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Survival of *Azospirillum brasilense* in liquid formulation amended with different chemical additives

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Abstract

Liquid bioinoculant formulation has become the preferred technology to solve the problems associated with shorter shelf life, high contamination, poor quality, low field performance and processing solid carrier in carrier based bioinoculant formulation. In this experiment, we evaluated different concentrations of six different chemical amendments viz, polyvinyl pyrollidone (PVP), glycerol, gum arabica, trehalose, polyethylene glycol (PEG) and polyvinyl alcohol (PVA) for their ability to support growth and promote survival of *Azospirillum brasilense* in N_2 free malic acid broth during the storage. Some concentrations of various additives to N_2 free malic acid broth promoted higher *Azospirillum* population compared to *Azospirillum* cells in N_2-free malic acid broth alone. Liquid *Azospirillum* bioinoculant formulated with trehalose (10mM) promoted long term survival of *Azospirillum* followed by glycerol (10 mM) gum arabica (0.3%) and PVP (2%) and they supported 10^8 cells/ml up to 11 months of storage under ambient temperature (28°C to 32°C), whereas PEG (1%), PVA (0.5%) and control (lignite carrier) recorded the same population upto 8 months, 6 months and 5 months respectively. The results of the present study clearly indicated that the liquid formulation of *Azospirillum* could be used more effectively than the carrier based formulation.

INTRODUCTION

Microbial inoculants represent an emerging technology designed to improve the productivity of Agricultural systems in the long run. They can be seen as a technology aligned with principles of sustainable agriculture, as opposed to the increased use of pesticides and fertilizers in recent times. *Azospirillum brasilense* is one of the potential plant growth promoting rhizobacteria (PGPR). Its positive impacts on plant growth through several mechanisms which include enhancement of root development, production of growth regulators and nitrogen fixation (Garcia *et.al.,* 1996 and Okon 1994). The content of nitrogen, phosphorus, potassium and various micronutrients is higher in plants inoculated with *Azospirillum* (Caballero-Mellado *et al.,* 1992 and Garcial *et al.,* 1996).

Azospirillum species have potentially been studied to the greatest extent and appeared to have significant potential for commercial application. FAO (1991) reported that most of the international producers of biofertilizers are engaged in the production of carrier-based inoculants. Peat is the most frequently used carrier for rhizobial inoculant industry because it has characteristics such as high water holding capacity and high surface area that support rhizobial growth and survival in large numbers. However, peat is not available in many countries, especially in tropics, and will be depleted in many areas in future (Smith 1992).

The carrier based microbial inoculants produced in India are generally lignite, coal (or) Charcoal based. The major disadvantages associated with these carriers are shorter shelflife, poor quality, high contamination and unpredictable field performance. The cost of solid carrier based inoculant production is high as it is labour and energy intensive process, involving milling, sieving and correcting pH (Somasegaran and Hoben, 1994).

Liquid inoculant formulation is one solution to the problems associated with processing of solid carriers. The use of various broth cultures amended with substance that promote cells survival in the package and after application for seed (or) soil. Additives to liquid inoculant formulations should have a role in protecting *Azospirillum* cells on seed at high temperature and during desiccation. Many kinds of polymers have been used for inoculant production because of their ability to limit heat transfer, their good rheological properties and high water activities (Mugnier and Jung 1985). In the present study, experiments were conducted to increase the survival of the liquid formulations of *Azospirillum brasilense* bioinoculant by the addition of different polymers like gum arabic, polyvinyl pyrollidone (PVP), glycerol, trahalose, polyethylene glycol (PEG) and poly vinyl alcohol (PVA).

MATERIALS AND METHODS

Microorganisms used

Azospirillum brasilense AZ15 isolate has been obtained from the screening studies and used in liquid formulation.

Medium

N_2-free malate (NFb) medium containg (g/l) 5.0 malic acid, 0.5 K_2HPO_4, 0.2 $MgSO_4$ $7H_2O$, 0.1 Nacl, 2.0 $CaCl_2$, 4.0 ml Fe-EDTA (1.64% w/v aquous), 2.0 ml trace element solution, 2.0 ml bromothymol blue (0.5% alcoholic solution), 1.0 ml vitamin solution, 4.0 KOH, 15.0 agar and pH 6.8 was used as a basal medium for liquid inoculant formulation with selected appropriate concentrations of additives.

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Chemical amendments

N_2 free malate (NFb) broth was tried in combination with different chemicals to increase the survival of *Azospirillum* cells in a liquid formulation. To standardize the optimum quantity of the amendments, the chemicals like PVP 1.0, 1.5, 2.0, 2.5 and 3.0%, glycerol 5.0, 7.5, 10, 12.5 and 15.0 mM, gum arabica 0.1, 0.3, 0.5, 0.8 and 1.0%, trechalose 1, 5, 10, 15 and 20 mM, PEG 0.1, 0.5, 1.0, 2.0, and 3.0% and PVA 0.1, 0.5, 1.0, 2.0 and 3.0 % were added to one liter of N_2 free malate broth separately. One ml of log phase culture of *Azospirillum brasilenese* was inoculated individually in each broth. Control (without any chemical addition) was also maintained and the flasks were incubated at room temperature. The broth cultures were analysed for viable cell population at 30 days interval upto six months.

Enumerating the viable cell population

The Nfb medium was prepared, sterilized and plated in sterile petriplates. The plates were kept at room temperature for 48h. Eight equal sectors on the outside bottom of the petridishes were radially marked. Four sectors were used for replication of one dilution and four for another, allowing two dilutions per plate. Serial dilutions were prepared by transfer of 1 ml each of inoculum into 9 ml sterile water blanks to get 10^{-1} dilutions. Similarly, the dilutions were made serially upto 10^{-10}. From the dilutions, 5 µl was pipetted out and placed on the respective quadrant in the Petri plate. The plates were incubated at 28 ± 2°C without any disturbance and individual colonies were counted through this drop plate method (Somasegaran and Hoben 1994).

Liquid inoculant production and survival of *Azospirillum* during prolonged storage

For developing liquid formulation of *Azospirillum,* NFb broth was prepared and standardized dosage of chemical amendments viz, PVP (2%), glycerol (10mM), gum arabica (0.3%), trehalose (10mM), PEG (1.0%) and PVA (0.5%) were added to one litre of broth separately. One ml of log phase culture of *A. brasilense* was inoculated individually in each broth and flasks were incubated at room temperature. The broth cultures were analyzed for viable cell population and pH at monthly intervals upto 12 months.

RESULTS & DISCUSSION

To enhance the shelf life of *Azospirillum* cells in liquid bioinoculant, certain chemicals viz., PVP, glycerol, gum arabica, trehalose, PEG and PVA were added as supplements to N_2 free malate broth. Experiments were carried out to standardize the optimum concentrations of different chemical additives in liquid formulation of *Azospirillum,*to support more viable population for longer period. The effects of different concentrations of chemical additives on the survival of *Azospirillum* are presented in Fig. 1 to 6. The results showed that maximum population was recorded in trehalose (10 mM) 4.00 x 10^9 followed by glycerol (10mM) 3.33 x 10^9, gum arabica (0.3%) 2.67x10^9, PVP (2%) 2.33x10^9, PEG (1.0)10.33x10^8 and PVA (0.5%) 5.00 x 10^8 cells/ml of *Azospirillum* liquid inoculants during 6th month of storage at room temperature (28 ± 2°C) whereas minimum population 6.33 x 10^2 was recorded in control (without chemical additives) N_2 free malate broth alone during 5th month. Hence, the concentration level of different chemical additives viz. PVP (2 %), glycerol (10 mM), gum arabic (0.3%), trehalose (10 mM), PEG (1.0%) and PVA (0.5%) were taken for further study.

Fig 1

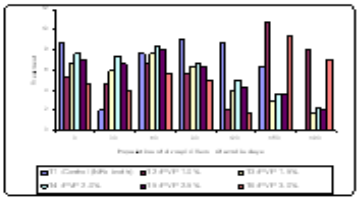


Fig 2

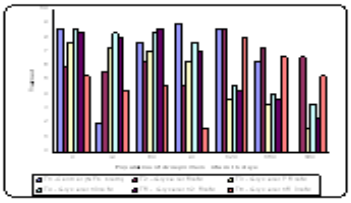


Fig 3

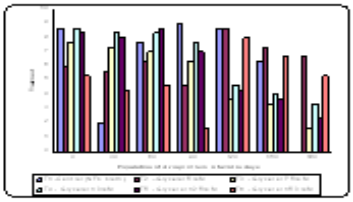


Fig 4

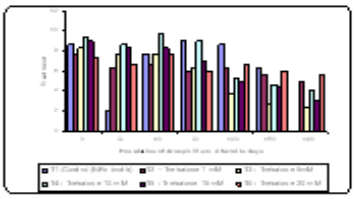


Fig 5

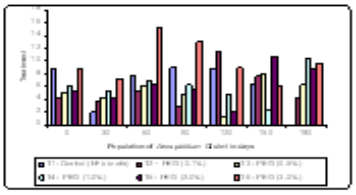
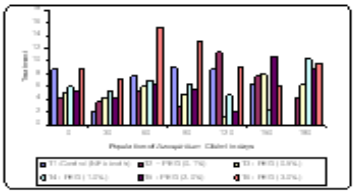


Fig 6



Effect of different chemical additives on survival of *Azospirillum brasilense* in liquid formulation.

Trehalose is an enigmatic compound which act as a reserve carbohydrate that may be mobilized during stress (Hounsa *et al.,* 1988). It is widely reported to enhance cell tolerance to desiccation, osmatic and temperature stress. It acts by stabilizing both enzymes and cell membranes (Fillinger *et al.,* 2001). The possible effect of trehalose's protective action is that it may be incorporated into the cell (or) may induce the synthesis of metabolites that protect against stress (Gomez *et al.,* 2003) which might be the reason for the higher population of *Azospirillum* cells in the trehalose treatments. Next to trehalose, 10 mM glycerol supported greater number of *Azospirillum* in liquid formulation. This may be due to high water binding capacity and may protect cells from the effect of desiccation by reducing the rate of drying (Lorda and Balatti 1996).

The 2% PVP treatment gave a marginal increase in population compared to control. Suresh Babu *et al.,* also found higher population of *Azospirillum* due to the addition of PVP at both 1 and 2% levels. It might be due to its high water binding capacity. Various polymers, such as PVP, PEG and gum arabic have adhesive properties. They have sticky consistency, which may enhance cell adherence to seed, and their viscous nature may slow the drying process of the bioinoculants (Temprano *et al.,* (2002). PVP also has a high water binding capacity, which could maintain water around the cells for their metabolism (Singleton *et al.,* 2002, Deaker *et al.,* 2004). PVP and gum arabic have been reported to protect cells against toxic seed coat factors. Biopolymers such as Cassava starch, alginate and gum arabic have the ability to limit heat transfer and also have high water activities (Mugnier and Jung 1985).

Liquid formulation of *Azospirillum,* was developed the N_2 free malate broth was amended with PVP (2.0%), glycerol (10 mM), gum arabic (0.3%), trehalsoe (10 mM), PEG (1.0%) and PVA (0.5%) separately. The addition of the chemical amendments like trehalose, glycerol, gum arabic and PVP allowed the maintence of 10^8 cells upto 11 months of storage, followed by, PEG upto 8 months and PVA upto 7 months where as the control (Lignite carrier based formulations) recorded the population level of 10^8 only up to 5 months. Among the amendments, trehalose supported highest number of *Azospirillum* cells throughout the observation period followed by glycerol, gum arabic, PVP, PEG and PVA (Table 1).

Table -1 Survival of *Azospirillum* in liquid formulation amended with different chemical additives

Days	Population of *Azospirillum* Cfu/ml in days						
	Control (Lignte carrier)	PVP (2 %)	Glycerol (10 mM)	Gum arabica (0.3%)	Trehalose (10 mM)	PEG (1.0 %)	PVA (0.5 %)
0	$8.67 X10^8$	$7.67 X10^{10}$	$8.67 X10^{10}$	$8.00 X10^{10}$	$9.33 X10^{10}$	$6.00 X10^{10}$	$4.67 X10^{10}$
30	$2.00 X10^9$	$7.33 X10^{10}$	$8.33 X10^{10}$	$7.67 X10^{10}$	$8.67 X10^{10}$	$5.33 X10^{10}$	$3.33 X10^{10}$
60	$7.67 X10^8$	$8.33 X10^9$	$8.67 X10^9$	$8.67 X10^9$	$9.67 X10^9$	$7.00 X10^9$	$7.67 X10^9$
90	$5.67 X10^8$	$6.67 X10^9$	$7.67 X10^9$	$7.33 X10^9$	$9.00 X10^9$	$6.33 X10^9$	$5.33 X10^9$
120	$3.67 X10^8$	$5.00 X10^9$	$4.67 X10^9$	$5.67 X10^9$	$5.33 X10^9$	$4.67 X10^9$	$9.00 X10^8$
150	$1.33 X10^8$	$3.67 X10^9$	$4.00 X10^9$	$4.00 X10^9$	$4.67 X10^9$	$2.33 X10^9$	$7.67 X10^8$
180	$8.00 X10^7$	$2.33 X10^9$	$3.33 X10^9$	$2.67 X10^9$	$4.00 X10^9$	$10.33 X10^8$	$5.00 X10^8$
210	$4.67 X10^6$	$2.00 X10^9$	$2.67 X10^9$	$2.00 X10^9$	$3.67 X10^9$	$6.67 X10^8$	$3.33 X10^8$
240	$2.33 X10^5$	$1.33 X10^9$	$2.00 X10^9$	$1.33 X10^9$	$2.33 X10^9$	$4.33 X10^8$	$9.67 X10^7$
270	$3.67 X10^4$	$9.00 X10^8$	$1.33 X10^9$	$9.67 X10^8$	$1.67 X10^9$	$9.67 X10^7$	$2.33 X10^7$
300	$2.33 X10^3$	$6.67 X10^8$	$7.67 X10^8$	$7.00 X10^8$	$8.67 X10^8$	$3.67 X10^7$	$8.67 X10^6$
330	$2.67 X 10^2$	$1.33 X10^8$	$2.33 X10^8$	$1.67 X10^8$	$4.00 X10^8$	$9.00 X10^6$	$9.33 X10^5$
360	-	$2.33 X10^7$	$6.67 X10^7$	$3.67 X10^7$	$8.67 X10^7$	$6.33 X10^5$	$8.00 X10^4$

Liquid inoculant formulation of cowpea rhizobia prepared with PVP as an osmoprotectant been observed to have higher shelf life than those without PVP amendment (Girisha *et al.,* 2006). Some of the polymers and chemicals which can be used as additives and protectants in liquid inoculants include PVP, methyl cellulose, gum arabica, trehalose, glycerol, sodium alginate, poly ethylene glycol, polyvinyl alcohol and tapioca flour (Panlada *et al.,* 2007). Vendan and Thangaraju (2006) developed liquid formulation of *Azospirillum brasilense* amended with trehalose, glycerol and PVP in NFb malate broth and reported 10^8 cells/ml upto 10 months storage under room temperature.

Singleton *et al.,* (2002) developed liquid formulations of *Rhizobium* by adding various additives in the yeast extract mannitol media and claimed cell numbers of 1 x 10^{10} cells/ml in the liquid inoculant. Enhanced survival of *Azospirillum* cells in the liquid formulation may be due to the action of chemical amendments added in the medium. Trehalose is capable of enhancing cell tolerance to desiccation, osmotic pressure and temperature stress (Streeter 1985) and stabilizing both enzymes and cell membranes (Fillinger *et al.,* 2001). Moreover, some polymeric additives such as

PVP,PVA and starch have polymeric properties. This protective property known as colloidal stabilization. The improvement of survival is analogous to the protective colloid effect where bacteria represent one colloid and the suspension the other (Deaker *et al.,* 2004).

Azospirillum liquid bioinoculant formulation could be produced by simple fermentation process with minimum labour, space and energy, as the culture from the fermentor is directly packed under aseptic conditions and stored. The cost of production of liquid formulation could be lesser than that of carrier formulation. From this study, it has been concluded that liquid formulation of *Azospirillum* bioinoculant has a shelf life of one year compared to the carrier based inoculant. Among the different chemical additives trehalose (10mM) performed well and hence this can be used in the formulation of liquid bioinoculant.

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