Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; SP2: 545-548

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Studies on developing PGPR consortium with improved shelf life

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Abstract
Plant growth promoting rhizobacteria (PGPR) are soil bacteria with some beneficial effects on soil properties. Plant growth promoting rhizobacteria are beneficial soil bacteria that colonize plant roots and enhance plant growth promotion activity by different mechanisms in various ways. The use of bio inoculants forms one of the vital components for a long-term sustainable agriculture system of any crop. Considering the potential of plant growth promoting rhizobacteria the knowledge on the association of PGPR will be of immense help for standardizing microbial inoculants to enhance the crop yield. In the present study an attempt has been made to develop a suitable consortium in a suitable formulation with improved shelf life to use as a prudential bio inoculants. An attempt was made to develop carrier based biofertilizer by using different isolates of PGPR viz., *A. lipoferum* TMAzs-13, *P. fluorescents* TMPs-19 + *B. megaterium* TMB-3 obtained in the present study with lignite, pressmud. The survivability was studied by monthly viable cell count of PGPR strains in single, dual inoculants, as well as in the consortium prepared with lignite and pressmud carrier materials upto six months of storage. The effect of different concentrations (1% and 2%) of Poly vinyl pyrollidone (PVP) on the survival of PGPR strains in lignite carrier was investigated.

Keywords: PGPR, Carrier Materials, Poly vinyl pyrollidone

Introduction
PGPR have been applied to various crops that enhance the growth, seed emergence and crop yield and some have been commercialized (Dey *et al.*, 2004; Herman *et al.*, 2008; Minorsky 2008) [4]. PGPR organisms like *Azospirillum*, *Azotobacter*, *Pseudomonas* and *Bacillus* that have been shown to colonize the roots of various plants, and to increase the height, flower number, fruit number and total weight of tomato plants (Minorsky, 2008) [4].

A frequent observation is that in carrier based inoculants, the number of viable cells decreases from 10^9 to 10^7 colony forming units (CFU) per g after 90 days of storage (Okon *et al.*, 1995). The most consistent feedback received from the farmers and inoculants producers is the concern about the the shelf life of carrier based inoculants, since they have shorter shelf life, which hardly ends beyond three to four month under normal storage conditions. The development of adequate formulations, which would ensure survival, 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 their goal. Hence techniques to increase the shelf life of inoculants thus become necessary to propagate their technology in large scale.

The use of bio inoculants forms one of the vital components for a long-term sustainable agriculture system of any crop (Tilak *et al.*, 2005) [12]. Considering the potential of Plant growth promoting rhizobacteria the knowledge on the association of PGPR will be of immense help for standardizing microbial inoculants to enhance the crop yield. In the present study an attempt has been made to develop a suitable consortium in a suitable formulation and studies to increase the shelf life of inoculants to use as a prudential bio inoculants for betterment of tomato.

Efficient strains of nitrogen fixing and phosphate solubilizing microorganisms are mass multiplied under laboratory condition and mixed with a carrier. These carrier based inoculants are supplied to farmers for crop inoculation. Carrier is a medium or matrix on which inoculant microorganisms grow to a reasonably higher population for an initial period and thereafter decline. The nature of the carrier often determines the subsequent performance of the inoculant. The criteria for a good carrier material are no toxicity to the introduced microorganisms, good absorption capacity, suitable pH, and fine particle size for better adherence to seed, good water holding capacity and availability of materials at cheaper cost. Different carriers have been tested and used for inoculation throughout the world. Some of the carriers used by different manufactures in the country and abroad are peat, lignite, vermiculite,

charcoal, pressmud, coal, polyacrylamide and alginates. The carrier based inoculant improves their shelf life and efficiency of biofertilizers (Palaniappan and Arangarasan, 1998) [1].

Lignite is the preferred and widely used carrier in most of the biofertilizer manufacturing plants all over India (Khungar, 1998) [3]. 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) [6]. Addition of various polymers, amendments and chemicals in both sterile and unsterile carriers resulted in increased shelf life of *A. lipoferum* (Sureshbabu *et al.*, 2002) [11].

Gopal (2004) [2] showed the better shelf life and effectiveness of lignite based rhizobacterial inoculant than other carrier based inoculants on Ashwagandha a commercially grown medicinal plant. Narendranath *et al.* (1996) [5] reported that higher survival of groundnut *Rhizobium* in peat followed by pressmud and lignite and suggested pressmud amended with soymeal as an alternative carrier to peat in inoculant preparation. Tilak and Subba Rao (1978) found that soil + FYM in 1:1 proportion had higher *Azospirillum* count followed by soil + FYM + Vermiculite in 5:3:2 proportion.

The addition of polymers and amendments improved the survival of *Azospirillum*. The hydrophilic polymers, Jalsakthi and terra cotton both at 2% and 4% levels and amendments, polyvinyl pyrrolidone (PVP) and skim milk at 1% and 2% levels were found suitable for retaining moisture and favouring the survival of *Azospirillum* cells in both sterilized and un-sterilized carrier based inoculants (Santhanakrishnan and Thangaraju, 2002) [9].

Sobhan Ardakani *et al.* (2010) [10] studied the formulation included a talc powder and bentonite-based powder as inorganic carriers for peat and rice bran as organic carriers for increasing stability in interaction between associated *Pseudomonas fluorescens* in different treatments for significantly promoting seedling height, root length, seedling dry weight and root dry weight of cotton seedling.

Saha *et al.* (2001) [6] studied the survival of *Rhizobium* in the paddy husk as carrier at different temperature using green fluorescent protein marker and reported that survival rate of *Rhizobium* transformant is less at higher temperature above 28ºC.

Sangeetha and Stella (2012) [8] reported the survival of plant growth promoting bacteria *viz.*, *Azospirillum lipoferum* VAZS-18, *Azotobacter chroococcum* VAZB-6, *Bacillus megaterium* VBA-2 and *Pseudomonas fluorescens* VPS-19 inoculants on different carrier materials *viz.*, lignite, vermiculite, pressmud and alginate bead based on the consortium treatments to survival of population at 25°C and 30°C upto six month storage period, respectively.

Materials and Methods

The rhizosphere soil samples of tomato were used for isolation and enumeration of *Azospirillum*, *Azotobacter*, *Pseudomonas* and *Bacillus* (phosphate solubilzing bacteria). Based on the efficiency of the plant growth promoting traits, *Azospirillum lipoferum* TMAzs-13, *Pseudomonas fluorescens* TMPs-19 and *Bacillus megaterium* TMB-3 were selected for developing consortium and inoculant production

Influence of Storage Temperature on the Survival of PGPR Strains in the Pressmud Carrier

Influence of storage temperature on the survival of PGPR strains in pressmud carrier was investigated and the results are given in Table 2. The inoculant population of PGPR strains in pressmud at room temperature was high after one month of packaging in single inoculant, dual inoculant and in consortium. The inoculant population was more at room temperature followed by 30°C, 40°C and 50°C. The inoculant population was maximum in the consortium (74.18×10^8 cfu g^{-1} for *Azospirillum lipoferum* TMAzs-13 + *Pseudomonas fluorescens* TMPs-19+*Bacillus megaterium* TMB-3) followed by the dual inoculant and single inoculant. The surviving population of PGPR strains in pressmud prepared with single, dual and consortium significantly reduced at 50°C.

Survival of plant growth promoting Rhizobacterial Inoculants in Different Carrier Materials

Based on the results of the performance of the plant growth promoting traits, three different PGPR isolates *viz.*, *Azospirillum lipoferum* TMAzs-13, *Pseudomonas fluorescens* TMPs-19 and *Bacillus megaterium* TMB-3 were selected for further studies. The survival of the above selected PGPR isolates was estimated in different carriers such as lignite, pressmud.

Preparation of carrier based inoculants

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 $1x10^9$ cfu ml^{-1} and were used for inoculants preparation. Lignite collected from Neyveli Lignite Corporation (NLC), Neyveli, Pressmud collected from EID Parry Ltd. Nellikuppam 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 h and allowed to cool over night and then mixed with the log phase culture ($1x10^9$ cfu ml^{-1}) of the selected plant growth promoting rhizobacterial isolates *viz.*, *A. lipoferum* TMAzs-13, *P. fluorescens* TMPs-19 and *B. megaterium* TMB-3 individually in separate quantities of sterile carrier in shallow trays. The moisture content was adjusted to 30-35 per cent. Curing in shallow trays for 24 h in aseptic rooms and packed in polythene bag (300 gauge) at the rate of 200 g bag^{-1} and sealed. Individual inoculants was prepared by mixing equal volumes of each culture broth with sterile carrier and combined inoculants was also prepared by mixing equal volumes of broth with the carrier materials. The populations of individual plant growth promoting bacteria in the inoculants carriers were assessed at monthly intervals up to six months.

Preparation of carrier based inoculants with different chemical amentmends

Nitrogen free malate broth, King's B broth and Nutrient broth were prepared for *Azospirillum*, *Pseudomonas* and *Bacillus* respectively which was mixed in combination with additives to increase the survival of plant growth promoting rhizo bacteria in different carriers. To standardize the optimum quantity of the chemical amendments, polyvinyl pyrollidone (PVP) were used as with 1% and 2% concentration. Individual inoculant was prepared by mixing equal volumes of each culture broth with sterile carrier and dual and consortium inoculant was also prepared by mixing equal volumes of broth with the carrier materials. The populations of individual, dual and consortium of plant growth promoting rhizobacteria in the carriers were analyzed for viable cell population at 1 month interval upto 12 month.

Results and Discussion

Influence of storage temperature on the survival of PGPR strains in lignite carrier

Influence of storage temperature on the survival of PGPR in lignite carrier was tested and the results are furnished in Table 1. The inoculant population of PGPR strains in lignite at room temperature was high after one month of packaging in single inoculant, dual inoculant and in consortium. The inoculant population was more at room temperature followed by 30°C, 40°C and 50°C. The inoculant population was maximum in the consortium (78.13×10^8 cfu g^{-1} for *Azospirillum lipoferum* TMAzs-13 + *Pseudomonas fluorescens* TMPs-19+*Bacillus megaterium* TMB-3) followed by the dual inoculant and single inoculant. The surviving population of PGPR strains in lignite prepared with single, dual as well as consortium significantly reduced at 50°C.

The carrier based inoculants of bacterial biofertilizer consortium have got several advantages such as increased shelf life, protection from adverse conditions, better survival on seed, etc. The carrier based individual inoculant effect on several crop plants has been studied (Rasal *et al*., 1994). The physico-chemical characters of carrier materials have got profound influence on the survival of inoculants. The ideal characteristics of an inoculant carrier include more surface area, rich in organic matter, high water holding capacity, neutral pH, easy availability and inexpensiveness (Arangarasan *et al.,* 1998) [1].

Formulation step is a crucial aspect for producing microbial inoculants and determines the success of a biological agent. Formulation typically consists of establishing viable bacteria in a suitable carrier together with additives that aid in stabilization and protection of microbial cell during storage, transport and at the target. The formulation should also be easy to handle and apply so that it is delivered to target in most appropriate manner and form, one that protects bacteria from harmful environmental factors and maintain or enhance the activity of the organisms in the field. Therefore, several critical factors including user preference have to be considered before delivery of a final product (Xavier *et al*., 2004) [13].

The factors that affect the longevity of the cells of bioinoculants include temperature, moisture, carrier material etc. The optimum moisture level of 35 per cent and a temperature of 30°C are required for maximum survival of the cells in the carrier based inoculants for longer period of storage and were found that up to 40°C there was no serious mortality (Bajpai *et al*., 1978; Sangeetha and Stella, 2012) [8].

Table 1: Influence of storage temperature on the survival of PGPR strains in lignite carrier

Storage Temperature	Inoculant population (Number of cfu × 10^8 g^{-1} of Lignite)						
	A. lipoferum TMAzs-13		*P. fluorescens* TMPs-19		*B. megaterium* TMB-3		Consortium
	Single Inoculant	Dual Inoculant	Single Inoculant	Dual Inoculant	Single Inoculant	Dual Inoculant	
Room Temperature	76.65 (9.88)	74.79 (9.87)	65.48 (9.81)	62.44 (9.79)	66.70 (9.82)	63.44 (9.80)	78.13 (9.89)
30°C	62.23 (9.79)	60.09 (9.78)	57.86 (9.76)	55.09 (9.74)	53.66 (9.73)	50.25 (9.70)	68.79 (9.85)
40°C	56.70 (9.75)	54.48 (9.73)	38.65 (9.58)	35.70 (9.55)	36.68 (9.56)	32.81 (9.51)	60.25 (9.77)
50°C	42.90 (9.63)	39.65 (9.59)	32.01 (9.50)	28.81 (9.45)	26.88 (9.42)	24.15 (9.38)	51.68 (9.71)
SED	0.03	0.03	0.01	0.01	0.05	0.07	0.01
CD (p=0.05)	0.08	0.08	0.04	0.04	0.08	0.18	0.03

Values in parenthesis are $\log_{10}$ transformed values

Table 2: Influence of storage temperature on the survival of PGPR strains in pressmud carrier

Storage Temperature	Inoculant population (Number of cfu × 10^8 g^{-1} of Pressmud)						
	A. lipoferum TMAzs-13		*P. fluorescens* TMPs-19		*B. megaterium* TMB-3		Consortium
	Single Inoculant	Dual Inoculant	Single Inoculant	Dual Inoculant	Single Inoculant	Dual Inoculant	
Room Temperature	72.44 (9.86)	70.13 (9.84)	60.79 (9.78)	58.44 (9.76)	57.18 (9.75)	55.09 (9.74)	74.18 (9.87)
30°C	58.18 (9.76)	56.20 (9.75)	53.56 (9.73)	50.65 (9.70)	48.09 (9.68)	46.54 (9.66)	64.06 (9.80)
40°C	52.11 (9.71)	50.54 (9.70)	35.86 (9.55)	31.65 (9.50)	34.95 (9.54)	28.68 (9.45)	55.65 (9.74)
50°C	38.81 (9.59)	35.65 (9.55)	28.30 (9.45)	26.01 (9.41)	24.11 (9.38)	21.81 (9.33)	47.81 (9.67)
SED	0.01	0.05	0.02	0.02	0.02	0.04	0.02
CD (p=0.05)	0.02	0.08	0.04	0.05	0.06	0.07	0.06

Values in parenthesis are $\log_{10}$ transformed values

Influence of storage temperature on the survival of PGPR strains in the pressmud carrier

Influence of storage temperature on the survival of PGPR strains in press mud carrier was investigated and the results are given in Table 2. The inoculant population of PGPR strains in press mud at room temperature was high after one month of packaging in single inoculant, dual inoculant and in consortium. The inoculant population was more at room temperature followed by 30°C, 40°C and 50°C. The inoculant population was maximum in the consortium (74.18×10^8 cfu g^{-1} for *Azospirillum lipoferum* TMAzs-13 + *Pseudomonas fluorescens* TMPs-19 + *Bacillus megaterium* TMB-3) followed by the dual inoculant and single inoculant. The surviving population of PGPR strains in press mud prepared with single, dual and consortium significantly reduced at 50°C.

Effect of different concentrations of Poly vinyl pyrollidone (PVP) on the survival of PGPR in lignite carrier

The effect of different concentrations (1% and 2%) of Poly

vinyl pyrollidone (PVP) on the survival of PGPR strains (*Azospirillum lipoferum* TMAzs-13, *Pseudomonas fluorescens* TMPs-19 and *Bacillus megaterium* TMB-3) was investigated and the findings are given in Table 3. The PGPR population was high in consortium at 1% of Poly vinyl pyrollidone (PVP) concentration (80.43×10^8 cfu g^{-1} at initial storage period and 70.13×10^8 cfu g^{-1} at 6^{th} month) followed by the 2% of Poly vinyl pyrollidone (PVP) (78.62×10^8 cfu g^{-1} at initial storage period and 69.44×10^8 cfu g^{-1} at 6^{th} month). The PGPR population was high in the consortium followed by the dual inoculation and single inoculation. The inoculant populations was high during the initial stage of storage and there after reduced on further storage, the cell populations were not significantly decreased up to six months of storage.

Kumaresan and Reetha (2011) evaluated the different concentrations of six different chemical amendments *viz*., polyvinyl pyrollid one (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 N2 free malic acid broth during the storage. 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 108 cells/ml upto 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.

Table 3: Effect of different concentration of Poly vinyl pyrollidone (PVP) on the survival of PGPR strains in lignite carrier

Population of inoculant (Number of cfu × 10^8 ml^{-1})								
Conc. of PVP	**Storage period in Months**	***A. lipoferum* TMAzs-13**	***P. fluorescens* TMPs-19**	***B. megaterium* TMB-3**	***A.lipoferum* TMAzs-13+ *P. fluorescens* TMPs-19**	***A.lipoferum* TMAzs-13 + *B. megaterium* TMB-3**	***P. fluorescens* TMPs-19 + *B. megaterium* TMB-3**	***A. lipoferum* TMAzs-13+ *P. fluorescens* TMPs-19+ *B. megaterium* TMB-3**
1% of PVP	Initial	(75.85) 9.82	(72.44) 9.86	(70.13) 9.84	(73.13) 9.86	(77.62) 9.89	(76.62) 9.89	(80.43) 9.90
	1st	(66.65) 9.64	(62.23) 9.79	(60.65) 9.78	(72.79) 9.86	(70.79) 9.85	(68.44) 9.84	(77.62) 9.89
	2nd	(55.11) 9.74	(53.65) 9.73	(51.41) 9.71	(67.60) 9.83	(65.06) 9.81	(63.18) 9.80	(76.62) 9.88
	3rd	(46.21) 9.66	(44.54) 9.65	(40.47) 9.60	(63.09) 9.80	(61.09) 9.79	(60.06) 9.79	(74.85) 9.87
	4th	(38.91) 9.59	(36.71) 9.56	(34.70) 9.54	(58.88) 9.77	(56.25) 9.75	(54.56) 9.74	(72.85) 9.86
	5th	(27.94) 9.44	(25.12) 9.40	(23.76) 9.37	(54.95) 9.74	(52.54) 9.72	(50.09) 9.70	(72.13) 9.86
	6th	(17.02) 9.23	(14.24) 9.15	(12.91) 9.11	(50.11) 9.70	(48.95) 9.67	(46.25) 9.66	(70.13) 9.84
2% of PVP	Initial	(74.13) 9.87	(64.56) 9.81	(62.62) 9.80	(76.44) 9.88	(74.13) 9.87	(73.85) 9.86	(78.62) 9.89
	1st	(52.81) 9.72	(50.97) 9.71	(46.77) 9.67	(65.18) 9.81	(64.60) 9.81	(62.79) 9.79	(76.62) 9.88
	2nd	(40.11) 9.72	(38.41) 9.58	(35.21) 9.55	(60.56) 9.78	(58.09) 9.76	(56.60) 9.75	(75.85) 9.88
	3rd	(34.22) 9.53	(31.23) 9.49	(28.77) 9.46	(58.25) 9.77	(55.65) 9.74	(53.56) 9.73	(74.85) 9.87
	4th	(23.54) 9.37	(20.31) 9.31	(17.41) 9.24	(56.54) 9.75	(53.88) 9.73	(50.09) 9.70	(73.85) 9.87
	5th	(14.12) 9.15	(12.41) 9.09	(10.76) 9.03	(52.70) 9.72	(50.23) 9.70	(48.65) 9.67	(73.13) 9.86
	6th	(10.60) 9.02	(08.60) 8.93	(06.30) 8.80	(48.11) 8.61	(45.95) 9.66	(43.88) 9.64	(72.44) 9.85
SED		0.01	0.01	0.01	0.01	0.01	0.01	0.01
CD (p=0.05)		0.04	0.04	0.03	0.02	0.02	0.03	0.02

Values in parenthesis are log_{10} transformed values

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