*Asian J. of Bio Sci. (2006) Vol. **1** No. 2 : 39-44*

Combined inoculation effect of pink pigmented facultative *Methylobacterium* (PPFM) and other bioinoculants on cotton

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(Accepted : March, 2006)

A field study was conducted to evaluate the individual and combined inoculation effect of PPFM and other bioinoculants on cotton. Initially a detailed survey was conduced in different agro climatic zones of Tamilnadu to isolate an efficient strain of *Methylobacterium.* Isolated PPFM strains were analyzed through polymerase chain reaction (PCR) for the presence of *mdh* gene using *mdh* gene specific probes. Only the isolates having the expected size of *mdh* gene product were chosen for further studies. Selected isolates were screened for plant growth promoting efficiency through vigor index studies. In the field trial conducted with cotton crop, PPFM was inoculated with a diazotroph and a phosphate solubilizing organism (PSB) as individual and combined inoculant treatments. PPFM inoculation has resulted in increased seedling vigor, drymatter production and yield. Inoculation of all the three inoculants together has resulted in increased rhizosphere population of the inoculants, drymatter production and yield.

Key words : Cotton, PPFM, Azospirillum, Phosphate solubilizing bacteria, *Mdh* gene, Vigor index, Field studies

INTRODUCTION

WORLD Cotton production for 2005 was estimated around 23000 metric tons, while the over all world cotton use has increased 2.5 % than 2004. With ever increasing cotton demand and dramatic increase in prices of chemical fertilizers, the widening gap between supply and demand and concern over environmental hazards caused by chemical fertilizers, now the focus has turned towards sustainable agriculture. Knowing the deleterious effects of using only the chemical fertilizers, use of soil microorganisms which can either fix atmospheric nitrogen or solubilize phosphorous or stimulate plant growth through synthesis of growth promoting substances will be environmentally benign approach for nutrient management and ecosystem function (Tilak and Annapurna, 1993).

The genus *Methylobacterium* commonly known as Pink Pigmented Facultative Methylotrophic (PPFM) bacteria are of ubiquitous in nature. These bacteria are widely found on seeds, plant phyllosphere and in plant rhizosphere (Ivanova *et al.,* 2001b). Scientific investigations have identified the close association of aerobic methylobacteria with plants through which the methanol synthesized and excreted by plants is used by *Methylobacteria* as a source of carbon and energy (Fall, 1996). In turn these bacteria secrete variety of auxins (Ivanova *et al.,* 2001a) and cytokinins (Long et al, 1997) which can be utilized by the plants leading to an increased plant growth and yield. The genus *Methylobacterium* includes a group of pink-pigmented facultatively methylotrophic bacteria with the ability to grow on one-carbon compounds such as formate, formaldehyde and methanol as sole carbon and energy sources, as well as on a wide range of multi-carbon growth substrates (Green, 1992). At present, this genus comprises 15 different species (Jourand et al., 2004 and Doronina et al., 2002). Utilizing PPFM for crop growth promotion is gaining imporance and exploiting the potential of the bacterium can lead to improved plant growth and yield in a sustainable way (Sundaram *et al.*, 2002).

Azospirillum, an associative symbiotic N_2-fixing bacterium, occurring abundantly in tropical conditions, can positively influence plant growth, crop yields and N-content; these properties may be attributed to its ability to fix nitrogen and synthesize plant growth regulators (PGR) such as auxins, gibberellins, cytokinins and ethylene (Strzelczyk *et al*., 1994).

Phosphorus is one of the major essential macronutrients for biological growth and development. About 98 per cent of Indian soils have inadequate supply of available phosphorus. It is present at levels of 400-1200 mg/kg of soil. Its cycle in the biosphere can be described as open or sedentary because there is no interchange with the atmosphere. Thus even if the total P is high and P fertilizers are applied regularly, the P is rapidly fixed to unavailable forms and accounts for low P use efficiency. Insoluble phosphate, which is not readily available for plant, is 95-99 per cent of the total soil phosphate (Walker, 1975). *Bacillus megaterium* is an efficient phosphate solubilizing bacterium (Rajarathinam *et al*., 1995) and significant increase in crop yield can be obtained through the inoculation of *Bacillus megaterium* (Thakuria *et al.*, 2004).

Counting the benefits of all the three bioinoculants together, the present experiment was aimed to evaluate individual and combined inoculation effect of PPFM and other inoculants on cotton crop through field trial study and biometric observations.

MATERIALS AND METHODS

Isolation of PPFM strains

A detailed survey was conducted in different agroclimatic zones of Tamilnadu (Aruppukottai, Kovilpatti, Paiyur and Coimbatore) for isolation of an efficient strain of PPFM. About 25 different PPFM isolates from the rhizosphere and phyllosphere of cotton and cotton based cropping systems were obtained. AMS medium (Ammonia Mineral Salts medium) which contains methanol as sole carbon source was used for the isolation of *Methylobacterium* strains (Whittenbery *et al*., 1970).

Authentication of PPFM isolates

Identification of methanol dehydrogenase (mxaF) gene by PCR

The presence of *mxaF* gene is unique to *Methylobacterium* and detected by PCR amplification of *mxaF* gene using specific probes. The total genomic DNA from the PPFM isolates was extracted according to the protocol given by Ivanova *et al.,* (2000) with slight modifications. The primers *mxa* f1003 5'GCG GCA CCA ACT GGG GCT GGT 3' and *mxa* r1561 5'GGG CAG CAT GAA GGG CTC CC3'(Mc Donald and Murrell, 1997) which target the highly conserved region of *mxaF* gene in *Methylobacterium* genera was used in the PCR reactions.

Vigor index studies

These PPFM isolates were screened for growth promoting efficiency of cotton crop through vigor index studies as described by ISTA (1993). The most efficient strain of PPFM isolate with

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maximum plant growth promoting efficiency was selected and used as the PPFM inoculam through mass multiplication.

Other inoculants

Standard strains of the bacterial inoculants *viz*., *Bacillus megaterium* (Phosphate Solubilizing Bacterium – 1) and *Azospirillum lipoferum* - Az204) obtained from Department of Agricultural Microbiology, TNAU, Coimbatore were used in the study.

Field trial

The field trial was conducted at the Department of cotton, Tamil Nadu Agricultural University. The soil type of the experimental soil was clay loam soil and had nutrient status of nitrogen - 97.0 Kg ha^{-1}, phosphorus – 30.0 Kg ha^{-1} and potassium - 387.5 Kg ha^{-1}. All the bioinoculants were applied with 75% recommended dose of fertilizers (RDF) except control. Bioinoculants were applied as individual and combined inoculant application treatments as follows,

Treatment details

T_1- 75% RDF + No bio - inoculant
T_2- 75% RDF + *Azospirillum*[a]
T_3- 75% RDF + PSB[b]
T_4- 75% RDF + PPFM[c]
T_5- 75% RDF+ *Azospirillum* TNAU + PSB
T_6- 75% RDF+ *Azospirillum* TNAU + PPFM
T_7- 75% RDF + PSB + PPFM
T_8- 75% RDF *Azospirillum* TNAU + PSB + PPFM
T_9- 100 % RDF only

Where as a - *Azospirillum lipoferum* - Az204, b - *Bacillus megaterium* – PSB,
c – Pink Pigmented Facultative Methylotroph isolate PYR2 (Selected from survey and vigor index studies).

Biometric observations

Root length was measured from the point of attachment of the stem base to the tip of the root. Shoot length was measured from the stem base to the tip of the shoot stretched at 45th, 90th and 135th days after sowing.

Dry matter production (mg seedling^{-1})

Ten normal plants per replicate were placed in a brown paper cover and dried under shade for 24 h and then in the hot air oven maintained at 85 ±1°C for 24 h. Dry weights of the plant samples were recorded in mg plant^{-1}.

Yield

Cotton was picked up two times from each plant at 110th and 140th days after sowing. Average yield was calculated for each treatment from three replications.

Soil nutrient analysis

The nitrogen content in the soil samples were analyzed by micro Kjeldahl method (Humphries, 1956). The total phosphorus content of the soil samples were estimated by Vanado-molybdophosphoric yellow color development method as described by Jackson, 1973 where as potassium content was estimated as described by Jackson (1973).

Population enumeration from the experimental field

Population of *Azospirillum lipoferum* was estimated through serial dilution and most probable number technique in nitrogen free semi solid medium as described by Dobereiner (1980). Population of *Bacillus megaterium* (PSB) and PPFM was estimated by serial dilution and plating technique (Allen, 1953) using Pikovaskya medium for PSB (Pikovaskya, 1948) and Methanol Mineral Salts medium for PPFM (Whittenbery *et al*., 1970).

RESULTS AND DISCUSSION

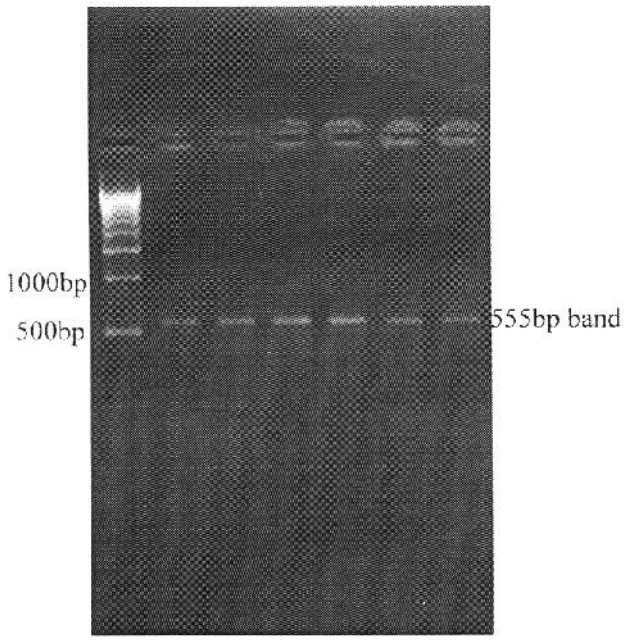
Isolation and screening of PPFM strains

Twenty-five PPFM strains were isolated from different agro climatic regions of cotton and cotton based cropping systems of Tamilnadu, using AMS medium.

Authentication of PPFM isolates

The non-degenerate primers *mxa* f1003 and *mxa* r1561 indeed produced an amplification product of expected 555bp band in PPFM isolates. Thirteen out of the 25 isolates which does not produced the expected 555bp band were not chosen for further studies. Only 12 isolates that produced the 555bp amplification product mdh gene were used in further studies (Fig 1).

Fig. 1 : *mxaf* gene of 555 bp size sequence amplified in *Methylobacterium* isolates.



Lane 1 : 500 bp marker
Lane 2 : Methylobacterium extroquens AM - 1
Lane 3 : Methylobacterium isolate PYR - 2
Lane 4 : Methylobacterium isolate APK - 1
Lane 6 : Methylobacterium isolate CBE - 1
Lane 7 : Methylobacterium isolate KP - 1

Screening of PPFM strains

Isolates were screened for plant growth promoting efficiency through vigor index studies. In all the cases, Inoculation of PPFM isolates promoted higher shoot length, root length and germination per centage. Among the 50 isolates screened for growth promoting efficiency, isolate PYR-2, from cotton based cropping system of Paiyur region significantly promoted highest root length (3.78cm), shoot length (5.8cm), germination per cent (90%) and expressed high growth promoting efficiency (results presented in Table 1). This isolate was mass multiplied and used for inoculation of cotton in the field study.

Biometric observations

Significant difference in plant height and dry matter production were noticed among the different treatments. On all days of observation, maximum plant height and drymatter production was recorded in treatment T8. Biometric results indicated higher

Table 1 : Screening of PPFM isolates through vigor index studies

S.No	Isolate No.	Germination %	Root length (Cm)	Shoot length (Cm)	Vigor Index
1	APK – 1	80	3.5	5.4	712
2	APK – 2	80	3.2	5.3	680
3	APK – 3	90	3.8	5.7	855
4	KP – 1	80	2.5	4.2	536
5	KP – 2	80	3.7	5.6	744
6	KP – 3	90	3.2	5.1	747
7	PYR - 1	70	2.5	4.0	455
8	PYR - 2	90	3.8	5.8	864
9	PYR - 3	90	3.5	5.2	783
10	CBE - 1	70	3.2	4.8	560
11	CBE – 2	90	3.5	4.3	702
12	CBE - 3	90	3.0	4.2	648

Table 2 : Biometric observation at 45th, 90th and 135th day

S. No.	Treatments	Plant height			Dry matter production		
		45th Day	90th Day	135th Day	45th Day	90th Day	135th Day
1	T_1- 75% RDF + No bioinoculant	16	53	68	8.56	48.65	68.30
2	T_2- 75% RDF + Azo. TNAU	17	60	71	12.38	55.60	73.60
3	T_3- 75% RDF + PSB	17	59	68	13.65	59.60	72.30
4	T_4- 75% RDF + PPFM	17	61	74	15.68	55.36	68.36
5	T_5- 75% RDF+ Azo. TNAU + PSB	18	62	72	17.63	57.48	75.80
6	T_6- 75% RDF+ Azo.TNAU + PPFM	19	59	72	16.45	56.38	79.55
7	T_7- 75% RDF + PSB + PPFM	19	59	75	16.58	54.75	75.60
8	T_8- 75% RDF Azo. TNAU + PSB + PPFM	19	59	78	16.90	65.55	83.42
9	T_9- 100 % RDF only	17	55	73	16.80	53.27	75.41
SEd		1.14	3.87	4.76	0.90	3.63	4.85
CD at 0.5%		2.42	8.21	10.09	1.91	7.70	10.29

Table 3 : Soil nutrient analysis after harvest

S. No.	Treatments	N (Kg ha^{-1})	P (Kg ha^{-1})	K (Kg ha^{-1})
1	T_1- 75% RDF + No bio- inoculant	118.60	46.35	440.65
2	T_2- 75% RDF + *Azospirillum* TNAU	128.33	65.80	450.30
3	T_3- 75% RDF + PSB	115.20	85.60	445.60
4	T_4- 75% RDF + PPFM	125.60	58.60	458.30
5	T_5- 75% RDF+ *Azospirillum* TNAU + PSB	128.60	80.67	456.30
6	T_6- 75% RDF+ *Azospirillum* TNAU + PPFM	107.20	65.30	448.28
7	T_7- 75% RDF + PSB + PPFM	98.60	75.66	440.23
8	T_8- 75% RDF *Azospirillum* TNAU + PSB + PPFM	136.75	87.66	455.60
9	T_9- 100 % RDF only	103.67	73.65	503.60
Sed		8.03	4.42	29.99
CD at 0.5%		17.03	9.37	NS

significant difference in plant drymatter production compared to plant height (Table 2). Maximum drymatter production was recorded in T8 at 135th DAS followed by T6 (79.55), T5 (75.80) and T7 (75.60).

Soil nutrient content

Nutrient content analysis of the soil after harvest of field trial has indicated an overall increase in soil nitrogen and phosphorus content from initial level (Table 3). However, there was no significant difference in soil potassium content except in 100% RDF applied treatment. Significant increase in soil nitrogen content was recorded when *Azospirillum* was inoculated. Maximum nitrogen content was recorded in T8 (136.75 Kg ha^{-1}) followed by T5 (128.60 Kg ha^{-1}) and T2 (128.33Kg ha^{-1}). Least soil nitrogen

content was recorded in uninoculated control. Inoculation of phosphate solubilizing bacterium resulted in significant increase in soil phosphorus content. Maximum soil phosphorus content (87.66 Kg ha^{-1}) was recorded when PSB was inoculated with *Azospirillum* and PPFM (T8).

Table 4 : Rhizosphere colonization (CFU per gram of soil)

S. No.	Treatments	*Azospirillum* count (10^5 CFU)			Phosphobacteria (10^5 CFU)			PPFM (10^4 CFU)		
		45th day	90th day	135th day	45th day	90th day	135th day	45th day	90th day	135th day
1	T_1- 75% RDF + No bioinoculant	0.02	0.3	0.2	0.10	1.0	3.0	0.01	0.10	0.01
2	T_2- 75% RDF + *Azospirillum*	4.0	16.0	15.0	0.20	5.0	4.0	0.02	0.50	0.08
3	T_3- 75% RDF + PSB	0.06	0.7	0.5	16.0	23.0	20.0	0.02	0.70	0.30
4	T_4- 75% RDF + PPFM	0.05	0.6	0.4	0.20	6.0	5.0	2.0	14.0	13.0
5	T_5- 75% RDF+ *Azospirillum* + PSB	7.0	18.0	16.0	18.0	26.0	19.0	0.05	0.60	0.40
6	T_6- 75% RDF+ *Azospirillum* + PPFM	6.0	17.0	15.0	0.30	5.0	3.0	5.0	17.0	16.0
7	T_7- 75% RDF + PSB + PPFM	0.04	0.5	0.3	20.0	23.0	18.0	3.0	15.0	12.0
8	T_8- 75% RDF *Azospirillum* + PSB + PPFM	8.0	18.0	15.0	18.0	27.0	16.0	3.0	16.0	16.0
9	T_9- 100 % RDF only	0.01	0.6	0.5	0.30	3.0	2.0	0.03	0.20	0.30
SEd		0.28	0.87	0.81	0.79	0.97	0.75	0.14	0.69	0.62
CD at 0.5%		0.60	1.85	1.73	1.68	2.05	1.59	0.30	1.47	1.33

Population enumeration studies

Significant increase in population of the inoculants was found due to bioinoculants application (Table 4). Drastic increase in the population of inoculants was found up to 90 days after inoculation and then there was no significant increase. Maximum population of *Azospirillum*, Phosphobacteria and PPFM was recorded in T8 (18.0×10^5, 27.0×10^5, 16×10^4 CFU g^{-1} of soil respectively) followed by T5. When *Azospirillum* and PSB were inoculated together (T5), significant increase in population of the both inoculants was found than in individual inoculants treatments (T2 and T3).

Yield attributes

PPFM inoculation has brought considerable increase in cotton yield in individual and combined inoculation treatments (Table 5, Fig 2). Highest boll number per plant, boll weight per plant and cotton yield (1020 kg ha^{-1}) was recorded when PPFM was inoculated along with PSB and *Azospirillum*. Among the individual inoculation application treatments (T2, T3 and T4), PPFM inoculation has brought maximum increase in boll number (13.90 $plant^{-1}$) and boll weight (3.50 g $plant^{-1}$) and PPFM recorded higher per cent increase over control (23.68 per cent) than *Azospirillum* (18.42 per cent) or PSB (10.43 per cent) inoculation (Table 6).

A quinoprotein methanol dehydrogenase (*mdh*) enzyme found in the Gram-negative methylotrophs (Goodwin & Anthony 1998) facilitates this group of bacteria to utilize methanol as source of carbon. This enables the isolation of *Methylobacterium* from environmental samples using methanol as sole source of carbon. Bacteria belonging *Methylobacterium* genus has methanol dehydrogenase gene (*mdh* gene - *mxaF*). Methanol dehydrogenase enzyme encoded by *mxaF* gene is required for methanol oxidation and utilization as carbon source for *Methylobacterium* genera (Lidstrom *et al*., 1994). As the MMS medium has only the methanol as sole source of carbon, only *Methylobacterium* strains with characteristic typical pink color were able to grow. PPFM strains isolated using MMS medium were further authenticated using specific primers and PCR amplification of a 555bp *mxaF* gene sequence. This 555 bp region of *mxaF* gene is a highly conserved region in all the PPFM species and amplification of this particular region in all the PPFM isolates authenticates their genus. Using this technique, 12 isolates out of 25 isolates studied were chosen for further studies. This has enabled the study to choose PPFM isolates using a quick, highly reliable and precise technique.

Auxins are a group of plant hormones, indole derivatives that stimulates the division, extension, and differentiation of plant cells, enhances root formation by promoting the conversion of parenchyma into xylem and phloem. The balance between auxin and cytokinin levels controls cellular differentiation and organo genesis in tissue and organ culture. Shoot proliferation to root formation takes place as the ratio of auxin/cytokinin increases (Skoog and Schmitz, 1972). Facultative methylotrophic bacteria with different pathways of C1 metabolism are able to produce auxins, particularly indole-3-acetic acid (IAA) in amounts of 3–100 mg/ml (Ivanova et al.,2001) and different cytokinin fractions like zeatin and zeatin riboside in amounts of 50 to 400 ng/g dry weight (Long *et al.,* 1997). *Methylobacterium* genera primarily promote the plant growth through auxin and cytokinin production. Hence vigor index studies which includes germination percentage, root length and shoot length provides an excellent mechanism for the screening PPFM isolates. PPFM inoculation has recorded positive results with all the 50 isolates tested. Through vigor index studies, PPFM strain PYR 2 selected was able to promote higher plant growth and vigor index. The same mechanism of plant growth promotion may also be attributed to the higher plant growth promotion and yield increase when PPFM was inoculated in the field trial study.

In the field trial conducted, combined inoculation of PPFM, *Azospirillum* and PSB has resulted in higher plant growth parameters like maximum plant height, plant dry matter production, yield attributes like higher boll number, boll weight per plant and seed cotton yield per ha. The combined inoculation of PPFM, *Azospirillum* and PSB has resulted in higher increase in plant growth parameters. Numerous studies have suggested that free-living heterotrophic N_2-fixers are potentially important source of N_2 fixation (Mahadevappa and Shenoy, 2000). Excluding *Methylobacterium nodulans*, all other species of PPFM are not nitrogen fixers. Hence coinoculation of PPFM along with diazotroph – *Azospirillum* has benefited not only the crop growth but also PPFM population through biological nitrogen fixation. Most efficient phosphate solublizers are reported from *Bacillus* genera and a yield increase of 10 to 30 % was recorded due to PSB inoculation to crop plants (Dubey and Gupta, 1996). In the present study, coinoculation of PPFM with other bioinoculants like *Azospirillum* and PSB has benefited plants through biological

Table 5 : Yield attributes

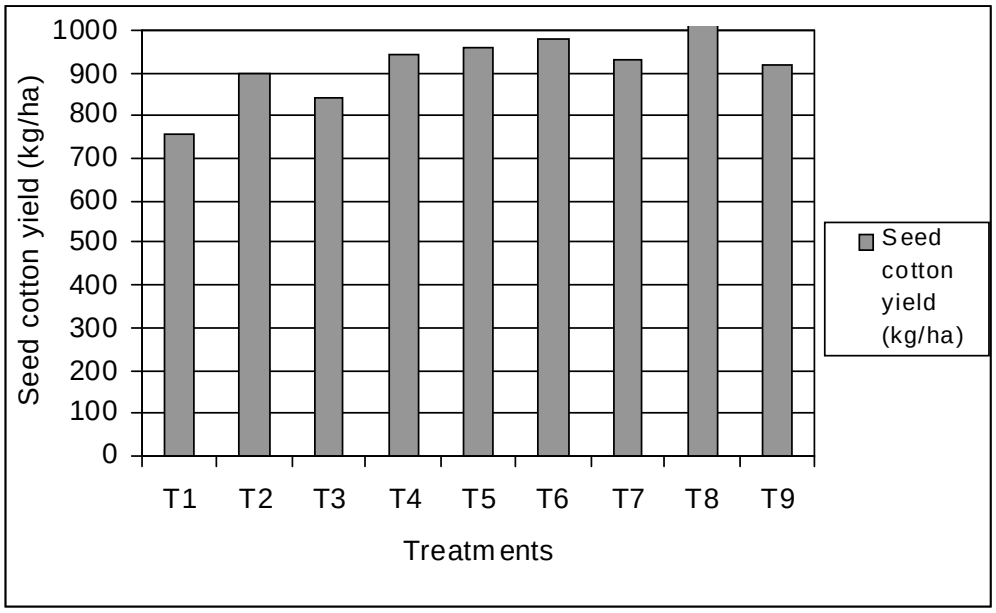
Treatments	Boll number (plant^{-1})	Boll weight(g)	Seed cotton yield (Kg ha^{-1})	Seed cotton yield (g plant^{-1})	Per cent increase over control
T_1- 75% RDF + No bioinoculant	9.0	2.75	760	42.1	-- --
T_2- 75% RDF + *Azospirillum* TNAU	11.45	3.30	900	47.6	18.42
T_3- 75% RDF + PSB	10.60	3.25	840	44.65	10.53
T_4- 75% RDF + PPFM	13.90	3.50	940	46.8	23.68
T_5- 75% RDF+ *Azospirillum* TNAU + PSB	12.75	3.60	960	49.6	26.32
T_6- 75% RDF+ *Azospirillum* TNAU + PPFM	13.50	3.80	980	50.1	28.95
T_7- 75% RDF + PSB + PPFM	12.80	3.30	930	49.4	22.37
T_8- 75% RDF +*Azospirillum* TNAU + PSB + PPFM	14.30	3.90	1020	51.6	34.21
T_9- 100 % RDF only	12.0	3.40	920	47.2	-- --
SEm	0.78	0.23	59.70	3.11	
CD at 0.5%	1.65	0.48	126.56	NS	

nitrogen fixation by diazotrophs, phosphate solubilization by PSB and plant growth promoting hormones production by PPFM.

Bio-inoculants application has also resulted in significant increase in the rhizosphere population of respective inoculants. In comparison with root-free soil, the rhizosphere forms a nutrient-rich niche for bacteria as a result of exudation of compounds such as organic acids, sugars and amino acids through the roots. Microorganisms are found at elevated levels in the rhizosphere (Brazin *et al.,* 1990). In turn microorganisms benefits plants through secretion of plant growth hormones, biological nitrogen fixation, phosphates solubilization, antibiosis, etc., The beneficial interaction between plant system and applied inoculants as reported earlier (Watanabe and Lin, 1984) favorably influenced the microbial population as well as crop biometric observations and yield. The decrease in root rhizosphere activity after flowering also has influenced the bacterial population to decrease after 90 days.

Dual inoculation or combined inoculation of microbial inoculants has several advantages over conventional single inoculant application (Shivathar *et al*., 2000). Combined inoculation of *Azospirillum lipoferum* and *Bacillus megaterium* (Arangrasan *et al.,* 1998) was reported to bring favorable results than respective single inoculant application. PPFM has been reported to promote plant growth of soybean (Holland, 1997), sugarcane (Madhaiyan *et al.,* 2005) etc. However when PPFM was inoculated along with *Azospirillum* and PSB, plant growth promotion occurs through tripartite relationship between these three inoculants and through three different mechanisms *viz*., plant growth harmones production by PPFM, biological nitrogen fixation by *Azospirillum* and solubilization of insoluble form of soil phosphorus into plant available form by phosphate solubilizing bacteria. Our study indicates that all the three inoculants are capable of promoting plant growth when applied individually, but when all the three inoculants were coinoculated, higher plant growth promotion and yield increase was recorded which may be correlated to the synergistic effect of all the three inoculants together. The research finding which supports the potential of pink pigmented facultative *Methylobacterium* (PPFM) as the bioinoculants for cotton could be exploited well along with other bioinoculants to maximize the cotton yield in a sustainable and

Fig. 2 : Yield increase due to PPFM and other bioino culants application.



cost effective way.

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