UTILIZATION OF FREE LIVING DIAZOTROPHIC BACTERIA FROM WAYANAD AS A BIOFERTILIZER

by

FATHIMA SHAHEENA K. M.

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"UTILIZATION OF FREE LIVING DIAZOTROPHIC BACTERIA FROM WAYANAD AS A BIOFERTILIZER"** 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 to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society

Vellanikkara,

Date 19-10-2015

Fathima Shaheena K. M.

(2013-11-162)

CERTIFICATE

Certified that this thesis entitled **"UTILIZATION OF FREE LIVING DIAZOTROPHIC BACTERIA FROM WAYANAD AS A BIOFERTILIZER"** is a record of research work done independently by **Mrs. Fathima Shaheena K. M.** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her

Vellanikkara,
Date 19-10-2015

Dr. D. Girija
(Major Advisor, Advisory Committee)
Professor and Head (Agricultural Microbiology)
College of Horticulture
Vellanikkara

CERTIFICATE

We, the undersigned members of the advisory committee of Ms Fathima Shaheena K M, a candidate for the degree of **Master of Science in Agriculture** with major in Agricultural Microbiology, agree that the thesis entitled **"UTILIZATION OF FREE LIVING DIAZOTROPHIC BACTERIA FROM WAYANAD AS A BIOFERTILIZER"** may be submitted by **Mrs Fathima Shaheena K. M.** in partial fulfilment of the requirement for the degree

16/10/15

Dr. D. Girija
(Chairman, Advisory Committee)
Professor and Head
Department of Agricultural Microbiology
College of Horticulture, Vellanikkara

16/10/15

Dr. K. Surendra Gopal
(Member, Advisory Committee)
Associate Professor
Department of Agricultural Microbiology
College of Horticulture, Vellanikkara

16/10/15

Dr P Sureshkumar
(Member, Advisory Committee)
Professor and Head
Radiotracer Laboratory
College of Horticulture, Vellanikkara

16/10/15

Dr P. G Sadhankumar
(Member, Advisory Committee)
Professor and Head (AICVIP)
Department of Olericulture
College of Horticulture, Vellanikkara

16/10/15

EXTERNAL EXAMINER

Murali Gopal

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ABBREVIATIONS

AEU	Agro Ecological unit
BNF	Biological nitrogen fixation
cfu	Colony forming unit
CRD	Complete randomized design
DAS	Days After Sowing
DNA	Deoxyribo Nucleic Acid
FYM	Farm Yard Manure
h	Hour(s)
HCN	Hydrogen cyanide
IAA	Indole Acetic Acid
INM	Integrated Nutrient Management
K	Potassium
KAU	Kerala Agricultural University
MAP	Month After Planting
MSL	Mean sea level
N	Nitrogen
NCBI	National Centre for Biotechnology Information
P	Phosphorus
PCR	Polymerase chain reaction
PGI	Percent Growth Inhibition
PGPR	Plant growth promoting Rhizobacteria
pH	Hydrogen ion concentration
POP	Package of Practices
RARS	Regional Agricultural Research Station
RD	Recommended Dose
TAE	Tris Acetate EDTA

Introduction

1. INTRODUCTION

Fertilizers are important inputs in agriculture and are currently responsible for 40-60% of the world's food supply. It is estimated that by 2020, to achieve the targeted production of 321 million tonnes of food grain, the requirement of nutrients will rise to 28.8 million tonnes. It has been also estimated that their availability will be only 21.6 million tonnes, the deficit being about 7.2 million tonnes (Sathyapal, 2013).

With the introduction of green revolution technologies, modern agriculture is getting more and more dependent upon the steady supply of the synthetic inputs, mainly nitrogenous fertilizers. The excessive and imbalanced use of chemical fertilizers has adversely affected the soil, causing decrease in organic carbon, reduction in microbial flora of soil and deterioration of soil properties. Excessive use of nitrogenous fertilizersleads to contamination of water bodies, thus affecting the fauna and causing health hazards for human beings and animals. In addition to this,nitrogenous fertilizers are costly and are manufactured from non-renewable petroleum feed stock which is gradually diminishing. Dependence on chemical fertilizers for future agricultural growth would mean further loss in soil quality, possibilities of water contamination and unsustainable burden on the fiscal system.

To overcome the deficit in nutrient supply and adverse effect of chemical fertilizers, it is suggested that efforts should be made to exploit all the available resources of nutrients under the theme of integrated nutrient management (INM). Under this approach the best available option lies in the complementary use of biofertilizers and organic manures in suitable combination with chemical fertilizers. It not only ensures higher productivity, but also good health of soil and environment.

Biofertilizers capable of fixing atmospheric nitrogen are essential components of integrated nutrient management. Nitrogen biofertilizers convert elemental nitrogen in the atmosphere into plant usable form through biological nitrogen fixation

(BNF)by using the microbial enzyme nitrogenase. It can increase crop yield by 20-30% and can replace nitrogen requirement by 25% (Evans and Furlong, 2010). These potential biological fertilizers could play a key role in productivity and sustainability of soil and protect the environment. These are relatively cheap, convenientfor use andhave lower manufacturing cost compared to chemical fertilizers. Theseare renewable source of plant nutrients, to supplement chemical fertilizers in sustainable agricultural system.

Nitrogen biofertilizers can be symbiotic or free living. Symbiotic nitrogen fixers form association with a specific host plant (eg: *Rhizobium*). Being non-specific, free living nitrogen fixers could be used for any crop.

Since Kerala soils are generally acidic, potential and effective free living diazotrophs adaptable to acidic conditions are required to develop an efficient biofertilizer. In addition to already commercialized nitrogen biofertilizers like *Azotobacter* and *Azospirillum*, other free living diazotrophs such as *Beijerinckia* and *Derxia*, which have not been exploited so far could be used as biofertilizers in future.

In this context, a study was conducted on 'Utilization of free-living diazotrophic bacteria from Wayand as a biofertilizer' with the following objective:

Isolation, screening and evaluation of free living diazotrophic bacteria from the phylloplane and rhizosphere of black pepper from Wayanad to develop an efficient biofertilizer.

Review of literature

2. REVIEW OF LITERATURE

Agriculture is heavily dependent on the use of chemical fertilizers, growth regulators and pesticides to increase yield. This dependency is associated with problems such as environmental pollution, health hazards, interruption of natural ecological nutrient cycling and destruction of biological communities. Hence, crop improvement and disease management have to be achieved in shorter intervals of time with fewer detrimental inputs. The excessive use of nitrogenous fertilizer is not judicial for soil health and crop production. Most of the soils of the world are deficient in nitrogen and applications of fertilizer nitrogen are essential for good yield. Generally, urea is the most convenient nitrogen source. But unfortunately less than 50% of the applied urea is used by plants (Halvorson *et al.*, 2002). This low efficiency of use is mainly caused by NH_3 volatilisation, denitrification, and losses from leaching. Volatilisation and denitrification pollute the atmosphere through the evolution of greenhouse gases like N_2O, NO and NH_3. Leaching of NO_3^{2-} nitrogen causes groundwater toxicity. In addition to these environmental problems, tillage systems making long term use of urea may deplete soil organic matter content (Wairiu and Lal, 2003). In the developing countries, the increasing price of fertilizers is hitting the small and marginal farmers. The application of high input technologies such as chemical fertilizers and pesticides have improved the production, but there is concern over its adverse effects on soil productivity and environment quality.

Hence, application of organic manure and biofertilizer in judicious combination with chemical fertilizers aid profitable and sustainable crop production along with maintenance of soil fertility (Singh and Sinsinwar, 2006). The use of bioresources to replace chemical pesticides, growth regulators and fertilizers is growing (Gore and Altin, 2006). Biofertilizers are gaining importance as they are ecofriendly, non hazardous and nontoxic.

2.1. BIOFERTILIZER

Biofertilizer refers to products consisting of selected and beneficial living microbes, which provides nutrients required by the plants and helps to increase the quality of the soil (Sharma *et al.*, 2007) or they are products containing living cells of different types of microorganisms which when, applied to seed, plant surface or soil, colonize the rhizosphere or the interior of the plant and promotes growth by converting unavailable nutritionally important elements, to available form through biological process such as nitrogen fixation and solubilization of rock phosphate (Rokhzadi *et al.*, 2008).

Beneficial microorganisms in biofertilizers accelerate and improve plant growth and protect plants from pests and diseases (El-yazeid *et al.*, 2007). A small dose of biofertilizer is sufficient to produce desirable results because each gram of carrier of biofertilizers contains at least 10 million viable cells of a specific strain (Anandaraj and Delapierre, 2010).

The important biofertilizers which help the plant to grow at different levels of its growth are, nitrogen fixing biofertilizers, phosphatic biofertilizers and potassic biofertilizers. Nitrogen fixing biofertilizers increase soil nitrogen level by fixing the atmospheric nitrogen and make it available to the plants. They transform inert atmospheric N_2 to organic compounds (Bakulin *et al.*, 2007).

Phosphatic biofertilizers can bephosphorous solubilizing or phosphorus mobilizing biofertilizers. Phosphorous solubilizing biofertilizers bring insoluble phosphate in soil into soluble forms by secreting organic acids. These acids lower the soil pH and bring about the dissolution of bound forms of phosphate (Gupta, 2004). Examples are Species of *Bacillus*, *Pseudomonas* and *Aspergillus*. Phosphorus mobilizing biofertilizers transfer phosphorus from the soil to the root cortex by increasing the root surface area. Example: Arbuscular Mycorrhiza (AM fungi).

Potassium solubilizing biofertilizers contains microorganisms which solubilize insoluble potassium minerals into soluble forms. Example: *Bacillus, Aspergillus.*Potassium mobilizing biofertilizers are responsible for the movement of potassium in soil and increase its availability. Example: *Frateuria aurantia*

2.2. NITROGEN FIXING MICROORGANISMS (DIAZOTROPHS)

Diazotrophs are the prokaryotic organisms, which have the ability to fix atmospheric nitrogen. At least 90 genera of specialized microorganisms are known to have the enzyme nitrogenase and can fix atmospheric N_2 into NH_3 (Murray and Jeff, 2008). These include, a) symbiotic b) associative and c) free-living nitrogen fixing forms.

2.2.1. Symbiotic nitrogen fixers

Symbiotic nitrogen fixers harbor within the tissue of host plants. Here nitrogen fixing bacteria directly provide the host plant with fixed nitrogen in exchange for carbohydrates and other nutrients. Symbiotic N_2 fixing bacteria include members of the family rhizobiaceae which forms symbiosis with leguminous plants like *Rhizobium* (Ahemad and Khan, 2012) and non-leguminous trees (e.g. Frankia).

2.2.2. Associative nitrogen fixers

Associative nitrogen fixing bacteria form a loose association with roots of several agricultural crops as well as wild plants (Rawia *et al.*, 2009). They grow on the surface of roots and may also colonise the outer layers of a root by entering between epidermal cells. A common genus forming associative nitrogen fixation is *Azospirillum.*

2.2.3. Free living nitrogen fixers

Some important free-living nitrogen-fixing bacteria include, *Achromobacter*, *Acetobacter*, *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Azomonas*,

Bacillus, *Beijerinckia*, *Clostridium*, *Derxia*, *Enterobacter* and *Klebsiella* (Saxena and Tilak, 1998). Recently, nitrogen fixing ability has been reported in some species of genera like *Microbacterium* (Gtari *et al.*, 2012), *Cellulosimicrobium* and *Brevundimonas* (Kumar and Gera, 2014).

2.2.3.1. Azotobacter

In 1901, Dutch microbiologist M. W. Beijerinck, using an enrichment culture technique with a medium devoid of a combined nitrogen source, discovered an aerobic microorganism capable of fixing molecular nitrogen to which the name of *Azotobacter chroococcum* was given.*Azotobacter* species are free-living, aerobic, Gram negative heterotrophic diazotrophs that depend on an adequate supply of reduced C compounds such as sugars for energy. The cell size is usually large ovoid and are polymorphic. They derive food from the organic matter present in the soil and root exudates and fix atmospheric N.

Tandon (1991) estimated the fertilizer equivalent of important biofertilizers. According to the estimate, for *Azotobacter* 20 kg N ha^{-1} of fertilizer equivalent was obtained.

2.2.3.2. Beijerinckia

The genus*Beijerinckia* are aerobic, Gram negative having a strictly respiratory type of metabolism. The cells occur singly as straight or slightly curved rods. They are non-motile or motile by means of peritrichous flagella. They fix molecular nitrogen under aerobic conditions and also under microaerobic conditions. Optimum temperature for the growth is 20-30°C (Derx, 1950).

2.2.3.3. Derxia

The genus *Derxia* consists of two species, *Derxia gummosa* and *Derxia indica*. They are Gram negative, rod-shaped with rounded ends, 1.0–1.2 mm in width

and motile due to the presence of short polar flagellum. Molecular nitrogen can be fixed under both aerobic and microaerobic condiditions(Jensen *et al.*, 1961). The optimumtemperature for growth is 25–35°C.

2.3. OCCURRENCE OF DIAZOTROPHS IN SOIL

Rhizosphere is the zone of soil which surrounds the plant root. It is the area of intense microbial activity compared to non-rhizosphere soil due to presence of root exudates. Through the exudation of a wide variety of compounds, root regulates soil microbial community and changes the physical and chemical properties of soil.

The occurrence of *Azotobacter* has been reported from the rhizosphere of a number of crop plants such as rice, maize, sugarcane, bajra, vegetables and plantation crops (Arun, 2007). Ahemad *et al.* (2008) isolated 47 *Azotobacter* strainsfrom rhizosphere of different crops like wheat, brinjal, sugarcane, cabbage and cauliflower. *Azotobacter* was isolated from rhizosphere of tomato (Ibiene *et al.*, 2013)

The occurrence of *Beijerinckia* was mentioned for the first time in Brazil and it was demonstrated that the number of the populations was related to vegetation, physical, and chemical characteristics of the soil (Dobereiner and Castro, 1955). Out of total tropical soil analysed by Becking (1961) 48% of samples were found to be positive for this genus. Veena (1999) reported the occurrence of *Beijerinckia* in rhizosphere soils of sorghum plants. Sudhakar *et al.* (2000) isolated *Beijerinckia indica* from mulberry rhizosphere along with other nitrogen fixing bacteria. A new strain of *Beijerinckia mobilis* was isolated from the pea rhizosphere by Polyanskaya *et al.* (2002). Naikar (2003) isolated *Beijerinckia* from diverse crop plants grown in different river belts in Karnataka. *Beijerinckia* in rhizosphere soil of potato and in forest areas in Colombia was reported by Moratto *et al.* (2005).

Dobereiner (1968) isolated *Derxia* from twenty rhizosphere soil collected from different forage grasses. Xie *et al.* (2004) isolated and characterized thirty

heterotrophic diazotrophs from paddy field. High acetylene reduction activity was noted among the isolates and one of the strains was identified as *Derxia*. Pathania *et al.* (2014)isolated and characterized *Derxia* from wheat rhizosphere.

Microbacterium has been isolated from surface sterilized stem of sugarcane (Lin *et al.*, 2012), roots of *Arabidopsis thaliana* (Schwachtje *et al.*, 2012) and stem, root and leaf of different rice cultivars (Ji *et al.*, 2014). Pontes *et al.* (2015) isolated *Microbacterium* from rhizosphere soil and roots of barley.

Stella and Suhaimi (2010) conducted a study on selection of suitable growth medium for free living diazotrophs. They isolated four strains of free living diazotrophs and observed that *Beijerinckia* medium was the most suitable growth medium for all four isolates, followed by Derxia medium and Ashby's medium. Sharma and Rai (2013) used Jensen's medium and Ashby's medium for the isolation of *Azotobacter* from soil. Jensen's medium was used for the isolation of *Azotobacter* from soil (Pathania *et al.*, 2014).

2.4. OCCURRENCE OF DIAZOTROPHS IN PHYLLOPLANE

Zone or area on leaves which is inhabited by microorganisms is known as phylloplane. Exposure of leaves to air current, dust etc. containing lots of microorganisms result in establishment of a typical microflora on leaf surface. Bacteria are by far the most numerous colonists of leaves, often being found in numbers averaging 10^6 to 10^7 cells/cm^2 (up to 10^8 cells/g) of leaf. Leaves constitute a very large microbial habitat. These bacteria are sufficiently numerous to contribute in many important processes globally, as well as to the behavior of the individual plants on which they live.

Gajendiran and Mahadevan (1989) isolated *Azotobacter* sp., *Beijerinckia* sp., *Derxia* sp fromplant species growing in tropical rain forest.

Azotobacterchroococcum is reported to occur in leaf sheath and root cortex (Tippannawar and Reddy, 1989).

Strains of the species *Microbacterium lacticum*, *Microbacterium liquefaciens* and *Microbacterium saperdae* were isolated from the phyllosphere of sugar beet and spring wheat (Thompson *et al.*, 1993). Santosa *et al.* (2003) isolated*Microbacterium lacticum* from phyllosphere of rice. Kumar and Gera (2014) isolated four strains of *Brevundimonas* sp. from the rhizosphere ofsugarcane.

2.5. EFFECT ON GROWTH, DEVELOPMENT AND YIELD OF CROPS

2.5.1. *Azotobacter*

Favourable response of *Azotobacter* has been noticed by many workers in vegetable crops. Eklund (1970) demonstrated that the presence of *Azotobacter chroococcum* in the rhizosphere of tomato and cucumber is correlated with increased germination and growth of seedlings. *Glomus fasciculatum* as well as *Azotobacter vinelandii* significantly increased leaf area, shoot dry weight, nitrogen content, phosphorus content and yield in tomato (Mohandas, 1987). Dry weight of tomato plants inoculated with *Azotobacterchroococcum* was significantly greater than that of non inoculated plants (Puertas and Gonzales, 1999). Bowen and Rovira (1999) reviewed the biology of the rhizosphere and its management to improve plant growth. Their review commenced with the increasesin growth when tomatoes were inoculated with *Azotobacter*. Subramanian *et al.* (2006) reported that biofertilizer containing *Azotobacter* increased number of fruit and flowers per plant and yield of tomato. El-yazeid *et al.* (2007) indicated that inoculation of tomato seedling with *Azotobacter* increased plant height, leaf number per plant, fruit mean weight and yield compared to control (without biofertilizers).

Pandey and Kumar (1989) observed the yield increase ranges from 2 to 45 per cent in vegetables, 9 to 24 per cent in sugarcane, 0 to 31 per cent in maize, sorghum,

mustard etc. on *Azotobacter* inoculation. Dutta and Singh (2002) reported a significant increase in seed yield (7.86q ha-1) in rapeseed and mustard due to inoculation with *Azotobacter.* Khalequzzaman and Hossain (2007) showed application of *Azotobacter* increased germination, plant height and yield of bush bean.Similarly, positive reports on application of *Azotobacter* and *Azospirillum* on the yield of mustard (*Brassica juncea*) are available (Tilak and Sharma, 2007).

Ravikumar *et al.* (2004) found that*Azotobacter* strains, which were inoculated with rhizophora (*Rhizophora mangle*) seedlings, increased significantly the average root biomass up to 98.2%, the root length by 48.45%, the leaf area by 277.86%, the shoot biomass by 29.49% as compared to control.

Rovira (1965) reported yield increase in wheat after inoculation with *Azotobacter chroococcum.* Inoculation with *Azotobacter* increased the number of root hairs, tillering ratio, dry matter contents, N-uptake or yields of wheat (Rai and Gaur, 1988). Inoculation with *Azotobacter* replaced up to 50% of the urea-N for wheat in greenhouse trials under aseptic (gnotobiotic) conditions (Hegazi *et al.*, 1998). Yields of rice in field trials increased significantly up to 0.9 t ha^{-1} (20% increase) with applications of *Azotobacter* (Yanni and El-Fattah, 1999).

Inoculation with *Azotobacter* can increase cotton yield by 15–28% (Iruthayaraj, 1981).Patil and Patil (1984) observed that seed inoculation with *A.chroococcum* along with 50–100 kg urea-N ha^{-1} gave higher cotton dry matter yield, N uptake and soil N content than those obtained with N alone (50–100 kg urea-N ha^{-1}) in greenhouse conditions using non-sterilised soils. Inoculation with the *Azotobacter* sp. augmented root dry weight, root length and growth in cotton (Hafeez *et al.*, 2004).Under green house conditions plant height, leaf number/plant, number of primary and secondary branches/plant, fresh and dry weight of whole plant, number of siliqua/plant, seeds/siliqua of brown sarson increased significantly with *Azotobacter* inoculation than no inoculation with seed (Wani, 2012).

The study at Kerala Agricultural University showed that plant height, LAI and yield were significantly high in treatment receiving 75% nitrogen along with FYM and *Azotobacter* inoculation in pot culture experiment with amaranthus (Arunkumar, 1997). In field condition maximum yield for amaranthus was obtained in *Azotobacter* treatment with FYM and 75% nitrogen which also had a significant increase in plant height. For brinjal also *Azotobacter* treatment receiving FYM and 75% nitrogen produced maximum plant height, no.of branches and yield. Keeping quality of fruits was significantly high in *Azotobacter* treatment with full dose of vemicompost and 75% nitrogen. According to Das and Saha (2007), combined inoculation of *Azotobacter* and *Azospirillum* in presence of partial application of farmyard manure increase crop productivity. Sepat *et al.* (2012) observed the treatment of *Azotobacter* alonedid not improve growth attributes significantly. The highest values of all growth attributes were observed due to an integrated application of 100% NPK and FYM + *Azotobacter* followed by 50% NPK + FYM + *Azotobacter* and 100% NPK + FYM.

Azotobacter has been applied by various methods such as dipping seed into microbial cultures before planting, dipping seedling roots into broth cultures, soil application at nursery or main field stages, top dressing or foliar application (Singh *et al.*, 1999).

2.5.2. *Beijerinckia*

Inoculation of rice seeds with *Beijerinckia* showed that this genus is able to multiply in the soil, establishing itself in large numbers, reducing the number of other competing microorganisms as well as an increase in the yield (Dobereiner and Ruschel, 1961). Nandi and Sen (1981) observed that *Beijerinckia* sp. isolated from sources other than leaf surface when sprayed on wheat and rice plants, as a substitute for nitrogenous fertilizers resulted in a marked improvement in yield and growth of the plants. Polyanskaya *et al.* (2002) studied the growth promoting effect of two new strains of *Beijerinckia mobilis* and *Clostridium* sp. isolated from pea rhizosphere on

some agricultural crops and reported that application of *B. mobilis* and *Clostridium* sp. cultures in combination with mineral fertilizers increased the crop yields by 1.5 to 2.5 times. Enhanced shoot growth and nitrogen content of the whole plant of tomato was observed by Meunchang *et al.* (2006) in soil amended with sugar mill byproduct compost inoculated with N2 fixing bacteria, *Azotobacter vinelandii, Bejerinckia derxii* and *Azospirillm* sp. than uninoculated control.

When *Beijerinckia* was inoculated into the rhizosphere of the Paspalum grass, the plants had a higher N content than the non-inoculated controls, suggesting that the microorganism may contribute to the development of this grass (Ruschel and Britto, 1966). *B. indica* significantly increased the dry weight andtotal N content of the plant and also colonized therhizosphere (Souto and Dobereiner, 1967).

Souto and Dobereiner (1967) observed a small but significant increase in growth of *Pennisetum pupureum* grown under green house condition when inoculated with *Derxia.*

2.5.4. Others

Strains of *Microbacterium* sp. Isolated from phyllosphere of rice significantly increased the plant height, upper parts biomass as well as root biomass of rice after inoculation (Santosa *et al.,* 2003). Pretreatment of soil with *Microbacterium* sp. 15 days before sowing showed a maximum increase in growth and biomass in terms of root length (93%), plant height (94%), dry root biomass (99%), and dry shoot biomass (99%) in *Pisum sativum* under pot culture experiment (Soni *et al.,* 2014). They also reported that inoculation of the same strain of *Microbacterium* sp. improved the growth and yield of maize.

Brevundimonas sp. has been used as PGPR in enhancing growth of wheat plants (Rana *et al.,* 2011). In a pot experiments conducted by Kumar and Gera (2014), inoculation of cotton seeds with *Brevundimonas* sp. enhanced the growth of

plants as shown by significant increase in plant height (68.41 %), shoot dry weight (58.44 %) and root dry weight (64.81 %) over untreated control.

A strain of *Cellulosimicrobium cellulans* promoted the growth of chilli plants (Chatterjee *et al.,* 2009).Brassica seeds bacterized with rhizospheric isolates of *Cellulosimicrobium* sp. showed significant increase in shoot length, root length, fresh weight, and dry weight over control (Singh *et al.,* 2014). Nabti *et al.* (2014) reported that four isolates of *Cellulosimicrobium* sp. significantly stimulated germination and growth of barley seedlings over untreated control.

2.6. EFFECT OF pH ON GROWTH AND NITROGEN FIXATION

Soil pH plays an important role in the occurrence and survival of diazotrophic bacteria. Biological nitrogen fixation by free-living bacteria in rice soils has been reported from alluvial, laterite, acid saline and acid sulphate saline soils (Sethunathan *et al.,* 1983). The degree of soil acidity or alkalinity influences the population of soil diazotrophs.

The presence of *Azotobacter* spp. and their rate of multiplication and nitrogen fixation are governed by many factors including soil pH (Jensen, 1961).Some strains of *Azotobacter beijerinckii* and *A. macrocytogenes* has been isolated which are able to grow and fix nitrogen at pH values of 4.5 to 5.0 (Becking, 1961). Optimum pH required for growth and nitrogen fixation of *Azotobacter* spp. was near or slightly above neutrality (Rangaswami and Sadasivan, 1965). Dobereiner (1968) observed a very frequent occurrence of *Azotobacter chroococcum* in 22 out of 27 acid soil samples. In a similar study conducted by Ninawe and Paulraj (1997) *Azotobacter* species isolated from paddy fields of Kerala were found to had the optimum pH at near or slightly above neutrality (*i.e.,*7-8.5) for growth and nitrogen fixation. Roy and Deb (2010) found that optimum pH for the growth and nitrogen fixation by

Azotobacter was 7-7.5 but in presence of combined nitrogen the pH range for growth was 4.8 – 8.5.

A preliminary analysis on the occurrence of nitrogen-fixing bacteria with grass showed that *Beijerinckia* and *Azotobacter* were the predominant bacteria in the rhizosphere, even with plants grown in acid soils (Ruschel and Dobereiner, 1965). Soil pH of rice fields of Southern Assam was below 6.0 which support the occurrence of acid tolerant strains of diazotrophs like *Azotobacter chroococcum*, *Beijerinckia indica* and *Derxia gummosa* (Andre *et al.*, 2007)

Becking (1961) showed that pH 3.0 is the lowest value in which the genus *Beijerinckia* is able to grow and fix nitrogen. He also observed that *Beijerinckia* occurred in soils within the pH range 4.3 to 7.0, in a wide range of soil types (forest, grass, cultivated) and under varying climatic conditions.Most isolation of *Beijerinckia* has been made from acid soils (Becking, 1961).A study on the occurrence of free living, N-fixing bacteria in Brazilian soils revealed the presence of *Beijerinckia* in 92 soil samples out of 158 all with a pH above 6.0 (Dobereiner, 1968). *Beijerinckia* occurred in soil samples in the pH range of 4.5 -6.9, but most frequently in samples from pH 5.0 – 6.4 (Becking,2006). *Beijerinckia* grew between pH 3-9 whereas Derxia grew between 5.5 – 9 (Roy and Deb, 2010) and in the case of *Beijerinckia*no growth was observed at pH 4.4 (Lasker *et al.*, 2010).

Derxia gummosa was capable of forming an effective nitrogen fixing association under a wide range of pH and oxygen concentration (Xie and Yokota, 2004).

2.7. PGPR

Bacteria associated with plants can be harmful and beneficial. Plant growth promoting (PGP) bacteria may promote growth directly, e.g. by fixation of atmospheric nitrogen, solubilization of minerals such as phosphorus, production of

siderophores that solubilize and sequester iron, or production of plant growth regulators or hormones (Kloepper, 1997). Indirect growth promotion occurs via the removal of pathogens by the production of secondary metabolites such as hydrogen cyanide and siderophores.

The use of PGPR offers an attractive way to replace chemical fertilizer, pesticides, and supplements; most of the isolates result in a significant increase in plant height, root length, and dry matter production of shoot and root of plants. PGPR help in the disease control in plants. Some PGPR especially if they are inoculated on the seed before planting, are able to establish themselves on the crop roots. PGPR is a component in integrated management systems in which reduced rates of agrochemicals and cultural control practices are used as biocontrol agents. Such an integrated system could be used for transplanted vegetables to produce more vigorous transplants that would be tolerant to nematodes and other diseases for at least a few weeks after transplanting to the field. They have the potential to contribute in the development of sustainable agricultural systems (Schippers *et al.*, 1995).

Yield increases obtained in inoculated plants were attributed to the production of plant growth substances by the root-colonizing bacteria (Kennedy and Tchan, 1992). Promotion of root growth after application of PGPR, resulted in enhanced nutrient and water uptake from the soil (Bhattarai and Hess, 1993) and thus the increased growth and yield. Treatment with plant growth promoting rhizobacteria increase LAI, chlorophyll content, (Chandrasekar *et al.*, 2005; Amujoyegbe *et al.*, 2007) germination percentage, seedling vigor and emergence, root and shoot growth, total biomass of plants, seed weight, early flowering, grains, fodder and fruit yields (Berova and Karanatsidis, 2008),tolerance to drought, salt stress and delayed leaf senescence.

In last few decades a large array of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Beijerinckia,*

Burkholderia, Bacillus, Derxia and *Serratia* have reported to enhance plant growth (Murphy *et al.*, 2003; Esitken *et al.*, 2006; Lugtenberg and Kamilova, 2009).

The mechanism of action of PGPR comprises direct and indirect method. The direct promotion by PGPR entails either providing the plant with plant growth promoting substances that are synthesized by the bacterium or facilitating the uptake of certain plant nutrients from the environment. The indirect promotion of plant growth occurs when PGPR prevent deleterious effects of one or more phytopathogenic microorganisms.

2.7.1. Direct mechanism of action of PGPR

2.7.1.1. Biological nitrogen fixation

Nitrogen is an essential nutrient for plant growth and productivity. Eventhough, 78% of nitrogen is present in the atmosphere, it can't be used by the plants. The atmospheric N_2 is converted into plant-utilizable forms by biological nitrogen fixation (BNF) which changes nitrogen to ammonia by nitrogen fixing microorganisms using a complex enzyme system known as nitrogenase (Kim and Rees, 1994). Biological nitrogen fixation occurs, generally at mild temperatures, by nitrogen fixing microorganisms, which are widely distributed in nature (Raymond *et al.*, 2004). In fact, BNF accounts for approximately two-thirds of the nitrogen fixed globally, while the rest of the nitrogen is industrially synthesized by the Haber–Bosch process (Rubio and Ludden, 2008).

Biological nitrogen fixation contributes about 60% of the earth's available nitrogen and it is an economically beneficial and environmentally sound alternative to chemical fertilizers (Ladha *et al.*, 1997).Biological nitrogen fixation is estimated to contribute 180 $\times 10^6$ metric tons/year globally (Postgate, 1998), of which eighty percent comes from symbiotic associations and the rest from free-living or associative systems (Graham, 1998). In natural ecosystems biological nitrogen

fixation by free living, associated and symbiotic diazotrophs is the most important source of nitrogen (Clevend *et al.*, 1999)

Patil and Patil (1984) observed that seed inoculation with *A.chroococcum* along with 50–100 kg urea-N ha^{-1} gave higher N uptake and soil N content than those obtained with N alone (50 – 100 kg urea - N ha^{-1}) in greenhouse conditions using non-sterilised soils in cotton. Inoculation of corn with *Azotobacter*, *Beijerinckia*, *Escherichia*, *Derxia*, and *Klebsiella* increased the concentration of nitrogen in the aboveground plant parts (Saric *et al.*, 1987).

Inoculation of *Beijerinckia* resulted in higher N-content of the plant compared to non-inoculated controls in Paspalum grass (Ruschel and Britto, 1966). Veena (1999) compared the nitrogen fixing ability of four diazotrophs *viz.*, *Azospirillum*, *Acetobacter*, *Azotobacter* and *Beijerinckia* isolatesshowed lowest nitrogen fixation which ranged from 3.46 to 8.36 mg N per g of glucose added.Naikar (2003) reported that the amount of nitrogen fixed by *Beijerinckia* isolates was found tovary between 2.55 to 4.74 mg per gram of 'C' source used. Nitrogen content of the whole plant of tomato was found to be higher by Meunchang *et al.* (2006) in soil amended with sugar mill byproduct compost inoculated with nitrogen fixing bacteria, *Azotobacter vinelandii, Bejerinckia derxii* and *Azospirillm* sp. than uninoulated control.

2.7.1.4. Production of phytohormones

Growth substances, or plant hormones, are natural substances that are produced by microorganisms and plants alike. They have stimulatory or inhibitory effects on certain physiological-biochemical processes in plants and microorganisms. The phytohormones production by PGPR is now being deliberated as one of the most important mechanisms through which several rhizobacteria promote plant growth (Spaepen *et al.*, 2008). Phytohormones are indicator molecules that act as chemical messengers and show a vital role as growth and development managers in the plants.

The capability of producing phytohormones widely disseminated among bacteria related with soil and plants.

Studies have verified that the PGPR can stimulate plant growth by regulating the high levels of endogenous ethylene in the plant or through the production of cytokinins (Timmusk *et al.*, 1999), gibberellines (Bottini *et al.*, 2004) and auxins (indole acetic acid) (Spaepen *et al.*, 2008).

The auxin (IAA) play role in division, expansion and differentiation of plant cells and tissues and inspires root elongation. The IAA synthesizing capacity has been detected in many symbiotic and free living bacterial species present in rhizosphere (Tsavkelova *et al.*, 2006). Microbial IAA has been implicated in the stimulation of growth or pathogenesis of plants. Among PGPR species capable of improving growth and development in several crops by producing these beneficial phytohormones, belonging to *Azotobacter* (Ahemad *et al.*, 2008), *Burkholderia, Enterobacter* etc. have been isolated from different rhizosphere soils (Shoebitz *et al.*, 2009).

Besides nitrogen fixation, *Azotobacter* synthesizes and secretes considerable amounts of biologically active substances like auxin, nicotinic acid, pantothenic acid, biotin, gibberelins etc. which enhance root growth of plants (Rao, 1986). The beneficial effect of *Azotobacter* on growth and yield of various crops include their potential to fix atmospheric nitrogen, production of growth substances including IAA, cytokines and gibberellins (Verma *et al.*, 2001).

Azcorn and Barea (1975) reported that *Azotobacter* inoculation enhanced the growth of tomato due to the synthesis of auxins, cytokinins, and GA-like substances. In a similar study conducted by Joseph *et al.* (2007) IAA production was detected in *Azotobacter* spp. Fatima *et al.* (2009) isolated seven PGPR strain including *Azotobacter, Pseudomonas and Azospirillum.* IAA production by *Azotobacter chroococcum* was reported by Wani(2012).Among the seven isolates highest

concentration of IAA was detected in one of the *Azotobacter* isolate. IAA production was detected in isolates of *Azotobacter* (Ibiene *et al.*, 2013).

Cacciari *et al.* (1980) found in his study that the excretion of IAA by *Beijerinckia* occurred during the stationary growth phase. IAA production was found to be higher when *Beijerinckia derxii* grown under agitation compared to culture under non agitated condition (Thuler *et al.*, 2003). They concluded that *Beijerinckia derxii* is a potential producer of IAA. Sheng *et al.* (2009) isolated a strain of *Microbacterium* which had plant growth promoting characteristics of producing indole acetic acid. Production of IAA by *Brevundimonas* sp. was detected by Kumar and Gera (2014). Significant amount of IAA production was detected in isolates of *Cellulosimicrobium* sp. by Singh *et al.* (2014). Pontes *et al.* (2015) reported that 74% of the isolated strains of *Microbacterium* produced indolic compound.

2.7.2. Indirect mechanism of action of PGPR

2.7.2.1. Suppression of plant diseases by PGPR

The use of environmental friendly microorganisms has proved useful in plant-growth promotion and disease control in modern agriculture (Weller, 1988). Biological control not only suppresses the disease and increases the crop yield but will be important in preventing the environmental pollution due to pesticides. PGPR inoculation is a promising agricultural approach that plays a vital role in crop protection, growth promotion or biological disease control (Dilantha *et al.*, 2006). Recently, PGPRs are increasingly and extensively used as inoculants in biological control of bacterial, viral and fungal plant diseases (Aliye *et al.*, 2008; Akgul and Mirik, 2008).

In general, competition for nutrients and niche, parasitism and antibiosis through the production of antibiotics, siderophores and other metabolites have been suggested as the major mechanisms of action of antagonistic bacteria and other

microorganisms in the biological control of various plant diseases (Pal and Jalali, 1998).

Azotobacter produces growth promoting substances which have anti microbial and fungicidal properties (Pal and Jalali, 1998).*Azotobacter chroococcum* produces an antibiotic which inhibits the growth of several pathogenic fungi in rhizosphere thereby seedling mortality (Subbarao, 2001).

The mechanisms of suppression of plant diseases are siderophore production and production of antimicrobial metabolites like ammonia and HCN.

2.7.2.1.1. Siderophore production

In the aerobic environment, iron occurs principally as Fe^{3+} and is likely to form insoluble hydroxides and oxyhydroxides, thus making it generally inaccessible to both plants and microorganisms (Rajkumar *et al.*, 2010).

Siderophores are low molecular weight iron-binding ligands which can bind to ferric ion and make it available to the producer microorganism (Neilands, 1981). In both Gram-negative and Gram-positive rhizobacteria, iron (Fe^{3+}) in Fe^{3+}- siderophore complex on bacterial membrane is reduced to Fe^{2+} which is further released into the cell from the siderophore via a gating mechanism linking the inner and outer membranes. During this reduction process, the siderophore may be destroyed/recycled and binding of the siderophore to a metal increases the soluble metal concentration (Rajkumar *et al.*, 2010). Hence, bacterial siderophores help to alleviate the stresses imposed on plants by high soil levels of heavy metals. Numerous studies of the plant growth promotion about siderophore-mediated iron uptake as a result of siderophore producing rhizobacterial inoculations have been reported (Rajkumar *et al.*, 2010).

Crowley and Kraemer (2007) revealed a siderophore mediated iron transport system in oat plants and inferred that siderophores produced by rhizosphere microorganisms deliver iron to oat, which has mechanisms for using Fe-siderophore complexes under iron-limited conditions.

The increase in yield on inoculation with *Azotobacter* isas a result of biological nitrogen fixation, production of antibacterial and antifungal compounds, growth regulators and siderophores (Pandey and Kumar, 1989). Saikia and Bezbaruah (1995) reported increased seed germination of *Cicer arietinum, Phaseolus mungo, Vigna catjung* and *Zea mays*. However, yield improvement is attributed more to the ability of *Azotobacter* to produce plant growth promoting substances such as phytohormone IAA and siderophore azotobactin, rather than to diazotrophic activity. Similarly Suneja and Lekshminarayana (1998) reported production of siderophores by *A. chroococcum* which solubilize Fe^{3+} and suppress plant pathogens through iron deprivation. Wani *et al.* (2007) reported siderophore production by *Azotobacter chroococcum.* Siderophore production was detected among some isolates of *Azotobacter* and demonstrated broad spectrum antifungal activity against the five tested fungi (Ahemad *et al.*, 2008).They also reported the production of siderophore by *Azotobacter* sp. *Azotobacter* inhibit phytopathogenic fungi through the production of antifungal substance and siderophores (Mali and Bodhankar, 2009). Siderophore production was detected in strains of *Microbacterium* (Sheng *et al.*, 2009; Pontes *et al.*, 2015). Singh *et al.*(2014) reported the ability of *Cellulosimicrobium* isolates to produce siderophore.

2.7.2.1.2. Productionof antimicrobial metabolites

Production and release of certain toxic/inhibitory metabolites by antagonistic bacteria has been recognized as a major factor in suppression of root pathogens. These toxic or antimicrobial metabolites mainly include the antibiotics, bacteriocins,

hydrocyanic acid (HCN) and several other extracellular metabolites (Pal and Jalali, 1998).

HCN inhibits the electron transport, thereby energy supply to the cell is disrupted leading to the death of the organism. It inhibits proper functioning of enzymes and natural receptors by reversible mechanism of inhibition (Corbett, 1974). Ahemad *et al.* (2008) reported production of HCN by *Azotobacter* sp.Raval and Desai (2012), detected HCN production in some isolates of PGPR isolated from rhizosphere of sunflower.

Azotobacter association with crop improvement is secretion of ammonia in the rhizosphere in the presence of root exudates, which helps in modification of nutrient uptake by the plants (Narula and Gupta, 1986). In a study conducted by Joseph *et al.* (2007) ammonia production was detected in *Azotobacter* spp. Ahemad *et al.* (2008) reported that *Azotobacter* sp. is able to produce ammonia. Singh *et al.*(2014) reported the ability of *Cellulosimicrobium* isolates to produce ammonia.

2.8. BIOLOGICAL CONTROL OF PLANT PATHOGENS

Biological control of plant diseases is gaining attention due to increased pollution concerns because of pesticides use for crop protection and development of pathogen resistance (Wisniiewski and Wilson, 1992).

2.8.1. *Rhizoctonia solani*

Rhizoctonia solani, a soil borne plant pathogen causes root rot, and damages a wide range of host plants. The pathogen reduces plant growth by rotting the roots and thus reducing the ability of the plants to take up water and nutrients (Wallwork, 1996).

Agarwal and Singh (2002) reported antagonistic activity of *Azotobacter* against*Rhizoctonia solani.* Fatima *et al.* (2009) inoculated two isolates of *Azotobacter*

in wheat infected with *R. solani*. When tested in pot experiment they found increased germination, biomass as well as root-shoot length of wheat as strains hinder *Rhizoctonia solani.* In a similar study conducted by Chauhan *et al.* (2012) *in vitro* results were reproduced under laboratory conditions in pot experiments in the greenhouse and selected ten isolates of *A. chroococcum* were found to be effective biocontrol agents against *R. solani* incotton and six against *R. solani* in rice.

2.8.2. *Fusarium oxysporum*

Fusarium oxysporum has received a considerable attention over the last few decades because of its ability to cause vascular diseases on a wide range of crops including non legumes, fruits and vegetables. The main target crop plants are tomato, cucumber, water melon, pepper, bean, cotton and groundnut (Rasheed *et al.*, 2004). The genus *Fusarium* is considered as an important group of fungi due to its diversity, cosmopolitan nature and ability to cause plant diseases and storage rots (Summerell *etal.*, 2006).

Agarwal and Singh (2002) reported antifungal activity of *Azotobacter* sp. against *Fusarium oxysporum* and *Aspergillus* sp. Cavaglieri *et al.* (2005) studied the effect of *Azotobacter* sp. and *Arthrobacter* sp. on root colonization of *Fusarium verticillioides* They also reported the growth inhibition and *in vitro* suppression of fumonisin B1 production by *Azotobacter armaniacus*. Similiarly Mali and Bodhankar (2009) showed the inhibition of *Fusarium oxysporum, Aspergillus flavus* by *Azotobacter chroococcum* isolated from rhizosphere soil of groundnut plant. *Azotobacter* has an ability to produce antifungal antibiotics and fungalstatic compounds against pathogens like *Fusarium, Alternaria and Helminthosporium* (Belhekar and Bhosale, 2010). Chauhan *et al.* (2012) found that twelve isolates of *Azotobacter* were inhibitory against *F. oxysporum* in tomato. Bhosale *et al.* (2013) isolated promising antifungal activity exhibiting strain of *Azotobacter vinelandii* and concluded that these strains can be used as bio control agent against phytopathogenic

F. oxysporum. Cellulosimicrobium was found to inhibit the mycelia growth of *Fusarium oxysporum* (Nabti *et al.*, 2014).

2.8.3. *Ralstonia solanacearum*

This bacterium has an unusual wide host range and highly susceptible crops are tomato, potato, egg plant, chilli, bell pepper and peanut. Tomato bacterial wilt disease is an important tomato disease that is widely distributed in tropical, subtropical and temperate regions. The disease is caused by *Ralstonia solanacearum* (Yabuuchi *et al.*, 1995). *R. solanacearum* can live in the soil for a long time in non-hosts (Grey and Steck, 2001) which results in ineffectiveness for the control of bacterial wilt disease by crop rotation. The use of resistant varieties is thought to be the most effective way of controlling tomato bacterial wilt, however the development of resistant varieties takes a long time and their use is also limited by strain resistance specificity. Therefore, developing effective biological control agents is very important for the control of tomato bacterial wilt.

Three antagonists were found to be very promising for the control of bacterial wilt in tomato (Nguyen and Ranamukhaarachchi, 2010). These bioinoculants were also found to increase the fruit weight, biomass and plant height. Seleim *et al.* (2011) reported that PGPR (*Pseudomonas fluorescens, P. putida* and *Bacillus subtilis)* could suppress bacterial wilt in tomato under field conditions. Maji and Chakrabartty (2014) found that *Pseudomonas* spp. obtained from rhizosphere could be used as biocontrol agents against bacterial wilt disease in tomato.

2.9. EFFECT OF BIOINOCULANTS ON GROWTH AND YIELD OF TOMATO

Tomato (*Lycopersicun esculentum* Mill) is one of the most widely grown vegetable crops in the world. The popularity of tomato among consumers has made it an important source of vitamins A and C in diets (Madhavi and Salunke, 1998). It is

the most popular vegetable crop of the world due to its wider adaptability and multifarious uses.

The cultivation of tomato has spread throughout the world occupying an area of 3.5×10^6 ha with the production of 1×10^6 tons (FAO, 2010). In addition to its economic importance, tomato consumption has recently been demonstrated to be beneficial to human health, because of its content of phytochemicals such as lycopene, β-carotene, flavonoids, vitamin C and many essential nutrients (Ordookhani *etal.*, 2010).

Tomato is highly responsive to nitrogen fertilizer application, where N availability may be limited and time of the application is critical (Taber, 2001). Being a heavy feeder and exhaustive crop, requires large quantities of inorganic and organic nutrient inputs (Sepat *et al.*, 2012).The yield response of tomato to the added nitrogen through inorganic fertilizers was evident to a considerable level.

Effect of biofertilizers on the growth, yield and quality of tomato has been reported by a few researchers. Stimulation of growth and yield of tomato in red ferrallitic soils was reported by inoculation of *Azotobacter chroococcum.*(Martinez *et al.*, 1993). They also reported maximum fresh weight, dry weight, ascorbic acid, TSS and minimum fruit cracking in seedling treatment with*Azotobacter*. A combination of nitrogen and seedling inoculation with *Azotobacter* minimized incidence of fruit cracking in tomato, as compared to N application alone or soil inoculation with *Azotobacter* (Kamili *et al.*, 2002). Bhadoria *et al.* (2005) observed maximum fresh weight and ascorbic acid content in treatment with 75 kg N/ha + seedling inoculation with *Azotobacter*.

Materials and methods

3. MATERIALS AND METHODS

The present study entitled, 'Utilization of free-living diazotrophic bacteria from Wayanad as a biofertilizer' was carried out at the Department of Agricultural Microbiology, College of Horticulture, Vellanikkara, during 2013-15. Details of materials used and the methods followed are presented below.

3.1. MATERIALS

3.1.1. Chemicals, glasswares and plastic wares

The chemicals used for the study were obtained from agencies like HIMEDIA, Merck India Ltd., Sisco Research Laboratory (SRL). Molecular biology reagents and buffers were purchased from Bangalore Genei Ltd. and Sigma-Aldrich India Ltd. All plastic wares used were obtained from Tarson India Ltd.Jensens agar, Ashby's agar and *Beijerinckia* agar were used for the isolation of free-living diazotrophicbacteria. The composition of these three media and various reagents used for the study are given in Appendix I and II.

3.1.2. Equipments and machinery

The equipment items available at the Department of Agricultural Microbiology, College of Horticulture, Vellanikkara were used for the study. Sterilization of culture media and glass wares were carried out in autoclave Equitron-7440 SLEFA (Eutech Instruments India). pH of the culture media was checked by using pH meter (Cyberscan-Eutech, Eutech Instruments, India). Inoculation of microorganisms was carried out in sterile condition using laminar air flow chamber (Rotek, Mumbai). Cultures were incubated in incubator-shaker (Merck-Genei-OS 250, Merck India Ltd.).Microbial cultures were stored in ultra-low temperature deep freezer (Haier DW-86L90, Haier International Co. Ltd., China). A compound binocular microscope (Leica-DM500) was used for viewing the morphology of

microorganisms and for photomicrography. Estimation of nitrogen was done using microkjeldahl apparatus (Kelplus, Pelican equipments). DNA amplification was carried out in Eppendorf Mastercycler (Eppendorf, Germany). Centrifugation was carried out in centrifuge (Eppendorf-5804R, Eppendorf, Germany). Visualization of DNA on agarose gel was carried out using UV transilluminator (UVP-Benchtop Transilluminator, USA). Altitude, latitude and longitude of the locations for sample collection were recorded with the help of Global Positioning System GPS (eTrex Vista HCX).

3.2. METHODOLOGY

3.2.1.Collection of rhizosphere soil and plant samples from black pepper growing areas of Wayanad district, Kerala

Rhizosphere soil and plant samples were collected from ten healthy black pepper gardens of Wayanad district, spread over two agro-ecological units (AEU 20: Wayanad Central Plateau and AEU 21: Wayand Eastern Plateau), as per the classification of Nair *et al.* (2011). The locations were identified at random, based on general crop stand at the time of visit. From each location, five random rhizosphere samples were collected and pooled together to get a representative composite soil sample by quartering technique. About 100 g of soil from each location was properly tagged, sealed and stored in refrigerator for further studies. Healthy leaves without any disease symptom or discolouration were also collected in polythene covers and brought to the laboratory.

3.2.2. Isolation and enumeration of free living diazotrophic bacteria from soil

The total diazotrophs present in the samples were isolated and enumerated by serial dilution and plating technique (Johnson and Curl, 1972).

Ten gram of soil sample was added to 90 ml of sterile water to get 10^{-1} dilution and was shaken for 15 minutes in orbital shaker. One ml of suspension from

this dilution was then transferred to a test tube containing 9 ml of sterile distilled water to get 10^{-2} dilution. This procedure was repeated till 10^{-6} dilution was obtained. One ml suspension from 10^{-4}, 10^{-5} and 10^{-6} dilutions was transferred to a sterile Petri dish and 20 ml molten culture media at 42^{O}C (N-free Jensen's agar, Ashby's agar and *Beijerinckia*agar) was poured. The suspension was then mixed with media by rotating the Petri dish in clockwise and anticlockwise directions. After solidification, the plates were incubated for 5 days at room temperature for the development of microbial colonies. After incubation, the number of colonies formed was counted. The final data were expressed as the mean of three replications in terms of number of colony forming unit (cfu) per gram of soil sample. Population of morphotypes of diazotrophs from rhizosphere was also recorded. Predominant colonies were purified and preserved for further studies.

3.2.2.1. Analysis of physico-chemical properties of soil

Ten locations for soil sample collection were grouped under two Agro-Ecological Units (AEUs). Two soil samples, one from each AEU (Kalpetta and Bathery) were analyzed for physico-chemical properties, as detailed in Table 1.

Table 1. Methods used for analysis of physico–chemical properties of soil

Particulars	**Method**	**Reference**
Soil reaction (pH)	Soil water suspension of 1:25 and read in pH meter	(Jackson, 1958)
Organic carbon	Walkley and Black method	(Walkley and Black, 1934)
Available P	Ascorbic acid reduced molybdo phosphoric blue colour method	(Bray and Kurtz, 1945)
Available K	Neutral normal ammonium acetateextract using flame photometer	(Jackson, 1958)
Texture	International Pipette Method	(Piper, 1966)

3.2.3. Isolation and enumeration of free-living diazotrophic bacteria from phylloplane

The isolation of diazotrophs from phylloplane was carried out by leaf impression method (Lamb and Brown, 1970).

In order to isolate diazotrophs on the abaxial and adaxial leaf surface, leaf imprints were made on N-free Jensen's agar, Ashby's agar and *Beijerinckia*agar. An intact leaf was placed on the agar surface. After bacterial isolation, each leaf was used to measure the area, using graph paper method. Plates were incubated at room temperature for 5 days. Population of phylloplane diazotrophs was recorded and expressed in terms of cfu/ cm^2leaf area. In order to compare the suitability of media, average of total population of diazotrophs was calculated separately for rhizosphere and phylloplane samples.

3.2.4. Purification and maintenance of predominant isolates

Twenty predominant isolates were selected based on their population on Jensen's agar, for further screening. Raised and mucoid colonies of diazotrophs were purified by streak plate method. A series of parallel non- overlapping streaks were made on the N-free medium with a sterilized inoculation loop containing bacterial isolate. The single colonies that developed at the end of the streaks were again picked and maintainedas agar slants and stabs in refrigerator and as glycerol stock in deep freezer.

3.2.5. Cultural and morphological characterization

Cultural characteristics of twenty isolates were studied on both nitrogen free Jensen's agar and nutrient agar. Observations on colony characters included size, surface, colour, form, elevation and margin.

For morphological studies, 24 h old culture of twenty selected isolates was used. Gram staining was employed to study the Gram reaction (Gram, 1884). The shape of the bacteria was observed under oil immersion objective of the microscope. Endospore staining was carried out for Gram positive isolates (Cappuccino and Sherman, 1992).

3.2.6. *In vitro* screening of selected isolates for growth and nitrogen fixation

Twenty selected isolates were screened for their ability to grow and fix nitrogen at different levels of pH (5.0, 5.5, 6.0, 6.5 and 7.0).

3.2.6.1.Evaluation of growth of isolates at different pH levels

A preliminaryexperiment was conducted to standardise the time period for obtaining maximum growth in nitrogen free Jensen's broth at pH 7.0 for all the isolates. Population was estimated at 0, 48, 72, 96, 120 and 144 hafter inoculation by serial dilution and plate count method. Since maximum growth was observed at 96 h, growth was evaluated at 96h for remaining pH levels. Twenty selected isolates were screened for theirgrowth and nitrogen fixation, in Jensen's broth adjusted to pH 7.0, 6.5, 6.0, 5.5 and 5.0. pH was adjusted using 0.1N NaOH or 0.1 N HCl. Forty eight hour old culture was inoculated in 50 ml sterile Jensen's broth and incubated at 28°C. Population was estimated after 96h of incubation.

3.2.6.2.Estimation of nitrogen fixation of the isolates at different pH levels

Twenty selected isolates were screened for their efficiency to fix nitrogen in Jensen's broth. 50 ml sterile broth was inoculated with a loopful of 48 h culture. Triplicate samples were maintained for each isolate. The flasks were incubated at room temperature for 15 days, under stationary conditions. At the end of incubation, the cultures were homogenized by shaking and nitrogen fixation was estimated using microkjeldahl method (Jackson, 1958 and Bremner, 1960). Ten ml of the

homogenized culture was drawn and transferred to Kjeldahl's digestion tube and it was kept for pre-digestion overnight after adding 10 ml concentrated H_2SO_4 and 1g of digestion mixture (K_2SO_4:$CuSO_4$:Selenium powder in the ratio100:10:1). It was kept on the digestion unit at 300^0C till it became a clear solution. After cooling, the volume was made upto 25 ml with distilled water. Then it was transferred to microkjeldahl distillation unit. An aliquot of 10 ml of 40% NaOH was added and condensed ammonia evolved was collected in boric acid-indicator mixture in 100ml conical flask kept at delivery end. When colour of the solution changed from reddish pink to bluish green, it was back titrated against 0.05 N H_2SO_4.Amount of nitrogen fixed was determined and the results were expressed as mg of N fixed per gram of carbon source utilized.

$$\text{mg of N/ g of C source} = \frac{(TV - BV) \times N \times 0.014 \times 1000}{V}$$

Where,

TV = Titre value

BV = Blank value

N = Normality of H_2SO_4

Y = Weight of C source

3.2.7. *In vitro* screening of predominant isolates forplant growth promoting activities

Twenty selected isolates were screened forvarious PGP activities like production of indole acetic acid (IAA), siderophore, HCN and ammonia.

3.2.7.1. Screening of isolates for Indole Acetic Acid (IAA) Production

Selectedisolates were screened for the production of plant growth hormone IAA (Brick *et al.*, 1991). Luria Bertani (LB) agar supplemented with 0.06 per cent sodium dodecyl sulphate (SDS) and one percent glycerol was poured in sterile Petri

plates. Overnight cultures of the isolates were spot inoculated and immediately overlaid with sterile discs of Whatman No. 1 filter paper (2cm×2cm). Plates were incubated for four days. Thefilter paper discs were removed from plates and soaked in Salkowski reagent. Bacteria producing IAA were identified by the formation of characteristic pink to red halo around the colony on filter paper.

3.2.7.2.Screening of isolates for hydrogen cyanide (HCN) production

HCN production by isolates was tested using the method suggested by Bakker and Schippers (1987). 48 h old cultures of all the isolates were streaked in a zig zag manner on King's B agar media supplemented with 4.4g/lglycine. Sterile Whatman No. 1 filter paper soaked with picric acid solution was placed in the lid of each Petri plate and incubated at room temperature for 5 days. The isolates were grouped in to three categories, based on their reaction, as follows:

Yellow to light brown	: Weak (+)
Brown	: Moderate (++)
Reddish-brown	: Good (+++)

3.2.7.3.Screening of isolates for siderophore production

Selected isolates were screened for siderophore production using chrome azurol S (CAS) agar medium (Schwyn and Neilands, 1987). 48h old cultures were spot inoculated on CAS agar and incubated at room temperature for 7 days. Development of yellow-orange halo around the colony was considered as positive for siderophore production.

3.2.7.4.Screening of isolates for Ammonia (NH_3) production

Isolates were screened for ammonia production by inoculating 48h old culture into 4 per cent peptone water in test tubes and incubated at room temperature. After

incubation, Nessler's reagent (0.5 ml) was added in each test tube. Development of yellow to brown colour indicates ammonia production by the isolates (Cappuccino and Sherman, 1992). Based on the intensity of colour produced, the reaction was rated as follows:

Yellow: Weak (+)

Orange: Moderate (++)

Brown: Good (+++)

3.2.7.5. In vitro screening for antagonistic activity

The antagonistic activity of all the twenty isolates was tested against three soil borne plant pathogens including two fungi (*Fusarium oxysporum* and*Rhizoctonia solani*)by dual culture methodand one bacterium (*Ralstonia solanacearum*) by cross streaking method. In the dual culture method, 8 mm discs of the fungi were placed in potato dextrose agar (PDA) medium, leaving 2 cm from the edge of Petri plate and incubated at room temperature for 48 hours. After incubation, bacterial antagonists were streaked at opposite side of the same plate by leaving 3-4 cm from the pathogen. The plates were then incubated at room temperature for 2-7 days for inhibition of fungal growth. Three replications were maintained for each isolates. Plates containing pathogen alone kept as control (Dennis and Webster, 1971).

Radial growth of the fungal pathogens was measured when growth of the pathogen in control plate reached maximum. The percent growth inhibition (PGI) was calculated using the formula,

$$PGI\ (\%) = \frac{C\text{-}T}{C} \times 100$$

C - Distance of fungal growth from the point of inoculation to the colony margin in control plate

T - Distance of fungal growth from the point of inoculation to the colony margin in the direction of antagonist

For testing the antagonism against bacterial pathogen *R. solanacearum*, cross-streak assay method (Crawford *et al.*, 1993)on nutrient agar medium was adopted. Selectedisolates were streaked at one end of the Petri plate as a single streak and the pathogen was streaked vertical to the test organism. Plates were incubated at room temperature for 5-6 days and observed for inhibition of growth at junctions.

3.2.8. 16S rDNA sequence analysis of selected isolates

Based on growth, nitrogen fixation, tolerance to acidic pH and PGPR activities, two isolates from rhizosphere soiland two from phylloplane were selected as the promising isolates. Before the evaluation of these four diazotrophs under pot culture experiment, identification was carried out by16S rDNA sequencing.

3.2.8.1.Amplification of 16S rDNA gene

Using micropipette, single colony of the isolate was mixed with 10 µl of sterile water. 2µl of this suspension was used as template for amplification of 16S rRNA gene. The details of primers (Siddapura *et al,* 2010) used are given in Table 2.

Polymerase chain reaction was carried out in Eppendorf Master Cycler (Gradient) using PCR master mix 'Emerald Amp GT PCR'. The composition of the reaction mixture for PCR is given in Table 3.

The reaction was set in 200 µl microfuge tube chilled over ice flakes. A momentary spin was given to mix completely all reagents and set in master cycler for amplification. The details of master cycler programme are given in Table 4.

Table 2. Primers used for 16S rDNA gene amplification

Primer details	Sequence 5'- 3'	Length in bp
8 F	AGAGTTTGATCCTGGCTCAG	20
1522 R	AAG GAG GTG ATC CAG CCG CA	20

Table 3.Composition of PCR reaction mixture

Component	Per reaction volume required
Master mix	12.5 µl
Template	2.0 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
dH_2O	9.5 µl
Total	25.0 µl

Table 4.Details of master cycle programme

No.	Step	Temperature (^{0}C)	Time (min)
1	Initial denaturation	95	3.00
2	Denaturation	94	1.30
3	Annealing	55	0.40
4	Primer extension	72	01.30
5	Steps 2-4	34 cycles	-
6	Final extension	72	20.00
7	Final hold	4	10.00

3.2.8.2. Agarose gel electrophoresis

The quality of isolated DNA was evaluated though agarose gel electrophoresis (Sambrook *et al.*, 1989). 100 ml of 1X TAE buffer was prepared from the 50X TAE (pH 8.0) stock solutions. 1.0 g of agarose (Genei, Low EEO) was added to the 1X TAE buffer in conical flask. Agarose was dissolved in buffer by heating and cooled to 42-45^{0}C. Ethidium bromide prepared from a stock of 10 mg ml^{-1} was added to it at a concentration of 0.5 µg ml^{-1} and mixed well without the formation of bubbles. After wiping the gel casting tray and comb with alcohol, the comb was placed properly in the casting apparatus. Prepared agarose was poured into the tray and left for solidification for 30-45 minutes. To make the well, the comb was pulled out and gel was placed in the buffer tank containing 1X TAE buffer with well side directed towards the cathode. 2 µl of the PCR product mixed with gel loading dye was carefully loaded into the wells using a micro pipette. The Gene Ruler 1 kb DNA ladder was used as the molecular weight marker. The cathode and anode of the electrophoresis unit were connected to the power pack and the gel was run at constant voltage of 100 V. Thepower was turned off when the tracking dye reached at about 3 cm from the anode end.

3.2.8.3. Gel documentation

The DNA bands separated by electrophoresis were viewed and photographed using gel documentation imaging system.

3.2.8.4. Purification and sequencing of PCR product

The PCR product was purified and sequenced at Vision Scientific Services Angamaly, using the primers 8F and 1522r.

3.2.8.5. Nucleotide sequence analysis

The Base Local Alignment Search Tool for Nucleotides (blastn) programme (http: //blast.ncbi.nlm.nih.gov/Blast) was used to find out the homology of the nucleotide sequences.

3.2.9. *In planta* evaluation of selected isolates

Four most promisingisolates of diazotrophs including two isolates from rhizosphere soil and two from phylloplane were selected based on growth, nitrogen fixation, tolerance to acidic pH and PGPR activities for *in planta* evaluation using tomato as the test crop. The experiment was conducted during January 29 to June 2, 2015 in the net house at the Department of Agricultural Microbiology, College of Horticulture, Vellanikkara. KAU commercial formulation of *Azotobacter* served as positive control. Treatment without any bioinoculant, chemical nitrogen and FYM was the absolute control. The experiment included two levels of fertilizer nitrogen (0% and 75% of recommended dose) and two levels of FYM (0% and 50% of recommended dose). P & K was provided as per POP recommendations for all the treatments. POP recommendation of fertilizer dose for tomato is 75: 40:25 N: P_2O_5: K_2O. Half the dose of N, full P and half K were applied as basal dose. One fourth N and half K were applied 20-30 DAP. Balance N was applied 2 MAP. 50% of the POP Recommended dose of FYM (20-25 t/ha) was also applied at the time of land preparation in treatments T_{11} to T_{16}.

The treatment details of experiment were as follows

Design : CRD
Replications : 3*
Treatments : 18
Variety : Anagha

* Each replication with four pots, each pot having one plant.

Treatments:

T_1 : Promising diazotroph-1 (from soil)

T_2 : Promising diazotroph-2(from soil)

T_3 : Promising diazotroph-3 (from phylloplane)

T_4 : Promising diazotroph-4 (from phylloplane)

T_5 : Commercial formulation of *Azotobacter* (KAU)

T_6 : T_1+ 75 per cent of RD of N

T_7 : T_2 + 75 per cent of RD of N

T_8 : T_3+ 75 per cent of RD of N

T_9 : T_4+ 75 per cent of RD of N

T_{10} : T_5+ 75 per cent of RD of N

T_{11} : T_6 + 50 per cent of RD of FYM

T_{12} : T_7+ 50 per cent of RD of FYM

T_{13} : T_8+ 50 per cent of RD of FYM

T_{14} : T_9+ 50 per cent of RD of FYM

T_{15} : T_5+ 50 per cent of RD of FYM

T_{16} : T_{10}+ 50 per cent of RD of FYM

T_{17} : 100 per cent N and FYM

T_{18} : Absolute control (No bioinoculant/ chemical N/ FYM)

3.2.9.1. Preparation of potting mixture and planting

The potting mixture was prepared with sand: soil: cow dung (1:1:1) and it was sterilized by adding 2% formaldehyde solution. This mixture was then covered with polythene film to retain vapours in potting mixture. After 15 days the mixture was raked thoroughly and left opens for 7 days. This sterile potting mixture was filled in pots. Disease free seeds of tomato obtained from Olericulture Department, College of Horticulture, Vellanikkara, were used for pot culture study.

3.2.9.2. Preparation of talc based formulation of the inoculums

Single colonies of each isolate were inoculated into 300ml Jensen's broth. After incubation at room temperature for four days, broth was mixed with sterilized talc@ 250 ml per kg talc so that the moisture content in the final product was 30-40%. This was kept for curing for 24 h. Before application of the inoculum, microbial population was enumerated by serial dilution and plate count method and microbial count was standardized for application in each treatment.

3.2.9.3. Seed treatment

A thick paste of the carrier based inoculums was prepared @ 500g culture for 5kg seed.Seeds were moistened by sprinkling rice gruel water and then gently mixed with the slurry without damaging seed coat. Treated seeds were spread evenly and dried under shade for 30 minutes and were sown immediately.

3.2.9.4. Seedling dip

A slurry of the culture was prepared by mixing carrier based inoculum with water in the ratio 1:1.5and roots of the seedlings were dipped in the slurry for 20 minutes at the time of transplanting.

3.2.9.5. Soil and foliar application

Inoculum was applied at monthly interval as soil application and foliar spray for isolate from soil and phylloplane respectively.

3.2.9.6. Observations

Biometric characters like germination, plant height, number of leaves, stem girth, leaf area, days to flowering, fresh weight and volume of root, root length, fresh and dry weight of plant were recorded. Yield parameters like average fruit weight,

number of fruits per plant, and yield per plant were also recorded. Observations on incidence of pest and disease were also made.

3.2.9.6.1. Germination

Number of seeds germinated was counted and percentage germination was calculated using the following formula:

$$\text{Percentage germination} = \frac{\text{No. of seeds germinated}}{\text{Total no. of seeds treated}} \times 100$$

3.2.9.6.2. Plant height

Height of plants was measured from ground level i.e., from base of plant to the tip of the topmost leaf at 30 days interval and expressed in centimeters.

3.2.9.6.3. Number of leaves

Total number of leaves per each plant was counted at 30 days interval and mean number of leaves was obtained.

3.2.9.6.4 Girth of stem

Girth of the stem of each plant was measured at a height of 5cm from the soil surface at 30 days interval and average was found out.

3.2.9.6.5. Leaf area

Second, third and fourth leaves of the primary branch were selected to measure the leaf area and average was found out. Three sets of leaves were taken from each plant.

3.2.9.6.6. Days to flowering

Number of days taken from sowing to opening of first flower was recorded in all the plants and mean was found out.

3.2.9.6.7. Root length

Plants were uprooted and length of roots of each plant was recorded in centimeters at the time of last harvest and average was found out.

3.2.9.6.8. Fresh weight of root

After uprooting the plants, roots were separated, washed and fresh weight of root was taken.

3.2.9.6.9. Volume of root

Volume of root was recorded by water displacement method. Amount of increase in water level was measured and expressed in cm^3.

3.2.9.6.10. Fresh and dry weight of plants

Plants were uprooted, cleaned and fresh weight was taken. Dry weight was also recorded after drying in oven at 60 ± 5^0C.

3.2.9.6.11. Number of fruits per plant

Total numbers of fruits harvested from each plant from the various harvest were counted and mean was obtained.

3.2.9.6.12. Average fruit weight

Weight of fruits harvested from each plant from the various harvests was recorded and average weight was worked out.

3.2.9.6.13. Yield per plant

Fruit yield was recorded from each plant and expressed in g/plant.

3.2.9.6.14. Pest and disease incidence

Incidence of pest and diseases on plants were recorded throughout the period of study.

3.2.9.6.15. Bacterial wilt disease incidence

Incidence of bacterial wilt was also observed during the growth period of crop.

3.2.10. Enumeration of bioinoculants at monthly interval

Population of bioinoculants in soil (for rhizosphere isolates) and phylloplane (for phylloplane isolates) was assessed at monthly interval by serial dilution and plating technique and leaf impression method respectively.

3.2.11. Enumeration of endophytic nitrogen fixers

Endophytic nitrogen fixers were isolated and enumerated from leaf, stem and root samples (McInroy and Kloepper, 1995). The whole plants were uprooted and washed thoroughly under running tap water. Leaf, stem and root were cut into small bits with a sterile knife and one gram of each was weighed. The samples were surface sterilized under aseptic condition using 1% sodium hypochlorite solution for three minutes and washed three times in sterile water. After that, samples were washed in 0.02 M sterile potassium phosphate buffer with pH 7.0. 1ml aliquot from the final buffer wash was transferred into a sterile Petri plate and molten, cooled Jensen's agar was poured over it, which served as sterility check. The samples were ground with a sterile mortar and pestle in 9 ml of sterile buffer. From this 1 ml was transferred to 9

ml sterile buffer in test tube. The dilution was serially repeated to get 10^{-3} dilution. From this 1 ml was transferred to Petri plate and molten, cooled Jensen's agar was poured. Plates were incubated at room temperature for five days.

3.2.12. Analysis of nitrogen content in plant at harvest and index leaf

Index leaves were collected from the plant for estimation of nitrogen content at 60DAS. Fully expanded leaves, just below the last opened flower cluster was taken for analysis. Samples from individual treatment were dried in an oven at 60^0C till constant weight was observed and further ground to fine powder using pestle and mortar. The powdered samples were used for nitrogen estimation.

Estimation of nitrogen content in plant at the time of harvest was also done. Plants were uprooted and dried in oven at 60^0Ctill constant weight was observed. Powdered samples were used for analysis of nitrogen.

Nitrogen content in index leaf and plant (at harvest) was estimated by modified microkjeldhal method (Jackson, 1958).

3.2.13. Estimation of total nitrogen in soil

Total nitrogen content in soil from each treatment was estimated at 60 DAS and harvest by micro kjeldahl method (Jackson, 1958).

3.2.14. Statistical analysis

Analysis of variance was done on the data collected using the statistical package MSTAT (Freed, 1986). Multiple comparisons among the treatment means were done using DMRT

Results

4. RESULTS

The results of the study on 'Utilization of free-living diazotrophic bacteria from Wayanad as a biofertilizer' conducted during the period 2013-2015 at Department of Agricultural Microbiology, College of Horticulture, Vellanikkara are presented in this chapter.

4.1. ENUMERATION OF FREE-LIVING DIAZOTROPHIC BACTERIA FROM SOIL

Details of the locationsofrhizosphere soil and plant samples collected from ten healthy black pepper gardens in different locations of Wayanad district are given in Table 5 and Plate 1.

Population of diazotrophic bacteria in rhizosphere soil varied from 9.3×10^4 to 19.6×10^4 cfu/g of soil (Table 6 and Plate 2). Out of the ten locations, maximum population of nitrogen fixers (19.6×10^4 cfu/g of soil) was obtained from Kochangode and minimum population was obtained from Pazhaya Vythiri (9.3×10^4 cfu/g of soil). Among the three media, maximum population was obtained from Jensen's agar (19.6×10^4 cfu/g of soil) and minimum population was obtained from Ashby's agar (9.3×10^4 cfu/g of soil).

Mean population of diazotrophs on the three media is given in Table 7. Based on this, Jensen's agar was found to be better since highest mean population of 16.23 cfu/g soil was obtained. This was followed by *Beijerinckia* agar (13.93×10^4 cfu/g soil) and Ashby's agar (12.81×10^4 cfu/g soil). Therefore Jensen's medium was used for further experiments with the nitrogen fixing bacteria.

Population of morphotypes of diazotrophs in rhizosphere soil from different locations is given in Table 9. A total of six different morphotypes were obtained from rhizosphere soil. Among these, medium sized, mucoid, circular colony was the

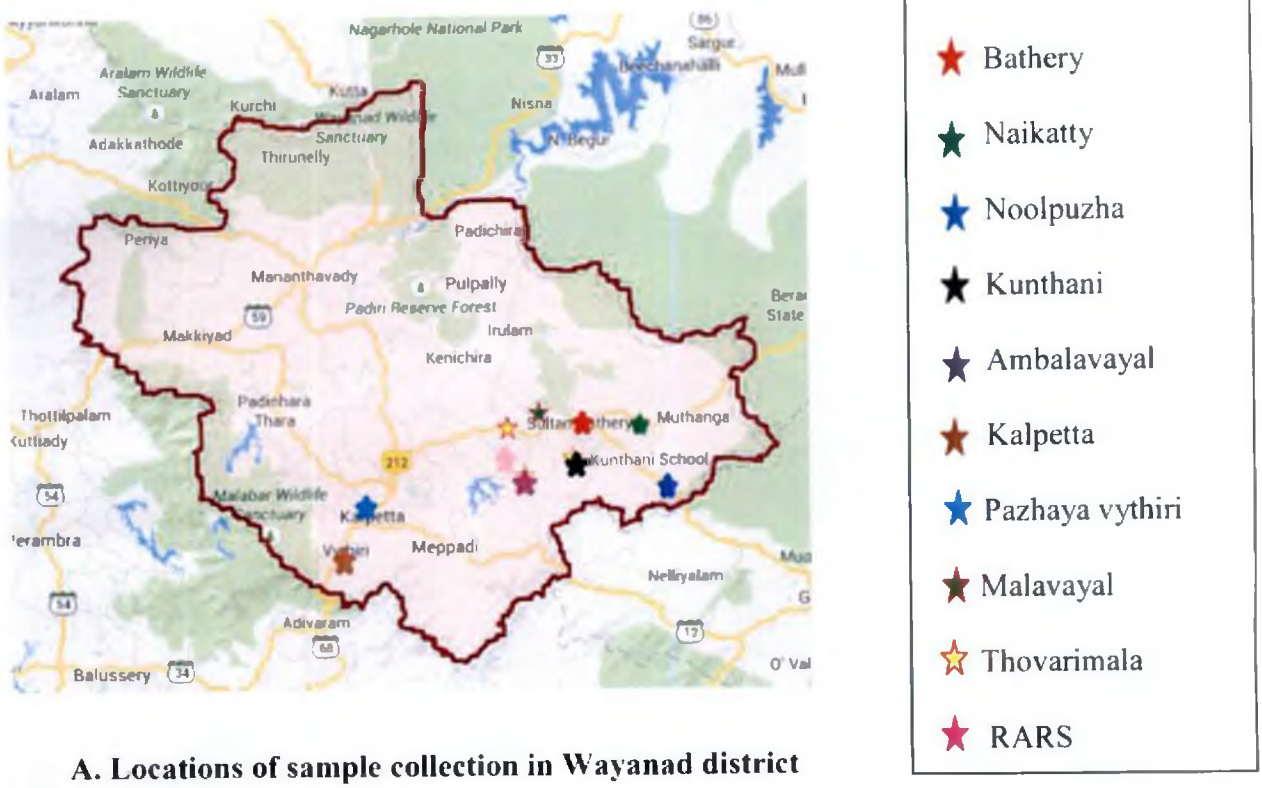
predominant one. Six morphotypes were obtained from Bathery and Kochangode. Only one type of morphotype was obtained from Naikatty. Among the three media maximum number (6) of morphotypes was obtained on Jensen's agar followed by *Beijerinckia* agar (5) and least on Ashby's agar (4).

4.1.1. Analysis of physico-chemical properties of soil samples

Physico-chemicalanalysis oftwo soil samples representing two agro-ecological units (AEU 20: Kalpetta and AEU 21: Bathery) revealed that the texture was sandy loam. All the soil samples were acidic in nature with pH below 5.0. Organic carbon content was 1.5% in Kalpetta and 1.7% in Bathery. Details are given in Table 8.

Table 5. Details of soil and leaf samples collected from black pepper

Sl. No.	Location	Code	Geographical position		
			Latitude (^{0}N)	Longitude (^{0}E)	Altitude (ft)
1.	Ambalavayal	A	11.60739	076.21736	3003
2.	Bathery	B	11.62914	076.26125	2914
3.	Kochangod	Kd	11.61738	076.22922	2951
4.	Kalpetta	K	11.63171	076.22413	3048
5.	Malavayal	M	11.63152	076.24893	2723
6.	Naikatty	Nk	11.67329	076.30635	3077
7.	Noolpuzha	N	11.67428	076.30541	3030
8.	Pazhaya Vythiri	P	11.53868	076.30516	20575
9.	RARS	R	11.61048	076.21178	3027
10.	Thovarimala	T	11.63073	076.25735	2918



A. Locations of sample collection in Wayanad district

Ambalavayal

Pazhaya vythiri

B. Collection of rhizosphere soil and leaf samples

Plate 1: Black pepper gardens in Wayanad selected for sample collection

Table 6. Population of freeliving diazotrophs in rhizosphere of black pepper

Location	Population of free living diazotrophs ($\times 10^4$ cfu per gram of soil)*		
	Jensen's agar	***Beijerinckia* agar**	**Ashby's agar**
Ambalavayal	17.3^{c}	15.0^{c}	10.0^{h}
Bathery	15.3^{h}	13.3^{f}	14.3^{c}
Kochangod	19.6^{a}	16.0^{a}	15.6^{a}
Kalpetta	16.3^{f}	13.3^{f}	12.3^{f}
Malavayal	16.6^{e}	14.6^{d}	12.0^{g}
Naikatty	15.6^{g}	14.6^{d}	13.0^{e}
Noolpuzha	19.0^{b}	15.6^{b}	15.3^{b}
Pazhaya Vythiri	12.6^{j}	11.3^{h}	9.3^{i}
RARS	13.0^{i}	13.3^{e}	13.3^{d}
Thovarimala	17.0^{d}	12.3^{g}	13.0^{e}

* Mean of three replications

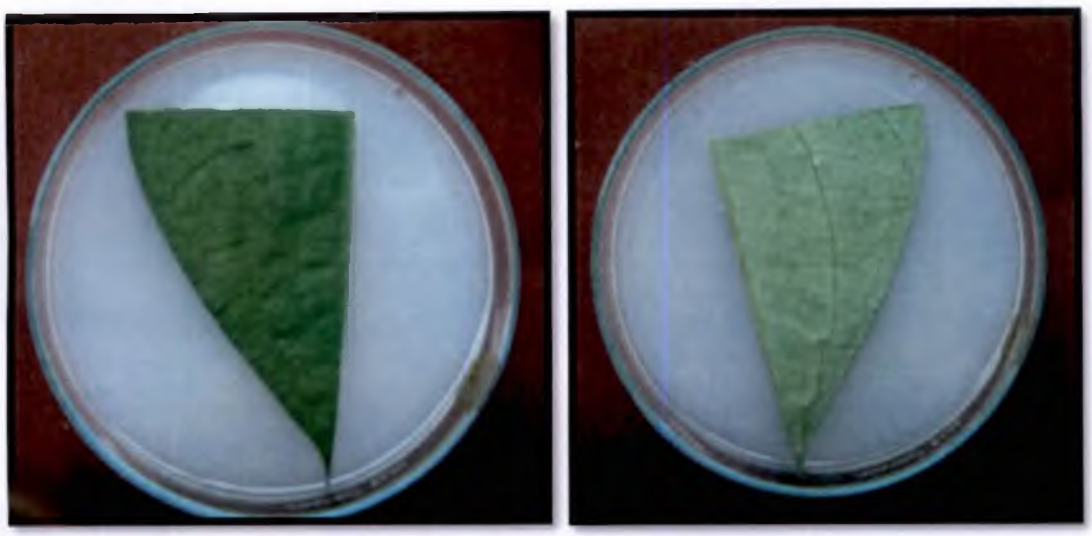
Table 7. Mean population of free living diazotrophs in rhizosphere soil on three media

Media	Mean population ($\times 10^4$ cfu/g of soil)
Jensen's agar	16.23
Beijerinckia agar	13.93
Ashby's agar	12.81

Table 8. Chemical properties of soil sample collected

Parameters	Kalpetta (AEU 20)	Remarks	Bathery (AEU 21)	Remarks
pH	4.5-5	Very strongly acidic	3.5-5	Very strongly acidic
Organic carbon (%)	1.5	Medium	1.7	High
Available phosphorus	25 kg/ha	High	25kg/ha	High
Available potassium	195 kg/ha	Medium	195kg/ha	Medium

A. Isolation from rhizosphere soil



Ventral side **Dorsal side**

B. Isolation from phylloplane

Plate 2: Isolation of free living diazotrophs

Table 9. Population of different morphotypes of diazotrophs in rhizosphere soil at different locations

Location	($\times 10^4$ cfu per gram of soil)*																	
	Morphotype																	
	A			B			C			D			E			F		
	Jen	Bei	Ash	Jen	Bei	Ash	Jen	Bei	Ash	Jen	Bei	Ash	Jen	Bei	Ash	Jen	Bei	Ash
Ambalavayal	10.3	-	-	7.1	-	-	-	-	-	-	-	-	-	15.0	5.2	-	-	4.8
Bathery	6.4	9.4	-	4.2	8.1	-	-	-	5.8	-	9.1	-	-	-	8.5	-	9.1	-
Kochangod	16.4	-	-	2.3	-	-	0.9	-	-	-	6.2	-	-	9.8	15.6	-	6.2	-
Kalpetta	-	-	-	10.4	13.3	-	-	-	-	-	-	-	5.9	-	-	-	-	12.3
Malavayal	16.6	-	-	-	14.6	-	-	-	12	-	-	-	-	-	-	-	-	-
Naikatty	-	-	-	15.6	-	-	-	-	-	-	-	-	-	14.6	13	-	-	-
Noolpuzha	10.2	6.4	-	1.8	-	-	3.2	-	15.3	5.0	-	-	1.3	9.2	-	-	-	-
Pazhaya Vythiri	-	-	-	12.6	-	-	-	-	9.3	-	11.3	-	-	-	-	-	11.3	-
RARS	-	-	-	7.1	-	-	-	-	-	3.2	-	-	2.7	13.3	13.3	3.2	-	-
Thovarimala	17.0	-	-	-	12.3	-	-	-	-	-	-	13.0	-	-	-	-	-	13.0

* Observations were made at 10^{-4} dilution

99Jen : Jensen's agar

Bei : *Beijerinckia* agar

Ash : Ashby's agar

A: Medium, mucoid, circular

B: Large, mucoid, circular

C: Small, watery, circular

D: Medium, watery, circular

E: Medium, watery, irregular

F: Large, watery, irregular

4.2. ENUMERATION OF FREE-LIVING DIAZOTROPHIC BACTERIA FROM PHYLLOPLANE

For the isolation of free living diazotrophs from phylloplane, leaf imprints of both dorsal and ventral surface were made on N-free Jensen's agar, *Beijerinckia* agar and Ashby's agar (Plate 2). Details are given in Table 10.

Population of diazotrophs on dorsal surface ranged from 0.47 to 1.23 per cm^2 leaf area and on ventral surface population ranged from 0.41 to 1.38 per cm^2 leaf area. On the dorsal surface, highest population was observed in samples from Malavayal (1.23 cfu/cm^2 leaf area) and lowest population was recorded from Thovarimala (0.47 cfu/cm^2 leaf area). On ventral surface of leaf, highest population was obtained from RARS (1.38 cfu/cm^2 leaf area) and lowest population was obtained from Pazhaya Vythiri (0.41 cfu/cm^2 leaf area).

Kendall coefficient of concordance was performed to assess differential colonization of diazotrophs on dorsal and ventral surface of leaf on the three media (Table 11). It was observed that Kendall's W was found to be significant when a differential rating based on mean rank scores was made. Colonization of diazotrophs was found to be more on dorsal surface, when Jensen's agar and *Beijerinckia* agar was used. However, on Ashby's agar colonization was more on ventral surface of leaf.

Mean population of the diazotrophs on dorsal and ventral surfaces of leaf on the three media is given in Table 12. Among the three media, maximum mean population was obtained on Jensen's agar followed by *Beijerinckia* agar and least on Ashby's agar.

Population of morphotypes of diazotrophs in phylloplane from different locations is given in Table 13. On ventral surface maximum number of five morphotypes was obtained from Bathery. On dorsal surface, maximum number of

Table 10. Population of free living diazotrophs in phylloplane of black pepper

Location	Population of nitrogen fixers (cfu per cm^2 area of leaf)*					
	Jensen's agar		*Beijerinckia* agar		Ashby's agar	
	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral
Ambalavayal	0.93^{a}	0.91^{d}	0.66^{f}	0.84^{c}	0.60^{b}	0.68^{e}
Bathery	1.22^{a}	1.35^{a}	0.90^{c}	1.13^{a}	0.81^{a}	0.48^{i}
Kochangod	1.16^{b}	0.90^{e}	1.07^{b}	0.81^{e}	0.65^{f}	0.75^{c}
Kalpetta	0.92^{d}	1.02^{c}	0.74^{e}	0.73^{g}	0.51^{i}	0.67^{f}
Malavayal	1.23^{a}	1.13^{b}	1.11^{a}	1.13^{a}	0.70^{d}	0.69^{d}
Naikatty	0.89^{e}	0.77^{f}	0.78^{d}	0.76^{f}	0.76^{c}	0.81^{b}
Noolpuzha	1.22^{a}	1.14^{b}	1.13^{a}	0.96^{b}	0.67^{e}	0.86^{a}
Pazhaya Vythiri	0.98^{c}	1.05^{c}	0.65^{f}	0.72^{h}	0.64^{g}	0.41^{j}
RARS	1.22^{a}	1.38^{a}	0.85^{c}	0.83^{d}	0.77^{b}	0.60^{g}
Thovarimala	0.70^{f}	0.89^{de}	0.85^{c}	0.66^{j}	0.47^{j}	0.52^{h}

* Mean of three replications

Table 11. Colonization of diazotrophs on dorsal and ventral surface of leaf

Media	Dorsal/Vental	Mean rank (cfu/ cm^2 area of leaf)
Jensen's agar	Dorsal	5.40
	Ventral	5.15
Beijerinckia agar	Dorsal	3.70
	Ventral	3.30
Ashby's agar	Dorsal	1.45
	Ventral	2.00

Kendall's W **0.739

Table 12. Mean population of free living diazotrophs on phylloplane on three media (cfu/cm^2 area of leaf)

Dorsal/ Ventral	Jensen's agar	*Beijerinckia* agar	Ashby's agar
Dorsal	1.04	0.87	0.65
Ventral	1.05	0.85	0.64

Table 13. Population of different morphotypes of diazotrophs in phylloplane

Location	Dorsal/ Ventral	Morphotype (cfu/cm^2 leaf area)														
		a			b			c			d			e		
		Jen	Bei	Ash	Jen	Bei	Ash	Jen	Bei	Ash	Jen	Bei	Ash	Jen	Bei	Ash
Ambalavayal	D	0.46	-	-	0.32	0.30	-	-	0.36	-	-	-	-	0.15	-	0.60
	V	0.55	-	-	-	0.84	-	-	-	-	-	-	-	0.36	-	0.68
Bathery	D	0.60	-	-	-	0.64	0.23	0.40	-	-	0.22	0.26	0.58	-	-	-
	V	0.32	-	-	0.48	-	-	-	0.73	-	0.54	-	0.48	-	0.40	-
Kochangode	D	0.53	-	-	-	-	-	0.24	-	-	0.39	0.56	0.65	-	0.51	-
	V	0.61	-	-	-	-	-	-	-	-	-	-	0.90	0.20	0.75	-
Kalpetta	D	0.38	-	-	0.19	-	-	-	-	-	-	-	0.74	0.35	0.51	-
	V	0.41	-	-	0.15	-	-	-	-	-	-	-	-	0.46	0.67	0.73
Malavayal	D	0.82	-	-	-	0.43	-	-	0.10	-	0.29	0.70	0.70	-	-	-
	V	0.73	-	-	-	0.20	-	-	-	-	0.4	0.93	0.69	-	-	-
Naikatty	D	-	-	-	0.89	-	-	-	-	-	-	0.78	0.76	-	-	-
	V	0.20	-	-	0.57	-	0.46	-	-	-	-	0.76	0.35	-	-	-
Noolpuzha	D	1.04	-	-	0.38	-	0.67	-	-	-	-	-	-	0.09	0.84	-
	V	0.27	-	-	0.87	-	-	-	-	-	-	0.55	0.85	-	0.41	-
Pazhaya Vythiri	D	-	-	-	-	-	-	-	0.20	-	0.83	-	0.64	-	0.78	-
	V	-	-	-	-	0.42	-	0.35	-	-	0.50	-	0.47	-	0.63	-
RARS	D	-	-	-	0.65	0.38	-	-	-	-	-	0.84	0.41	-	-	-
	V	0.21	-	-	0.51	0.19	-	-	-	-	-	1.02	0.60	-	-	-
Thovarimala	D	0.52	-	-	-	-	-	-	-	-	-	0.85	-	-	-	0.77
	V	0.31	-	-	-	-	-	-	-	-	0.39	0.66	0.35	-	-	0.54

Jen : Jensen's agar

Bei : *Beijerinckia* agar

Ash : Ashby's agarc: Large, watery,irregular

a: Large, mucoid, circular

b: Medium, mucoid, circular

d: Large, watery, circular

e: Small, watery, circular

four morphotypes was obtained from five locations (Ambalavayal, Bathery, Kochangode, Kalpetta, Malavayal). Medium sized, mucoid, circular colony was the predominant morphotype in phylloplane. Among the three media maximum number (5) of morphotypes was obtained in Jensen's agar followed by *Beijerinckia* agar (4) and least in Ashby's agar (3).

In the present study, a total of forty three isolates of diazotrophic bacteria were obtained, including twenty five isolates from rhizosphere soil and eighteen from phylloplane. From these twenty predominant isolates, including eleven isolates from rhizosphere soil and nine isolates from phylloplane were selected for further screening based their population on Jensen's agar.

4.3. CHARACTERIZATION OF PREDOMINANT ISOLATES

Twenty isolates selected for further studies were named by a 3-5 letter isolate code. The first letter used for all the isolates was 'N' (nitrogen fixer). This was followed by alphabet representing the location. 'S' and 'P' was given for representing isolates from rhizosphere soil and phylloplane respectively. Isolates from dorsal and ventral surface of leaf were represented by 'D' and 'V' respectively.

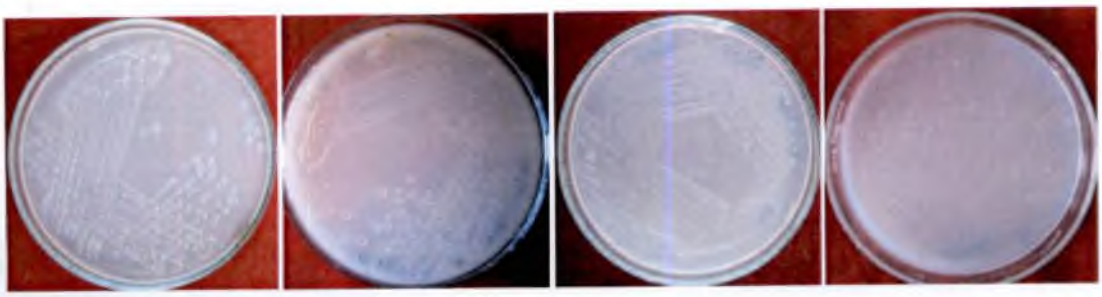
All the twenty isolates were characterized based on morphological and cultural tests. Colony characters of the isolates were observed on Jensen's agar (Table 14; Plates3 and 4) and nutrient agar (Table 15; Plates 5 and 6). On Jensen's agar, surface of the colonies were found to be watery or mucoidal, circular in shape and raised or flat in elevation. Size of the isolates ranged from small (0.3 cm) to large (>0.5 cm). Surface of most of the isolates was observed to be mucoidal (15) than watery (5). All the twenty isolates were found to be circular with entire margin. Most of the isolates (17) had raised colonies except three isolates which were flat. Morphological characterization of the isolates revealed that, majority of the isolates (15) were Gram positive rods. Only five isolates were found to be Gram negative rods. None of the isolates produced endospores.

Table 14. Colony and morphological characters of selected diazotrophs on Jensen's agar medium

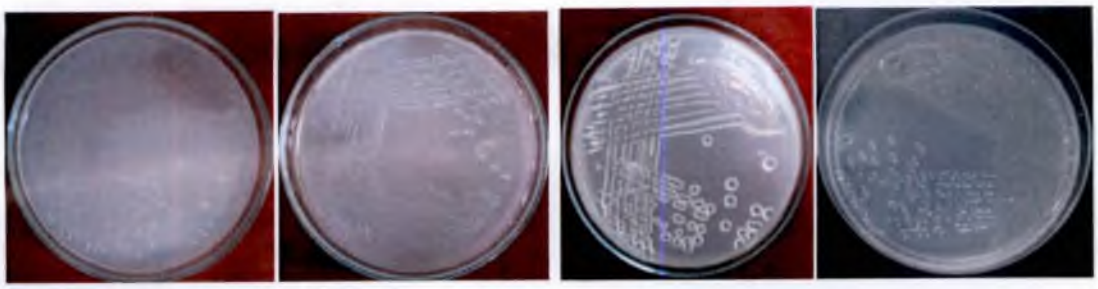
Location	Isolates	Size	Surface	Form	Elevation	Margin	Gram reaction	Shape of cell
Thovarimala	NTS-1	Small	Watery	Circular	Raised	Entire	+ve	Rod
	NTS-2	Medium	Mucoid	Circular	Raised	Entire	+ve	Rod
	NTS-3	Medium	Mucoid	Circular	Flat	Entire	+ve	Rod
Kalpetta	NKS-1	Medium	Mucoid	Circular	Raised	Entire	+ve	Rod
	NKS-2	Medium	Watery	Circular	Raised	Entire	+ve	Rod
Bathery	NBS	Medium	Mucoid	Circular	Raised	Entire	-ve	Rod
Kochangode	NKdS	Large	Mucoid	Circular	Raised	Entire	+ve	Rod
Ambalavayal	NAS	Medium	Watery	Circular	Flat	Entire	+ve	Rod
Pazhaya Vythiri	NPS-1	Large	Mucoid	Circular	Raised	Entire	+ve	Rod
	NPS-2	Medium	Mucoid	Circular	Raised	Entire	+ve	Rod
	NPS-3	Medium	Mucoid	Circular	Raised	Entire	+ve	Rod
Kalpetta	NKPD	Medium	Watery	Circular	Flat	Entire	+ve	Rod
	NKPV-1	Medium	Mucoid	Circular	Raised	Entire	-ve	Rod
	NKPV-2	Medium	Mucoid	Circular	Raised	Entire	+ve	Rod
Kochangode	NKdPV	Medium	Mucoid	Circular	Raised	Entire	+ve	Rod
Noolpuzha	NNPD	Medium	Watery	Circular	Raised	Entire	+ve	Rod
	NNPV	Medium	Mucoid	Circular	Raised	Entire	-ve	Rod
Naikatty	NNkPV	Medium	Mucoid	Circular	Raised	Entire	+ve	Rod
Pazhaya Vythiri	NPPV	Medium	Mucoid	Circular	Raised	Entire	+ve	Rod
Ambalavayal	NAPV	Medium	Mucoid	Circular	Raised	Entire	-ve	Rod

N: Nitrogen fixer S: Rhizosphere soil P: Phylloplane D: Dorsal V: Ventral

<0.3 cm: Small 0.3-0.5 cm: Medium >0.5 cm: Large



NTS-1 NTS-2 NTS-3 NKS-1



NKS-2 NBS NKdS NAS

NPS-1 NPS-2 NPS-3

Plate 3: Colony morphology of diazotrophs from rhizosphere soil on Jensen's agar

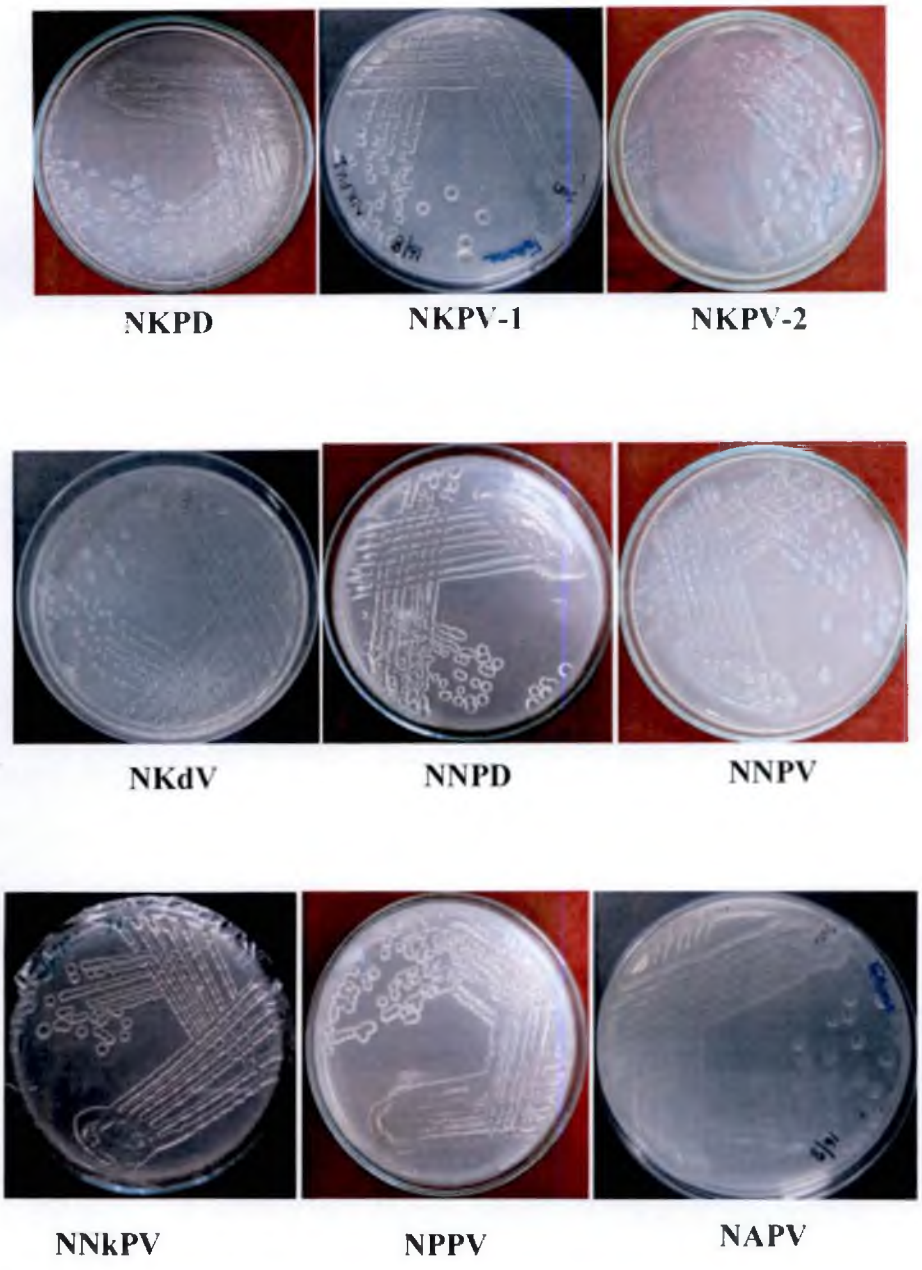


Plate 4: Colony morphology of diazotrophs from phylloplane on Jensen's agar

Table 15. Colony characters of selected diazotrophs on nutrient agar medium

Isolates	Size	Surface	Colour	Form	Elevation	Margin
NTS-1	Medium	Smooth	White	Circular	Flat	Entire
NTS-2	Large	Smooth	White	Circular	Flat	Entire
NTS-3	Large	Smooth	Cream	Circular	Flat	Entire
NKS-1	Medium	Smooth	Yellow	Circular	Flat	Entire
NKS-2	Medium	Smooth	Yellow	Circular	Flat	Entire
NBS	Medium	Smooth	White	Circular	Flat	Entire
NKdS	Medium	Smooth	White	Circular	Flat	Entire
NAS	Large	Smooth	White	Circular	Flat	Entire
NPS-1	Medium	Smooth	White	Circular	Flat	Entire
NPS-3	Small	Smooth	Cream	Circular	Flat	Entire
NKPD	Large	Smooth	Cream	Circular	Flat	Entire
NKPV-1	Medium	Smooth	White	Circular	Flat	Entire
NKPV-2	Small	Smooth	White	Circular	Flat	Entire
NKdPV	Large	Smooth	White	Circular	Flat	Entire
NNPD	Large	Smooth	White	Circular	Flat	Entire
NNPV	Medium	Smooth	Yellow	Circular	Flat	Entire
NNkPV	Medium	Smooth	Yellow	Circular	Flat	Entire
NPPV	Small	Smooth	Yellow	Circular	Flat	Entire
NAPV	Large	Smooth	White	Circular	Flat	Entire

<0.2 cm: Small 0.2-0.3 cm: Medium 0.3-0.6 cm: Large

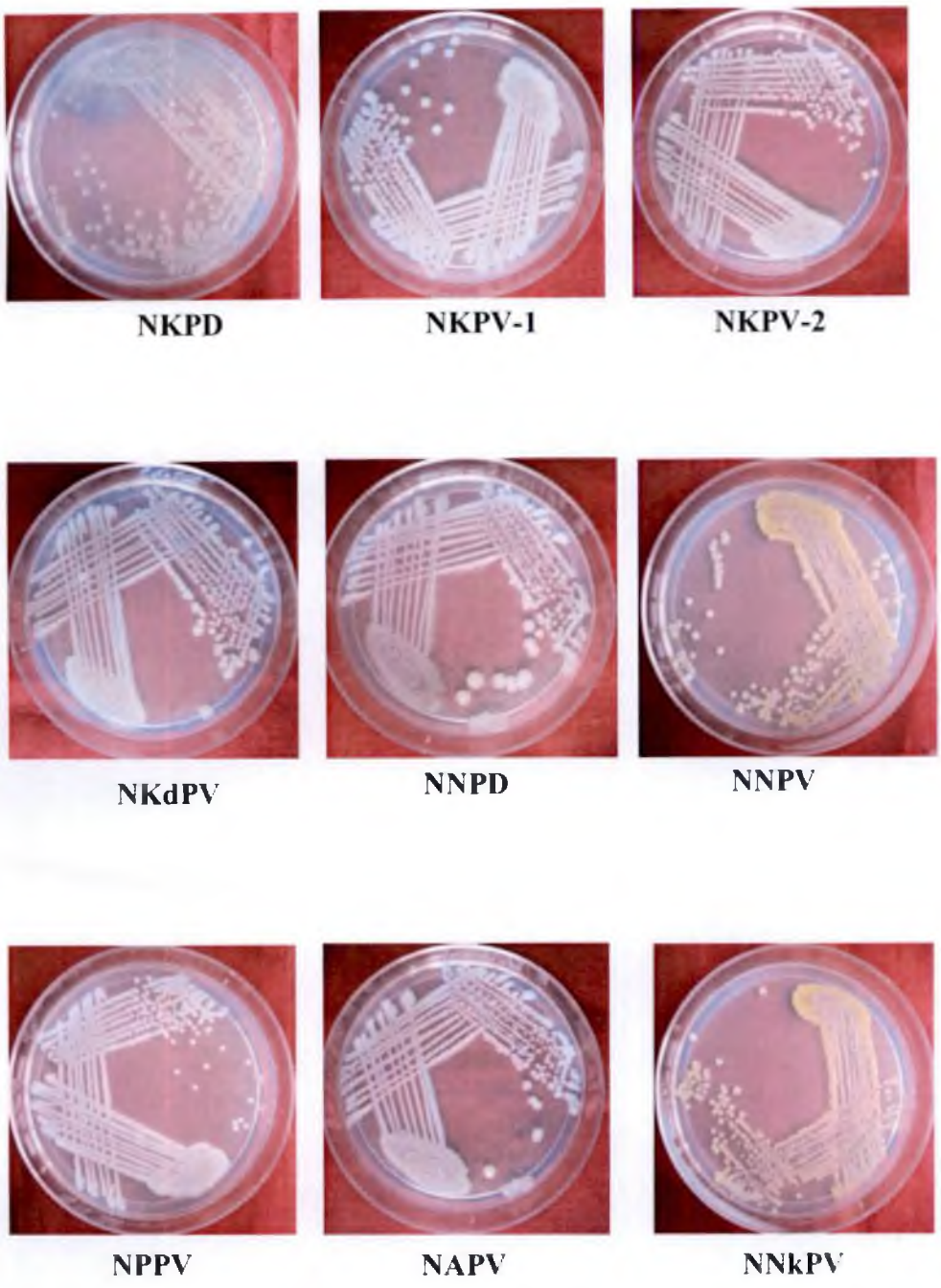


Plate 6: Colony morphology of diazotrophs from phylloplane on nutrient agar

On nutrient agar, all the isolates produced circular, flat colonies with entire margin and exhibited variation in colony size and pigmentation. Size of colony on nutrient agar ranged from small (0.2 cm) to large (0.6 cm). Most of the isolates (12) produced white pigmentation, three isolates produced cream pigmentation and five produced yellow pigmentation. Compared to Jensen's agar, colonies were smalleron nutrient agar.

4.4. *IN VITRO* SCREENING OF PREDOMINANT ISOLATES FOR ACID TOLERANCE AND NITROGEN FIXATION

A preliminary screening of isolates for standardization of time period at which maximum growth occur was carried out in Jensen'sbroth adjusted to pH 7.0 (Table 16 and Fig. 1). For this, population of all the isolates were checked by using serial dilution and pour plate at 0h, 48h, 72h, 96h, 120h and 144h after inoculation. Maximum growth was observed at 96h for all the isolates and it was standardized for evaluation of growth of isolates at remaining pH levels.

4.4.1. Evaluation of growth of isolates at different pH levels

All the twenty selected isolates were found to grow at all the pH levels tested including 7.0, 6.5, 6.0, 5.5, and 5.0. Maximum growth of all the selected isolates was observed at pH 7.0.In general, growth declined as pH was lowered. Data showing the results are given in Table 17; Figs. 2 and 3).

Maximum growth at pH 7.0 was observed for NKdS (200.00× 10^5cfu/ml). Compared to growth of all other isolates this was statistically superior. Second highest growth was observed for NPPV (177.33 × 10^5cfu/ml) followed by NKPV-2 which was on par with NNPD, NPS-1 and NTS-2. Lowest growth at neutral pH was observed for NBS (38.00× 10^5cfu/ml).

At pH 6.5, 6.0, 5.5 and 5.0 maximum growth was observed for NPPV. This was statistically superior to growth of all other isolates. Second highest growth was

Table 16.Population of diazotrophs at different time interval at pH 7.0

Isolate	Population (10^5 × cfu/ml of broth)					
	0h	48h	96h	72h	120h	144h
NTS-1	0.23	12.77	78.91	100.00	90.27	84.33
NTS-2	0.26	17.51	83.12	136.33	103.92	78.00
NTS-3	0.29	13.00	47.28	79.00	45.67	28.94
NKS-1	0.31	17.84	72.57	122.33	93.55	64.39
NKS-2	0.42	26.20	97.89	101.33	94.21	72.30
NBS	0.49	25.61	31.10	38.00	18.00	15.77
NKdS	0.38	27.81	92.00	200.00	94.53	89.54
NAS	0.32	17.79	74.18	114.66	77.03	71.29
NPS-1	0.40	27.97	95.21	134.33	113.28	95.40
NPS-2	0.32	20.42	39.21	67.00	41.95	35.76
NPS-3	0.21	18.31	69.54	115.00	88.97	63.29
NKPD	0.31	19.80	48.27	79.33	43.28	33.00
NKPV-1	0.34	24.28	99.75	101.00	85.67	40.37
NKPV-2	0.33	20.92	86.00	138.33	104.56	69.78
NKdPV	0.31	16.95	92.91	117.33	97.48	55.00
NNPD	0.25	32.71	91.24	134.00	102.33	88.92
NNPV	0.26	35.32	42.64	88.33	64.00	48.53
NNkPV	0.37	31.46	54.69	88.00	37.89	18.71
NPPV	0.28	11.14	127.48	177.33	112.86	82.22
NAPV	0.37	0.92	63.15	83.33	67.90	38.54

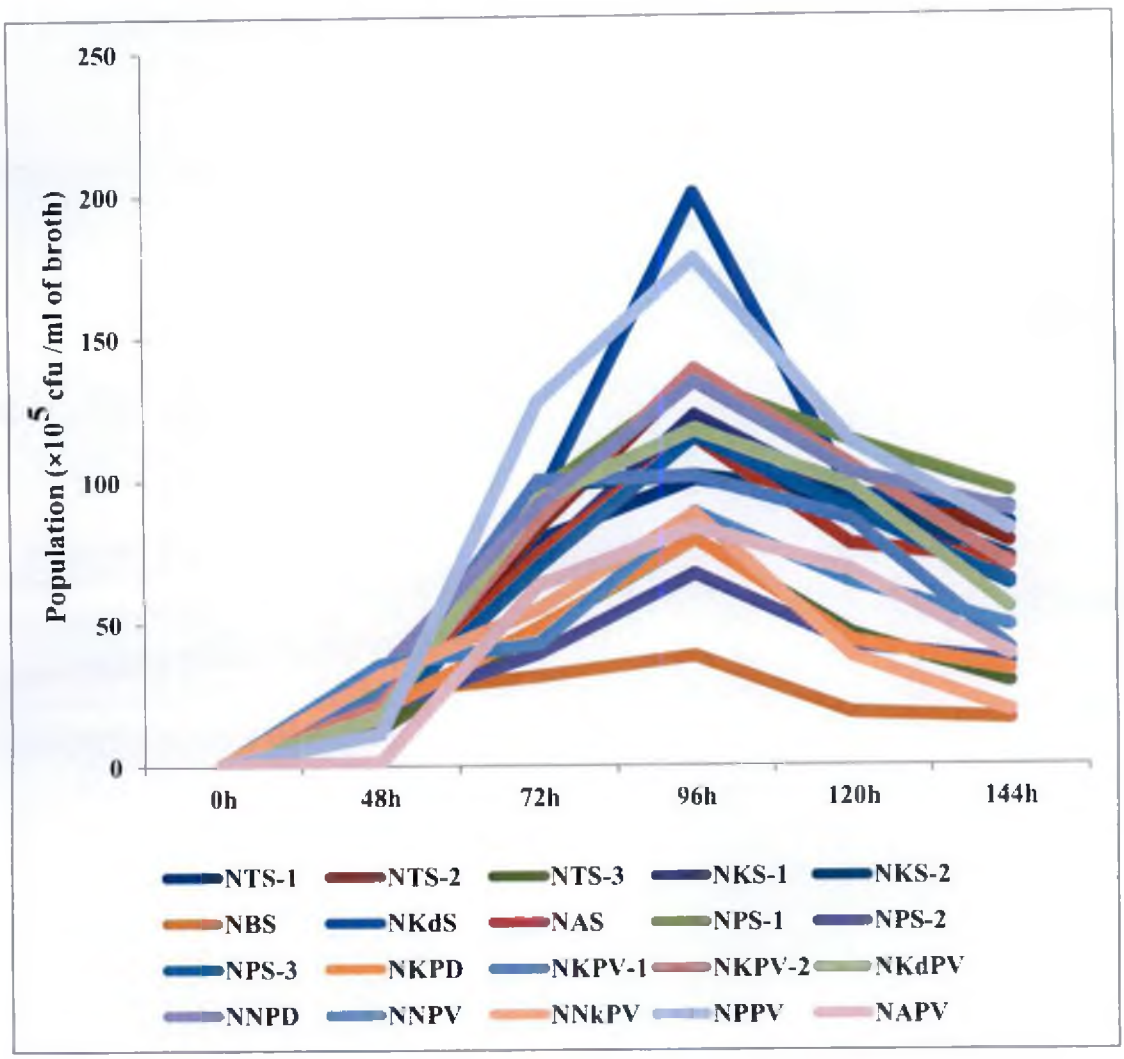


Fig.1. Population of diazotrophs at different time interval at pH 7.0

Table 17. Population of free living diazotrophs at different pH levels after 96h

Isolate	Population (× 10^5 cfu/ml)				
	pH 7.0	**pH 6.5**	**pH 6.0**	**pH 5.5**	**pH 5.0**
NTS-1	100.00^{f}	48.33^{j}	8.33^{jk}	3.00^{jk}	0.00^{k}
NTS-2	136.33^{c}	62.00^{i}	35.33^{h}	29.33^{d}	9.00^{de}
NTS-3	79.00^{h}	7.33^{m}	5.00^{k}	2.33^{k}	1.66^{ijk}
NKS-1	122.33^{d}	67.00^{ghi}	18.33^{l}	9.33^{hij}	7.33^{ef}
NKS-2	101.33^{f}	90.66^{de}	43.00^{fg}	26.00^{de}	5.33^{fg}
NBS	38.00^{j}	37.00^{k}	13.33^{ij}	8.00^{ijk}	4.00^{ghi}
NKdS	200.00^{a}	125.33^{b}	84.00^{b}	70.33^{b}	13.66^{c}
NAS	114.66^{e}	79.33^{f}	17.00^{l}	5.33^{jk}	4.33^{gh}
NPS-1	134.33^{c}	105.33^{c}	72.00^{c}	39.00^{c}	16.66^{b}
NPS-2	67.00^{i}	19.33^{l}	16.33^{l}	1.33^{k}	1.00^{jk}
NPS-3	115.00^{e}	79.33^{f}	39.33^{gh}	17.00^{fg}	5.00^{fg}
NKPD	79.33^{h}	76.33^{fg}	71.00^{cd}	3.33^{jk}	2.33^{hijk}
NKPV-1	101.00^{f}	81.33^{ef}	49.00^{f}	16.33^{fg}	4.00^{ghi}
NKPV-2	138.33^{c}	118.00^{b}	71.33^{cd}	30.00^{d}	11.00^{d}
NKdPV	117.33^{de}	100.00^{cd}	49.33^{f}	12.33^{ghi}	7.00^{ef}
NNPD	134.00^{c}	73.33^{fgh}	65.33^{de}	19.00^{fg}	10.00^{d}
NNPV	88.33^{g}	79.33^{f}	61.00^{e}	22.00^{ef}	3.00^{ghij}
NNkPV	88.00^{g}	63.66^{hi}	59.00^{e}	6.00^{ijk}	0.33^{k}
NPPV	177.33^{b}	158.00^{a}	133.33^{a}	82.33^{a}	33.00^{a}
NAPV	83.33^{gh}	58.00^{ij}	49.33^{f}	15.00^{gh}	9.33^{de}

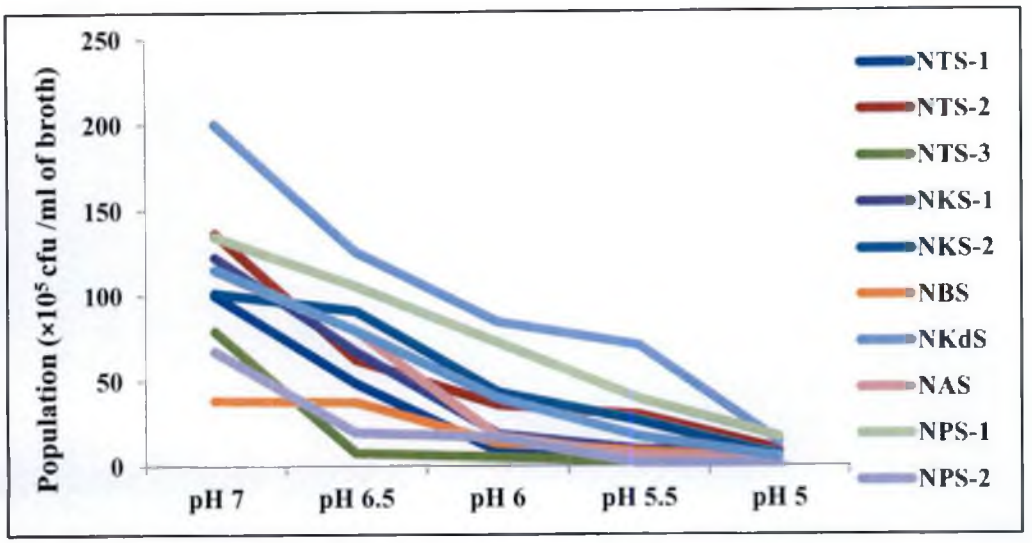


Fig 2. Growth of rhizosphere isolates at different pH levels

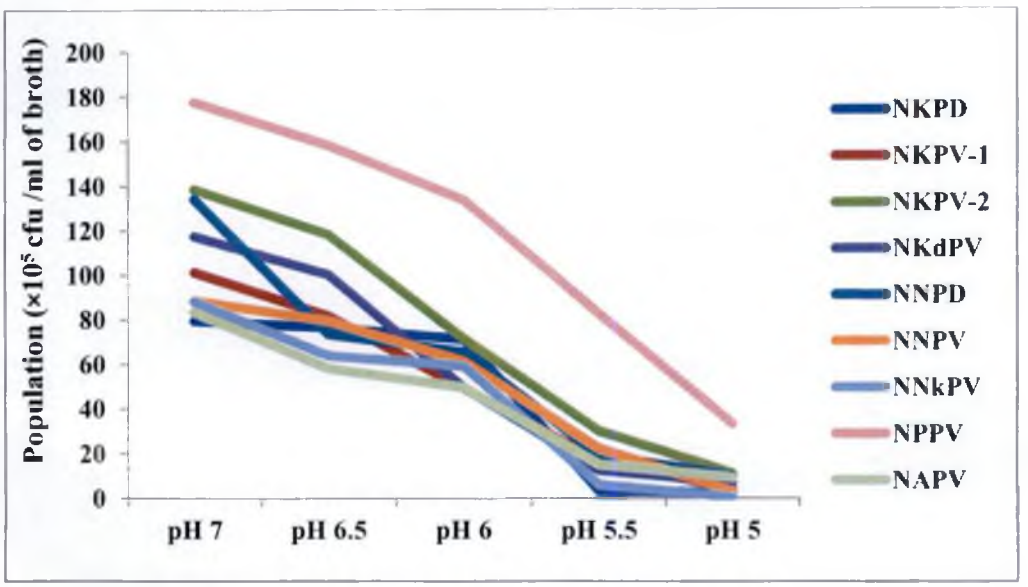


Fig 3. Growth of phylloplane isolates at different pH levels

observed for NKdS at pH 6.5, 6.0 and 5.5. However, at lowest pH 5.0 second highest growth was observed for NPS-1. Lowest growth at pH 6.5 and 6.0 was recorded in NTS-3 (7.33×10^5cfu/ml and 5.0×10^5cfu/ml respectively). At pH 5.5, minimum growth was recorded in NPS-2 and this was on par with NTS-3. At lowest pH of 5.0, no growth was observed for the isolate NTS-1.

4.4.2. Estimation of nitrogen fixation of the isolates at different pH levels

Amount of nitrogen fixed by all the twenty isolates was estimated using microkjeldahl method (Table 18; Fig. 4 and 5). NPS-1 was found to fix maximum amount of nitrogen (40.60 mg of nitrogen per gram of sucrose utilized) at neutral pH which was on par with NPPV (39.66 mg of nitrogen per gram of sucrose utilized). At pH 6.5 also NPS-1 fixed highest amount of nitrogen (35.00 mg of nitrogen per gram of sucrose utilized). NPPV was found to fix highest amount of nitrogen (25.66 mg of nitrogen per gram of sucrose utilized) at pH 6.0 which was on par with NPS-1, NKPV-2, and NAPV. NKdS fixed highest amount of nitrogen at pH 5.5 (21.00 mg of nitrogen per gram of sucrose utilized) which was on par with NPPV. At the lowest pH of 5.0, NKdS, NKPV-2 and NPPV fixed highest amount of nitrogen, which were on par.

Hence, NKdS and NPS-1 from rhizosphere soil and NKPV-2 and NPPV from phylloplane were found to perform better, in terms of growth and nitrogen fixation at different levels of pH.

Table 18. Amount of nitrogen fixed by the free living diazotrophs at different pH levels

Isolate	Amount of nitrogen fixed (mg of N/ g of sucrose utilized)				
	pH 7.0	pH 6.5	pH 6.0	pH 5.5	pH 5.0
NTS-1	5.60^{n}	4.70^{k}	3.27^{l}	4.66^{ij}	1.40^{h}
NTS-2	11.66^{l}	11.20^{j}	6.06^{l}	4.20^{ij}	3.26^{gh}
NTS-3	30.80^{g}	20.06^{g}	16.80^{c}	11.66^{g}	7.46^{de}
NKS-1	16.80^{j}	8.86^{j}	3.26^{j}	1.86^{k}	1.40^{gh}
NKS-2	35.00^{de}	28.00^{bcd}	15.40^{cd}	14.00^{ef}	6.06^{ef}
NBS	14.46^{k}	11.20^{j}	8.86^{h}	7.00^{h}	4.66^{fg}
NKdS	36.86^{cd}	28.46^{bcd}	22.86^{b}	21.00^{a}	16.00^{a}
NAS	22.86^{i}	14.46^{l}	9.80^{gh}	6.06^{hi}	1.86^{h}
NPS-1	40.60^{a}	35.00^{a}	24.26^{ab}	17.26^{cd}	11.66^{b}
NPS-2	21.00^{i}	18.66^{gh}	14.46^{de}	7.50^{ijk}	3.30^{de}
NPS-3	32.66^{ef}	22.86^{i}	14.46^{de}	12.60^{fg}	8.40^{cd}
NKPD	34.06^{ef}	26.60^{de}	15.86^{cd}	13.06^{fg}	9.33^{cd}
NKPV-1	18.66^{j}	17.30^{gh}	13.06^{ef}	7.50^{h}	2.80^{gh}
NKPV-2	37.80^{bc}	29.86^{b}	23.80^{ab}	18.66^{bc}	14.60^{a}
NKdPV	26.60^{h}	14.46^{l}	12.60^{ef}	5.60^{hi}	1.40^{h}
NNPD	34.06^{ef}	27.06^{cde}	17.26^{c}	15.86^{de}	10.26^{bc}
NNPV	18.66^{j}	16.80^{hi}	11.66^{fg}	7.46^{h}	4.66^{fg}
NNkPV	8.86^{m}	9.80^{j}	2.80^{j}	1.86^{k}	1.33^{gh}
NPPV	39.66^{ab}	29.40^{bc}	25.66^{a}	20.06^{ab}	14.73^{a}
NAPV	34.06^{ef}	25.20^{ef}	23.80^{ab}	13.06^{fg}	6.06^{ef}

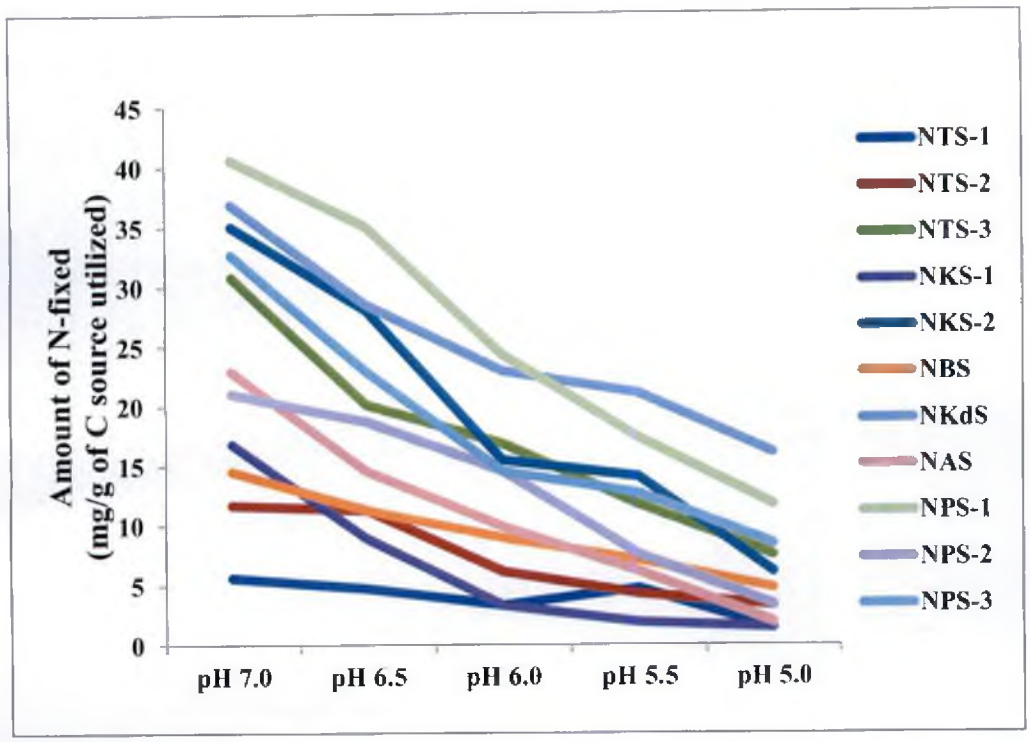


Fig 4. Amount of nitrogen fixed by the rhizosphere isolates at different pH levels

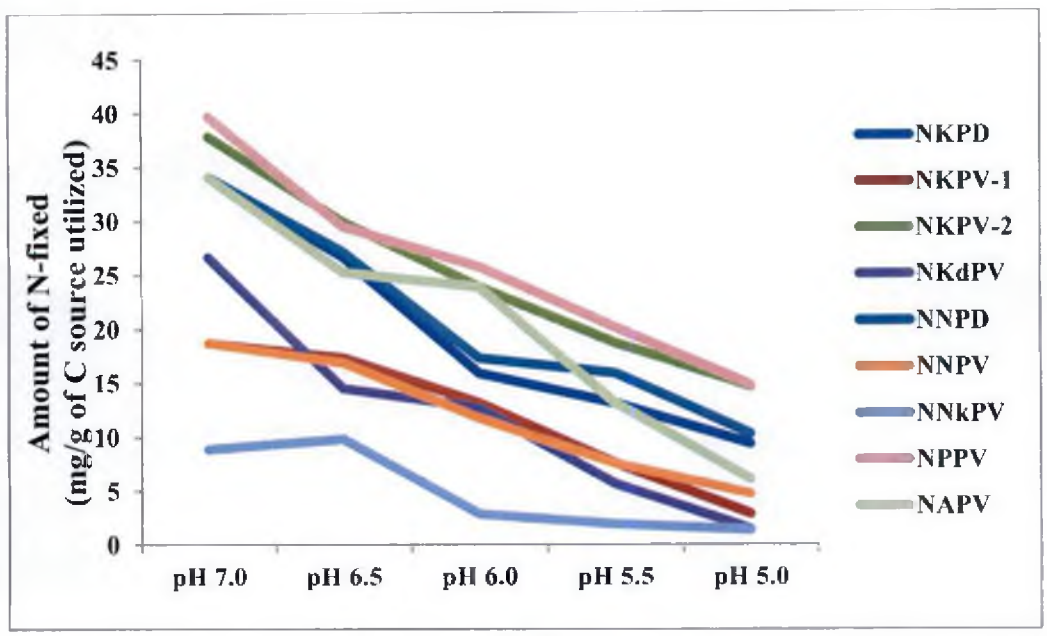


Fig 5. Amount of nitrogen fixed by the phylloplane isolates at different pH levels

4.5. *IN VITRO* SCREENING OF PREDOMINANT ISOLATES FOR PLANT GROWTH PROMOTING ACTIVITIES

4.5.1. Screening of isolates for indole acetic acid (IAA) Production

All the twenty selected isolates were screened for IAA production using Salkowski's assay method. Based on the development of pink to red colour on the filter paper after soaking in Salkowski's reagent, four isolates found to be positive for IAA production. They are NTS-3, NKdS, NKPV-1 and NPS-1 (Plate 7).

4.5.2. Screening of isolates for hydrogen cyanide (HCN) production

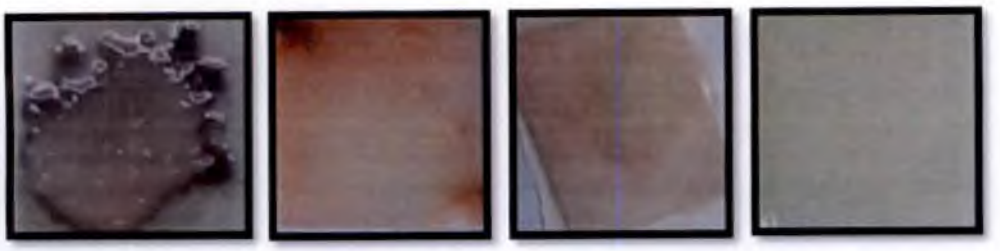
HCNproduction by the isolates was assessed based on the development of yellow to reddish brownish colour on the filter paper impregnated with picric acid solution (Table 19 andPlate 7). Out of twenty isolates screened, HCN production was shown by five isolates namely NBS, NTS-1, NPS-1, NPPV, and NAS. Among these NTS-1 was observed to be good HCN producers. HCN production was moderate in isolates NPS-1, NBS and NPPV whereas, NAS was found to be a weak producer of HCN.

4.5.3. Siderophore production

Isolates were screened for their ability for siderophore production. None of the isolates produced yellow-orange halo around the colony, indicating that there was no siderophore production.

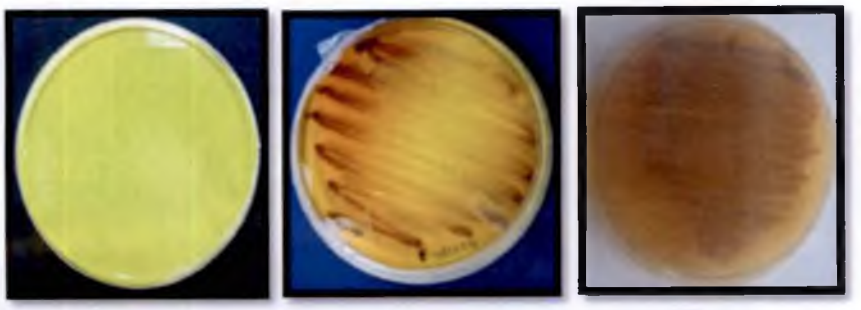
4.5.4. Ammonia (NH_3) production

Ammonia production by the twenty selected isolates was examined based on the development of yellow to brown colour when Nessler's reagent was added (Table 20 and Plate 7). Sixteen isolates were observed to be positive for ammonia production. Out of sixteen isolates eight isolates (NTS-1, NPS-1, NKdS, NKPV-2,

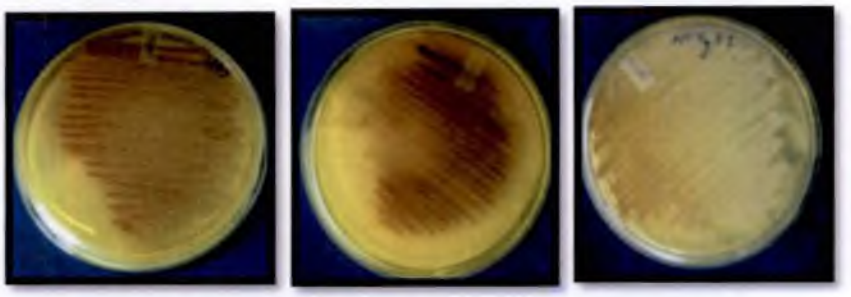


NKdS NKPV-1 NPS-1 Control

A. IAA production



Control NTS-1 NPS-1



NBS NPPV NAS

B. HCN production

NPS-1 NKdS NTS-1 NPPV NNPV NKPV-2 NPS-3 NBS Uninoculated control

NPS-1
NKdS
NTS-1
NPPV
NNPV
NKPV-2
} Good

NPS-3
NBS
} Moderate

C. Ammonia production

Plate 7: Screening of diazotrophs for PGPR traits

Table 19. HCN production by diazotrophic bacteria

Sl.no.	Isolate	HCN production
1.	NPS-1	+++
2.	NBS	++
3.	NTS-1	+++
4.	NAS	+
5.	NPPV	++

+++ Good (Reddish brown) ++ Moderate (Brown) + Weak (Yellow-light brown)

Table 20.Ammonia production by diazotrophic bacteria

Sl.no.	Isolate	Ammonia production
1.	NPS-1	+++
2.	NKdS	+++
3.	NTS-1	+++
4.	NPPV	+++
5.	NNPV	+++
6.	NKPV-2	+++
7.	NNkPV	+++
8.	NNPD	+++
9.	NPS-3	++
10.	NBS	++
11.	NKS-1	+
12.	NTS-2	+
13.	NAS	+
14.	NKPD	+
15.	NKPV-1	+
16.	NAPV	+

+++ Good (Brown) ++ Moderate (Orange) + Weak (Yellow)

Table 21.Antagonistic activity of diazotrophs against *Rhizoctonia solani*

Sl. no.	Isolate	Per cent inhibition
1.	NKdS	45.00
2.	NPPV	47.22
3.	NKS-1	56.94

NNPV, NPPV, NNkPV, and NNPD) were found to be good ammonia producers since brown colour was developed after adding Nessler's reagent. Two isolates (NPS-3 and NBS) were categorized as moderate producers of ammonia and six isolates as weak producers (NKS-1, NTS-2, NAS, NKPD, NKPV-1 and NAPV).

4.5.5. Screening for antagonistic activity

4.5.5.1.Screening of diazotrophs against Rhizoctonia solani

Twenty selected diazotrophs were tested against *R. solani* for their antagonistic property by dual culture method (Table 21 and Plate 8). Per cent inhibition of growth was calculated after five days of incubation. Three isolates viz. NkdS, NPPV and NKS-1 were found to exhibit antagonistic action to *Rhizoctonia solani.* NKS-1 showed maximum per cent inhibition of 56.94.

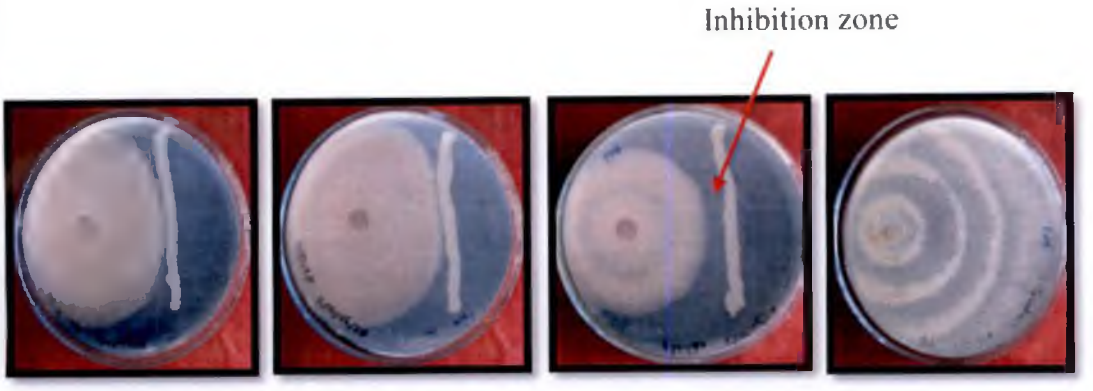
4.5.5.2.Screening of diazotrophs against Fusarium oxysporum

Results of the dual culture experiment revealed that none of the isolates exhibited antagonistic activity against *Fusarium oxysporum*, which was indicated by the absence of inhibition zone.

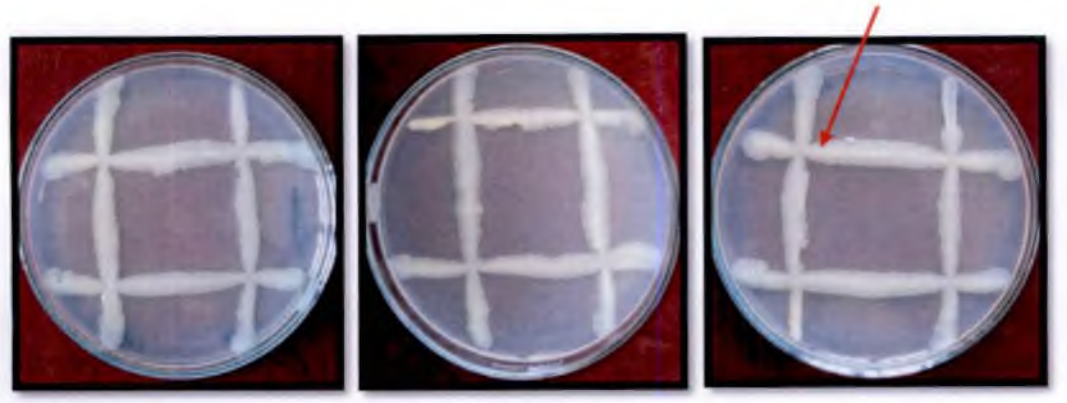
4.5.5.3.Screening of diazotrophs against Ralstonia solanacearum

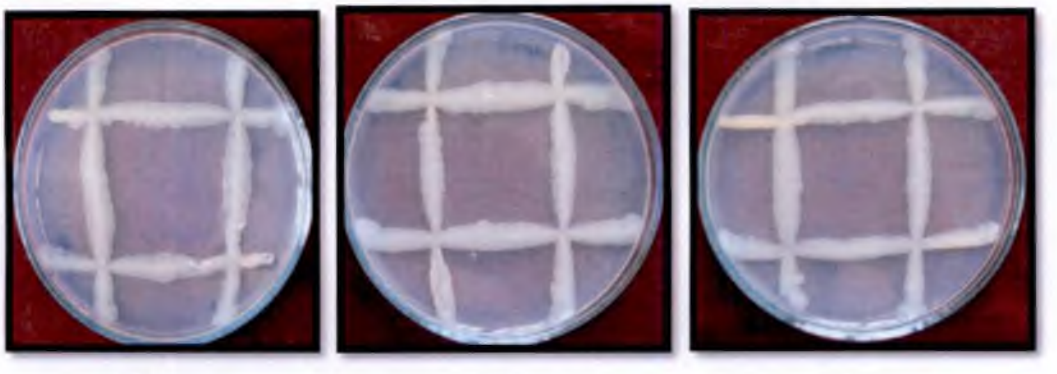
The antagonistic activity of all the twenty selected isolates against the bacterial plant pathogen *R. solanacearum* was tested by cross streakmethod. Lysis at the junction of isolate and pathogen was observed for six isolates. The isolates which exhibited antagonistic activity against the bacterial pathogen included NPS-2, NPS-3, NNPV, NNkPV, NKPV-2 and NKPD (Plate 8).

Based on growth, nitrogen fixation, tolerance to acidic pH and PGPR activities under *in vitro* condition, four promising diazotrophs, including two



A. *Rhizoctoia solani*





B. *Ralstonia solanacearum*

Plate 8: Screening for antagonistic activity against soil borne plant pathogens

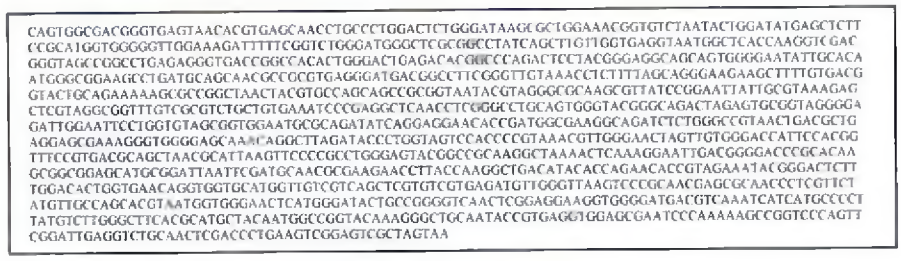
rhizosphere isolates (NPS-1 and NKdS) and two phylloplane isolates (NPPV and NKPV-2) were selected for *in planta* evaluation.

4.6. IDENTIFICATION OF THE SELECTED FREE-LIVING DIAZOTROPHIC BACTERIA

Identification of four promising diazotrophs, selected for *in planta* evaluation were carried out by 16S rDNA sequencing. 1500bp amplicon was obtained on amplification of the 16S rDNA. Homology of nucleotide sequences in NCBI database, obtained from blastn outputis presented in Table 22. The isolate NKdS and NPPV showed maximum homology with *Microbacterium* sp., NPS-1 with *Cellulosimicrobium* sp. and NKPV-2 with *Brevundimonas* sp. (Plate 9 and 10).Photomicrographs of the four selected isolates are provided in Plate 11.

4.7. *IN PLANTA* EVALUATION OF MOST PROMISING ISOLATES FOR PLANT GROWTH PROMOTION

Based on the growth, nitrogen fixation, tolerance to acidic pHand PGPR activities under *in vitro* conditions, four promising diazotrophs including, two rhizosphere isolates, *Microbacterium* sp. (NKdS) and *Cellulosimicrobium* sp. (NPS-1) and two phylloplane isolates, *Microbacterium* sp. (NPPV) and *Brevundimonas* sp. (NKPV-2) were selected for evaluation under *in planta* condition using Anagha variety of tomato. The experiment included two levels of fertilizer nitrogen (0% and 75%) and two levels of FYM (0% and 50%). KAU *Azotobacter* formulation was the positive control of the experiment. Treatment without any bioinoculant, chemical nitrogen and FYM served as the absolute control.

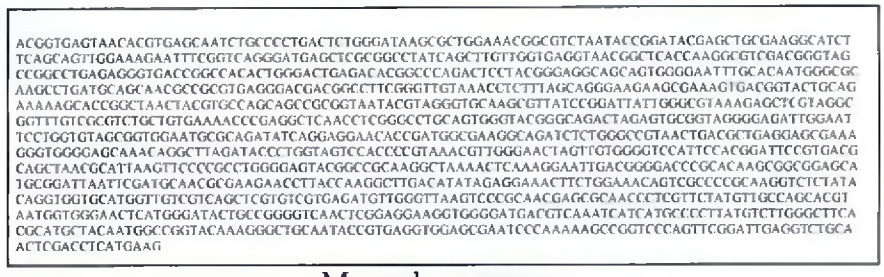


Merged sequence

Isolate	NCBI accessions showing maximum homology		Maximum score	Query coverage %	Identity %	e-value
	Accession no.	Name				
NPS-1	KJ024047.1	*Cellulosimicrobium* sp.	1796	94	97	0.0
	KC685574.1	*Cellulosimicrobium* sp.	1796	94	97	0.0
	KC429587.1	*Cellulosimicrobium funkei* strain	1796	94	97	0.0
	JQ659848.1	*Cellulosimicrobium funkei* strain	1796	94	97	0.0

Blastn result

A. Sequence analysis of isolate NPS-1



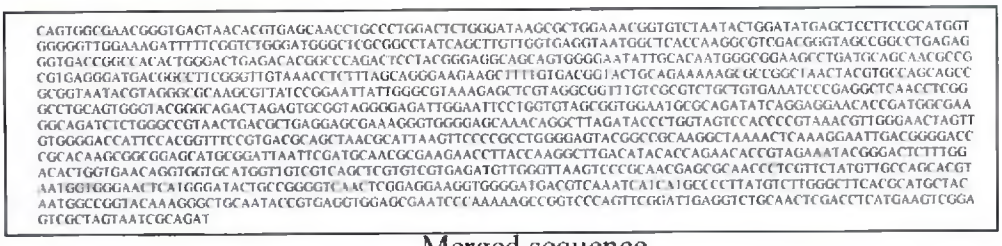
Merged sequence

Isolate	NCBI accessions showing maximum homology		Maximum score	Query coverage %	Identity %	e-value
	Accession no.	Name				
NKdS	KC853186.1	*Microbacterium* sp.	2270	100	99	0.0
	HQ530521.1	Uncultured*Microbacterium* sp.	2242	100	99	0.0
	KR265734.1	*Microbacterium* sp.	2231	100	99	0.0
	KM507678.1	*Microbacterium* sp.	2231	100	99	0.0

Blastn result

B. Sequence analysis of isolate NKdS

Plate 9: 16S rDNA sequence analysis

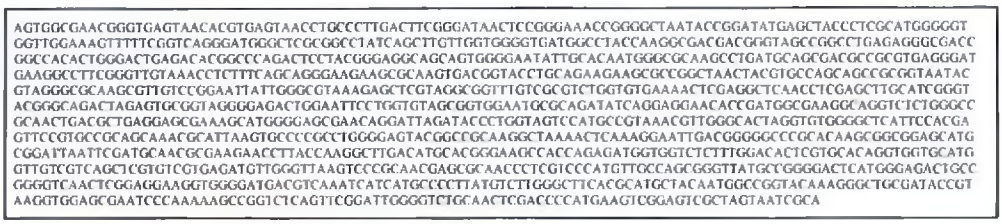


Merged sequence

Isolate	NCBI accessions showing maximum homology		Maximum score	Query coverage %	Identity %	e-value
	Accession no.	Name				
NPPV	KC853186.1	*Microbacterium* sp.	1606	94	95	0.0
	EU977655.1	*Microbacterium hominis* strain	1592	97	93	0.0
	JQ622211.1	Uncultured *Microbacterium* sp.	1588	97	93	0.0
	KJ631291.1	*Microbacterium trichothecenolyticum*	1586	97	93	0.0

Blastn result

A. Sequence analysis of isolate NPPV



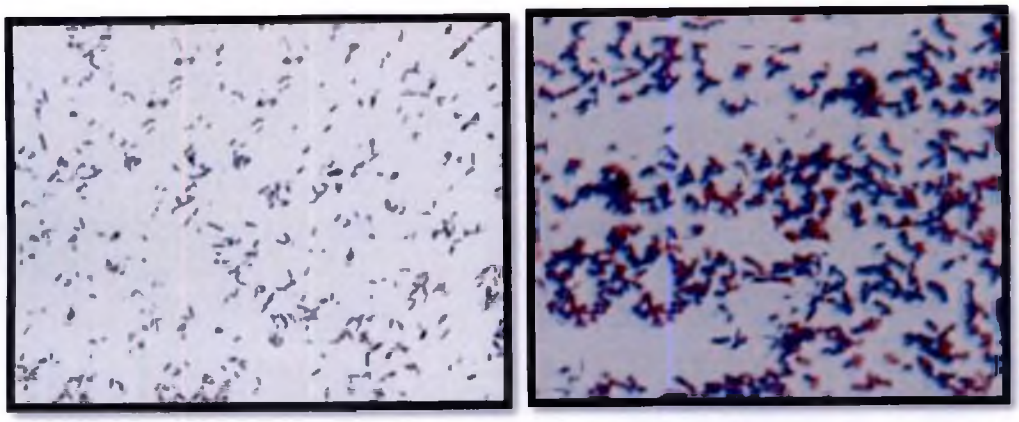
Merged sequence

Isolate	NCBI accessions showing maximum homology		Maximum score	Query coverage %	Identity %	e-value
	Accession no.	Name				
NKPV-2	KJ482719.1	*Brevundimonas* sp.	1719	86	99	0.0
	HG917242.1	Uncultured *Brevundimonas* sp.	1719	86	99	0.0
	KC634247.1	*Brevundimonas naejangsanensis*	1719	86	99	0.0
	KF017644.1	*Brevundimonas sp.*	1719	86	99	0.0

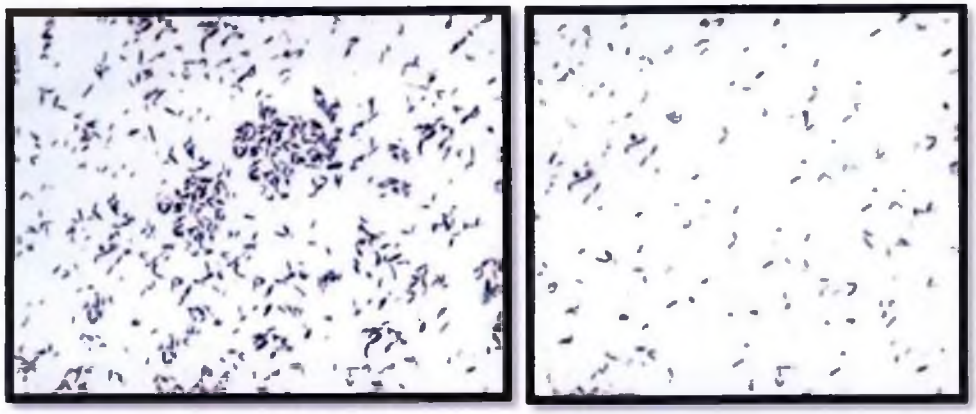
Blastn result

B. Sequence analysis of isolate NKPV-2

Plate 10: 16S rDNA sequence analysis



NKdS: ***Microbacterium*** **NPS-1:*Cellulosimicrobium***



NPPV: ***Microbacterium*** **NKPV-2:** ***Brevundimonas***

Plate 11: Microscopic view of promising diazotrophs

Table 22. 16S rDNA sequence analysis of promisingdiazotrophs

Isolate	NCBI accessions showing maximum homology		Maximum score	Query coverage %	Identity %	e-value
	Accession no.	Name				
NKdS	KC853186.1	*Microbacterium* sp.	2270	100	99	0.0
	HQ530521.1	Uncultured *Microbacterium* sp.	2242	100	99	0.0
	KR265734.1	*Microbacterium* sp.	2231	100	99	0.0
	KM507678.1	*Microbacterium* sp.	2231	100	99	0.0
	KM507654.1	*Microbacterium* sp.	2231	100	99	0.0
NPS-1	KJ024047.1	*Cellulosimicrobium* sp.	1796	94	97	0.0
	KC685574.1	*Cellulosimicrobium* sp.	1796	94	97	0.0
	KC429587.1	*Cellulosimicrobium funkei* strain	1796	94	97	0.0
	JQ659848.1	*Cellulosimicrobium funkei* strain	1796	94	97	0.0
	JQ659839.1	*Cellulosimicrobium funkei* strain	1796	94	97	0.0
NPPV	KC853186.1	*Microbacterium* sp.	1606	94	95	0.0
	EU977655.1	*Microbacterium hominis* strain	1592	97	93	0.0
	JQ622211.1	Uncultured *Microbacterium* sp.	1588	97	93	0.0
	KJ631291.1	*Microbacterium trichothecenolyticum*	1586	97	93	0.0
	HG796193.1	*Microbacterium* sp.	1586	97	93	0.0
NKPV-2	KJ482719.1	*Brevundimonas* sp.	1719	86	99	0.0
	HG917242.1	Uncultured *Brevundimonas* sp.	1719	86	99	0.0
	KC634247.1	*Brevundimonas naejangsanensis*	1719	86	99	0.0
	KF017644.1	*Brevundimonas sp.*	1719	86	99	0.0
	KC294075.1	*Brevundimonas sp.*	1719	86	99	0.0

4.7.1. Biometric observations

4.7.1.1. Germination

The data on germination are presented inTable 23. Seed germination was enhanced when seeds were pre-treated with the bioinoculants, as compared to uninoculated control. Number of days taken for 100% germination ranged between 9 to 11 days. Seeds treated with KAU *Azotobacter* formulation triggered faster germination compared to all other treatments. Upto six DAS, a similar trend was noticed. However, after 7 days of sowing maximum number of seedling emergence (90.24%) was recorded in seeds treated with *Cellulosimicrobium* sp. (NPS-1) followed by KAU *Azotobacter* formulation (88.52%). Lowest germination was observed in absolute control (62.85%). The isolate *Cellulosimicrobium* sp. (NPS-1) recorded 100% germination at 9 DAS and all other isolates recorded 100% germination at 10 DAS.

4.7.1.2. Height of plants

Plant height was recorded at30 DAS,60 DAS, 90 DAS and at the time of harvest (Table 24, Plate 12). The data revealed that microbial inoculants had a significant effect on plant height of tomato compared to control. In general, treatments with microbial inoculants + 75% RD of N + 50% RD of FYM recorded significantly higher plant height than treatments with isolate alone and isolate + 75% RD of N.

At the time of transplanting (30 DAS), T_7*Microbacterium* sp. (NKdS) recorded maximum plant height (6.19cm). This was statistically on par with T_{12}, T_2 and T_9. At 60 DAS, 90 DAS and harvest, *Microbacterium* sp. (NKdS) + 75% RD of N + 50% RD of FYM (T_{12}) recorded maximum plant height of 60.29cm, 127.64cm and 134.28cm respectively and this was statistically superior to all other treatments. This treatment was found to be significantly superior to POP recommendation of

Table 23.Effect of free living diazotrophs on germination

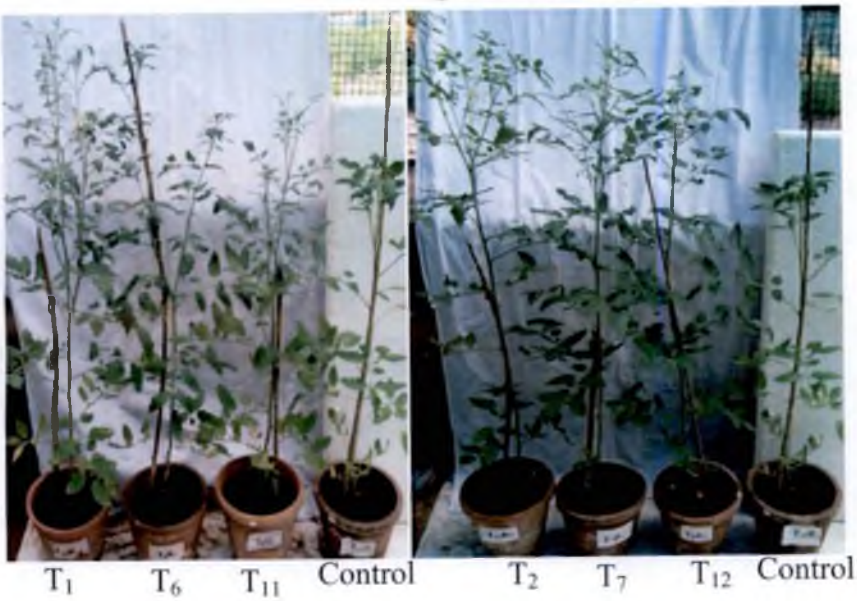
Treatments	Germination (%)								
	3 DAS	4 DAS	5 DAS	6 DAS	7 DAS	8 DAS	9 DAS	10 DAS	11 DAS
NPS-1	0	24.39	36.58	70.73	90.24	96.00	100.00	100.00	100.00
NKdS	0	40.00	46.00	76.00	84.00	95.12	97.56	100.00	100.00
NKPV-2	0	26.00	52.00	74.00	84.00	94.00	98.00	100.00	100.00
NPPV	0	25.71	37.14	68.57	80.00	85.71	97.14	100.00	100.00
KAU *Azotobacter* formulation	14.75	55.73	62.29	83.62	88.52	91.83	96.72	100.00	100.00
Control	0	25.71	37.14	48.57	62.85	74.28	85.71	97.14	100.00

Table 24. Effect of free living diazotrophs on plant height of tomato

Treatment	Plant height (cm)			
	30 DAP	60 DAP	90 DAP	Harvesting
T_1 (*Microbacterium* sp.)	3.04^{defgh}	31.11^{g}	99.78^{j}	101.21^{jk}
T_2 (*Cellulosimicrobium* sp.)	5.86^{ab}	32.78^{fg}	102.23^{h}	104.68^{ij}
T_3 (*Microbacterium* sp.)	3.54^{def}	27.54^{h}	88.55^{l}	99.18^{k}
T_4 (*Brevundimonas* sp.)	5.03^{c}	25.84^{hi}	97.61^{j}	100.13^{k}
T_5 (*Azotobacter* formulation)	2.75^{fgh}	22.66^{j}	94.84^{k}	95.53^{l}
T_6 (T_1 + 75% N)	3.18^{defg}	40.86^{e}	109.04^{g}	112.15^{ef}
T_7 (T_2 + 75% N)	6.19^{a}	48.39^{d}	111.03^{f}	112.38^{ef}
T_8 (T_3 + 75% N)	3.76^{d}	40.28^{e}	108.49^{g}	110.05^{fg}
T_9 (T_4 + 75% N)	5.43^{abc}	38.38^{e}	107.52^{g}	108.50^{gh}
T_{10} (T_5 + 75% N)	2.70^{gh}	22.27^{j}	104.19^{h}	105.93^{hi}
T_{11} (T_6 + 75% N + 50% FYM)	3.04^{defgh}	53.28^{b}	124.06^{b}	126.56^{b}
T_{12} (T_7 + 75% N + 50% FYM)	5.94^{ab}	60.29^{a}	127.64^{a}	134.28^{a}
T_{13} (T_8 + 75% N + 50% FYM)	3.72^{de}	51.28^{bc}	119.87^{c}	121.27^{cd}
T_{14} (T_9 + 75% N + 50% FYM)	5.14^{bc}	49.71^{cd}	117.03^{d}	118.86^{d}
T_{15} (T_5 + + 50% FYM)	2.64^{gh}	24.70^{ij}	103.69^{h}	108.06^{ghi}
T_{16} (T_5 + 75% N + 50% FYM)	2.91^{efgh}	34.13^{f}	113.05^{e}	114.18^{e}
T_{17} (POP Recommendation)	2.35^{h}	31.54^{g}	121.27^{c}	123.81^{bc}
T_{18} (Absolute control)	2.35^{h}	18.71^{k}	89.98^{l}	90.63^{m}

A. Nursery

A. During transplanting



T_1: *Cellulosimicrobium* sp.

T_6: T_1 + 75% N

T_{11}: T_6 + 50% FYM

T_2: *Microbacterium* sp.

T_7: T_2 + 75% N

T_{12}: T_7 + 50% FYM

C. Two months after planting

D. Overview of pot culture experiment

Plate 12: Effect of free living diazotrophs on plant height

KAU also. All the four isolates performed better than KAU *Azotobacter* formulation in all treatment combinations. Plant height was minimum (90.63cm) in T_{18} (absolute control).

T_2 (NKdS: *Microbacterium* sp.) recorded significantly higher plant height, among the treatments with bioinoculants alone. When 75% N was given along with the isolates, T_6 (NPS-1: *Cellulosimicrobium*sp.) and T_7 (NKdS: *Microbacterium* sp.) recorded maximum plant height.

4.7.1.3. Number of leaves

Number of leaves at 30 DAS, 60 DAS, 90 DAS and harvest is given in Table 25. For all isolates, treatment with 75% N + 50% FYM was superior to treatments with inoculant alone or inoculant + 75% N. At 30 DAS, T_2(NKdS: *Microbacterium* sp.) recorded significantly highest number of leaves (31.08) followed by T_{12} and T_7. The treatment *Microbacterium* sp. (NKdS) + 75% N + 50% FYM (T_{12})showed maximum number of leaves at 60 DAS, 90 DAS as well as at the time of harvest (81.50, 156.38 and 169.66 respectively). At 60 DAP and harvest, this was statistically superior to all other treatments. At the time of harvest T_{12} was followed by *Cellulosimicrobium* sp.(NPS-1) + 75% N + 50% FYM (T_{11}) which was on par with T_{17} (POP recommendation). Least number of leaves was observed in T_{18} (absolute control). All the four isolates performed better, compared to KAU *Azotobacter* formulation in all treatment combinations.

NKdS (*Microbacterium* sp.) recorded maximum number of leaves when bioinoculants alone were applied (T_2) as well as bioinoculants were applied along with 75% N (T_7).

Table 25. Effect of free living diazotrophs on number of leaves in tomato

Treatment	No. of leaves per plant			
	30 DAP	60 DAP	90 DAP	Harvesting
T_1 (*Microbacterium* sp.)	16.33^{f}	33.08^{gh}	88.75^{h}	100.83^{ij}
T_2 (*Cellulosimicrobium* sp.)	31.08^{a}	40.56^{ef}	94.30^{gh}	103.33^{hi}
T_3 (*Microbacterium* sp.)	19.91^{e}	31.16^{h}	88.38^{h}	95.33^{j}
T_4 (*Brevundimonas* sp.)	25.75^{c}	32.50^{gh}	73.55^{i}	85.33^{k}
T_5 (*Azotobacter* formulation)	12.25^{g}	23.33^{j}	71.41^{ij}	82.50^{k}
T_6 (T_1 + 75% N)	16.25^{f}	51.08^{c}	108.83^{ef}	116.00^{ef}
T_7 (T_2 + 75% N)	29.25^{b}	51.75^{c}	113.08^{e}	119.33^{e}
T_8 (T_3 + 75% N)	20.83^{e}	47.16^{cd}	105.41^{f}	113.83^{efg}
T_9 (T_4 + 75% N)	24.66^{cd}	42.08^{def}	97.16^{g}	108.83^{fgh}
T_{10} (T_5 + 75% N)	11.33^{g}	25.41^{ij}	97.41^{g}	107.00^{ghi}
T_{11} (T_6 + 75% N + 50% FYM)	15.75^{f}	70.25^{b}	154.00^{ab}	156.83^{b}
T_{12} (T_7 + 75% N + 50% FYM)	29.25^{b}	81.50^{a}	156.38^{a}	169.66^{a}
T_{13} (T_8 + 75% N + 50% FYM)	19.41^{e}	67.75^{b}	142.69^{cd}	147.87^{cd}
T_{14} (T_9 + 75% N + 50% FYM)	24.00^{d}	66.58^{b}	138.61^{d}	141.50^{d}
T_{15} (T_5 + + 50% FYM)	11.83^{g}	30.58^{hi}	105.25^{f}	110.33^{fgh}
T_{16} (T_5 + 75% N + 50% FYM)	12.25^{g}	43.75^{de}	113.83^{e}	116.00^{ef}
T_{17} (POP Recommendation)	6.58^{h}	37.00^{fg}	148.16^{bc}	153.00^{bc}
T_{18} (Absolute control)	6.83^{h}	20.33^{j}	65.83^{j}	74.50^{l}

4.7.1.4. Girth of stem

Treatments with isolate + 75% N + 50% FYM, recorded significantly higher girth compared to treatments with isolate alone and isolate + 75% N (Table 26). Stem girth at 60 DAS was highest in *Cellulosimicrobium* sp. (NPS-1)+ 75% N + 50% FYM (T_{11}) with 2.36cm, which was on par with *Microbacterium* sp.(NKdS) + 75% N + 50% FYM (T_{12}) and*Microbacterium* sp.(NPPV) + 75% N + 50% FYM (T_{14}). T_{11} at 90 DAS and harvest was found to be significantly superior to all other treatments, and recorded girth of 2.87cm and 3.38cm respectively. Absolute control (T_{18}) recorded lowest values for stem girth during all growth stages (1.01cm, 1.60cm, 1.74cm respectively).*Microbacterium* sp. recorded maximum girth when bioinoculants alone were applied (T_2) as well as bioinoculants were applied along with 75% N (T_7). In all the treatment combinations, the four native isolates performed better than KAU *Azotobacter* formulation.

4.7.1.5. Days to flowering

The data on days taken for first flowering are presented in Table 27. Number of days taken for flowering ranged from 59 to 68 days. Minimum number of days (59.75) for first blooming was recorded in treatment with isolate *Cellulosimicrobium* sp. (NPS-1) + 75% N and 50% FYM (T_{11}) followed by *Microbacterium* sp. (NKdS) + 75% N + 50% FYM (T_{12}). Maximum number of days (69.66) to first blooming was recorded in T_5 (KAU *Azotobacter* formulation) which was statistically on par with T_1, T_3, T_{10}, T_{15}, T_{16} and T_{18}.

4.7.1.6. Leaf area

Combined application of isolate NPS-1(*Cellulosimicrobium* sp.) along with 75% N + 50% FYM (T_{11}) recorded highest leaf area of 32.77cm^2(Table 27). This was statistically on par with all other treatments except T_1, T_3, T_4, T_5 and T_{18}. Among

Table 26. Effect of free living diazotrophs on girth of stem in tomato

Treatment	Girth of stem (cm)		
	60 DAP	90 DAP	Harvesting
T_1 (*Microbacterium* sp.)	1.86^{g}	2.22^{gh}	2.44^{hij}
T_2 (*Cellulosimicrobium* sp.)	1.98^{efg}	2.37^{efg}	2.47^{ghi}
T_3 (*Microbacterium* sp.)	1.41^{h}	2.18^{h}	2.39^{ij}
T_4 (*Brevundimonas* sp.)	1.41^{h}	2.16^{h}	2.25^{j}
T_5 (*Azotobacter* formulation)	0.95^{i}	1.71^{i}	1.84^{k}
T_6 (T_1 + 75% N)	2.06^{de}	2.40^{def}	2.56^{fghi}
T_7 (T_2 + 75% N)	2.09^{cde}	2.43^{de}	2.64^{efgh}
T_8 (T_3 + 75% N)	1.99^{efg}	2.43^{de}	2.56^{fghi}
T_9 (T_4 + 75% N)	2.03^{ef}	2.36^{efg}	2.55^{fghi}
T_{10} (T_5 + 75% N)	1.49^{h}	2.22^{gh}	2.40^{ij}
T_{11} (T_6 + 75% N + 50% FYM)	2.36^{a}	2.87^{a}	3.38^{a}
T_{12} (T_7 + 75% N + 50% FYM)	2.32^{ab}	2.66^{b}	3.10^{b}
T_{13} (T_8 + 75% N + 50% FYM)	2.21^{bc}	2.47^{cde}	2.85^{cd}
T_{14} (T_9 + 75% N + 50% FYM)	2.34^{ab}	2.54^{bcd}	2.82^{cde}
T_{15} (T_5 + + 50% FYM)	1.91^{fg}	2.25^{fgh}	2.67^{defg}
T_{16} (T_5 + 75% N + 50% FYM)	2.07^{cde}	2.44^{de}	2.70^{def}
T_{17} (POP Recommendation)	2.20^{bcd}	2.61^{bc}	2.98^{bc}
T_{18} (Absolute control)	1.01^{i}	1.60^{i}	1.74^{k}

Table 27. Effect of free living diazotrophs on days to flowering and leaf area

Treatments	Days taken for first flowering	Leaf area (cm^2)
T_1 (*Microbacterium* sp.)	66.83^{abcd}	27.68^{bcd}
T_2 (*Cellulosimicrobium* sp.)	64.16^{cdef}	28.24^{abcd}
T_3 (*Microbacterium* sp.)	66.41^{abcde}	27.50^{bcd}
T_4 (*Brevundimonas* sp.)	64.25^{cdef}	26.21^{cd}
T_5 (*Azotobacter* formulation)	67.58^{a}	26.42^{cd}
T_6 (T_1 + 75% N)	65.41^{cdef}	28.77^{abcd}
T_7 (T_2 + 75% N)	63.41^{defg}	30.74^{abc}
T_8 (T_3 + 75% N)	64.58^{bcdef}	28.68^{abcd}
T_9 (T_4 + 75% N)	64.50^{cdef}	29.94^{abc}
T_{10} (T_5 + 75% N)	69.66^{abc}	28.65^{abcd}
T_{11} (T_6 + 75% N + 50% FYM)	59.75^{g}	32.70^{a}
T_{12} (T_7 + 75% N + 50% FYM)	61.66^{fg}	32.01^{ab}
T_{13} (T_8 + 75% N + 50% FYM)	62.91^{defg}	31.98^{ab}
T_{14} (T_9 + 75% N + 50% FYM)	62.75^{efg}	30.67^{abc}
T_{15} (T_5 + + 50% FYM)	66.58^{abcde}	28.25^{abcd}
T_{16} (T_5 + 75% N + 50% FYM)	66.16^{abcde}	29.85^{abc}
T_{17} (POP Recommendation)	64.16^{cdef}	32.34^{a}
T_{18} (Absolute control)	68.50^{ab}	25.23^{d}

treatments with isolate alone, NKdS (*Microbacterium* sp.) recorded maximum leaf area. Minimum leaf area (25.23 cm^2) was recorded in absolute control (T_{18}).

4.7.1.7. Fresh weight of root

Treatment effects on fresh weight of root at harvest are given in Table 28 and Plate 13. Results revealed that inoculants had a significant effect on fresh weight of root. Treatments with microbial inoculant + 75% N + 50% FYM resulted in significantly higher fresh weight of root than treatments with isolate alone or isolate + 75% N. Fresh weight of root was highest (48.907g) in NKdS (*Microbacterium* sp.) + 75% N + 50% FYM (T_{12}) followed by POP recommendation of KAU (41.160g). Least fresh weight of root (3.98g) was obtained in T_4 (*Brevundimonas* sp.) which was statistically on par with T_5 (KAU *Azotobacter* formulation) andT_{18} (absolute control). *Microbacterium* sp. (NKdS) recorded maximum fresh weight of root, among the treatments with isolate alone. The same isolate performed better along with 75% N also. The effect of KAU *Azotobacter* formulation on root fresh weight was less, compared to the effect of other four isolates and POP recommendation.

4.7.1.8. Volume of root

The highest root volume of 40.66cm^3 was recorded in *Microbacterium* sp. (NKdS) + 75% N+ 50% FYM (T_{12}) and this was significantly superior to all other treatment (Table 28). This was followed by POP recommendation (T_{17})with a root volume of 34.83cm^3. Lowest root volume (1.84cm^3) was obtained in absolute control (T_{18}). Among the treatments with isolate alone, NKdS (*Microbacterium* sp.) recorded highest root volume which was on par with T_1 (*Cellulosimicrobium* sp.).

4.7.1.9. Root length

The effects of application of different treatments on root length are presented in Table 28. Application of NPPV (*Brevundimonas* sp.) along with 75% N (T_9)

Table 28. Effect of free living diazotrophs on root parameters in tomato

Treatment	Fresh weight of root (g)	Volume of root (cm^3)	Root length (cm)
T_1 (*Microbacterium* sp.)	6.91^{jk}	8.83^{i}	14.26^{e}
T_2 (*Cellulosimicrobium* sp.)	7.74^{ij}	9.66^{i}	17.60^{de}
T_3 (*Microbacterium* sp.)	5.11^{kl}	5.00^{j}	17.90^{de}
T_4 (*Brevundimonas* sp.)	3.98^{l}	3.16^{jk}	13.43^{e}
T_5 (*Azotobacter* formulation)	4.42^{l}	2.66^{jk}	17.23^{de}
T_6 (T_1 + 75% N)	9.13^{hij}	13.00^{gh}	36.63^{abc}
T_7 (T_2 + 75% N)	11.40^{gh}	15.33^{fg}	18.33^{de}
T_8 (T_3 + 75% N)	9.69^{hi}	13.00^{gh}	22.90^{abcde}
T_9 (T_4 + 75% N)	8.16^{ij}	10.83^{hi}	39.03^{a}
T_{10} (T_5 + 75% N)	6.83^{jk}	9.33^{i}	20.23^{cde}
T_{11} (T_6 + 75% N + 50% FYM)	36.67^{c}	29.00^{c}	36.73^{abc}
T_{12} (T_7 + 75% N + 50% FYM)	48.90^{a}	40.66^{a}	35.80^{abc}
T_{13} (T_8 + 75% N + 50% FYM)	25.39^{d}	22.83^{d}	32.66^{abcd}
T_{14} (T_9 + 75% N + 50% FYM)	22.65^{e}	21.33^{d}	22.60^{abcde}
T_{15} (T_5 + + 50% FYM)	12.62^{g}	18.33^{e}	16.10^{de}
T_{16} (T_5 + 75% N + 50% FYM)	17.57^{f}	16.83^{ef}	22.43^{bcde}
T_{17} (POP Recommendation)	41.16^{b}	34.83^{b}	37.40^{ab}
T_{18} (Absolute control)	4.11^{l}	1.84^{k}	11.50^{e}

T_{11} T_{12} T_{13} T_{14} T_{15} T_{16} T_{17} Control

A. Isolates + 75% N + 50% FYM

T_{11}: *Cellulosimicrobium* sp.

T_{12}: *Microbacterium* sp. (Soil)

T_{13}: *Microbacterium* sp. (Phylloplan

T_{14}: *Brevundimonas* sp.

T_{16}: *Azotobacter* formulation

T_{17}: POP Recommendations

T_2 T_7 T_{12} Control

T_2: *Microbacterium* sp.

T_7: T_2 + 75% N

T_{12}: T_7 + 50% FYM

B. *Microbacterium* (NKdS)

Plate 13: Effect of free living diazotrophs on root growth

recorded maximum root length of 39.03cm which was statistically on par with T_6, T_8, T_{11}, T_{12}, T_{13}, T_{14}, T_{17}. Absolute control recorded minimum root length of 11.50cm, which was on par with T_1 (*Cellulosimicrobium*) and T_4 (NPPV: *Microbacterium*). Root length was found to be maximum in T_3*Microbacterium* sp. (NPPV) among treatments with isolate alone and T_9 (*Brevundimonas* sp.) recorded maximum root length, among treatments with isolates + 75% N. In presence of 75% N and 50% FYM, *Cellulosimicrobium* sp. (NPS-1) recorded maximum root length.

4.7.1.10. Fresh weight of plants

The treatment effects on plant fresh weight are presented in Table 29. Combined application of microbial inoculants *Microbacterium* sp. (NKdS) + 75% N + 50% FYM (T_{12}) was found to be statistically superior to all other treatments with a fresh weight of 133.06g. Lowest fresh weight was noted in absolute control with 82.63g. The effect of KAU *Azotobacter* formulation on fresh weight of plant was less, compared to the effect of other treatments.

4.7.1.11. Dry weight of plants

The effect on plant dry weight at harvest, by the application of different treatments is given in Table 29. Dry weight of plant was highest (32.46g) in *Microbacterium* sp. (NKdS) + 75% N + 50% FYM (T_{12})which was on par with *Cellulosimicrobium* sp. (NPS-1) +75% N + 50% FYM (T_{11}) and T_{17} (POP recommendation). Lowest dry weight of 21.11g was observed in absolute control and this was on par with T_5 (KAU *Azotobacter* formulation).

Table 29. Effect of free living diazotrophs on fresh and dry weight of plants

Treatment	Fresh weight of plant (g)	Dry weight of plant (g)
T_1 (*Microbacterium* sp.)	97.97^{h}	26.58^{def}
T_2 (*Cellulosimicrobium* sp.)	99.27^{h}	24.49^{fg}
T_3 (*Microbacterium* sp.)	97.84^{h}	24.51^{fg}
T_4 (*Brevundimonas* sp.)	91.32^{i}	22.80^{gh}
T_5 (*Azotobacter* formulation)	86.85^{j}	21.10^{h}
T_6 (T_1 + 75% N)	111.92^{ef}	27.60^{cde}
T_7 (T_2 + 75% N)	113.53^{e}	29.34^{bcd}
T_8 (T_3 + 75% N)	112.28^{ef}	28.76^{bcde}
T_9 (T_4 + 75% N)	111.93^{ef}	26.91^{def}
T_{10} (T_5 + 75% N)	105.16^{g}	27.55^{cdef}
T_{11} (T_6 + 75% N + 50% FYM)	124.24^{b}	30.44^{abc}
T_{12} (T_7 + 75% N + 50% FYM)	133.06^{a}	32.46^{a}
T_{13} (T_8 + 75% N + 50% FYM)	122.12^{bc}	28.01^{bcde}
T_{14} (T_9 + 75% N + 50% FYM)	115.17^{de}	28.82^{bcde}
T_{15} (T_5 + + 50% FYM)	108.98^{fg}	26.04^{ef}
T_{16} (T_5 + 75% N + 50% FYM)	112.19^{ef}	28.04^{bcde}
T_{17} (POP Recommendation)	119.28^{cd}	30.94^{ab}
T_{18} (Absolute control)	82.63^{j}	21.11^{h}

4.7.2. Yield attributes

4.7.2.1. Average fruit weight

The data on average fruit weight is given in Table 30. Highest average fruit weight of 28.60g was recorded in *Microbacterium* sp. (NKdS) + 75% N + 50% FYM (T_{12}) which was on par with T_7, T_{11}, T_{13}, T_{14}, T_{16} and T_{17} (POP recommendation). NKdS (*Microbacterium* sp.) recorded highest average fruit weight, among the treatments with isolates alone and isolates + 75% N. Minimum fruit weight was observed in absolute control (21.01g).

4.7.2.2. Yield per plant

The effect of application of different treatments on yield per plant is given in Table 30. In general, application of isolates along with 75% N + 50% FYM recorded significantly higher yield per plant than isolate alone and isolate + 75% N. *Microbacterium* sp. (NKdS) + 75% N + 50% FYM (T_{12}) recorded highest per plant yield of 136.90g and this was statistically superior to all other treatments. Second highest per plant yield was observed in *Cellulosimicrobium* sp. (NPS-1) +75% N + 50% FYM (T_{11}). Lowest yield per plant (29.93g) was recorded in absolute control (T_{18}).

4.7.2.3. Number of fruits

The data on effect of treatment on number of fruits (Table 30) shows that highest number of fruits was produced in treatment *Microbacterium* sp. (NKdS) + 75% N + 50% FYM (T_{12}). This was statistically on par with POP recommendation and other bioinoculants + 75% N + 50% FYM. There was no significant difference in number of fruits, among treatments with isolate + 75% N. Minimum number of fruits (2.33) was recorded in absolute control and T_5 (KAU *Azotobacter* formulation).

Table 30. Effect of free living diazotrophs on yield characteristics

Treatment	Number of fruits	Average fruit weight (g)	Yield per plant (Kg)
T_1 (*Microbacterium* sp.)	3.66^{bcde}	23.62^{de}	50.37^{g}
T_2 (*Cellulosimicrobium* sp.)	3.66^{bcde}	24.06^{cde}	47.16^{gh}
T_3 (*Microbacterium* sp.)	3.00^{cde}	23.22^{de}	42.04^{hi}
T_4 (*Brevundimonas* sp.)	2.66^{de}	23.23^{de}	38.14^{i}
T_5 (*Azotobacter* formulation)	2.33^{e}	23.17^{de}	41.80^{hi}
T_6 (T_1 + 75% N)	4.33^{bcde}	25.73^{bcde}	66.50^{f}
T_7 (T_2 + 75% N)	4.66^{bcde}	26.02^{abcd}	76.03^{e}
T_8 (T_3 + 75% N)	4.33^{bcde}	25.34^{bcde}	60.66^{f}
T_9 (T_4 + 75% N)	4.00^{bcde}	25.05^{bcde}	50.80^{g}
T_{10} (T_5 + 75% N)	4.00^{bcde}	24.72^{bcde}	48.13^{gh}
T_{11} (T_6 + 75% N + 50% FYM)	6.00^{ab}	26.03^{ab}	129.88^{b}
T_{12} (T_7 + 75% N + 50% FYM)	7.33^{a}	28.60^{a}	136.90^{a}
T_{13} (T_8 + 75% N + 50% FYM)	5.00^{abcd}	27.49^{abc}	88.09^{d}
T_{14} (T_9 + 75% N + 50% FYM)	5.33^{abc}	26.18^{abcd}	85.49^{d}
T_{15} (T_5 + + 50% FYM)	4.00^{bcde}	24.01^{cde}	60.31^{f}
T_{16} (T_5 + 75% N + 50% FYM)	5.00^{abcd}	26.06^{abcd}	82.60^{de}
T_{17} (POP Recommendation)	5.33^{abc}	27.94^{abcd}	98.64^{c}
T_{18} (Absolute control)	2.33^{e}	21.01^{e}	29.93^{j}

4.8. Estimation of total nitrogen content

4.8.1. Estimation of total nitrogen content of plant

Nitrogen content of index leaf (60 DAS) and plant (at harvest) is presented in Table 31. Combined application of isolates along with 75% N + 50% FYM recorded higher nitrogen content compared to treatments with isolate alone and isolate + 75% N. Nitrogen content in index leaf was higher compared to nitrogen content in the plant at the time of harvest.

In index leaf, highest nitrogen content of 3.06% was recorded in *Microbacterium* sp. (NKdS) + 75% N + 50% FYM (T_{12}) which was statistically superior to all other treatments. T_{12} was followed by *Cellulosimicrobium* sp. (NPS-1)+75% N + 50% FYM (T_{11})which was on par with T_{17} (POP recommendation). Nitrogen content in index leaf was lowest (1.57%) in absolute control (T_{18}).

At harvest, nitrogen content in *Microbacterium* sp. (NKdS) + 75% RD of N + 50% RD of FYM (T_{12}) recorded highest value (1.31%) which was on par with T_{11} (1.22%). Lowest nitrogen content was recorded in absolute control (0.35%). Treatment with NKdS (*Microbacterium* sp.) recorded higher nitrogen content when applied alone as well as in combination with 75% N.

Nitrogen content in index leaf and plant at harvest was higherin treatment with NKdS (*Microbacterium* sp.), when applied alone and as well as in combination with 75% N.

4.8.2. Estimation of total nitrogen content in soil

Total nitrogen content in the soil at 60 DAS and harvest is given in Table 31. Initial nitrogen status of the potting mixture was 1.0g/kg. In general, nitrogen content was higher in treatment with isolates + 75% N + 50% FYM compared to treatments with isolate alone and isolate + 75% N. At 60 DAS, highest nitrogen content of

Table 31.Effect of free living diazotrophs on nitrogen content in plant and soil

Treatment	Nitrogen content			
	60 DAS		At Harvest	
	Index leaf (%)	Soil (g/kg)	Plant (%)	Soil (g/kg)
T_1 (*Microbacterium* sp.)	1.83^{jk}	1.00^{h}	0.61^{hi}	0.90^{h}
T_2 (*Cellulosimicrobium* sp.)	1.92^{j}	1.00^{h}	0.70^{gh}	0.90^{h}
T_3 (*Microbacterium* sp.)	1.75^{kl}	0.96^{h}	0.61^{hi}	0.93^{h}
T_4 (*Brevundimonas* sp.)	1.75^{kl}	1.00^{h}	0.52^{ij}	0.83^{i}
T_5 (*Azotobacter* formulation)	1.66^{lm}	0.83^{j}	0.43^{jk}	0.73^{j}
T_6 (T_1 + 75% N)	2.36^{gh}	1.73^{d}	0.87^{ef}	1.30^{e}
T_7 (T_2 + 75% N)	2.45^{fg}	1.70^{d}	0.96^{de}	1.40^{d}
T_8 (T_3 + 75% N)	2.27^{hi}	1.53^{e}	0.87^{ef}	1.23^{f}
T_9 (T_4 + 75% N)	2.18^{i}	1.30^{f}	0.78^{fg}	1.20^{f}
T_{10} (T_5 + 75% N)	2.27^{hi}	1.13^{g}	0.78^{fg}	1.00^{g}
T_{11} (T_6 + 75% N + 50% FYM)	2.88^{b}	2.10^{b}	1.22^{ab}	1.93^{a}
T_{12} (T_7 + 75% N + 50% FYM)	3.06^{a}	2.20^{a}	1.31^{a}	1.90^{a}
T_{13} (T_8 + 75% N + 50% FYM)	2.71^{cd}	1.93^{c}	1.13^{bc}	1.80^{b}
T_{14} (T_9 + 75% N + 50% FYM)	2.62^{de}	1.90^{c}	1.05^{cd}	1.40^{d}
T_{15} (T_5 + + 50% FYM)	1.83^{jk}	0.90^{i}	0.70^{gh}	0.83^{i}
T_{16} (T_5 + 75% N + 50% FYM)	2.53^{ef}	1.70^{d}	0.96^{de}	1.50^{c}
T_{17} (POP Recommendation)	2.80^{bc}	2.10^{b}	1.13^{bc}	1.90^{a}
T_{18} (Absolute control)	1.57^{m}	0.60^{k}	0.35^{k}	0.20^{k}

2.20g/kg was obtained in *Microbacterium* sp. (NKdS) + 75% N + 50% FYM (T_{12}) which was significantly superior to nitrogen content of all other treatment. Although 100% N was provided in POP recommendation $T_{12}$was found to be significantly superior. Nitrogen content was lowest (0.60g/kg) in control (T_{18}). There was no significant difference in nitrogen content among the treatments, when bioinoculants were applied alone. Within the treatment combination (isolate + 75% N and isolate + 75% N + 50% FYM), nitrogen content in treatments with KAU *Azotobacter* formulation was found to be less.

At the time of harvest, nitrogen content was maximum (1.93g/kg) in *Cellulosimicrobium* sp. (NPS-1)+75% N + 50% FYM (T_{11}) which was on par with *Microbacterium* sp. (NKdS) + 75% N + 50% FYM (T_{12}) and T_{17} (POP recommendation). Lowest nitrogen (0.20g/kg) content was obtained in absolute control (T_{18}).

4.7.4. Population of bioinoculants in soil

Population of bioinoculants in soil (for rhizosphere isolates) was assessed prior to the monthly application of bioinoculants (Table 32). At 1 MAP, 2 MAP, 3 MAP and 4 MAP all the bioinoculants recorded significantly higher population compared to uninoculated control and POP recommendations. Population was significantly higher in treatments with bioinoculant + 75% N + 50% FYM than treatments with bioinoculant alone or bioinoculant + 75% N.

At 1 MAP, *Cellulosimicrobium* sp. + 75% N + 50% FYM (T_{11})recorded maximum population (49.22×10^5 cfu/g soil). This was statistically superior to population of all other bioinoculants. T_{11} recorded highest population at 2 MAP (41.10×10^5 cfu/g soil)and this was on par with T_{15} (*Azotobacter* formulation + 75% N) and T_{16} (*Azotobacter* formulation + 75% N + 50% FYM). At 3 MAP and 4 MAP, *Microbacterium* sp. + 75% N + 50% FYM (T_{12}) recorded maximum population

(47.83× 10^5 cfu/g soil and 45× 10^5 cfu/g soil respectively) which was on par with T_{11} (*Cellulosimicrobium* sp. + 75% N + 50% FYM). Minimum population was recorded in absolute control (T_{18}) and POP recommendations (T_{17}).

4.7.5. Population of bioinoculants in phylloplane

Population of bioinoculants in phylloplane (for phylloplane isolates) was assessed before monthly application of foliar spray (Table 33).At 1 MAP, population on dorsal and ventral side of leaf was observed only in T_3 (*Microbacterium* sp.) and T_9 (*Brevundimonas* sp. + 75% N). Maximum population of all the isolates was recorded at 2 MAP on ventral and dorsal side of leaf in all the treatments except in T_3 (*Microbacterium* sp.) on dorsal side and T_9 (*Microbacterium* +75% N) on ventral side. At 2 MAP highest population of 1.25/cm^2leaf area was recorded in T_{13} and T_{14} on dorsal side and 1.40/cm^2leaf area in T_{13} on ventral side. Minimum population on both dorsal (0.98/cm^2leaf area) and ventral (0.90/cm^2leaf area) side was observed in T_8. At 3 MAP, highest population on dorsal surface was observed in T_8 (0.89/cm^2leaf area) which was on par with T_3. On ventral surface highest population of 0.89/cm^2leaf area was recorded in T_3 which was on par with T_8. Minimum population on dorsal surface was observed in T_{13} (0.75/cm^2leaf area). T_{13} recorded minimum population on ventral side which was on par with T_4 and T_{14}. At 4 MAP T_3 recorded highest population of 1.07/cm^2leaf area which was on par with T_9 on dorsal surface. On ventral surface T_{14} recorded maximum population (1.08 /cm^2leaf area) which was on par with T_{13} and T_9. Minimum population on dorsal side was observed in T_{13} (0.93/cm^2leaf area) which was on par with T_8. T_3 recorded lowest population on ventral side.

Table 32. Population of bioinoculants in soil at monthly interval

Treatment	Population of bioinoculants in soil ($\times 10^5$ cfu/g soil)			
	1 MAP	2 MAP	3 MAP	4 MAP
T_1 (*Microbacterium* sp.)	21.50^{c}	28.44^{c}	21.99^{d}	23.66^{d}
T_2 (*Cellulosimicrobium* sp.)	20.55^{c}	20.88^{d}	25.22^{d}	24.55^{d}
T_5 (*Azotobacter* formulation)	10.33^{d}	12.22^{e}	10.77^{e}	10.55^{e}
T_6 (T_1 + 75% N)	26.88^{c}	20.32^{d}	29.77^{c}	23.66^{d}
T_7 (T_2 + 75% N)	25.22^{c}	23.99^{cd}	21.77^{d}	24.70^{d}
T_{10} (T_5 + 75% N)	11.44^{d}	11.33^{e}	10.44^{e}	10.55^{e}
T_{11} (T_6 + 75% N + 50% FYM)	49.22^{a}	41.10^{a}	47.66^{a}	44.99^{a}
T_{12} (T_7 + 75% N + 50% FYM)	37.33^{b}	34.89^{b}	47.83^{a}	45.00^{a}
T_{15} (T_5 + + 50% FYM)	26.21^{c}	37.66^{ab}	38.66^{b}	35.55^{c}
T_{16} (T_5 + 75% N + 50% FYM)	37.88^{b}	38.11^{ab}	38.88^{b}	41.33^{b}
T_{17} (POP Recommendation)	1.44^{e}	2.43^{f}	3.77^{f}	1.99^{f}
T_{18} (Absolute control)	2.10^{e}	1.99^{f}	3.33^{f}	0.88^{f}

Table 33. Population of bioinoculants in phylloplane

Treatments	Population of bioinoculant (cfu/cm^2)							
	Dorsal				Ventral			
	1 MAP	2 MAP	3 MAP	4 MAP	1 MAP	2 MAP	3 MAP	4 MAP
T_3 (*Microbacterium* sp.)	0.27^{b}	1.00^{cd}	0.88^{a}	1.07^{a}	0.29^{b}	1.11^{b}	0.89^{a}	0.96^{c}
T_4 (*Brevundimonas* sp.)	0.0	1.25^{a}	0.85^{b}	1.00^{b}	0.0	1.19^{b}	0.74^{c}	1.01^{b}
T_8 (T_3 + 75% N)	0.0	0.98^{d}	0.89^{a}	0.95^{c}	0.0	0.90^{c}	0.88^{a}	0.97^{bc}
T_9 (T_4 + 75% N)	0.55^{a}	1.04^{c}	0.80^{c}	1.03^{ab}	0.47^{a}	1.09^{b}	0.80^{b}	1.05^{a}
T_{13} (T_8 + 75% N + 50% FYM)	0.0	1.25^{a}	0.75^{d}	0.93^{c}	0.0	1.40^{a}	0.72^{c}	1.06^{a}
T_{14} (T_9 + 75% N + 50% FYM)	0.0	1.19^{b}	0.79^{c}	1.01^{b}	0.0	1.20^{b}	0.74^{c}	1.08^{a}

4.7.6. Population of endophytes

Population of endophytes in stem, root and leaf is presented in Table 34. In general, when bioinoculants were applied as soil application, population build up of the isolates was found to be more in root followed by stem and least in leaf. Population of endophytes in leaf was more when bioinoculants were applied as foliar spray. Maximum endophytes from stem (12.7×10^4 cfu/g) was recorded in *Cellulosimicrobium* (NPS-1) + 75% N +50% FYM (T_{11})which was on par with *Microbacterium* (NKdS) + 75% N +50% FYM (T_{12})and T_6 (*Cellulosimicrobium* + 75% N). In root also, maximum population (16.4×10^4 cfu/g) was obtained in T_{11} which was on par with T_{12}. In the case of leaf, *Brevundimonas* (NKPV-2) + 75% N + 50% FYM (T_{14}) recorded maximum population of 20.4×10^4 cfu/g. This was statistically superior to all other treatments. In stem, root and leaf, minimum population was observed in control (T_{18}), POP recommendations (T_{17}) and KAU *Azotobacter* formulation (T_5).

Table 34. Population of endophytes in plants at harvest

Treatment	Population of endophytes ($\times 10^4$ cfu/g)		
	Stem	Root	Leaf
T_1(*Microbacterium* sp.)	5.66^{efg}	8.00^{de}	2.33^{fg}
T_2(*Cellulosimicrobium* sp.)	6.66^{def}	9.33^{d}	1.00^{fgh}
T_3(*Microbacterium* sp.)	3.33^{ghi}	6.00^{e}	10.00^{cd}
T_4 (*Brevundimonas* sp.)	5.00^{efg}	5.66^{e}	7.70^{e}
T_5 (*Azotobacter* formulation)	3.33^{ij}	2.00^{f}	0.00^{h}
T_6 (T_1 + 75% N)	12.66^{a}	10.33^{cd}	8.33^{cde}
T_7 (T_2 + 75% N)	9.7^{bc}	12.4^{bc}	3.00^{f}
T_8 (T_3 + 75% N)	4.33^{fgh}	6.00^{e}	10.40^{c}
T_9 (T_4 + 75% N)	6.66^{def}	5.66^{e}	8.00^{de}
T_{10} (T_5 + 75% N)	2.00^{hij}	9.33^{d}	0.33^{gh}
T_{11} (T_6 + 75% N + 50% FYM)	12.7^{a}	16.40^{a}	7.00^{e}
T_{12} (T_7 + 75% N + 50% FYM)	11.33^{ab}	14.7^{ab}	2.33^{fg}
T_{13} (T_8 + 75% N + 50% FYM)	8.66^{bcd}	10.40^{cd}	16.33^{b}
T_{14} (T_9 + 75% N + 50% FYM)	6.33^{def}	9.33^{d}	20.40^{a}
T_{15} (T_5 + + 50% FYM)	3.33^{ghi}	9.66^{cd}	0.33^{gh}
T_{16} (T_5 + 75% N + 50% FYM)	7.33^{cde}	10.00^{cd}	1.33^{fgh}
T_{17} (POP Recommendation)	0.66^{ij}	1.33^{f}	1.00^{fgh}
T_{18} (Absolute control)	1.00^{ij}	0.33^{f}	0.00^{h}

Discussion

5. DISCUSSION

Nitrogen is one of the most important nutrients required for plant growth and productivity, as it forms an integral part of proteins, nucleic acids and other essential biomolecules (Bockman, 1997). Although the atmosphere contains 78% nitrogen, it is in the elemental form and unavailable to plants. It needs to be converted into ammonium, which is the form available to plants and other eukaryotes. Atmospheric nitrogen is converted into forms utilized by plants by biological nitrogen fixation, which involves the conversion of nitrogen into ammonium by microorganisms, using a complex enzyme system identified as nitrogenase (Kim and Rees, 1994). Biological nitrogen fixation contributes about 60% of the earth's available nitrogen and represents an economically beneficial and environmentally sound alternative to chemical fertilizers (Ladha *et al.*, 1997). The availability of nitrogen often limits plant growth in terrestrial ecosystem. In agriculture, nitrogen is one of the most widely used fertilizers, with an ever-increasing demand. The only biological reaction counterbalancing the loss of N from soils or ecosystems is biological nitrogen fixation.In agricultural soils, except for anthropogenic sources, nitrogen fixing microorganisms are the main source of nitrogen. Biological fixation offers a non-polluting source of nitrogen and could improve crop production and decrease the global use of synthetic fertilizers. Non-symbiotic bacterial diazotrophs can bring about economic and environmental benefits including increased income from high yields, reduced fertilizer costs and reduced emissions of the greenhouse gas N_2O, as well as reduced leaching of nitrate to ground water (Kennedy *et al.*, 2004). Besides their role in atmospheric nitrogen fixation, they also provide better nutrient uptake, increased tolerance towards drought and moisture stress and antagonistic activity against pathogens.

Kerala being in the high rainfall region, is predominantly an acid soil tract. About 88% of the Kerala soils are acidic in nature. Strongly acidic environment leads to a stressed environment for plant growth. It impairs the absorption of nutrients and

inhibits the microbial activity in soil. Hence, efficient biofertilizers have to be developed which are adaptable to the acidic soils of Kerala. Biofertilizers capable of fixing atmospheric nitrogen can replace chemical nitrogen by 25% (Evans and Furlong, 2010).Being non-specific, free living nitrogen fixers could be used for any crop.Nitrogenous biofertilizers like *Azotobacter* and *Azospirillum* have been commercialized but, other nitrogen fixers like *Derxia* and *Beijerinckia* have not been exploited in Kerala. Reports indicate that soil pH below 6.0 supports the occurrence of acid tolerant strains of diazotrophs like *Azotobacter chroococcum, Beijerinckia indica,* and *Derxia gummosa* (Andre *et al.*, 2007).

Black pepper is an important spice crop of Kerala. It is one of the oldest spices known to mankind.In India, Kerala ranks first in black pepper production, contributing about 97%. Wayanad and Idukki are the main pepper growing tracts in Kerala. In Wayanad, black pepper is cultivated in 36,488ha area with a production of 9828 tonnes (FIB, 2009).

Rhizosphere is the region around the root and it is the most active site of microbial activity (Pathania *et al.*, 2014). Colonization of microorganisms is more in rhizosphere compared to non-rhizosphere region, due to the presence of root exudates which are the main source of food to the microorganisms. Area on leaves which is inhabited by microorganisms is known as phylloplane. Exposure of leaves to air current, dust etc. containing lots of microorganisms result in establishment of a typical microflora on leaf surface. A wide range of microorganisms like nitrogen fixers, phosphate solubilizers, potassium solubilizing and mobilizing microorganismswhich are beneficial to the plants, are present in the rhizosphere and phylloplane.

Presence of beneficial microorganisms in the rhizosphere, could be one of the reasons for a plant to remain healthy. Hence in the present investigation, diversity of

beneficial nitrogen fixers present in the rhizosphere soil and phylloplane of healthy black pepper garden was studied.

In the present study, rhizosphere soil and leaf samples from ten healthy black pepper gardens of Wayanad district were collected for the isolation of free living diazotrophs. Analysis of physico-chemical properties of soil samples indicated that, soil samples were sandy loamand very strongly acidic with pH below 5.0. Organic carbon content was medium (1.5%) in Kalpetta and high (1.7%) in Bathery.

Population of diazotrophic bacteria in rhizosphere soil varied from 9.3×10^4 to 19.6×10^4 cfu/g of soil. Maximum population of nitrogen fixers (19.6×10^4 cfu/g of soil) in rhizosphere soil was obtained from Kochangode and lowest population from Pazhaya Vythiri (9.3×10^4 cfu/g of soil). It is generally known that, the microbial population in any rhizosphere soil is influenced by several biotic and abiotic factors. Soil physico-chemical properties influence the nature and type of microflora, including the diazotrophs (Naher *et al.*, 2009). Among the soil properties, soil pH is one of the strong determinants of microbial community structure in soil (Pathania *et al.*, 2014). Organic carbon present in the soil influences the status of food materials available to the microorganisms. Microbial population was found to be higher, when organic carbon is higher in the soil (Sahoo *et al.*, 2009). The root exudates of the plant also have a profound effect on the rhizoflora. Hence, the variation in population of diazotrophs in rhizosphere soils collected from various locations in the present study could be due to soil chemical and physical properties.

Population of diazotrophs in phylloplane was enumerated from both dorsal and ventral surfaces of leaf. On the dorsal surface of leaf, highest population was observed in samples from Malavayal (1.23 cfu/cm^2 leaf area) and on ventral surface, highest population was obtained from RARS (1.38 cfu/cm^2 leaf area). The variation in population of the free living diazotrophs in different locations might be due to the difference in stage of the plant growth at the time of sample collection(Pathania *et al.*,

2014). In this study, leaf samples were collected from black pepper vines at different growth stages and hence this could have also influenced the population of diazotrophs. In the present study,colonization of diazotrophs was found to be more on dorsal surface, when Jensen's agar and *Beijerinckia* agar was used. However, on Ashby's agar colonization was more on ventral surface of leaf.

The microbial diversity is influenced by both the environment and choice of the nutrient media (Bhromsiri and Bhromsiri, 2010). Earlier studies indicated that, population of diazotrophs vary with media used for isolation. The nitrogen free media for isolation of nitrogen fixers include Jensen's, *Beijerinckia*, Burk's, Ashby's and Dobereiner's media. In the present study, three nitrogen free media namely Jensen's agar, *Beijerinckia* agar and Ashby's agar were used for the isolation of free living diazorophs. When the mean population of diazotrophs in rhizosphere soil on these three media was compared, maximum mean population (16.23×10^4 cfu/ g of soil)was recorded on Jensen's agarand minimum mean population (12.81×10^4 cfu/ g of soil) was recorded on Ashby's agar (Fig. 6). In phylloplane also, maximum mean population among the three media was obtained on Jensen's agar and minimum population was obtained on Ashby's agar (Fig. 7). Pathania *et al.* (2014) also compared different nitrogen free media and found that Jensen's medium performed better than*Beijerinckia* medium. This could be due to the low pH of *Beijerinckia* medium (6.5). $CaCO_3$ is one of the ingredients of the former medium and this could have neutralized the acidity produced by diazotrophs during their growth. Stella and Suhaimi (2010) compared the performance of *Beijerinckia* agar with Ashby's agar for supporting the growth of four diazotrophs. They found that sucrose containing medium (*Beijerinckia* agar) was most ideal for isolation, growth and nitrogenase activity of free living diazotrophs. The carbon source in Ashby's agar was glucose and this could be the reason for poor growth.

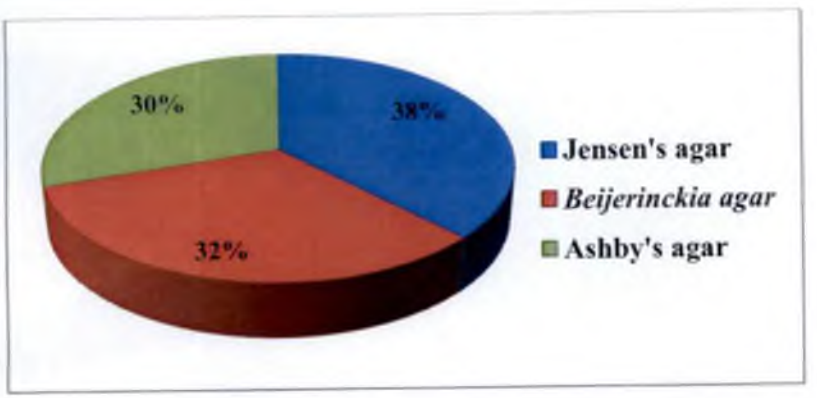


Fig. 6.Mean population of diazotrophs from rhizosphere soil on three media

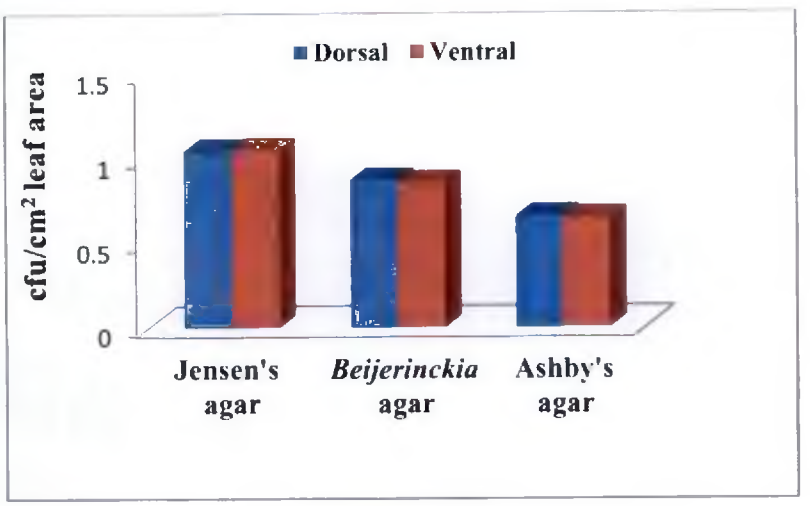


Fig. 7. Mean population of diazotrophs from phylloplane on the three media

In the present study, pH of the soil sample collected and media used for the isolation was different. Population of acid tolerant strains isolated, might be less compared to the actual population present in the soil, since the pH of the media used was higher. The soil samples collected were very strongly acidic (pH below 5.0) and the pH of the three media used for the isolation was 6.5 (*Beijerinckia* agar), 7.4(Ashby's agar) and 7.2 (Jensen's agar). If the media adjusted to lower pH was used, more acid tolerant strains could have been isolated.

A group of bacterial strains that are distinguishable from other strains based on morphological characteristics are called morphotype. Six different morphotypes were obtained from rhizosphere soil, in which medium sized, mucoid, circular colony was the predominant one (Fig. 8). From phylloplane, five morphotypes were obtained and predominant morphotype was large sized, mucoid, circular colony (Figs. 9 and 10). Maximum number of morphotypes from rhizosphere soil and phylloplane was obtained from Bathery. This could be due to the higher organic carbon content in Bathery soil (1.7%, rated as high), as compared to Kalpetta soil (1.5%, rated as medium). Pathania *et al.* (2014) reported that the number of morphotypes in different locations may vary due to variation in soil characters like pH, EC, presence of NH_4^+,NO_3^- and organic carbon.

In the present study, forty three isolates of diazotrophic bacteria were obtained, including twenty five from rhizosphere soil and eighteen from phylloplane. From these, twenty predominant isolates including eleven from rhizosphere soil and nine from phylloplane were selected based on their population on Jensen's agar for further evaluation.

Cultural and morphological characteristics of the twenty selected isolates were studied on Jensen's agar. Colony characters were also recorded in a complete medium, nutrient agar (NA). Most of the colonies were raised, circular and mucoidal,

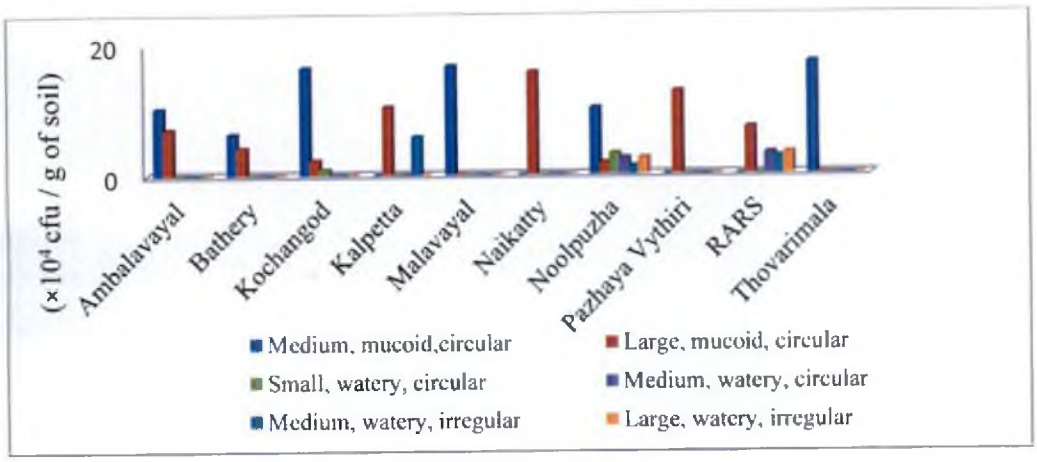


Fig. 8.Population of morphotypes in rhizosphere soil from different locations

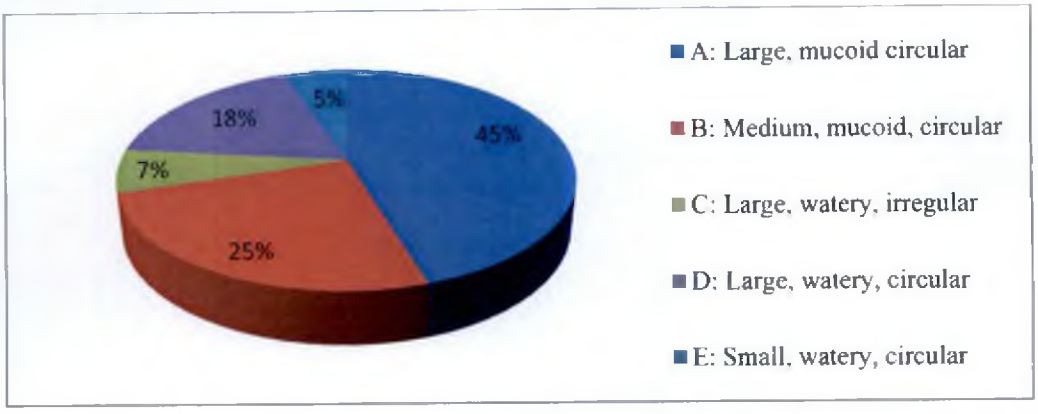


Fig. 9.Population of morphotypes on dorsal surface of leaves from different locations

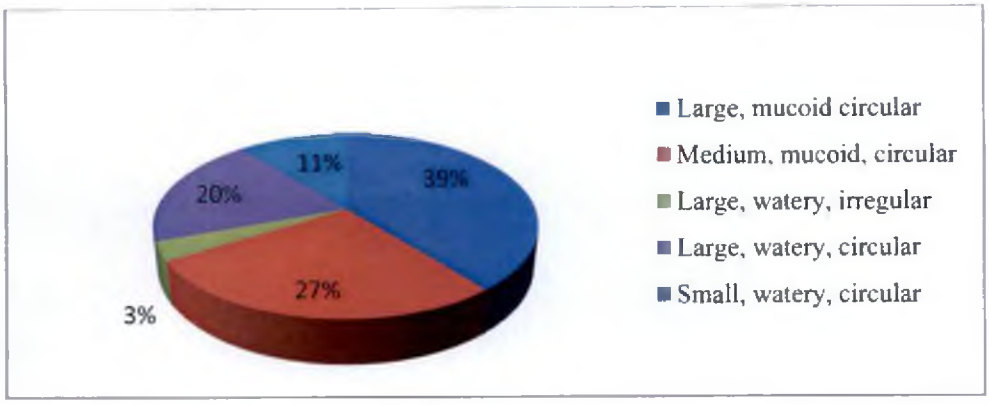


Fig. 10.Population of morphotypes on ventral surface of leaf from different locations

with entire margin. Jensen's medium is a selective medium that contains no source of nitrogen. This could be the reason for absence of pigmentation on this medium. However, on nutrient agar, most of the isolates produced white or coloured, smooth colonies. Nutrient agar is a complex medium that contains beef extract providing all the required nutrients to bacteria and peptone which is a source of organic nitrogen.

Gram staining is one of the major tests carried out for characterization of bacterial isolates. It is a differential staining to classify bacteria into either Gram positive and negative. The variation in cell wall composition of Gram positive and negative bacteria accounts for the difference in staining. Cell wall of Gram positive bacteria contain thick layer of peptidoglycan with numerous teichoic acid cross linking, which resist the decolourization and remains purple. In the present study, fifteen isolates were identified as Gram positive rods and five were Gram negative rods.

All the twenty selected isolates were screened *in vitro* for their ability to grow and fix nitrogen at different levels of pH (7.0, 6.5, 6.0, 5.5, and 5.0). A preliminary experiment was conducted to standardize the time interval at which maximum growth is obtained for all the isolates, in liquid culture. Jensen's broth at a pH of 7.0 was used and population was estimated at 0h, 48h, 72h, 96h, 120h and 144h after inoculation. Maximum growth was observed at 96h for all the isolates (Fig.1).Hence, in further evaluation of growth of isolates at varying pH levels, enumeration was carried out at 96h. All the selected isolates were found to grow at all the pH levels tested. However, among the twenty isolates, NKdS (*Microbacterium* sp.), NPS-1 (*Cellulosimicrobium* sp.), NPPV(*Microbacterium* sp.) and NKPV-2 (*Brevundimonas* sp.) performed better at all the pH levels tested. In all the isolates, highest growth and nitrogen fixation was observed at pH 7.0 and there was a gradual decline as pH was lowered (Fig.2-5). A similar trend was reported earlier by Ninawe and Paulraj (1997). They reported that population and nitrogen fixation of *Azotobacter*

*vinelandii*was maximum at near or slightly above neutrality. With the increasing level of pH, a gradual increasing trend of growth was observed upto neutral pH, which was followed by maximum growth at pH 7.0. Further increase in pH resulted in a sharp decline of growth. They observed decrease of nitrogen fixation at more acidic pH and complete inhibition below pH 5.0. They also noticed that all isolates recorded growth and nitrogen fixation at pH 7.0, 6.5, 6.0, 5.5, and 5.0. Other reports also indicated that maximum growth of *Azotobacter* was obtained at pH 7.0 and growth diminished at acidic pH 4.0 (Saribay, 2003; Carpa *et al.*, 2010). The results of the present investigation revealed the significance of native, acid tolerant strains of biofertilizers and also amelioration of soil acidity by liming.

PGPM provide beneficial effects on the plant directly or indirectly. Direct promotion of growth includes, production of metabolites that enhances plant growth such as auxins, cytokinins, gibberellins, fixation of atmospheric nitrogen, and through the solubilization of phosphate minerals. Indirect growth promotion occurs through the suppression of pathogens by the production of secondary metabolites such as hydrogen cyanide, ammonia and siderophores.

Indole-3-acetic acid (IAA) is an important plant growth regulator and growth stimulator produced by the plants as well as bacteria. IAA produced by bacteria improves plant growth by increasing the number of root hairs and lateral roots(Okon and Kapulnik, 1986). In the present study, all the twenty selected isolates were screened, for their IAA production using Salkowski assay method (Brick *et al.*, 1991). Based on the development of pink to red colour on the filter paper after soaking in Salkowski reagent, four isolates namely NTS-3, NKdS, NKPV-1 and NPS-1 found to be positive for IAA production. Among these, NPS-1 (*Cellulosimicrobium* sp.) was found to be an excellent producer of IAA while NKdS (*Microbacterium* sp.) was a moderate IAA producer. In a similar study by Egamberdieva (2008),IAA production was detected in some strains of

Microbacterium and *Cellulosimicrobium*.IAA production was detected in a strain of *Cellulosimicrobiumcellulans* (Chatterjee *et al.*, 2009; Nabti *et al.*, 2014).Datta *et al.* (2011) reported that majority of the rhizospheric isolates regularly produced IAA and production of IAA is a most common mechanism of action in PGPR. Similar results have been reported byIbiene *et al.* (2013), who observed that strains of *Azotobacter* sp.produced IAA. Al-Shah *et al.* (2013) reported that isolates of *Microbacterium* produced higher concentration of IAA compared to all other isolates tested.

Volatile compounds such as HCN is produced by many bacterial strains and has been considered as an important metabolite in disease control. The toxicity of HCN is attributed to its ability to form strong bond with iron of cytochrome oxidase, inhibiting electron transfer in respiratory chain and thus disrupting cellular respiration. HCN production by the selected isolates was assessed based on the development of yellow to reddish brown colour around the colony (Bakker and Schippers, 1987). Out of twenty isolates screened, HCN production was shown by five isolates namely NBS, NTS-1, NPS-1, NPPV, and NAS. Among these NTS-1 was observed as a good HCN producer. HCN production was moderate in isolates NPS-1 (*Cellulosimicrobium* sp.), NBS and NPPV (*Microbacterium* sp.) whereas, NAS was found to be a weak producer of HCN. In a similar study conducted by Raval and Desai (2012), HCN production was detected in some isolates of PGPR isolated from rhizosphere of sunflower.

Ammonia production is another important trait of PGPR that indirectly influences plant growth. Production of ammonia helps to control plant diseases by way of inhibiting the growth of plant pathogens. Martin (1982) reported that accumulation of ammonia results in increase in pH even upto 9-9.5, which leads to the inhibition of growth of other microorganisms. In the present investigation, production of ammonia by the selected isolates was detected by the formation of yellow to brown colour when Nessler's reagent was added. Sixteen isolates were

observed to be positive for ammonia production. Eight isolates (NPS-1 (*Cellulosimicrobium* sp.), NKdS (*Microbacterium* sp.), NTS-1, NKPV-2 (*Brevundimonas* sp.) NNPV, NPPV (*Microbacterium* sp.), NNkPV, and NNPD were found to be good ammonia producers,developing brown colour when Nessler's reagent was added. Two isolates (NPS-3 and NBS) were categorized as moderate producers of ammonia. Kumar *et al.* (2015) reported the production of ammonia by some strains of PGPR isolated from rhizosphere soil of french bean. Ammonia production was detected in *Cellulosimicrobium* sp. (Singh *et al.,* 2014).

Siderophore production is another important attribute of PGPR. Siderophores are low molecular weight, iron chelating compounds secreted by microorganisms which bind to the available form of iron in the rhizosphere, thus making it unavailable to the phytopathogens.In the present study, siderophore production by the twenty selected isolates was screened based on the formation of yellow-orange halo around the colony in CAS medium. However, none of the isolates produced siderophore.

Antagonistic activity is an important trait of PGPR, which provide beneficial effect on the plant indirectly, through the production of HCN, ammonia, siderophore, antibiotics etc. All the twenty selected isolates were screened for their antagonistic activity against important soil borne pathogens including, two fungi*Rhizoctonia solani* and *Fusarium oxysporum* and one bacterium*Ralstonia solanacearum.* Dual culture technique was used for the screening of isolates against two fungi and cross streaking method for the screening of isolates against the bacterium. In dual culture technique, antagonistic activity was measured in terms of per cent inhibition. Three isolates viz. NkdS (*Microbacterium*), NPPV (*Microbacterium*) and NKS-1 were found to exhibit antagonistic activity against *Rhizoctonia solani.*Maximum per cent inhibition of 56.94 was recorded inNKS-1. Under *in vitro* screening NPPV was found to be a moderate HCN producer and ammonia production was detected in isolates

NKdS and NKS-1. Hence, production of HCN and ammonia could be the mechanism behind the antagonistic activity of the isolates. Similar findings have been reported earlier (Paul *et al.*, 2005; Trivedi *et al.*, 2008). Ji *et al.* (2014) also reported antagonistic activity of three isolates of *Microbacterium* sp against *R. solani*. Chauhan*et al.* (2012) reported that isolates of *Azotobacter chroococcum* were found to be effective biocontrol agents against *R. solani* in cotton.

In the present investigation, none of the isolates exhibited antagonism against *Fusarium oxysporum*. Egamberdieva (2008) reported that isolates of *Microbacterium* and *Cellulosimicrobium* did not exhibited antagonistic activity against *Fusarium culmorum*.

Bacterial wilt is an importantdisease in tomatocaused by *Ralstonia solanacearum* (Yabuuchi *et al.*, 1995). In the present study, six isolates namely NPS-2, NPS-3, NNPV, NNkPV, NKPV-2 and NKPD exhibited antagonistic activity against *Ralstonia solanacearum*. All these six isolates were found to be positive for ammonia production under *in vitro* condition and this could be one of the mechanisms behind their antagonistic activity against *Ralstonia solanacearum*. It has been reported that PGPR isolated from leaves of mulberry exhibited strong antagonistic activity against *R. solanacearum* and reduced the disease incidence (Ji *et al.*, 2008).

Based on the growth, nitrogen fixation, tolerance to acidic pHand PGPR activities under *in vitro* conditions, four promising diazotrophs, including two rhizosphere isolates (NKdS and NPS-1) and two phylloplane isolates (NPPV and NKPV-2) were selected and evaluated under*in planta* conditions.

Apart from cultural and morphological characterization, the four isolates selected for *in planta* evaluation were identified by 16S rDNA sequence analysis. The isolates were identified based on the accession in NCBI database, showing maximum

homology with query sequence. Isolate NKdS and NPPV showed maximum homology with *Microbacterium* sp., NPS-1 with *Cellulosimicrobium* sp. and NKPV-2 with *Brevundimonas* sp.

After *in vitro* screening of beneficial organisms, and before going to the field experiment, evaluation of their efficiency under controlled conditions is essential. Tomato was used as the test crop in the present study, as it is highly responsive to the application of nitrogenous fertilizers (Taber, 2001). Tomato is a heavy feeder and exhaustive crop which requires large quantities of inorganic and organic nutrient inputs (Sepat *et al.*, 2012). The variety 'Anagha' was selected for the pot culture experiment. It is a high yielding tomato variety with built-in resistance to bacterial wilt, released from Kerala Agricultural University.A similar study was conducted by Egamberdieva (2008) who isolated *Microbacterium* from phyllosphere of pea and *Cellulosimicrobium* from rhizosphere of wheat and evaluated these in pot culture experiment. In the present study, the four promising isolates were applied alone, in combination with 75% N of the recommended dose, as per the Package of Practices Recommendations (KAU) and with 75% N + 50% FYM.Activity of diazotrophs will be less, when sufficient amount of nitrogen is present in the soil. Lower dose of nitrogen (75%) was thus used for the present study, assuming that 25% will be supplied by diazotrophs.Sharma and Thakur (2001), conducted a field trial to study the effect of *Azotobacter* with different levels of nitrogen (0, 50, 75 and100 kg N/ha) on growth and yield of tomato. In the present investigation, KAU commercial formulation of *Azotobacter* served as positive control. Treatment without any bioinoculant, chemical nitrogen and FYM servedas the absolute control.

In the present study, diazotrophs were inoculated as seed treatment, seedling dip, soil application for rhizosphere isolates and foliar spray for phylloplane isolates. Germination was enhanced in all treatments that received seed treatment with bioinoculants, as compared to uninoculated control (Fig. 11). Earlier reports indicated

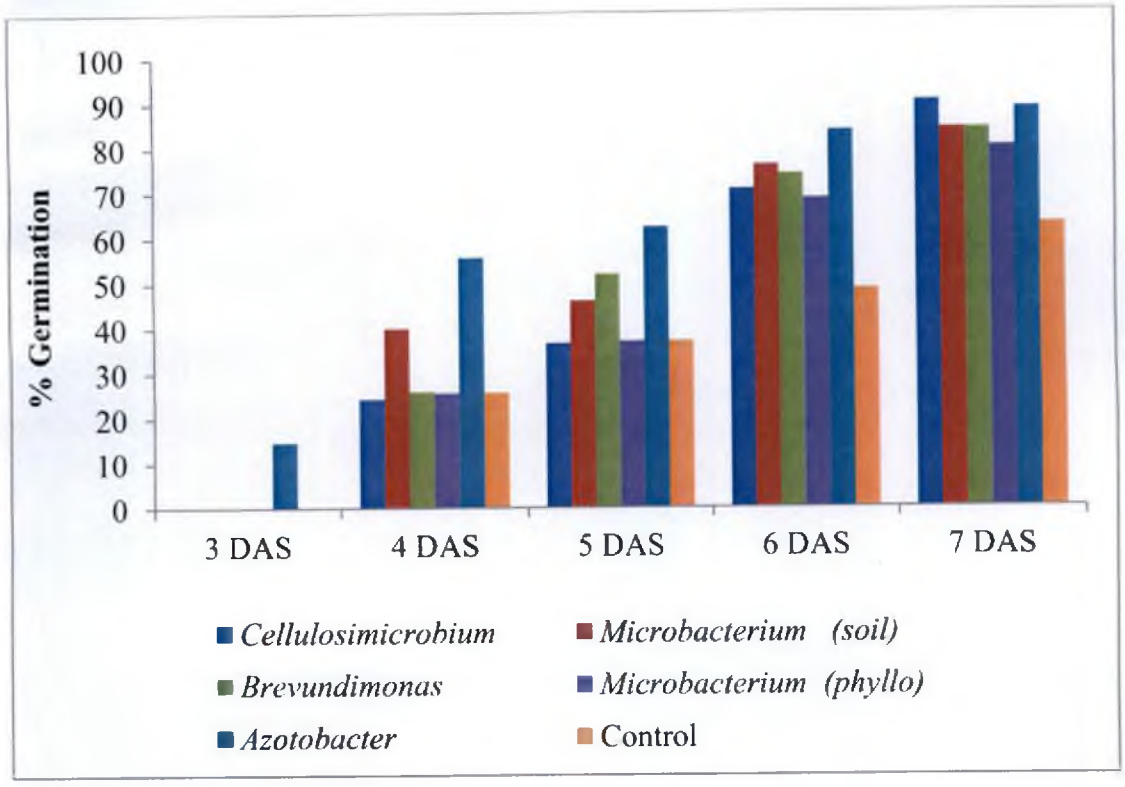


Fig. 11. Effect of diazotrophs on seed germination in tomato

that bacterial inoculants are able to increase plant growth and germination rate, improve seedling emergence, respond to external stress factors and protect plants from diseases (Biswas *et al.,* 2000). In another report by Ibiene *et al.* (2013) bacterial inoculants (*Azotobacter* sp, *Nitrobacter* sp and *Nitrosomonas* sp) improved germination rate and seedling emergence. In this study, the number of days taken for 100% germination ranged between 9 to11 days. Delayed germination might be due to the longer storage of the seeds used for planting. Seeds treated with KAU *Azotobacter* formulation triggered faster germination compared to all other treatments, up to six DAS. However, at seven DAS, highest per cent germination was observed in NPS-1 (*Cellulosimicrobium* sp.). The same isolate recorded 100% germination at 9 DAS. Under *in vitro* screening of the isolates, NPS-1 was found to be positive for IAA production. The possible reason for increased germination could be attributed to the production of IAA, which is known to play a key role in plant growth regulation. According to Zimmer *et al.* (1995),the phytohormone IAApromotes seed germination and root elongation. Plant growth promoting strain of *Cellulosimicrobium cellulans* wasearlier described byChatterjee *et al.* (2009) as a potential IAA producer.

In the *in planta* experiment, biometric characters and yield parameters were recorded at monthly intervals. With respect to plant height and number of leaves, microbial inoculants along with 75% N + 50% FYM performed better than other treatments with isolate alone or isolate with 75% nitrogen. This might be due to the direct supply of nutrients from FYM as well as increased population build up of bioinoculants, in presence of FYM which might have resulted in better nutrient uptake. Influenceof organic matter present in the soil for multiplication and population build up of microorganisms has been reported by several workers (Dev and Tilak, 1976, Niawska *et al.* 2000). For effective nitrogen fixation by free living organisms, presence of considerable amounts of organic matter is important

maximum girth. However, *Microbacterium* sp. recorded maximum girth when bioinoculants alone were applied as well as bioinoculants were applied along with 75% N. Previous studies on *Cellulosimicrobium cellulans* revealed that, it colonized the rhizosphere of chilli plant after inoculation and subsequently promoted the growth. The study suggested the potentiality of this strain as a plant growth promoter (Chatterjee *et al*., 2009). Earlier report indicated that application of PGPR increased the stem girth of tea under green house condition (Trivedi *et al.*, 2005). Similiarly, Ibiene *et al.* (2013) reported that PGPR isolates significantly increased the stem girth over untreated control in tomato.

In the present investigation, number of days taken for flowering ranged from 59 to 68 DAS.*Cellulosimicrobium* sp. + 75% N + 50% FYM (T_{11}) recorded the minimum number of days taken for flowering (Fig. 12). Earlier reports indicate that PGPR can induce early blooming in tomato (Brown *et al.*, 1964; Raj *et al.*, -2005;Ramamoorthy *et al.*, 2001).

Leaf area was also maximumin the above treatment and this was on par with POP recommendation of KAU (Fig. 13). However, among the treatments with isolatesalone and isolates + 75% N, NKdS (*Microbacterium* sp.).recorded early flowering and maximum leaf area. Increase in leaf area by the application of PGPR has been reported earlier (Karakurt and Aslantas, 2010; Mia *et al.*, 2010).

The diazotrophs had a significant effect on root growth of tomato plant. Treatments with bioinoculants + 75% N + 50% FYM, had significantly higher root fresh weight and volume compared to treatments with isolate alone or isolate + 75% N. Among the different isolates, *Microbacterium* sp. (NKdS) performed better, whether alone (T_2), in combination with N (T_7) or N + FYM (T_{12}), in enhancing root weight and volume. Under *in vitro* condition, this isolate recorded IAA production which might have attributed to the increased root growth.This is in line with the report of Egamberdieva and Jabborova (2012), who reported that treatments with a

(Kizilkaya, 2009).These results stress upon the need for application of FYM along with biofertilizer application.

Plant height and number of leaves was significantly higher in plants treated with NKdS (*Microbacterium* sp.) + 75% N + 50% FYM, compared to all other treatment. All the test isolates recorded higher plant height and number of leaves than KAU *Azotobacter* formulation, in all treatment combinations. NKdS (*Microbacterium*sp.) performed better, in the absence (T_2) and also in combination with N and FYM (T_{12}). Under *in vitro* screening for growth and nitrogen fixation, *Microbacterium* sp. obtained from rhizosphere soilperformed better. This isolate was also found to produce IAA. Plant growthpromotion through hormonal stimulation as well as through nitrogen fixation by this isolate could have contributed to the increase in plant height and number of leaves. These results are in agreement with the observations made by Egamberdieva and Jabborova (2012). They reported that in*in planta* evaluation,*Microbacterium* sp. increased plant height in maize, wheat and cotton. Similiarly Kumar *et al.* (2015) reported that field inoculation of *Microbacterium arborescence* significantly increased plant height in maize by 42% over uninoculated control. Similiar results have been reported earlier (Ji *et al*., 2014). It is well established that IAA have a role in shoot and root elongation as well as in enhancing plant growth (Vikram, 2007). However, plant height of tomato plants in the present experiment was higher in general, because of the partially shaded condition in the net house, where the pots were kept. This also could have influenced plant height and hence the effect of inoculants on plant height needs to be further evaluated under open conditions.

The bioinoculants were found to have a profound influence on the girth of stem. Treatments with isolate + 75% N + 50% FYM, had significantly higher stem girth compared to treatments with isolate alone and isolate + 75% N. NPS-1 (*Cellulosimicrobium* sp.) along with 75% N + 50% FYM was found to produce

maximum girth. However, *Microbacterium* sp. recorded maximum girth when bioinoculants alone were applied as well as bioinoculants were applied along with 75% N. Previous studies on *Cellulosimicrobium cellulans* revealed that, it colonized the rhizosphere of chilli plant after inoculation and subsequently promoted the growth. The study suggested the potentiality of this strain as a plant growth promoter (Chatterjee *et al.*, 2009). Earlier report indicated that application of PGPR increased the stem girth of tea under green house condition (Trivedi *et al.*, 2005). Similiarly, Ibiene *et al.* (2013) reported that PGPR isolates significantly increased the stem girth over untreated control in tomato.

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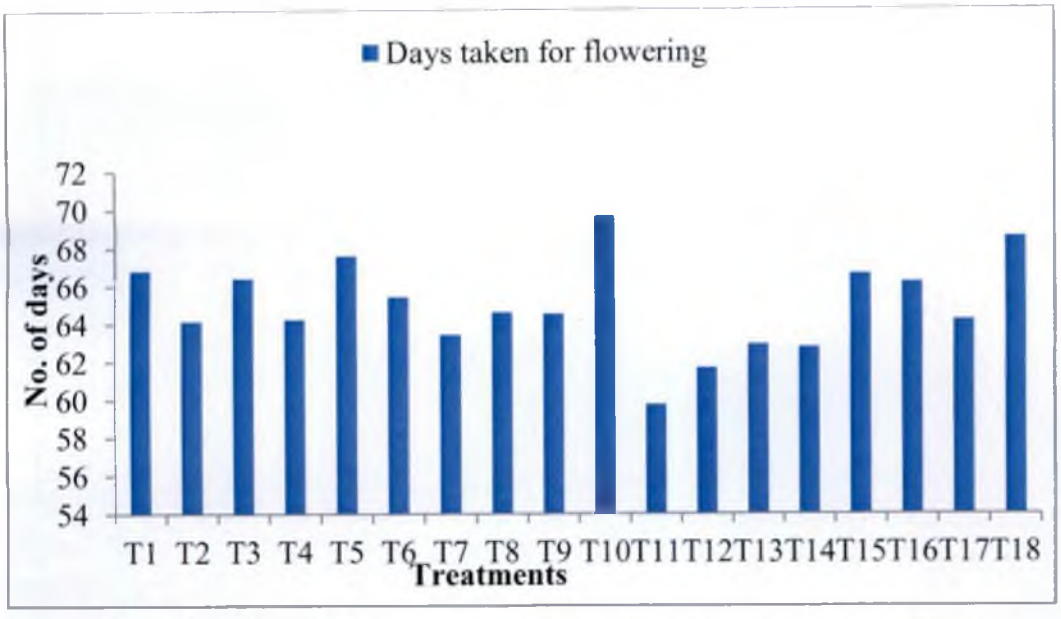

Fig. 12. Effect of diazotrophs on days taken for flowering in tomato

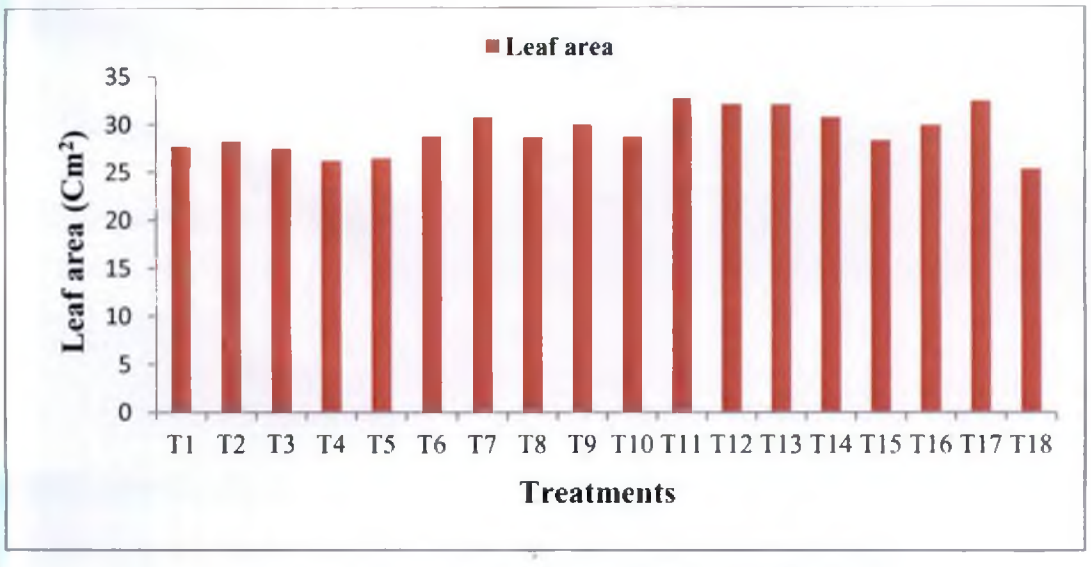

Fig. 13. Effect of diazotrophs on leaf area in tomato

strain of *Microbacterium* sp. significantly increased the root growth of wheat, maize and cotton. Ji *et al.* (2014) also reported that, rice seeds treated with *Microbacterium* sp. improved the root growth.In a similiar study conducted byVessey (2003), IAA produced by the diazotrophic PGPR resulted in bigger and more branched roots, thus providing a larger root surface area. Among all the treatments, *Brevundimonas* + 75% N (T_9) recorded maximum rootlength.

Plant fresh weight and dry weight were found to be superior in treatments with isolates + 75% N + 50% FYM compared to treatments with isolate alone or isolate + 75% N. Maximum plant fresh weight was recorded in*Microbacterium* sp.(NKdS) + 75% N + 50% FYM(T_{12}).The same isolate recorded maximum fresh weight among treatments with isolate alone and isolate along with 75% N. Maximum plant dry weight along with 75% N and 75% N + 50% FYM was also recorded in treatments with the sameisolate. Mohandas (1987) studied the effect of *Azotobacter vinelandii* on the plant growth promotion and revealed that it can significantlyincrease the shoot dry weight and leaf area in tomato. Dry weight of tomato plantsinoculated with *Azotobacterchroococcum* was significantly greater than that of non inoculated plants (Puertas and Gonzales, 1999).Earlier reports indicated that, significant response of isolates of *Microbacterium* sp. was observed in the increment of shoot fresh weight (Karnwal, 2012).

In the present study, yield per plant and average fruit weight were found to be superior in treatments with, isolates + 75% N + 50% FYM compared to treatments with isolate alone or isolate + 75% N. These results are in agreement with the study of Akbari *et al.* (2011), who reported that grain yield in sunflower was found to be maximum when nitrogenous biofertilizers were applied in combination with chemical nitrogen and FYM. They concluded that application of biofertilizers along with 50% N and 50% FYM was the most appropriate combination.

In the present study, yield per plant, average fruit weight and number of fruits were maximum in *Microbacterium* sp.(NKdS) + 75% N + 50% FYM(T_{12}) (Fig. 14). Earlier reports also indicated that biofertilizers increase the growth and yield of crop plants. Mohandas(1987) reported yield increase in tomato on inoculation with *Azotobacter vinelandii.* In a similar study by Subramanian *et al.* (2006), biofertilizer containing *Azotobacter* increased the number of fruit, flowers per plant and yield of tomato. *Microbacterium* sp. has been reported to increase the yield by 26-28% in apple, in a similar study by Karlidag *et al.* (2007).

Total nitrogen content of index leaf (at 60 DAS) and plant (at harvest) was analyzed and maximum nitrogen content was recorded in *Microbacterium* sp.(NKdS) + 75% N + 50% FYM (T_{12}). Treatments with same isolate recorded maximum nitrogen content in index leaf and in plant at the time of harvest, when isolates were applied alone (T_2) as well as isolates applied along with 75% N (T_7). Field inoculation of *Azotobacter vinelandii* on tomato resulted in significant increase in nitrogen content of plant (Mohandas, 1987).Shehata *et al.* (2006) also reported that treatment with nitrogenous biofertilizer in squash, increased the nitrogen percentage in plant leaves compared to untreated seeds.Lin *et al.* (2012) reported that inoculation of a strain of *Microbacterium*, increased the nitrogen content in sugarcane.

Total nitrogen content in the soil at 60 DAS and harvest was analysed in all the treatments. Initial nitrogen status of thepotting mixture was 1.0g/kg.There was an increase in nitrogen content of soil in all the treatments, except in absolute control. The decrease in nitrogen content absolute control might be due to the uptake of nitrogen in the potting mixture by the plants. At 60 DAS, highest nitrogen content of 2.20g/kg was obtained in *Microbacterium* sp. (NKdS) + 75% N + 50% FYM (T_{12}). However, at the time of harvest, nitrogen content was maximum (1.93g/kg) in *Cellulosimicrobium* sp.(NPS-1) +75% N + 50% FYM(T_{11}). This treatment was found to be on par with POP recommendation in which 100% N was provided. Increase in

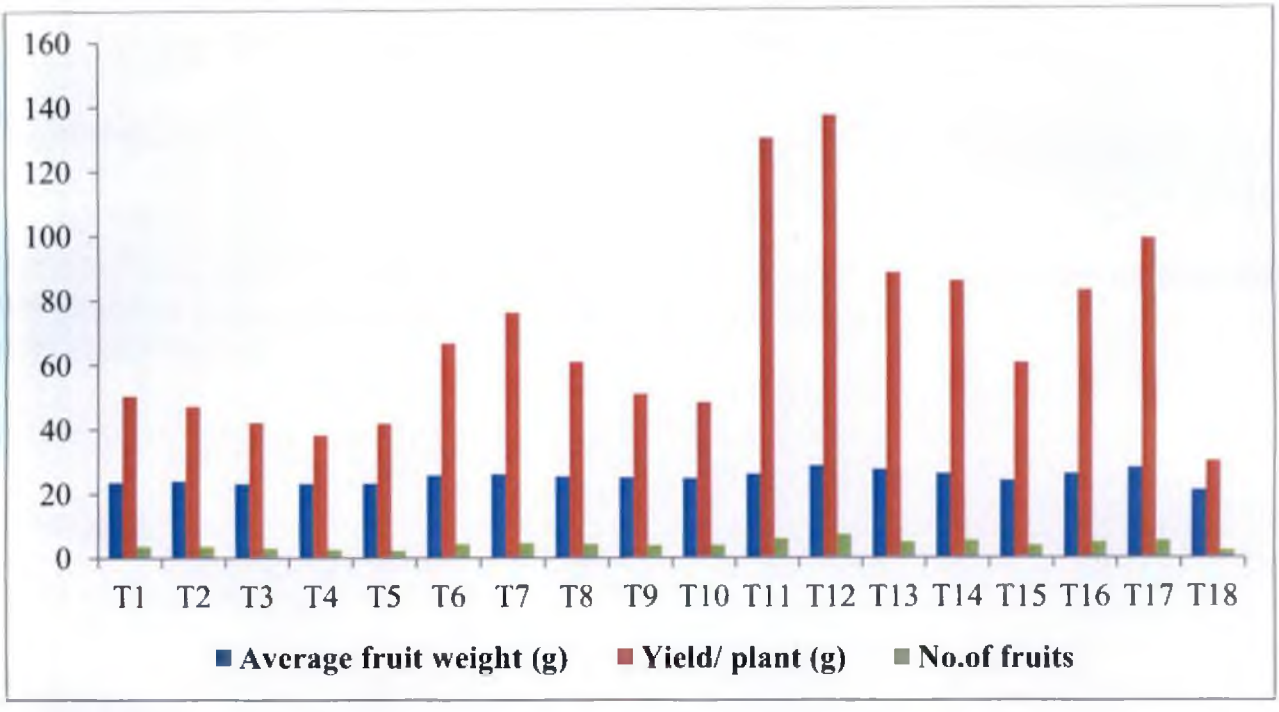

Fig. 14. Effect of diazotrophs on yield parameters in tomato

nitrogen content of soil by the application of nitrogenous biofertilizers has been reported earlier (Kennedy and Rangarajan, 2001). Akbari *et al.* (2011) reported that total nitrogen content in soil increased after application of *Azospirillum* along with inorganic nitrogenous fertilizers.

To be an effective biofertilizer, the microorganism must be able to colonize root environment because they need to establish themselves in the rhizosphere at population densities sufficient to produce the beneficial effects.Enumerationof population of the diazotrophs in soil was done prior to the monthly application of bioinoculants.Population was significantly higher when bioinoculants were applied, along with 75% nitrogen and 50% FYM. At 1 MAP and 2 MAP, *Cellulosimicrobium* sp. (NPS-1) + 75% N + 50% FYM (T_{11})recorded maximum population. At 3 MAP and 4 MAP, population of*Microbacterium* sp.(NKdS)and *Cellulosimicrobium* sp. along with 75% N + 50% FYMhad comparable value. Maximum population of *Cellulosimicrobium* sp. and*Microbacterium* sp. might be due to the efficient utilization of the nutrients in soil from the root exudates as well as applied through fertilizers. Bowen and Foster (1978) reported that abundance of *Pseudomonas* in the rhizosphere might be due to the existence of more favourable environmental condition for their growth. Lilinares*et al.* (1994) stated that predominance of *Bacillus* may be due to its ability to produce substances that inhibit the growth of other microorganisms in their vicinity.

Analysis of population of the bioinoculants applied in soil indicated that diazotrophs were also present in T_{17} (POP Recommendations), although bioinoculant was not applied in this treatment. This might have come from FYM, which was not sterilized before application. Hence, in treatments with bioinoculants + FYM also, diazotrophs from FYM might be present. If FYM was sterilized before application, data on population of bioinoculants at monthly interval could be more realistic.

Population of bioinoculants in phylloplane, applied as foliar spray was also analysed, before monthly application of foliar spray (Fig. 15 and 16). At 1 MAP, population on dorsal and ventral side of leaf was observed only in T_3(*Microbacterium* sp.)and T_9 (*Brevundimonas* sp. + 75% N). Maximum population of all the isolates was recorded at 2 MAP, except in *Microbacterium* sp. (T_3) on dorsal side and *Microbacterium* +75% N (T_9) on ventral side. After 2 MAP, population of the bioinoculants generally showed a decreasing trend. Maximum colonization of bioinoculants at 2 MAP, coincide with the flowering stage of the tomato, where maximum vegetative growth occur. This might have provided a favourable condition in the phyllosphere for growth of diazotrophs. At 2 MAP,*Microbacterium* (NPPV)+75% N+50% FYM (T_{13}) recorded highest population on dorsal and ventral surface of leaf. Increased colonization of diazotrophs on phylloplane might be due to theincrease in general health of plant by the application of FYM.

In the present study, enumeration of bioinoculants applied in soil and phylloplane at monthly interval was done by serial dilution and plating technique and they were identified based on the colony characters on the N-free media. Antibiotic resistant marker could also be used for the exact identification of the isolates applied.

Population of endophytes in root, stem and leaf revealed that bioinoculants applied as soil application colonized more in root followed by stem and least in leaf. Population of endophytes in leaf was more when bioinoculants were applied as foliarspray. Similar results were reported by Egamberdieva (2008) that colonization of bacteria in the phyllosphere was significantly lower compared to rhizosphere colonization in both wheat and pea. *Cellulosimicrobium* sp. (NPS-1) along with 75% N + 50% FYM recorded maximum population in stem as well as root. In leaf, maximum population was recorded in *Brevundimonas* sp.+ 75% N + 50% FYM.

One of the reasons for limited success of biofertilizers could be the non-availability of native isolates, specific to an agro-ecological zone or location.

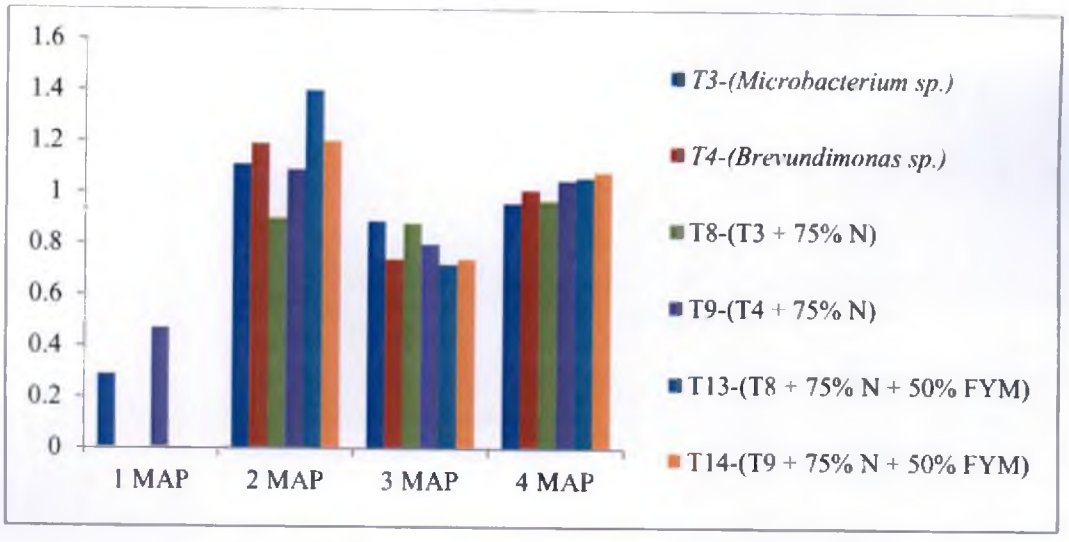

Fig. 15. Survival of diazotrophs on dorsal surface of leaf

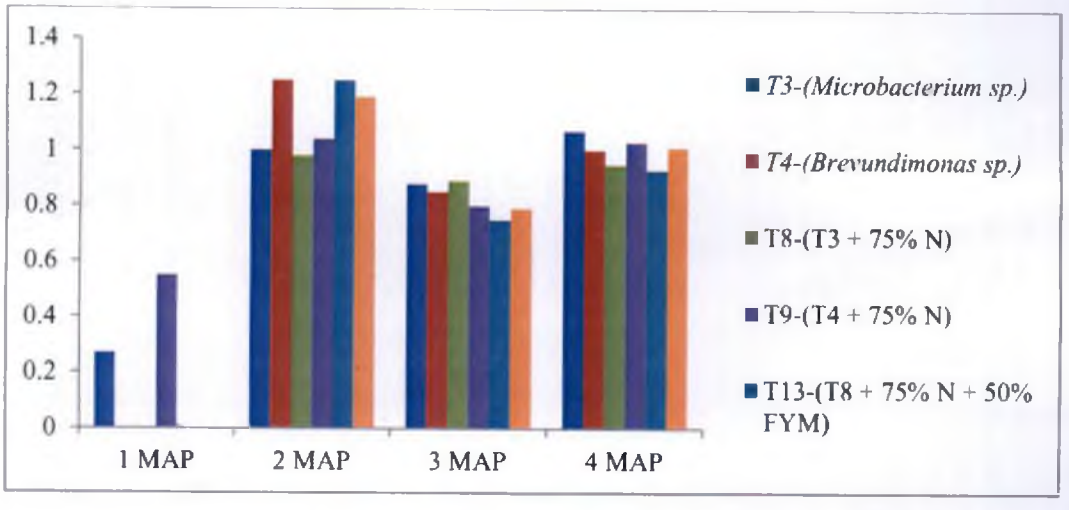

Fig. 16. Survival of diazotrophs on ventral surface of leaf

The present study revealed that, apart from already commercialized nitrogenous biofertilizers like *Azotobacter* and *Azospirillum*, novel, free living diazotrophs like *Microbacterium* and *Cellulosimicrobium*, suitable for acidic soils of Kerala could be exploited as biofertilizers, in future. *Microbacterium* is a Gram positive, non-sporulating rod-shaped bacterium, reported to be present in a broad range of environmental habitats. It is an endophytic actinobacteria, abundant in common bean leaves (Costa *et al.*, 2012). *Cellulosimicrobium* is also a Gram positive, non-endospore forming bacterium reported to promote plant growth through IAA production and phosphate solubilization (Chatterjee *et al.*, 2009). Since these are non-symbiotic and non-specific, these could be used for any crop. In general, in the *in planta* experiment, isolates along with 75% N + 50% FYM recorded highest value in all the biometric and yield parameters, compared to isolate alone or isolate along with 75% N. It indicates the importance of application of FYM along with biofertilizersfor their better performance. The results of the study, stress upon the amelioration of soil through lime application.

Before commercialization, the promising isolates *Microbacterium* sp. (soil) and *Cellulosimicrobium* sp. may be further evaluated for plant growth promotion under field condition with lower doses of nitrogen (25% or 50%) and different levels of FYM. The isolates in combination with 100% nitrogen may also be assessed to compare with isolate + 75% nitrogen. Growth, tolerance and nitrogen fixation of the isolates under *in vitro* condition at higher levels of pH may also be assessed. In addition to this, effect of phylloplane isolates on plant growth promotion when inoculated as soil application can also be assessed.

Summary

6. SUMMARY

Agriculture is heavily dependent on the use of chemical fertilizers.Although chemical fertilizers contribute a lot infulfilling the nutrient requirement, their excessive and unbalanced use may lead to deterioration of soil properties and environmental pollution. Biofertilizers are potential biological fertilizers which play a key role in maintaining productivityand sustainability of soil and protecting the environment.

The study entitled 'Utilization of freeliving diazotrophic bacteria from Wayand as a biofertilizer' was carried out in theDepartment of Agricultural Microbiology, College of Horticulture, Vellanikkaraduring 2013-2015. The main objective of the study was isolation, screening and evaluation of free living diazotrophic bacteria from the rhizosphere and phylloplane of black pepper to develop an efficient biofertilizer. The important findings of the study are summarizedbelow:

- A total of forty three free living diazotrophic bacteria were isolated on three different N-free media (Jensen's agar, *Beijerinckia* agar and Ashby's agar). Maximum population was obtained on Jensen's agar.
- Twenty predominant isolates were selected, including eleven isolates from rhizosphere soil and nine isolates from phylloplane, based on their growth characteristics on Jensen's agar for further screening.
- All the twenty selected isolates were screened *in vitro* for their ability to grow and fix nitrogen at different levels of pH (7.0, 6.5, 6.0, 5.5, and 5.0).A preliminary experiment was conducted to standardize the time interval at which maximum growth is obtained for all the isolates.Jensen's broth at a pH of 7.0 was used and population was estimated at 0h, 48h, 72h, 96h, 120h and 144h after inoculation. Maximum growth was observed at 96h for all the isolates and it was

standardized for evaluation of growth of isolates at remaining pH levels.

- Maximum growth and nitrogen fixation of all the selected isolates were observed at neutral pHand there was a gradual decline as pH was lowered. Among the isolates screened, two rhizosphere isolates (NKdS and NPS-1) and two phylloplane isolates (NKPV-2 and NPPV) performed better.
- Twenty selected isolates were screened under *in vitro* condition for plant growth promoting activities like production of IAA, HCN, siderophore and ammonia. IAA production was found to be positive for isolates NTS-3, NKdS, NKPV-1 and NPS-1. HCN production was detected in isolates NBS, NTS-1, NPS-1, NPPV, and NAS. All the isolates were observed to be positive for ammonia production except NTS-3, NKS-2, NPS-2 and NKdPV. Siderophore production was not detected in none of the isolates.
- The antagonistic activity of all the twenty isolates were tested against three soil borne plant pathogens including two fungi(*Fusarium oxysporum, Rhizoctonia solani*)and one bacterium (*Ralstonia solanacearum*). Isolates NkdS, NPPV and NKS-1 were found to exhibit antagonistic activity against*Rhizoctonia solani*,in which,NKS-1 showed maximum per cent inhibition of 56.94.The isolates which exhibited antagonistic activity against *Ralstonia solanacearum* are NPS-2, NPS-3, NNPV, NNkPV, NKPV-2 andNKPD. None of the isolatesinhibited the growth of*Fusarium oxysporum*.
- Based on the growth, nitrogen fixation, tolerance to acidic pHand PGPR activities under *in vitro* conditions, four promising diazotrophs, including two rhizosphere isolates (NKdS and NPS-1) and two phylloplane isolates (NPPV and NKPV-2) were selected for *in planta*

evaluation using tomato as the test crop. These isolates were applied alone, in combination with 75% N and with 75% N and 50% FYM.

- 16S rDNA sequence analysis was carried out for the four promising isolates. The isolate NKdS and NPPV showed maximum homology with *Microbacterium* sp., NPS-1 with *Cellulosimicrobium* sp. and NKPV-2 with *Brevundimonas* sp.
- Diazotrophs were inoculated as seed treatment, seedling dip, soil application for rhizosphere isolates and foliar spray for phylloplane isolates.Seeds treated withKAU *Azotobacter* formulation triggered faster germination compared to all other treatments. However, after 7 days of sowing maximum number of seedling emergence (90.24%) was recorded in seeds treated with NPS-1 (*Cellulosimicrobium*) followed by KAU *Azotobacter* formulation (88.52%).
- In general, treatments with microbial inoculants + 75% RD of N + 50% RD of FYM recorded higher biometric and yield parameters compared to treatments with isolate alone and isolate + 75% RD of N.
- Plant height, number of leaves, fresh weight and volume of root, fresh weight and dry weight of plants were observed to be maximum in treatment *Microbacterium* sp. (NKdS) + 75% N + 50% FYM (T_{12}).
- *Cellulosimicrobium* sp. (NPS-1) +75% N + 50% FYM (T_{11}) recorded maximum girth of stem, leaf area and early flowering.
- Yield per plant, average fruit weight and number of fruits were recorded maximum in treatment *Microbacterium* sp. (NKdS) + 75% N + 50% FYM (T_{12}).
- Population build up of bioinoculants in soil was higher in presence of FYM. At 1 MAP and 2 MAP, *Cellulosimicrobium* sp. (NPS-1) + 75% N + 50% FYM (T_{11})recorded maximum population. At 3 MAP and 4

MAP,*Microbacterium* sp. (NKdS) + 75% N + 50% FYM (T_{12}) recorded maximum population which was on par with T_{11}.

- Population of bioinoculants in phylloplane applied as foliar spray was also analysed. In general,Maximum population of all the isolates was recorded at 2 MAP and after that a decreasing trend was noticed.
- Population of endophytes in root, stem and leaf revealed that bioinoculants applied as soil application colonized more in root followed by stem and least in leaf. Bioinoculants applied as foliar spray, colonized more in leaf compared to root and stem.
- Combined application of isolates along with 75% nitrogen and 50% FYM recorded higher nitrogen content in both plants and soil compared to treatments with isolate + 75% nitrogen and isolate alone. Total nitrogen content in index leaf was maximum in *Microbacterium* sp. (NKdS) + 75% N + 50% FYM (T_{12}). Same treatment recorded maximum total nitrogen content in soil and plant after harvest.
- The present study revealed that, apart from already commercialized nitrogenous biofertilizers like *Azotobacter* and *Azospirillum*, novel, free living diazotrophic bacteria like*Microbacterium* and *Cellulosimicrobium*, suitable for acidic soils of Kerala could be exploited as biofertilizers, in future.

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Appendices

APPENDIX I

MEDIA USED AND COMPOSITION

a) Ashby's agar

Glucose	20.00 g
Dipotassium phosphate	0.20 g
Magnesium sulphate	0.20 g
Sodium chloride	0.20 g
Potassium sulphate	0.10 g
Calcium carbonate	5.00 g
Agar	20.00 g
Distilled water	1000 ml
pH	7.4±0.2

b) *Beijerinckia* agar

Sucrose	20.00 g
Dipotassium phosphate	0.20 g
Monopotassium phosphate	0.80 g
Magnesium sulphate	0.50 g
Ferric chloride	0.10 g
Sodium molybdate	0.005 g
Agar	20.00 g
Distilled water	1000 ml
pH	6.5

c) Jensen's agar

Sucrose	20.00 g
Dipotassium phosphate	1.00 g
Magnesium sulphate	0.50 g

Sodium chloride	0.50 g
Ferrous sulphate	0.10 g
Sodium molybdate	0.005 g
Calcium carbonate	2.00 g
Agar	20.00 g
Distilled water	1000 ml

d) Nutrient agar

Beef extract	3.00 g
Peptone	5.00 g
NaCl	5.00 g
Agar	20.00g
Distilled water	1000 ml

e) Potato dextrose agar

Potato infusion	200.00 g
Glucose	20.00 g
Agar	20.00 g
Distilled water	1000 ml
pH	5.1

APPENDIX II

REAGENTS USED

a) **Boric acid-indicator mixture**

4 ml of mixed indicator solution (2% bromocresol green + 0.2% methyl red in alcohol in 5:1 ratio)is added to 1L of 4% boric acid solution prepared in hot water

b) **Salkowski reagent**

2 % of 0.5 M $FeCl_3$ in 35 % perchloric acid

c) **Picric acid solution**

2.5 g of picric acid

12.5 g of Na_2CO_3

1000 ml of distilled water

UTILIZATION OF FREE LIVING DIAZOTROPHIC BACTERIA FROM WAYANAD AS A BIOFERTILIZER

by

FATHIMA SHAHEENA K. M.

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ABSTRACT OF THE THESIS

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ABSTRACT

Agriculture is heavily dependent on the use of chemical fertilizers. However, excessive and unbalanced use of these inputs may lead to environmentalpollution and health hazards. Biofertilizers are biological fertilizers which play a key role in maintaining productivityand sustainability of soil, thereby protecting the environment. Hence, a study was undertaken to isolate, screen and evaluate free living diazotrophic bacteria to develop an efficient biofertilizer.

Rhizosphere soils and plant samples were collected from ten healthy black pepper gardens of different locations of Wayanad district. A total of 43 free living diazotrophic bacteria were isolated on three different N-free media (Jensen's agar, *Beijerinckia* agar and Ashby's agar). Maximum population of nitrogen fixers was obtained on Jensen's agar. Twenty predominant isolates were selected, including eleven isolates from rhizosphere soil and nine isolates from phylloplane, based on their growth characteristics on N-free media for further evaluation.

Twenty selected isolates were screened under *in vitro* condition for growth, nitrogen fixation and acid tolerance. As pH was lowered from 7.0 to 5.0, a reduction in growth and nitrogen fixation was observed. However, among the isolates, two from the rhizosphere (NKdS and NPS-1) and two from phylloplane (NKPV-2 and NPPV) performed better.

Twenty selected isolates were screened *in vitro* for plant growth promoting activities like production of IAA, HCN, siderophore and ammonia.The antagonistic activity of all the twenty isolates was tested against three soil borne plant pathogens *Fusarium oxysporum, Rhizoctonia solani*and *Ralstonia solanacearum.* Isolates NkdS, NPPV and NKS-1 were found to exhibit antagonistic activityagainst *Rhizoctonia solani*.Six isolatesinhibited the growth of*Ralstonia solanacearum.* None of the isolatesrecorded antagonistic activity against *Fusarium oxysporum.*

Four most promising diazotrophs including, two rhizosphere isolates (NKdS and NPS-1) and two phylloplane isolates (NPPV and NKPV-2) were selectedfor *in*

planta evaluation, based on nitrogen fixation ability, tolerance to acidic pHand PGPR activities. 16S rDNA sequence analysis was carried out and promising isolates were identified as*Microbacterium* sp. (NKdS and NPPV); *Cellulosimicrobium* sp. (NPS-1) and *Brevundimonas* sp. (NKPV-2).

In planta evaluation was carried out with tomato as the test crop. These isolates were applied alone, in combination with 75% N and with 75% N + 50% FYM. Diazotrophs were inoculated as seed treatment, seedling dip and soil/foliar application. Seeds treated withKAU *Azotobacter* formulation triggered faster germination compared to all other treatments. However, after 7 days of sowing, maximum number of seedling emergence (90.24%) was recorded in seeds treated with *Cellulosimicrobium* sp.

Plant height, number of leaves, fresh weight and volume of root, fresh weight and dry weight of plants,yield per plant, average fruit weight and number of fruits were observed to be maximum in treatment T_{12} (*Microbacterium* sp.+ 75% N + 50% FYM). T_{11} (*Cellulosimicrobium* sp. +75% N + 50% FYM) recorded maximum girth of stem, leaf area and minimum days to flowering.

Total nitrogen content of index leaf and plant was maximum in T_{12} (*Microbacterium* sp. + 75% N + 50% FYM). The same treatment recorded maximum nitrogen content in soil at 60DAS. At harvest, T_{11} (*Cellulosimicrobium* sp. + 75% N + 50% FYM) recorded highest nitrogen content in soil.

Population build up of bioinoculants in soil was higher in presence of FYM.Endophytic colonization was higher in root, in case of soil application of bioinoculants and higher in leaves, in case of foliar spray.

The study revealed that novel, free living diazotrophic bacteria like *Microbacterium* and *Cellulosimicrobium* could be exploited as biofertilizer formeeting 25% of the nitrogen requirement. Results also indicated the importance of application of organic manure along with the isolates, for their best performance.