Standardization of liquid formulation for enhancing the shelf life of *Azospirillum* and phosphate solubilizing bacteria (PSB)

Plan Project

(State Planning Board, Government of Kerala)

Final Report

(June, 2014- March, 2017)

(As per order No. R8/64463/13 dt: 19-03-2016 of the DR, KAU)

(KAU PC group code: BO-01-00-09-2014-VKA (24)-KAU-P)

Dr.K.Surendra Gopal

Professor (Microbiology)

Department of Agricultural Microbiology

Kerala Agricultural University

Vellanikkara. Thrissur-680656

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Contents

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1. **Project title**: Standardization of liquid formulation for enhancing the shelf life of *Azospirillum* and phosphate solubilizing bacteria (PSB)
2. **Location**: Dept. of Agricultural Microbiology College of Horticulture.
3. **Principal Investigator** : Dr. K. Surendra Gopal, Professor (Microbiology)
4. **Co-PI/Associates** : Dr. D. Girija, Professor & Head
   Dr. Sally K. Mathew, Professor (Retd)
5. **AS&TS details (GO & KAU orders)**: R8/61046/14 dt: 28/03/2014
6. **Financial Sanction details (of Comptroller)** :(As per order No.EP/B1/6220/14 dt. 26-5-2014)

Head of account 206-31-9554	Amount sanctioned by GoK for 2013-2014 (in Lakhs)	Amount sanctioned by GoK for 2014-2015 (in Lakhs)	Total provision (2014-2015) (in Lakhs)	Balance amount revalidated from 2014-15 (in Lakhs)	Amount sanctioned by GoK for 2015-2016 (in Lakhs)	Total provision for 2015-16 (lakhs)	Total provision for 2016-17 (Lakhs)
142- Labour cost	0.20	0.20	0.40000	0.19900	0.300	0.49900	0.34600
153- Contra staff	1.08	1.08	2.16000	1.38990	1.080	2.46990	1.37800
210- Research materials	0.30	0.20	0. 50,000	0.16870	0.300	0.46870	0.08100
420- Equipments	0.50			0.07235	0.00	0.07235	0.07200
Total	**2,08,000**	**1,48,000**	**3,56,000**	**1,82,995**	**1,68,000**	**3,50,995**	**1,87,700**

Budget provision for 2016-2017

Head of Account 206-31-9554	Balance amount revalidated from 2016-17 (lakhs)	Amount sanctioned by GoK for 2016-2017 (in Lakhs)	Total provision for 2016-17 (lakhs)
142- Labour cost	0.34600	0	0.34600
153- Contractual staff	1.37790	0	1.37800
210- Research materials	0.08079	0	0.08100
420- Equipments	0.07235	0	0.07200
	1,87,704	**0**	**1,87,700**

7 **Date of commencement**: June, 2014

8. **Date of completion**: March, 2017

9. **Total budget and total expenditure of the project**: 5.24 lakhs

10. **Year-wise budget and expenditure (item/ head wise):**

Head of account 206-31-9554	Total provision (2014-2015) (Lakhs)	Expendit-ure (2014-2015) (Lakhs)	Provision for (2015-2016) (Lakhs)	Total provision (2015-2016) (Inclusive of balance from 2014-2015) (Lakhs)	Expen-diture (2015-2016) (Lakhs)	Total Expen-diture (2014-2016) (Lakhs)	Total provision (2016-2017) (Lakhs)	Expen-diture (2016-2017) (Lakhs)	Balance (Lakhs)
142- Labour cost	0. 40,000	0.05100	0.30000	0.49900	0.15300	0.20400	0.34600	0.20500	0.14100
153- Contra staff	2.16000	0.77010	1.08000	2.46990	1.09200	1.77410	1.37800	0.85110	0.52690
210- Research materials	0.50000	0.48130	0.30000	0.46870	0.38791	0.86921	0.08100	0.07439	0.00661
420- Equipment	0.50000	0.42765	0	0.07235	0	0.42765	0.07200	0.07000	0.00200
Total	3,56,000	1,73005	1,68,000	3.50995	1,63,291	3,27,496	1,87,700	1,20,049	67,651

456345 + 67651 = 523996

67655

11. **Back ground of the project**

The success of any biofertilizers depends on the quality of bioformulations. At present, most of the biofertilizers produced are of inferior quality. Another problem in biofertilizer inoculant production is the survival of organisms up to field application. The carrier-based formulations (solid substrates) have certain limitations like short-shelf life, unavailability of good quality carrier material in the local area, labour intensive, sensitivity to temperature, contamination etc.To overcome the problems of carrier based formulations, liquid formulations are preferred due to its advantages like no contamination, better survival on seeds, longer shelf-life, less dosage than carrier based formulations and easy to use.

Liquid biofertilizers (LB) contain a desired organism and their nutrients with special cell protectants or substances that encourage longer shelf life and tolerance to adverse conditions. *Azospirillum* and phosphate solubilizing bacteria (PSB) are the two popular biofertilizers in Kerala but no systematic work has been done in Kerala on developing liquid formulations of *Azospirillum* and PSB. Literature on the liquid formulations of biofertilizers are scanty. Therefore, this study was undertaken to enhance the shelf-life of *Azospirillum* and PSB through liquid formulations. .

12. **Objectives**

- To standardize liquid formulation and enhance the shelf-life of *Azospirillum* and PSB
- To evaluate the liquid formulations under field condition using a test crop.

13. **Technical programme**

13.1 The native isolates of *Azospirillum* and PSB were obtained from the Dept. of Agricultural Microbiology, CoA, Vellayani and CoH, Vellannikkara respectively.

13.2. Okon's broth *(Azospirillum)* and Pikovskaya's broth (Phosphate solubilizing bacteria) were prepared using standard protocol.

13.3. Okon's broth *(Azospirillum)* and Pikovskaya's broth (phosphate solubilizing bacteria) were supplemented with chemical additives such as trehalose (Control, 5 mM, 10 mM, 15 mM and 20 mM), glycerol (Control, 5 mM, 10 mM,15 mM and 20 mM) and PVP (Control,1,1.5,2,2.5 %) at different concentrations with 10 replications each .

13.4 Trehalose was added separately into sterilized Okon's broth (100 ml) and Pikovskaya's broth (100 ml) by filter sterilization.

13.5. Polyvinylpyrrolidone (PVP) and glycerol were added to Okon's broth (100 ml) and Pikovskaya's broth (100 ml) separately at different concentrations and then sterilized.

13.6. The enumeration of *Azospirillum* and PSB were done at monthly interval using serial dilution and plate count method (Johnson and Curl, 1952) under aseptic conditions.

13.7. Field evaluation of standardized liquid formulations of *Azospirillum* and PSB were carried out using amaranth as the test crop. The details of the experiment are presented below:

13.7.1. **Preparation of land**: The field experiment in randomized block design with eight treatments and four replications were conducted at department of Agronomy, College of Horticulture, Vellanikkara from October, 2016 to January, 2017. The soil of the experimental site was lateritic (Oxisol), belonging to Vellanikkara series, having 75.26 (Low), 40.33 (Low) and 454.74 (Medium) kg ha^{-1} of available N, P and K respectively. The pH of the soil was 5.52 and organic carbon was 1.14 (Medium) %. Each experimental plot of 160 sq.m (4 cents) was ploughed and weeds were removed from the field. A total of 32 plots were prepared with each plot area measuring 2 m x 2.25 m including a control.

13.7.2. **Nursery preparation:** The amaranth seeds (Variety: Kannara Local Arun) were obtained from the Department of Olericulture, KAU. The seeds were surface sterilized with 0.1% sodium hypochlorite for 3 minutes and repeatedly washed under sterile water till the residue of sodium hypochlorite was removed. The seeds were sown in a tray containing sterilized soil and sand mixture (1:1). The 25 day-old-seedlings were transplanted to the main field.

13.7.3. **Field evaluation**: The liquid biofertilizers along with carrier-based biofertilizers were evaluated under field condition using amaranth as the test crop.

Treatment details

Variety: Kannara Local Arun

Design: RCBD

Treatments: 8

Replication: 4 (30 plants/replication)

Amaranth seedlings were transplanted in rows with 20 cm gap between 2 rows and each row had 10 plants.

T_1 :KAU POP recommendations (FYM: 50 t as basal dose, N: P: K @ 50:50:50 kg/ha, Spraying urea @ 1 % after each harvest)

T_2 :*Azospirillum* (Liquid formulation)

T_3 : Phosphate Solubilizing Bacteria (Liquid formulation))

T_4 : *Azospirillum* (Liquid formulation) + PSB (Liquid formulation)

T_5 : *Azospirillum* (Carrier based formulation)

T_6 : Phosphate Solubilizing Bacteria (Career based formulation)

T_7 : *Azospirillum* (Career based) + PSB (Carrier based formulation)

T_8 : Absolute control

13.7.4. **Preparation of liquid formulations**

- Mother culture preparation: The Okon's and Pikovaskaya's broth (100 ml each) were prepared for *Azospirillum* sp. and PSB respectively in a conical flask and sterilized. Two loopful of pure *Azospirillum* and PSB cultures were inoculated in respective media and incubated at 28 ^{0}C +/- 2 for 48 hours.

- *Azospirillum* liquid formulation: From 100 ml mother culture broth of *Azospirillum*, 3 ml (@ 10^8 cfu/ml) of the culture suspension was inoculated to 300 ml Okon's broth supplemented with filter sterilized trehalose (15 mM) and incubated for 48 h at 28° C +/- 2

- PSB liquid formulation: From 100 ml mother culture broth of PSB, 3 ml (@ 10^8 cfu/ml) of the culture suspension was inoculated to 300 ml Pikovskaya's broth supplemented with 2.5% PVP and incubated for 48 h at 28°C +/- 2 .

In the case of consortia, 150 ml each of *Azospirillum*, and PSB broth were mixed to obtain liquid formulation.

13.7.5. **Preparation of carrier based formulations**

- Mother culture preparation: The Okon's and Pikovaskaya's broth (100 ml each) were prepared for *Azospirillum* and PSB respectively in a conical flask and sterilized. Two loopful of pure *Azospirillum* and PSB cultures were inoculated to respective media and incubated at 28 ^{0}C +/- 2 for 48 hours.

- Carrier based *Azospirillum* and PSB inoculant: From 100 ml mother culture broth of *Azospirillum* and PSB , 3 ml (@ 10^8 cfu/ml) of the respective culture suspension were inoculated to 300 ml of the Okon's and Pikovskaya's broth separately and incubated for 48 h at 28° C +/- 2.

- Mixing with carrier material: After incubation, the respective culture broth (300 ml) containing *Azospirillum* sp. and PSB were mixed separately with 1 kg of sterilized talc powder and air dried. In the case of consortia, 150 ml each of *Azospirillum* and PSB were mixed with 1 kg of sterilized talc powder.

13.7.6. **Method of inoculum application**

Seedling dip method was used for the application of biofertilizer at the time of transplanting the crop. In the case of liquid formulations from T_2 to T_4 treatments, the *Azospirillum* and PSB were applied @ 100 ml / ha. The 3 weeks old seedlings were dipped for 30 minutes in respective liquid formulations and planted based on the treatments. For combination of biofertilizers, the consortia containing 150 ml of *Azospirillum* and 150 ml of PSB were used. In the case of treatments T_5 to T_7, the carrier-based *Azospirillum* and PSB were applied @ 500 g / ha by seedling dip method. For consortia of biofertilizers, seedlings were dipped for 30 minutes in the talc based formulation.

13.7.7. **Observations recorded**

The plant height and number of leaves were recorded at fortnightly interval whereas, the number of days taken for flowering were recorded as and when the flowers appeared. The population of *Azospirillum* and PSB were recorded at 30 DAP and 60 DAP.

· The fresh biomass was recorded at 50, 75 and 100 days after planting. The root-fresh and dry biomass were recorded at the time of harvest.

14. Results and discussion

14.1. Effect of chemical additives in Okon's broth on the *Azospirillum* population

In general, the population of *Azospirillum* sp. declined from x 10^{10} cfu /ml to x 10^{5} cfu/ml at 14 MAI (Table 1.). At 1 MAI, the highest population of *Azospirillum* sp. (0.64 x 10^{10} cfu/ml) was recorded in the case of glycerol (5 mM). At 2 MAI, glycerol (5 mM) recorded the highest population of *Azospirillum* sp. (0.60 x 10^{10} cfu/ml) when compared with other additives. However, in the 3 MAI, the maximum population was recorded in the case of Trehalose (20 mM) (71×10^8 cfu/ml). At 4 MAI, the highest population of PSB was recorded in the case of PVP (1.5 %) (1.66×10^8 cfu /ml) where as, at the 5 MAI, the highest PSB population was recorded in the case of Trehalose (15 mM and 20 mM) (0.66×10^8 cfu/ml). At 6 MAI, the highest population of *Azospirillum* (300×10^8 cfu/ml) was found in Trehalose (15 mM) and PVP (1 %). At 7 MAI, Trehalose (20 mM) recorded the highest population of *Azospirillum* (7.98 $\times10^8$ cfu/ml). At 8 MAI, the highest population of *Azopsirillum* sp. was recorded in the case of Trehalose (15 mM) (26.5 $\times10^8$ cfu/ml)

Table 1: Effect of different chemical additives on the population of *Azospirillum* in Okon's broth at monthly interval

Chemical Additives	Population of *Azospirillum* (cfu/ml)															
	1 MAI	2 MAI	3 MAI	4 MAI	5 MAI	6 MAI	7 MAI	8 MAI	9 MAI	10 MAI	11 MAI	12 MAI	13 MAI	14 MAI	15 MAI	16 MAI
Trehalose (5mM)	0.06×10^{7}	0.332×10^{7}	1×10^{7}	0.66×10^{8}	0.99×10^{7}	300×10^{8}	6×10^{8}	1.44×10^{6}	0.33×10^{5}	0.11×10^{5}	0	0	0	0	0	0
Trehalose (10mM)	0.462×10^{7}	19.6×10^{8}	1.6×10^{8}	2.33×10^{7}	0.33×10^{7}	0.44×10^{8}	0.11×10^{8}	0.11×10^{8}	0	0	0	0	0	0	0	0
Trehalose (15mM)	0.66×10^{9}	0.33×10^{7}	10.6×10^{8}	1.33×10^{8}	0.66×10^{8}	0.66×10^{8}	1.43×10^{8}	26.5×10^{8}	1.77×10^{8}	0.33×10^{6}	0.55×10^{5}	0	0	0	0	0
Trehalose (20mM)	0.66×10^{9}	0.332×10^{7}	71×10^{8}	2×10^{7}	0.66×10^{8}	0.33×10^{8}	7.98×10^{8}	0.44×10^{8}	0.22×10^{7}	0.11×10^{7}	1.55×10^{5}	1.33×10^{5}	0	0	0	0
PVP (1%)	0.266×10^{9}	0.52×10^{8}	23.3×10^{8}	3.66×10^{6}	0.33×10^{6}	300×10^{8}	1.11×10^{8}	0.11×10^{8}	0.55×10^{5}	0.33×10^{4}	0.66×10^{3}	0.77×10^{4}	0.22×10^{3}	0	0	0
PVP (1.5%)	0.66×10^{9}	0.80×10^{10}	0.66×10^{8}	$1.66x10^{8}$	4×10^{6}	0.33×10^{8}	0.11×10^{8}	0.11×10^{8}	0.11×10^{4}	0.22×10^{4}	0.66×10^{3}	1.55×10^{4}	0.11×10^{4}	1.99×10^{3}	0	0
PVP (2%)	0.264×10^{7}	0.32×10^{7}	2.33×10^{7}	1.33×10^{7}	1.66×10^{6}	0.66×10^{8}	1.33×10^{8}	0.33×10^{5}	0.22×10^{4}	0.22×10^{4}	0.66×10^{4}	0	0	0	0	0
PVP (2.5%)	0.38×10^{7}	0.32×10^{7}	0.66×10^{8}	1×10^{7}	1.66×10^{6}	1.88×10^{8}	0.55×10^{8}	0.11×10^{4}	0.33×10^{4}	0.55×10^{3}	0	0	0	0	0	0
Glycerol (5 mM)	0.64×10^{10}	0.6×10^{10}	5.33×10^{7}	0.66×10^{7}	0.33×10^{6}	2×10^{7}	0.55×10^{8}	0.22×10^{7}	0.66×10^{6}	0.11×10^{5}	0.18×10^{5}	0.11×10^{5}	0.11×10^{5}	2.11×10^{4}	0	0
Glycerol (10mM)	0.26×10^{10}	0.38×10^{10}	6.33×10^{7}	0.33×10^{8}	0.33×10^{7}	0.11×10^{8}	0.11×10^{6}	0.11×10^{5}	0.22×10^{5}	4.22×10^{5}	0..22×10^{5}	0.33×10^{5}	0	0	0	0
Glycerol (15 mM)	0.12×10^{10}	0.3×10^{7}	2.66×10^{7}	1×10^{8}	1.66×10^{7}	8.44×10^{8}	0.33×10^{4}	0.77×10^{5}	0.44×10^{4}	1.44×10^{4}	0.22×10^{5}	0	0	0	0	0
Glycerol (20 mM)	0.18×10^{10}	0.06×10^{8}	0.66×10^{8}	7.66×10^{7}	1.33×10^{7}	3.33×10^{6}	0.22×10^{7}	0.22×10^{7}	0.11×10^{7}	0.22×10^{4}	3.1×10^{5}	2.66×10^{5}	0.11×10^{5}	0.55×10^{5}	0	0
Control without additives	$3.67 x10^{7}$	0.47×10^{7}	$8.00 x 10^{4}$	0	0	0	0	0	0	0	0	0	0	0	0	0

PVP-Poly Vinyl Pyrollidine

and at 9 MAI also, the highest population of *Azospirillum* sp. was recorded in the case of Trehalose (15 mM) (1.77×10^8 cfu/ml), which was the same population as prescribed in the quality standards for liquid biofertilizers and in subsequent month,the population declined. At 10 MAI, the highest population was recorded in the case of Trehalose (20 mM). (0.11×10^7 cfu/ml). However, at 11 MAI, the population of PSB was highest (55×10^5 cfu/ml) in the case of Trehalose (20 mM). At 12 MAI,the population of PSB was highest (2.66×10^5 cfu/ml) in the case of glycerol (20 mM) whereas , highest population of *Azospirillum* sp. (0.11×10^5 cfu/ml) was recorded in glycerol (5 & 20 mM) at 13 MAI. At 14 MAI,the population of PSB was highest (0.55×10^5 cfu/ml) in the case of glycerol (20 mM)

In general, the population of *Azospirillum* sp. was highest in trehalose and glycerol when compared with PVP as supplement. The highest population of *Azospirillum* (1.77×10^8 cfu/ml) was maintained in the case of trehalose (15 mM) upto 9 MAI and after that the population declined to 10^6 cfu/ml by 14 MAI. Hence, trehalose (15 mM) was the most suitable supplement for enhancing the shelf life of *Azospirillum* sp. and maintaining the population at 10^8cfu/ml, which is also the quality standard for liquid formulation of *Azospirillum* sp. These results are in concurrence with earlier studies where enhanced survival of *Azospirillum* cells in the liquid formulation has been reported due to the action of chemical amendments added in the medium. In the present studies, the trehalose. Trehalose is capable of enhancing cell tolerance to desiccation, osmotic pressure and temperature stress (Streeter, 1985) and stabilizing both enzymes and cell membranes (Fillinger, *et al.*, 2001). Moreover, some polymeric additives such as PVP, PVA and starch have polymeric properties. This protective property known as colloidal stabilization. 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 storage under room temperature.

14.2. Effect of chemical additives in Pikovskaya's broth on the PSB population

In general, the population of PSB declined from 10^{10} cfu /ml at 1 MAI to 10^6 cfu/ml at 16 MAI (Table 2.). At 1 month after inoculation (MAI) of PSB in Pikovskaya's broth supplemented with different chemical additives, the media supplemented with

Table 2: Effect of different chemical additives on the population of phosphate solubilizing bacteria in Pikovskaya's broth at monthly interval

Chemical Additives	Population of PSB (cfu/ml)															
	1 MAI	2 MAI	3 MAI	4 MAI	5 MAI	6 MAI	7 MAI	8 MAI	9 MAI	10 MAI	11 MAI	12 MAI	13 MAI	14 MAI	15 MAI	16 MAI
Trehalose (5mM)	0.11×10^{9}	6.66×10^{7}	0.44×10^{8}	0.77×10^{8}	1.88×10^{5}	0.44×10^{5}	0.11×10^{5}	0	0	0	0	0	0	0	0	0
Trehalose (10mM)	0.33×10^{7}	0.33×10^{7}	0.99×10^{8}	0	0	0	0	0	0	0	0	0	0	0	0	0
Trehalose (15mM)	0.33×10^{8}	0.33×10^{6}	1.66×10^{7}	0.77×10^{8}	0.66×10^{7}	0.11×10^{5}	0.22×10^{5}	0	0	0	0	0	0	0	0	0
Trehalose (20mM)	0.11×10^{8}	0.66×10^{8}	0.11×10^{8}	0.11×10^{5}	0	0	0	0	0	0	0	0	0	0	0	0
PVP (1%)	0.33×10^{6}	0.33×10^{8}	1.33×10^{7}	0.11×10^{8}	0.11×10^{8}	0.22×10^{6}	0.55×10^{4}	0.33×10^{5}	0	0	0	0	0	0	0	0
PVP (1.5%)	0.33×10^{8}	1×10^{8}	0.33×10^{8}	0.33×10^{8}	0	0	0	0	0	0	0	0	0	0	0	0
PVP (2%)	0.33×10^{10}	2.33×10^{7}	1×10^{8}	0.66×10^{8}	0.77×10^{8}	0.55×10^{8}	0.22×10^{8}	0.55×10^{6}	6.44×10^{6}	1.10×10^{6}	6.55×10^{6}	5.22×10^{6}	5.2×10^{6}	0.66×10^{6}	0	0
PVP (2.5%)	1.33×10^{10}	0.33×10^{9}	0.11×10^{9}	6.66×10^{7}	0.33×10^{8}	0.33×10^{7}	0.33×10^{7}	0.33×10^{8}	3.77×10^{8}	0.44×10^{5}	1.33×10^{6}	2.88×10^{6}	0.44×10^{4}	0	0	0
Glycerol (5 mM)	0.55×10^{4}	4×10^{8}	0.33×10^{7}	0.11×10^{8}	0.11×10^{8}	0.11×10^{7}	0.33×10^{6}	0.11×10^{6}	0.33×10^{6}	0.11×10^{6}	0	0	0	0	0	0
Glycerol (10mM)	4×10^{8}	2.33×10^{7}	0.66×10^{8}	0.33×10^{8}	0.11×10^{8}	0.88×10^{8}	1.22×10^{7}	0.22×10^{7}	16.22×10^{6}	0.22×10^{6}	0	0	0	0	0	0
Glycerol (15 mM)	0.22×10^{5}	0.33×10^{8}	1.33×10^{7}	1.33×10^{6}	0.44×10^{8}	0.99×10^{8}	1.55×10^{7}	1.88×10^{6}	2.33×10^{6}	0.11×10^{6}	1.66×10^{5}	0	0	0	0	0
Glycerol (20 mM)	0.11×10^{9}	2×10^{6}	1.66×10^{8}	1.33×10^{8}	0.33×10^{8}	1.21×10^{8}	0.11×10^{8}	18.33×10^{6}	0	0	0	0	0	0	0	0
Control without additives	2.67×10^{9}	8.67×10^{6}	6.33×10^{5}	9.33×10^{5}	0	0	0	0	0	0	0	0	0	0	0	0

PVP-Poly Vinyl Pyrollidine

PVP (2.5%) recorded the highest population (1.33 x 10^{10} cfu/ml) of PSB when compared with other additives. However, in the 2 MAI, the maximum population was recorded in the case of PVP (2.5 %) (0.33 $\times 10^9$ cfu/ml). At 3 MAI, the highest population of PSB was recorded in the case of PVP (2.5 %) (0.11$\times 10^9$ cfu /ml) where as the highest PSB population was recorded in the case of Trehalose (5 and 15 mM) (0.77$\times 10^8$) cfu/ml) at 4 MAI. At 5 MAI, the highest population of PSB was recorded in the case of PVP (2%) (0.77$\times 10^8$cfu/ml) and at 6 MAI, highest population of PSB sp. (0.99 $\times 10^8$cfu/ml) was found in glycerol (15 mM). At 7 MAI, the highest population was recorded in the case of PVP (2 %) (0.22 $\times 10^8$cfu/ml). The highest population (0.33 $\times 10^8$cfu/ml) was recorded in the case of PVP (2.5 %) at 8 MAI. However, at 9 MAI, the population of PSB was highest in the case of PVP (2.5 %) with a population of (3.77 $\times 10^8$cfu/ml) and it stabilized to 10^8 cfu/ml in the case of PVP (2.5 %) which was same population as prescribed in the quality standards for liquid biofertilizers. At 10 MAI,the population of PSB declined to 1.10 $\times 10^6$ cfu/ml but it was the highest in the case of PVP (2%). After 11 MAI, the highest population was recorded in the case of PVP (2 %) with a population of 6.55 $\times 10^6$ cfu/ml). At 12, 13, 14 MAI, the population declined but was highest in the case of PVP (2 %). The population of PSB was absent at 15 and 16 MAI.

In general, the population of PSB was highest in PVP when compared with other supplements added. The highest population of PSB (3.77$\times 10^8$ cfu /ml) was maintained in the case of PVP (2.5 %) upto 9 MAI and after that the population declined to 10^6 cfu/ml by 14 MAI. Hence, PVP (2.5 %) was the most suitable supplement for enhancing the shelf life of PSB at 10^8 cfu/ml which is also the quality standard for liquid formulation of PSB. Liquid inoculant formulation of cowpea rhizobia prepared with PVP as an osmoprotectant been observed to have higher shelf life than those without PVP amendment (Girisha *et al.,* 2006). 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). Enhanced survival in liquid inoculant-2 was recorded due to the presence of PVP that maintained the moisture and protected the cell from desiccation (Somasegaran and Hoben, 1985). Sridhar *et.al.* (2004) reported that liquid inoculant-2 containing osmoprotectants *viz.,* Polyvinyl Pyrrolidone (PVP), glycerol and glucose supported higher viable population (log 10 10.50 CFU/ml) and endospores (log 10 9.21 CFU/ml) up to a storage period of 6 months. It also supported higher viable cells on cowpea seed (log 10 4.50 CFU/ml) and also enhanced the P-uptake and total biomass of cowpea significantly. These results are in agreement with present studies.

14.3. Evaluation of liquid formulations of *Azospirillum* sp. and PSB for plant growth promotion under field condition

An experiment was conducted to evaluate the standardized liquid formulations of *Azospirillum* sp. and PSB for growth promotion of amaranth under field condition.

14.3.1. Effect of different treatments on the plant height

There were significant differences among the treatments.The plant height showed increasing trend from 15 DAP to 60 DAP (Table 3). At 60 DAP, maximum plant height (34.19 cm / plant) was recorded in the case of PSB (Liquid formulation) followed by *Azospirillum* sp. (Liquid Formulation) (28.16 cm / plant). The lowest plant height was in the case of T_1 (21.19 cm / plant). In a study by Mofunanya (2014), it was reported that organic fertilizer produced higher nutritional values on *Amaranthus spinosus* when compared with inorganic fertilizer. Hima Bindu (2016) investigated the effects of liquid and carrier based biofertilizers of *Bradyrhizobium* and phosphate solubilizing bacteria (PSB) on soybean yield. The studies showed that treatment with 75 % RDF + liquid based biofertilizers (soil application) had more, seeds per pod, test weight and seed yield per hectare. The results are in agreement with the present studies where liquid biofertilizer of PSB performed better than all other treatments. Currently, liquid biofertilizers are considered to be the best alternative for the conventional carrier based formulations which will help in the enhanced crop yield, soil health and sustainable food production.

14.3.2. Effect of different treatments on the number of leaves

There were significant differences among the treatments at 15, 30 and 45 DAP. However, no significant differences were observed among the treatments at 60 DAP (Table 4).The number of leaves showed increasing trend from 15 DAP to 60 DAP. The highest number of leaves (98.66) were recorded in the case of PSB (Liquid formulation) followed by *Azospirillum* sp. (Liquid formulation) (76.04) at the time of final harvest. The number of leaves were less in the case of control plants (53 cm).

Table 3: Effect of different treatments on the plant height of amaranth under field condition

Treatment details	Plant height (cm)			
	15 DAP	30 DAP	45 DAP	60 DAP (10 days after first harvest at 50 DAP)
T_1 : KAU POP Recommendations (FYM: 50 t as basal dose, N: P: K @ 50:50:50 kg/ha, Spraying urea @ 1 % after each harvest)	1.75	7.43	23.58	21.19
T_2:*Azospirillum*(LF)	4.52	13.06	29.16	28.16
T_3: Phosphate Solubilizing Bacteria (LF)	5.24	19.62	57.10	34.19
T_4: *Azospirillum* (LF) + PSB (LF)	3.01	9.89	23.68	24.21
T_5: *Azospirillum*(CB)	2.89	9.47	30.68	20.83
T_6: Phosphate Solubilizing Bacteria (CB)	2.75	9.88	38.62	25.81
T_7: *Azospirillum* (CB) + PSB (CB)	2.81	5.78	16.40	22.55
T_8 : Control	2.27	7.85	21.93	22.32
C.D	1.39	5.52	16.06	6.82
S.E (M)	0.47	1.86	5.42	2.30

Each value represents a mean of 40 plants

LF: Liquid formulations

CF: Carrier based formulations

DAP: Days after planting

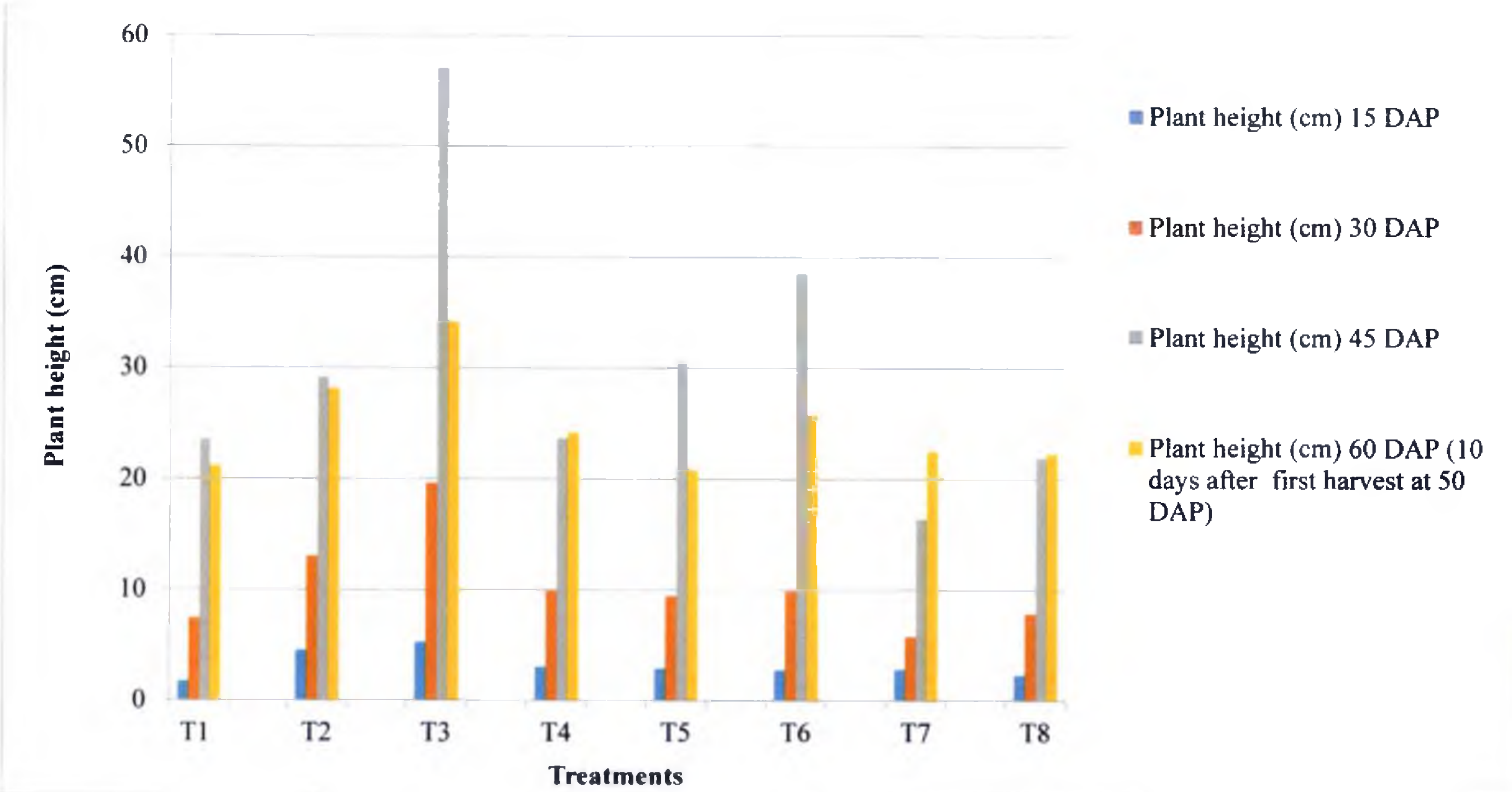

Figure 3: Effect of different treatments on the plant height of amaranth under field condition

Table 4: Effect of different treatments on the number of leaves in amaranth under field condition

Treatment details	Number of leaves			
	15 DAP	30 DAP	45 DAP	60 DAP (10 days after first harvest)
T_1 : KAU POP Recommendations (FYM: 50 t as basal dose, N: P: K @ 50:50:50 kg/ha, Spraying urea @ 1 % after each harvest)	14.0	15.50	54.38	57.81
T_2:*Azospirillum*(LF)	5.91	37.67	63.09	76.04
T_3: Phosphate Solubilizing Bacteria (LF)	7.17	64.81	129.83	98.66
T_4: *Azospirillum* (LF) + PSB (LF)	4.38	21.50	66.92	71.96
T_5: *Azospirillum*(CB)	4.60	23.50	53.48	62.94
T_6: Phosphate Solubilizing Bacteria (CB)	5.52	18.70	92.97	71.79
T_7: *Azospirillum* (CB) + PSB (CB)	5.47	13.45	42.46	74.78
T_8 : Control	5.15	18.24	51.49	53.03
C.D	1.69	16.41	50.78	NS
S.E (M)	0.57	5.54	17.15	10.97

Each value represents a mean of 40 plants

LF: Liquid formulation

CF: Carrier based formulations

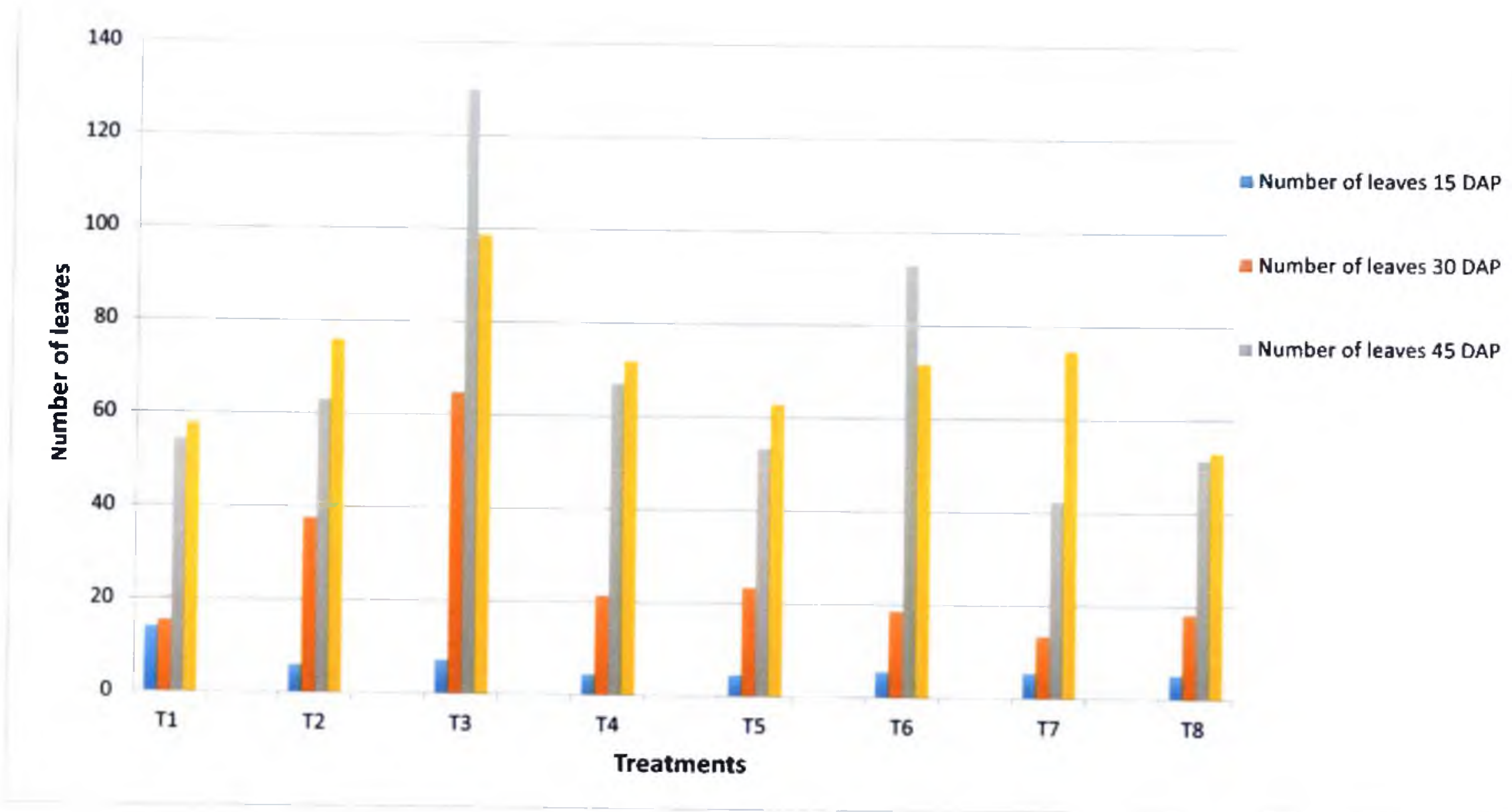

Figure 4: Effect of different treatments on the number of leaves in amaranth under field condition

14.3.3. Effect of different treatments on the number of days taken for flowering

There were significant differences among the treatments. The number of days taken for the flowering ranged from 31.75 to 48.75 (Table.5). The minimum number of days taken for flowering (31.75) was recorded with PSB (Liquid Formulation) followed by *Azospirillum* sp. (Liquid Formulation) (36.75). It has been reported by Hegde (2008) that liquid biofertilizers not only contains the desired microorganisms and their nutrients but also special cell protectants or chemicals that promote formation of resting spores or cysts for longer shelf life and tolerance to adverse conditions. In the present studies, the minimum number of days taken for flowering was recorded in the case of liquid biofertilizer of PSB, whereas, the maximum number of days taken for flowering was in the case of carrier based formulation of *Azospirillum* + PSB indicating that the liquid biofertilizers perform better than carrier based formulations against adverse stress conditions with respect to early flowering in plants.

14.3.4. Effect of different treatments on the fresh leaf biomass

There were no significant differences among the treatments (Table.6). However, the mean total fresh biomass was highest (130.85 g / plant) in the case of PSB (Liquid formulations) followed by PSB (Carried based formulation) (130.55 g / plant). The total fresh leaf biomass of plant was lowest in the case of T_1 (KAU POP). It has been reported earlier that inoculation with PSB such as *Pseudomonas, Bacillus, Rhizobium, Micrococcus, Flavobacterium, Achromobacter, Erwinia* and *Agrobacterium* increased the solubilization of fixed P which ensured high crop yields (Rodriguez and Fraga, 1999). In another study,(Zehra, 2010) reported that application of PSB such as *Bacillus* (M-13), with and without varying amounts of phosphorus resulted in highest yield of sunflower seeds under field conditions. *Bacillus* sp. (PSB) also enhanced the growth and yield of cotton (Qureshi *et al.*, 2012).In the present studies, liquid formulation of PSB recorded highest biomass due to the increased shelf-life and tolerance to adverse conditions which is in agreement with earlier studies by Sridhar (2004), who reported that *Bacillus megaterium* (PSB) containing polyvinyl pyrrolidone (PVP), glycerol and glucose supported higher viable population (log 10 10.50 CFU/ml) and endospores (log 10 9.21 CFU/ml) up to a storage period of 6 months which also supported higher viable cells on cowpea seed (log 10 4.50 CFU/ml) and enhanced the P-uptake and

Table 5: Effect of different treatments on the number of days taken for flowering in amaranth under field conditions

Treatment details	No of days taken for flowering
T_1 : KAU POP Recommendations (FYM: 50 t as basal dose, N: P: K @ 50:50:50 kg/ha, Spraying urea @ 1 % after each harvest)	47.50
T_2:*Azospirillum*(LF)	36.75
T_3: Phosphate Solubilizing Bacteria (LF)	31.75
T_4: *Azospirillum* (LF) + PSB (LF)	43.75
T_5: *Azospirillum*(CB)	44.0
T_6: Phosphate Solubilizing Bacteria (CB)	45.25
T_7: *Azospirillum* (CB) + PSB (CB)	48.75
T_8 : Control	48.0
C.D	6.74
S.E (M)	2.27

Each value represents a mean of 40 plants

LF: Liquid formulations

CF: Carrier based formulations

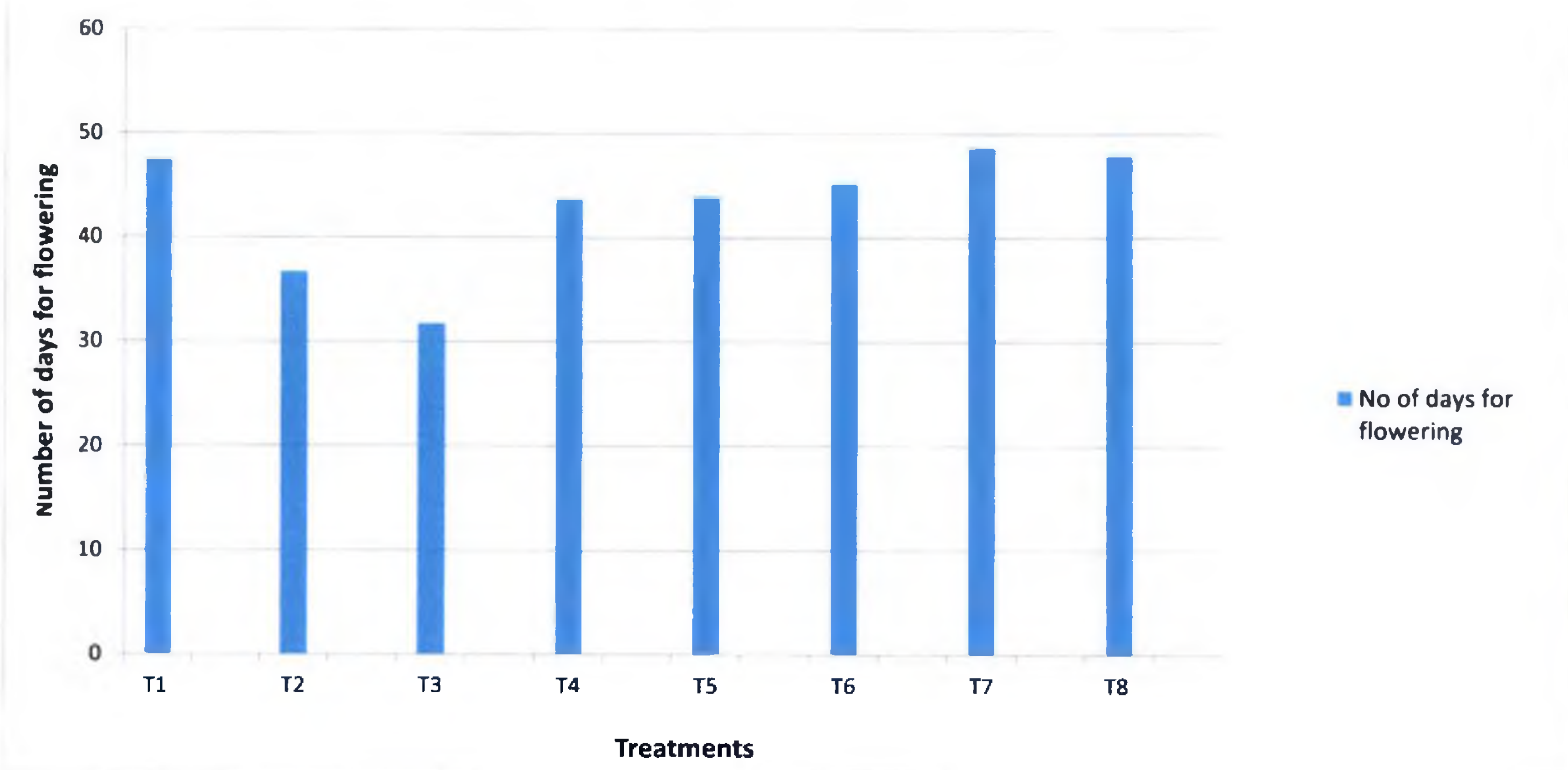

Figure 5: Effect of different treatments on the number of days taken for flowering in amaranth under field conditions

Table 6: Effect of different treatments on the fresh biomass of amaranth at different intervals under field condition

Treatment details	Fresh biomass (g / plant)			
	50 DAP (First Harvest)	75 DAP (Second Harvest)	100 DAP (Third Harvest)	Total biomass (Mean of 1st,2nd and 3rd harvest)
T_1 : KAU POP Recommendations (FYM: 50 t as basal dose, N: P: K @ 50:50:50 kg/ha, Spraying urea @ 1 % after each harvest)	105.77	91.47	55.69	84.31
T_2:*Azospirillum*(LF)	106.80	115.45	65.93	96.06
T_3: Phosphate Solubilizing Bacteria (LF)	205.72	87.48	99.36	130.85
T_4: *Azospirillum* (LF) + PSB (LF)	111.23	103.86	65.26	93.45
T_5: *Azospirillum*(CB)	113.81	115.86	69.07	99.58
T_6: Phosphate Solubilizing Bacteria (CB)	176.05	124.36	91.24	130.55
T_7: *Azospirillum* (CB) + PSB (CB)	124.47	124.76	68.50	105.91
T_8 : Control	130.07	90.93	66.80	95.94
C.D	NS	NS	NS	NS
S.E (M)	40.38	15.67	15.57	12.37

Each value represents a mean of 40 plants

LF: Liquid formulations

CF: Carrier based formulations

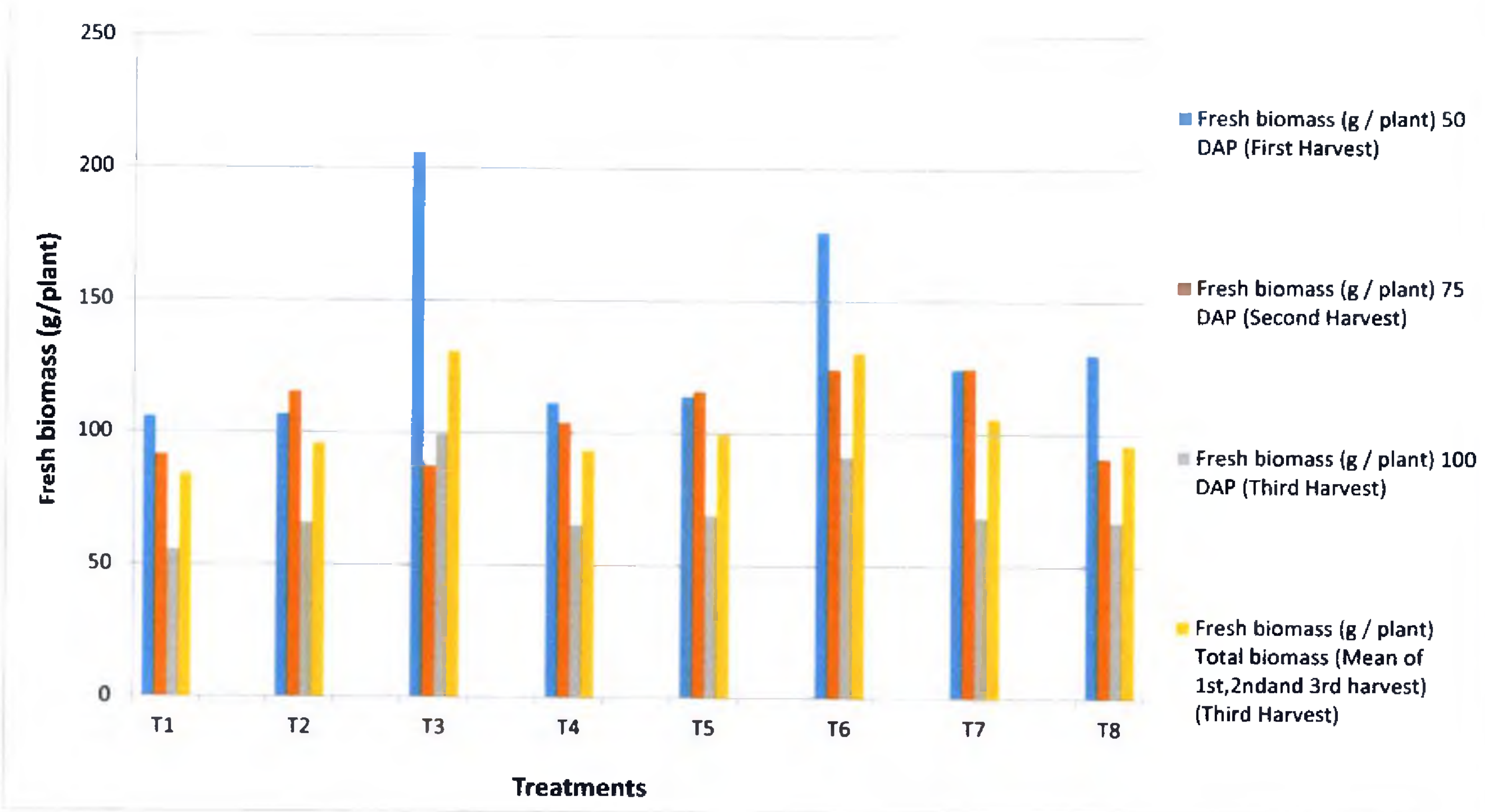

Figure 6: Effect of different treatments on the fresh biomass of amaranth at different intervals under field condition

total biomass of cowpea significantly. Similarly, Vanithamani (2017) reported that cyanobacteria, phosphobacteria and *Azospirillum* combined with half dose of inorganic fertilizer (NPK) enhanced the nutritional status of leafy vegetable *Amaranthus polygonoids* compared to control. Islam (2011) also reported that application of chemical fertilizer or organic manure alone to amaranth gave poor result compared to the integrated use of organic manure coupled with a reduced dose of chemical fertilizers in the study.

14.3.5. Effect of different treatments on the rhizosphere population of *Azosprillum* sp. at different intervals.

Native population of *Azospirillum* sp. in the field soil was found to be absent before the start of experiment. Even after the inoculation, *Azospirillum* population was not observed at 30 DAP and 60 DAP (Table.7) indicating that the isolate could not tolerate acidic pH and soil edaphic factors. The *Azospirillum* sp. preponderance and colonization vary with various factors such as soil types, fertilizer application, soil moisture content, crop rotation etc. (Bashan et al. 2004; Garbeva et al. 2008). Verma *et.al.* (2011) reported that no tillage, growing traditional maize cultivar, land use history, soil organic carbon (>1%) and intercrop with oat supported the prevalence of *Azospirillum* in maize rhizosphere. Various studies have also indicated that the extent of *Azospirillum* sp. responses varied with location, season, product quality etc. (Bashan, *et al.* 2004). In the present studies, there were no *Azospirillum* sp. recorded in the soil which indicated that soil edaphic factors and the season during the crop growth period might have affected the survivability of inoculated *Azospirillum* sp.

14.3.6 Effect of different treatments on the rhizosphere population of PSB at different intervals

Native population of PSB in the field soil was not observed before the start of the experiment. After the inoculation of PSB, there were no significant differences among the treatments at 30 DAP, with highest population recorded in the case of PSB (Liquid formulation) (1.62×10^{3} cfu/g) (Table.8). At 60 DAP, there were significant differences among treatment. The PSB population was highest (2.29×10^{3} cfu/g) in the case of PSP (Liquid formulation) treated plants at the time of harvest. The present studies clearly indicated that the population of inoculated PSB (Liquid formulation) survived in the soil despite low soil pH, as

Table 7: Population of *Azospirillum* sp. in the rhizosphere of amaranth under field condition

Treatment details	Population (cfu/g)	
	30 DAP	60 DAP
T_1 : KAU POP Recommendations (FYM: 50 t as basal dose, N: P: K @ 50:50:50 kg/ha, Spraying urea @ 1 % after each harvest)	a	a
T_2:*Azospirillum* (LF)	a	a
T_3: Phosphate Solubilizing Bacteria (LF)	a	a
T_4: *Azospirillum* (LF) + PSB (LF)	a	a
T_5: *Azospirillum*(CB)	a	a
T_6: Phosphate Solubilizing Bacteria (CB)	a	a
T_7: *Azospirillum* (CB) + PSB (CB)	a	a
T_8 : Control	a	a

Initial population of *Azospirillum* sp. in the field soil was zero

a: absent

LF: Liquid formulations

CF: Carrier based formulations

Table 8: Population of phosphate solubilizing bacteria (PSB) in rhizosphere soil of amaranth under field condition

Treatment details	Population (x 10^3 cfu/ g)	
	30 DAP	60 DAP
T_1 : KAU POP Recommendations (FYM: 50 t as basal dose, N: P: K @ 50:50:50 kg/ha, Spraying urea @ 1 % after each harvest)	1.0	1.0
T_2:*Azospirillum* (LF)	1.13	1.0
T_3: Phosphate Solubilizing Bacteria (LF)	1.62	2.29
T_4: *Azospirillum* (LF) + PSB (LF)	1.33	1.13
T_5: *Azospirillum*(CB)	1.24	1.0
T_6: Phosphate Solubilizing Bacteria (CB)	1.0	1.0
T_7: *Azospirillum* (CB) + PSB (CB)	1.13	1.24
T_8 : Control	1.24	1.0
C.D.	NS	0.316
SE(m)	0.18	0.10

Initial population of phosphate solubilizing bacteria in the field soil was zero

LF: Liquid formulations

CF: Carrier based formulations

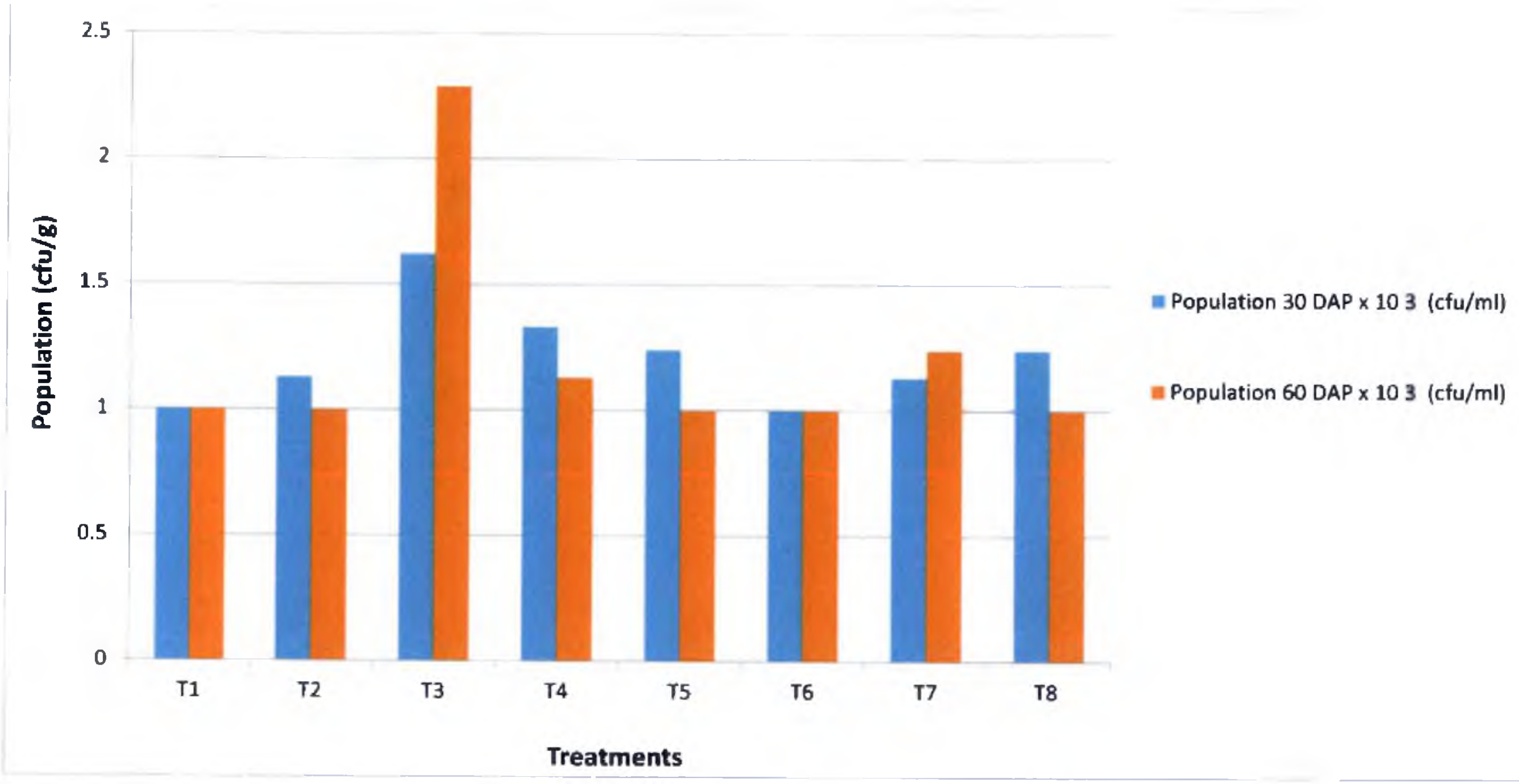

Figure 8: Population of phosphate solubilizing bacteria in rhizosphere soil of amaranth under field condition

the liquid biofertilizers could tolerate adverse conditions due to addition of cell protectants in the formulation. PSB are known to be ubiquitous and their population varies in different soils which depends on chemical and physical properties, organic matter and P content of the soil (Kim, 1998). Gupta *et al.* (1986) reported that population of PSB was generally low in arid and semi-arid regions, possibly due to the low level of organic matter and high temperature regime but the population was higher in soils under mild and moist climates (Subba Rao, 1982).It has also been reported earlier that calcium phosphate dissolving microorganisms were larger in number than microorganisms that dissolve other mineral phosphate compounds (Banik and Dey 1983; Dharmwal, *et al.* 1989). In the present studies, the native population of PSB was not observed before the start of the experiment but the inoculated PSB (Liquid formulation) maintained highest population at the time of harvest when compared with carrier based formulations of PSB and *Azospirillum,* indicating that liquid formulations are better adapted to adverse conditions in soil compared with *Azospirillum* sp.

14.3.7. Effect of different treatments on fresh and dry weight of roots at harvest

There were no significant differences among the treatments (Table 9.).The fresh weight of root was highest (16.49 g/plant) in the case of *Azospirillum* (Liquid formulation) followed by PSB (Carrier-based formulation) (15.80 g/ plant). The lowest total fresh weight was observed in the case of consortia of *Azospirillum* (Liquid formulations) + PSB (Liquid formulations) indicating that there is a possibility of two isolates becoming incompatible over a period of time in soil.

Similarly, there were no significant differences among the treatments with respect to dry weight of roots. The highest dry weight (7.88 g /plant) was recorded in the case of *Azospirillum* (Liquid formulation) followed by PSB (Carrier based formulation) (7.63 g / plant).*Azospirillum* sp. not only fixes atmospheric nitrogen but also produces phytohormones which enhances the growth of plant (Motsara,1995) which was evident in the present studies.

Although, chemical fertilizers offer benefits to the plant, their extensive use might damage the soil health and further, there will be no increase in the crop production. The use of chemical fertilizers is also an environmental concern. Therefore, biofertilzers promise a better alternative to this problem, particularly liquid formulations of biofertilizers which have better shelf-life. It is an eco-

Table 9: Effect of different treatments on the fresh and dry weight of roots at the time of harvest under field condition

Treatment details	Fresh weight of root (g/plant)	Dry weight of root (g/plant)
T_1 : KAU POP Recommendations (FYM: 50 t as basal dose, N: P: K @ 50:50:50 kg/ha, Spraying urea @ 1 % after each harvest)	14.41	4.04
T_2:*Azospirillum* (LF)	16.49	7.88
T_3: Phosphate Solubilizing Bacteria (LF)	13.27	4.34
T_4: *Azospirillum* (LF) + PSB (LF)	9.48	3.50
T_5: *Azospirillum* (CB)	11.66	3.85
T_6: Phosphate Solubilizing Bacteria (CB)	15.80	7.63
T_7: *Azospirillum* (CB) + PSB (CB)	12.02	3.94
T_8 : Control	11.64	3.61
C.D	NS	NS
S.E (M)	3.12	2.04

Each value represents a mean of 40 plants

LF: Liquid formulations

CF: Carrier based formulations

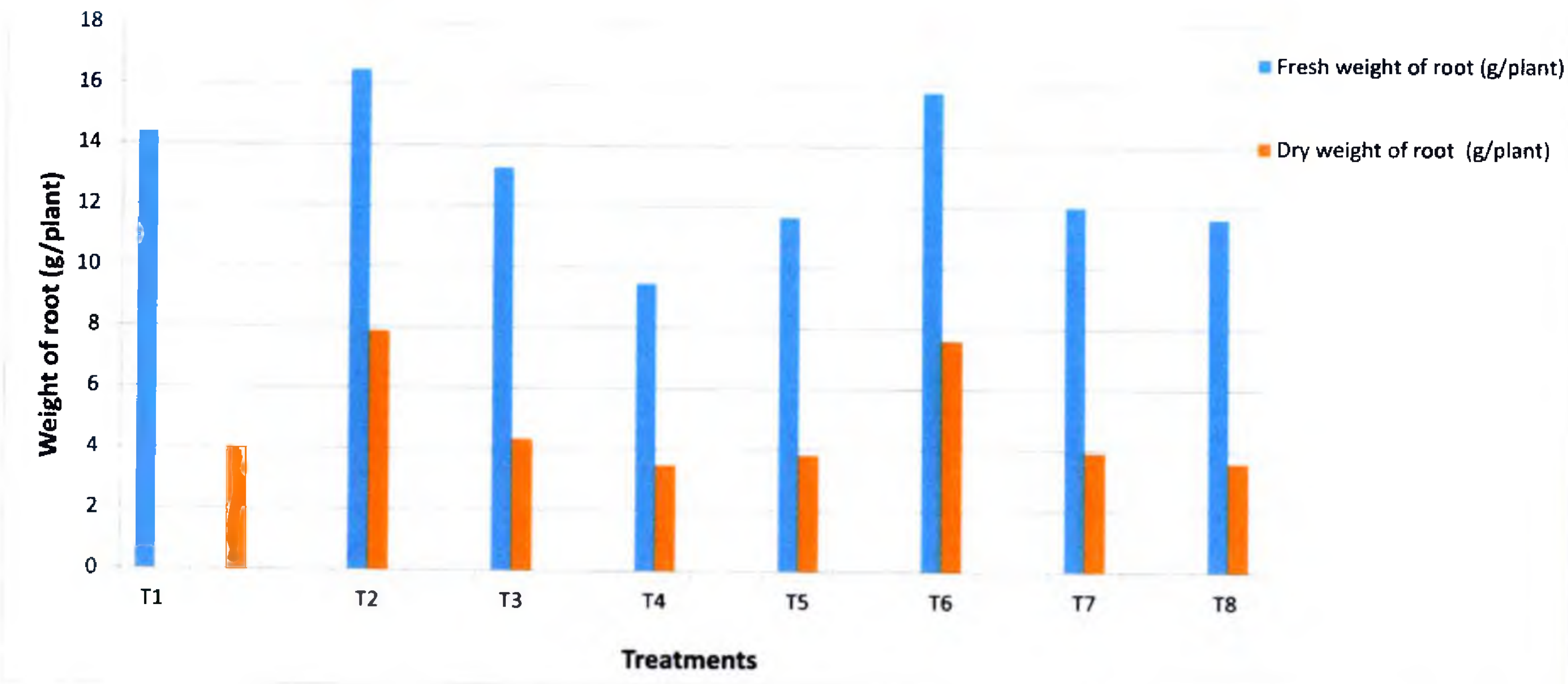

Figure 9: Effect of different treatments on the fresh and dry weight of roots at the time of harvest under field condition

friendly and cost effective agro technology to improve crop production. Hence, there is a need to develop the liquid biofertilzers on a large scale so that good quality biofertilzers are available to the farmers and use of chemical fertilizers are minimized.

15. **Major equipment's purchased**: Hot air oven

16. **Major infrastructure created**: Not applicable

17. **Major outcome of the project** (Not more than one paragraph)

The highest population of *Azospirillum* (1.77×10^8 cfu/ml) was recorded in the case of trehalose (15 mM) whereas, highest population of PSB (3.77×10^8 cfu /ml) was in the case of PVP (2.5 %) upto 9 MAI. Hence, trehalose (15 mM) and PVP (2.5 %) were the most suitable chemical additive for enhancing the shelf life of *Azospirillum* sp. and PSB respectively with a population of 10^8 cfu/ml, which is the quality standard prescribed for liquid formulations of *Azospirillum* sp and phosphate solubilizing bacteria. These results indicated that the shelf-life of *Azospirillum* sp. and PSB could be enhanced upto 9 months at room temperature when compared to shelf-life of carrier based inoculant which is about 5-6 months.

In order to assess the performance of liquid formulations of *Azospirillum* sp. and PSB, a field experiment was conducted using amaranth as a test crop. Based on the plant height, number of leaves, number of days taken for flowering and total biomass of plant, the PSB (liquid formulation) was the most promising biofertilizer for enhancing the growth of amaranth under field conditions.While comparing the performance of liquid and carrier based formulations of *Azospirillum* sp. and PSB, the liquid formulations performed better than carrier based formulations.

18. **Major technological outcome i.e., useful for the farming community as a whole** (Not more than three sentences):

- Shelf-life of *Azospirillum* sp. and PSB could be enhanced upto 9 months at room temperature when compared to shelf-life of carrier based inoculant which is about 5-6 months.
- The liquid formulation of PSB performed better than *Azospirillum* sp under field condition

- Among all the treatments, PSB (liquid formulation) was the most promising biofertilizer for enhancing the growth and yield of amaranth under field condition.

These outcomes will help the farming community to get the liquid formulation of phosphate solubilizing bacteria with increased shelf-life.

Research paper published (Under the research project)

Surendra Gopal, K. and Akhila Baby.2016. Enhanced shelf-life of *Azospirillum* and PSB through addition of chemicals in liquid formulation. *International Journal of Science, Environment and Technology.* 5(4). 2023-2029 **(NAAS rating: 3.98)**

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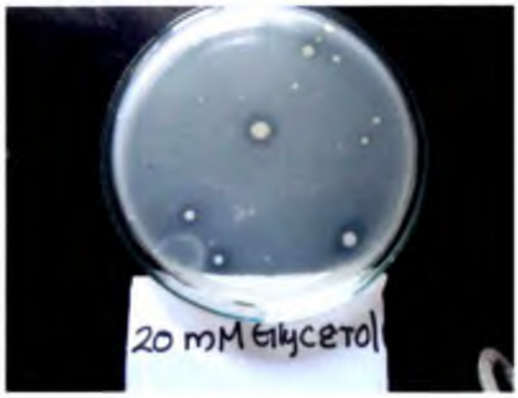
Name and Signature of PI

Dr. K.SURENDRA GOPAL, M.Sc (Agri), Ph.D
Professor (Microbiology)
COH, Kerala Agricultural University
Vellanikkara - 680 656, Thrissur

Signature of Heads of Station

ASSOCIATE DEAN
College of Horticulture
Kerala Agricultural University
K.A.U. P.O., Thrissur-680 656



1. **Effect of Glycerol (20 mM) on the population of PSB in Pikovskaya's agar media**

2. ***Azospirillum* culture in Okon's broth**

3. PSB culture in Pikovskaya's broth

4. **Overview of the experimental field at 2 weeks after planting**

T_3 R_1 (PSB-Liquid)

T_3 R_2 (PSB-Liquid)

T_3 R_3 (PSB-Liquid)

T_8 R_1 (Control)

T_8 R_2 (Control)

T_8 R_3 (Control)

5. Effect of liquid formulation of phosphate solubilizing bacteria (T_3) on the biomass of amaranth and control plants (T_8) under field conditions at 12 weeks after planting

6. Effect of liquid formulation of phosphate solubilizing bacteria (T3) on the yield of amaranth and control plants (T8) at the time of harvest under field conditions.