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Influence of Abiotic Factors on Multigeneric Coaggregation Among Diazotrophic Bacteria

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The effect of different abiotic factors *viz*., growth temperature, pH, divalent cations and chelating agents, on the development of 'Multigeneric diazotrophic coaggregates", consisting of triple efficient diazotrophic isolates *viz*., *Azospirillum* (AZS-3), *Azotobacter* (AZT-3) and *Rhizobium* (RZB-3), was studied with a view to optimize the abiotic factors for the mass scale production of the novel formulation of bioinoculant. In the present study, it was observed that the diazotrophic cells of the triple genera grown at 35°C temperature exhibited more coaggregation followed by 40, 45, 30 and 25 degrees of temperature. pH at a level of 7.5 exhibited more coaggregation than other pH levels. Addition of divalent cation *viz*., Ca^{2+} at 0.1 mM concentration augmented more coaggregation followed by Mg^{2+} and Ba^{2+}. Interestingly, addition of chelating agent *viz*., EDTA drastically reduced the coaggregation percentage of diazotrophic cells to a higher level than EGTA. It was concluded that maximization of "Multigeneric diazotrophic coaggregates" could be possible at 35°C growth temperature of diazotrophic cells at a pH level of 7.5 in the presence of Ca^{2+} cation.

Key words: Multigeneric diazotrophic coaggregates, growth temperature, pH, cation, coaggregation percentage.

Nitrogen is one of the major nutrient essential for plant growth. Global agriculture, now, relies heavily on synthetic chemical nitrogenous fertilizers. The escalating prices and the environmental hazards due to the persistent and injudicious use of the same pose many problems and there is an increasing need for harnessing the atmospheric dinitrogen through the agency of microorganisms for sustainable crop production (Mudahar, 1987).

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Plant growth promoting rhizobacterial (PGPR) are rhizosphere bacteria that directly affect plant growth by producing and secreting plant growth regulators or by eliciting root metabolic activities by supplying biologically fixed nitrogen. The well known PGPR include bacterial genera, namely, *Azospirillum*, *Azotobacter* and *Rhizobium* on non-legumes.

Higher degree of stress tolerance, longer shelf life, enhanced survivability in soils and on seeds and consistent plant responses to inoculation are the important characteristics of agricultural bioinocula (Neyra *et al*., 1995). Okon and Labandera-Gonzalez (1994) suggested the importance of the physiological status of microorganisms in bioinoculant preparation rather than their cell numbers. Olubayi *et al.*

(1998) reported the use of flocculated cell forms of microorganisms with high poly-b-hydroxybutyrate content, as agricultural bioinoculant, to achieve the abovesaid attributes.

van Veen *et al*. (1997) critically reviewed the reasons for the poor performance of agricultural bioinocula in natural environments and suggested that instead of trying single strain with a single trait, as agricultural bioinoculant, trying to use microbial consortia for harnessing multiple benefits. Neyra *et al*. (1999) and Nikitina *et al*. (2001) studied the intergeneric coaggregation among *Azospirillum* with *Rhizobium* and *Azospirillum brasilense* Sp_7 with *Micrococcus citrus* 27/1 M, respectively and proposed the concept of 'Intergeneric microbial coaggregates' for the production of multipurpose agricultural bioinoculant with multiple benefits. In our laboratory, Rubiya (2006) successfully developed "Multigeneric diazotrophic coaggregates" consisting of triple genera *viz*., *Azotobacter*, *Azospirillum* and *Rhizobium* and reported the positive influence of the same on the enhancement of growth and yield parameters in lowland rice cv. BPT-5804. However, optimization of the abiotic factors that critically controls the mass scale production of 'Multigeneric diazotrophic coaggregates' has not been revealed, so far. Hence the present study has been undertaken with an aim to reveal the influence of abiotic factors *viz*., growth temperature, pH, divalent cations and chelating agents, on the development of 'Multigeneric diazotrophic coaggregates' consisting of triple diazotrophic genera.

MATERIAL AND METHODS

Diazotrophic strains used

Strains of *Azospirillum brasilense* (AZS-3) *Rhizobium* sp. (RZB-3) and *Azotobacter chroococcum* (AZT-3), isolated from the rhizosphere of rice cv.

BPT-5804, were maintained in nutrient agar slants at 35°C with monthly transfer and used throughout the study.

Studies on the Coaggregation mechanism of diazotrophic isolates

Preparation of inoculum

All the three diazotrophic isolates, namely, *Azospirillum*, *Azotobacter* and *Rhizobium* were grown in synthetic malate broth (Day and Dobereiner, 1976) supplemented with 0.05 per cent yeast extract (w/v), base 77 broth and yeast extract mannitol broth, respectively, in a shaking bath at 30±2°C for 5 days. Then, the medium was centrifuged at 5000xg 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 cells were resuspended in the same buffer to a cell concentration of $1x10^7$ CFU/mL by measuring the absorbance of 420 nm and used as inoculum.

Preparation of Co-AG buffer

The Co-AG buffer was prepared according to Grimaudo and Nesbitt (1997).

Estimation of coaggregation percentage

The coaggregation percentage of *Azospirillum*, *Azotobacter* and *Rhizobium* cell was made according to the procedure of Madi and Henis (1989).

Factors affecting the coaggregation of diazotrophs

Effect of temperature on coaggregation of diazotrophic isolates

All the three diazotrophic isolates were grown for 120 h and the temperature was maintained at different levels, namely, 25, 30, 35, 40 and 45°C for the growth of the isolates. After 120 h incubation, the coaggregation percentage was estimated according to Madi and Henis (1989) in coaggregation buffer (Grimaudo and Nesbitt, 1997).

Effect of pH on coaggregation of diazotrophic isolates

All the three diazotrophic isolates were grown for 120 h. After 120 h incubation, the cells of each diazotrophic isolats were harvested and the coaggregation percentage was estimated according to Madi and Henis (1989) in coaggregation buffer (Grimaudo and Nesbitt, 1997) maintained at different pH levels, namely, 6.0, 6.5, 7.0 and 7.5.

Effect of divalent cations on coaggregation of diazotrophic isolates

All the three diazotrophic isolates were grown in malate broth, base 77 broth and YEMB, respectively at 35°C for 120 h. Then, the cells were harvested and the coaggregation percentage was estimated in 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 coaggregation, at 0.1 mM level.

Effect of chelating agents on coaggregation of diazotrophic isolates

All the three diazotrophic isolates were grown and the coaggregation percentage was estimated in coaggregation buffer (Grimaudo and Nesbitt, 1997) maintained at pH 7.0 with the addition of EDTA (Ethylene diamine tetraacetic acid) at 1mM level.

Statistical analysis

The experimental results were statistically analysed in Duncan's Multiple Range Test (DMRT) as per the procedure described by Gomez and Gomez (1984).

RESULTS AND DISCUSSION

The effect of different levels of growth temperatures namely 25, 30, 35, 40 and 45°C on the coaggregation percentage of diazotrophs are presented in Table 1. It was observed that the increasing levels of growth temperature increased the coaggregation percentage of diazotrophs upto a level of 35°C and thereby a reduction in the growth was recorded. Burdman *et al*. (1998) reported the positive effect of growth temperature on coaggregation of *Azospirillum brasilense* cells. They reported that *Azospirillum* grown under high C:N ratio recorded higher aggregation at higher temperature level whereas the highest temperature beyond the optimum temperature level caused dispersion of the coaggregates.

Among the different buffer pH levels tested, the 7.5 level of buffer pH recorded the highest coaggregation percentage followed by 6.5, 6.0 and 7.0 buffer pH levels (Table 2). Sadasivan and Neyra (1985) and Madi and Henis (1989) reported the positive effect of pH on coaggregation of *Azospirillum* cells and added that there was dispersion of *Azospirillum* cell at neutral pH (pH 7.0) while any increase or decrease to this pH level augmented the coaggregation of *Azospirillum* cells. Addition of divalent cations to the Co-AG buffer augmented coaggregation percentage of diazotrophs, positively. Among the different divalent cations tested, Ca^{2+} was found to augment the phenomenon to a higher level followed by Mg^{2+} and Ba^{2+} (Table 3). Smit *et al*. (1992) reported that no flocculation occurred in the absence of Ca^{2+} in *Saccharomyces cerevisiae*.

Addition of chelating agents, namely, EDTA and EGTA to Co-AG buffer reduced the coaggregation percentage of diazotrophic cells significantly. Between the two chelating agents

Table 1. Effect of different levels of growth temperature on coaggregation[d] of diazotrophic cells (inoculums level of $10^7 : 10^7 : 10^7$ cells ml^{-1})

Diazotroph, culture medium and growth phase[a]	Growth temperature (°C)[b]	Percentage of coaggregation[c]
Azotobacter		
Base 77 broth		
Stationary	25	87.5 ± 0.9[d]
Azospirillum	30	90.8 ± 0.5[c]
'N' free malate Broth	35	98.2 ± 0.2[a]
Stationary	40	97.8 ± 0.4[a]
Rhizobium	45	96.1 ± 0.7[b]
Yeast extract mannitol broth		
Stationary		

[a] medium from which diazotrophic cells are harvested at stationary phase and utilized for coaggregation assay

[b] temperature at which the diazotrophic cells are cultured.

[c] assayed according to Madi and Henis (1985) after 24 h incubation time.

[d] values followed by different letters are significantly differed at 5% level according to Student 't' test.

tested, the addition of EDTA to the Co-AG buffer reduced the coaggregation percentage to a marked level followed by EGTA (Table 4). Burdman *et al*. (1998) reported the effect of EDTA and EGTA on the dispersion of *Azospirillum* coaggregation. They suggested the involvement of outer membrane proteins of microbial cells in cell-to-cell adhesion. They also added that higher concentration of these compounds drastically reduced the cell viability and caused partial lysis of bacteria.

It was concluded that abiotic factors such as growth, temperature, pH, cations and chelating agents played a key role in determining the degree

Table 2. Effect of different levels of pH on coaggregation[d] of diazotrophic cells

Diazotroph, culture medium and growth phase[a]	pH levels of buffer[b]	Percentage of coaggregation[c]
Azotobacter		
Base 77 broth		
Stationary		
Azospirillum	6.0	90.64 ± 1.10[c]
'N' free malate broth	6.5	90.74 ± 1.14[b]
Stationary	7.0	80.30 ± 1.52[a]
Rhizobium	7.5	92.84 ± 0.92[a]
Yeast extract mannitol broth		
Stationary		

[a] medium from which diazotrophic cells are harvested at stationary phase and utilized for coaggregation assay
[b] pH levels of buffer at which the diazotrophic cells are coaggregated.
[c] assayed according to Madi and Henis (1985) after 24 h incubation time.
[d] values followed by different letters are significantly differed at 5% level according to Student 't' test.

Table 3. Effect of addition of divalent cations on coaggregation[d] of diazotrophic cells

Diazotroph, culture medium and growth phase[a]	Addition of divalent cations[b]	Percentage of coaggregation[c]
Azotobacter		
Base 77 broth		
Stationary		
Azospirillum	Control	88.20 ± 0.70[d]
'N' free malate Broth	Ca^{2+}	98.90 ± 0.20[a]
Stationary	Mg^{2+}	96.40 ± 0.50[b]
Rhizobium	Ba^{2+}	92.84 ± 0.92[c]
Yeast extract mannitol broth		
Stationary		

[a] medium from which diazotrophic cells are harvested at stationary phase and utilized for coaggregation assay
[b] Addition of divalent cation at a concentration of 0.1 mM to the buffer.
[c] assayed according to Madi and Henis (1985) after 24 h incubation time.
[d] values followed by different letters are significantly differed at 5% level according to Student 't' test.

Table 4. Effect of addition of chelating agents on coaggregation[d] of diazotrophic cells

Diazotroph, culture medium and growth phase[a]	Addition of chelating agent[b]	Percentage of coaggregation[c]
Azotobacter		
Base 77 broth		
Stationary		
Azospirillum	Control	98.20 ± 0.40[a]
'N' free malate Broth	EDTA	74.60 ± 0.80[c]
Stationary	EGTA	78.40 ± 0.70[b]
Rhizobium		
Yeast extract mannitol broth		
Stationary		

EDTA – Ethylene diamine tetraacetic acid; EGTA – Ethylene glycol-bis (B amino ethyl ether)N, N'-tetra acetic acid

[a] medium from which diazotrophic cells are harvested at stationary phase and utilized for coaggregation assay

[b] Addition of chelating agents at a concentration of 0.1 mM to the buffer.

[c] assayed according to Madi and Henis (1985) after 24 h incubation time.

[d] values followed by different letters are significantly differed at 5% level according to Student 't' test.

of multigeneric microbial coaggregates. In the present study, diazotrophic cells *viz*., *Azotobacter*, *Azospirillum* and *Rhizobium* grown at 35°C could form coaggregates at pH level of 7.5 and in the presence of Ca^{2+} cation. Interestingly, addition of chelating agents *viz*., EDTA and EGTA was found to reduce the coaggregation of diazotrophic cells, drastically. The findings might be considered in the mass scale production of Multigeneric diazotrophic coaggregates.

REFERENCES

1. Mudahar, M.S., Energy requirements, technology and resources in fertilizers sector. In: Energy in plant nutrition and pest control. Elsevier Publications, New York, 1987; 29-50.
2. Neyra, C.A., A. Atkinson and O. Olubayi., Coaggregation of *Azospirillum* with other bacteria : Basis for functional diversity. NATO Asi Ser. 1995; G**37**: 429-439.
3. Okon, Y. and C.A. Labandera-Gonzalez., Agronomic applications of *Azospirillum*: An evaluation of 20 years worldwide field inoculation. *Soil Biol. Biochem.*, 1994; **26**: 1591-1601.
4. Olubayi, O., R. Caudales, A. Atkinson and C.A. Neyra., Differences in chemical composition between non-flocculated and flocculated *Azospirillum brasilense* Cd. *Can. J. Microbiol.,* 1998; **10**: 802-806.
5. van Veen, A.J., Van, L.S. Overbeak and J.D. Van Elsas., Fate and activity of microorganism introduced into soil. *Microbiol. Mol. Biol. Rev*., 1997; **61**: 121-135.
6. Neyra, C.A., L.A. Atkinson, O. Olubayi, L. Sadasivan, D. Zaurov and E. Zappi., Novel microbial technologies for the enhancement of plant growth and biocontrol of fungal diseases in crops. *Cawers Optimo Mediterraneans*, 1999; **31**: 447-456.
7. Nikitina, V.E., E.G. Ponomareva, S.A. Aienkina and S.A. Konnova., The role of cell surface lectins in the aggregation of Azospirilla. *Microbiology*, (Moscow, Russ), 2001; **70**: 408-412.
8. Rubiya, E., Coaggregated diazotrophic cultures – A novel delivery system of bioinocula for low land rice (*Oryza sativa* L.). M.Sc.(Ag.) Thesis submitted to Annamalai University 2006.
9. Day, J.M. and J. Dobereiner., Physiological aspects of nitrogen fiation by a *Spirillum* from digitaria roots. *Soil Biol. Biochem.*, 1976; **8**: 45-50.
10. Grimaudo, N.J. and W.E. Nesbitt., Coaggregation of *Candida albicans* with oral

Fusabacterium sp. *Oral Microbiol. Immunol*., 1997 **12**: 168-173.

11. Gomez, K.A. and A.A. Gomez., Statistical procedures for agricultural research. John Wiley and Sons, Inc., New York, 1984; 20-29.
12. Madi, L. and Y. Henis., Aggregation in *Azospirillum brasilense* cd. conditions and factors involved in cell-to-cell adhesion. *Plant and Soil*, 1989; **115**: 89-98.
13. Burdaman, S., E. Jurkevitch, B. Schrartsburd, M. Hampel and Y. Okon., Aggregation in *Azospirillum brasilense*: Effects of chemical and physical factors and involvement of extracellular components. *Microbiol*., 1998; **144**: 1989-1999.
14. Sadasivan, L. and C.A. Neyra., Flocculation in *Azospirillum brasilense*, *Azospirillum lipoferum*: Exopolysaccharides and cyst formation. *J. Bacteriol.,* 1985; **163**: 716-723.
15. Smit, G., M.H. Straver, B.J. Luteniberg and J.W. Kijne., Flocculence of *Saccharomyces cerevisiae* cells induced by nutrient limitation with cell surface hydropohobicity as a major determinant. *Appl. Environ. Microbiol*., 1992; **58**: 3709-3714.