STANDARDIZATION OF LIQUID FORMULATION OF PGPR MIX-I AND ITS EVALUATION FOR PLANT GROWTH PROMOTION IN AMARANTHUS (*Amaranthus tricolor* L.)

by

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"STANDARDIZATION OF LIQUID FORMULATION OF PGPR MIX–I AND ITS EVALUATION FOR PLANT GROWTH PROMOTION IN AMARANTHUS (*Amaranthus tricolor* L.)"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

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LIST OF ABBREVIATIONS AND SYMBOLS USED

et al.	And other co-workers
cm	Centimetre
cfu	Colony forming units
CRD	Completely Randomised Design
©	Copy Right
CD	Critical Difference
DAT	Days After Transplanting
°C	Degree Celsius
Fig.	Figure
g	Gram
ha	Hectare
hrs	Hours
IAA	Indole-3- Acetic Acid
kg	Kilogram
LAI	Leaf Area Index
µg	Microgram
mg	Milligram
mL	Millilitre
mM	Millimolar
mm	Millimetre
viz.	Namely
nm	Nanometre
NBRIP	National Botanical Research Institute Phosphate Growth Medium
N	Nitrogen

NFB	Nitrogen Free Bromothymol Blue Medium
No.	Number
OD	Optical Density
ppm	Parts per million
%	Per cent
P	Phosphorous
PGPR	Plant Growth Promoting Rhizobacteria
PEG	Poly Ethylene Glycol
PVP	Polyvinyl Pyrrollidone
K	Potassium
sp.	Species
SE	Standard Error
i.e.	That is
TM	Trade Mark
TCP	Tricalcium Phosphate
var.	Variety

INTRODUCTION

1. INTRODUCTION

Organic farming has emerged as an important priority area globally in view of the growing demand for safe and healthy food. It has also been concerned with long term sustainability and environmental pollution associated with indiscriminate use of agrochemicals. Though the use of chemical inputs in agriculture is inevitable to meet the growing demand for food in world, there are opportunities to use effective microorganisms as a viable alternative for reliable and sustainable productivity.

Plant growth promoting rhizobacteria (PGPR) are microorganisms having the ability to aggressively colonize plant roots and stimulate growth of plants. Strains with plant growth promoting activity, belonging to genera *Azoarcus, Azospirillum, Azotobacter, Arthrobacter, Bacillus, Clostridium, Enterobacter, Gluconacetobacter, Pseudomonas,* and *Serratia*, have been reported to have PGPR activity (Hurek and Hurek, 2003). In recent years considerable attention has been paid to PGPR to replace agrochemicals (fertilizers and pesticides) for plant growth promotion by a variety of mechanisms. These mechanisms involve soil structure formation, recycling of essential elements, solubilization of mineral nutrients, decomposition of organic matter, producing numerous plant growth regulators, degrading organic pollutants, stimulation of root growth and soil fertility, biocontrol of soil and seed borne plant pathogens and in promoting changes in vegetation.

Microbial Inoculant Technology has emerged as a potential tool to enhance productivity of agricultural systems. The technology has been designed with beneficial microorganisms which could be exploited for crop nutrition and protection. A major purpose of bacterial inoculant formulation is to offer more suitable microhabitat for survival in the soil ecosystem. Traditionally carrier based inoculants, especially talc, are being used widely since they increase the survival rate of bacteria by protecting it from desiccation and death of cells. Other organic carriers used for formulation development include peat, turf, lignite, kaolinite,

pyrophyllite, zeolite, montmorillonite, alginate, pressmud, sawdust and vermiculite, etc. The shelf life of bacteria, however, varies depending upon bacterial genera, carriers and their particle size. PGPR mix-I is a talc based compatible consortium of efficient NPK biofertilizer organisms such as *Azospirillum lipoferum* and *Azotobacter chroococcum* (Nitrogen fixers), *Bacillus megaterium* (P solubilizer) and *Bacillus sporothermodurans* (K solubilizer) developed by Kerala Agricultural University which has been widely accepted by the farmers of Kerala (KAU, 2009). Results obtained by several workers suggested the use of PGPR mix-I to reduce the use of chemical fertilizers considerably since it acts as a viable alternative for inorganic chemical fertilizers and also suggested its use as an economic and effective method.

Many researches have already reported the advantages of liquid based formulations over carrier based formulations. Talc based powder formulations which have shorter shelf-life and reduced efficacy during longer storage periods necessitates the development of alternate formulations with longer shelf life. Further, the application of talc based bioformulations through micro irrigation techniques encountered problems such as blockage of nozzles and uneven distribution of bio-inoculants.

The development of liquid formulation has several advantages including high cell count, zero contamination, longer shelf life, greater protection against environmental stresses and increased field efficacy. Moreover, liquid cultures containing cell protectants like trehalose, glycerol, polyvinyl pyrrolidone, polyethylene glycol, gum arabic, sodium alginate etc. help to maintain high microbial numbers and also promote the formation of resting cells such as cysts and spores which offer higher resistance to abiotic stresses, thus increasing the survivability of bacteria. These chemical additives were found to have considerable effect on shelf life and protection against environmental stresses.

Considering the merits of liquid formulations with chemical additives over carrier based formuation, an attempt was made to standardize liquid formulation of PGPR mix–I.

Hence the present programme was undertaken with major thrust on the following aspects:

1. Standardization of liquid formulation of PGPR mix–I.
2. Evaluation of standardized liquid formulation of PGPR mix–I along with saving of fertilizers for plant growth promotion in Amaranthus as test crop.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Organic farming has got worldwide attention in concern with increasing demand for safe and healthy food and sustainable production. The indiscriminate use of agrochemicals has adversely affected soil quality and health to an extent which threaten our food security.

The use of agrochemicals is the need of the present day as there is an increasing demand for food globally to meet food security. But the approach should be integrated nutrient management giving more emphasis to soil health. Soil organic matter is the key indicator of soil health and an integral part of organic farming. Organic production has got wide acceptance in domestic and international market with regard to food safety, eco-friendly nature and sustainable productivity.

Organic matter enrichment in soil for long term basis through organic farming maintain soil health with adequate beneficial microorganisms which attribute to soil fertility and productivity and also stimulate the immune system of crop plants. Soil microorganisms have emerged as effective alternative for reliable and sustainable productivity in the global food chain.

2.1 Plant Growth Promoting Rhizobacteria (PGPR)

Rhizosphere is the narrow zone of soil specifically influenced by the root system (Dobbelaere *et al.*, 2003). Accumulation of diverse plant exudates such as amino acids and sugars made this region rich zone of carbon source which promote growth of different microbes involved in nutrient transformation (Gray and Smith, 2005).

It is found that microbial population around the rhizospheric region is generally 10 to 100 times higher than that the bulk soil (Weller and Thomashow, 1994).

Rhizosphere is inhabited by a diverse range of beneficial microorganisms and bacterial population called rhizobacteria (Schroth and Hancock, 1982).

Based on the effects on plant growth, bacterial population can be classified as beneficial, deleterious and neutral groups (Dobbelaere *et al.*, 2003).

Beneficial free-living soil bacteria are usually referred to as plant growth-promoting rhizobacteria (PGPR) and found to be beneficial for plant growth, yield and crop quality (Kloepper *et al.*, 1989). These bacterial species include strains in the genera, *Serratia, Pseudomonas, Burkholderia, Agrobacterium, Erwinia, Xanthomonas, Azospirillum, Bacillus, Enterobacter, Rhizobium, Alcanigenes, Arthrobacter, Acetobacter, Acinetobacter, Achromobacter, Aerobacter, Artrobacter, Azotobacter, Clostridium, Klebsiellla, Micrococcus, Rhodobacter, Rhodospirrilum* and *Flavobacterium* (Rodriguez and Fraga, 1999; Bloemberg and Lugtenberg, 2001; Esitken *et al.*, 2003).

In recent years considerable attention has been paid to PGPR to replace agrochemicals (fertilizers and pesticides) for plant growth promotion by a variety of mechanisms. These mechanisms involve soil structure formation, decomposition of organic matter, recycling of essential elements, solubilization of mineral nutrients, producing numerous plant growth regulators, degrading organic pollutants, stimulation of root growth, crucial for soil fertility, biocontrol of soil and seed borne plant pathogens and in promoting changes in vegetation.

An understanding of plant growth promoting rhizobacteria and their interactions with biotic and abiotic factors is indispensable for sustainable crop production.

2.2 Plant Growth Promoting Rhizobacteria as Biofertilizers

Plant growth promoting rhizobacteria are well known for enhancement of plant growth which is due to certain rhizobacterial traits. For promoting plant growth and development, PGPR employ various mechanisms in different environmental conditions. In case of PGPR mediated plant growth promotion,

modification of the total microbial population in the rhizosphere occurs by production of various substances.

Many PGPR have the ability to fix nitrogen which include *Azospirillum* (Garcia *et al.*, 1996), *Azotobacter* (Jnawali *et al.*, 2015), *Azoarcus* sp. (Hurek *et al.*, 1994), *Beijerinckia* sp. (Baldani *et al.*, 1997), *Klebsiella pneumoniae* (Riggs *et al.*, 2001), *Pantoea agglomerans* (Riggs *et al.*, 2001), and *Rhizobium* sp. (Antoun *et al.*, 1998; Yanni *et al.*, 2001).

Among these various plant growth promoting rhizospheric organisms, mostly studied, *Azospirillum* is a bacterium which is capable of improving growth and yield of several plant species due to its ability to produce various phytohormones (Dobbelaere *et al.*, 2001).

El- Komy (2004) reported that *Azospirillum* spp. have multiple effect on plants including synthesis of phytohormones, nitrogen fixation, nitrate reductase activity and enhancing mineral uptake which ultimately enhance plant growth.

The beneficial effect of *Azospirillum* can be accrued from its nitrogen fixation and stimulating effect on root development (Noshin *et al.*, 2008). It has also been reported that *Azospirillum* - plant association is accompanied by biochemical changes in roots, which in turn promote plant growth and tolerance to low soil moisture. The bacteria stimulate plant growth even under stressed conditions such as drought.

Pandiarajan *et al.* (2012) reported that strains of *Azospirillum* will help the plants in utilization of various soil resources for better growth and these strains are used as very efficient biofertilizers in crop plants all over the world.

Faruq *et al.* (2015) investigated the potential of *Azospirillum spp.* for improving shoot and root of a Malaysian sweet corn variety (j 58) under *in vitro* conditions and it was found that, *A. brasilense* strains inoculated corn seedlings

produced longer roots, highest number of roots, lateral and tertiary root formation and biomass.

Many reports suggest that *Azotobacter chroococcum,* a plant growth promoting rhizobacterium, can act as potential plant growth promoter. Eklund (1970) reported that germination and growth of seedlings of tomato and cucumber was increased due to the presence of *A. chroococcum* in the rhizosphere.

Azotobacter spp. are non-symbiotic heterotrophic bacteria capable of fixing an average of 20 kg N/ha/year. Bacterization helps to improve plant growth and to increase soil nitrogen through nitrogen fixation by utilizing carbon for its metabolism (Monib *et al.*, 1979).

Rajaee *et al.* (2007) observed that inoculation of wheat seeds with *Azotobacter*, helped in uptake of N, P and micronutrients like Fe and Zn.

Besides nitrogen fixation, *Azotobacter* produces plant growth promoting substances like thiomin, riboflavin, nicotin, indole acetic acid and gibberellin. Maize seeds inoculated with *Azotobacter* enhanced the germination to a significant level (Brakel and Hilger, 1965).

In addition, there are several reports which firmly support plant growth promoting activities of *Azospirillum* and *Azotobacter* (Okon *et al.*, 1976; Sasikumar, 1996; Kavitha, 2001; Kizilkaya, 2009).

PGPR enhance the nutrient availability to host plants by solubilization of phosphorus in the rhizosphere. Phosphate-solubilizing bacteria are common in rhizospheres (Nautiyal *et al.*, 2000; Vazquez *et al.*, 2000; Illmeer and Schinner, 1982). According to Banerjee *et al.*, (2005), the most powerful phosphate solubilizers belong to the the genera *Bacillus, Rhizobium* and *Pseudomonas*.

The P solubilizers isolated from Kerala soils were highly efficient in releasing the soil phosphorus (Meenakumari *et al.*, 2008).

Among the soil bacterial communities, the most important P solubilizers include *Bacillus megaterium*, *B. circulans*, *B. subtilis*, *B. polymyxa*, *B. sircalmous*, *Pseudomonas striata*, and *Enterobacter* (Subbarao, 1988; Kucey *et al.*, 1989).

PSM inoculated plants showed increased plant growth and yield under glasshouse conditions (Zaidi *et al.*, 2009; Khan *et al.*, 2010).

Study conducted by Singh and Reddy (2011) on wheat and maize under field condition revealed that PSMs reduced the need of chemical or organic fertilizers.

The highest Phosphate Solubilization Index (PSI) was observed for 8 microbial isolates of *Pseudomonas, Bacillus* and *Rhizobium* out of 37 isolates and it ranged from 1.13 - 3.0 (Karpagam and Nagalakshmi, 2014).

Plant growth promoting rhizobacteria can produce organic acids which in turn solubilize potassium rock. PGPR like *Acidothiobacillus ferrooxidans*, *Bacillus edaphicus*, *Bacillus mucilaginosus*, *Burkholderia*, *Paenibacillus* sp. and *Pseudomonas* are potassium solubilizers and are able to release potassium in available form. Thus, application of potassium solubilizing plant growth promoting rhizobacteria as biofertilizer for agriculture improvement can reduce the use of agrochemicals and support ecofriendly crop production (Sakthidharan, 2011; Shanware *et al.*, 2014).

Application of K solubilizers developed by KAU increased beta carotene, vitamin C and crude protein content in amaranthus (Sakthidharan 2011).

Archana (2013) isolated potassium solubilizing bacteria from rhizosphere soil of different crops from Dharwad and Belgaum districts. These isolates were tested for K solubilization and the amount of K released ranged from 2.41 to 44.49 g mL^{-1}. The differential ability of K solubilizers to solubilize insoluble inorganic potassium could be due to differences in their ability to release organic acids.

Similarly, Kumar and Sindhu (2013) isolated 137 bacterial cultures from wheat rhizosphere on modified Aleksandrov medium containing mica powder as potassium source. Twenty bacterial strains, among 137 cultures tested, showed significant potassium solubilization on mica powder supplemented plates and the amount of K released by different strains varied from 15 to 48 mg L^{-1}. Bacterial strain WPS73 caused maximm solubilization (49.0 mg L^{-1}) at 25°C whereas bacterial strain NNY43 caused maximum solubilization at 30°C.

Chandra *et al.* (2005) observed that application of potash solubilizer in combination with other biofertilizers like *Rhizobium*, *Azospirillum*, *Azotobacter*, *Acetobacter* and PSM in yam and tapioca increased the yield by 15-20 per cent.

Inoculation of potash solubilizing bacteria in brinjal significantly enhanced the yield, plant height and K uptake compared to control (Ramarethinam and Chandra, 2005).

The most reported mechanism predominantly used to explain the positive PGPB effects on plant growth is their ability to produce auxin. Patten and Glick (1996) reported that about 80% of rhizosphere microbes could synthesize and release auxin as a secondary metabolite.

The colorimetric Salkowski assay and HPLC-based isotopic dilution were used to quantify the IAA production of 20 strains of *Azospirillum* isolated from roots of maize and teosinte. There was little correlation between the estimates obtained with the two procedures. The Salkowski assay observed that the culture medium of *A. brasilense* 703Ebc contained mazimum quantity of 26.1 µg ml^{-1} of IAA and minimum of 1.0 µg ml^{-1} of IAA whereas, HPLC-based isotopic dilution recorded a maximum quantity of 4.1 µg ml^{-1} of IAA and minimum of 0.04 µg ml^{-1} of IAA (Crozier *et al.*, 1988).

Brakel and Hilger (1965) reported that *Azotobacter* produced indol-3-acetic acid (IAA) in a medium containing tryptophan. Hennequin and Blachere

(1966) found only small amounts of IAA in old cultures of *Azotobacter* to which no tryptophan was added.

Kavitha (2001) reported that the IAA production by *Azospirillum* sp. isolated from chilli roots under *in vitro conditions* ranged between 21 and 55 µg ml^{-1}.

Recently, Meenakumari *et al.*, (2018) isolated 25 isolates of *Azospirillum* and 12 isolates of *Azotobacter* from the soil samples collected from undisturbed forest areas of Attappady hill tracts. All the isolates have shown to produce IAA and it ranged from 14.83 to 49.74 µg ml^{-1} and 28.95 to 49.81 µg ml^{-1} of culture filtrate for *Azospirillum* sp. and *Azotobacter* sp. respectively.

In addition to the aforementioned plant growth promoting mechanisms PGPR also produce several other growth promoting substances including GA3, zeatin, ABA (Perrig *et al.*, 2007) and siderophores (Beneduzi, 2014). Furthermore, they promote plant growth through production of antibiotics (Hill *et al.*, 1994; Souza *et al.*, 2003; Woeng *et al.*, 2003), hydrolytic enzyme production (Neeraja *et al.*, 2010; Maksimov *et al.*, 2011), induced systemic resistance (Kloepper, 1993; Van Loon *et al.*, 1998) and exo polysaccharides production (Lloret *et al.*, 1996; Rehm and Valla, 1996).

2.3. PGPR mix-I, A Consortium of Efficient Plant Growth Promoters

PGPR mix-I is a talc based consortium of nitrogen fixers, P and K solubilizers developed by the Department of Agricultural Microbiology, College of Agriculture, Vellayani. It contains strains of *Azospirillum lipoferum, Azotobacter chroococcum, Bacillus megaterium* and *Bacillus sporothermodurans* (KAU, 2017). The product has been widely accepted by the farmers of Kerala.

Talc, a widely used carrier material, owing to its inert nature and easy availability as raw material from soapstone industries is used as a carrier for

formulation development. Kloepper and Schroth (1981) demonstrated the potentiality of talc to be used as a carrier for formulating rhizobacteria.

Raj *et al.,* (2012) conducted a field experiment on rice which could establish that basal application (2 kg ha^{-1}) of PGPR mix-I with recommended half the dose of chemical fertilizers (45-22.5-7.5 kg ha^{-1} NPK) and lime top dressing (250 kg ha^{-1}) had significant effect in terms of increasing yield in paddy and it can also be used as a viable alternative for chemical fertilizer thereby saving chemical fertilizers.

Furthermore, Sathyan (2013) conducted an investigation on effect of integrated plant nutrient systems (IPNS) on the soil biological regimes in red loam soil. The study conclusively selected the treatment PGPR mix-I enriched vermicompost + N, P & K as the best treatment both in sustaining soil biological fertility and economic returns. The same treatment has also recorded highest values for enzyme activity number as well as for B:C ratio.

In addition, Yadav (2017) conducted an on farm trial to rejuvenate the soil microbial population for enhancing the response to the nutrients applied and protecting the plants from many pathogens using the plant growth promoting rhizobacteria consortium developed by KAU. This study proved that application of soil test based liming + PGPR mix-I consortium + Mix-II as an economic and effective management method to reduce chemical fertilizer and pesticide load in crop production with the advantages of growth promoting effect and disease control.

More recently, Mohanan (2016) found that application of PGPR mix-I increased the leaf breadth (9.66 cm), number of suckers per plant (4.25), number of ray florets (69.6) and length of ray florets (5.51 cm) in *Gerbera jamesonii.*

2.4 Carrier Based Bioformulations of PGPR

Bioformulations refers to blending of active ingredients such as microbial cells with the inert materials like carriers and adjuvants in order to alter the physical characteristics of the active ingredients to a more desirable form. The function of formulation is to improve spore harvesting, survival in store, application and post-application survival (Burges *et al.*, 1998). The benefits of bioformulations of PGPR certainly are huge in respect to the sustainability they provide and being eco-friendly.

The potential PGPR isolates are formulated using different organic and inorganic carriers either through solid or liquid fermentation technologies. Carriers increase the survival rate of bacteria by protecting it from desiccation and death of cells. The shelf life of bacteria varies depending upon bacterial genera, carriers and their particle size. The organic carriers used for formulation development include peat,turf, talc, lignite, kaolinite, pyrophyllite, zeolite, montmorillonite, alginate, pressmud, sawdust, and vermiculite, etc.

An investigation by Vendan and Thangaraju (2006) reported that solid carrier based preparations generally suffer from short shelf-life, poor quality, high contamination and low and unpredictable field performances.

Talc based powder formulations which have shorter shelf-life and reduced efficacy during longer storage periods necessitates the development of alternate formulations with longer shelf life. Further, the application of talc based bioformulations through micro irrigation techniques encountered problems such as blockage of nozzles and uneven distribution of bio-inoculants. The major disadvantages associated with talc based inoculants are shorter shelf life, poor quality, high contamination and unpredictable field performance. The cost of solid carrier based inoculant production is high as it is labour and energy intensive process, involving milling, sieving and correcting pH (Somasegaran and Hoben, 1994). Considering the demerits of carrier based bioformulations many researches have already been conducted on development of liquid formulation of

biofertilizers. Liquid biofertilizers have the capacity to replace carrier based biofertilizers and play a major role in restoring the soil health.

2.5 Liquid formulations of PGPR

Liquid biofertilizers are microbial preparations containing specific beneficial microorganisms which are capable of fixing or solubilizing or mobilizing plant nutrients by their biological activity. Liquid biofertilizers are special liquid formulation containing not only the desired beneficial microorganisms and their biological secretions, but also special cell protectants or substances that encourage the formation of dormant spores or cysts for longer shelf life and tolerance to adverse conditions.

Liquid formulation is a budding technology in India and has very specific characteristics and uniqueness in its production methods. Liquid biofertilizers are microbial preparations containing specific beneficial microorganisms which are capable of fixing or solubilizing or mobilizing plant nutrients by their biological activity (Mahdi *et al.,* 2010).

Liquid biofertilizers (LB) contain desired organisms and their nutrients with special cell protectants or substances that encourage longer shelf life and tolerance to adverse conditions (Krishan *et al.*, 2005).

The development of liquid formulation has several advantages including high cell count, zero contamination, longer shelf life, greater protection against environmental stresses and increased field efficacy (Vendan and Thangaraju, 2006). In liquid formulation, the microbial organisms are present in a dormant cyst form and after application in the field; the dormant form gives rise to active cells. This helps to increase the shelf life of liquid bioformulation for more than 1 year (Vendan and Thangaraju, 2007). Many earlier works proved the high performance of liquid based bioformulations containing efficient strains of PGPR.

Mugilan *et al.*, (2011) carried out a study to improve the shelf life and survivability of inoculants in liquid form. *Pseudomonas striata* was used as an inoculant which having phosphate solubilizing capacity Three different treatment like vermiculite, lignite and liquid inoculants were used for survival of *Pseudomonas stiata*. Upon that liquid inoculant showed high phosphate solubilizing efficiency than other two treatments and also liquid inoculant showed more survivability of *Pseudomonas striata* than other two treatments. Based on the results, liquid inoculant is considered as best bioinoculants in growth of paddy than control and other two treatments.

The effect of biopriming with liquid biofertilizers (*Azospirillum* and *Phosphobacterium*) in stored seeds of *Pongamia pinnata* to improve the seed and seedling quality characters revealed that seed treatment with liquid *Phosphobacterium* at 1.5% recorded higher germination (35%) followed by *Azospirillum* 0.5% (30%) after six months of storage (Mariappan, 2014).

Recently, Vijendrakumar (2014) based on pot culture experiment reported the effect of different liquid bio fertilizers on growth, yield and survival of seedlings in garden rue (*Ruta graveolens* Linn.). Liquid biofertilizers *viz.*; *Azospirillum lipoferum*, *Pseudomonas striata* and *Pseudomonas fluorescens* were used to treat root system of seedlings in single and combination before transplanting. The results revealed that, the treatments differed significantly with respect to growth, yield and survival of seedlings. Among the various treatments used seedlings treated with the combinations of *Azospirillum lipoferum*, *Pseudomonas striata* and *Pseudomonas fluorescens* recorded highest values for plant height (51.40cm), number of branches (13.27), number of compound leaves (44.50), stem girth (8.27mm) and fresh bio-mass yield (73.73g) compare to single inoculation and control.

Liquid formulations contain not only the desired beneficial microorganisms and their biological secretions, but also special cell protectants or substances that encourage the formation of dormant spores or cysts for longer

shelf life and tolerance to adverse conditions. There are many chemical additives which found to have profound influence on shelf life and protection against stresses of microbial cells even though researches focus on the most efficient one for the last few years.

Similar *in vivo* studies on different liquid biofertilizer have been reported by many workers (Maheswari and Elakkiya, 2014; Leksono and Yanuwiadi, 2014; Barat *et al.*, 2016).

2.5.1 Liquid Formulations of PGPR with Chemical Additives

The liquid formulation of efficient PGPR with the addition different protective substances helps in maintaining cell viability during storage for long time.

Streeter (1985) suggested that enhanced survival of *Azospirillum* cells in the liquid formulation may be due to the action of chemical amendments added in the medium. Trehalose is capable of enhancing cell tolerance to desiccation, osmotic pressure and temperature stress. The possible effect of trehalose's protective action may be due to induced synthesis of metabolites that protect against stress.

Lorda and Balatti (1996) reported greater number of *Azospirillum* cells in 10mM glycerol amended medium which may be due to its high water holding capacity and protect the cells from the effect of dessication by reducing the rate of drying.

Similarly, Sridhar *et al.*, (2004) found that PVP, glyecerol and glucose amended liquid medium of *Bacillus megaterium* supported higher viable population and endospores up to 6 months storage period.

Kumaresan and Reetha (2011) who reported that liquid *Azospirillum* bioinoculant formulated with trehalose (10mM) promoted long term survival of *Azospirillum* compared to glycerol (10 mM), gum arabica (0.3%) and PVP (2%)

and they supported 10^8 cells ml^{-1} up to 11 months of storage under ambient temperature (28°C to 32°C).

Furthermore, Velineni and Brahmaprakash (2011) reported that liquid formulation supplemented with PVP and glycerol supported higher viable population of *Bacillus megaterium* up to a period of four weeks.

Trehalose, gum Arabica and PEG (300) provided better protective effects for *Acetobacter diazotrophicus* than other protective substances (Nita *et al.*, 2012).

Anith *et al.*, (2016) studied the population dynamics of *Pseudomonas fluorescens* AMB-8 in coconut water based liquid formulation along with nutrient broth (NB) and King's B broth (KB) in the presence of different preservatives, and their effect on the seedling growth of chilli and tomato. It was observed that the rate of decline in population was less in coconut water amended with PVP (2% w/v) and glycerol (2% v/v) during six months of storage. In plant growth promotion experiments, all biometric parameters had higher values when freshly grown bacterial strain in KB with population density of 10^9 cfu ml^{-1} was used for seed bacterization. High bacterial population density in the formulations had a positive effect on chilli and tomato seedlings. Liquid bio-formulations are more acceptable than solid bioformulations as they have improved shelf life and better field performance.

A recent investigation by Gopal and Baby (2016) to standardize liquid formulation for *Azospirillum* (KAU isolate) and phosphate solubilizing bacteria (KAU isolate) with chemical amendments enhance the shelf-life of the inoculants for the benefit of farmers in Kerala recorded the highest population of *Azospirillum* (1.77×10^8 cfu ml^{-1}) in trehalose (15 mM) amended medium whereas, PSB population (3.77×10^8 cfu ml^{-1}) was highest in the case of PVP (2.5%). Hence, trehalose (15 mM) and PVP (2.5%) were found to be the most suitable chemical additive for enhancing the shelf life of *Azospirillum* sp. and PSB respectively upto 9 months with a population of 10^8 cfu ml^{-1}. These results

indicated that the shelf-life of *Azospirillum* sp. and PSB could be enhanced upto 9 months at room temperature.

Manimekalai and Kannahi (2018) studied the effect of four different cell protective substances and selected trehalose (1%) as the potential additive because it could maintain a relatively high population and conferred greater microbial vitality.

Similar results on effect of trehalose as cell protectant in liquid bioformulations was reported by Karunya and Reetha (2014) and Gupta *et al.*, (2016).

2.6 PGPR as an Integral Component of INM Strategy

Presently, biofertilizers serve as an integral part of Integrated Nutrient Management practices. Application of PGPR mix-I along with the recommended dose of chemical fertilizer enhances growth and yield of crop plants and hence the product PGPR mix-I could be advocated to the farmers as an integral part of INM strategy.

The high cost of fertilizers and unstable crop production call for substituting part of the inorganic fertilizers by locally available low cost organic sources viz., manures, green manures, biofertilizers etc. in an integrated manner for sustainable production and to maintain soil health (Acharya, 2002).

Integrated nutrient approach by the combination of organics, chemical fertilizers and biofertilizers have numerous environmental benefits over chemical sources of nitrogen alone. It also helps in maintaining stability in crop production and productivity (Swaminathan, 1987).

Use of FYM, green manure or other organic amendments enhanced the benefits from inoculation (Wani, 1990). Application of *Eudrillus* compost enriched with both *Azospirillum* and P solubilizing organisms to plants gave maximum per plant yield in chilli (Zachariah, 1994).

An investigation on Integrated Nutrient Management in brinjal by Rekha (1999) reported the effect of organic manures, chemical fertilizers and biofertilizers on the productivity and quality of brinjal. *Azospirillum* application increased the plant height and number of branches during early stages of growth and it also increased the number of flowers and fruits per plant when compared to control plants. The study observed that beneficial effect of *Azospirillum* is more pronounced when it is applied along with 100 per cent organic manure.

Akshay (2011) based on the study on standardization of organic nutrient schedule for chilly suggested FYM @ 20 t ha^{-1} along with 75 Kg N ha^{-1} applied through a combination of FYM and neem cake in 1:1 ratio+ *Pseudomonas* + *Trichoderma* and PGPR mix –I and Adhoc POP recommendation of KAU-FYM @ 25 t ha^{-1} along with poultry manure @ 5 t ha^{-1} + *Pseudomonas* + *Trichoderma* and PGPR mix-I, each @ 2.5kg as best nutrient schedule for realizing maximum yield from chilli.

Similar results were also reported in many of the previous studies (Mariappan, 2014; Raja and Takankhar 2017).

2.7 PGPR Formulations as a Viable Alternative for Chemical Fertilizer

Application of chemical fertilizers and pesticides is resulting in serious issues such as depletion of soil fertility and environmental pollution. There is an immediate need to go for the biological alternatives to enhance crop productivity and replace the harmful chemicals. It is well known now that the indiscriminate use of chemicals has resulted in loss of beneficial soil microorganisms. Here comes the importance of biological alternatives which save the soil fertility and productivity without causing environmental pollution. Experiments which conclusively established PGPR formulations as a viable alternative for chemical fertilizer are given hereunder.

A field experiment on rice could establish that basal application (2 Kg ha^{-1}) of PGPR mix-I with recommended half the dose of chemical fertilizers (45-

22.5-7.5 Kg ha^{-1} NPK) and lime top dressing (250 Kg ha^{-1}) has significant effect in terms of increasing yield and it can also be used as a viable alternative for chemical fertilizer thereby saving chemical fertilizers (Raj *et al.*, 2012).

A similar recent study conducted by Vanithamani (2016) to assess the impact of exposure of *Amaranthus polygonoides* to biofertilizer, chemical fertilizer and vermicompost individually and in combination. The plants samples were analysed with five days of intervals with morphological parameters and biochemical constituents such as total chlorophyll, carotenoids, total amino acid, protein, carbohydrates on 10th ,20th and 30th day old plants and cellular levels of calcium and iron on 20th and 30th day old plants. The result suggested that the bio-fertilizers *Cyanobacteria, Phosphobacteria and Azospirillum* combined with half dose of inorganic fertilizer (NPK) can lead to enhancement in growth and nutritional status of leafy vegetable *Amaranthus polygonoids* compared to control (without fertilizer).

Yazdani and Pirdashti (2011) suggested that application of Phosphate Solubilizing microorganism and PGPR together reduces P application by 50 per cent.

Based on an On Farm Trial Yadav (2017) suggested the use of PGPRs to reduce the use of chemical fertilizers considerably since it act as a viable alternative for inorganic chemical fertilizers.

Wu *et al.*, (2013) conducted a pot trial to investigate the single, dual, and triple inoculation of earthworms or plant growth–promoting rhizobacteria (PGPR), including nitrogen-fixing bacteria (NFB) (*Azotobacter chroococcum* HKN-5) and phosphate-solubilizing bacteria (PSB) (*Bacillus megaterium* HKP-1), on the growth of *Brassica parachinenesis* and nitrogen (N) and phosphorus (P) availability in soils. This study could establish that triple inoculation of earthworms or plant growth promoting rhizobacteria (PGPR), including nitrogen-fixing bacteria (NFB) (*Azotobacter chroococcum* HKN-5) and phosphate-solubilizing bacteria (PSB) (*Bacillus megaterium* HKP-1) may be a

promising approach for reducing the need for chemical fertilizers in growing vegetables.

2.8 Effect of PGPR Formulations on Growth and Yield of Amaranthus

Amaranthus (*Amaranthus tricolor* L.) is the most popular and widely grown leafy vegetable in Kerala due to its attractive colour, taste and nutritional value. Amaranthus is a dicotyledonous herbaceous plant which belongs to the family *Amaranthaceae*. It includes approximately 70 species, of which 17 produce edible leaves and three produce food grains. Because of its valuable nutrition, many farmers grow amaranth today. Traditional green vegetables occupy an important role in household nutrition throughout the world particularly in rural areas. Effect of PGPR Formulations on growth and yield of amaranthus has already been studied by many of the workers and suggested the use of potential PGPR organisms based on their results.

Significant effect of PGPR mix-I on biochemical properties of Amaranthus plants was reported by Sakthidharan, (2011). These effects might be mediated by the production of beta carotene, vitamin C and crude protein

Sandeep *et al.*, (2011) reported that amaranthus plants inoculated with *A. chroococcum* showed better growth response, biomass yield and nutrient content when compared with uninoculated control plants.

Bio-fertilizers, *Cyanobacteria, Phosphobacteria and Azospirillum* combined with half dose of inorganic fertilizer (NPK) can lead to enhancement in growth and nutritional status of leafy vegetable *Amaranthus polygonoids* compared to control (without fertilizer) (Vanithamani, 2016).

Gopal (2018) recently studied the effect of liquid formulations of *Azospirillum* sp. and Phosphate Solubilizing Bacteria on growth and yield of amaranthus plant. The results of these experiments concluded that PSB (liquid formulation) was the most promising liquid biofertilizer for enhancing growth of

amaranthus. Moreover, the performance of liquid based formulations of *Azospirillum* sp. and Phosphate Solubilizing Bacteria were better than carrier based formulations as the liquid formulations have better shelf life and higher population of the bacterial isolate.

2.9 Effect of PGPR Formulations on N, P and K content of soil

Application of PGPR formulations significantly influences the level of soil available NPK content. Inoculation of PGPR increases the availability of nutrients through various mechanisms which in turn results in increased uptake of nutrients by plants.

Archana (2007) reported the increased uptake of potassium in maize plants receiving KSB inoculation compared to the absolute control.

Based on field experiment using bhindi as test crop, Sathyan (2011) reported that treatment N (75 %), as *Azospirillum* enriched vermicompost + N (25 %), P & K significantly increased soil nitrogen content, whereas P (75 %), as PSB enriched vermicompost + P (25 %), N & K recorded the highest available P content and the treatment N, P, K (75 %) as PGPR mix-I enriched vermicompost + N, P & K (25 %) recorded the highest available K content.

Furthermore, Fan *et al.*, (2017) observed that inoculation with PGPR increased plant growth and N and P uptake by tomato grown on calcareous soils.

Similar results on enhanced uptake of K were obtained by Zhang *et al.* (2004), Han and Wer (2005), Ramarethinam and Chandra (2005) and Sheng (2005).

2.10 Effect of PGPR Formulations on Soil Microbial Population

For better understanding of how PGPR application affects the soil properties, the changes in rhizosphere population of microorganisms should also

be taken into account. In this regard several researchers studied the effect of application of PGPR formulations on rhizosphere microflora.

Inoculation of *Azospirillum, Azotobacter and Glomus fasciculatum* were found to increase the soil beneficial microbial population which in turn results in increased activities of dehydrogenase and phosphatase enzymes (Aseri and Rao, 2005).

Increase in rhizosphere population of bacteria, fungi and actinomycetes was observed when soil was inoculated with PSB enriched vermicompost and PGPR mix-I enriched vermicompost with different doses of NPK fertilizers (Sathyan, 2011).

Vijendrakumar and Hanumaiah (2014) reported that dual and triple inoculation of bio-fertilizers resulted in maximum CFU g^{-1} soil with respect to both beneficial and general micro-flora.

Mary *et al.*, (2015) reported luxuriant growth of bacteria in all the biofertizer treated rhizosphere in the order of FYM <*Azospirillum* < Phosphobacteria < Vermicompost.

Khipla *et al.*, (2017) reported that highest soil microbial population and enzyme activities were observed on application of 100 per cent chemical N and P along with consortium of *Azotobacter* and PSB.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The experiment on "Standardization of liquid formulation of PGPR mix–I and its evaluation for plant growth promotion in Amaranthus (*Amaranthus tricolor* L)" was carried out during the period from 2016 - 18 in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram. The details of the materials used and methods followed in the present study are presented in this chapter.

3.1 *IN VITRO* ASSESSMENT OF IAA OF THE ISOLATES IN PGPR MIX–I

3.1.1 Procurement of All the Cultures of PGPR Mix- I and Maintenance in Specific Medium

The component cultures of PGPR-Mix-I– *Azospirillum lipoferum, Azotobacter chroococcum, Bacillus megaterium* and *Bacillus sporothermodurans* were procured from the Department of Agricultural Microbiology, College of Agriculture, Vellayani and the cultures were maintained in Nitrogen Free Bromothymol Blue (NFB) medium (Dobereiner and Day, 1976), Jenson's medium (Jenson, 1942), Pikovaskaya's medium (Rao and sinha, 1963), Nutrient Agar medium (Salfinger and Tortorello, 2015) respectively. All the cultures were preserved on slants of the respective selective medium at 4°C in a refrigerator for further use.

3.1.2 Quantification of IAA of Different Isolates of PGPR mix- I.

Indole Acetic Acid was estimated as per the procedure described by Gordon and Weber (1951).

100 ml each of NFB broth medium, Jenson's broth medium, Pikovaskaya's broth medium and Nutrient agar broth medium was prepared in 250 ml flasks. To each medium, 0.1 g tryptophan was added. The medium was inoculated with one ml of respective isolates containing $4.3x10^9$, $5.1x10^9$, $3.6x10^9$ and $3.3x10^9$ cfu ml^{-1} respectively under aseptic conditions. Flasks were kept in

shaker for incubation at 30^0 C for 5 days. After incubation, the culture was centrifuged at 10000 rpm for 10 minutes. To the 10 ml of culture supernatant, 2 ml of the Salkowski reagent was added and incubated at room temperature for 25 minutes and then read at OD_{530}. Using the standard curve for IAA, the amount of IAA was calculated.

3.2 *IN VITRO* ESTIMATION OF NITROGEN RELEASED BY THE NITROGEN FIXING ORGANISMS IN PGPR MIX-I

One hundred ml of NFB medium for *Azospirillum* and Jenson's medium for *Azotobacter* supplemented with 50 mg yeast extract was taken in 200 ml conical flask and 1 ml of inoculum was added aseptically. After seven days of incubation, the medium was concentrated to 5 ml by keeping in sand bath and it was digested with 10 ml concentrated H_2SO_4 and digestion mixture. The digestion was carried out until the contents were clear. After cooling, the aliquot was transferred to volumetric flask and the volume was made upto 100 ml. Ten ml of the aliquot was taken in the Kjeltec digestion tube and total nitrogen was estimated volumetrically (Humphries, 1956). The results were expressed as mg nitrogen released per gram carbon source.

3.3 QUANTITATIVE AND QUALITATIVE ASSESSMENT OF SOLUBILIZATION OF P AND K BY *Bacillus megaterium* AND *Bacillus sporothermodurans* PRESENT IN PGPR MIX–I UNDER *IN VITRO* CONDITIONS

3.3.1 Quantitative Assessment of Solubilization of P by *Bacillus megaterium* Present in PGPR mix–I under *In vitro* Conditions

Phosphorous solubilization by *Bacillus megaterium* in PGPR mix-I was assessed quantitatively using method described by Clescerie *et al.* (1998).

For this, cultures were inoculated to 50 ml of Pikovaskaya's broth and incubated at 30 °C for 48 h. The broth was centrifuged and 5 ml of the supernatant

was collected in a screw capped vial. 5 ml of Vanadomolybdate solution was added to the supernatant. The volume was made up to 25 ml and incubated overnight for the development of yellow color. The absorbance was measured by using spectrophotometer at 430 nm wavelength. Using the standard curve for phosphorous, the amount of phosphorous was calculated.

3.3.2 Qualitative Assessment of Solubilization of P by *Bacillus megaterium* Present in PGPR mix–I under *In vitro* Conditions

3.3.2.1 Solubilization of P by Bacillus megaterium in Pikovaskaya's Medium

The isolate *Bacillus megaterium* was tested for its ability to solubilize tricalcium phosphate present in the Pikovaskaya's medium (Pikovaskaya, 1948; Gupta *et al.*, 1994). A loopful of pure culture was placed at the center of the agar plates and incubated for 27±2°C for 5 days. Colonies exhibiting clearing zone of P release were noticed and the diameter of clearing zone in mm was recorded.

3.3.2.2 Solubilization of P by Bacillus megaterium in NBRIP Medium

In addition, *Bacillus megaterium* was tested for their ability to solubilize tricalcium phosphate in NBRIP (National Botanical Research Institute Phosphate) medium (Nautiyal, 1999) supplemented with $Ca_3 (PO_4)_2$ (5.0 g L^{-1}) as per the procedure described by Gupta *et al.*, (1994). A loopful of pure culture of *Bacillus megaterium* was placed at the center of the agar plates and incubated for 27±2°C for 5 days. The diameter of solubilization zone produced was measured in mm.

3.3.3 Quantitative Assessment of Solubilization of K by *Bacillus sporothermodurans* Present in PGPR mix–I under *In vitro* Conditions

The solubilization of K by *Bacillus sporothermodurans* was assessed by flame photometry described by Sugumaran and Janarthanam (2007).

One ml of overnight culture of each isolate was inoculated to 25 ml of Aleksandrov broth (Hu *et al.*, 2006) in three replicates and then incubated for two

weeks at 28±2°C. The amount of K released in the broth and control was estimated 15 days after incubation. The broth cultures were centrifuged at 10,000 rpm for 10 minutes to separate the supernatant from the cell growth and insoluble potassium. One ml of the culture supernatant was taken in a 50 ml volumetric flask and the volume was made to 50 ml with distilled water and mixed thoroughly. After that the solution was fed to flame photometer and K content was determined. Using the standard curve for potassium, the amount of potassium was calculated.

3.3.4 Qualitative Assessment of Solubilization of K by *Bacillus sporothermodurans* Present in PGPR mix–I under *In vitro* Conditions

The ability of bacterial cultures to release K in Glucose Yeast extract Agar medium was assessed as per the procedure described by Prajapati and Modi (2012).

For this, sterilized Glucose Yeast extract Agar medium containing feldspar (0.5 %) as the sole source of potash was poured into sterilized Petri plates. After solidification of the media, the plates were spot inoculated with the bacterial strain and incubated at 28-30°C and assayed visually up to 7 days. Colonies exhibiting clearing zone of K release was noticed and the diameter of clearing zone in mm was recorded.

3.4 CHARACTERIZATION OF THE ISOLATES

The component cultures in PGPR mix–I were characterized based on morphological and biochemical characteristics.

3.4.1 Morphological Characterization of the Isolates

The following morphological tests viz., cell shape, gram reaction and motility were carried out to characterize the component cultures in PGPR mix–I.

3.4.1.1 Cell Shape

The purified cultures at log phase were observed microscopically for the cell morphological characters (Becking, 1974).

3.4.1.2 Cell Motility

The 72 h old cells were observed microscopically using cavity slide for their motility.

3.4.1.3 Gram Reaction

Gram staining was carried out as per modified Hucker's method (Rangaswami and Bagyaraj, 1993) and observed under the microscope.

3.4.2 Biochemical Characterization of the Isolates

Biochemical characterization of isolates of PGPR mix-I was done by performing various biochemical tests and carbohydrate utilization tests by using readymade Himedia© kits (HiCarbo™, Part A, Band C, Hi25™ Enterobacteriaceae) and readymade Himedia© kits (HiCarbo Part A, B and C, Hi-Bacillus and Hi- Assorted) as per the manufacturer's instructions (Plate 1). Colour change observed on the biochemical amended media of the kit after spot inoculating culture suspensions of selected isolates followed by incubation for 48 h indicated the reaction with respect to different biochemicals or carbohydrates as positive or negative. Various biochemical tests performed were Oxidase, Lysine utilization, Ornithine utilization, Urease, Nitrate reduction, Catalase, Starch hydrolysis, Gelatin hydrolysis, Arginine Lyase, Malonate utilization, Voges Proskauer, Casein and Urea. Different carbohydrate utilization tests performed were Glucose, Lactose, ONPG, Sucrose, Mannitol, Trehalose, Xylose, Maltose, Fructose, Galactose, Dulcitol, Rhamnose, Cellobiose, Arbutin, Esculin hydrolysis and D- arabinose. The results of biochemical tests were utilized to arrive at a tentative genus level identification of isolates.

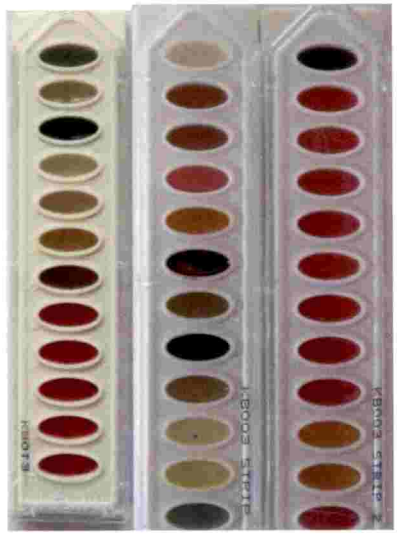


Plate 1. Biochemical kit

3.5 STANDARDIZATION OF PROTOCOL FOR THE PREPARATION OF LIQUID FORMULATION OF PGPR MIX-I

An experiment was carried out in completely randomized design with the following additive treatments with four replications each. Appropriate control treatment without additives was also maintained. Survival of the cultures in talc based formulation was also monitored in their respective medium.

3.5.1 Details of the Experiment

Design : Complete Randomized Design

Treatments : 7

Replications : 4

Treatments

T_1 - 2% Glycerol (Anith *et al.*, 2016)

T_2 - 2% Polyvinylpyrrolidone (Anith *et al.*, 2016)

T_3 - 15mM Trehalose (Surendragopal, 2016)

T_4 - 1% Glycerol and 1% Polyvinylpyrrolidone (Sivaprasad, 2011)

T_5 - Glycerol (2%), Trehalose (1%), Yeast extract (1%), Polyvinylpyrrolidone (1%), Proline (1%) (Bhumannavar, 2008)

T_6 - Control without any additives

T_7 - Talc based formulation

The bacterial cultures were inoculated to 100 ml of the PGPR medium (Sivaprasad, 2011). The population of each of the isolates was enumerated at monthly intervals by serial dilution technique (Timonin, 1940) for a minimum period of 10 months in appropriate medium.

3.6 POT CULTURE STUDIES TO EVALUATE THE EFFICIENCY OF LIQUID FORMULATION OF PGPR MIX-I

An experiment was conducted in Completely Randomized Design under glass house conditions at College of Agriculture, Vellayani to test the efficacy of the best treatment obtained from experiment 3.5 with Amaranthus as the test crop (Plate 2).

3.6.1 Preparation of Potting Mixture

Potting mixture was prepared by mixing sand, soil and farmyard manure in the ratio 1:1:1 and was sterilized by autoclaving at 121^{0}C for 1 h for three consecutive days. Sterilized potting mixture was filled into earthen pots of dimension 23x28 cm at the rate of 7.5 kg per pot.

3.6.2 Fertilizer Application

NPK fertilizers were applied as per POP recommendations for Amaranthus (KAU, 2016). Fertilizers were applied as per the recommended dose of 100:50:50 kg NPK per hectare. N and P in the form of Factamfos and K in the form of Muriate of Potash were applied to soil.

3.6.3 Preparation of PGPR mix-I Inoculum

The PGPR mix-I bioinoculant was prepared by inoculating 72 h old log phase culture in PGPR medium (Sivaprasad, 2011). The flasks were incubated in shaker at room temperature for 5 days.

3.6.4 Raising Seedlings

Coir pith was sterilized by autoclaving at 121^{0}C for 1 h for three consecutive days. Sterilized coir pith was filled into protrays containing 98 cells having a diameter of 3.2 cm each. Seeds of amaranthus were surface sterilized in one per cent sodium hypochlorite aqueous solution for 3 minutes under aseptic conditions. The seeds were further washed in sterile distilled water thrice. The

Plate 2. General view of pot culture experiment

surface sterilized seeds of amaranthus were sown in protrays and were kept in poly house. Twenty one days old seedlings were used for transplanting to the pots maintaining one seedling per pot. Sterile water was used for irrigation.

3.6.5 Details of Pot Culture Experiment

Location	: Instructional Farm, College of Agriculture, Vellayani.
Crop	: Amaranthus
Variety	: Amt-1
Design	: Completely Randomized Design
Treatments	: 9
Replications	: 3
Number of plants/replication	: 5

Treatments

T_1- Chemical fertilizer @ 100% NPK as per POP, KAU (KAU, 2016)

T_2- Talc based formulation of PGPR mix–I alone

T_3- Talc based formulation of PGPR mix–I + 100% NPK as per POP, KAU (KAU, 2016)

T_4- Talc based formulation of PGPR mix–I + 50% NPK as per POP, KAU (KAU, 2016)

T_5- Liquid biofertilizer formulation of PGPR mix–I alone

T_6- Liquid biofertilizer formulation of PGPR mix–I+ 100% NPK as per POP, KAU (KAU, 2016)

T_7- Liquid biofertilizer formulation of PGPR mix–I+ 50%NPKas per POP, KAU (KAU, 2016)

T_8- Control with additives without PGPR microorganisms

T_9- Absolute control

3.6.6 Seedling Dip Method

The seedlings were uprooted from protray and dipped in two per cent liquid formulation of PGPR mix-I containing a population of 3.4×10^9, 3.5×10^9, 4.2×10^9 and 5.4×10^9 cfu ml^{-1} of *Azospirillum lipoferum*, *Azotobacter chroococcum*, *Bacillus megaterium* and *Bacillus sporothermodurans* respectively at the time of transplanting.

3.6.7 Soil Drenching

Soil drenching of 50 ml with 2 per cent liquid formulation of PGPR mix-I containing a population of 2.9×10^9, 3.3×10^9, 2.2×10^9 and 4.8×10^9 cfu ml^{-1} of *Azospirillum lipoferum*, *Azotobacter chroococcum*, *Bacillus megaterium* and *Bacillus sporothermodurans* respectively was performed two weeks after transplanting.

3.6.8 Observations

3.6.8.1 Plant Height (cm)

The height of the plant was measured from the base to the growing tip of the shoot in cm at 20 DAT and at harvest.

3.6.8.2 Fresh Weight of Shoot (g)

The fresh weight of shoot (g) was taken in an electronic single pan balance immediately after uprooting the plants at harvest.

3.6.8.3 Fresh Weight of Root (g)

The fresh weight of root (g) was taken in an electronic single pan balance immediately after uprooting the plants at harvest.

3.6.8.4 Dry Weight of Shoot (g)

The dry weight of shoot (g) was taken after drying the samples to a constant weight at 60^0C in a drying oven.

3.6.8.5 Dry Weight of Root (g)

The dry weight of root (g) was taken after drying the samples to a constant weight at 60^0C in a drying oven.

3.6.8.6 Leaf Area Index

Leaf Area Index was calculated at the harvest of plants. Leaf area of the whole sampled plants was determined by measuring the individual leaf length and width and multiplied by 0.64 (Kolawole and Sarah, 2009). This is the total area of leaves to the ground area occupied by the crop (Forbes and Watson, 1992). From the recorded data Leaf Area Index was computed using the following formula:

$$\text{Leaf Area Index} = \frac{\text{Leaf area}}{\text{Ground area}}$$

3.6.8.7 Oxalate Content

Estimation of oxalate was done by method suggested by A.O.A.C (1984).

Five leaf samples from each plant were collected at harvest. The samples were shade dried for one day and kept in a drying oven at 60 °C for three days. To the one gram of dried powder 0.25 N hydrochloric acid was added and kept in water bath for one hour. After one hour again 0.25 N hydrochloric acid was added and in water bath for one hour. After water bath supernatant was collected in a conical flask. To this supernatant 5 ml of tungsto phosphoric acid was added and

kept overnight. This was neutralized with dilute ammonia solution in 1:1 ratio. Precipitation was done by using 5 ml acetate buffer with calcium chloride (pH 4.5). Centrifuged and washed the precipitate two times each with 6 ml wash liquid. Precipitate was transferred into 100 ml conical flask by dissolving 10-15 ml 2N Sulphuric acid and titrated against 0.01N potassium permanganate solution at 60°C.

$$\text{Percentage Oxalate} = \frac{0.063 \times V}{1g}$$

V- Titre value

3.6.8.8 Estimation of Initial and Final Soil N, P and K Content

3.6.8.8.1 Available Nitrogen

Available Nitrogen in the soil was determined as per the alkaline permanganate method described by Subbiah and Asija (1956).

3.6.8.8.2 Available Phosphorous

Available phosphorus in the soil was estimated as per the Bray No.1 extraction and ascorbic acid reduced molybdo-phosphoric blue colour method (Bray and Kurtz, 1945).

3.6.8.8.2 Available Potassium

Ammonium acetate soil extract was used for the determination of potassium using a flame photometer (Jackson, 1973).

3.6.8.9 Population of *Azospirillum lipoferum, Azotobacter chroococcum, Bacillus megaterium* and *Bacillus sporothermodurans* in Soil.

The population of each of the isolates in soil was enumerated by serial dilution technique described by Timonin (1940) in their respective medium.

3.7 STATISTICAL ANALYSIS

The data generated from the experiments were statistically analyzed using Analysis of Variance techniques (ANOVA) as applied to Completely Randomized Design described by Panse and Sukhatme (1985).

RESULTS

4. RESULTS

The present study on "Standardization of liquid formulation of PGPR mix–I and its evaluation for plant growth promotion in Amaranthus (*Amaranthus tricolor* L.)" was conducted during the period from 2016-18 in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala. The results based on statistically analyzed data pertaining to the experiment conducted during the course of investigation are presented below:

4.1 *IN VITRO* ASSESSMENT OF IAA OF THE ISOLATES IN PGPR MIX–I

IAA produced by the isolates in PGPR mix-I (Plate 3) was estimated five days after inoculation. All the isolates produced significant quantity of IAA under *in vitro* conditions. The nitrogen fixers, *Azospirillum lipoferum and Azotobacter chroococcum* produced 40.31 and 36.43 ppm of IAA respectively, whereas *Bacillus megaterium* and *Bacillus sporothermodurans* produced 1.28 ppm and 3.36 ppm of IAA respectively (Table 1).

4.2 *IN VITRO* ESTIMATION OF NITROGEN RELEASED BY THE NITROGEN FIXING ORGANISMS IN PGPR MIX-I

The *in vitro* estimation of nitrogen fixation by nitrogen fixing organisms namely, *Azospirillum lipoferum and Azotobacter chroococcum* recorded a significant quantity of 21 and 14 mg N g^{-1} of carbon source respectively (Table 2).

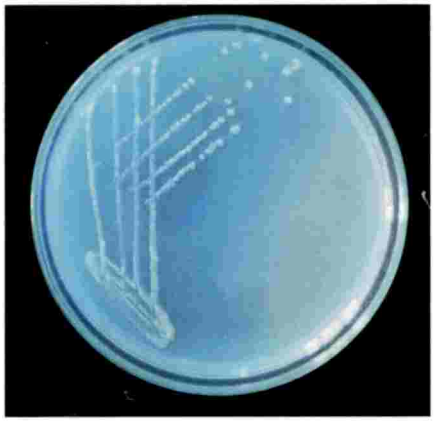
4.3 QUANTITATIVE AND QUALITATIVE ASSESSMENT OF SOLUBILIZATION OF P AND K BY *Bacillus megaterium* AND *Bacillus sporothermodurans* PRESENT IN PGPR MIX–I UNDER *IN VITRO* CONDITIONS

Table 1. Quantification of IAA production by different isolates of PGPR mix-I

Isolate	IAA (ppm)
Azospirillum lipoferum,	40.31
Azotobacter chroococcum	36.43
Bacillus megaterium	1.28
Bacillus sporothermodurans	3.36

Table 2. *In vitro* estimation of nitrogen released by the nitrogen fixing organisms of PGPR mix-1

Isolate	Nitrogen fixation (mg N g^{-1} of carbon source)
Azospirillum lipoferum	21
Azotobacter chroococcum	14



Azospirillum lipoferum

Azotobacter chroococcum

Bacillus megaterium

Bacillus sporothermodurans

Plate 3. Component cultures of PGPR mix- I

4.3.1 Quantitative and Qualitative Assessment of Solubilization of P by *Bacillus megaterium* Present in PGPR mix–I under *In Vitro* Conditions

Quantitative assessment of solubilization of P by *Bacillus megaterium* present in PGPR mix–I under *in vitro* conditions recorded 69.36 ppm (Table 3), whereas qualitative assessment recorded a clearing zone of 8 mm and 12 mm diameter in NBRIP and Pikovaskaya's medium respectively (Table 4) (Plate 4).

4.3.2 Quantitative and Qualitative Assessment of Solubilization of K by *Bacillus sporothermodurans* Present in PGPR mix–I under *In Vitro* Conditions

In vitro assessment of K solubilization by *Bacillus sporothermodurans* recorded a K content of 12.18 ppm of potassium on solubilization (Table 5) and a clearing zone of 18 mm diameter in Glucose Yeast Agar medium (Table 6) (Plate 5).

4.4 CHARACTERIZATION OF THE ISOLATES

The component cultures in PGPR mix–I were characterized based on morphological and biochemical characteristics.

4.4.1 Morphological Characterization of the Isolates

The component cultures in PGPR mix–I was subjected to morphological characterization and the results are presented in Table 7. All the isolates in PGPR mix–I were motile and they varied in morphological characteristics such as colony morphology, size, margin, texture, colour and cell shape. The nitrogen fixers, *Azospirillum lipoferum* and *Azotobacter chroococcum* stained Gram negative, whereas *Bacillus megaterium* and *Bacillus sporothermodurans* stained Gram positive (Plate 6).

Table 3. Quantitative assessment of solubilization of P by *Bacillus megaterium* present in PGPR mix-1 under *in vitro* conditions

Isolate	Phosphorous (ppm)
Bacillus megaterium	69.36

Table 4. Qualitative assessment of solubilization of P by *Bacillus megaterium* present in PGPR mix-1 under *in vitro* conditions

Medium	Diameter of clearing zone (mm)
NBRIP	8
Pikovaskaya's medium	12

Table 5. Quantitative assessment of solubilization of K by *Bacillus sporothermodurans* present in PGPR mix-1 under *in vitro* conditions

Isolate	Potassium (ppm)
Bacillus sporothermodurans	12.18

Table 6. Qualitative assessment of solubilization of K by *Bacillus sporothermodurans* present in PGPR mix-1 under *in vitro* conditions

Medium	Diameter of clearing zone (mm)
Glucose Yeast Agar medium	18

Table 7. Morphological characterization of component cultures of PGPR mix-1

Isolate	Colony Morphology	Size	Margin	Texture	Colour	Motility	Cell shape	Gram reaction
Azospirillum lipoferum	Round	Moderate	Entire	Smooth	Off-white	Motile	Curved rod	Gram negative
Azotobacter chroococcum	Ovoid	Moderate	Entire	Slimy	Transparent during early phase, turns dark brown	Motile	Rod	Gram negative
Bacillus megaterium	Round	Moderate	Undulated	Rough	Creamy white	Motile	Rod	Gram positive
Bacillus sporothermodurans	Round	Large	Entire	Rough	Greyish white	Motile	Rod	Gram positive

(A) **(B)**

Plate 4. Phosphorous solubilization by *Bacillus megaterium* in **(A)** NBRIP and **(B)** Pikovaskaya's medium

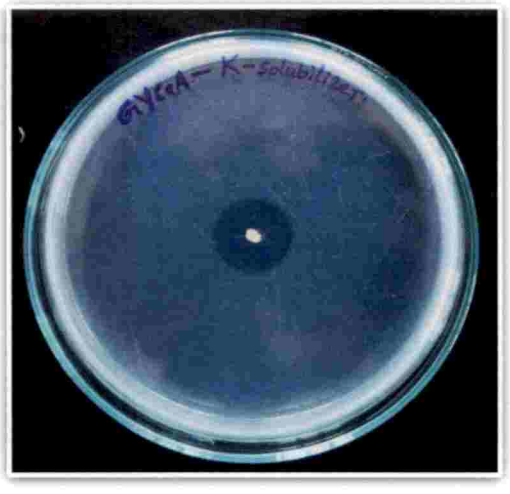
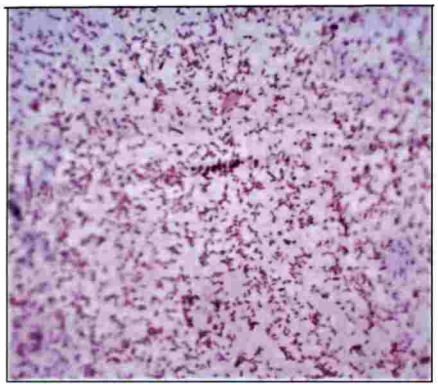
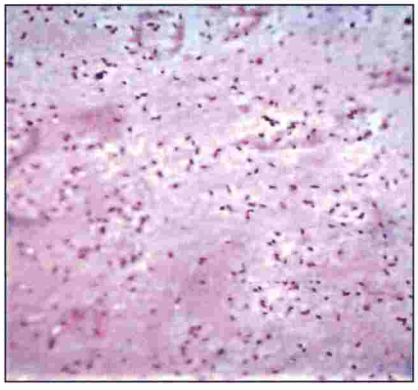


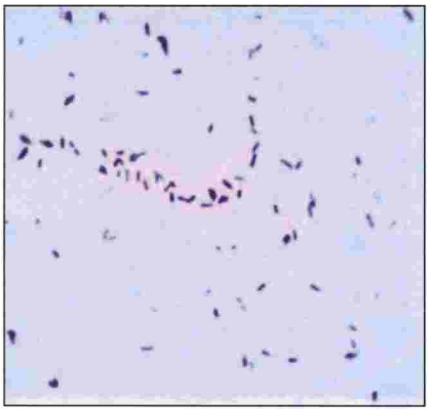
Plate 5. Potassium solubilization by *Bacillus sporothermodurans* in Glucose Yeast Agar medium



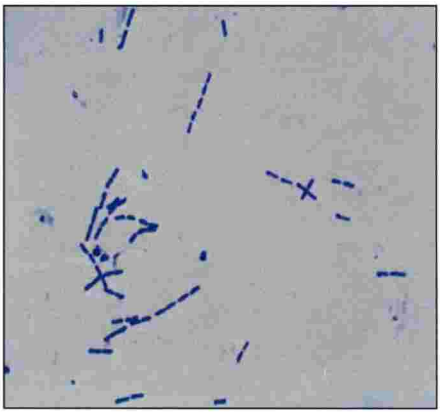
Azospirillum lipoferum



Azotobacter chroococcum



Bacillus megaterium



Bacillus sporothermodurans

Plate 6. Gram reaction of component cultures of PGPR mix-I

4.4.2 Biochemical Characterization of the Isolates

For further characterization, these four isolates were subjected to a series of biochemical tests. The results of various biochemical tests supported the genus level identification of the isolates. The results are presented in Table 8-11.

4.5 STANDARDIZATION OF PROTOCOL FOR THE PREPARATION OF LIQUID FORMULATION OF PGPR MIX-I

4.5.1 Population of *Azospirillum lipoferum*, *Azotobacter chroococcum*, *Bacillus megaterium* and *Bacillus sporothermodurans* in PGPR Mix-I (cfu ml^{-1})

4.5.1.1 Population at the Time of Inoculation

On zeroth day after inoculation, the treatment T_1 recorded the maximum significant population of *Azospirillum lipoferum, Azotobacter chroococcum,* P solubilizer and K solubilizer of 1.83×10^8 cfu ml^{-1}, 1.44×10^8 cfu ml^{-1}, 1.48×10^8 cfu ml^{-1} and 1.52×10^8 cfu ml^{-1} respectively, which was significantly superior to all other treatments. The control treatment and talc based formulation recorded 2.25×10^6 and 1.09×10^8, 2.0×10^6 and 2.1×10^7, 3.62×10^6 and 4.38×10^7, 4.87×10^6 and 7.35×10^7 cfu ml^{-1} of *Azospirillum lipoferum, Azotobacter chroococcum,* P solubilizer and K solubilizer respectively (Table 12).

4.5.1.2 First Month after Inoculation

First month after inoculation the treatment T_3 recorded the maximum total viable count of *Azospirillum lipoferum* (2.34×10^{10} cfu ml^{-1}) which was statistically on par with treatments T_1 (1.55×10^{10} cfu ml^{-1}) and T_2 (1.54×10^{10} cfu ml^{-1}), which was significantly superior to the control treatment T_6 which recorded 1.93×10^9 cfu ml^{-1}. The population of *Azospirillum lipoferum* in talc based formulation recorded 4.8×10^9 cfu g^{-1} (Plate 7).

The maximum colony count of *Azotobacter chroococcum* was observed in treatment T_3 (1.68×10^{10} cfu ml^{-1}) which was statistically on par with treatments T_1

Table 8. Biochemical characterization of *Azospirillum lipoferum*

BIOCHEMICAL TESTS	
Oxidase	-
Catalase	+
Urease	-
Nitrate Reduction	-
Starch Hydrolysis	-
Gelatin Hydrolysis	-

CARBOHYDRATE UTILISATION TESTS	
Glucose	+
Sucrose	-
Mannitol	-
Cellobiose	-
Lactose	+
Fructose	-
Dulcitol	-
Galactose	-
Xylose	-
Maltose	-
Glucose as sole carbon source	+
Sucrose as sole carbon source	-

+ = Positive reaction - = Negative reaction

Table 9. Biochemical characterization of *Azotobacter chroococcum*

BIOCHEMICAL TESTS	
Oxidase	+
Catalase	+
Urease	+
Nitrate Reduction	+
Starch Hydrolysis	-
Gelatin Hydrolysis	-
Ornithine	+
Lysine	+

CARBOHYDRATE UTILISATION TESTS	
Glucose	-
Sucrose	-
Mannitol	+
Malonate	+
Cellobiose	-
Lactose	+
Fructose	-
Rhamnose	-
Galactose	-
Xylose	-
Maltose	+
Dulcitol	-

+ = Positive reaction, - = Negative reaction

Table 10. Biochemical characterization of *Bacillus megaterium*

BIOCHEMICAL TESTS	
Voges Proskauers	-
Catalase	+
ONPG	+
Nitrate Reduction	+
Arginine	-
Gelatin Hydrolysis	+
Ornithine	-
Lysine	-

CARBOHYDRATE UTILISATION TESTS	
Glucose	+
Sucrose	+
Mannitol	+
Arabinose	+
Trehalose	+

+ = positive reaction - = negative reaction

Table 11. Biochemical characterization of *Bacillus sporothermodurans*

BIOCHEMICAL TESTS	
Voges Proskauers	-
Catalase	+
ONPG	-
Nitrate Reduction	-
Arginine	-
Gelatin Hydrolysis	-
Ornithine	+
Lysine	+
Esculin	+
Arbutin	-
Casein	-
Urea	-
Starch	-
Oxidase	+

CARBOHYDRATE UTILISATION TESTS	
Glucose	+
Sucrose	-
Mannitol	-
Arabinose	-
Trehalose	+

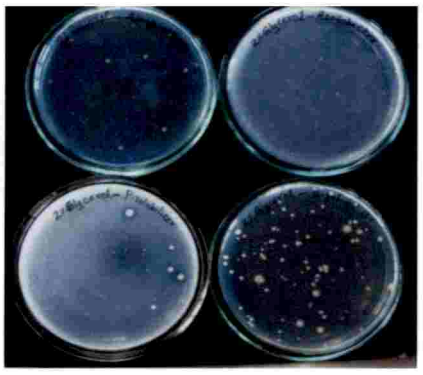
+ = positive reaction - = negative reaction

Table 12. Population of component organisms of PGPR mix-1 in liquid formulation with different additives on the day of inoculation

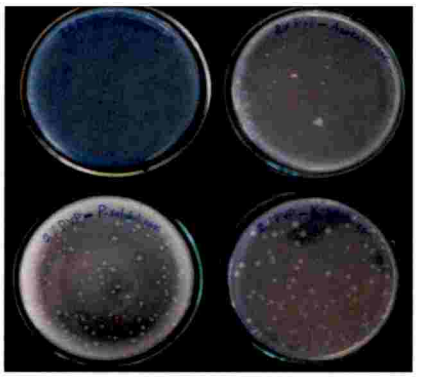
TREATMENTS	ZEROTH DAY (log cfu ml^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K Solubilizer (*Bacillus sporothermodurans*)
T1- 2% Glycerol	8.25^{a}	8.15^{a}	8.15^{a}	8.16^{a}
T2- 2% Poly Vinyl Pyrrolidone	7.04^{c}	7.01^{d}	7.14^{d}	7.47^{c}
T3- 15Mm Trehalose	8.05^{b}	7.85^{b}	7.82^{b}	7.89^{b}
T4- 1% Glycerol and 1% PVP	6.73^{d}	6.84^{e}	6.58^{f}	6.75^{e}
T5- Glycerol (2%), Trehalose (1%), Yeast extract (1%), PVP (1%) and Proline (1%)	6.82^{d}	6.62^{f}	6.8^{e}	6.94^{d}
T6- Control without any additives	6.33^{e}	6.29^{g}	6.55^{f}	6.65^{e}
T7- Talc based formulation*	8.03^{b}	7.22^{c}	7.63^{c}	7.86^{b}
SEm (±)	.036	0.05	0.33	.034
CD (0.05)	0.107	0.153	0.098	0.101

* Log cfu g^{-1} Each value represents a mean of 4 replication

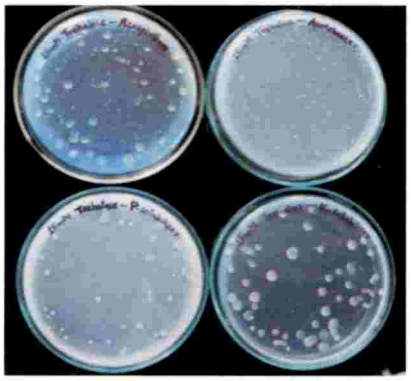
Figures in a column followed by same letters do not differ significantly at $P \geq 0.05$



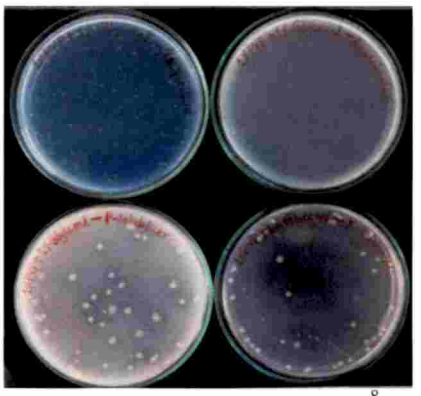
T1- 2% Glycerol (10^8 dilution)



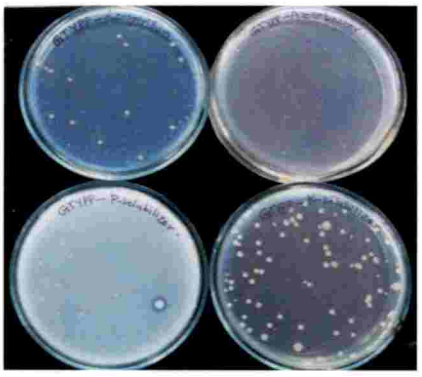
T2 -2% Poly Vinyl Pyrrolidone (10^8 dilution)



T3 -15mM Trehalose (10^8 dilution)



T4- 1% Glycerol and 1% PVP (10^8 dilution)



T5- Glycerol (2%), Trehalose (1%), Yeast extract (1%), PVP(1%) and Proline (1%) (10^8 dilution)

T6- Control without any additive (10^7 dilution)

T7- Talc based formulation (10^8 dilution)

Plate 7. Population of component organisms of PGPR mix-1 in liquid formulation with different additives one month after inoculation

(1.36×10^{10} cfu ml^{-1}) and T_2 (1.21×10^{10} cfu ml^{-1}). These were significantly superior to the control treatment T_6 which recorded 8.4×10^{8} cfu ml^{-1}. The population of *Azotobacter chroococcum* in talc based formulation recorded 2.35×10^{9} cfu g^{-1}.

The treatment T_3 (1.58×10^{10} cfu ml^{-1}) recorded the highest population of P solubilizer which was statistically on par with treatments T_1 (1.48×10^{10} cfu ml^{-1}) and T_2 (1.45×10^{10} cfu ml^{-1}). These treatments were found significantly to be superior to the control treatment T_6 which recorded 1.48×10^{9} cfu ml^{-1}. The population of P solubilizer in talc based formulation recorded 3.4×10^{9} cfu g^{-1}.

The maximum colony count of K solubilizer was observed in treatment T_3 (1.83×10^{10} cfu ml^{-1}) which was statistically on par with treatment T_2 (1.78×10^{10} cfu ml^{-1}). These treatments were significantly superior to the control treatment T_6 which recorded 1.64×10^{9} cfu ml^{-1}. The population of K solubilizer in talc formulation recorded 2.80×10^{9} cfu g^{-1} (Table 13).

4.5.1.3 Second Month after Inoculation

From the observation on second month after inoculation it was noticed that the, treatment T_4 (2.45×10^{10} cfu ml^{-1}) recorded the highest population of *Azospirillum lipoferum* which was statistically on par with treatments T7 (2.43×10^{10} cfu ml^{-1}) and T_3 (2.41×10^{10} cfu ml^{-1}), T_5 (1.95×10^{10} cfu ml^{-1}), T_2 (1.91×10^{10} cfu ml^{-1}) and T_1 (1.80×10^{10} cfu ml^{-1}), which was significantly superior to the control treatment T_6 which recorded 1.69×10^{8} cfu g^{-1}.

The maximum viable count of *Azotobacter chroococcum* was observed in treatment T_4 (2.22×10^{10} cfu ml^{-1}) which was statistically on par with the treatments T_5 (1.72×10^{10} cfu ml^{-1}) and T_3 (1.54×10^{10} cfu ml^{-1}). Both of these treatments were significantly superior to the control treatment T_6 (1.85×10^{8} cfu ml^{-1}), whereas the population of *Azotobacter chroococcum* in talc formulation recorded 1.46×10^{10} cfu g^{-1}.

The treatment T_2 (2.07×10^{10} cfu ml^{-1}) recorded the highest population of P solubilizer which was statistically on par with treatments T_5 (1.98×10^{10} cfu ml^{-1}),

Table 13. Population of component organisms of PGPR mix-1 in liquid formulation with different additives one month after inoculation

TREATMENTS	FIRST MONTH (log cfu ml^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K Solubilizer (*Bacillus sporothermodurans*)
T1- 2% Glycerol	10.19^{a}	10.13^{a}	10.17^{a}	9.4^{cd}
T2- 2% Poly Vinyl Pyrrolidone	10.16^{a}	10.08^{a}	10.15^{a}	10.25^{a}
T3- 15Mm Trehalose	10.36^{a}	10.21^{a}	10.19^{a}	10.24^{a}
T4- 1% Glycerol and 1% PVP	9.67^{b}	9.59^{b}	9.75^{b}	9.78^{b}
T5- Glycerol (2%), Trehalose (1%), Yeast extract (1%), PVP (1%) and Proline (1%)	9.42^{c}	9.07^{d}	9.57^{bc}	9.55^{c}
T6- Control without any additives	9.25^{c}	8.89^{d}	9.12^{d}	9.2^{d}
T7- Talc based formulation*	9.68^{b}	9.36^{c}	9.53^{c}	9.44^{c}
SEm (±)	0.075	0.068	0.073	0.08
CD (0.05)	0.221	0.202	0.215	0.237

* Log cfu g^{-1} Each value represents a mean of 4 replication

Figures in a column followed by same letters do not differ significantly at $P \geq 0.05$

T_3 (2.02×10^{10} cfu ml^{-1}), T_1 (1.84×10^{10} cfu ml^{-1}) and T_4 (1.82×10^{10} cfu ml^{-1}). All these treatments were significantly superior to the control treatment T_6 (2.27×10^{8} cfu ml^{-1}) and talc based formulation (1.18×10^{10} cfu g^{-1}).

Population of K solubilizer was highest in treatment T_1 (2.63×10^{10} cfu ml^{-1}). This was followed by T_3 (2.34×10^{10} cfu ml^{-1}), T_2 (2.26×10^{10} cfu ml^{-1}) T_4 (2.16×10^{10} cfu ml^{-1}), T_5 (2.11×10^{10} cfu ml^{-1}) and T_7 (1.71×10^{10} cfu ml^{-1}). All these treatments were statistically on par with each other, these were also significantly superior to control treatment T_6 (1.44×10^{8} cfu ml^{-1}) (Table 14).

4.5.1.4 Third Month after Inoculation

Observation on third month after inoculation indicated that the treatment T_5 (2.47×10^{10} cfu ml^{-1}) recorded the highest population of *Azospirillum lipoferum* which was statistically on par with treatments T_1 (2.17×10^{10} cfu ml^{-1}), T_3 (1.90×10^{10} cfu ml^{-1}) and T_2 (1.83×10^{10} cfu ml^{-1}) which were significantly superior to the control treatment T_6 which recorded 7.0×10^{7} cfu ml^{-1}, whereas *Azospirillum lipoferum* in talc based formulation recorded 1.3×10^{9} cfu g^{-1}. Similarly, maximum population of *Azotobacter chroococcum* was observed in treatment T_5 (2.32×10^{10} cfu ml^{-1}) followed by T_1 (1.69×10^{10} cfu ml^{-1}) and T_3 (1.55×10^{10} cfu ml^{-1}). All these treatments were significantly superior to control T_6 (1.77×10^{8} cfu ml^{-1}) and talc formulation (2.15×10^{9} cfu g^{-1}).

The treatment T_3 (2.03×10^{10} cfu ml^{-1}) recorded the highest population of P solubilizer which was statistically on par with treatments T_5 (1.98×10^{10} cfu ml^{-1}), T_2 (1.78×10^{10} cfu ml^{-1}) and T_1 (1.50×10^{10} cfu ml^{-1}) which were significantly superior to the control treatment T_6 (1.73×10^{8} cfu ml^{-1}). The population of P solubilizer in talc based formulation recorded a value of 2.31×10^{9} cfu g^{-1}.

The maximum colony count of K solubilizer was observed in treatment T_3 (2.25×10^{10} cfu ml^{-1}) which was statistically on par with treatments T_5 (2.17×10^{10} cfu ml^{-1}), T_1 (1.72×10^{10} cfu ml^{-1}) and T_2 (1.57×10^{10} cfu ml^{-1}) which was significantly superior to the control treatment T_6 which recorded 8.86×10^{7} cfu

Table 14. Population of component organisms of PGPR mix-1 in liquid formulation with different additives two months after inoculation

TREATMENTS	SECOND MONTH (log cfu ml^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K Solubilizer (*Bacillus sporothermodurans*)
T1- 2% Glycerol	10.25^{a}	10.1^{b}	10.26^{a}	10.31^{a}
T2- 2% Poly Vinyl Pyrrolidone	10.28^{a}	10.14^{b}	10.31^{a}	10.34^{a}
T3- 15Mm Trehalose	10.37^{a}	10.18^{ab}	10.28^{a}	10.36^{a}
T4- 1% Glycerol and 1% PVP	10.39^{a}	10.34^{a}	10.21^{ab}	10.31^{a}
T5- Glycerol (2%), Trehalose (1%), Yeast extract (1%), PVP (1%) and Proline (1%)	10.24^{a}	10.22^{ab}	10.29^{a}	10.31^{a}
T6- Control without any additives	8.16^{b}	8.23^{c}	8.35^{c}	8.11^{b}
T7- Talc based formulation*	10.37^{a}	10.12^{b}	10.06^{b}	10.21^{a}
SEm (±)	0.052	0.062	0.064	0.08
CD (0.05)	0.052	0.062	0.064	0.08

* Log cfu g^{-1} Each value represents a mean of 4 replication

Figures in a column followed by same letters do not differ significantly at $P \geq 0.05$

ml^{-1}. The population of K solubilizer in talc formulation recorded 3.05×10^9 cfu g^{-1} (Table 15).

4.5.1.5 Fourth Month after Inoculation

After four months, *Azospirillum lipoferum* population was highest in T_3 (1.86×10^{10} cfu ml^{-1}) which exhibited significant population. Treatment T_2 (1.64×10^{10} cfu ml^{-1}) and T_1 (1.49×10^{10} cfu ml^{-1}) recorded the second highest values for *Azospirillum lipoferum* population. All these treatments were statistically on par with each other. The population of *Azospirillum lipoferum* recorded a value of 5.6×10^8 cfu g^{-1} in talc based formulation, whereas 1.25×10^7 cfu ml^{-1} in control treatment.

The total viable count of *Azotobacter chroococcum* was maximum in treatment T_3 (1.86×10^{10} cfu ml^{-1}) which was statistically on par with treatments T_2 (1.58×10^{10} cfu ml^{-1}) and T_1 (1.48×10^{10} cfu ml^{-1}). These treatments were significantly superior to the control treatment T_6 which recorded 7.91×10^6 cfu ml^{-1}. The population of *Azotobacter chroococcum* in talc formulation recorded a value of 1.48×10^9 cfu g^{-1}.

The treatment T_2 (2.49×10^{10} cfu ml^{-1}) recorded the highest population of P solubilizer and it was significantly superior to control and talc based formulations which recorded 1.04×10^7 and 6.6×10^8 cfu g^{-1} respectively.

Population of K solubilizer was maximum in T_3 (1.89×10^{10} cfu ml^{-1}) followed by treatment T_1 (1.88×10^{10} cfu ml^{-1}). These treatments were superior to the control treatment T_6 which recorded 1.0×10^7cfu ml^{-1}. The population of K solubilizer in talc formulation recorded 2.71×10^9 cfu g^{-1} (Table 16).

4.5.1.6 Fifth Month after Inoculation

On fifth month after inoculation, treatment T_1 (1.82×10^{10} cfu ml^{-1}) recorded the highest population of *Azospirillum lipoferum* which was statistically on par with the treatment T_3 (1.41×10^{10} cfu ml^{-1}). However, the control treatment

Table 15. Population of component organisms of PGPR mix-1 in liquid formulation with different additives three months after inoculation

TREATMENTS	THIRD MONTH (log cfu ml^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K Solubilizer (*Bacillus sporothermodurans*)
T1- 2% Glycerol	10.33^{a}	10.22^{ab}	10.17^{a}	10.23^{a}
T2- 2% Poly Vinyl Pyrrolidone	10.26^{a}	10.01^{b}	10.24^{a}	10.17^{a}
T3- 15Mm Trehalose	10.27^{a}	10.19^{ab}	10.29^{a}	10.34^{a}
T4- 1% Glycerol and 1% PVP	9.75^{b}	9.37^{c}	9.69^{b}	9.34^{b}
T5- Glycerol (2%), Trehalose (1%), Yeast extract (1%), PVP (1%) and Proline (1%)	10.39^{a}	10.34^{a}	10.29^{a}	10.31^{a}
T6- Control without any additives	7.82^{a}	8.21^{d}	8.23^{d}	7.9^{c}
T7- Talc based formulation*	9.05^{d}	9.28^{c}	9.35^{c}	9.47^{b}
SEm (±)	0.07	0.07	0.061	0.07
CD (0.05)	0.207	0.216	0.179	0.208

* Log cfu g^{-1} Each value represents a mean of 4 replication

Figures in a column followed by same letters do not differ significantly at P ≥0.05

Table 16. Population of component organisms of PGPR mix-1 in liquid formulation with different additives four months after inoculation

TREATMENTS	FOURTH MONTH (log cfu ml^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K Solubilizer (*Bacillus sporothermodurans)*
T1- 2% Glycerol	10.17^{a}	10.16^{a}	10.32^{a}	10.27^{a}
T2- 2% Poly Vinyl Pyrrolidone	10.21^{a}	10.19^{a}	10.39^{a}	10.07^{bc}
T3- 15Mm Trehalose	10.26^{a}	10.27^{a}	10.29^{a}	10.25^{ab}
T4- 1% Glycerol and 1% PVP	9.84^{b}	9.7^{b}	9.9^{b}	9.88^{c}
T5- Glycerol (2%), Trehalose (1%), Yeast extract (1%), PVP (1%) and Proline (1%)	9.26^{c}	9.44^{c}	9.61^{c}	8.98
T6- Control without any additives	7.09^{e}	6.89^{e}	7.01^{e}	6.98
T7- Talc based formulation*	8.74^{d}	9.15^{d}	8.8^{d}	9.42^{d}
SEm (±)	0.064	0.06	0.059	0.067
CD (0.05)	0.189	0.201	0.176	0.198

* Log cfu g^{-1} Each value represents a mean of 4 replication

Figures in a column followed by same letters do not differ significantly at P ≥0.05

T_6 and talc based formulation recorded 1.3×10^7 cfu ml^{-1} and 1.5×10^9 cfu g^{-1} respectively.

The maximum colony count of *Azotobacter chroococcum* was observed in treatment T_1 (1.41×10^{10} cfu ml^{-1}) which was followed by the treatment T_3 (9.92×10^9 cfu ml^{-1}). These two treatments were significantly superior to the control treatment T_6 which recorded 1.32×10^7 cfu ml^{-1}. The population of *Azotobacter chroococcum* in talc formulation recorded 2.58×10^9 cfu g^{-1}.

The treatment T_1 (1.20×10^{10} cfu ml^{-1}) recorded the highest population of P solubilizer which was statistically on par with treatments T_3 (1.14×10^{10} cfu ml^{-1}) and T_2 (9.13×10^9 cfu ml^{-1}), whereas control treatment T_6 recorded 1.60×10^7 cfu ml^{-1} and talc based formulation recorded 2.27×10^9 cfu g^{-1}.

The maximum colony count of K solubilizer was observed in treatment T_1 (2.26×10^{10} cfu ml^{-1}) which was significantly superior to the control treatment T_6 which recorded 1.95×10^7 cfu ml^{-1}. The population of K solubilizer in talc formulation recorded 3.02×10^9 cfu g^{-1} (Table 17).

4.5.1.7 Sixth Month after Inoculation

Total viable count of *Azospirillum lipoferum* after sixth month of inoculation was maximum in T_3 (1.21×10^{10} cfu ml^{-1}). This was followed by the treatment T_1 which recorded (1.19×10^{10} cfu ml^{-1}). However, the control and talc based formulation recorded 1.86×10^7 cfu ml^{-1} and 5.2×10^8 cfu g^{-1} respectively.

On the same month, population of *Azotobacter chroococcum* was maximum in treatment T_3 (1.54×10^{10} cfu ml^{-1}) which was statistically on par with the treatment T_1 (1.29×10^{10} cfu ml^{-1}). Moreover, these treatments were statistically superior to control and talc based formulation recorded 7.52×10^6 cfu ml^{-1} and 7.6×10^8 cfu g^{-1} respectively.

The treatment T_3 (8.45×10^9 cfu ml^{-1}) recorded the highest population of P solubilizer which was statistically on par with treatment T_1 (4.97×10^9 cfu ml^{-1}).

Table 17. Population of component organisms of PGPR mix-1 in liquid formulation with different additives five months after inoculation

TREATMENTS	FIFTH MONTH (log cfu ml^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K Solubilizer (*Bacillus sporothermodurans*)
T1- 2% Glycerol	10.25^{a}	10.14^{a}	10.06^{a}	10.37^{a}
T2- 2% Poly Vinyl Pyrrolidone	9.83^{b}	9.57^{b}	9.95^{a}	9.65^{cd}
T3- 15Mm Trehalose	10.14^{a}	9.98^{a}	10.05^{a}	10.04^{b}
T4- 1% Glycerol and 1% PVP	9.53^{c}	9.07^{d}	9.09^{c}	8.77^{e}
T5- Glycerol (2%), Trehalose (1%), Yeast extract (1%), PVP (1%) and Proline (1%)	9.8^{b}	9.24^{cd}	9.31^{bc}	9.71^{c}
T6- Control without any additives	7.09^{e}	7.14^{e}	7.18^{d}	7.25^{f}
T7- Talc based formulation*	9.16^{d}	9.38^{bc}	9.43^{b}	9.46^{d}
SEm (±)	0.062	0.066	0.072	0.065
CD (0.05)	0.184	0.195	0.212	0.192

* Log cfu g^{-1} Each value represents a mean of 4 replication

Figures in a column followed by same letters do not differ significantly at $P \geq 0.05$

These were also significantly superior to the control treatment T_6 which recorded 1.92×10^7 cfu ml^{-1}. The population of P solubilizer in talc based formulation recorded 7.3×10^8 cfu g^{-1}.

The maximum colony count of K solubilizer was observed in treatment T_3 (1.90×10^{10} cfu ml^{-1}) as against the control treatment T_6 which recorded 1.88×10^7 cfu ml^{-1}, whereas the population of K solubilizer in talc formulation recorded 3.6×10^8 cfu g^{-1} (Table 18) (Plate 8).

4.5.1.8 Seventh Month after Inoculation

The treatment T_3 recorded the highest population of *Azospirillum lipoferum* (1.67×10^{10} cfu ml^{-1}) after seven month of inoculation. This was followed by the treatment T_1 (1.50×10^{10} cfu ml^{-1}) which was found to be statistically on par with each other. These treatments were statistically superior to control treatment and talc based formulation which recorded 1.30×10^7 cfu ml^{-1} and 5.0×10^7 cfu g^{-1} of viable count respectively.

Maximum significant population of *Azotobacter chroococcum* was observed in treatment T_1 (1.69×10^{10} cfu ml^{-1}) which was statistically on par with the treatment T_3 (9.72×10^{10} cfu ml^{-1}). These treatments were significantly superior to the control treatment T_6 which recorded 4.71×10^6 cfu ml^{-1}. The population of *Azotobacter chroococcum* in talc formulation recorded 1.1×10^8 cfu g^{-1}.

The treatment T_3 (1.22×10^{10} cfu ml^{-1}) recorded the highest population of P solubilizer followed by T_1 (1.03×10^{10} cfu ml^{-1}).These treatments were significantly superior to the control treatment T_6 which recorded 6.73×10^6 cfu ml^{-1}. The population of P solubilizer in talc based formulation recorded 1.1×10^8 cfu g^{-1}.

The maximum colony count of K solubilizer was observed in treatment T_1 (2.44×10^{10} cfu ml^{-1}) which was significantly superior to all the treatments including the control treatment T_6 which recorded 1.12×10^7 cfu ml^{-1}. The

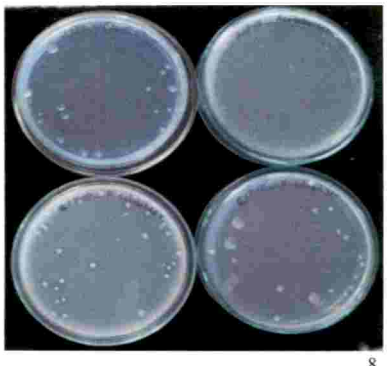
Table 18. Population of component organisms of PGPR mix-1 in liquid formulation with different additives six months after inoculation

TREATMENTS	SIXTH MONTH (log cfu ml^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K Solubilizer (*Bacillus sporothermodurans)*
T1- 2% Glycerol	10.07^{a}	10.11^{a}	9.68^{a}	9.96^{b}
T2- 2% Poly Vinyl Pyrrolidone	9.49^{bc}	9.21^{c}	9.29^{b}	9.64^{c}
T3- 15Mm Trehalose	10.08^{a}	10.18^{a}	9.91^{a}	10.27^{a}
T4- 1% Glycerol and 1% PVP	9.31^{c}	9.65^{b}	8.88^{c}	9.78^{bc}
T5- Glycerol (2%), Trehalose (1%), Yeast extract (1%), PVP (1%) and Proline (1%)	9.66^{b}	9.21^{c}	9.34^{b}	9.70^{c}
T6- Control without any additives	7.2^{e}	6.85^{e}	7.21^{d}	7.26^{e}
T7- Talc based formulation*	8.65^{d}	8.85^{d}	8.84^{c}	8.53^{d}
SEm (±)	0.096	0.061	0.09	0.063
CD (0.05)	0.286	0.182	0.266	0.185

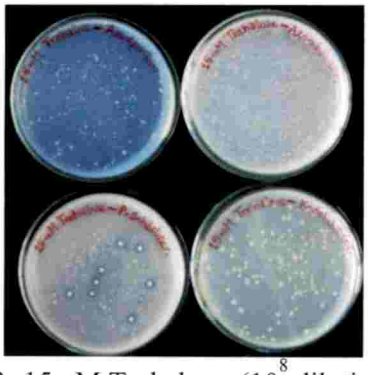
* Log cfu g^{-1} Each value represents a mean of 4 replication

Figures in a column followed by same letters do not differ significantly at P ≥0.05

T1- 2% Glycerol (10^{8} dilution)



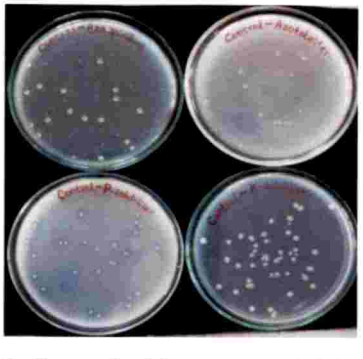
T2 -2% Poly Vinyl Pyrrolidone (10^{8} dilution)



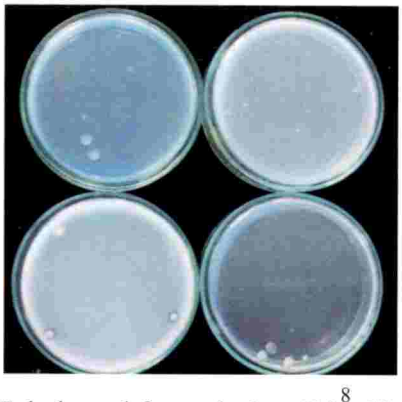
T3 -15mM Trehalose (10^{8} dilution)

T4- 1% Glycerol and 1% PVP (10^{8} dilution)

T5- Glycerol (2%), Trehalose (1%), Yeast extract (1%), PVP (1%) and Proline (1%) (10^{8} dilution)



T6- Control without any additives (10^{5} dilution)



T8- Talc based formulation (10^{8} dilution)

Plate 8. Population of component organisms of PGPR mix-1 in liquid formulation with different additives six months after inoculation

population of K solubilizer in talc formulation recorded 1.2×10^8 cfu g^{-1} (Table 19).

4.5.1.9 Eighth Month after Inoculation

On the eighth month after inoculation, treatment T_3 (7.67×10^9 cfu ml^{-1}) recorded the highest population of *Azospirillum lipoferum* which was significantly superior to all the treatments including the control treatment T_6 and talc based formulation which recorded 6.01×10^6 cfu ml^{-1} and 2.0×10^8 cfu g^{-1} respectively.

The population of *Azotobacter chroococcum* was observed maximum in the treatment T_3 (5.31×10^9 cfu ml^{-1}) which was significantly superior to all the treatments including the control treatment T_6 which recorded 1.86×10^6 cfu ml^{-1}. The population of *Azotobacter chroococcum* in talc formulation recorded 7.0×10^7 cfu g^{-1}.

The treatment T_3 (4.10×10^9 cfu ml^{-1}) recorded the highest population of P solubilizer which was significantly superior to all the treatments. However, the control treatment T_6 recorded 5.57×10^6 cfu ml^{-1} and the talc based formulation recorded 3.5×10^8 cfu g^{-1}.

The maximum colony count of K solubilizer was observed in the treatment T_3 (7.4×10^9 cfu ml^{-1}) which was significantly superior to all the treatments including the control treatment T_6 which recorded 1.98×10^6 cfu ml^{-1}. The population of K solubilizer in talc formulation recorded 1.5×10^8 cfu g^{-1} (Table 20).

4.5.1.10 Ninth Month after Inoculation

From ninth month onwards formulation amended with 15mM Trehalose (T_3) exhibited maximum viable count until fourteenth month. Treatment T_3 recorded the highest population of *Azospirillum lipoferum* (2.46×10^9 cfu ml^{-1}) which was significantly superior to all the treatments including the control treatment T_6 and talc based formulation which recorded 1.45×10^6 cfu ml^{-1}.and

Table 19. Population of component organisms of PGPR mix-1 in liquid formulation with different additives seven months after inoculation

TREATMENTS	SEVENTH MONTH (log cfu ml^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K Solubilizer (*Bacillus sporothermodurans*)
T1- 2% Glycerol	10.17^{a}	10.21^{a}	9.97^{a}	10.38^{a}
T2- 2% Poly Vinyl Pyrrolidone	8.88^{d}	8.54^{c}	8.55^{c}	8.86^{d}
T3- 15Mm Trehalose	10.22^{a}	9.98^{a}	10.08^{a}	10.09^{b}
T4- 1% Glycerol and 1% PVP	9.12^{c}	8.77^{c}	9.09^{b}	9.02^{d}
T5- Glycerol (2%), Trehalose (1%), Yeast extract (1%), PVP (1%) and Proline (1%)	9.85^{b}	9.23^{b}	9.28^{b}	9.71^{c}
T6- Control without any additives	7.09^{f}	6.65^{e}	6.82^{e}	7.01^{f}
T7- Talc based formulation*	7.85^{e}	8.07^{d}	8.00^{d}	8.15^{e}
SEm (±)	0.062	0.106	0.074	0.098
CD (0.05)	0.185	0.313	0.219	0.291

* Log cfu g^{-1} Each value represents a mean of 4 replication

Figures in a column followed by same letters do not differ significantly at $P \geq 0.05$

Table 20. Population of component organisms of PGPR mix-1 in liquid formulation with different additives eight months after inoculation

TREATMENTS	EIGHTH MONTH (log cfu ml^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K Solubilizer (*Bacillus sporothermodurans*)
T1- 2% Glycerol	9.46^{b}	9.37^{b}	9.39^{a}	9.49^{b}
T2- 2% Poly Vinyl Pyrrolidone	8.54^{d}	8.09^{d}	8.53^{c}	8.55^{d}
T3- 15Mm Trehalose	9.88^{a}	9.72^{a}	9.61^{a}	9.86^{a}
T4- 1% Glycerol and 1% PVP	9.38^{b}	8.93^{d}	9.08^{b}	9.19^{c}
T5- Glycerol (2%), Trehalose (1%), Yeast extract (1%), PVP (1%) and Proline (1%)	8.8^{c}	8.04^{c}	8.62^{c}	8.24^{e}
T6- Control without any additives	6.73^{e}	6.25^{f}	6.72^{d}	6.54^{f}
T7- Talc based formulation*	8.38^{d}	7.88^{e}	8.53^{c}	8.26^{e}
SEm (±)	0.073	0.083	0.09	0.096
CD (0.05)	0.217	0.246	0.27	0.283

* Log cfu g^{-1} Each value represents a mean of 4 replication

Figures in a column followed by same letters do not differ significantly at $P \geq 0.05$

2.3×10^7 cfu g^{-1} respectively. Similar trend was observed in total viable count of *Azotobacter chroococcum,* P solubilizer and K solubilizer up to fourteenth month. Same treatment recorded the highest population of *Azotobacter chroococcum* (1.45×10^9 cfu ml^{-1}), P solubilizer (1.72×10^9 cfu ml^{-1}) and K solubilizer (2.41×10^9 cfu ml^{-1}). All these treatments were significantly superior to the control treatment and talc based formulation which recorded 1.0×10^6, 2.18×10^6 and 1.96×10^6 cfu ml^{-1}, 2.7×10^7 and 2.5×10^8, 4.3×107 cfu g^{-1} respectively (Table 21).

4.5.1.11 Tenth Month after Inoculation

Population at tenth month indicated that the treatment T_3 was significantly superior to all other treatments. Here the total viable count of *Azospirillum lipoferum* was 1.07×10^9 cfu ml^{-1} was recorded as against control treatment and talc based formulation which recorded 1.67×10^6 cfu ml^{-1} and 3.0×10^6 cfu g^{-1} respectively. Population of the component culture *Azotobacter chroococcum* recorded 1.27×10^9 cfu ml^{-1} compared to control and talc based formulation which recorded 1.15×10^6 cfu ml^{-1} and 5.6×10^6cfu g^{-1} respectively. Same treatment recorded the maximum population of P solubilizer (7.7×10^8 cfu ml^{-1}) and K solubilizer (1.71×10^9 cfu ml^{-1}) and was significantly superior to control and talc based formulation (Table 22).

4.5.1.12 Eleventh Month after Inoculation

After eleven month, the total viable count of *Azospirillum lipoferum* (1.1×10^9 cfu ml^{-1}), *Azotobacter chroococcum* (1.06×10^9 cfu ml^{-1}), P solubilizer (1.68×10^9 cfu ml^{-1}) and K solubilizer (1.53×10^9 cfu ml^{-1}) in T_3 was found significantly superior to control treatment and talc based formulation. Significant decline of population was observed in talc based formulation which recorded a population of 3.22×10^6 cfu ml^{-1}, 2.86×10^6 cfu ml^{-1}, 6.05×10^6 cfu ml^{-1} and 7.23×10^6 cfu ml^{-1} of *Azospirillum lipoferum, Azotobacter chroococcum,* P solubilizer and K solubilizer (Table 23).

Table 21. Population of component organisms of PGPR mix-1 in liquid formulation with different additives nine months after inoculation

TREATMENTS	NINTH MONTH (log cfu ml^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K Solubilizer (*Bacillus sporothermodurans*)
T1- 2% Glycerol	8.57^{b}	8.06^{b}	8.14^{b}	8.36^{b}
T2- 2% Poly Vinyl Pyrrolidone	8.07^{d}	7.66^{c}	7.69^{c}	7.88^{d}
T3- 15Mm Trehalose	9.37^{a}	9.14^{a}	9.22^{a}	9.37^{a}
T4- 1% Glycerol and 1% PVP	8.02^{d}	8.07^{b}	8.16^{b}	8.05^{c}
T5- Glycerol (2%), Trehalose (1%), Yeast extract (1%), PVP (1%) and Proline (1%)	8.35^{c}	8^{b}	7.85^{c}	8.24^{b}
T6- Control without any additives	6.11^{f}	5.98^{d}	6.3^{e}	6.26^{f}
T7- Talc based formulation*	7.35^{e}	7.4^{c}	7.38^{d}	7.62^{e}
SEm (±)	0.07	0.098	0.079	0.051
CD (0.05)	0.219	0.29	0.235	0.152

* Log cfu g^{-1} Each value represents a mean of 4 replication

Figures in a column followed by same letters do not differ significantly at $P \geq 0.05$

Table 22. Population of component organisms of PGPR mix-1 in liquid formulation with different additives ten months after inoculation

TREATMENTS	TENTH MONTH (log cfu ml^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K Solubilizer (*Bacillus sporothermodurans*)
T1- 2% Glycerol	8.59^{b}	8.29^{b}	8.07^{b}	8.49^{b}
T2- 2% Poly Vinyl Pyrrolidone	7.38^{e}	7.35^{d}	7.25^{d}	7.33^{d}
T3- 15Mm Trehalose	9.02^{a}	9.09^{a}	8.88^{a}	9.22^{a}
T4- 1% Glycerol and 1% PVP	7.95^{d}	7.48^{e}	7.72^{e}	7.43^{e}
T5- Glycerol (2%), Trehalose (1%), Yeast extract (1%), PVP (1%) and Proline (1%)	7.69^{c}	7.7^{c}	7.79^{d}	7.89^{c}
T6- Control without any additives	6.19^{g}	6.04^{f}	6.06^{f}	6.13^{f}
T7- Talc based formulation*	6.46^{f}	6.71^{e}	6.7^{e}	6.81^{e}
SEm (±)	0.063	0.063	0.064	0.066
CD (0.05)	0.186	0.186	0.19	0.19

* Log cfu g^{-1} Each value represents a mean of 4 replication

Figures in a column followed by same letters do not differ significantly at $P \geq 0.05$

Table 23. Population of component organisms of PGPR mix-1 in liquid formulation with different additives eleven months after inoculation

TREATMENTS	ELEVENTH MONTH (log cfu ml^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K Solubilizer (*Bacillus sporothermodurans)*
T1- 2% Glycerol	8.11^{b}	8.11^{b}	8.26^{b}	8.47^{b}
T2- 2% Poly Vinyl Pyrrolidone	7.77	6.55^{d}	7.78^{c}	7.76^{c}
T3- 15Mm Trehalose	9.02^{a}	8.54^{a}	9.28^{a}	9.16^{a}
T4- 1% Glycerol and 1% PVP	6.34^{d}	6.3^{e}	6.64^{e}	6.52^{e}
T5- Glycerol (2%), Trehalose (1%), Yeast extract (1%), PVP (1%) and Proline (1%)	7.78^{c}	7.13^{c}	7.57^{d}	7.76^{c}
T6- Control without any additives	6.39^{d}	5.2^{g}	6.19^{f}	6.36^{f}
T7- Talc based formulation*	6.48^{d}	5.42^{f}	6.75^{e}	6.84^{d}
SEm (±)	0.056	0.048	0.046	0.035
CD (0.05)	0.167	0.132	0.188	0.149

* Log cfu g^{-1} Each value represents a mean of 4 replication

Figures in a column followed by same letters do not differ significantly at $P \geq 0.05$

4.5.1.13 Twelfth Month after Inoculation

On twelfth month after inoculation, treatment T_3 recorded the highest population of *Azospirillum lipoferum* (6.22×10^8 cfu ml^{-1}), *Azotobacter chroococcum* (3.63×10^8 cfu ml^{-1}), P solubilizer (4.23×10^8 cfu ml^{-1}) and K solubilizer (5.16×10^8 cfu ml^{-1}), whereas the control recorded 1.83×10^5 cfu ml^{-1}, 1.62×10^5 cfu ml^{-1}, 1.60×10^5 cfu ml^{-1} and 1.52×10^5 cfu ml^{-1} respectively. The population of talc based formulation was declined significantly which recorded 2.7×10^7 cfu g^{-1}, 2.7×10^5 cfu g^{-1}, 1.80×10^5 cfu ml^{-1} and 4.7×10^5 cfu g^{-1} of *Azospirillum lipoferum*, *Azotobacter chroococcum*, P solubilizer and K solubilizer respectively (Table 24).

4.5.1.14 Thirteenth Month after Inoculation

On thirteenth month, total viable count of component cultures of PGPR mix-1 was significantly superior to other treatments and control treatments in T_3 as in last few months. Here significant population of *Azospirillum lipoferum* (3.31×10^8 cfu ml^{-1}), *Azotobacter chroococcum* (2.72×10^8 cfu ml^{-1}), P solubilizer (2.46×10^8 cfu ml^{-1}) and K solubilizer (3.40×10^8 cfu ml^{-1}) compared to control treatment and talc based formulation was recorded. The population of all the cultures of PGPR mix-1 in talc based formulation was reduced considerably compared to the treatment T_3 (Table 25).

4.5.1.15 Fourteenth Month after Inoculation

Even after thirteen month significant viable count of cultures was seen in the treatment T_3, which recorded a population of 4.17×10^8 cfu ml^{-1} of *Azospirillum lipoferum*, 3.03×10^8 cfu ml^{-1} of *Azotobacter chroococcum*, 3.50×10^8 cfu ml^{-1} of P solubilizer and 3.96×10^8 cfu ml^{-1} of K solubilizer (Plate 9). However, colonies observed in talc based formulation declined significantly. The population in talc based formulation recorded 2.0×10^4, 1.23×10^4, 1.31×10^4 and 1.21×10^4 cfu ml^{-1} of *Azospirillum lipoferum, Azotobacter chroococcum,* P solubilizer and K solubilizer respectively (Table 26) (Plate 10 and 11).

Table 24. Population of component organisms of PGPR mix-1 in liquid formulation with different additives twelve months after inoculation

TREATMENTS	TWELFTH MONTH (log cfu ml^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K Solubilizer (*Bacillus sporothermodurans)*
T1- 2% Glycerol	8.26^{b}	8.08^{b}	8.06^{b}	8.18^{b}
T2- 2% Poly Vinyl Pyrrolidone	6.63^{d}	6.55^{d}	6.25^{d}	6.40^{d}
T3- 15Mm Trehalose	8.77^{a}	8.54^{a}	8.61^{a}	8.70^{a}
T4- 1% Glycerol and 1% PVP	6.24^{e}	6.30^{e}	6.11^{e}	6.25^{e}
T5- Glycerol (2%), Trehalose (1%), Yeast extract (1%), PVP (1%) and Proline (1%)	7.57^{c}	7.13^{c}	7.11^{c}	7.69^{c}
T6- Control without any additives	5.26^{f}	5.20^{f}	5.20^{f}	5.17^{g}
T7- Talc based formulation*	5.42^{f}	6.4^{e}	5.25^{f}	5.66^{f}
SEm (±)	0.048	0.050	0.045	0.042
CD (0.05)	0.278	0.147	0.132	0.126

* Log cfu g^{-1} Each value represents a mean of 4 replication

Figures in a column followed by same letters do not differ significantly at $P \geq 0.05$

Table 25. Population of component organisms of PGPR mix-1 in liquid formulation with different additives thirteen months after inoculation

TREATMENTS	THIRTEENTH MONTH (log cfu ml^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K Solubilizer (*Bacillus sporothermodurans*)
T1- 2% Glycerol	7.43^{c}	7.14^{c}	7.09^{c}	7.5^{b}
T2- 2% Poly Vinyl Pyrrolidone	6.36^{d}	6.25^{d}	6.27^{d}	6.36^{c}
T3- 15Mm Trehalose	8.5^{a}	8.42^{a}	8.38^{a}	8.61^{a}
T4- 1% Glycerol and 1% PVP	6.08^{e}	5.89^{e}	5.94^{e}	6.11^{d}
T5- Glycerol (2%), Trehalose (1%), Yeast extract (1%), PVP (1%) and Proline (1%)	7.6^{b}	7.25^{b}	7.23^{b}	7.61^{b}
T6- Control without any additives	5.22^{g}	5.27^{f}	5.17^{g}	5.27^{e}
T7- Talc based formulation*	5.51^{f}	5.43^{f}	5.44^{f}	5.41^{e}
SEm (±)	0.042	0.057	0.043	0.048
CD (0.05)	0.122	0.162	0.129	0.148

* Log cfu g^{-1} Each value represents a mean of 4 replication

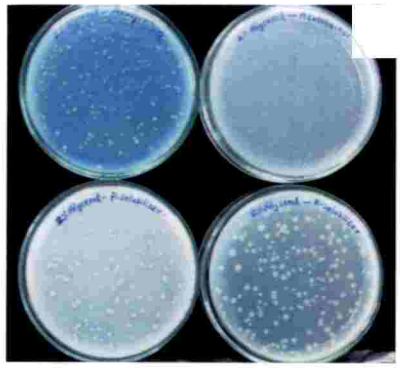
Figures in a column followed by same letters do not differ significantly at $P \geq 0.05$

Table 26. Population of component organisms of PGPR mix-1 in liquid formulation with different additives fourteen months after inoculation

TREATMENTS	FOURTEENTH MONTH (log cfu ml^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K Solubilizer (*Bacillus sporothermodurans*)
T1- 2% Glycerol	6.98^{c}	6.88^{b}	7.01^{c}	7.05^{c}
T2- 2% Poly Vinyl Pyrrolidone	6.06^{d}	5.86^{c}	5.95^{d}	6.06^{d}
T3- 15Mm Trehalose	8.61^{a}	8.47^{a}	8.52^{a}	8.58^{a}
T4- 1% Glycerol and 1% PVP	5.46^{e}	5.55^{c}	5.42^{e}	5.40^{e}
T5- Glycerol (2%), Trehalose (1%), Yeast extract (1%), PVP (1%) and Proline (1%)	7.63^{b}	7.37^{b}	7.43^{b}	7.83^{b}
T6- Control without any additives	4.79^{f}	5.55^{c}	4.81^{f}	5.00^{f}
T7- Talc based formulation*	4.19^{g}	4.09^{g}	4.11^{g}	4.08^{g}
SEm (±)	0.035	0.046	0.054	0.038
CD (0.05)	0.105	0.982	0.235	0.113

* Log cfu g^{-1} Each value represents a mean of 4 replication

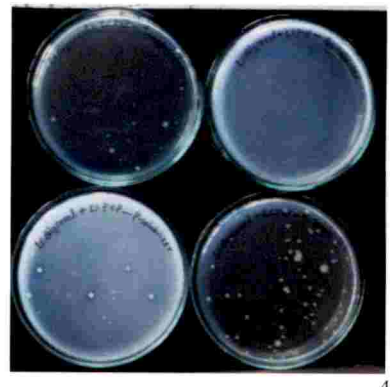
Figures in a column followed by same letters do not differ significantly at $P \geq 0.05$



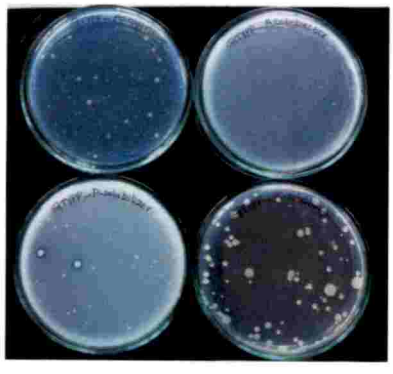
T1- 2% Glycerol (10^5 dilution)

T2 -2% Poly Vinyl Pyrrolidone (10^4 dilution)

T3 -15mM Trehalose (10^7 dilution)



T4- 1% Glycerol and 1% PVP (10^4 dilution)

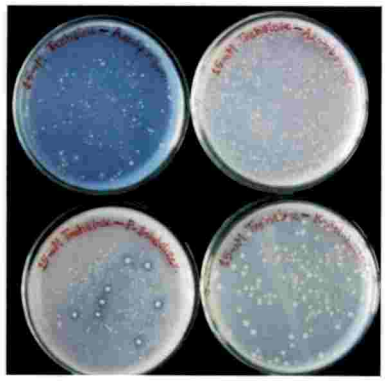


T5- Glycerol (2%), Trehalose (1%), Yeast extract (1%), PVP(1%) and Proline (1%) (10^6 dilution)

T6- Control without any additives (10^3 dilution)

Plate 9. Population of component organisms of PGPR mix-1 in liquid formulation with different additives fourteen months after inoculation

First month (10^{8} dilution)



Sixth month (10^{8} dilution)

Fourteenth month (10^{7} dilution)

T3-15mM Trehalose

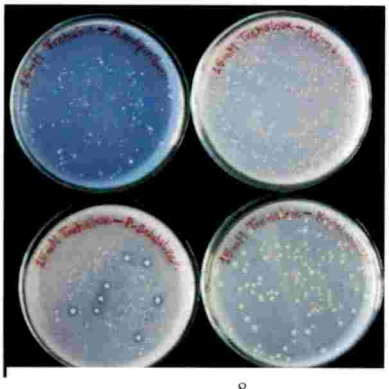
First month (10^{8} dilution)

Sixth month (10^{8} dilution)

Talc

Plate 10. Comparison of best treatment (15mM Trehalose) with talc formulation of PGPR Mix-I at different intervals

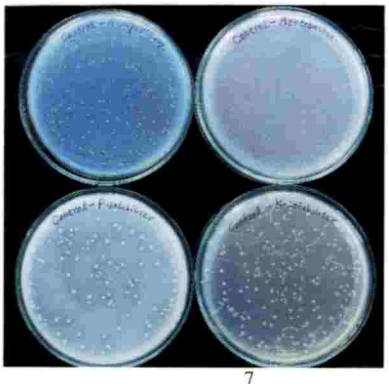
First month (10^{8} dilution)



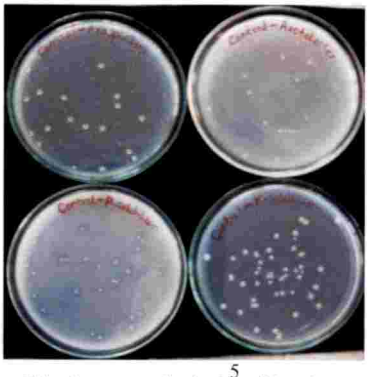
Sixth month (10^{8} dilution)

Fourteenth month (10^{7} dilution)

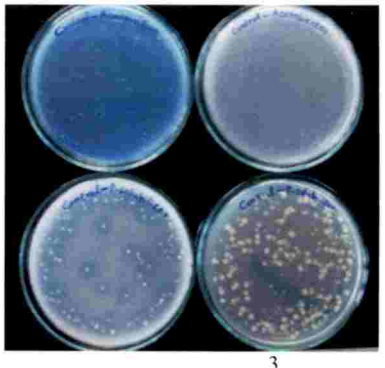
T3-15mM Trehalose



First month (10^{7} dilution)



Sixth month (10^{5} dilution)



Fourteenth month(10^{3} dilution)

Control

Plate 11. Comparison of best treatment (15mM Treahalose) with control at different intervals

4.6 POT CULTURE STUDIES TO EVALUATE THE EFFICIENCY OF LIQUID FORMULATION OF PGPR MIX-I

The best treatment selected from experiment 5, viz., 15mM Trehalose was evaluated under pot culture conditions using sterilized soil in Completely Randomized Design with Amaranthus variety Amt-1 as the target crop.

4.6.1 Effect of Liquid Formulation of PGPR Mix-1 on Plant Height of Amaranthus at 20 DAT

Preliminary observation on plant height at 20th day after transplanting (Plate 12 and 13) indicated that maximum plant height of 15.16 cm was observed in treatment T_6 (Liquid biofertilizer formulation of PGPR mix–I+ 100% NPK) followed by T_3 (Talc based formulation of PGPR mix–I + 100% NPK) which recorded a plant height of 14.92 cm and these were found to be statistically on par with each other. The absolute control recorded a plant height of 9.64 cm (Table 27).

4.6.2 Effect of Liquid Formulation of PGPR Mix-1 on Plant Height of Amaranthus at Harvest

Maximum plant height of 37.54 cm was observed in treatment T_6 (Liquid biofertilizer formulation of PGPR mix–I+ 100% NPK) followed by T_3 (Talc based formulation of PGPR mix–I + 100% NPK) which recorded a plant height of 37.38 cm and these were found to be statistically on par with each other. The treatment T_6 and T_3 were found to be significantly superior to all other treatments and the treatment T_7 (Liquid biofertilizer formulation of PGPR mix -I+50% NPK) was found to be on par with T_1 (chemical fertilizer 100% NPK) and these two showed significantly higher plant height compared to treatment T_5 (Liquid biofertilizer formulation of PGPR mix–I alone) and absolute control (Table 28) (Plate14 and 15).

Table 27. Effect of different treatments on plant height at 20 DAT

TREATMENT	Plant height (20 DAT) (cm)*
T_1- Chemical fertilizer @ 100% NPK	13.06^{b}
T_2- Talc based formulation of PGPR mix–I alone	12.31^{bc}
T_3- Talc based formulation of PGPR mix–I + 100% NPK	14.92^{a}
T_4- Talc based formulation of PGPR mix–I + 50% NPK	13.02^{b}
T_5- Liquid biofertilizer formulation of PGPR mix–I alone	11.61^{c}
T_6- Liquid biofertilizer formulation of PGPR mix–I+ 100% NPK	15.16^{a}
T_7- Liquid biofertilizer formulation of PGPR mix–I+ 50% NPK	13.18^{b}
T_8- Control with additives without PGPR microorganisms	8.91^{d}
T_9- Absolute control	9.64^{d}
SE(m)	0.495
CD(0.05)	1.467

*Mean of 3 observations

Figures in a column followed by same letters do not differ significantly at $P \geq 0.05$

T1- 100% NPK T6- Liquid + 100% NPK T7 -Liquid + 50% NPK

T5-Liquid Alone T9-Absolute Control

Plate 12. Effect of different treatments on plant height of *Amaranthus tricolor* at 20 DAT

T1- 100% NPK	T7 -Liquid + 50% NPK	T9-Absolute Control

Plate 13. Comparison of different treatments on plant height of *Amaranthus tricolor* at 20 DAT

T1 T6 T7 T5 T9

T1- 100% NPK T6- Liquid + 100% NPK T7 -Liquid + 50% NPK

T5-Liquid Alone T9-Absolute Control

Plate 14. Effect of different treatments on plant height of *Amaranthus tricolor* at harvest

T1- 100% NPK | T7 -Liquid + 50% NPK | T9-Absolute Control

Plate 15. Comparison of different treatments on plant height of *Amaranthus tricolor* at harvest

4.6.3 Effect of Liquid Formulation of PGPR Mix-1 on Fresh Weight of Shoot of Amaranthus at Harvest

The highest shoot fresh weight of 55.41 g was observed in plants treated with treatment T_6 (Liquid biofertilizer formulation of PGPR mix–I+ 100% NPK) which was found to be statistically on par with T_3 (Talc based formulation of PGPR mix–I + 100% NPK) which recorded shoot fresh weight of 54.76 g. These treatments were significantly superior to absolute control, which recorded a shoot fresh weight of 23.60 g plant^{-1} (Table 28).

4.6.4 Effect of Liquid Formulation of PGPR Mix-1 on Fresh Weight of Root of Amaranthus at Harvest

The maximum root fresh weight of 6.6 g was observed in T_3 (Talc based formulation of PGPR mix–I+ 100% NPK) and this was statistically on par with treatments T_6 (Liquid biofertilizer formulation of PGPR mix–I+ 100% NPK), T_7 (Liquid biofertilizer formulation of PGPR mix–I+ 50% NPK), T_4 (Talc based formulation of PGPR mix–I+ 50% NPK) and T_1 (chemical fertilizer 100% NPK) which recorded a root fresh weight of 6.21, 6.21, 5.82 and 5.73 g plant^{1} respectively. All these treatments were significantly superior to the absolute control which recorded a root fresh weight of 2.39 g plant^{1} (Table 28).

4.6.5 Effect of Liquid Formulation of PGPR Mix-1 on Dry Weight of Shoot of Amaranthus at Harvest

The dry weight of shoot was significantly higher in plants treated with T_3 (Talc based formulation of PGPR mix–I + 100% NPK) which recorded a shoot dry weight of 4.97 g plant^{-1}. The next best treatment in improving shoot dry weight was T_6 (Liquid biofertilizer formulation of PGPR mix–I+ 100% NPK) which recorded 4.83 g plant^{-1} of shoot dry weight. These treatments were found to be statistically on par with each other and superior to the absolute control (1.50 g plant^{-1}) (Table 28).

4.6.6 Effect of Liquid Formulation of PGPR Mix-1 on Dry Weight of Root of Amaranthus at Harvest

Significantly superior dry weight of root was observed in plants treated with T_3 (Talc based formulation of PGPR mix–I+ 100% NPK) which recorded 0.56 g plant^{-1} (Table 28). This was statistically on par with treatments T_6 (Liquid biofertilizer formulation of PGPR mix–I+ 100% NPK), T_4 (Talc based formulation of PGPR mix–I+ 50% NPK), T_1 (chemical fertilizer 100% NPK) and T_7 (Liquid biofertilizer formulation of PGPR mix–I+ 50% NPK) which recorded a root dry weight of 0.55, 0.49, 0.48 and 0.47 g plant^{-1} respectively. The absolute control recorded a root dry weight of 0.27 g plant^{-1}.

4.6.7 Effect of Liquid Formulation of PGPR Mix-1 on Leaf Area Index of Amaranthus at Harvest

Significant increase in Leaf Area Index (LAI) was observed in plants treated with T_6 (Liquid biofertilizer formulation of PGPR mix–I+ 100% NPK) which recorded a LAI of 4.97 and this was statistically on par with the treatment T_3 (Talc based formulation of PGPR mix–I+ 100% NPK) which recorded the LAI of 4.94. These treatments recorded significantly superior LAI as against control (2.08) (Table 29).

4.6.8 Effect of Liquid Formulation of PGPR Mix-1 on Number of Leaves of Amaranthus at Harvest

The maximum number of leaves of 44.44 was observed in plants treated with treatment T_6 (Liquid biofertilizer formulation of PGPR mix–I+ 100% NPK) which was found to be statistically on par with T_3 which recorded a number of leaves of 40.11. However, the absolute control recorded 18.77 numbers of leaves (Table 29).

Table 28. Effect of different treatments on biometric characters of *Amaranthus tricolor* at harvest

Treatment	Plant height (at the time of harvest)* (cm)	Fresh weight of shoot (g plant^{-1})*	Dry weight of shoot (g plant^{-1})*	Fresh weight of root (g plant^{-1})*	Dry weight of root (g plant^{-1})*
T_1- Chemical fertilizer @ 100% NPK	32.95^{b}	43.50^{b}	3.95^{b}	5.73^{ab}	0.48^{ab}
T_2- Talc based formulation of PGPR mix–I alone	27.28^{c}	34.55^{c}	3.64^{b}	4.29^{c}	0.42^{b}
T_3-Talc based formulation of PGPR mix–I+100% NPK	37.38^{a}	54.76^{a}	4.97^{a}	6.60^{a}	0.56^{a}
T_4-Talc based formulation of PGPR mix–I + 50% NPK	32.34^{b}	41.19^{b}	3.77^{b}	5.82^{ab}	0.49^{ab}
T_5-Liquid biofertilizer formulation of PGPR mix–I alone	27.91^{c}	34.54^{c}	3.68^{b}	5.13^{bc}	0.41^{b}
T_6-Liquid biofertilizer formulation of PGPR mix-I + 100% NPK	37.54^{a}	55.41^{a}	4.83^{a}	6.21^{ab}	0.55^{a}
T_7- Liquid biofertilizer formulation of PGPR mix-I + 50% NPK	32.88^{b}	42.43^{b}	3.75^{b}	6.21^{ab}	0.47^{ab}
T_8- Control with additives without PGPR microorganisms	19.10^{d}	20.82^{d}	1.50^{c}	2.11^{d}	0.30^{c}
T_9- Absolute control	21.92^{d}	23.60^{d}	2.01^{c}	2.39^{d}	0.27^{c}
SE(m)	1.391	2.206	0.267	0.435	0.034
CD(0.05)	4.130	6.550	0.797	1.295	0.100

* Mean of 3 observations Figures in a column followed by same letters do not differ significantly at $P \geq 0.05$

4.6.9 Effect of Liquid Formulation of PGPR Mix-1 on Oxalate Content of Amaranthus at Harvest

Among all the treatments, T_6 (Liquid biofertilizer formulation of PGPR mix–I+ 100% NPK) recorded the least oxalate content of 0.39 per cent similar to the oxalate content of absolute control which also recorded 0.39 per cent (Table 29). This was followed by the treatments T_8 (Control with additives without PGPR microorganisms), T_1 (chemical fertilizer 100% NPK) and T_7 (Liquid biofertilizer formulation of PGPR mix–I+ 50% NPK) which recorded an oxalate content of 0.41, 0.42 and 0.45 per cent respectively.

4.6.10 Effect of Liquid Formulation of PGPR Mix-1 on Initial and Final Available Soil N, P and K Content

The available NPK content of soil before treatment were 238.48, 27.4 and 856.91 kg ha^{-1} respectively (Table 30). Significant soil nitrogen content was observed in T_6 (Liquid biofertilizer formulation of PGPR mix–I+ 100% NPK) which recorded 288.51 kg ha^{-1} of nitrogen, which was followed by treatment T_1 (chemical fertilizer 100% NPK) and T_3 (Talc based formulation of PGPR mix–I+ 100% NPK) which recorded a nitrogen content of 275.96 and 269.69 kg ha^{-1} respectively. The absolute control recorded a nitrogen content of 194.43 kg ha^{-1}.

Significant phosphorous content was observed in treatment T_6 (Liquid biofertilizer formulation of PGPR mix–I+ 100% NPK) which recorded 61.6 kg ha^{-1} which was followed by T_3 (Talc based formulation of PGPR mix–I+ 100% NPK), T_4 (Talc based formulation of PGPR mix–I+ 50% NPK), T_7 (Liquid biofertilizer formulation of PGPR mix–I+ 50% NPK) and T_1 (chemical fertilizer 100% NPK), which recorded a phosphorous content of 59.70, 57.86, 57.80 and 56.49 kg ha^{-1} respectively. The absolute control recorded a phosphorous content of 26.13 kg ha^{-1}.

Table 29. Effect of different treatments on Leaf Area Index, number of leaves and oxalate content of amaranthus at harvest

Treatment	Leaf Area Index	Number of Leaves	Oxalate Content (%)
T_1-Chemical fertilizer @ 100% NPK	3.98^{b}	32.00^{bc}	0.42^{cde}
T_2-Talc based formulation of PGPR mix–I alone	3.14^{c}	29.55^{c}	0.48^{bcb}
T_3-Talc based formulation of PGPR mix–I + 100% NPK	4.94^{a}	40.11^{ab}	0.47^{bcd}
T_4-Talc based formulation of PGPR mix–I + 50% NPK	3.72^{bc}	33.44^{bc}	0.52^{ab}
T_5-Liquid biofertilizer formulation of PGPR mix–I alone	3.17^{bc}	30.11^{c}	0.57^{a}
T_6-Liquid biofertilizer formulation of PGPR mix–I +100% NPK	4.97^{a}	44.44^{a}	0.39^{e}
T_7-Liquid biofertilizer formulation of PGPR mix–I + 50% NPK	3.88^{bc}	31.55^{c}	0.45^{cde}
T_8-Control with additives without PGPR microorganisms	1.83^{d}	18.44^{d}	0.41^{de}
T_9-Absolute control	2.08^{d}	18.77^{d}	0.39^{e}
SE(m)	0.283	2.810	0.021
CD(0.05)	0.828	8.329	0.062

*Mean of 3 observations

Figures in a column followed by same letters do not differ significantly at $P \geq 0.05$

The result indicated that potassium content (1012.10kg ha^{-1}) in the treatment T_6 was significantly superior to all other treatments, whereas the absolute control recorded a potassium content of 363.17kg ha^{-1} (Table 31).

4.6.11 Population of *Azospirillum lipoferum*, *Azotobacter chroococcum*, *Bacillus megaterium* and *Bacillus sporothermodurans* in Soil at Harvest (log cfu g^{-1})

The population of *Azospirillum lipoferum* was maximum in treatment liquid biofertilizer formulation of PGPR mix–I+ 50% NPK (1.30×10^8 cfu ml^{-1}) which was statistically on par with liquid biofertilizer formulation of PGPR mix–I alone (1.07×10^8 cfu ml^{-1}) (Plate 16). However, maximum population of *Azotobacter chroococcum* was observed in liquid biofertilizer formulation of PGPR mix–I alone (1.03×10^8 cfu ml^{-1}) which was statistically on par with the treatments liquid biofertilizer formulation of PGPR mix–I+ 50% NPK (7.77×10^7 cfu ml^{-1}), talc based formulation of PGPR mix-I alone (7.17×10^7 cfu ml^{-1}), liquid biofertilizer formulation of PGPR mix–I+ 100% NPK (7.16×10^7). Population of phosphorous solubilizer, *Bacillus megaterium* was maximum in liquid biofertilizer formulation of PGPR mix–I alone (9.33×10^7 cfu ml^{-1}) which was statistically on par with treatments liquid biofertilizer formulation of PGPR mix–I+ 50% NPK (7.77×10^7 cfu ml^{-1}) and liquid biofertilizer formulation of PGPR mix–I+ 100% NPK (7.16×10^7 cfu ml^{-1}). Maximum population of potassium solubilizer, *Bacillus sporothermodurans* was observed in treatment liquid biofertilizer formulation of PGPR mix–I alone (1.03×10^8 cfu ml^{-1}) which was statistically on par with the treatments liquid biofertilizer formulation of PGPR mix–I+ 100% NPK (9.33×10^7 cfu ml^{-1}) and liquid biofertilizer formulation of PGPR mix–I+ 50% NPK (9.08×10^7 cfu ml^{-1}) (Table 32).

Table 30. Initial available N, P and K content of the experimental soil

Sample	Available N (kg ha^{-1})	Available P (kg ha^{-1})	Available K (kg ha^{-1})
Initial available NPK	238.48	27.4	856.91

Table 31. Effect of different treatments on final available soil N, P and K content

TREATMENT	N (kg ha^{-1})	P (kg ha^{-1})	K (kg ha^{-1})
T_1- Chemical fertilizer @ 100% NPK	275.96^{a}	56.49^{ab}	351.08^{e}
T_2- Talc based formulation of PGPR mix–I alone	213.24^{bc}	44.8^{b}	301.39^{f}
T_3- Talc based formulation of PGPR mix–I + 100% NPK	269.69^{a}	59.7^{a}	451.74^{d}
T_4- Talc based formulation of PGPR mix–I + 50% NPK	200.70^{cd}	57.86^{a}	360.08^{e}
T_5- Liquid biofertilizer formulation of PGPR mix–I alone	213.66^{bc}	44.8^{b}	851.68^{b}
T_6- Liquid biofertilizer formulation of PGPR mix–I+ 100% NPK	288.51^{a}	61.6^{a}	1012.10^{a}
T_7- Liquid biofertilizer formulation of PGPR mix–I+ 50%	238.33^{b}	57.8^{a}	868.52^{b}
T_8- Control with additives without PGPR microorganisms	181.05^{d}	24.2^{c}	562.01^{c}
T_9- Absolute control	$194.43c^{d}$	26.13^{c}	363.17^{e}
SEm (±)	11.290	4.190	32.100
CD(0.05)	33.800	12.359	96.103

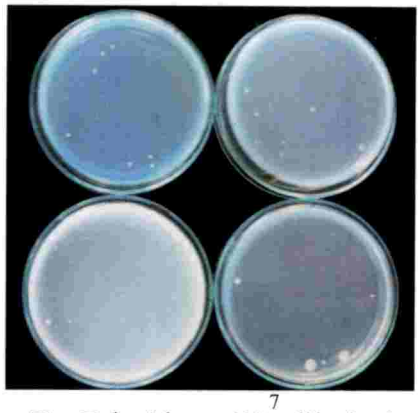
Figures in a column followed by same letters do not differ significantly at P ≥0.05

Table 32. Effect of different treatments on population of *Azospirillum lipoferum, Azotobacter chroococcum, Bacillus megaterium* and *Bacillus sporothermodurans* in soil at harvest (log cfu g^{-1})

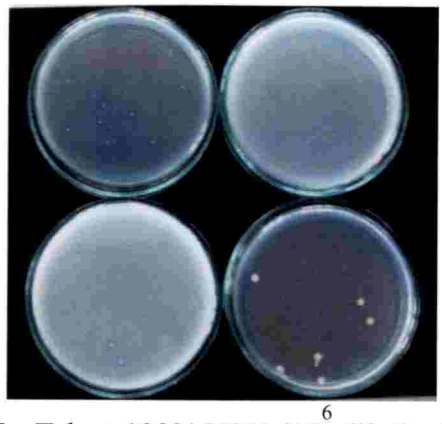
Treatments	*Azospirillum lipoferum*	*Azotobacter chroococcm*	*Bacilus megaterium*	*Bacillus sporothermodurans*
Talc based formulation of PGPR mix–I alone	7.57^{c}	7.85^{a}	7.71^{b}	7.73^{b}
Talc based formulation of PGPR mix–I + 100% NPK	6.96^{d}	6.97^{b}	6.93^{c}	6.96^{c}
Talc based formulation of PGPR mix–I + 50% NPK	6.73^{e}	6.49^{c}	6.67^{d}	6.80^{c}
Liquid biofertilizer formulation of PGPR mix–I alone	8.02^{ab}	8.01^{a}	7.96^{a}	8.01^{a}
Liquid biofertilizer formulation of PGPR mix–I+ 100% NPK	7.92^{b}	7.85^{a}	7.85^{ab}	7.98^{a}
Liquid biofertilizer formulation of PGPR mix–I+ 50%	8.11^{a}	7.87^{a}	7.88^{a}	7.95^{a}
SEm (±)	0.041	0.052	0.056	0.055
CD (0.05)	0.125	0.160	0.172	0.170

Each value represents a mean of 3 replication

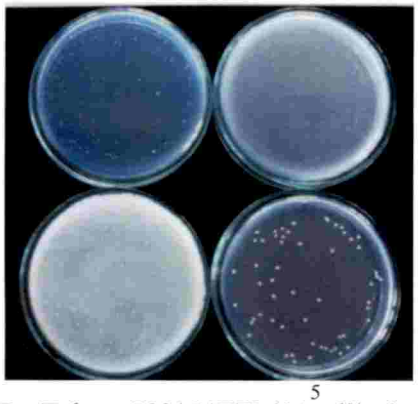
Figures in a column followed by same letters do not differ significantly at P ≥0.05



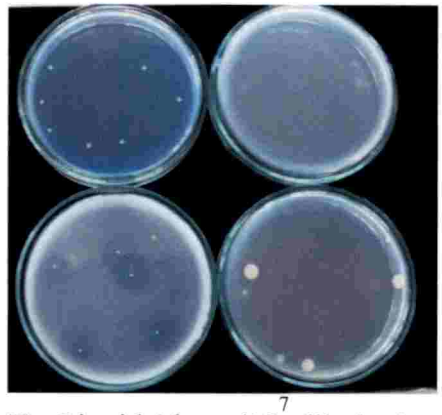
T_2- Talc Alone (10^{7} dilution)



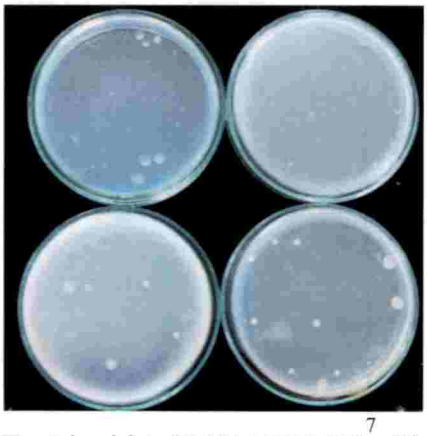
T_3- Talc + 100% NPK (10^{6} dilution)



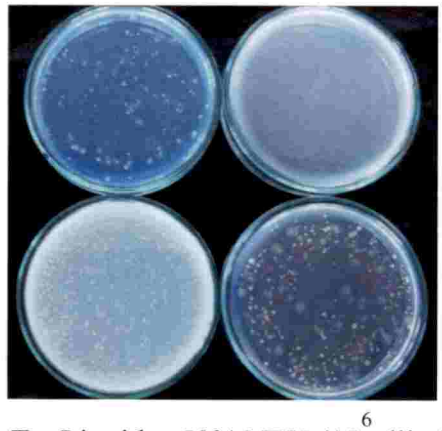
T_4- Talc + 50% NPK (10^{5} dilution)



T_5- Liquid Alone (10^{7} dilution)



T_6- Liquid + 100% NPK (10^{7} dilution)



T_7- Liquid + 50% NPK (10^{6} dilution)

Plate 16. Effect of different treatments on population of *Azospirillum lipoferum, Azotobacter chroococcum*, *Bacillus megaterium* and *Bacillus sporothermodurans* in soil at harvest (log cfu g^{-1})

DISCUSSION

5. DISCUSSION

Excessive use of chemical fertilizers to increase production from available land has resulted in deterioration of soil quality. The injudicious application of chemical fertilizers is not only costly but also hazardous for both environment and humans. Moreover, manufacturing of such fertilizers causes depletion of non renewable natural resources. Hence, a viable alternative for chemical fertilizers that will efficiently provide nitrogen, phosphorous, potassium and various phytohormones to plants and prevent the depletion of soil fertility and soil quality is highly essential. To prevent further soil deterioration, the use of beneficial microbes which have the ability to colonize different habitats, including soil, sediment, water and both epiphytes and endophytes as host plants, has been suggested for sustainable agriculture.

Plant growth promoting rhizobacteria (PGPR) are beneficial bacteria which have the ability to colonize the roots and either promotes plant growth through direct action or via biological control of plant diseases (Kloepper and Schroth, 1978). This group of bacteria plays a significant role in the biogeochemical cycle in soil ecosystems, ultimately fortifying plants and sustaining agriculture. PGPR include strains in the genera *Serratia, Pseudomonas, Burkholderia, Agrobacterium, Xanthomonas, Azospirillum, Bacillus, Enterobacter, Rhizobium, Arthrobacter, Acetobacter, Acinetobacter, Achromobacter, Aerobacter, Artrobacter, Azotobacter, Micrococcus, Rhodospirrilum, Flavobacterium* etc. (Rodriguez and Fraga, 1999; Bloemberg and Lugtenberg, 2001; Esitken *et al*., 2003).

Among the different PGPRs, *Azospirillum lipoferum* is the most efficient plant growth promoter which is capable of improving growth and yield of several plant species due to its ability to produce various phytohormones (Dobbelaere *et al.*, 2001). Many reports suggest that *Azotobacter chroococcum,* a plant growth promoting rhizobacterium, can act as efficient plant growth promoter. Increased germination and growth of seedlings of tomato and cucumber has been reported

due to the presence of *Azotobacter chroococcum* in the rhizosphere of plants (Eklund, 1970). Several studies proved the efficiency of P solubilizer of plant rhizosphere and could be better exploited for sustainable agriculture. Meenakumari *et al.*, (2008) revealed the high efficiency of P solubilizers in releasing the soil phosphorous. They have been reported to influence plant biochemical properties in Amaranthus (Sakthidharan, 2011). These effects might be mediated by the production of beta carotene, vitamin C and crude protein.

It is reported that a variety of rhizobacteria have been found to solubilize silicate from insoluble mineral source, thereby increasing the availability to crop plants (Bunt and Rovira, 1955). Hu *et al.* (2006) reported that *Bacillus megatherium* and *B. mucilaginosus* were capable of solubilizing both rock phosphate and potassium. They also reported that co-inoculation of these two *Bacillus* species were potential in solubilizing potassium rocks. Greater release of K from muscovite by *Bacillus muciloginosus* was documented by Sugumaran and Janarthanam (2007). Use of these potential agents can enhance crop growth and productivity without losing soil quality.

Bioinoculants have been used as a commercial alternative to chemical fertilizers to reduce environmental effects and diseases and promote plant growth. Bioinoculants are microbial preparations of a single or consortia of living microorganisms. Because of importance in the rhizosphere, potential for plant growth promotion and substituting chemical fertilizers, PGPR have gained great attention as bioinoculants for use in agriculture. PGPR mix-I is a talc based consortium of efficient NPK biofertilizer organisms such as *Azospirillum lipoferum* and *Azotobacter chroococcum* (Nitrogen fixers), *Bacillus megaterium* (P solubilizer) and *Bacillus sporothermodurans* (K solubilizer) developed by Kerala Agricultural University which has been widely accepted by the farmers of Kerala (KAU, 2017). Several studies in KAU have established the positive effect of talc based formulation of PGPR mix-I in various crops (Akshay, 2011; Raj *et al.*, 2012; Sathyan, 2013; Yadav, 2013; Mohanan, 2016). Many of the recent works, however, reported advantages of liquid based biofertilizer formulations

over carrier based biofertilizers. Considering the merits of liquid based formulation over carrier based one, the present investigation was designed to standardize the liquid formulation of PGPR mix–I and its evaluation for plant growth promotion along with saving of chemical fertilizers in Amaranthus. The results obtained on these investigations are discussed hereunder.

In the present investigation it was found that all the component cultures of PGPR mix-I produced significant quantity of IAA under *in vitro* conditions. The nitrogen fixers, *Azospirillum lipoferum and Azotobacter chroococcum* produced 40.31 and 36.43 ppm of IAA respectively, whereas *Bacillus megaterium* and *Bacillus sporothermodurans* produced 1.28 ppm and 3.36 ppm of IAA respectively. The results were in agreement with the findings of Crozier *et al.*, (1988) who analyzed the IAA production of 20 strains of *Azospirillum* isolated from roots of maize and teosinte by colorimetric Salkowski assay. The Salkowski assay suggested that the culture medium of *Azospirillum* contained 1.0 to 26.1 µg ml^{-1} of IAA. The production of IAA by *Azospirillum* sp isolated from chilli roots under *in vitro conditions* ranged between 21 and 55 µg ml^{-1} (Kavitha, 2001). Bashan *et al.*, (2008) reported the production of IAA by wild type *Azospirillum* spp. in promoting growth of the microalga *Chlorella vulgaris* Beij. The quantity of IAA produced ranged from 13.0 to 126.6 µg ml^{-1} under *in vitro* conditions. Similar results were also obtained by Meenakumari *et al.*, (2018) who isolated 25 isolates of *Azospirillum* and 12 isolates of *Azotobacter* from the soil samples collected from undisturbed forest areas of Attappady hill tracts. The IAA production of *Azospirillum* sp and *Azotobacter* sp ranged from 14.83 to 49.74 µg ml^{-1} of culture filtrate and 28.95 to 49.81 µg ml^{-1} of culture filtrate respectively.

In the present study, the nitrogen fixers, *Azospirillum lipoferum and Azotobacter chroococcum* of PGPR mix-I were subsequently tested for the ability to fix nitrogen under *in vitro* conditions. The *in vitro* estimation recorded a significant quantity of 21 and 14 mg N g^{-1} of carbon source respectively.

The quantity of nitrogen fixed by *Azospirillum lipoferum and Azotobacter chroococcum* were similar to that obtained by Rao *et al.*, (1984) who estimated the range of N_2 fixation by *Azospirillum* isolated from soil samples and it ranged from 2.0 to 14.0 mg g^{-1} of malate. Nitrogen fixation capacity of *Azotobacter* spp. strains inoculated with clayey soil, loam soil, and sandy clay loam soil during eight week incubation period were 4.78-15.91 µg N g^{-1} of carbon source ,9.03- 13.47 µg N g^{-1} of carbon source and 6.51-16.60 µg N g^{-1} of carbon source respectively (Kizilkaya, 2009). Meenakumari (2018) reported the nitrogen fixing capacity of *Azospirillum* sp and *Azotobacter* sp isolated from soil samples collected from undisturbed forest areas of Attappady hill tracts which ranged between 10.38 to 19.97 mg N g^{-1} of carbon source and 11.40 to 15.31 mg N g^{-1} of carbon source respectively. The results were also in line with several other studies (Okon *et al.*, 1976; Sasikumar, 1996; Kavitha, 2001).

In addition to nitrogen fixation by plant growth promoting rhizobacteria, phosphorous and potassium solubilization are other important desirable properties as it plays an important role in plant growth promotion. Hence, the present study assessed the quantitative solubilization of P by *Bacillus megaterium* present in PGPR mix–I under *in vitro* conditions.

Selvi *et al.*, (2011) isolated *Bacillus* sp from rhizosphere soil sample of leguminous plant. The isolated bacterium was used to test for solubilization of insoluble inorganic phosphate sources under *in vitro* conditions. The release of P from insoluble inorganic phosphate sources by the isolated bacteria recorded a maximum quantity of 69.26 mg P_2O_5 from KH_2PO_4. Karpagam and Nagalakshmi (2014) isolated a total of 37 phosphate solubilizing microbial colonies on the Pikovaskaya's agar medium, containing insoluble tricalcium phosphate (TCP) from agricultural soil and the colonies showing clear halo zones around the microbial growth were considered as phosphate solubilization. The maximum clearing zone produced by *Bacillus* sp recorded 2.3 cm in diameter.

All the above mentioned studies strongly support the results of this study which recorded a solubilization of 69.36 ppm of phosphorous, whereas qualitative assessment recorded a clearing zone of 8 mm and 12 mm diameter in NBRIP and Pikovaskaya's medium respectively. The NBRIP and Pikovaskaya's medium contain tricalcium phosphate as the phosphorus source. The release of P from insoluble inorganic phosphate could be due to the release of organic acid anions, phosphatase, phytase, C–P lyase and protons hydroxyl ions (Kwak *et al.*, 2014).

In the present investigation the results of *in vitro* assessment of K solubilization by *Bacillus sporothermodurans* indicated the high efficiency of the bacterium to solubilize insoluble inorganic potassium. The amount of potassium solubilized by *Bacillus sporothermodurans* was 12.18 ppm, and it also produced a clearing zone of 18 mm diameter in Glucose Yeast Agar medium. The ability of K solubilizers to solubilize insoluble inorganic potassium could be due to their inherent ability to release organic acids.

Similar findings were also noticed by Archana (2013) who isolated potassium solubilizing bacteria from rhizosphere soil of different crops from Dharwad and Belgaum districts. These isolates were tested for K solubilization and the quantity of K released ranged from 2.41 to 44.49 g mL^{-1}. The study on K solubilization ability of rhizospheric bacteria by Prajapati and Modi (2012) reported that out of 14 isolates, 5 bacterial strains exhibited highest potassium solubilization on solid medium and the diameter of clearing zone ranged from 6 to 13 mm.

The present investigation also confirmed the genus of the component cultures of PGPR mix-I based on morphological and biochemical characterization. The results of both morphological and biochemical characterization indicated the differential ability of the bacteria in the utilization of different nutrient sources. All the isolates in PGPR mix–I was motile and they varied in morphological characteristics such as colony morphology, size, margin, texture, colour and cell shape. The nitrogen fixers, *Azospirillum lipoferum* and *Azotobacter chroococcum*

stained Gram negative, whereas *Bacillus megaterium* and *Bacillus sporothermodurans* stained Gram positive. For further characterization, these four isolates were subjected to a series of biochemical tests. The results of various biochemical tests supported the genus level identification of the isolates.

Similar results on morphological and biochemical characterization of *Azospirillum lipoferum* were obtained by Ilyas *et al.,* (2012), Pandiarajan (2012) and Hossain *et al.,* (2015), whereas similar observations on characterization of *Azotobacter* and *Bacillus* sp were reported by Prajapati and Modi (2012), Parmar and Sindhu (2013) and Vijendrakumar *et al.* (2014).

The potential PGPR isolates are formulated using different organic and inorganic carriers either through solid or liquid fermentation technologies. Talc, a widely used carrier material, owing to its inert nature and easy availability as raw material from soapstone industries it is used as a carrier for formulation development. Kloepper and Schroth (1981) demonstrated the potentiality of talc to be used as a carrier for formulating rhizobacteria. Vendan and Thangaraju (2006) reported that solid carrier based preparations generally suffer from short shelf-life, poor quality, high contamination and low and unpredictable field performances.

Talc based powder formulations which have shorter shelf-life and reduced efficacy during longer storage periods necessitates the development of alternate formulations with longer shelf life. Further, the application of talc based bioformulations through micro irrigation techniques encountered problems such as blockage of nozzles and uneven distribution of bio-inoculants. Considering the demerits of carrier based bioformulations many researches have already been conducted investigations on development of alternative formulations such as liquid formulation of biofertilizers using different additives (Lorda and Balatti, 1996; Sridhar *et al.*, 2004; Santhosh, 2015; Velineni and Brahmaprakash, 2011; Gopal and Baby, 2016).

Liquid biofertilizers have the capacity to replace carrier based biofertilizers and plays a major role in restoring the soil health. Liquid biofertilizers are special liquid formulation containing not only the desired microorganisms and their nutrients, but also special cell protectants or substances that encourage formation of resting spores or cysts for longer shelf life and tolerance to adverse conditions. The development of liquid formulation has several advantages including high cell count, zero contamination, longer shelf life, greater protection against environmental stresses and increased field efficacy (Vendan and Thangaraju, 2006). Liquid cultures containing cell protectants not only maintain high microbial numbers but also promote the formation of resting cells such as, cysts and spores which offer higher resistance to abiotic stresses, thus increasing the survivability of bacteria. The cell protectants like glycerol, polyvinyl pyrrolidone, polyethylene glycol, gum arabic, sodium alginate etc. were found to have considerable effect on shelf life and protection against environmental stresses (Santhosh, 2015).

Hence, the present investigation focused to standardize the protocol for the preparation of liquid formulation of PGPR mix–I in completely randomized design with different amendments such as 2% Glycerol, 2% Polyvinylpyrrolidone, 15mM Trehalose, 1% Glycerol + 1% PVP and a combination of Glycerol (2%), Trehalose (1%), Yeast extract (1%), PVP (1%) and Proline (1%).

It was observed that on zeroth day of inoculation total viable count of all bacterial cultures was maximum in formulation amended with Glycerol (2%) (Figure 1). This result was in line with the study of Lorda and Balatti (1996) who reported greater number of *Azospirillum* cells in 10mM glycerol amended medium which may be due to its high water holding capacity and protect the cells from the effect of dessication by reducing the rate of drying. Similarly, Sridhar *et al.*, (2004) found that PVP, glycerol and glucose amended liquid medium of *Bacillus megaterium* supported higher viable population and endospores up to 6 months storage period. Furthermore, Velineni and Brahmaprakash (2011) reported that liquid formulation supplemented with PVP and glycerol supported higher viable

population of *Bacillus megaterium* up to a period of four weeks. Anith *et al.*, (2016) observed that the rate of decline in population of *Pseudomonas fluorescens* AMB-8 was less in coconut water amended with PVP (2% w/v) and glycerol (2% v/v) compared to nutrient broth (NB) and King's B broth (KB) with different amendments during six months of storage.

It was interesting to note that in the present investigation great variation could be observed in the total viable count of component cultures of PGPR mix-I in different treatments till eighth month (Figure 2 to 9). However, from ninth month onwards, formulation amended with 15mM Trehalose exhibited maximum viable count until fourteenth month consistently (Figure 10 to 15). Enhanced survival of cells in the liquid formulation may be due to the action of chemical amendments added in the medium. Trehalose is a disaccharide which is capable of enhancing cell tolerance to desiccation, osmotic pressure and temperature stress. The possible effect of trehalose's protective action may be due to induced synthesis of metabolites that protect against stress. From this study, it has been concluded that among the different chemical additives trehalose (15mM) performed best (Figure 16). Moreover, the survival of bacterial cultures in talc based formulation was found to decrease gradually in each month. A significant decline of total viable population in talc based formulation was observed in each month compared to 15mM Trehalose amended formulation up to fourteenth month. Since a significant total viable count was observed in 15mM Trehalose amended liquid formulation even after fourteen months, the survival study is being continued (Figure 17).

The results of the present study was also in agreement with the study of Kumaresan and Reetha (2011) who reported that liquid *Azospirillum* bioinoculant formulated with trehalose (10mM) promoted long term survival of Azospirillum compared to glycerol (10 mM), gum arabica (0.3%) and PVP (2%) and they supported 10^8 cells ml^{-1} up to 11 months of storage under ambient temperature (28°C to 32°C). In confirmation with the study Neta *et al.*, (2012) reported that trehalose, gum Arabica and PEG (300) provided better protective effects for

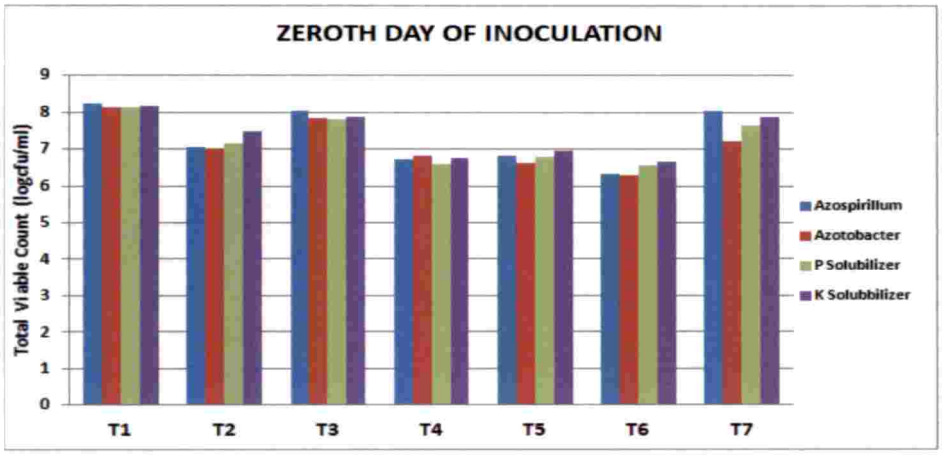


Fig. 1. Population of component organisms of PGPR mix-1 in liquid formulation with different additives on the day of inoculation

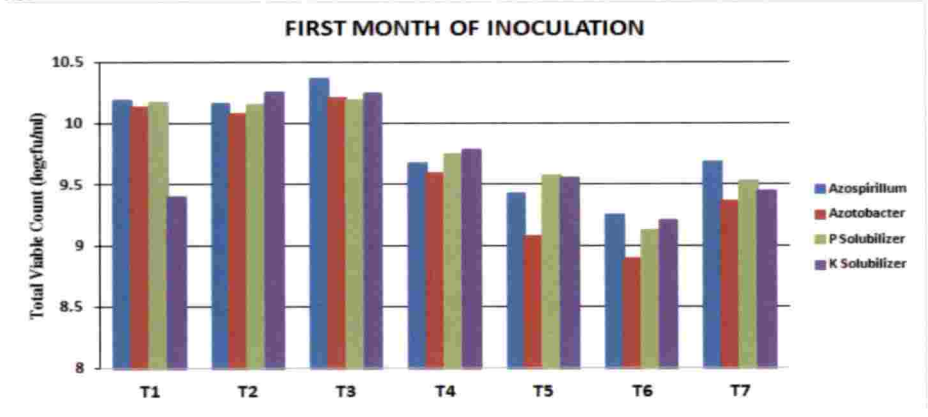


Fig. 2. Population of component organisms of PGPR mix-1 in liquid formulation with different additives one month after inoculation

T1- 2 % Glycerol **T2**- 2 % poly vinyl pyrrolidone **T3**–15 mM Trehalose **T4**–1 % Glycerol and 1 % PVP

T5-Glycerol (2%), Trehalose (1%), Yeast Extract (1%), PVP (1%) and Proline (1%)

T6- Control without any additives **T7**- Talc based formulation

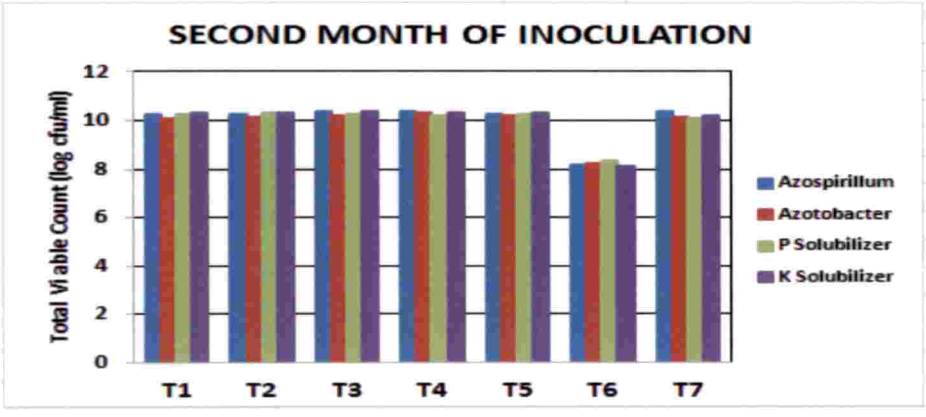


Fig. 3. Population of component organisms of PGPR mix-1 in liquid formulation with different additives two months after inoculation

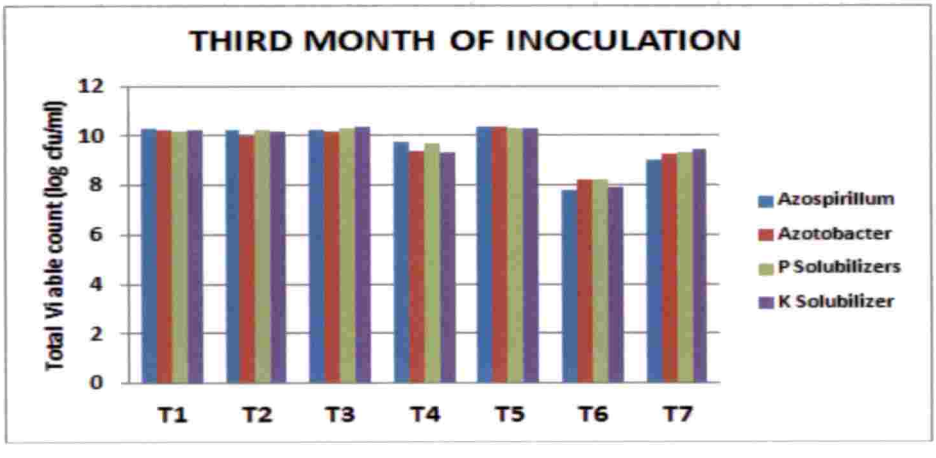


Fig. 4. Population of component organisms of PGPR mix-1 in liquid formulation with different additives three months after inoculation

T1- 2 % Glycerol **T2**- 2 % poly vinyl pyrrolidone **T3**–15 mM Trehalose **T4**–1 % Glycerol and 1 % PVP

T5-Glycerol (2%), Trehalose (1%), Yeast Extract (1%), PVP (1%) and Proline (1%)

T6- Control without any additives **T7**- Talc based formulation

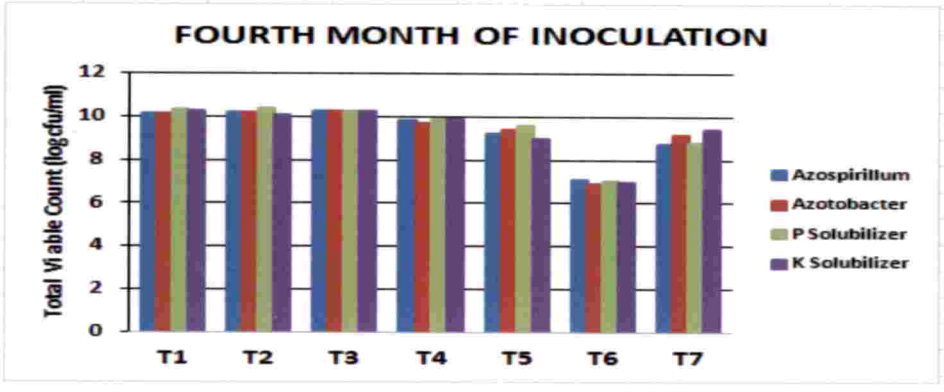


Fig. 5. Population of component organisms of PGPR mix-1 in liquid formulation with different additives four months after inoculation

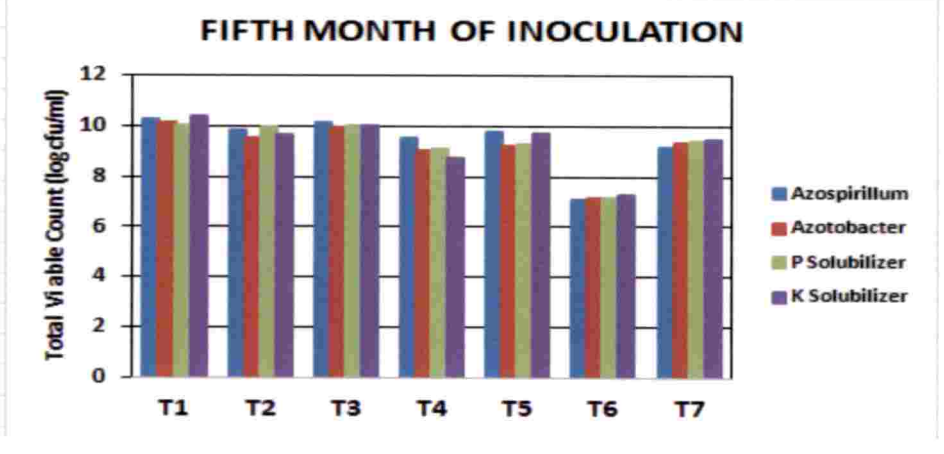


Fig. 6. Population of component organisms of PGPR mix-1 in liquid formulation with different additives five months after inoculation

T1- 2 % Glycerol **T2**- 2 % poly vinyl pyrrolidone **T3**–15 mM Trehalose **T4**–1 % Glycerol and 1 % PVP

T5-Glycerol (2%), Trehalose (1%), Yeast Extract (1%), PVP (1%) and Proline (1%)

T6- Control without any additives **T7**- Talc based formulation

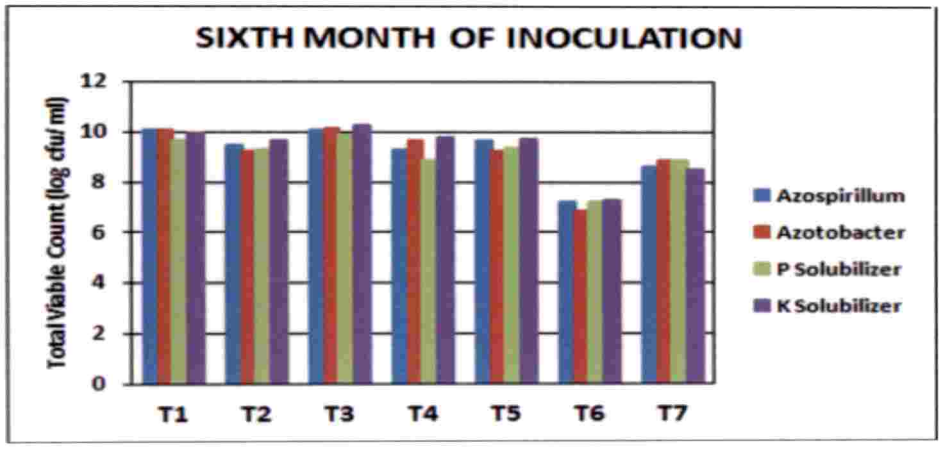


Fig. 7. Population of component organisms of PGPR mix-1 in liquid formulation with different additives six months after inoculation

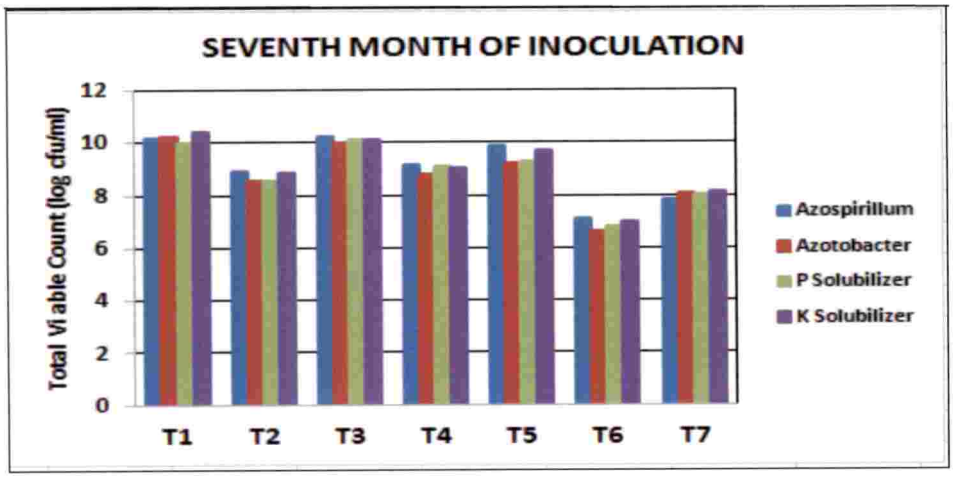


Fig. 8. Population of component organisms of PGPR mix-1 in liquid formulation with different additives seven months after inoculation

T1- 2 % Glycerol **T2**- 2 % poly vinyl pyrrolidone **T3**–15 mM Trehalose **T4**–1 % Glycerol and 1 % PVP

T5-Glycerol (2%), Trehalose (1%), Yeast Extract (1%), PVP (1%) and Proline (1%)

T6- Control without any additives **T7**- Talc based formulation

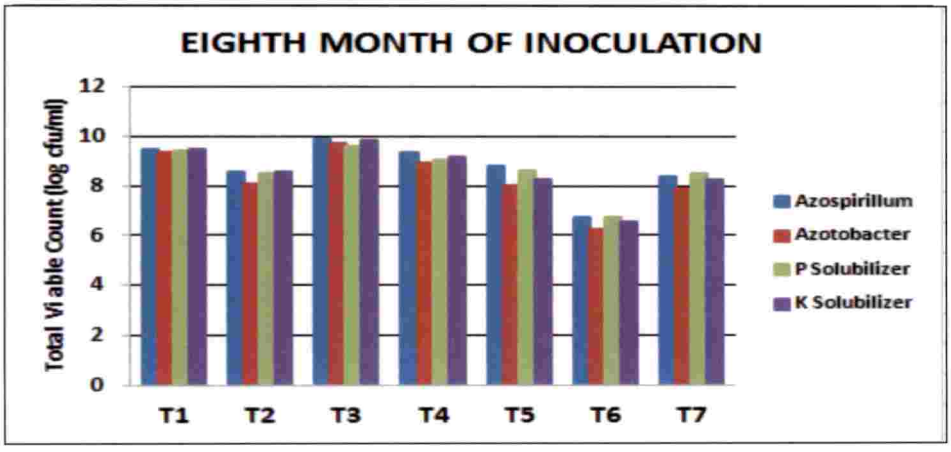


Fig. 9. Population of component organisms of PGPR mix-1 in liquid formulation with different additives eight months after inoculation

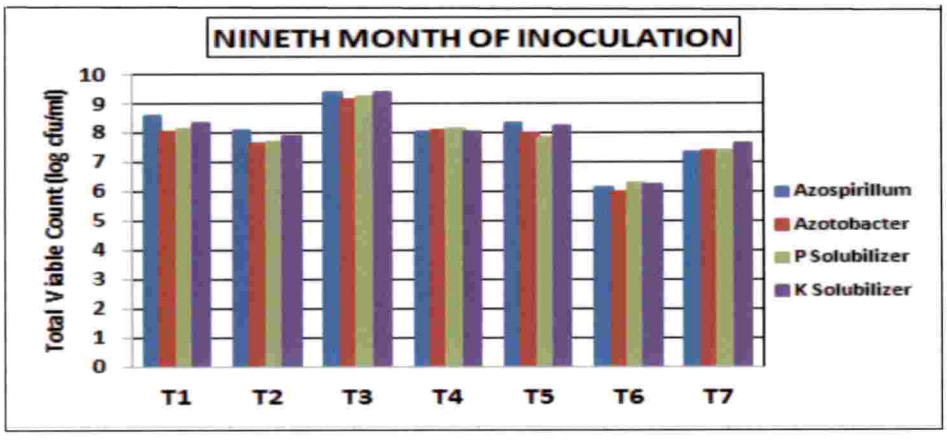


Fig. 10. Population of component organisms of PGPR mix-1 in liquid formulation with different additives nine months after inoculation

T1- 2 % Glycerol **T2**- 2 % poly vinyl pyrrolidone **T3**–15 mM Trehalose **T4**–1 % Glycerol and 1 % PVP

T5-Glycerol (2%), Trehalose (1%), Yeast Extract (1%), PVP (1%) and Proline (1%)

T6- Control without any additives **T7**- Talc based formulation

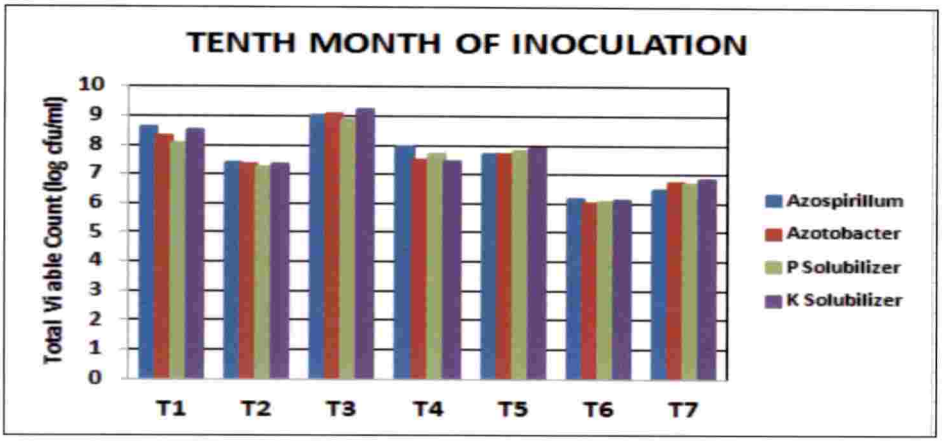


Fig. 11. Population of component organisms of PGPR mix-1 in liquid formulation with different additives ten months after inoculation

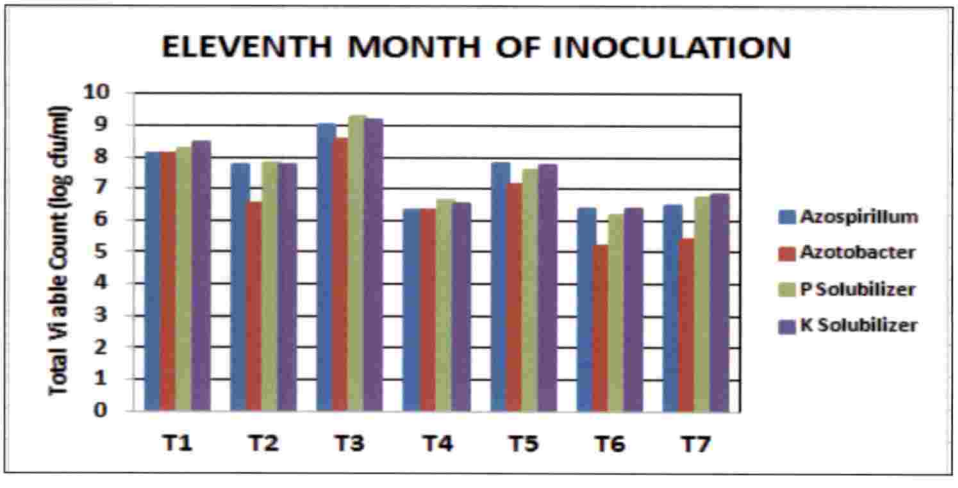


Fig. 12. Population of component organisms of PGPR mix-1 in liquid formulation with different additives eleven months after inoculation

T1- 2 % Glycerol **T2**- 2 % poly vinyl pyrrolidone **T3**–15 mM Trehalose **T4**–1 % Glycerol and 1 % PVP

T5-Glycerol (2%), Trehalose (1%), Yeast Extract (1%), PVP (1%) and Proline (1%)

T6- Control without any additives **T7**- Talc based formulation

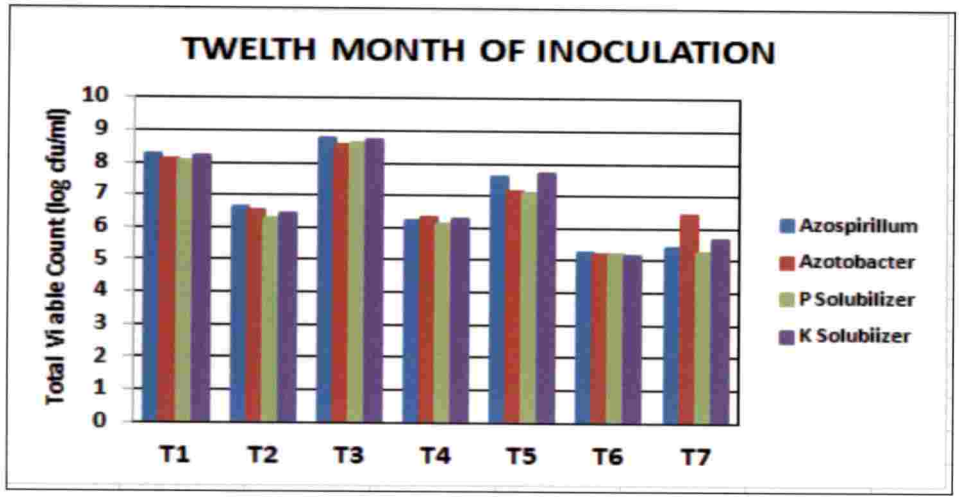


Fig. 13. Population of component organisms of PGPR mix-1 in liquid formulation with different additives twelve months after inoculation

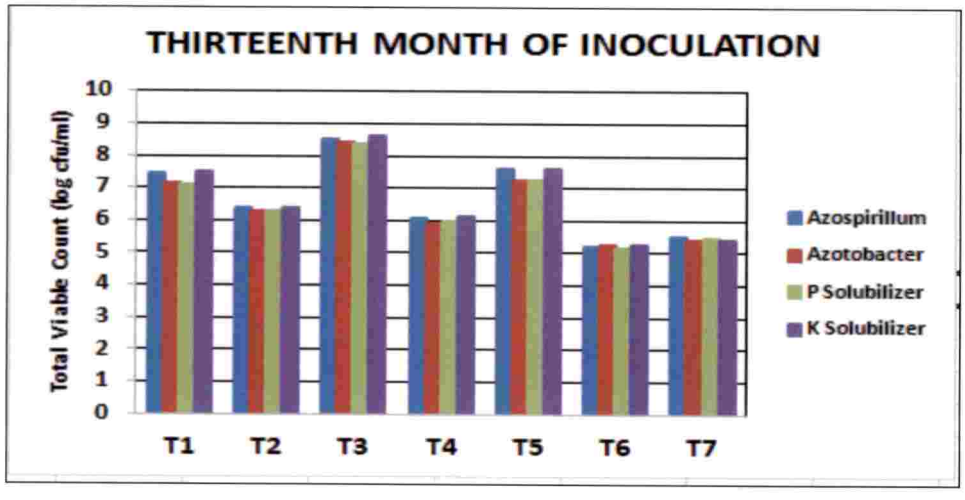


Fig. 14. Population of component organisms of PGPR mix-1 in liquid formulation with different additives thirteen months after inoculation

T1- 2 % Glycerol **T2**- 2 % poly vinyl pyrrolidone **T3**–15 mM Trehalose **T4**–1 % Glycerol and 1 % PVP

T5-Glycerol (2%), Trehalose (1%), Yeast Extract (1%), PVP (1%) and Proline (1%)

T6- Control without any additives **T7**- Talc based formulation

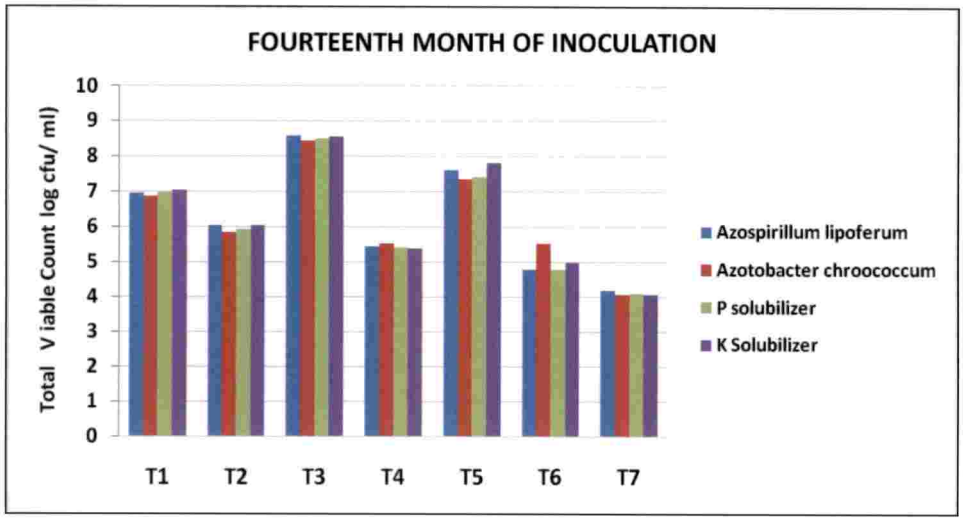


Fig. 15. Population of component organisms of PGPR mix-1 in liquid formulation with different additives fourteen months after inoculation

T1- 2 % Glycerol **T2**- 2 % poly vinyl pyrrolidone **T3**–15 mM Trehalose **T4**–1 % Glycerol and 1 % PVP

T5-Glycerol (2%), Trehalose (1%), Yeast Extract (1%), PVP (1%) and Proline (1%)

T6- Control without any additives **T7**- Talc based formulation

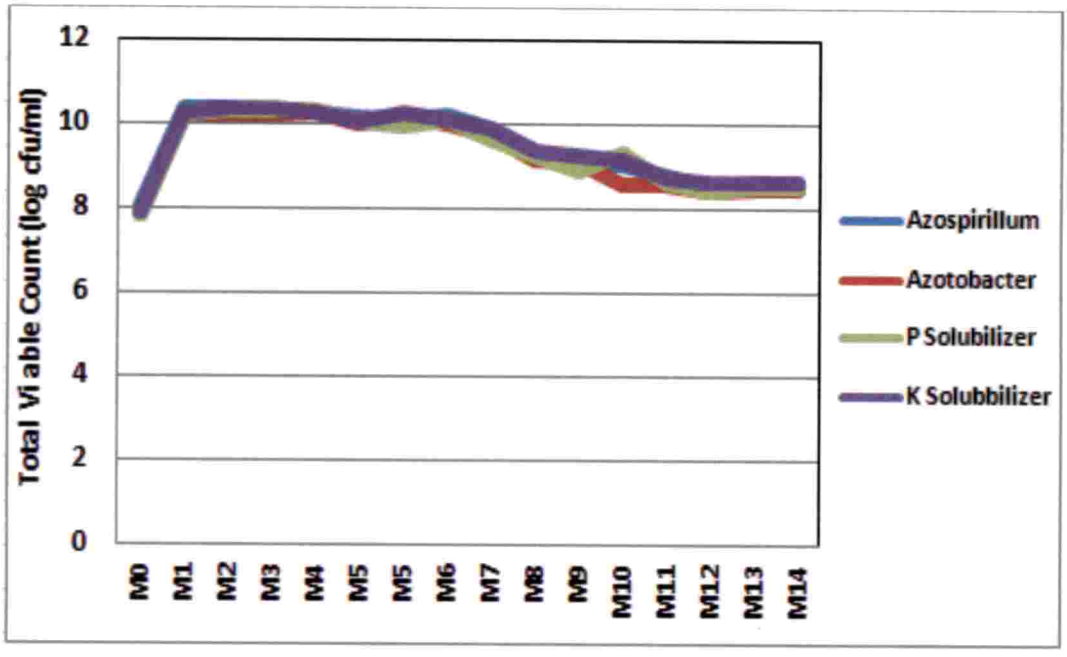


Fig. 16. Performance of the best treatment (15mm trehalose) at monthly intervals

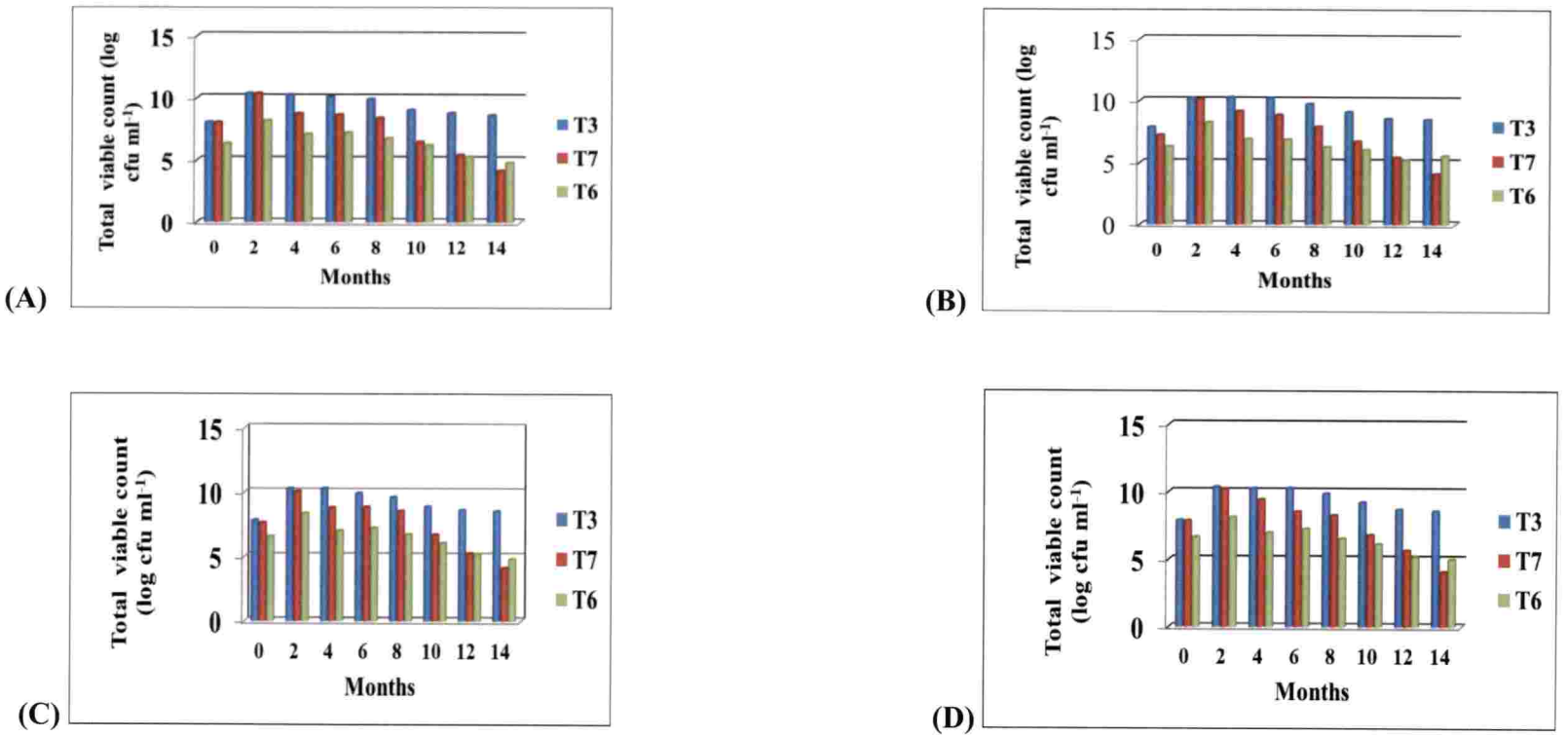


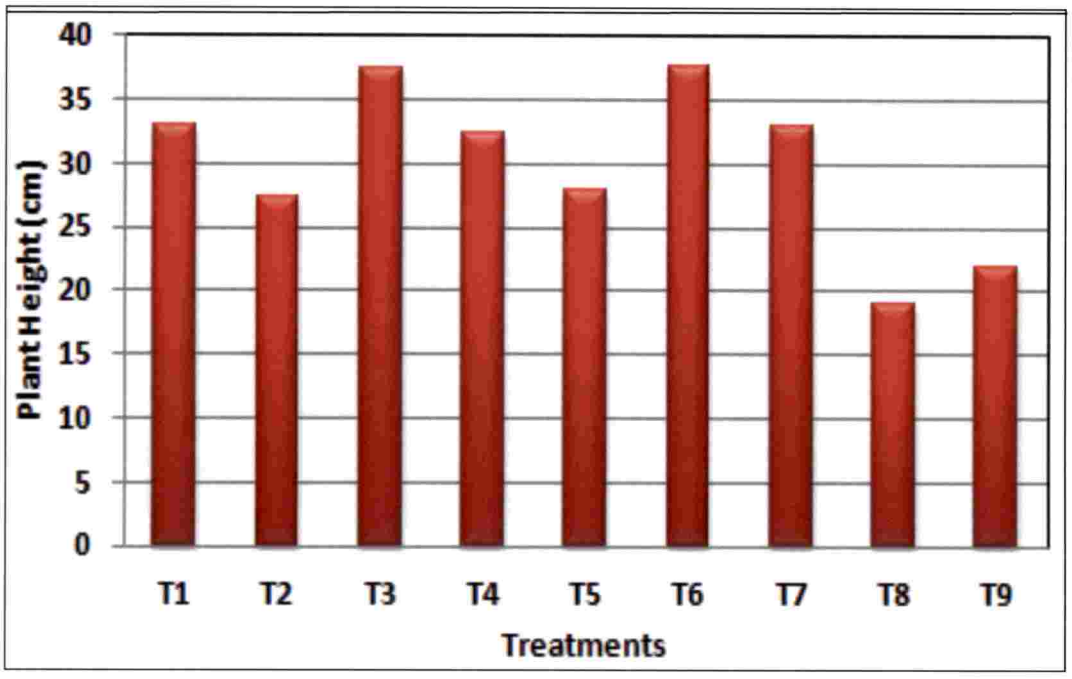
Fig. 17. Population of **(A)** *Azospirillum lipoferum,* **(B)** *Azotobacter chroococcum,* **(C)** *Bacillus megaterium* and **(D)** *Bacillus sporothermodurans* in liquid formulation with different additives at bimonthly intervals (log cfu ml^{-1})

Acetobacter diazotrophicus than other protective substances. Gopal and Baby (2016) could standardize liquid formulation for *Azospirillum* (KAU isolate) and phosphate solubilizing bacteria (KAU isolate) along with chemical amendments. The highest population of *Azospirillum* (1.77×10^8 cfu ml^{-1}) was recorded in the case of trehalose (15 mM) amended medium. Manimekalai and Kannahi (2018) studied the effect of four different cell protective substances and selected trehalose (1%) as the potential additive because it could maintain a relatively high population and conferred greater microbial vitality. Similar results on effect of trehalose as cell protectant in liquid bioformulations was obtained by Karunya and Reetha (2014) and Gupta *et al.*, (2016).

All the above mentioned reviews and the present study strongly support the positive effect of liquid formulation amended with trehalose over talc based formulation.

Hence in the present investigation on standardization of the protocol for the preparation of liquid formulation of PGPR mix–I with different chemical additives, trehalose (15mM) was adjudged as the best additive. In order to test the efficiency of the developed liquid formulation amended with trehalose (15mM) a pot culture experiment was conducted with amaranthus as the test crop.

In the pot culture study, preliminary observations on plant height at 20 DAT indicated that liquid biofertilizer formulation of PGPR mix–I+ 100% NPK was the superior treatment over other treatments. Furthermore, the same treatment recorded maximum plant height, shoot fresh weight, leaf area index, number of leaves, dry weight of shoot, fresh weight of root and dry weight of root of 7.54 cm, 55.41 g $plant^{-1}$, 4.97, 44.44, 4.97 g $plant^{-1}$, 6.60 g $plant^{-1}$ and 0.56 g $plant^{-1}$ respectively at harvest also (Figure 18 and 19). The treatment, liquid biofertilizer formulation of PGPR mix–I+ 100% NPK also recorded the least oxalate content of 0.39 per cent similar to control plants. Low levels of oxalate content are often a highly desirable character in amaranthus. Despite of great accumulation of anti-nutrients such as oxalates, low levels of oxalate content are a highly desirable



T_1-Chemical fertilizer @ 100% NPK

T_2-Talc based formulation of PGPR mix–I alone

T_3-Talc based formulation of PGPR mix–I + 100% NPK

T_4-Talc based formulation of PGPR mix–I + 50% NPK

T_5-Liquid biofertilizer formulation of PGPR mix–I alone

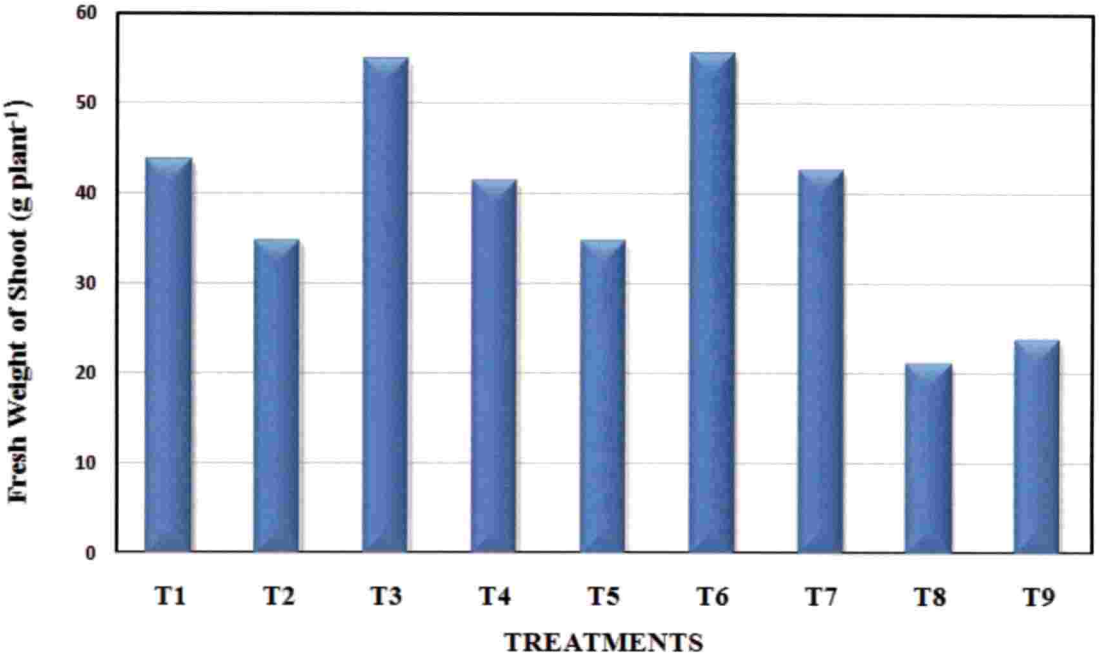
T_6-Liquid biofertilizer formulation of PGPR mix–I+100% NPK

T_7-Liquid biofertilizer formulation of PGPR mix–I+ 50% NPK

T_8-Control with additives without PGPR microorganisms

T_9-Absolute control

Fig. 18. Effect of different treatments on plant height of amaranthus at harvest



T_1-Chemical fertilizer @ 100% NPK

T_2-Talc based formulation of PGPR mix–I alone

T_3-Talc based formulation of PGPR mix–I + 100% NPK

T_4-Talc based formulation of PGPR mix–I + 50% NPK

T_5-Liquid biofertilizer formulation of PGPR mix–I alone

T_6-Liquid biofertilizer formulation of PGPR mix–I+100% NPK

T_7-Liquid biofertilizer formulation of PGPR mix–I+ 50% NPK

T_8-Control with additives without PGPR microorganisms

T_9-Absolute control

Fig. 19. Effect of different treatments on fresh weight of shoot of amaranthus at harvest

character in amaranthus plants. Kelly *et al.,* (2008) found that high levels of oxalic acid and nitrates have been reported to reduce the availability of certain minerals in the body, most notably calcium. The pot culture study also revealed that liquid formulation of PGPR mix–I is equally effective as talc based formulation of PGPR mix–I along with 100% NPK chemical fertilizers in enhancing yield and other biometric parameters of amaranthus. At present PGPR mix-I is being marketed as talc based formulation. Since it contains the same organisms, in the present investigation the efficiency of both talc and liquid formulations are statistically on par with respect to all the biometric parameters studied.

In the current agricultural scenario biofertilizers serve as an integral component of Integrated Nutrient Management practices. The present study could establish that application of PGPR mix-I along with the recommended dose of chemical fertilizer ie., 100:50:50 Kg NPK could enhance the yield, plant height and other biometric parameters of amaranthus and hence the product PGPR mix-I could be advocated to the farmers as an integral part of INM strategy. The results are in tune with many of the earlier studies conducted.

An investigation on Integrated Nutrient Management in brinjal by Rekha (1999) reported the effect of organic manures, chemical fertilizers and biofertilizers on the productivity and quality of brinjal. *Azospirillum* application increased the plant height and number of branches during early stages of growth and it also increased the number of flowers and fruits per plant when compared to control plants. Akshay (2011) based on the study on standardization of organic nutrient schedule for chilly suggested FYM @ 20 t ha^{-1} along with 75 Kg N ha^{-1} applied through a combination of FYM and neem cake in 1:1 ratio+ *Pseudomonas* + *Trichoderma* and PGPR mix-I as best nutrient schedule for realizing maximum yield from chilli. An investigation on the effect of integrated plant nutrient systems (IPNS) on the soil biological regimes in red loam soil was conducted at College of Agriculture, Vellayani. The study conclusively selected the treatment PGPR mix-I enriched vermicompost + N, P & K as the best treatment both in

sustaining soil biological fertility and economic returns (Sathyan, 2013). Vijendrakumar (2014) reported that combined application of liquid biofertilizers *viz.*; *Azospirillum lipoferum*, *Pseudomonas striata* and *Pseudomonas fluorescens* significantly increased growth, yield and survival of seedlings in garden rue (*Ruta graveolens* Linn.). Recently, Gopal (2018) suggested that PSB (liquid formulation) was the most promising liquid biofertilizer for enhancing growth of amaranthus. Similar results were also reported in many of the previous studies (Mariappan, 2014; Raja and Takankhar 2017).

Remarkably, the present study could identify that the treatment liquid biofertilizer formulation of PGPR mix–I+ 50 % NPK was found to be on par with chemical fertilizer @ 100 % NPK in all the parameters observed such as plant height at 20 DAT and harvest and fresh and dry weight of shoot and root, number of leaves, leaf area index and oxalate content of plants at harvest. The effective contribution of nitrogen by two diazotrophs present in PGPR mix-I and P and K solubilization by *Bacillus* sp has helped to save 50 per cent chemical fertilizers.

Similar observations on saving of chemical fertilizers using PGPR mix-I has already been reported by many earlier workers (Yazdani and Pirdashti 2011; Raj *et al.,* 2012; Sathyan, 2013; Wu *et al.*, 2013; Yadav, 2017; Vanithamani, 2016).

Raj *et al.,* (2012) conducted a field experiment on rice which could establish that basal application (2 Kg ha^{-1}) of PGPR mix-I with recommended half the dose of chemical fertilizers (45-22.5-7.5 Kg ha^{-1} NPK) and lime top dressing (250 Kg ha^{-1}) has significant effect in terms of increasing yield and it can also be used as a viable alternative for chemical fertilizer thereby saving chemical fertilizers. Based on an On Farm Trial, Yadav (2013) suggested the use of PGPR mix-I to reduce the use of chemical fertilizers considerably since it act as a viable alternative for inorganic chemical fertilizers and also suggested its use as an economic and effective management method. Vanithamani (2016) suggested that the bio-fertilizers *Cyanobacteria, Phosphobacteria and Azospirillum* combined

with half dose of inorganic fertilizer (NPK) can lead to enhancement in growth and nutritional status of leafy vegetable *Amaranthus polygonoids* compared to control (without fertilizer). Moreover, in support of these findings Yazdani and Pirdashti (2011) suggested that application of Phosphate Solubilizing microorganism and PGPR together reduces P application by 50 per cent. Wu *et al.*, (2013) could establish that triple inoculation of earthworms or plant growth–promoting rhizobacteria (PGPR), including nitrogen-fixing bacteria (NFB) (*Azotobacter chroococcum* HKN-5) and phosphate-solubilizing bacteria (PSB) (*Bacillus megaterium* HKP-1) may be a promising approach for reducing the need for chemical fertilizers in growing vegetables. Similar *in vivo* studies on different liquid biofertilizers have already been reported by many workers (Leksono and Yanuwiadi, 2014; Maheswari and Elakkiya, 2014; Barat *et al.*, 2016).

The present investigation noticed that significant increase in available NPK content in soil could be obtained due to application of liquid formulation of PGPR mix-I + 100% NPK. However, the results of soil studies have to be furthet tested under field conditions.

In support of our result Archana (2007) reported the increased uptake of potassium in maize plants receiving KSB inoculation compared to the absolute control. Furthermore, Fan *et al.*, (2017) observed that inoculation with PGPR may increase plant growth and N and P uptake by tomato grown on calcareous soils. Similar results on enhanced uptake of K were obtained by Zhang *et al.* (2004), Han and Lee (2005), Ramarethinam and Chandra (2005) and Sheng (2005).

Analysis of rhizosphere population after application of PGPR Mix-I revealed successful colonization of organisms of PGPR Mix-I in the rhizosphere of amaranthus. The results are in corroboration with the report of Vijendrakumar and Hanumaiah (2014) who reported that dual and triple inoculation of bio-fertilizers resulted in maximum CFU g^{-1} soil with respect to both beneficial and general micro-flora. Mary *et al.*, (2015) reported luxuriant growth of bacteria in all the biofertizer treated rhizosphere in the order FYM <Azospirillum <

Phosphobacteria < Vermicompost. Highest soil microbial population and enzyme activities were observed on application of 100 per cent chemical N and P along with consortium of *Azotobacter* and PSB (Khipla *et al.*, 2017). Similar results of improved soil microbial population and activity were obtained in many of the earlier studies (Verma, 1993; Manonmani, 1992; Shivakrishnaswamy, 2001).

Many of the researchers who have investigated the plant growth promoting activity of PGPR mix-I, suggested that the concerted effect of component cultures of PGPR mix-I significantly improve growth and yield of different crops. At present the product is being marketed as a talc based formulation. Taking into account the merits of liquid formulation over talc based formulation the present investigation was designed to develop liquid formulation of PGPR mix-I amended with most effective chemical additive that support prolonged shelf life and stress tolerance of component cultures. The present study could standardize the protocol for liquid formulation of PGPR mix-I with 15 mM Trehalose as the best amendment. The study revealed that liquid formulation of PGPR mix–I is equally effective as talc based formulation of PGPR mix–I along with 100% NPK chemical fertilizers in enhancing yield and other biometric parameters of amaranthus suggesting its role in INM. Compared to talc based formulation, the liquid formulation of PGPR mix–I showed higher shelf life beyond fourteenth month. The study also indicated that liquid formulation of PGPR mix–I + 50% NPK as chemical fertilizers was on par with chemical fertilizer @ 100% NPK in all the biometric parameters of amaranthus. The investigation, however, conclusively established that the treatment liquid biofertilizer formulation of PGPR mix–I+ 50 % NPK can also be used as a viable alternative for chemical fertilizer thereby saving 50% of chemical fertilizers. The present study could also establish the effective colonization of all the cultures of PGPR mix-I in the rhizosphere of amaranthus.

SUMMARY

6. SUMMARY

Intensive agriculture relies on the use of chemical fertilizers to promote plant growth and yield. On the other hand, excessive use of chemical fertilizers causes problems not only in terms of financial cost but also in terms of depletion of soil health. The interest in sustainable agriculture recently has increased. The development and application of sustainable agricultural techniques and biofertilization are vital to alleviating environmental pollution. Hence, a viable alternative for chemical fertilizers that will efficiently provide nitrogen, phosphorous, potassium and various phytohormones to plants is highly essential. Many bacterial species, mostly associated with plant rhizosphere, have been tested and found to be beneficial for plant growth, yield, and crop quality. They have been designated as "plant growth promoting rhizobacteria (PGPR)". Use of carrier based formulations of PGPR has gained considerable attention among farmers of Kerala. However, many of the recent works reported advantages of liquid based biofertilizer formulations over carrier based biofertilizer (Vendan and Thangaraju, 2006; Vendan and Thangaraju, 2007; Maheswari and Elakkiya, 2014; Leksono and Yanuwiadi, 2014; Barat *et al.*, 2016). Moreover, amendment of liquid based biofertilizer formulations with different chemical additives was found to increase the shelf life and protection against environmental stresses. Hence the development of liquid based biofertilizer formulations with most effective chemical additive which helps to reduce the use of chemical fertilizer is highly essential. In this context the programme entitled "Standardization of liquid formulation of PGPR mix–I and its evaluation for plant growth promotion in Amaranthus (*Amaranthus tricolor* L.)" was undertaken in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram.

The main objectives of the present study were standardization of liquid formulation of PGPR mix–I and its evaluation for plant growth promotion along

with saving of chemical fertilizers in Amaranthus. The salient findings of the present study are summarized below.

The component cultures of PGPR mix-I, *Azospirillum lipoferum, Azotobacter chroococcum, Bacillus megaterium* and *Bacillus sporothermodurans* were procured from the Department of Agricultural Microbiology, College of Agriculture, Vellayani. All the isolates produced significant quantity of IAA under *in vitro* conditions. The nitrogen (N) fixers, *Azospirillum lipoferum* and *Azotobacter chroococcum* produced 40.31 and 36.43 ppm of IAA respectively, whereas *Bacillus megaterium* and *Bacillus sporothermodurans* produced 1.28 ppm and 3.36 ppm of IAA respectively.

The *in vitro* estimation of N fixation by N fixing organisms namely, *Azospirillum lipoferum* and *Azotobacter chroococcum* recorded significant quantity of 21 and 14 mg N g^{-1} of carbon source respectively.

Quantitative assessment of solubilization of phosphorus (P) by *Bacillus megaterium* present in PGPR mix–I under *in vitro* conditions recorded 69.36 ppm, whereas qualitative assessment recorded a clearing zone of 8 mm and 12 mm diameter in NBRIP and Pikovaskaya's medium respectively. Similarly, *in vitro* assessment of K solubilization by *Bacillus sporothermodurans* recorded 12.18 ppm of potassium (K) and a clearing zone of 18 mm diameter in Glucose Yeast Agar medium.

The component cultures in PGPR mix–I was subjected to morphological characterization. All the isolates in PGPR mix–I was motile and they varied in morphological characteristics such as colony morphology, size, margin, texture, colour and cell shape. The nitrogen fixers, *Azospirillum lipoferum* and *Azotobacter chroococcum* stained Gram negative, whereas *Bacillus megaterium* and *Bacillus sporothermodurans* stained Gram positive. For further characterization, these four isolates were subjected to a series of biochemical tests. Morphological and biochemical characterization of the isolates supported the genus level identification of the isolates.

An experiment was carried out to standardize the protocol for the preparation of liquid formulation of PGPR mix–I in completely randomized design with different treatments such as 2% Glycerol, 2% Polyvinylpyrrolidone (PVP), 15mM Trehalose, 1% Glycerol + 1% PVP, Glycerol (2%) + Trehalose (1%) + Yeast extract (1%) + PVP (1%) + Proline (1%) and control without any additives in four replications. Talc based formulation was kept as a standard. In spite of inconsistent viable count recorded in different treatments till eighth month, from ninth month onwards, formulation amended with 15mM Trehalose exhibited maximum viable count until fourteenth month. A significant decline of total viable population in talc based formulation was observed each month compared to 15mM Trehalose amended formulation. Based on the population study, 15mM Trehalose amended formulation was adjudged as the best liquid biofertilizer formulation. Even after fourteen months significant population was observed in formulation amended with 15mM Trehalose and hence the shelf life studies of the same have to be continued.

A pot culture experiment was conducted in completely randomized design using sterilized soil under glass house conditions to test the efficacy of the best treatment (liquid formulation amended with 15mM Trehalose) with amaranthus as the test crop. The seven treatments included 100% NPK as per KAU recommendation as chemical fertilizer alone, talc based or liquid formulation of PGPR mix–I each alone and with 100% and 50% NPK, control with additives without PGPR microorganisms and absolute control in three replications. Roots of seedlings were dipped in 2 per cent of the freshly prepared liquid formulation at the time of transplanting and 50 ml of 2 per cent liquid was drenched in the soil in each pot two weeks after transplanting.

The results indicated that the treatment T_6- liquid formulation of PGPR mix–I+ 100% NPK recorded, maximum plant height of 37.54 cm, leaf number of 44.44 and leaf area index of 4.97 at harvest, but was on par with T_3- talc based formulation of PGPR mix–I+ 100% NPK. The treatment T_6 also recorded maximum fresh and dry weight of shoot (55.41 and 4.97 g plant^{-1} respectively)

and fresh and dry weight of root (6.6 and 0.56 g plant^{-1} respectively). However, the treatment T_6 was found to be on par with T_3 for these characters also. But T_6 recorded the least oxalate content of 0.39 per cent as well. The treatment liquid formulation of PGPR mix–I + 50% NPK (T_7) was found to be statistically on par with chemical fertilizer @ 100% NPK (T_1) in parameters such as plant height, number of leaves, leaf area index, fresh and dry weight of shoot and root and oxalate content.

Application of treatments had significant effect on the soil available NPK content. Analysis of rhizosphere population after application of PGPR mix-I revealed successful colonization of organisms of PGPR mix-I in the rhizosphere of amaranthus.

In the present investigation, liquid formulation of PGPR mix-I with 15 mM Trehalose was selected as the best amendment. The study revealed that liquid formulation of PGPR mix–I is equally effective as talc based formulation of PGPR mix–I along with 100% NPK chemical fertilizers in enhancing yield and other biometric parameters of amaranthus. Compared to talc based formulation, the liquid formulation of PGPR mix–I showed higher shelf life beyond fourteenth month.

Further studies on the effect of liquid formulation of PGPR mix-I with 15 mM Trehalose on crop plants are required before developing commercial formulations. Hence, the future studies may be focused on the following aspects:

1. Continuation of shelf life studies of liquid formulation of PGPR Mix-I.
2. Field evaluation of liquid formulation of PGPR Mix-I in different crops including paddy in different agro climatic zones of Kerala.

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STANDARDIZATION OF LIQUID FORMULATION OF PGPR MIX-I AND ITS EVALUATION FOR PLANT GROWTH PROMOTION IN AMARANTHUS (*Amaranthus tricolor* L.)

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ABSTRACT

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ABSTRACT

The study entitled "Standardization of liquid formulation of PGPR mix–I and its evaluation for plant growth promotion in Amaranthus (*Amaranthus tricolor* L.)" was conducted in the Department of Agricultural Microbiology, College of Agriculture, Vellayani during the period 2016-2018. The main objectives of the study were standardization of liquid formulation of PGPR mix–I and its evaluation for plant growth promotion along with saving of chemical fertilizers in amaranthus.

The component cultures of PGPR mix-I, *Azospirillum lipoferum, Azotobacter chroococcum, Bacillus megaterium* and *Bacillus sporothermodurans* were procured from the Department of Agricultural Microbiology, College of Agriculture, Vellayani. All the isolates produced significant quantity of IAA under *in vitro* conditions. The nitrogen (N) fixers, *Azospirillum lipoferum* and *Azotobacter chroococcum* produced 40.31 and 36.43 ppm of IAA respectively, whereas *Bacillus megaterium* and *Bacillus sporothermodurans* produced 1.28 ppm and 3.36 ppm of IAA respectively.

The *in vitro* estimation of N fixation by N fixing organisms namely, *Azospirillum lipoferum* and *Azotobacter chroococcum* recorded a significant quantity of 21 and 14 mg N g^{-1} of carbon source respectively.

Quantitative assessment of solubilization of phosphorus (P) by *Bacillus megaterium* present in PGPR mix–I under *in vitro* conditions recorded 69.36 ppm, whereas qualitative assessment recorded a clearing zone of 8 mm and 12 mm diameter in NBRIP and Pikovaskaya's medium respectively. Similarly, *in vitro* assessment of K solubilization by *Bacillus sporothermodurans* recorded 12.18 ppm of potassium (K) and a clearing zone of 18 mm diameter in Glucose Yeast Agar medium.

Morphological and biochemical characterization of the isolates supported the genus level identification of the isolates.

An experiment was carried out to standardize the protocol for the preparation of liquid formulation of PGPR mix–I in completely randomized design with different treatments such as 2% Glycerol, 2% Polyvinylpyrrolidone (PVP), 15mM Trehalose, 1% Glycerol + 1% PVP, Glycerol (2%) + Trehalose (1%) + Yeast extract (1%) + PVP (1%) + Proline (1%) and control without any additives in four replications. Talc based formulation was kept as a standard. In spite of inconsistent viable count recorded in different treatments till eighth month, from ninth month onwards, formulation amended with 15mM Trehalose exhibited maximum viable count until fourteenth month. A significant decline of total viable population in talc based formulation was observed in each month compared to 15mM Trehalose amended formulation. Based on the population study, 15mM Trehalose amended formulation was adjudged as the best liquid biofertilizer formulation. Even after fourteenth month significant population was observed in 15mM Trehalose amended formulation and hence the shelf life studies of the same have to be continued.

A pot culture experiment was conducted in completely randomized design using sterilized soil under glass house conditions to test the efficacy of the best treatment (liquid formulation amended with 15mM Trehalose) with amaranthus as the test crop The seven treatments included 100% NPK as per KAU recommendation as chemical fertilizer alone, talc based or liquid formulation of PGPR mix–I each alone and with 100% and 50% NPK, control with additives without PGPR microorganisms and absolute control in three replications. Roots of seedlings were dipped in 2 per cent of the freshly prepared liquid formulation at the time of transplanting and 50 ml of 2 per cent liquid was drenched in the soil in each pot two weeks after transplanting.

The results indicated that the treatment T_6- liquid formulation of PGPR mix–I+ 100% NPK recorded, maximum plant height of 37.54 cm, leaf number of

44.44 and leaf area index of 4.97 at harvest, but was on par with T_3- talc based formulation of PGPR mix–I+ 100% NPK. The treatment T_6 also recorded maximum fresh and dry weight of shoot (55.41 and 4.97 g plant^{-1} respectively) and fresh and dry weight of root (6.6 and 0.56 g plant^{-1} respectively). However, the treatment T_6 was found to be on par with T_3 in these characters also. But T_6 recorded the least oxalate content of 0.39 per cent as well. The treatment liquid formulation of PGPR mix–I + 50% NPK (T_7) was found to be statistically on par with chemical fertilizer @ 100% NPK (T_1) in parameters such as plant height, number of leaves, leaf area index, fresh and dry weight of shoot and root and oxalate content.

Application of treatments had significant effect on the soil available NPK content. Analysis of rhizosphere population after application of PGPR mix-I revealed successful colonization of organisms of PGPR mix-I in the rhizosphere of amaranthus.

The present investigation could standardize the protocol for liquid formulation of PGPR mix-I with 15 mM Trehalose as the best amendment. The study revealed that liquid formulation of PGPR mix–I is equally effective as talc based formulation of PGPR mix–I along with 100% NPK chemical fertilizers in enhancing yield and other biometric parameters of amaranthus. Compared to talc based formulation, the liquid formulation of PGPR mix–I showed higher shelf life beyond fourteenth month. The study also indicated that liquid formulation of PGPR mix–I + 50% NPK as chemical fertilizers was on par with chemical fertilizer @ 100% NPK and hence a saving of 50 per cent of chemical fertilizers could be advocated after confirmatory field trials.

APPENDICES

APPENDIX - I

COMPOSITION OF MEDIA USED

1. Nitrogen free Bromothymol media

Malic acid	- 5g
K_2HPO_4	- 0.5g
$MgSO_4.7H_2O$	- 0.2g
NaCl	- 0.1g
$CaCl_2$	- 0.02g
Trace element solution	- 2ml
BTB	- 2ml
$FeSO_4$	- 0.05g
Vitamin solution	- 4ml
KOH	- 4g
Agar-agar	- 20g
Distilled water	- 1000 ml

Malic acid, K_2HPO_4, $MgSO_4.7H_2O$, NaCl, $CaCl_2$, trace element solution, BTB, $FeSO_4$, Vitamin solution and KOH were dissolved in 500 ml distilled water and volume made up to 1000 ml. 20 g agar-agar was added into this mixture and autoclaved at 15 lbs pressure and 121 °C for 15 min.

2. Jenson's medium

Sucrose	- 20g
K_2HPO_4	- 1g
$MgSO_4$	- 0.5g
NaCl	- 0.5g
$FeSO_4$	- 0.1

$NaMoO_4$	- 0.005g
$CaCO_3$	- 2g
Distilled water	- 1000 ml
pH	- 7 to 7.3

Sucrose, K_2HPO_4, $MgSO_4$, NaCl, $FeSO_4$, $NaMoO_4$ and $CaCO_3$ were dissolved in 500 ml distilled water and volume made up to 1000 ml. 20 g agar-agar was added into this mixture and autoclaved at 15 lbs pressure and 121 °C for 15 min.

3. Pikovaskaya's media

Glucose	- 10g
$Ca(PO_4)_2$	- 5g
$(NH_4)_2SO_4$	- 0.5g
KCl	- 0.2g
$MgSO_4$	- trace
$FeSO_4$	- trace
Yeast extract	- 0.5g
Agar-agar	- 15g
Distilled water	- 1000 ml

Glucose, $Ca(PO_4)_2$, $(NH_4)_2SO_4$, KCl, $MgSO_4$, Yeast extract and $FeSO_4$ were dissolved in 500 ml distilled water and volume made up to 1000 ml. 20 g agar-agar was added into this mixture and autoclaved at 15 lbs pressure and 121 °C for 15 min.

4. Nutrient Agar

Peptone	- 5g
Beef extract	- 3g
NaCl	- 5g
Agar-agar	- 20g
Distilled water	- 1000 ml
pH	- 7

Peptone, Beef extract and NaCl were dissolved in 500 ml distilled water and volume made up to 1000 ml. 20 g agar-agar was added into this mixture and autoclaved at 15 lbs pressure and 121 °C for 15 min.

5. PGPR MEDIA

Malic acid	- 5g
Sucrose	- 10g
KH_2PO_4	- 1g
$MgSO_4$	- 0.4g
NaCl	- 0.2 g
$CaCl_2$	- 0.02g
$CaCO_3$	- 0.75g
$NaMoO_4$	- 5mg
NH_4Cl	- 100mg
Trace element solution	- 2ml
$FeSO_4$	- 0.05g
Vitamin solution	- 4 ml

KOH	- 4g
pH	- 6.8
Agar-agar	- 20g

Malic acid, Sucrose, KH_2PO_4, $MgSO_4$, NaCl, $CaCl_2$, $CaCO_3$, $NaMoO_4$, NH_4Cl, Trace element solution, $FeSO_4$, Vitamin solution, KOH were dissolved in 500 ml distilled water and volume made up to 1000 ml and autoclaved at 15 lbs pressure and 121 °C for 15 min.

APPENDIX - II

COMPOSITION OF STAIN USED

1. Crystal violet

One volume saturated alcohol solution of crystal violet in four volumes of one per cent aqueous ammonium oxalate.

2. Gram's iodine

Iodine crystals	- 1.0g
Potassium iodide	- 2.0g
Distilled water	- 300ml

3. Safranin

Ten ml saturated solution of safranin in 100 ml distilled water.