Available Online at http://www.recentscientific.com

ISSN: 0976-3031

International Journal of Recent Scientific Research
Vol. 5, Issue, 6, pp.1218-1221, June, 2014

International Journal of Recent Scientific Research

RESEARCH ARTICLE

LONG-TERM SURVIVABILITY OF STRESS TOLERANT *AZOSPIRILLUM* STRAINS IN DIFFERENT INOCULANT CARRIERS AND ITS FIELD LEVEL EVALUATION AT GRADED N LEVELS

***Kumaran, S and Elango, R**

Department of Microbiology, Annamalai University, India

ARTICLE INFO

Article History:

Received 17th, May, 2014
Received in revised form 24th, May, 2014
Accepted 19th, June, 2014
Published online 28th, June, 2014

Key words:

Azospirillum. Survival. Carrier. Sunflower

ABSTRACT

Stress tolerant*Azospirillum* strains (ATT-3 and AST-1) on comparison with reference strain (MTCC-125) were found to superior in survival in different inoculant carriers. However the degree of survivability varied among the different carriers. Sterilized lignite sustained the highest number of viable *Azospirillum* cells, followed by unsterilized lignite, sterilized pressmud and unsterilized pressmud. A field trail was also conducted to study the efficiency of these strains at graded nitrogen levels. *Azospirillum* cells were also found to be superior in augmenting the growth and yield of sunflower.

INTRODUCTION

Azospirilla are free-living nitrogen-fixing proteobacteria that enhances the growth and yield many important crop plants through the production of survival of the cells within the floc, they may have 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 (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 the stress tolerant cells rich in PHB survive better than those without PHB [10-12]. Because of the survival advantages of the stress tolerant cells of *Azospirillum* over vegetative cells Neyra *et al.* [13]suggested that flocs can be produced readily on a large scale and of 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 inoculants 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 inoculant.

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 ATT-3 and AST-1. The bacterial strain was maintained at-20°C in NA both of which 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 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 Cl (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 sterilized lignite as an Inoculant

Carrier: Sterilized 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 *Azospirillum* co-aggregates (minimum 10^9 CFU

* Corresponding author: **S. Kumaran**
Department of Microbiology, Annamalai University, India

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.

Details of the Experimental Field

The experiment was conducted in a farmer field, Adoor village, cuddalore district, Tamilnadu, India, during June-September 2011 with 8 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 26.2 to 29.4°C. The mean minimum monthly values never come below 17.2°C and the mean maximum monthly value rise to 37.7°C in July. The wind velocity did not exceed 2Km h 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 60th 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 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 was expressed in g plant.

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 analyzed 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 decline of introduced bacteria in the soil. Introduction of sufficient cell numbers in the immediate surroundings 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 stress tolerant *Azospirillum* cells were compared for their survivability in different locally available carrier materials including sterilized lignite, unsterilized lignite sterilized pressmud and sterilized pressmud (Fig. 1).

Table 1 Physico chemical properties of experimental field soil

S.No.	Properties	Result
1.	pH	7.92
2.	Electrical conductivity (dSm^{-1})	1.80
3.	Soil organic carbon (%)	0.47
4.	Soil N (kg ha^{-1})	11.56
5.	Soil P (kg ha^{-1})	12.88
6.	Soil K (kg ha^{-1})	245.52

A closer look at the Fig. 1 clear reveals the high level survivability of the stress tolerant *Azospirillum* cells, when compared 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.

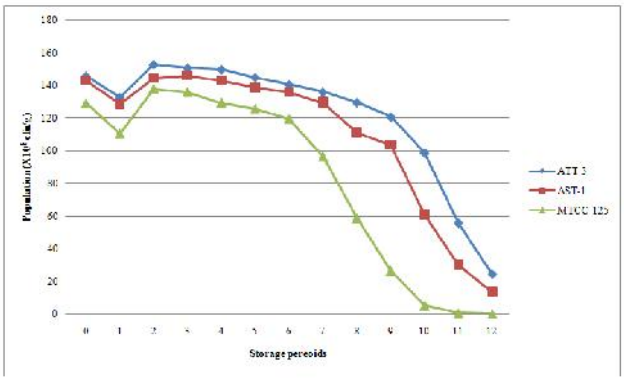

Figure 1 Survival of stress tolerant *Azospirillum* strains in sterilized lignite

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

The results pertaining to the bioinoculation effect of the stress tolerant *Azospirillum* cells at graded levels of N 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 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 75 per cent of recommended N. The better performance of *Azospirillum* with

Table 2 Effect of *Azospirillum brasilense*ATT-3 inoculation on the Plant height, dry matter production, seed yield, oil content and protein content of sunflower var. modern

S. No.	Treatments	Plant height (cm)	Dry matter production (kg ha^{-1})	Seed yield (kg ha)	Oil content (%)	Protein content (%)
1.	100% NPK	125.25	4618.00	1341.7	38.85	11.85
2.	75% N + Temperature tolerant *Azospirillum* (ATT-3)	144.50	4767.00	1410.86	39.25	12.25
3.	50% N + Temperature tolerant *Azospirillum* (ATT-3)	139.20	4593.86	1350.60	38.95	11.95
4.	75% N + Salt tolerant *Azospirillum* (AST-1)	141.50	4722.58	1386.45	39.10	12.15
5.	50% N + Salt tolerant *Azospirillum* (AST-1)	136.50	4588.50	1200.8	38.65	11.70
6.	75% N + Reference strain	135.50	4526.42	1135.7	38.20	10.35
7.	50% N + Reference strain	118.00	4252.00	1119.3	38.10	10.16
8.	Control	97.88	3217.00	956.5	37.51	9.16
	SED	5.50	179.07	56.77	0.26	0.40
	CD (P=0.05)	11.02	348.56	112.18	0.54	0.80

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*to rhizobiocoenosis is most effective at moderate level of N. They further indicated poor crop root association of *Azospirillum* at higher level of N.

References

1. Okon, Y. and C.A. Laberandera-Gonzalez, 1994. Agronomic application of *Azospirillum*: An evaluation of 20 years worldwide field inoculation. Soil Biol. Biochem., 26: 1591-1601.
2. Bashan, Y. And H. Levanony, 1990. Curent status of *Azospirillum* inoculation technology: *Azospirillum* as a challenge for agriculture. Canadian J. Microbiol., 36: 591, 1990.
3. Van Elsas, J.D., A.R. Kijkstra, J.M. Govaert and J.A. van Veen, 1986. Survival of *Pseudomonas fluorescens* and *Bacillus subtilis* introduced into two 15. Kenney, D.S., 1997. Commercialization of biological soils of different texture in field microplots. FEMS control products in the chemical pesticide world.Microbiol. Ecol., 38: 151-160.
4. Van Elsas, J.D. and L.S. van Overbeek, 1993. Bacterial responses to soil stimuli. *In* Starvation in bacteria, S. Kjelleberg (ed.), pp: 55-79, PlenumPress, New York.
5. Cochran, W.G., 1959. Estimation of bacterial densities by means of 'most probable number'. Biometrics, 6:105-116.
6. Sadasivan, L. and C.A. Neyra, 1987. Cyst production and brown pigment formation in aging cultures of *Azospirillum brasilense* ATCC, 29145. J. Bacteriol., 169: 1670-1677.
7. Bleaky, B.H., M.H. Gaskins, D.H. Hubbel and S.G. Zam, 1988. Floc formation by *zospirillum lipoferum* grown on poly- -hydroxy butyrate. Appl. Environ. Microbiol., 54: 2986-2990.
8. Gowri, P.M. and S. Srivastava, 1996. Encapsulation as a response of *Azospirillum brasilense sp7* to zinc stress. World J. Microbiol. Biotechnol., 12: 319-322.
9. Bashan, Y., G. Mitiku, R.E. Whitmoyer and H. Levanony, 1991b. Evidence that fibrillar anchoring is essential for *Azospirillum brasilense* Cd attachment to sand. Plant Soil, 132: 73-83.
10. Assmus, B., P. Hutzler, G. Kirchhof, R. Amann, J.R. Lawrence and A. Hartmann, 1995. In situlocalization of *Azospirillum brasilense* in the rhizosphere of wheat with fluorescently labeled, rRNA targeted oligonucleotide probes and scanning confocal laser microscopy. Appl. Environ. Microbiol., 61: 1013-1019.
11. Oliveira, R.G.B. and M.L. de Souza, 1991. Partial characterization of a brown pigment produced by *Azospirillum lipoferum.* Rev. Microbiol., 22: 340-344.
12. Zaady, E., A. Perevolotsky and Y. Okon, 1993. Promotion of plant growth by inoculum with aggregated and single cell suspensions of *Azospirillum brasilense* Cd. Soil Biol. Biochem., 25:819-823.
13. Neyra, C.A., A. Atkinson and O. Olubayi, 1995. Coaggregation of *Azospirillum* with other bacteria:basis for functional diversity. In: Fendrik I, Del GalloM, Vanderleyden J, de Zamaroczy M (eds). *Azospirillum* VI and related microorganisms, genetics- physiology-ecology. NATO ASI series, series G.Springer, Berlin Heidelberg New York, pp: 429-439.
14. Lethbridge, G., 1989. An industrial view of microbiala challenge for agriculture. Canadian J. Microbiol., inoculants for crop plants. *In* Microbial inoculation ofcrop plants, R. Campbell and R.M. Macdonald (eds.),pp: 11-28, Special publication of the society of generalmicrobiology, vol. 25, IRL Press, Oxford, New York.
15. Kenney, D.S., 1997. Commercialization of biologicalcontrol products in the chemical pesticide world. In Plant Growth-Promoting Rhizobacteria-presentstatus and future prospects, A. Ogoshi,K. Kobayashi, Y. Homma, F. Kodama, N. Kondo and S. Akino (eds.), pp: 12G-127, Faculty of Agriculture,Hokkaido University, Sapporo, Japan.
16. Sambrook J, Fritisch EF, Manialis T. Molecular. cloning; a laboratory manual. 1989; 2 ed. Cold springHarbor, NY: Cold Spring Harbor Laboratory.
17. Somasegaran, P. And H.J. Hoben, 1994. Handbook forRhizobia: Methods in Legume-*Rhizobium*Technology. NIFTAL Project, University of Hawaii,Paia, pp: 450.
18. Nieuwenhove, C.V., L.V. Holm, S.A. Kulasooriya and. K. Vlassak, 2000. Establishment of

Azorhizobiumcaulinodans in the rhizosphere of wetland rice (*Oryza sativa L.*). Biol. Fertil. Soil, 31: 143-149.

19. Bashan, Y., 1998. inoculants of plant growth promoting bacteria use in agriculture. Iotechnol Adv. 16: 729-770.
20. Bremner, J.M., 1960. Determination of nitrogen byKjeldhal method. J. Agric. Sci., 55: 11-33.
21. Humphries, E.C., 1996. Mineral composition and ash analysis. pp: 468-502. In: Modern methods of plant analysis. Vol. 1. K. Peach and M.V. Tracey (Eds.),Spriger-Verlag, Berlin.
22. Gomez, K.A. and A.A. Gomez, 1984. Statisticalprocedures for Agriculture Research. John Wiley andSons. Inc, New York, pp: 20-29.
23. Olubayi, O., R. Caudules, A. Atkinson and C.A.Neyra, 1998.''Differences in Chemical CompositionBetween Nonflocculated and Flocculated*Azospirillum brasilense* cd,'' Can. J. Microbiol., 44: 386-390.
24. Bashan, Y. and G. Holguin, 1997. *Azospirillum*-plantrelationships: environmental and physiological advances (1990-1996). Can. J. Microbiol., 43: 103-121.
25. Bashan Y, Y. Ream, H. Levanony and A. Sade, 1998. Nonspecific responses in plant growth, yield, and root colonization of noncereal crop plants to inoculation with *Azospirillum brasilense* Cd. J. Bot., 67: 1317-1324.
26. Fulchieri, M. and L. Frioni, 1994. *Azospirillum* inoculation on maize (Zea mays): effect of yield in a field experiment in Central Argentina. Soil Biol. Biochem., 26: 921-924.
27. Colnaghi, R., A. Green, L. He, P. Rudmick andKennedy, 1997. Strategies for increased ammoniumproduction in free living or plant associated nitrogenfixingbacteria. Plant Soil, 194: 145-154.
28. Tripathi, A.K., T. Nagarajan, S.C. Verma andD.L. Rudulier, 2002. Inhibition of biosynthesis andactivity of nitrogenase in *Azospirillum brasilense* inrelation to plant root attachment. Microbiol. Ecol.,32: 149-169.
29. Vasuvat, Y., B. Fragcham, S. Simpin, D. Fusang and P. Cannaram, 1986. Yield maximization of food grainsby associative N fixing bacteria pp: 12-16. In: Can. Proceedings of an international seminar on yield maximization of food grains through soil and fertilizer management Bangkok, Thailand.
30. Gopal, N.O., 1998. Diazotrophic association of Azospirillum with coriander (*Coriandrum sativum* L) Ph.D. Thesis, Tamil Nadu Agricultural Univ., India.

****