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Standardization of media for co-culturing of *Azospirillum*, phosphobacteria and *Methylobacterium* (Azophosmet)

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Nitrogen fixers, phosphate solubilizers and a plant growth hormone synthesizer were cocultured in a suitable single medium The compatibility nature of *Azospirillum brasilense* Sp7, *Bacillus megaterium* var phosphaticum PB1 and *Methylobacterium extorquens* CO 47 in a common growth medium was investigated to coculture the bioinoculants (Azophosmet). All the three had better growth in YEMA. During coculturing the survival of *Azospirillum* Sp7, PSB PB1 and PPFM CO 47 was maximum in YEMB supplemented with 0.5% methanol. Biochemical characters of YEMB culture filtrates (0.5% methanol) recorded the highest amount of ammonia secretion, cell protein and polysaccharide content when compared to nutrient broth and glycerol peptone broth. The shelf life studies of Azophosmet in lignite carrier based formulation assessed for four months indicated that *Azospirillum* Sp7, PSB PB1 and PPFM CO 47 had cell load of 10^8 cfu g^{-1}. The cotton seeds treated with the Azophosmet revealed the surviving ability of *Azospirillum* Sp7, PSB PB1 and PPFM CO 47 noticed up to 24 hr on the seeds.

Key words : *Azospirillum*, *Bacillus*, Methylobacterium *and coculturing*.

INTRODUCTION

There is world wide consensus now that sole dependence on chemical inputs based agriculture is not sustainable in the long run and only integrated nutrient system involving a combination of fertilizers, organic acid or green manures and biofertilizer are essential to sustain crop production, preserve soil health and soil biodiversity. The high cost of chemical fertilizer, the widening gap between supply and demand and their adverse effect on environmental has led agricultural scientists to look for new strategies. Biofertilizers are the alternative sources to meet the nutrient requirement of crops and to bridge the future gaps in chemical fertilizers production. But the key constraint in successful commercialization of biofertilizer is overcoming difficulties in formulating a viable, cost effective and user friendly final product. The development of new microbial formulations is a challenging task and requires greater efforts (Jones and Burges, 1998). The inoculation of consortium containing more than one microbial inoculants is more advantageous than single inoculants application, since the combined inoculation brings in the benefits of two or more inoculants together. The present study was aimed to develop a new formulation of the bioinoculants as a consortium of microbial inoculants with a view to improve the efficiency of bioinoculants, reduce cost of production and to facilitate easy application. *Azospirillum brasilense* as associative nitrogen fixing bacterium, *Bacillus megaterium* var phosphaticum a phosphate solubilizing bacteria and plant hormone synthesizer Pink pigmented facultative methylotrophs (PPFMs) belonging to the genus Methylobacterium are persistently present in the rhizosphere and phyllosphere regions of plants and even on the surface of the seeds of various plants. They were revealed on leaves of almost all plants (Corpe and Rheem, 1989). The three inoculants were selected to form a consortium of inoculants that can promote plant growth efficiently.

MATERIALS AND METHODS

Culture collection:

Azospirillum brasilense Sp7 (*Azospirillum* Sp7), *Bacillus megaterium* var phosphaticum PB1 (PSB PB1) were obtained from the culture collection center of the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore.

Invitro growth assay of Azospirillum brasilense, Bacillus megaterium and Methylobacterium extorquens on liquid and solid media:

The bioinoculants strains *Azospirillum, Bacillus megaterium* and *Methylobacterium extorquens* were tested in individual and coculture media. The *Azospirillum* Sp7 was tested in nitrogen free malic acid broth, Phosphobacteria PB1 in Pikovskya's broth and PPFM CO 47 in ammonium mineral salt broth. All the bioinoculants strains were inoculated simultaneously

into yeast extract mannitol broth, nutrient broth and glycerol peptone broth. Another set of treatments was also maintained with all three broths supplemented with 0.5% methanol. The population dynamics of *Azospirillum* Sp7, PSB PB1 and PPFM CO 47 in the cocultured liquid media was studied by spreading 1ml of serially diluted samples on Pikovskya's agar (Pikovskya's, 1948) and ammonium mineral salt agar (Whittenbury *et al*., 1970), respectively in weekly intervals upto 9 weeks for PSB and PPFM. The growth characteristic of PSB PB1 and PPFM CO 47 on their respective media were taken care of and colony forming units per ml was estimated. The population of *Azospirillum* was assessed by MPN technique on Nfb semisolid medium (Dobereiner, 1980).

Biochemical characters of culture broth of individual and cocultured inoculants :

Ammonia excretion:

The ammonia excretion was analysed as described by Solorzano, 1969.

Cell protein content:

The colory metric method of Lowry *et al*., 1951 was employed.

Polysaccharide:

The polysaccharide content in the cells was determined using the method of Dubois *et al*. (1951).

Shelf life studies of Azophosmet in lignite based formulation:

Preparation of carrier:

The lignite obtained from Neyveli Lignite Corporation Ltd. was shade dried and sieved through 106µ IS sieve. The pH of the carrier material was adjusted to neutral using commercial grade calcium carbonate. The carrier was packed in 20g lots in opaque low-density grade polythene bags of 75µ thickness and sterilized.

Inoculation of broth culture in the sterile carrier:

Log phase cultures of Azophosmet with the cell load of 10^9 cells ml^{-1} was inoculated in the carrier bags using a sterile, plastic syringe fitted with hypodermic needle until 40-45 per cent moisture content was obtained. The bags were thoroughly kneaded to ensure through absorption of the liquid culture in to the carrier.

Estimation of Azophosmet survival in carrier based formulation:

One gram of carrier inoculant was used for the serial dilution technique as described earlier, the populations of *Azospirillum*, PSB and PPFM were estimated using respective media. The plates were incubated at 28 ± 1^0C and the population was estimated at every 15 days interval upto 4 months.

Survival ability of individual and the co cultured Azophosmet on cotton seeds

The sterile carrier based inoculant of *Azospirillum*, phosphobacteria, PPFM and cocultured inoculant were used for seed bacterization. One gram of MCU- 12 cotton seeds was taken. The seeds were surface sterilized and coated with 0.1g of inoculant by preparing slurry to obtain a uniform coating. The treated seeds were shade dried. The number of viable cells adhering to each seed was estimated at regular interval of every six hours upto 38h *viz*., 0hr, 6hr, 12hr, 18hr upto 38hr following serial dilution and drop plating technique.

The treatment details of this experiment are as : T_1 – Seed treatment with *Azospirillum* Sp7, T_2 – Seed treatment with Phosphate solubilizing bacteria PB ,T_3 – Seed treatment with PPFM CO 47, T_4– Seed treatment with Azophosmet, T_5 – Seed imbibition with Azphosmet, T_6: Control,

RESULTS AND DISCUSSION

Compatibility test of the inoculants is prerequisite to achieve simultaneous growth of two or more of the inoculants together in a common growth medium. The compatibility of Methylobacterium sp with *Rhizobium*, *Azospirillum Pseudomonas* and *Bacillus* sp. and other biocontrol agents was already established in our laboratory (Senthilkumar *et al.*, 2005). Yeast extract mannitol liquid (YEMB) medium supported the simultaneous growth of *Azospirillum*, PSB and PPFM at a faster rate when compared to nutrient broth (NB) and glycerol peptone broth (GPB). But YEMB with 0.5% methanol was found to be the best suited medium for coculturing three bioinoculants. Though NB is a common growth medium that can support the growth of majority of the microorganisms when grown as individual cultures, the inoculants when cocultured in common nutrient rich medium like glucose yeast extract peptone broth resulted in decrease in population of the individual inoculants (Prasad and Chandra, 2003). But the present study clearly established the possibility of growing *Azospirillum*, PSB and PPFM in YEMB with 0.5% methanol without decreasing the cell load. Since PPFM preferably utilize methanol in the medium, it may not compete with PSB and *Azospirillum* for mannitol in YEMB(Table 1 and 2).

Table 1 : Population estimation of cocultured Azophosmet inoculants in various liquid media added with 0.5% methanol

Storage days	Yeast extract mannitol broth			Nutrient broth			Glycerol peptone broth		
	1	2	3	1	2	3	1	2	3
1	$7.44x10^7$	$7.58x10^8$	$7.66x10^7$	$6.35x10^7$	$8.28x10^8$	$4.67x10^7$	$6.46x10^7$	$7.24x10^7$	$4.52x10^8$
	(7.871)	(8.879)	(7.802)	(7.802)	(8.918)	(7.669)	(7.810)	(7.859)	(8.655)
3	$7.70x10^8$	$8.83x10^9$	$8.36x10^8$	$7.27x10^8$	$9.31x10^9$	$5.44x10^7$	$6.75x10^8$	$8.39x10^8$	$5.97x10^8$
	(8.886)	(9.945)	(8.922)	(8.861)	(9.968)	(7.735)	(8.829)	(8.923)	(8.775)
5	$8.64x10^9$	$7.42x10^9$	$9.49x10^9$	$7.59x10^9$	$8.73x10^{10}$	$7.65x10^8$	$7.29x10^8$	$7.20x10^8$	$7.38x10^9$
	(9.936)	(9.870)	(9.977)	(9.880)	(10.944)	(8.883)	(8.862)	(8.859)	(9.860)
7	$7.60x10^9$	$6.59x10^9$	$8.59x10^{10}$	$7.22x10^8$	$7.58x10^9$	$8.25x10^9$	$6.41x10^8$	$5.41x10^9$	$6.11x10^{10}$
	(9.880)	(9.818)	(10.858)	(8.858)	(9.879)	(9.916)	(8.806)	(9.733)	(10.786)
10	$9.87x10^{10}$	$6.38x10^9$	$7.80x10^{10}$	$6.39x10^8$	$7.41x10^9$	$7.77x10^9$	$6.05x10^8$	$4.99x10^9$	$5.68x10^9$
	(10.994)	(9.804)	(10.805)	(8.805)	(9.869)	(9.890)	(8.781)	(9.698)	(9.754)
15	$8.61x10^9$	$6.19x10^9$	$7.68x10^9$	$6.75x10^8$	$6.54x10^9$	$6.00x10^8$	$5.66x10^8$	$4.23x10^8$	$4.62x10^8$
	(9.935)	(9.791)	(9.885)	(8.829)	(9.815)	(8.778)	(8.752)	(8.626)	(8.664)
30	$7.65x10^8$	$5.51x10^8$	$7.02x10^9$	$6.41x10^8$	$6.65x10^8$	$4.57x10^7$	$3.56x10^8$	$2.44x10^8$	$3.46x10^8$
	(8.883)	(8.741)	(9.806)	(8.806)	(8.822)	(7.599)	(8.551)	(8.387)	(8.539)
45	$7.33x10^8$	$4.93x10^8$	$6.63x10^8$	$5.70x10^7$	$5.47x10^8$	$3.80x10^7$	$2.82x10^7$	$2.21x10^7$	$3.12x10^8$
	(8.865)	(8.692)	(8.821)	(7.755)	(8.737)	(7.579)	(7.450)	(7.344)	(8.494)
60	$6.61x10^8$	$4.44x10^8$	$6.12x10^8$	$5.25x10^7$	$4.48x10^8$	$3.40x10^7$	$2.516x10^7$	$1.63x10^7$	$2.83x10^7$
	(8.820)	(8.647)	(8.786)	(7.720)	(8.651)	(7.531)	(7.399)	(8.212)	(7.328)
SE.±	0.010	0.007	0.013	0.008	0.010	0.014	0.016	0.032	0.024
C.D. (P=0.05)	0.021	0.016	0.028	0.022	0.022	0.029	0.035	0.067	0.051

1. *A.brasilense* Sp7, 2. *Bacillus megaterium* PB1 3. *Methylobacterium extorquens* CO 47

Table 2 : Population estimation of cocultured Azophosmet inoculants in various liquid media with out methanol

Storage days	Yeast extract mannitol broth			Nutrient broth			Glycerol peptone broth		
	1	2	3	1	2	3	1	2	3
1	$5.45x10^7$	$4.48x10^7$	$5.78x10^7$	$4.25x10^7$	$5.24x10^8$	$4.52x10^7$	$4.25x10^6$	$5.37x10^6$	$4.71x10^7$
	(7.736)	(7.651)	(7.761)	(7.628)	(8.719)	(7.655)	(6.628)	(6.729)	(7.673)
3	$5.95x10^8$	$5.01x10^8$	$8.36x10^8$	$4.75x10^8$	$5.95x10^9$	$5.28x10^7$	$5.48x10^7$	$5.76x10^7$	$6.35x10^7$
	(8.774)	(8.699)	(8.922)	(8.676)	(9.774)	(7.722)	(7.738)	(7.760)	(7.802)
5	$6.25x10^9$	$5.27x10^9$	$7.24x10^9$	$5.57x10^9$	$6.72x10^{10}$	$5.69x10^9$	$6.05x10^8$	$5.94x10^8$	$7.24x10^8$
	(9.795)	(9.721)	(9.859)	(9.745)	(10.827)	(9.755)	(8.781)	(8.773)	(8.859)
7	$6.47x10^9$	$5.95x10^9$	$7.54x10^9$	$6.24x10^8$	$7.24x10^{10}$	$6.14x10^9$	$6.52x10^8$	$6.28x10^8$	$8.50x10^8$
	(9.810)	(9.774)	(9.877)	(8.795)	(10.859)	(9.788)	(8.814)	(8.797)	(8.929)
10	$5.82x10^8$	$4.47x10^8$	$6.24x10^8$	$4.725x10^8$	$5.728x10^9$	$5.147x10^8$	$6.14x10^7$	$5.43x10^7$	$6.40x10^8$
	(8.764)	(8.650)	(8.795)	(9.674)	(9.757)	(8.710)	(7.788)	(7.734)	(8.806)
15	$5.24x10^8$	$4.42x10^8$	$5.97x10^8$	$4.54x10^7$	$4.95x10^9$	$4.78x10^7$	$4.24x10^7$	$3.27x10^7$	$4.27x10^8$
	(8.719)	(8.645)	(8.755)	(9.657)	(9.694)	(7.679)	(7.627)	(7.514)	(8.630)
30	$4.78x10^8$	$4.27x10^8$	$5.45x10^8$	$4.27x10^7$	$5.27x10^8$	$4.12x10^7$	$2.40x10^7$	$1.96x10^7$	$2.56x10^8$
	(8.679)	(8.630)	(8.736)	(8.721)	(7.614)	(7.380)	(7.292)	(8.408)	(8.408)
45	$3.24x10^8$	$3.57x10^7$	$4.47x10^8$	$3.54x10^7$	$4.24x10^7$	$3.90x10^7$	$2.14x10^7$	$1.78x10^7$	$1.45x10^8$
	(8.510)	(7.552)	(8.650)	(7.549)	(7.621)	(7.591)	(7.330)	(7.250)	(8.161)
60	$2.95x10^7$	$2.87x10^7$	$4.25x10^7$	$3.44x10^6$	$3.47x10^7$	$3.25x10^6$	$1.95x10^6$	$1.50x10^6$	$2.50x10^7$
	(7.469)	(7.457)	(7.678)	(6.536)	(7.540)	(6.511)	(6.290)	(6.176)	(7.397)
S.E.±	0.356	0.349	0.358	0.351	0.377	0.334	0.311	0.315	0.339
C.D (P=0.05)	0.748	0.735	0.752	0.739	0.792	0.702	0.654	0.662	0.712

Table 3 : *In vitro* growth characters of *A.brasilense* Sp7, *Bacillus megaterium* PB1 and *Methylobacterium extorquens* CO 47 on various solid media

Time (Hours)	N free malic acid medium	Pikovskya's medium	Ammonium mineral salt medium	Yeast extract mannitol agar medium			Nutrient agar medium			Glycerol peptone agar mediaum		
	1	2	3	1	2	3	1	2	3	1	2	3
24	-	+	-	-	+	-	-	+	-	-	+	-
48	+	+ +	-	+	++	+	+	+ +	+	+	+	+
60	+ +	+ + +	-	+ +	+ + +	+ +	+ +	+ + +	+ +	+ +	+ +	+ +
84	+ + +	+ + +	+	+ + +	+ + +	+ + +	+ + +	+	+ +	+ +	+ +	+ +
96	+ +	+ +	+ +	+ + +	+ + +	+ + +	+	+ +	+ +	+ +	+ +	+ +

1. *A.brasilense* Sp7, 2. *Bacillus megaterium* PB1 3. *Methylobacterium extorquens* CO 47
- : No growth, + : Slight growth, + + : Moderate growth, + + + : Profuse growth

Our simultaneous experiments on culturing of all the three in YEMA solid medium further confirmed this result (Table 3). The already existing proof for good compatibility of these three bioinoculants having no antagonistic effect with each other is a valid support of these findings (Senthil kumar *et al.*, 2002).

Raja (2004) reported that *Azospirillum* recorded the maximum amount of ammonia excretion when cultured alone. But there was some marginal reduction in ammonia excretion in the cocultured media. He suggested the reduction in ammonia excretion might be due to the mixed population of the inoculants with inherent variations in ammonia excretion.The present study showed that PPFM had comparatively higher amount of ammonia excretion (0.24l ml^{-1}) among *Azospirillum*. Though there is a little reduction in ammonia excretion (0.21ml ml^{-1}) in the cocultured medium, it was statistically at par with PPFM. It is interesting to know the capability of PPFM to excrete comparatively more ammonia than *Azospirillum*. The maximum cell protein (1.98mg ml^{-1}) and polysaccharide content (1.09 mg ml^{-1}) due to coculturing of Azophosmet compared to the single inoculants in their respective selective media (Table 4)

Cocultured inoculants prepared as lignite carrier based formulation recorded comparatively lower population of individual inoculants when compared with

Table 4 : Biochemical characters of culture broth of individual and cocultured inoculants

Sr. No.	Inoculants	Ammonia excretion (µl of ammonium chloride ml^{-1})	Cell protein mg ml^{-1}	Polysaccharide (mg of glucose ml^{-1})
1.	*A.brasilense* Sp7 in NfB	0.07	1.18	0.93
2.	*Bacillus megaterium* PB1 in pikovskya's broth	0.02	1.05	0.90
3.	*Methylobacterium extorquens* CO 47 in AMSB	0.24	1.26	1.00
4.	Azophosmet in YEMB	0.21	1.98	1.09
5.	Azophosmet in NB	0.15	1.43	1.08
6.	Azophosmet in GPB	0.10	1.26	1.08
	S.E. ±	0.082	0.112	0.098
	C.D. (P=0.05)	0.016	0.232	0.203

YEMB : Yeast extract mannitol broth
NB : Nutrient broth
GPB : Glycerol peptone broth
NfB : Nitrogen free malic acid broth
AMSB : Ammonium mineral salt broth

Table 5 : Shelf life of individual bioinoculants in sterile lignite based Azophosmet preparation

Storage days	Population cfu g^{-1}		
	A.brasilense Sp7	*Bacillus megaterium* PB1	*Methylobacterium extorquens* CO 47
7	$8.36x10^9$ (9.922)	$8.40x10^9$ (9.924)	$8.10x10^9$ (9.908)
15	$9.40x10^9$ (9.973)	$9.42x10^9$ (9.974)	$8.34x10^9$ (9.921)
30	$7.40x10^9$ (9.869)	$7.93x10^9$ (9.899)	$8.62x10^9$ (9.935)
45	$7.10x10^9$ (9.851)	$7.27x10^9$ (9.861)	$8.36x10^9$ (9.922)
60	$6.50x10^8$ (8.881)	$6.42x10^9$ (10.807)	$7.05x10^8$ (8.848)
75	$5.58x10^8$ (8.746)	$6.18x10^9$ (9.790)	$6.18x10^8$ (8.790)
90	$6.08x10^8$ (8.783)	$5.67x10^8$ (8.753)	$5.23x10^8$ (8.718)
105	$4.55x10^8$ (8.658)	$3.47x10^8$ (8.540)	$4.41x10^8$ (8.644)
120	$3.27x10^8$ (8.487)	$4.07x10^8$ (8.848)	$3.94x10^8$ (8.482)
S.E.±	0.017	0.012	0.014
C.D. (P=0.05)	0.036	0.026	0.029

Table 6 : Survival of bioinoculants on cotton seeds treated with Azophosmet and individual inoculants

Bioinoculants	Organism tested for survival	Population (cfu seed^{-1})					
		0h	6h	12h	24h	30h	36h
A.brasilense Sp7	*Azospirillum*	$1.15x10^6$	$3.3x10^6$	$5.8x10^6$	$6.2x10^5$	$4.03x10^4$	$2.12x10^4$
		(6.060)	(6.518)	(6.763)	(4.792)	(4.605)	(4.326)
B. megaterium PB1	PSB	$1.6x10^6$	$4.8x10^6$	$5.5x10^6$	$4.47x10^5$	$3.23x10^4$	$2.24x10^4$
		(6.204)	(6.681)	(6.740)	(5.650)	(4.509)	(4.350)
Methylobacterium	PPFM	$1.05x10^6$	$4.5x10^6$	$6.0x10^6$	$5.28x10^5$	$5.78x10^4$	$4.47x10^4$
extorquens CO 47		(6.021)	(6.653)	(6.778)	(5.722)	(4.761)	(4.650)
Carrier based	*Azospirillum*	$4.27x10^6$	$5.23x10^6$	$4.24x10^6$	$6.79x10^5$	$3.58x10^5$	$1.25x10^5$
Azophosmet		(6.630)	(6.718)	(6.627)	(5.831)	(5.553)	(5.096)
inoculant	PSB	$4.87x10^6$	$5.96x10^6$	$6.88x10^6$	$5.67x10^5$	$4.78x10^5$	$3.85x10^5$
		(6.687)	(6.775)	(6.837)	(5.753)	(5.679)	(5.585)
	PPFM	$3.85x10^6$	$5.58x10^6$	$7.25x10^6$	$6.57x10^5$	$5.78x10^5$	$4.97x10^5$
		(6.585)	(6.746)	(6.860)	(5.817)	(5.761)	(5.696)
Liquid culture of	*Azospirillum*	$5.47x10^7$	$6.54x10^6$	$6.68x10^6$	$7.14x10^5$	$4.57x10^5$	$3.14x10^5$
Azophosmet		(7.737)	(6.815)	(6.824)	(5.853)	(5.659)	(5.496)
inoculant	PSB	$4.47x10^7$	$6.25x10^6$	$7.01x10^6$	$4.57x10^5$	$2.27x10^5$	$1.58x10^5$
		(7.650)	(6.795)	(6.845)	(5.659)	(5.356)	(5.198)
	PPFM	$4.07x10^7$	$5.74x10^6$	$6.78x10^6$	$8.40x10^5$	$5.49x10^5$	$5.27x10^5$
		(7.609)	(6.739)	(6.758)	(5.831)	(5.924)	(5.721)
S.E.±		0.623	0.557	0.566	0.465	0.453	0.435
C.D. (P=0.05)		1.321	1.180	1.199	0.987	0.961	0.922

(Figures in parenthesis indicate Log transformed values)

the pure cultures of the same formulation. In contrary to the above findings, the microbial components in the combined biofertilizers prepared as a single lignite carrier based formulation, were reported to survive without any inhibition of each other (Poonguzhali, 2002). The present study indicated that the cocultured inoculum in lignite based carrier had a very good shelf life up to four months. The respective shelf life of the individual bioinoculants of *Azospirillum* were at 3.27 $x10^8$ cfu ml^{-1} PSB at 4.07 $x10^8$ cfu ml^{-1} and PPFM at 3.94 $x10^8$ cfu ml^{-1}(Table 5). Lignite was already established as the best suited carrier material for biofertilizer production that facilitates convenient packing and ease of application (Smith, 1992).

Poongulazhali (2002) reported that the survival of *Azospirillum* and phosphobacteria on cumbu seeds was similar when treated with mixed inoculant and individual inoculants. She also revealed that *Rhizobium* and phosphobacteria on blackgram seeds as individual inoculants showed not much increase in population when compared to the mixed inoculants. The surviving ability of the individual bioinoculants on the cotton seed treated with the cocultured Azophosmet inoculant than the seeds treated with individual bioinoculants (Table 6). The present finding reported that Cocultured inoculants (Azophosmet) showed encouraging results compared to individual inoculant. However, further studies are required for testing their efficacy under field conditions.

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