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Research Article

Survival of saline tolerant PGPR in different carriers and liquid formulations

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Abstract

In the present scenario of increasing international concern for food and environmental quality, the use of plant growth promoting rhizobacteria (PGPR) for reducing chemical inputs in agriculture is a potentially important issue. In the present study three different saline tolerant PGPR strains *viz*., *Azospirillum brasilense* PA-17, *Bacillus subtilis* PB-15 and *Pseudomonas fluorescens* PP-15 were identified from the coastal soils of Tamil Nadu. The survival of these three stains were tested on different carriers *viz*., Lignite, pressmud and vermiculite for a period of six month storage. In the same way, the survival of these three PGPR strains were tested on the liquid formulation amended with different additives such as PVP, trehalose and glycerol for a period of six months storage. The results of the study revealed that the required population (1×10^8 cells /ml) of saline tolerant strains was maintained both in carriers and in liquid based formulation. Among the carriers, lignite supported higher population followed by pressmud and vermiculite. Among the different additives tested, poly vinyl pyrollidone (PVP) at 1 % supported more population of saline tolerant PGPR upto six months of storage period without any significant reduction. The study confirmed the better survival of saline tolerant PGPR strains on both carrier based and liquid based inoculants.

Keywords: *Catharanthus roseus*, Antimicrobial activity and Phytochemical analysis.

Introduction

Biofertilizers manufactured in India presently are carrier based and they suffer from short shelf life, poor quality, high contamination and unpredictable field performances (Hegde, 2002). Rice and Olsen (1992) suggested liquid inoculation as a better method than seed treatments with carrier inoculant. Alagawadi and Gaur (1992) observed that the combined inoculation of *A.brasilense* and *B.polymyxa* or *P.striata* had significant increase in the grain yield, dry matter yield, and N and P uptake of sorghum over single inoculation.

The development of suitable formulation, which would ensure survival and protection of the strain and the application technology, which would allow timely, easy and precise delivery in the field could be a major step towards this goal (Fages, 1994). There are many other constraints in using the carriers for manufacture of biofertilizers listed by Bhattacharya and Kumar (2000) included unavailability of good carriers, supporting poor cell number, poor moisture retention capacity, bulk sterilization problem, pollution hazards from carrier dust, high transportation cost, etc.

Lignite based inoculants are widely accepted and used for seed treatment of various crops (Rasal *et al.*, 1994). Thangaraju (1996) recommended the use of decomposed coir pith with lignite or peat (1:1) for better survival of *Rhizobium*. Govindarajan (1996) studied the growth and survival of *A. lipoferum* in peat, coir pith and mixture of peat and coir pith. The peat supported higher proliferation of the inoculated organisms than other carriers. Lignite is the preferred and widely used carrier in most of the bio fertilizer

manufacturing plants all over India (Khungar, 1998). Among the four different bioinoculant carriers (paddy husk, groundnut shell, lignite and sawdust). The population was maximum in lignite at all temperatures studied (Saha *et al.,* 2001). Addition of various polymers, amendments and chemicals in both sterile and unsterile carriers resulted in increased shelf life of *A. lipoferum* (Suresh babu *et al.,* 2002).

Materials and Methods

Preparation of different carrier based inoculant

The selected isolates were multiplied in large quantities in appropriate culture broths by incubating at 28±2ºC in an incubator shaker till they attained log phase with a cell load of $1x10^{10}$ cfu ml^{-1} and used for inoculant preparation. Lignite collected from Neyveli Lignite Corporation (NLC), Neyveli, Pressmud collected from EID Parry Ltd. Nellikuppam and Vermiculite collected from Tamilnadu Minerals Ltd. Chennai were used as carriers.

The individual carrier materials were powdered and the pH was brought to neutral by adding $CaCO_3$ if necessary and sterilized at 15 psi for 1 hour and allowed to cool over night and then mixed with the log phase culture ($1x10^{10}$ cfu ml^{-1}) of the selected saline tolerant PGPR straiins *viz., A. brasilense* PA-17, *B. subtilis* PB-15 and *P. fluorescens* PP-16 individually in separate quantities of sterile carrier in shallow trays. The moisture content was adjusted to 40-45 per cent. Curing in shallow trays for 24 hrs in aseptic rooms and packed in high density opaque polythene bag (300 gauge) at the rate of 200 g bag^{-1} and sealed. Individual inoculant was prepared by mixing equal volumes of each culture broth with sterile carrier and placed in polythene bags, and were stored at room temperature for a period of six months. The surviving population of saline tolerant PGPR was estimated at monthly intervals upto a period of six months. The population of halotolerant PGPR strains in the carriers were assessed at monthly intervals upto the period of six months.

Preparation of different liquid based inoculants

Nitrogen free malate broth, Nutrient broth, and King's B broth were prepared for *Azospirillum*, *Bacillus* and *Pseudomonas* respectively which was mixed in combination with different additives such as PVP, trehalose and glycerol to increase the survival of saline tolerant PGPR *viz*., *A. brasilense* PA-17, *B. subtilis* PB-15 and *P. fluorescens* PP-16 in a liquid formulation. To standardize the optimum quantity of the chemical amendments, Glycerol (5 mM), Trehalose (10 mM) and polyvinyl pyrollidone (PVP) at (1 %) were added to one litre of respective broth separately. One ml of log phase culture of *Azospirillum*, *Bacillus* and *Pseudomonas* were inoculated as single inoculant in respective broth and the flasks were incubated at room temperature. The formulation was analyzed for viable cell population at 1 month interval upto 6 months.

Results and Discussion

The survival of efficient saline tolerant PGPR isolates *viz., Azospirillum brasilense* PA-17, *Bacillus subtilis* PB-15 and *Pseudomonas fluorescens* PP-16 which was obtained in the present study was tried with different carrier materials *viz*., lignite, pressmud and vermiculite (Table – 1). The initial population of *Azospirillum brasilense* PA-17 was 74.22×10^8 cfu g^{-1}, 75.82×10^8 cfu g^{-1} and 75.85×10^8 cfu g^{-1} in lignite, pressmud and vermiculite respectively. While, the corresponding strains *Bacillus subtilis* PB-15 and *Pseudomonas fluorescens* PP-16 was 75.85×10^8 cfu g^{-1} and 54.22×10^8 cfu g^{-1} in lignite, 56.23×10^8 cfu g^{-1} and 41.68×10^8 cfu g^{-1} in pressmud and 67.60×10^8 cfu g^{-1} and 65.60×10^8 cfu g^{-1} in vermiculite, respectively. The surviving population of PGPR strains during 6^{th} month of storage was 2.33×10^8 cfu g^{-1} in lignite, 1.81×10^8 cfu g^{-1} in pressmud and 1.73×10^8 cfu g^{-1} in vermiculite for *Azospirillum brasilense* PA-17 followed by *Bacillus subtilis* PB-15 and *Pseudomonas fluorescens* PP-16 was 2.63×10^8 cfu g^{-1} and 1.88×10^8 cfu g^{-1} in lignite, 2.18×10^8 cfu g^{-1} and 1.69×10^8 cfu g^{-1} in pressmud and 1.81×10^8 cfu g^{-1} and 1.69×10^8 cfu g^{-1} in vermiculite, respectively.

Sangeetha and Stella (2012) and Sivasakthivelan and Saranraj (2013) studied the survival of PGPR isolates on different carrier materials. The carrier based PGPR consortium with four selected strains *viz*., *Azospirillum lipoferum*, *Azotobacter chroococcum*, *Bacillus megaterium*, *Pseudomonas fluorescens* was prepared and the shelf life for each inoculants was studied upto six months of storage.

The survival of efficient saline tolerant PGPR isolates *viz., Azospirillum brasilense* PA-17, *Bacillus subtilis*

PB-15 and *Pseudomonas fluorescens* PP-16 was studied in liquid formulation amended with poly vinyl pyrollidone, trehalose and glycerol (Table – 2). The initial population of PGPR strain *Azospirillum brasilense* PA-17 was 75.85 × 10^8 cfu g^{-1}, 72.04 × 10^8 cfu g^{-1} and 64.56 × 10^8 cfu g^{-1} in poly vinyl pyrollidone, trehalose and glycerol respectively. While, the corresponding strains *Bacillus subtilis* PB-15 and *Pseudomonas fluorescens* PP-16 was (72.44 × 10^8 cfu g^{-1} and 70.79 × 10^8 cfu g^{-1}) in polyvinyl pyrollidone (74.13 × 10^8 cfu g^{-1} and 64.56 × 10^8 cfu g^{-1}) in trehalose and (70.79 × 10^8 cfu g^{-1} and 72.62 × 10^8 cfu g^{-1}) in glycerol, respectively. The surviving population of PGPR strains during 6th month of storage was 07.94 × 10^8 cfu g^{-1} in poly vinyl pyrollidone, 06.30 × 10^8 cfu g^{-1} in trehalose and 07.24 × 10^8 cfu g^{-1} in glycerol for *Azospirillum brasilense* PA-17. Followed by *Bacillus subtilis* PB-15 and *Pseudomonas fluorescens* PP-16 was (07.76 × 10^8 cfu g^{-1} and 07.07 × 10^8 cfu g^{-1}) in poly vinyl pyrollidone (07.07 × 10^8 cfu g^{-1} and 06.91 × 10^8 cfu g^{-1}) in trehalose and (07.07 × 10^8 cfu g^{-1} and 07.41 × 10^8 cfu g^{-1}) in glycerol, respectively.

Table – 1: Survival of saline tolerant PGPR on different carrier materials

PGPR isolates	Inoculant population (Number of cfu x 10^8 g^{-1})						
	Initial	1st Month	2nd Month	3rd Month	4th month	5th Month	6th Month
	Lignite						
A. brasilense (PA-17)	74.22 (9.87)	72.55 (9.86)	63.09 (9.79)	22.66 (9.35)	16.22 (9.21)	5.33 (8.72)	2.33 (8.36)
B. subtilis (PB-15)	75.85 (9.87)	72.44 (9.85)	63.09 (9.79)	21.37 (9.32)	15.13 (9.17)	10.00 (9.00)	2.63 (8.14)
P. fluorescens (PP-16)	54.22 (9.73)	53.00 (9.72)	45.66 (9.65)	12.22 (9.08)	6.83 (8.83)	3.44 (8.53)	1.88 (8.27)
SEd	0.695	0.649	0.581	0.328	0.295	0.194	0.021
CD(p=0.05)	1.391	1.298	1.163	0.657	0.591	0.289	0.043
	Pressmud						
A. brasilense (PA-17)	75.82 (9.87)	74.13 (9.86)	52.48 (9.71)	33.88 (9.88)	20.89 (9.31)	17.37 (9.23)	1.81 (8.25)
B. subtilis (PB-15)	56.23 (9.74)	53.70 (9.72)	33.88 (9.52)	25.11 (9.39)	10.47 (9.01)	4.07 (8.60)	2.18 (8.33)
P. fluorescens (PP-16)	41.68 (9.61)	38.01 (9.57)	26.30 (9.41)	10.00 (9.00)	7.94 (8.89)	3.16 (8.49)	1.69 (8.22)
SEd	0.990	1.045	0.777	0.697	0.396	0.459	0.041
CD(p=0.05)	1.981	2.091	1.555	1.395	0.793	0.919	0.082
	Vermiculite						
A. brasilense (PA-17)	75.85 (9.87)	74.13 (9.86)	39.81 (9.59)	18.19 (9.25)	7.41 (8.86)	2.63 (8.41)	1.73 (8.23)
B. subtilis (PB-15)	67.60 (9.82)	63.09 (9.79)	46.77 (9.66)	10.96 (9.03)	3.98 (8.59)	2.45 (8.38)	1.81 (8.25)
P. fluorescens (PP-16)	65.60 (9.81)	56.23 (9.74)	13.18 (9.11)	7.94 (8.89)	3.16 (8.49)	2.18 (8.33)	1.69 (8.22)
SEd	0.275	0.521	1.023	0.304	0.130	0.013	0.003
CD(p=0.05)	0.551	1.042	2.046	0.609	0.261	0.026	0.007

Table – 2: Survival of saline tolerant PGPR in liquid formulation with different chemical additives

PGPR isolates	Inoculant population (Number of cfu x 10^8 g^{-1})						
	Initial	1st Month	2nd Month	3rd Month	4th month	5th Month	6th Month
	Poly vinyl pyrollidone						
A. brasilense (PA-17)	75.85 (9.87)	40.73 (9.60)	29.51 (9.46)	14.79 (9.16)	09.77 (8.98)	08.91 (8.94)	07.94 (8.88)
B. subtilis (PB-15)	72.44 (9.85)	42.65 (9.62)	32.35 (9.50)	17.78 (9.24)	13.18 (9.11)	09.33 (8.96)	07.76 (8.89)
P. fluorescens (PP-16)	70.79 (9.84)	54.95 (9.73)	32.35 (9.50)	19.45 (9.28)	10.47 (9.01)	08.51 (8.92)	07.07 (8.84)
SEd	0.148	0.445	0.094	0.136	0.103	0.023	0.026
CD(p=0.05)	0.296	0.891	0.189	0.273	0.207	0.047	0.053
	Trehalose						
A. brasilense (PA-17)	72.04 (9.85)	50.11 (9.69)	28.18 (9.44)	15.84 (9.19)	09.77 (9.98)	07.41 (8.86)	06.30 (8.79)
B. subtilis (PB-15)	74.13 (9.86)	33.11 (9.51)	24.54 (9.38)	11.22 (9.04)	09.33 (8.96)	07.94 (8.89)	07.07 (8.84)
P. fluorescens (PP-16)	64.56 (9.80)	46.77 (9.66)	16.98 (9.22)	12.58 (9.09)	09.54 (8.97)	07.76 (8.88)	06.91 (8.83)
SEd	0.290	0.520	0.329	0.137	0.012	0.015	0.023
CD(p=0.05)	0.581	1.041	0.659	0.275	0.015	0.031	0.047
	Glycerol						
A. brasilense (PA-17)	64.56 (9.80)	71.68 (9.85)	25.70 (9.40)	14.79 (9.16)	09.54 (8.97)	08.31 (8.91)	07.24 (8.85)
B. subtilis (PB-15)	70.79 (9.89)	40.73 (9.60)	22.38 (9.34)	11.74 (9.06)	09.12 (8.95)	07.76 (8.88)	07.07 (8.84)
P. fluorescens (PP-16)	72.62 (9.88)	46.77 (9.66)	26.97 (9.43)	15.84 (9.19)	09.77 (8.98)	08.12 (8.90)	07.41 (8.86)
SEd	0.377	0.947	0.136	0.122	0.019	0.016	0.009
CD(p=0.05)	0.756	1.895	0.273	0.245	0.038	0.033	0.019

Suresh Babu *et al.* (2002) found higher population of *Azospirillum* due to the addition of PVP at both 1 and 1.5% 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. 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).

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 and stabilizing both enzymes and cell membranes. Moreover, some polymeric additives such as PVP, PVA and starch have polymeric properties. 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). 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 of storage under room temperature.

Kumaresan and Reetha (2011) evaluated the different concentrations of chemical amendments *viz*., Gum arabica, 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.

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