Global Journal of Molecular Sciences 4 (2): 80-85, 2009
ISSN 1990-9241

Long-Term Survivability of *Azospirillum* Flocculated Cell in Different Inoculant Carriers and its Field Level Evaluation at Graded N Levels

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Abstract: *Azospirillum* flocculated cells on comparison with normal cells were found to superior in survival in different inoculant carriers. However the degree of survivability varied among the different carriers. Vermicultite sustained the highest number of viable *Azospirillum* cells, followed by lignite and cured compost. A field trail was also conducted to study the efficiency of these flocculated cells at graded nitrogen levels. *Azospirillum* flocculated cells were also found to be superior in augmenting the growth and yield of sunflower.

Keywords: *Azospirillum* · Flocculation · Survival · Carrier · Sunflower

INTRODUCTION

Azospirilla are free-living nitrogen-fixing α-proteobacteria that enhances the growth and yield of many important crop plants through the production of phytohormones [1]. However, despite this tremendous success in green house experiments, its commercial application on a large scale has been a failure. The main reason attributed to this failure is the unpredictability and inconsistency of field results [1].

In general, shortly after the bacteria are introduced into the soil, the bacterial population declines progressively [2, 3]. The main reason for this is that soil is a heterogeneous and unpredictable environment [4]. Moreover, the inoculated bacteria must compete with the often better-adapted native microflora and withstand predation by protozoans [5].

In this context the major role of inoculant formulation is to provide a suitable microenvironment to prevent the rapid decline of introduced bacteria in the soil. Inoculants have to be designed to provide a dependable source of beneficial bacteria that survive in the soil and become available to the plant.

It has well been documented that under various stress conditions, bacteria are capable of cyst and floc (macro, visible aggregates) formations, both of which improve survival. These phenomena can result from aging [6], culture conditions [7], toxic metals [8], or water stress [9].

Moreover these flocculated cells rich in PHB survive better than those without PHB [10-12]. Because of the survival advantages of flocculated cells of *Azospirillum* over vegetative cells, Neyra *et al*. [13] suggested that flocs can be produced readily on a large scale and separated easily from the growth medium with improved survival of the cells within the floc, they may have potential in inoculant preparation.

However to be considered as a successful inoculant formulation it has to satisfy the phenomenon given below: 1) long shelf life and stability 2) successful bioinoculation effect as evident by field level experiments.

Products lacking this above said characteristics will be unacceptable in the agricultural market [14, 15]. Hence the present experiment was undertaken to evaluate the above said phenomenon to recommend the use of flocculated cultures as inoculant delivery system.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions: The *Azospirillum brasilense* strain was isolated from the rhizosphere of sunflower. The isolate was identified and characterized in the Department of microbiology, Faculty of Agriculture, Annamalai University and designated as AZP-18

The bacterial strain was maintained at-20°C in NA broth containing 20% (v/v) glycerol and, before being used, they were grown overnight at 30°C and 120 rpm in Nutrient broth medium (Himedia) or on Nutrient agar medium (Himedia) at 30 °C for 24 h.

The bacterial inoculum was inoculated on M 9 salts minimal media as described by Sambrook *et al.* [16] in a

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Table 1: Soil characteristics of the experimental site

S. No.	Composition	Values
1.	Course sand (%)	13.02
2.	Fine sand (%)	36.72
3.	Clay (%)	37.12
4.	Textural class	Clay loom
5.	Available Nitrogen Kg ha^{-1} (Calcium hydroxide method)	74.4
6.	Available Phosphorous Kg ha^{-1} (Watanabe and Olsen, 1965)	63.5
7	Available Potassium Kg ha^{-1} (ammonium acetate extraction method)	76.5
8.	Organic Carbon (%) ''wet'' oxidation by acidified dichromate method	0.32
9.	Organic matter (%)	0.40
10	Soil reaction (pH)	8.0
11	Electrical conductivity dsm^{-1}	0.91

shaking bath at 30±2°C for 24 h to get the log phase cells. However for the induction of aggregation a slight modification was made to the minimal salt medium in which the carbon and nitrogen sources were replaced by fructose (6.67gL^{-1}) and NH_4Cl (0.214 gL^{-1}) in the ratio of 30:1. Then the medium was centrifuged at 5000-x g for 10 min to harvest the stationary phase cells and the pellets were washed three times with 0.1M-phosphate buffer (pH 6.8). Finally, the cells were re-suspended in the same buffer to a final concentration of 1 x 10^9 CFU/mL by measuring the absorbency at 650 nm and used as inoculum (OD value of 0.6).

Long-Term Survivability in Vermiculite as an Inoculant Carrier: Vermiculite, lignite and was selected for survival studies. Standard procedures for carrier preparation were followed [17]. Ten gram of selected carrier material was aseptically injected with buffer containing the *Azospirillum* co-aggregates (minimum 10^9 CFU mL^{-1} for each strains). The buffer: carrier ratio was chosen according to the water-holding capacity of substrate as per the procedures of Nieuwenhove, [18]. The treatments simulated realistic conditions of storage: room temperature (28±2°C). Sampling was done in three replicate bags per treatment. The total survival population, every two months up to a period 12 months after inoculation (MAI) was estimated by plating decimal dilution series in Phosphate buffer of 1 g stored material on Nutrient agar medium.

The individual *Azospirillum* population was determined by Most Probable Number (MPN) method [19].

Details of the Experimental Field: The experiment was conducted in a farmer field, manor village, Tirunelvelli district, Tamilnadu, India, during June-September 2008 with 12 treatments and 3 replications. The replications were made in a random throughout the plot. The Statistical Design adopted was RBD.

The temperature during this period has mean values ranging from 24.2 to 28.4°C. The mean minimum monthly values never come below 16.1°C and the mean maximum monthly value rise to 36.4°C in July. The wind velocity did not exceed 2Km h^{-1} during the experimental period. Soil characteristics of the experimental locations are shown in Table 1.

Plant Height: The height of the plants from each treatment was measured on 60^{th} day after sowing (DAS). The mean values of the plants from 5 replications were recorded.

Nitrogen Content of the Plant: The plant samples were washed in water, air dried and later dried to a constant weight in an oven at 50°C. Then they were ground, sieved and 100mg of sample was taken for analysis. The total nitrogen content was determined by microkjeldahl method [20].

Dry Matter Production: Five plants were randomly selected from each treatment and collected, washed and dried in an oven at 80°C till constant weight was observed. The plants were weighed and DMP was expressed in kg ha^{-1} on 60DAS.

Total Number of Seeds per Capitullum: Total number of seeds in the five representative samples was counted and the mean value per plant was recorded.

Seed yield

The seeds of the five representative samples were weighed and the mean value $plant^{-1}$ was expressed in g $plant^{-1}$.

Oil Content: The oil content of the seed was estimated using diethyl ether as extractant by soxhelt extractor and expressed in percentage.

Protein Content: Crude protein content of seed was calculated by multiplying the nitrogen content of the kernel with 6.25 [21].

Statistical Analysis: The experimental results were statistically analysed in randomized block design (RBD) and in Duncan's multiple range test (DMRT) as per the procedure described by Gomez and Gomez [22].

RESULTS AND DISCUSSION

A major role of inoculant formulation is to provide a more suitable microenvironment to prevent the rapid decline of introduced bacteria in the soil. Introduction of sufficient cell numbers in the immediate surrounding of the germinating seed can be done through the use of high-quality inoculants. Hence, the development of a reliable inoculation technology determines the potential success in agricultural production [1].

In the present study the *Azospirillum* flocculated cells were compared for their survivability in different locally available carrier materials including lignite, vermiculite and cure compost (Fig. 1).

A closer look at the Fig. 1 clear reveals the high level survivability of flocculated cells, when compared to the normal. A population > 7 log 10 cfu/mg dry was maintained for more than 12 months in all the carrier materials, while a drastic reduction was noticed in the case of normal cell after 7 months.

This increase in survivability exhibited by the *Azospirillum* flocculated cells is attributed to the fact that during flocculation PHA in flocculated cells reaches about 60-65 per cent of dry cell wt [23]. Earlier reports [24, 25] revealed that this energy and carbon storage compound is used under stress conditions, such as limitations of carbon and energy, a capacity that enhance the survival under stress conditions.

The results pertaining to the bioinoculation effect of *Azospirillum* flocculated cells at graded levels of N fertilizer are presented in Table 2. The growth and yield parameter of sunflower was increased significantly by bioinoculation over the uninoculted control. This yield increase obtained in the field experimented could be correlated with hormonal effects and nitrogen fixation of *Azospirillum*. Fulcheri and Frioni [26] obtained thrice the seed yield and 59 per cent increased seed dry wt of corn due to *Azospirillum* inoculation.

Sunflower crop response to *Azospirillum* was more pronounced at 50 per cent nitrogen and found to be higher, when compared to 100% N alone. However, the response to azospirillum declined with increased N levels from 50 to 100 per cent of recommended N. The better performance of *Azospirillum* with moderated doses of combined nitrogen is attributed to the congenial environment and ideal condition for the growth and multiplication of the bacterium in the rhizosphere. The reduced effect of *Azospirillum* under high levels of nitrogen is best explained with inhibitory effect of nitrogen on the nitrogenase activity [27, 28]. Further reports by Vasuvat *et al.* [29] and Gopal [30] explained that the *Azospirillum*-rhizobiocoenosis is most effective at moderate level of N. They further indicated poor crop root association of *Azospirillum* at higher level of N.

The *in-vitro* and field studies pertaining to the use of flocculated cultures are quite encouraging and advocates the use of flocculated cultures as inoculant delivery system.

Table 2: Effect of Azflc-18 inoculation at graded levels of nitrogen on plant growth and yield of sunflower var. sunbeam

Treatments	Plant height in cm[c]	'N' uptake[c]	Dry matter production (kg ha^{-1})	Seed yield (Kg ha)	Oil content (%)	Protein content (%)
Control	80.54	124.60	1791.24	956.50	37.12	9.14
Azp-18[a]	10554.00	154.20	2204.42	1120.80	38.14	10.24
Azflc-18[b]	109.74	162.40	2250.54	1174.60	38.44	10.32
50%	108.24	154.20	2114.24	1138.40	38.24	10.42
75%	112.24	168.40	2304.52	1204.60	38.68	11.74
100%	115.24	174.60	2442.52	1342.60	38.92	11.87
Azflc-18+50%N	120.56	186.40	2614.42	1364.50	39.12	11.96
Azflc-18+75%N	122.24	188.40	2624.42	1370.40	39.42	12.08
Azflc-18+100%N	124.24	190.60	2628.24	1472.20	39.94	12.17
LSD(P=0.05)	2.24	1.46	12.12	6.52	1.64	0.24

a) *Azospirillum* normal cells b) *Azospirillum* flocculated cells c) Observation at 60 DAS.

Mean values followed by different letters are differed significantly according to least significant difference test (P<0.05)

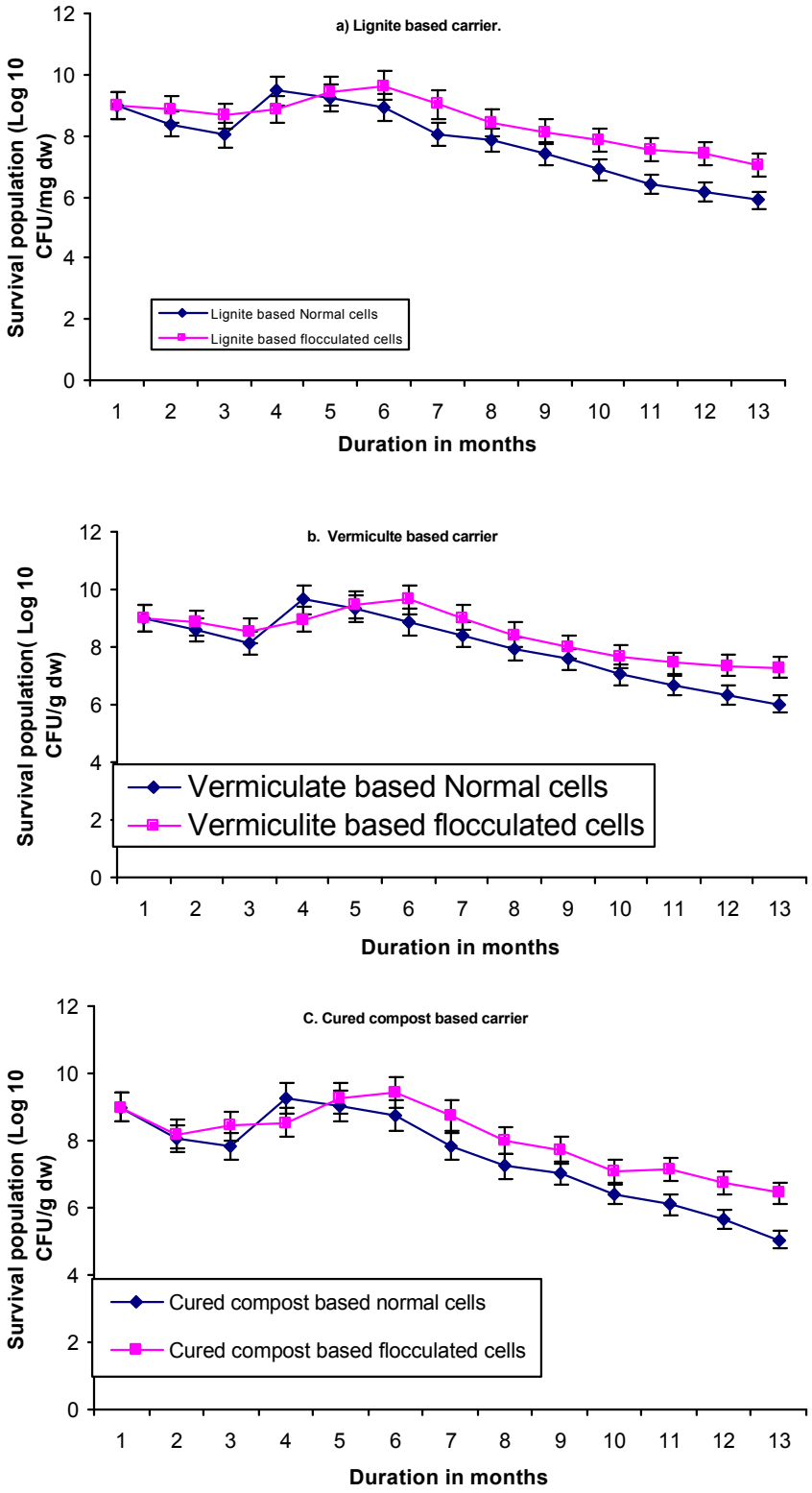

Fig. 1: Long term survival of *Azospirillum* flocculated cells (Log 10 cfu g^{-1} dry carrier) on seven sampling dates, in different inoculant carriers stored at 28±2°C. Error bars indicate the minimum significant difference (5%) for comparing treatments on each sampling date

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