level of PGPR cells played a critical role in determining the coaggregation percentage of the PGPR partners. The increasing level of growth temperature showed an increasing trend in coaggregation percentage upto 35°C and thereby a reduction in the same was observed (Fig-4). Kestrup and Funder-Nielsen (1974) reported the positive effect of growth temperature in determining the coaggregation of *Streptococci* with *Fusobacterium* and *Actinomyces*. Burdman *et al.* (1998) reported the positive effect of growth temperature on coaggregation of *Azospirillum brasilense* cd. cells. They reported that *Azospirillum* grown under high C:N ratio recorded a higher aggregation at higher growth temperature levels whereas the highest temperature beyond the optimum growth temperature level caused dispersion of the coaggregates.

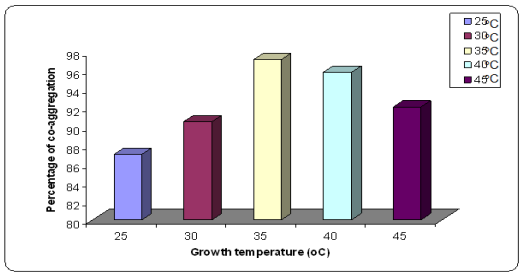

Fig 4 Effect of different growth temperature levels on co-aggregation[b] of *pseudomonas fluorescens* and *Paenibacillus polymyxa*

The effect of different levels of buffer pH on the coaggregation percentage of PGPR cells *viz.*, *Pseudomonas* (PFR-3) and *Paenibacillus* (PBR-3) revealed the positive role of buffer pH on the coaggregating PGPR partners to attain maximum co-aggregation percentage. Among the different buffer pH levels tested, the 7.5 level of buffer pH, recorded the highest coaggregation percentage of PGPR partners followed by 6.5, 6.0 and 7.0 buffer pH levels (Fig-5).

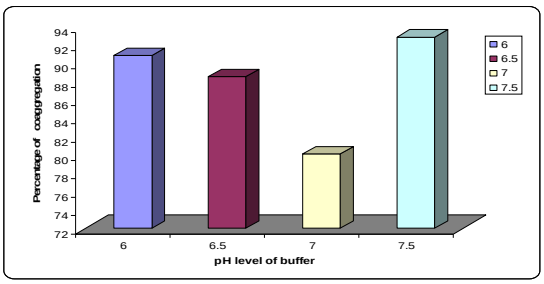

Fig 5 Effect of different levels of pH on co-aggregation of *Pseudomonas fluorescens* and *paenibacillus polymyxa*

reported the positive effect of pH on coaggregation of *Azosprillum* cells and added that there was dispersion of *Azospirillum* cells at neutral pH (pH 7.0) while any increase or decrease to this pH level augmented the coaggregation of *Azospirillum* cells. Burdman *et al.* (1998) reported the involvement of charged groups in this phenomenon and the *Azospirillum* strains Cd and FAJ 0204 responded differentially to the levels of pH. They also added that the negative ionized groups of bacterial cell surface could be neutralized by protonation and thus

reducing the strength of repulsive forces between the bacteria which lead to coaggregation. The results of the present study also revealed the differential response of PGPR cells to different pH levels for coaggregation.

The effect of addition of different divalent cations *viz.*, Ca^{2+}, Mg^{2+} and Ba^{2+} to Co-Ag buffer on the coaggregation percentage of PGPR cells *viz.*, *Pseudomonas* (PFR-3) and *Paenibacillus* (PBR-3) revealed the positive effect of divalent cations in augmenting coaggregation percentage of PGPR cells. Among the different divalent cations tested, the addition of Ca^{2+} was found to augment the phenomenon to a higher level followed by Mg^{2+} and Ba^{2+} (Fig-6).

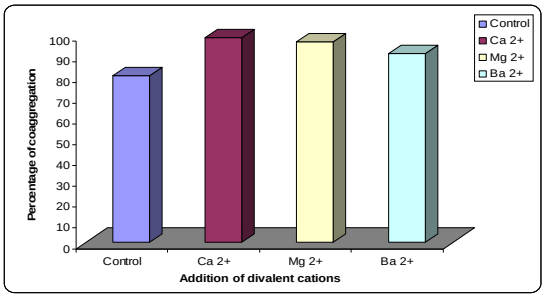

Fig 6 Effect of addition of divalent cations on coaggregation of *Pseudomonas fluorescens* and *Paenibacillus polymyxa*

Jana (1998) reported the positive role of Ca^{2+} ions in the augmentation of cell surface hydrophobicity in *Pseudomonas fluorescens*. Rose (1984) reported the positive role of divalent cations on the flocculating ability of *S. cerevisiae*. Mill (1964) reported that Ca^{2+} ions acting as bridges in the coaggregation of yeast cells. Miki *et al.* (1982) emphasized the importance of Ca^{2+} ions in yeast cell flocculation but in some cases magnesium and manganese ions may act as substitutes. The results of the present study clearly revealed the importance of Ca^{2+} ions in augmenting the coaggregation of PGPR cells.

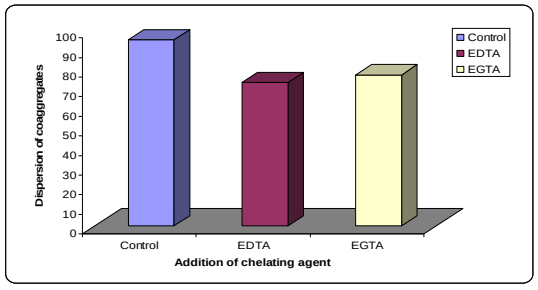

Fig 7 Effect of addition of chelating agents on coaggregation of *pseudomonas fluorescens* and *Paenibacillus polymyxa*

The effect of addition of chelating agents *viz.*, EDTA and EGTA to co-aggregation buffer on the coaggregation percentage of PGPR cells *viz.*, *Pseudomonas* (PFR-3) and *Paenibacillus* (PBR-3) revealed the positive role of chelating agents on the reduction of coaggregation percentage among PGPR cells. Between the two chelating agents tested, the addition of EDTA to the Co-Ag buffer reduced the coaggregation percentage of PGPR cells to a marked level followed by EGTA (Fig-7). Burdman *et al.* (1998) reported the effect of EDTA and EGTA on the dispersion of *Azospirillum* coaggregates. They suggested the involvement of outer membrane proteins of microbial cells in cell-to-cell adhesion. They also added that higher concentrations of these compounds drastically reduced the cell viability and caused partial lysis of bacteria. Madi and Henis (1989) showed the treatment with NaEDTA resulted in the dispersion of *Azospirillum* aggregates while the addition of dialyzed EDTA restored their aggregation capacity and suggested the role surface-located proteins involved in the coaggregation process. The results of the present study also revealed the positive influence of EDTA reducing the coaggregation of PGPR cells and emphasized the role of surface located protein in coaggregation.

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