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RESEARCH PAPER

Growth of *Pseudomonas striata* in liquid formulations as influenced by different concentration of additives, adjuvants and surfactants

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Abstract: An attempt was made to develop liquid formulations of *Pseudomonas striata*, a phosphate solubilizing bacteria using different concentrations of additives, adjuvants, surfactants and to study its survival on seeds. The results indicated that growth of *Pseudomonas striata* was higher in liquid formulations compared to unamended basal medium. The promising liquid formulation was used to treat maize and sorghum seeds at concentrations of 2 mL, 4 mL 6 mL and 8 mL kg^{-1} seed and the treatment effect was compared with treatment of seeds with lignite based formulation @ 20 g kg^{-1}. Liquid formulation treated @ 2 ml kg^{-1} maize and 4 ml kg^{-1} sorghum seeds resulted in higher mean viable population of $\log_{10}$ 6.72 CFU $seed^{-1}$ and $\log_{10}$ 5.36 CFU $seed^{-1}$ respectively. The least viable population was seen in treatment with lignite based formulation recording population of $\log_{10}$ 4.72 CFU ml^{-1} $seed^{-1}$ on maize seeds and $\log_{10}$ 2.508 CFU ml^{-1} $seed^{-1}$ on sorghum seeds respectively. The cost involved in production of the best formulation was rupees 149 $litre^{-1}$. This cost included the cost of chemicals, cell protectants, packing material, depreciation cost of instruments, electricity cost and the labour cost. Lignite based formulation coasted rupees 60 kg^{-1}, and was inoculated @ 20 g kg^{-1} seed. The cost of this technology was about rupees 1.2 kg^{-1} seed. As only 2 to 4 ml kg^{-1} seed of promising liquid formulation was required the total cost of using this technology was less than one rupees per kg seeds.

Key words: Cell protectants, Phosphorus, Solubilizer, Viable population

Introduction

Phosphorus is an essential element for plant growth and development, next only to nitrogen. Because of its sparingly soluble nature it is present in very less proportion in the soil for plant uptake (Rajesh, *et al.*, 2013). Phosphorus solubilizing microorganisms (PSM) are found useful especially to make P available to plants through solubilization. PSM can mineralize organic phosphorus into soluble form by their enzymatic activity and help to save P_2O_5 up to 30-50 kg/ha (Pindi and Satyanarayana, 2012). *Pseudomonas striata* is one of the popularly used P-solubilizing bacteria (PSB). This bacterium produces a variety of organic acids, including acetic, gluconic, formic, and propionic acids (Sharon**,** *et al.*, 2016). Use of PSB for mobilizing P is very cost effective considering the cost of soluble P fertilizers. Besides, mobilizing P the PSB are known to enhance the productivity of crop plants when inoculated.

Presently, biofertilizers are carrier-based (solid), with a shelf life of only six months. They are not tolerant to UV rays and temperatures of more than 30 degrees (Santhosh, 2015). Carrier based biofertilizers have some limitations such as short-shelf life, non-availability of good quality carrier material locally, more labour intensive and high transportation cost (Surendra and Akhila, 2016). Because of these constraints there is a need to develop an alternate formulation for wider application and commercialization of biofertilizer technology. Liquid bioinoculants are special formulations containing not only the desired microorganisms and their nutrients, but also possess, special cell protectants or substances that encourage the longer shelf life and tolerance to adverse conditions (Vora *et al.*, 2008). Some efforts have been made worldwide, to develop liquid formulations of phosphate solubilizing microorganisms. However, a concerted effort to determine the effective concentration of additives, adjuvants and surfactants to develop an effective formulation appears to be lacking. The present investigation aimed at evaluating the influence of different concentration of additives, adjuvants and surfactants on growth of *Pseudomonas striata* a phosphate solubilizing microorganism.

Material and methods

Liquid formulation of *Pseudomonas striata*, a P-solubilizing bacterium, was initiated at the Institute of Organic Farming, University of Agricultural Sciences, Dharwad during the year 2015.

Growth media

Pikovskaya's broth containing Glucose (10 g L^{-1}), Tri-calcium phosphate (5 g L^{-1}), $MgSO_4.7H_2O$ (2.5% solution @ 10 ml L^{-1}), $CaCl_2$ (1% solution @ 10 ml L^{-1}), Yeast extract powder (0.5 g L^{-1}), distilled water (1000 ml) adjusted to pH 7 were used to culture and mass multiply *P. striata*. All chemicals used were of analytical grade.

Screening for different concentrations of additives, adjuvants and surfactants

Pikovskaya's broth was amended with different concentration of additive, adjuvants and surfactants as shown in Table 1. A total of 32 different liquid formulations were developed and screened. The experiment was carried out in 250 ml Erlenmeyer flasks containing 100 ml Pikovskaya's broth

Table 1. Concentration of cell protectants, additives, adjuvants and surfactants used to develop liquid formulation

Different amendment used		Concentrations used in the final formulation (w/v)	
Additive	Glycerol (mM)	5	10
	Per cent Polyethylene glycol (PEG, MW 4000)	0.5	1
Adjuvants	Per cent Corboxy methyl cellulose (CMC)	0.05	0.1
	Per cent Gum arabica (GA)	0.15	0.3
Surfactants	Polysorbate 20 (ppm)	125	250

amended with cell protectants like additives, adjuvants and surfactants. A loopful of *P. striata* grown for 24 hr containing 4.4 x 10^8 CFU ml^{-1} was inoculated separately at the rate of five per cent to freshly prepared formulations and grown in an incubator shaker (100 rpm) at 30 ± 1^0C for 84 hr. The viable cell populations in the formulations were determined at 24 hr interval up to 84 hr by employing the standard dilution plate count method using Pikovskaya's agar **.**

Survival of *Pseudomonas striata* on seeds

Out of 32 formulations, the liquid formulation 18, that showed higher population (190 x 10^{10} CFU ml^{-1}) was selected for seed treatment (Table 2)**.** Prior to treatment, the seeds were surface sterilized with 70 per cent alcohol for one minute, followed by sodium hypochlorite (1% active chlorine) for three minutes. Later, seeds were washed six times with sterile distilled water. The seeds were allowed to dry under laminar air flow and then treated with promising formulation of *Pseudomonas striata* at the rate of 2, 4, 6 and 8 ml kg^{-1} of seeds and seeds treated with lignite based formulation at the rate of 20 g kg^{-1} seed served as check.

Immediately after seed treatment, ten treated seeds were randomly picked and placed in the conical flask containing 10 ml sterile distilled water and vertexed for 10 minute and viable count per ml of the diluent was determined by following serial dilution and standard plate count method. In the same manner, other ten seeds were assayed at 12, 24, 36, and 48 hr after treatment. Finally, the viable count per seed was calculated by following the procedure as described by Sridhar *et. al.* (2004).

Cost of developed liquid formulations

The cost in rupees involved in production of promising liquid formulation of *Pseudomonas striata* was calculated based on the costs of ingredients used, depreciation cost on the laboratory instruments, utilities cost and cost of labour involved.

All the data were analyzed statistically and the means were separated by the Duncan's Multiple Range Test (DMRT) at five per cent level of significance (Little and Hills, 1978).

Results and discussion

Among the 32 different liquid formulations screened, 18 formulations showed an excellent cell retention as compared to the un-amended control and all the other liquid formulations. Out of these 18 formulations, the one with 10 mM glycerol, 0.5 per cent PEG, 0.05 per cent CMC, 0.15 per cent GA and 250 ppm polysorbate 20 (Formulation No.18) recorded the highest population of 19 x 10^{11} CFU mL^{-1} after 84 hr of inoculation. This could have been achieved due to the favorable environment for growth and survival of *Pseudomonas striata* created by the combination of the amendments used. The environment probably protected cells of *Pseudomonas striata* from stresses and help their establishment in the formulation as reported in similar earlier works (Albareda *et al.*, 2008; Mugilan *et al.*, 2011). These amendment are also known to enhance cell tolerance to desiccation, osmotic pressure and temperature stress and thus stabilizing both enzymes and cell membranes (Kavi and Reetha, 2014). Possibly, because of all these favorable conditions in the formulation 18, the population of *Pseudomonas striata* was about 200 times higher than that recorded in unamended basal medium (8 x 10^9 CFU ml^{-1}) after 84 hr of incubation (Table 2). Earlier Daniel *et al.* (2013), demonstrated that some constituents in liquid biofertilizers such as CMC @ 0.1 per cent, and polysorbate 20 @ 0.025 per cent had excellent cell retention property. Kumaresan and Reetha (2011) also reported that, liquid *Azospirillum* bioinoculant formulated with glycerol (10 mM), and gum arabica (0.3 %) promoted upto 11 months survival of *Azospirillum* with a population of 10^8 cell mL^{-1}. In the present study the amendments such as glycerol (10 mM), PEG (0.5%), CMC (0.05%), Gum Arabica (0.15%) and Polysorbate 20 (250 ppm) at concentrations lower than those reported in earlier studies were found to be optimal in developing an effective liquid formulation.

The results from studies on survival of *Pseudomonas striata* on maize seeds (Table 3) showed that seeds treated with 2 mL kg^{-1} of liquid formulation recorded a higher mean viable population ($\log_{10}$ 6.72 CFU mL^{-1}) which was on par with seeds treated with 4 mL ($\log_{10}$ 6.67) and 6 mL ($\log_{10}$ 6.54 CFU mL^{-1}) respectively. The least population was on seeds treated with lignite based formulation ($\log_{10}$ 4.72 CFU mL^{-1}). Similarly, sorghum seeds (Table 4) treated with 4 mL kg^{-1} seeds ($\log_{10}$ 5.36 CFU ml^{-1}) recorded higher mean viable population which was on par with seeds treated @ 6 mL kg^{-1} ($\log_{10}$ 5.25 CFU mL^{-1}) and the least mean viable population was observed in seeds treated with lignite based formulation ($\log_{10}$ 2.50 CFU ml^{-1}). The enhanced survival of *Pseudomonas striata* on seeds could be attributed to presence of cell protectants which promoted rapid and even coating of PSB on seed, enhanced cell adherence to seed (Temprano *et al.*, 2002), favorable moisture maintained thus preventing cell desiccation (Somasegaran and Hoben, 1985). Similar results were also obtained in liquid inoculant- 2 containing glycerol and PVP as cell protectants (Sridhar *et al.*, 2004). They observed a higher viable cells of *Bacillus megaterium* on cowpea seed ($\log_{10}$ 4.50 CFU ml^{-1}) inoculated @ 3 mL kg^{-1} as compared to the lignite based formulations after 8 hr of incubation. From the present study it was evident that two mL of liquid formulation was optimum to coat one kg of bigger sized maize seeds, while four mL of the same was found optimal for smaller sorghum seeds. Sorghum seeds being smaller in size than maize seeds had higher surface area and hence

Table 2. Population of *P. striata* ($x10^{10}$ CFUmL^{-1}) as influenced by amending Pikovskaya's broth with various concentrations of additives, adjuvants and surfactants in the promising formulations

SI.No.	Formulations	Composition of formulation	Population in log transformed values at different intervals in hours				
			0	24	48	72	84
1	6	Basal medium+A1+B1+C2+D1+E2	7.678 (0.0050)	8.778 (0.060)	8.778 (0.366)	10.386 (2.433)	10.230 (1.766)
2	8	Basal medium+A1+B1+C2+D2+E2	7.699 (0.0050)	9.028 (0.106)	9.028 (0.633)	10.894 (7.833)	10.864 (7.333)
3	9	Basal medium+A1+B2+C1+D1+E1	7.719 (0.0053)	9.102 (0.126)	9.102 (0.900)	10.380 (2.433)	10.059 (1.166)
4	11	Basal medium+A1+B2+C1+D2+E1	7.100 (0.0013)	8.778 (0.060)	8.778 (0.833)	10.743 (5.533)	10.725 (5.333)
5	14	Basal medium+A1+B2+C2+D1+E2	7.725 (0.0053)	8.800 (0.063)	8.800 (1.166)	10.230 (1.700)	10.185 (1.533)
6	15	Basal medium+A1+B2+C2+D2+E1	7.699 (0.0050)	8.602 (0.066)	8.602 (3.033)	10.386 (2.433)	10.230 (1.766)
7	18	Basal medium+A2+B1+C1+D1+E2	7.477 (0.0030)	9.086 (0.123)	9.634 (0.433)	12.308 (203.333)	12.278 (190.000)
8	22	Basal medium+A2+B1+C2+D1+E2	7.634 (0.0043)	9.864 (0.733)	10.301 (2.000)	10.748 (5.666)	10.735 (5.433)
9	23	Basal medium+A2+B1+C2+D2+E1	7.360 (0.0023)	10.012 (1.033)	10.201 (1.666)	11.038 (11.000)	10.962 (9.333)
10	24	Basal medium+A2+B1+C2+D2+E2	7.519 (0.0033)	8.661 (0.046)	9.100 (0.133)	10.519 (3.333)	10.602 (4.000)
11	25	Basal medium+A2+B2+C1+D1+E1	7.519 (0.0033)	9.634 (0.433)	10.519 (3.333)	11.933 (85.666)	11.897 (79.000)
12	26	Basal medium+A2+B2+C1+D1+E2	7.201 (0.0017)	9.100 (0.133)	10.509 (3.233)	11.695 (49.666)	11.682 (48.333)
13	27	Basal medium+A2+B2+C1+D2+E1	7.634 (0.0043)	9.387 (0.246)	10.654 (4.533)	11.819 (66.000)	11.796 (62.666)
14	28	Basal medium+A2+B2+C1+D2+E2	7.360 (0.0023)	9.748 (0.566)	10.881 (7.666)	11.330 (21.666)	11.266 (19.000)
15	29	Basal medium+A2+B2+C2+D1+E1	7.360 (0.0023)	9.100 (0.133)	10.634 (4.333)	11.300 (20.000)	11.236 (17.333)
16	30	Basal medium+A2+B2+C2+D1+E2	7.360 (0.0023)	9.820 (0.666)	11.185 (15.333)	11.946 (88.333)	11.933 (85.666)
17	31	Basal medium+A2+B2+C2+D2+E1	7.418 (0.0027)	9.100 (0.133)	9.962 (0.933)	10.661 (4.666)	10.634 (4.333)
18	32	Basal medium+A2+B2+C2+D2+E2	7.560 (0.0037)	9.000 (0.100)	9.923 (0.866)	11.899 (79.333	11.879 (75.666)
19	Control	Basal medium	7.360 (0.0023)	9.536 (0.343)	9.957 (0.906)	9.954 (0.900)	9.901 (0.800)

Note :Values in parenthesis represent original CFU ($x10^{10}ml^{-1}$) CFU- Colony forming units A1 - 5 mM Glycerol A2 - 10 mM Glycerol
B1 - 0.5% Poly ethylene glycol B2 - 1% Poly ethylene glycol C1 - 0.05%Corboxy methyl cellulose C2 - 0.1%Corboxy methyl cellulose
D1 - 0.15% Gum arabica D2 - 0.3% Gum arabica E1 - 125 ppm Polysorbate 20
E2 - 250 ppm Polysorbate 20 Basal media – Pikovskaya's broth.

Table 3. Viable population (x 10^5 CFU $seed^{-1}$) of *P. striata* on maize seeds as influenced by different levels of inoculum and storage time

Treatments	Population (x10^5 CFU $seed^{-1}$) at different time interval(hr) after inoculation					
Level of inoculum	Mean of factor A and factor B interaction					Level of
(mL kg^{-1} seeds)	Incubation period in hours					inoculum (Mean
	0	12	24	36	48	of factor A)
T_1 – 2 ml of liquid formulation	39.333 (7.594^{bcd})	23.6600(7.372^{d})	9.000 (6.952^{e})	2.433 (6.383^{gh})	0.210 (5.335^{i})	14.920 (6.723^{a})
T_2 – 4 ml of liquid formulation	59.000 (7.770^{abc})	32.000 (7.505^{cd})	2.860 (6.538^{fg})	2.160 (6.341^{gh})	0.160 (5.201^{i})	19.356(6.671^{a})
T_3 – 6 ml of liquid formulation	64.600 (7.810^{ab})	26.660 (7.359^{d})	5.000 (6.673^{f})	1.333 (6.156^{h})	0.042 (4.719^{j})	19.526(6.543^{a})
T_4 – 8 ml of liquid formulation	58.300 (7.763^{abc})	43.330 (7.592^{bcd})	3.333 (6.433^{fg})	0.360 (5.460^{i})	0.033 (4.392^{k})	21.070(6.328^{b})
T_5 – 20 g of lignite formulation	77.500 (7.877^{a})	2.530 (6.403 ^{fgh})	0.063 (4.917^{j})	0.003 (3.418^{l})	(1^{m})	16.019(4.728^{c})
Mean of factor B Incubation time (hr)	59.746(7.763^{a})	25.630 (7.246^{b})	4.170(6.303^{c})	1.256(5.547^{d})	0.092 (4.129^{e})	
		S.Em ±			L.S.D (0.05)	
Level of inoculum (A)		0.056			0.192	
Incubation time (B)		0.056			0.192	
A x B		0.125			0.248	

Note: CFU- Colony forming units, Factor A – Levels of inoculum per kg seeds, Factor B - different time intervals in hours.
Means followed by the same alphabet/s within factors (A and B) and their interaction (A X B) do not differ significantly at P = 0.05 by DMRT
Values in parenthesis = (log of population +1) mL = milli liter

Table 4. Viable population (x 10^5 CFU $seed^{-1}$) of *P. striata* on sorghum seeds as influenced by different levels of inoculum and storage time

Treatments	Population (x10^5 CFU $seed^{-1}$) at different time interval (hr) after inoculation					
Level of inoculum	Mean of factor A and factor B interaction					Level of
(mlkg-1 seeds)	Incubation time in hours					inoculum (Mean
	0	12	24	36	48	of factor A)
T_1 - 2 ml of liquid formulation	0.336 (5.526d)	0.233 (5.361d)	0.014 (4.145f)	0.0033 (3.501g)	(1i)	0.116(3.906c)
T_2 - 4 ml of liquid formulation	8.333 (6.920a)	1.666 (6.159b)	0.346 (5.539d)	0.040 (4.592e)	0.043 (3.619e)	2.080 (5.366a)
T_3 - 6 ml of liquid formulation	10.666 (7.027a)	0.633 (5.793c)	0.240 (5.379d)	0.029 (4.471e)	0.0043 (3.592e)	2.313(5.253ab)
T_4 - 8 ml of liquid formulation	12.333 (7.090a)	0.733 (5.859c)	0.233 (5.367d)	0.025 (4.408e)	0.0023 (3.301h)	2.664(5.205b)
T_5 - 20 g of lignite formulation	0.296 (5.429d)	0.013 (4.113f)	(1i)	(1i)	(1i)	0.0504(2.508d)
Mean of factor B Incubation time (hr)		6.390 (6.398a)	0.653(5.457b)	0.166(4.286c)	0.0194(3.594d)	0.0178(2.502e)
		S.Em ±		L.S.D (0.05)		
Level of inoculum (A)		0.062		0.144		
Incubation time (B)		0.062		0.144		
A x B		0.140		0.187		

Note: CFU- Colony forming units, Factor A – Levels of inoculum per kg seeds, Factor B - different time intervals in hours.
Means followed by the same alphabet/s within factors (A and B) and their interaction (A X B) do not differ significantly at P = 0.05 by DMRT. Values in parenthesis = (log of population +1) ml = milli liter

Table 5. Cost on production of 75 L of the efficient liquid formulation in batch culture

List of particulars used for calculation	Cost of production (₹)
Cost on raw materials and chemical substances	8588
Depreciated cost of instruments	429
Electricity cost	1308
Labor cost	850
Total cost for 75 liters	11175

Calculated for a batch of 75 liters and from which cost $litre^{-1}$ was worked out.
The total cost for one liter of liquid formulation was ₹ 149.
Note:Raw materials and chemical substances includes the cost of chemicals used in preparing the Pikovskaya's broth, cell protectants, bottles used for packing and all other miscellaneous cost.
Depreciated cost includes depreciation cost of all the equipments (based on ten years of range).
Electricity cost includes cost incurred on electricity consumed to run all the equipments used in production of liquid formulation.
Labour cost includes the labour payment.

required double the volume of liquid formulation perhaps for coating on seeds.

The cost of developing formulation 18 in this study was about rupees 149 $litre^{-1}$ (Table 5) of which packing material alone accounted for rupees 90 $litre^{-1}$. The cost of the liquid formulation could be reduced substantially by replacing with a suitable cheaper packing bottle. Currently, the cost incurred was about two and half times higher than the cost of lignite based formulation (rupees 60 kg^{-1}). However, only $1/10^{th}$ or $1/5^{th}$ the quantity of lignite based formulation was required to treat seeds of different sizes, which worked out to be less bulky and low cost crop production technology over the present practice of dressing seeds with lignite based biofertilizers @ 20 g kg^{-1} seeds.

As many as 32 liquid formulations developed using two additives, two adjuvants and one surfactant each at two different concentrations were evaluated for their ability support

growth and survival of *Pseudomonas striata*. Among them formulation 18 in which very high population of *Pseudomonas striata* (19 x 10^{11} CFU ml^{-1}) could be achieved was the most promising one. Very low quantities of this formulation was found optimal (2ml in sorghum and 4 ml in maize) for treating one kg seeds. The findings of this study helped to identify right combinations and concentration of amendments to develop a low cost liquid biofertilizer of *Pseudomonas striata.*

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