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Azospirillum Biofloc, A new Generation of Agricultural Bioinoculant Suitable for Environmental Stesses

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***Azospirillum*, a well known plant growth, promoting rhizobacteria, has been widely used as an agricultural bioinoculant. The application of the bioinocualnt enhances the plant growth through the production of growth regulators and by supplying biologically fixed nitrogen to the host plant. However, the lack of stress tolerance and poor survivability in soil of the introduced bacteria results in poor performance of the bioinoculant under stress soils. To ensure the good quality bioinoculant production, physiological status of the microorganism play a vital role rather than the cell numbers. It has been proposed that flocculation and concomittant accumulation of poly-β-hydroxybutyrate, a cellular reserve material, in *Azospirillum* cells rendering more resistance during environmental stresses and use of the flocculated cell forms of *Azospirillum*, as agricultural bioinoculant, is considered to be the novel bioinoculant technology in stress soils.**

In the present study, it was observed that Fructose and KNO_3, as sole carbon and nitrogen source, physiologically augmented more flocculation in *Azospirillum* cells. Moreover, addition of *Strychnos potatorum* seed material, as plant seed flocculant, non-physiologically inducted more flocculation of *Azospirillum* cells in a shorter period. The harvested *Azospirillum* bioflocs, exhibited higher desiccation and thermal tolerance when compared to the vegetative cell forms.

It was concluded that *Azospirillum* biofloc, as a novel formulation of agricultural bioinoculant, exhibited more tolerance to temperature and desiccation which are the two critical environmental stress conditions responsible for the poor performance of agricultural bioinocula in natural environments.

Key words : *Azospirillum*, Bioflocs, Plant seed, flocculants, Desiccation and Thermal tolerance.

Plant growth promoting rhizobacteria (PGPR) are group of rhizosphere bacteria which enhances the plant growth through production of plant growth regulators or by supplying the plant with biologically fixed nitrogen and include genera, such as, *Azotobacter*, *Azospirillum*, *Bacillus*, *Klebsiella* and *Rhizobium* on non-legumes (Burdman *et al*., 2000). Enhancement of growth and yield of agriculturally important crops through bioinoculation of *Azospirillum* has long been under practice (Okon and Labaendra-Gonzalez, 1994). However, lack of stress

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tolerance and poor survivability in soil are the most important critical factors which prevented the successful bioinoculation of field crops (Triplet and Sadowsky, 1992).

Higher degree of stress tolerance, long shelflife, and enhanced survivability in soil and on seeds are the important characteristics of any agricultural bioinoculant (Fages, 1990). To ensure these objectives, more importance will be given to the physiological status of microorganisms rather than their cell number in the bioinoculant preparation (Okon and Labaendra-Gonzalez, 1994). It has been well documented that the physiological alterations in a microbial cell could modify the composition of cell outer membrane together with high accumulation of intracellular poly-β-hydroxybutyrate (PHB) content (Sadasivan and Neyra, 1985) which rendered the microbial cells more resistance to environmental stresses. Neyra *et al*. (1995) proposed the use of flocculated cell forms of *Azospirillum* with high content of PHB (40%), as a novel *Azospirillum* bioinoculant technology, for the improvement of stress tolerance and survivability in stress soils.

On the basis of Neyra *et al*. (1995), the present research work has been undertaken with an aim to develop and use *Azospirillum* bioflocs, as a new generation of agricultural bioinoculant, and with the following objectives.

1. To optimize the cultural conditions of *Azospirillum* biofertilizer for achieving maximum flocculation.
2. Induction of artificial flocculation in *Azospirillum* cells through plant seed flocculants and
3. To assess thermal and desiccation tolerance of *Azospirillum* bioflocs for their resistance against environmental stresses *viz*., thermal and desiccation tolerance in comparison with vegetative cell forms.

MATERIAL AND METHODS

Bacterial strain used

Azospirillum brasilense strain AZS-3, an isolate from the rhizosphere of rice cv. BPT-5804, was maintained in nutrient agar slants at 35°C with monthly transfer and used throughout the study.

Effect of carbon and nitrogen source on flocculation

The minimal medium as described by Sadasivan and Neyra (1985) was used for the flocculation studies by supplementing mannitol or fructose, as carbon source and potassium nitrate or ammonium chloride, as nitrogen source.

Preparation of inoculum

The *Azospirillum brasilense* strain AZS-3 was grown in synthetic malate broth (Day and Dobereiner, 1976) supplemented with 0.05 per cent yeast extract (w/v) in a shaking bath at 30±2°C for 24 h. Then, the medium were centrifuged at 5000xg for 10 min to harvest the log phase cells and the pellets washed 3 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 absorbancy at 420 nm and used as inoculum.

Preparation of plant seed extract

The following plant seed materials, namely, *Moringa oleifera*, *Strychnos potatorum*, *Allium cepa*, *Sappindus emaginatus* and *Aestracantha longifolia* were collected, crushed and sieved (0.8 mm mesh). The seed powder is mixed with sterile water to form paste and then diluted to required strength.

Preparation of Co-AG buffer

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

Estimation of flocculation percentage and floc yield

The flocculation percentage and floc yield of *Azospirillum* cells were done according to the procedure of Madi and Henis (1989) and Sadasivan and Neyra (1985), respectively.

Estimation of poly-β-hydroxybutyrate (PHB)

Two ml of encysted culture of *Azospirillum* were added to 8 ml of sodium hypochlorite solution. After the digestion, the residue from hypochlorite solution was washed twice with 10 ml each of distilled water, acetone and diethylether to remove soluble salts and not PHB lipids. The final pellet was dried and dissolved in 2 ml of concentrated H_2SO_4 in order to yield crotonic acid. The absorbance of crotonic acid at 235 nm was measured and sodium DL-β-hydroxybutyrate was used as standard.

Desiccation and thermal tolerance of *Azospirillum* bioflocs

The desiccation and thermal tolerance of *Azospirillum* bioflocs were done according to Sadasivan and Neyra (1985).

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 fructose or malic acid, as sole carbon source, together with NH_4Cl or KNO_3 as sole nitrogen source, on the induction of flocculation in *Azospirillum* cells was studied (Table 1). It was observed that fructose and potassium nitrate, as sole carbon and nitrogen source inducted more flocculation and concomittant accumulation of poly-b-hydroxybutyrate in *Azospirillum* cells when compared to other treatments. Interestingly, malic acid combination with two nitrogen sources *viz*., malic acid + ammonium chloride and malic acid + potassium nitrate recorded flocculation of *Azospirillum* cells in trace amount. Sadasivan and Neyra (1985) and Madi and Henis (1989) reported the efficiency of fructose and potassium nitrate, as sole carbon and nitrogen source, in augmenting the flocculation and concomittant accumulation of *Azospirillum* cells and the results of present study are also in conformity with the above findings.

The addition of plant seed flocculants to Co-AG buffer could augment the flocculation of

Table 1. Effect of carbon and nitrogen sources on yield of *Azospirillum*+ biofloc

Carbon and nitrogen++ sources	Floc yield (g/l)		PHB content (%)	pH
	Fresh weight	Dry weight		
Fructose + NH_4Cl	2.5	0.15	27.90	3.2
Fructose + KNO_3	4.7	0.65	39.50	6.2
Malate + NH_4Cl	Trace	Trace	Nil	8.2
Malate + KNO_3	0.025	Trace	Nil	7.7

+ At a inoculum level of $1x10^7$ cfu/ml
++ Carbon and nitrogen source at a concentration of 0.5 and 0.01 per cent, respectively.

Table 2. Effect of addition of plant seed materials on flocculation of *Azospirillum*+ cells

Addition of plant seed material++	Percentage of [a] flocculation+++
Moringa oleifera	94.1 ±0.2[c]
Strychnos potatorum	98.5 ± 0.1[a]
Sappindus emaginatus	93.8 ± 0.4[c]
Allium cepa	96.2 ± 0.3[b]
Aestracantha longifolia	93.2 ± 0.5[c]

+ Inoculum at a level of 1×10^7 cfu/ml
++ Addition of plant seed material at a concentration of 5% level.
+++ Assayed according to Madi and Henis (1989) after 1 h of incubation time.
[a] Values followed by different letters are significantly differed at 5% level according to student 't' test.

Table 3. Studies on the thermal tolerance of Azospirillum[a] biofloc

Temperature (°C)[a]	No. of viable cells /ml after 20 min treatment[b]	
	Vegetative cell	Biofloc
40	4.8×10^8	5.0×10^9
45	6.8×10^7	4.2×10^9
50	2.2×10^5	3.8×10^9
55	nd	8.7×10^8

a – At 1×10^{10} cfu/ml level of inoculum
b – Values are means of three replications
nd – Below detectable limit of 10^3

Azospirillum cells within a short period of time, namely, 1 h. Among the different plant seed materials, the seed materials from *Strychnos potatorum* could augment the flocculation of *Azospirillum* cells to a higher level followed by

Table 4. Studies on the desiccation tolerance of *Azospirillum* biofloc

Incubation time (days)[a]	No. of viable cells /ml[b]	
	Vegetative cell	Biofloc
5	5.7×10^{8}	5.5×10^{9}
10	4.4×10^{6}	4.8×10^{9}
15	nd	4.5×10^{9}
20	nd	8.8×10^{8}

a-At 1×10^{10} cfu/ml level of inoculums
b-Values are means of three replications
nd-below the detectable limit of 10^{3}.

Allium cepa, *Moringa oleifera*, *Sappindus emaginatus* and *Aestracantha longifolia* (Table 2) Okuda *et al*. (2000) characterized some of the traditionally used plant seed materials as plant seed flocculant and emphasized the role of Indian Nirmali seed (*Strychnos potatorum*), for primary water treatments. The water soluble protein released from the seed kernel of *Strychnos potatorum* might be the reason for the flocculation of *Azospirillum* cells in a shorter period and the mechanism is unknown.

The thermal and desiccation tolerance of *Azospirillum* bioflocs was found to be maximuim upto 50°C and therafter a reduction was recorded upto 55°C while the desiccation tolerance was maximum upto 15 days of incubation. Olubayi *et al*. (1997) emphasized the increase in the content of PHB during flocculation and their positive role on the enhancement of thermal and desiccation tolerance in *Azospirillum* cells (Table 3 & 4). The positive results of the present study might be due to the increase in the content of PHB, a cellular reserve material, of the diazotroph.

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