Optimization of biofloc production in *Azospirillum brasilense* (MTCC-125) and evaluation of its adherence with the roots of certain crops

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Abstract The phenomenon of flocculation in *Azospirillum brasilense* (MTCC-125) was studied under different combinations of carbon and nitrogen sources. Fructose and Potassium nitrate at a pH of 6.4 in the cultural medium favour a higher bio-floc production. The biofloc was studied for root adhesion and its survival efficiency in the rhizoplane and rhizosphere of certain crops such as sorghum and sunflower under dryland condition. It has been demonstrated that the flocculated cultures of *Azospirillum* were found to have maximum adhesion to the root surface and higher survival rate in the rhizoplane and rhizosphere under different moisture stressed conditions as compared to the log phase cells of *Azospirillum*.

Keywords *Azospirillum brasillense* (MTCC-125) · Flocculation · Poly- β -hydroxy-butyrate · Rhizosphere · Rhizoplane

Introduction

The free living N_2 fixing rhizobacterium *Azospirillum brasillense* lives in close association with many agriculturally important crops and exert beneficial effect on plant growth through the production of phytohormones [1, 2]. Though it has been suggested that polar flagella, extracellular polysaccharides and proteins are involved in the process of successful colonization [3–5]. Factors controlling the successful colonization of roots by *Azospirillum* species are not yet fully understood. *Azospirillum* cells have the ability to aggregate and flocculate [6] and these flocculated cells are generally formed by the modification of entire vegetative cells, which often accumulates additional cell wall or capsular material as well as intracellular polymers such as poly- β -hydroxy butyrate (PHB) [7], which in turn enhances its survival in the soil [8].

In addition to that, the flocs have increased resistance to environmental factors, such as starvation, desiccation and remain metabolically dormant as long as the stress conditions persist [9]. This phenomenon of aggregation is of great significance in the production, storage and survival of bacterial inoculants for agricultural application [10]. Flocculation may be induced by altering the nutrient culture conditions under which the cells are grown, or by the addition of various chemical agents [11].

This motivated the authors to undertake the present study that explored the positive influence of flocculation in *Azospirillum* cells, for its ability to be used as a bioinoculant under rainfed conditions. Experiments were conducted to achieve optimization of conditions to achieve maximum flocculation. The present study conducted to evaluate the ability of bioflocculated cultures for adhesion to the root

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surface and to survive in the rhizosphere and rhizoplane of these crops under different moisture stressed conditions.

Materials and methods

Culture and growth conditions

Azospirillum brasilense (MTCC-125) provided by IM-TECH Chandigarh, was used throughout the study. The strains were maintained on nutrient agar slants at 30°C. The stock cultures were routinely sub cultured in semisolid N-free malate medium for assessing N fixing potential.

The inoculant culture of *Azospirillum brasilense* MTCC-125 was separately multiplied for harvesting log phase cells (initial optical density of 0.4 in 660 nm) and for getting the bioflocs the detailed methodology as given below was followed

Induction of flocculation

The procedure outlined by Sadasivan and Neyra, 1985 has been used to obtain the flocs. The minimal salts medium was enriched by the addition of a trace amount of yeast extract (0.20 gL⁻¹), Vitamin solution (1 mL⁻¹) (containing Biotin-0.01 g and Pyridoxin-0.02g), Trace element solution (2 mL⁻¹) (containing Sodium molybdate -0.02 gL⁻¹, Manganese sulphate -0.02 gL⁻¹, Boric acid -0.02gL⁻¹, Copper sulphate-0.008 gL⁻¹, Zinc sulphate₄ -0.025gL⁻¹ and FeEDTA -4mL⁻¹). Iron salt was filter sterilized and separately added to the minimal salts medium, either fructose or mannitol at 8 mM concentration and potassium nitrate or ammonium nitrate at 5 mM concentration was used as the nitrogen sources respectively and the final volume was made to one litre and the pH was maintained at 6.8. Experiments were conducted in a 250 mL conical flask containing 100mL of enriched minimal salts medium and incubated on a shaker incubator (200 rpm) at 28±2°C.

Quantification of the flocs.

The flocs were harvested on the 3rd day of incubation by filtering the flocs through the filter paper (Whatman No-1).

For dry weight determination, the filter paper with the flocs were placed in a desiccator oven at 80°C for 2h and weighed. The weight of the filter paper was then excluded. Floc yield was expressed as mgL⁻¹.

Adsorption assay of *Azospirillum* bioflocs

The present study was conducted with sunflower variety JK-Chitra and the Sorghum variety Co-18. One germinated seeds of sunflower and sorghum were transferred to Semi-

solid Weavers medium separately [12] contained in a test tube. The log phase cultures of *Azospirillum* isolate MTCC-125 and its bioflocs were separately inoculated to the tube containing the germinated seed. The inoculated tubes were kept at 30±2°C with 10 h light and 14 h darkness for growth. Five replications were maintained for each treatment.

After 7 days of growth period one g of root sample from each treatment was serially diluted in 10 mL of sterile distilled water. The bacterial cell count was enumerated by MPN method [13].

Root surface colonization and rhizosphere survival of *Azospirillum* bioflocs

Pot culture experiment

A pot culture experiment was carried out at the green house, Department of Microbiology, Annamalai University, during Sep-Oct 2006. Rectangular cement pots of size 18" × 12" × 12" were filled with 45 kg of clay loam soil having a pH 7.4 and EC of 0.93 dSm⁻¹. The root surface colonization and survival of the bioflocs were tested against the log phase cells under moisture stressed conditions (withholding irrigation water for 3, 6, 9 and 12 days). The observations for rhizoplane and rhizosphere survival of *Azospirillum* from different treatments were enumerated by MPN method and expressed per g⁻¹ of root dry weight for root surface colonization and g⁻¹ for rhizosphere soil survival. The observations were made on 45 DAS.

Rhizoplane population

Random samples of root pieces of 5cm size, from each treatment were used to determine the rhizoplane population of *Azospirillum*. Root pieces were placed in a glass tube containing 5 mL of sterile phosphate buffer (0.2 mM; pH 7.0) and 2.5 g of 3 mm diameter glass beads the contents were shaken vigorously for 1 min. The phosphate buffer containing bacterial cells were serially diluted and its rhizoplane population was determined by MPN method [13].

Rhizosphere soil survival

Random of soil samples were collected from each of the pot treatment. The rhizosphere population of *Azospirillum* from the biofloc and the log phase treated ones were estimated by using MPN method [13].

Statistical analysis

The experiment results were statistically analyzed in the Duncans multiple range test (DMRT) using SPSS 7.0 sta-

tistical package. The triplicate sets of data (Table 3, pot culture experiment) for the various parameters evaluated were subjected to ANOVA (Analysis of Variance) in accordance with the experimental design (Completely Randomized Block Design) using NPROC statistical package to quantify and evaluate the source of variation, and CD (Critical Differences) values were calculated at P level of 0.05%.

Results and Discussion

The phenomenon of flocculation occurs in the case of *Azospirillum* log phase cultures grown in nutrient broth transferred to a minimal medium containing fructose or malate as carbon source and potassium nitrate or ammonium chloride as nitrogen source. Flocs were found to be visible after 4–5 h leaving behind a clear supernatant. The floc yield was found to be higher with a minimal medium containing fructose and potassium nitrate, followed by Fructose and ammonium nitrate (Table 1). In our present study the enriched medium containing the fructose as a carbon source and potassium nitrate as nitrogen source was able to induce more flocculation in *Azospirillum*. Previous reports have showed the efficiency of fructose and potassium nitrate as carbon and nitrogen plays a major role in the flocculation by enhancing the polysaccharide synthesis [7, 14, 15] and this

polysaccharide induce flocculation by bridging of polymers between the cells [16].

A closer look at the Table 1 clearly indicates that the formation of flocs was not pH dependent, since a medium containing fructose supplemented with potassium nitrate and ammonium chloride yielded more flocculation as compared to the medium containing malate supplemented with potassium nitrate and ammonium nitrate.

The *Azospirillum* bioflocs were tested for their root adhesion in dry land crops namely, sorghum and sunflower (Table 2). It has been found that the number of bacteria adhered to the roots were 68.75 per cent more in sorghum and 53.60 per cent more in sunflower with biofloc treatment as compared to the log phase treated ones (Table 2) This significant increase in the adhesion may be attributed to the fact that during flocculation the vegetative cells lose motility, assume an enlarged spherical form, and accumulate abundant poly- β -hydroxy butyrate granules, developing an outer layer (or) coat of polysaccharides [8] and the production of these exocellular polysaccharides, particularly arabinose rich polysaccharides participate in the cell-to-cell interaction with proteins or other molecules attached to bacterial envelope and this adhesive property is correlated with the ability of the bacteria to firmly attach to the root surface [10, 17].

The bioflocs were tested for their survival efficiency in the rhizoplane and rhizosphere of dry land crops such as

Table 1 Effect of different carbon* and nitrogen* sources on floc yields after flocculation of *A. brasilense*

Carbon/ Nitrogen source*	Floc yield (mg L ⁻¹)**		Final pH***
	Fresh weight	Dry weight	
Fructose + Ammonium chloride	4043.00 $\pm$ 41.00 ^b	554.2 $\pm$ 3.0 ^b	5.2 $\pm$ 0.2
Fructose + Pottasium nitrate	4922.00 $\pm$ 64.00 ^a	695.40 $\pm$ 5.42 ^a	6.4 $\pm$ 0.2
Malate+ Ammonium chloride	15.40 $\pm$ 0.64 ^d	0.26 $\pm$ 0.01 ^d	8.0 $\pm$ 0.2
Fructose + Pottasium nitrate	25.4 $\pm$ 2.64 ^c	0.44 $\pm$ 0.08 ^c	7.6 $\pm$ 0.1

The values are a mean of five replications $\pm$ SD. 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.

*Fructose or malate was added as the carbon source at 8 mM and KNO₃ or NH₄Cl as nitrogen source at 5 mM in the minimal salts medium

**Flocs were harvested after 24 h for wt determination.

***Final pH of the medium after 24 h.

Table2 Evaluation of log phase cells and bioflocs of *Azospirillum* for the root adhesion of sorghum and sunflower cultivars at 45DAS

Crop	Physiological form	No. of cells adhered (1 $\times$ 10 ⁶ h ⁻¹ g ⁻¹ root dry wt)	Percentage increase over the log phase cell treatment
Sunflower	Log phase cell	3.2 $\pm$ 0.14 ^b	68.75%
	Biofloc	5.4 $\pm$ 0.06 ^a	
Sorghum	Log phase cell	4.1 $\pm$ 0.17 ^b	53.60%
	Biofloc	6.3 $\pm$ 0.20 ^a	

The initial number of cells for each treatment was 1 $\times$ 10⁷ cfu mL⁻¹.

The values are a mean of five replications $\pm$ SD. 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.

Table 3 Evaluation of *Azospirillum* bioflocs for colonization in the rhizosphere soil and on the root surface under moisture stress condition

Crop	Physio-logical form	Rhizosphere population(1×10^6 g ⁻¹)				Rhizoplane population (1×10^6 g ⁻¹)			
		Days without watering after 15 DAS							
		3	6	9	12	3	6	9	12
Sunflower	Log phase cell	3.96 ^b	3.58 ^b	2.34 ^b	2.04 ^b	3.82 ^b	3.54 ^b	2.21 ^b	1.98 ^b
	Biofloc	4.68 ^a (18.18)*	4.20 ^a (20.01)*	3.18 ^a (35.89)*	2.98 ^a (46.07)*	4.42 ^a (15.70)*	4.2 ^a (18.64)*	3.0 ^a (39.89)*	2.86 ^a (44.44)*
Sorghum	Log phase cell	4.61 ^b	3.58 ^b	2.42 ^b	2.14 ^b	3.94 ^b	3.62 ^b	2.30 ^b	2.04 ^b
	Biofloc	5.84 ^a (27.0)*	4.80 ^a (34.0)*	3.37 ^a (39.0)*	3.04 ^a (42.0)*	4.64 ^a (17.70)*	4.3 ^a (19.44)*	3.24 ^a (40.86)*	2.94 ^a (44.11)*

The initial number of cells for each treatment was 1×10^7 cfu mL⁻¹.

*Figures in parenthesis refer to percent increase over log phase treated cells.

Within a column different letters after values indicate that there is a significant difference at p value of 0.05, as determined by DMRT.

sunflower and sorghum under different moisture stressed conditions. There has been a significant increase in the rhizoplane population of these two crops treated with biofloc, which showed a 40–45 per cent increase over the log phase cells (Table 3). The biofloc treated sunflower roots harbored 15.70, 18.64, 39.89 and 44.11 per cent more population with the progressive increase in the level of drought over log phase cells treatment. The biofloc treated sorghum roots also showed 17.70, 19.44, 40.86 and 44.11 per cent increase in rhizoplane population over log phase treated ones with the progressive increase in the level of drought. The studies on the survival population of *Azospirillum* in the biofloc treatment sunflower plants was 18.18, 20.01, 35.89 and 46.07 per cent more than the log phase cell treatment in the level of increase in moisture stress created by withholding irrigation for 3, 6, 9 and 12 days. Sorghum plants treated with biofloc also recorded higher rhizosphere population than log phase cell treated plants. The percent increase in survival was 27.0, 34.0, 39.0 and 42.0 per cent in the rhizosphere soil of sorghum.

The results of the present study clearly showed that survivability of *Azospirillum* bioflocs were higher than log phase treated cells at different moisture stressed conditions. Previous studies [18, 19 20] have showed that flocculated cells of *Azospirillum* are rich in PHB granules with a high degree of resistance to desiccation. This phenomenon has increased resistance to environmental stress as they remain metabolically dormant as long as the stress conditions persist [14]. This property has been positively utilized in the production of *Azospirillum* inoculants for coating turf grasses as well as agronomically important cereal crops, such as corn [21] as flocculation has provided a microenvironment, which is highly protective against physical and chemical stress and provides a safe niche for survival and cell release upon seed sowing in favorable surroundings, which enhances its surviv-

ability on dry surfaces[22]. Other genetic studies in which the ACC deaminase was proved to be an important factor in the stress resistant mechanisms in *Azospirillum* cd transformed from *Enterobacter cloacae* uw-4 ACC deaminase gene. The transformant found to have less metabolic load and improved fitness. In the present study Bioflocs of *Azospirillum* containing cyst found to have less metabolic load and would have and possessed increased ability to survive under stress in line with the report earlier [23].

These findings open up the possibilities for investigation of genetic basis of effective association and colonization of *Azospirillum* bioflocs and other mechanisms involved.

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