# STUDIES ON PINK PIGMENTED FACULTATIVE METHYLOTROPHS ON TOMATO (*Lycopersicum esculentum* L.) CROP

by

**Miss. Nakade Suchita Shashikant**
(Reg. No. 020/251)

A Thesis submitted to the
**MAHATMA PHULE KRISHI VIDYAPEETH,**
**RAHURI - 413 722, DIST-AHMEDNAGAR,**
**MAHARASHTRA, INDIA.**

In partial fulfilment of the requirements for the degree

of

**MASTER OF SCIENCE (AGRICULTURE)**

in

**AGRICULTURAL MICROBIOLOGY**

**DEPARTMENT OF PLANT PATHOLOGY AND AGRICULTURAL MICROBIOLOGY**
**POST GRADUATE INSTITUTE**
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**2023**

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APPROVED BY

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**MAHARASHTRA, INDIA**
**2023**

# CANDIDATE'S DECLARATION

I hereby declare that this thesis or part

there of has not been submitted

by me or other person to any

other University or Institute

for a Degree or

Diploma

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Date : / /2023

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The assistance and help received during the course of this investigation have been duly acknowledged.

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**Date :** / /2023 (Suchita S. Nakade)

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# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| % | : | Per cent |
| @ | : | At the rate of |
| µm | : | Micrometer |
| $^0$C | : | Degree celcius |
| a.i. | : | Active ingredient |
| C .V | : | Coefficient of variation |
| C.D. | : | Critical difference |
| *Cfu* | : | Colony forming unit (s) |
| cm | : | Centimeter (s) |
| DAS | : | Days After Sowing |
| EC | : | Emulsifiable Concentrate |
| *et al.* | : | And others (*et alli*) |
| etc. | : | And so forth (*et cetera*) |
| Fig. | : | Figure |
| g | : | Grams |
| ha | : | Hectare |
| Hr | : | Hour |
| *i.e.* | : | That is |
| Kg | : | Kilogram |
| Km | : | Kilometer |
| Max | : | Maximum |
| Min | : | Minimum |
| mm | : | Millimeter |
| Mt | : | Million tonne |
| N | : | Nitrogen |
| P | : | Phosphorous |
| pp | : | Page |
| PPFM | : | Pink Pigmented Facultative Methylotrophs |
| PVP | : | Poly-vinyl-pyrrolidone |
| RBD | : | Randomized Block Design |
| *Spp* | : | Species |
| Sr. no | : | Serial Number |
| qha$^{-1}$ | : | Quintal per hectare |
| Var. | : | Variety |
| *viz.* | : | Videlicet (Namely) |

# ABSTRACT

**STUDIES ON PINK PIGMENTED FACULTATIVE METHYLOTROPHIN TOMATO (*Lycopersicon esculentum*L.)**

by

**Miss. NAKADE SUCHITA SHASHIKANT**

A candidate for the degree

of

MASTER OF SCIENCE (AGRICULTURE)

in

AGRICULTURAL MICROBIOLOGY

2023

**Research Guide**: Dr. A.M. Navale

**Department** : Agricultural Microbiology

The current study entitled "Studies on Pink Pigmented Facultative Methylotrophs in Tomato (*Lycopersicon esculentum* L.)" was conducted in laboratory as well as a field experiment during the summer season of (2022) at the Department of Plant Pathology and Agricultural Microbiology, Post Graduate Institute Mahatma Phule Krishi Vidyapeeth, Rahuri, with an objective of isolating, characterising, and studying their effect on growth, nutrient uptake and yield of chilli under field condition.

Morphologically the cells of PPFM were found be rod shaped, motile and gram reaction was found to be negative for all isolates. Cell size of bacteria was recorded in the range of 0.6 x 1.3 µm to 0.9 x 1.5 µm dimension and shows light pink to dark pink in color. Biochemical test was carried out to identify the PPFM bacteria. All the isolates shown positive result towards oxidase test, urease test, catalase activity and citrate utilization test. On the basis of result obtained from various morphological and biochemical parameters it was confirmed that isolates bacteria was Pink Pigmented Facultative Methylotrophic bacteria. As per the result of indole production test only three isolates i.e. $PPFM_1$, $PPFM_2$ and $PPFM_3$ were chosen for further analysis.

The three isolates chosen as per the result of indole production test i.e $PPFM_1$, $PPFM_2$ and $PPFM_3$ were analyzed for nitrogen fixation ability by using N- free malate medium. The maximum nitrogen fixation was recorded by $PPFM_1$ isolate i. e. 1.097 mg N $g^{-1}$ of malate medium. It was found that PPFM bacteria have inferior ability towards nitrogen fixation.

For preparation of liquid consortium of PPFM media was standardized as per the procedure on the basis of characteristics pink pigmentation medium 4 was observed most suitable medium for PPFM growth. On the basis of nitrogen fixation capacity the most efficient isolate that is $PPFM_1$ was chosen for the preparation of liquid consortium on suitable standardized medium 4 as per protocol. For preparation of liquid consortia at different pH various cell protectant such as arabinose, trehalose, glycerol, PVP and Fe-EDTA and prepared liquid medium $LM_1$, $LM_2$, $LM_3$, $LM_4$ and $LM_5$. The maximum cell count was noted on $LM_4$ medium and chosen for consortium preparation.

After formulation of liquid consortia the shelf of PPFM formulation stored in UV sterilized high density polyethylene (HDPE) bottles were investigated for shelf life of consortium. Cell count was taken for monthly duration consecutively for upto 360 days. The maximum cell count was recorded at 180 days after storage and thereafter gently decreases upto 360 days.

The field trial was carried out in summer (2022) to assess the Combined effect of PPFM and *Azotobacter* and various treatment combination on various plant growth parameters in tomato like plant height, number of leaves, root length, fresh weight and dry weight, number of branches, number of fruits, yield, nutrient uptake in chilli and population dynamics of PPFMs recorded at flowering and harvesting phase under graded level of nitrogen in field condition.

Due to various combination of treatment of PPFM along with *Azotobacter* under various level of nitrogen all growth parameters of chilli were influenced by the treatment $T_6$ (Seed treatment of PPFM + *Azotobacter* + 100% N + Foliar spray of PPFM) which was significantly dominant over rest of the treatments and it was at par with treatment $T_5$ (Seed treatment of PPFM + *Azotobacter* + 75% N + Foliar spray of PPFM)

Total nutrient uptake were also influenced by application of treatment $T_6$ (Seed treatment of PPFM + Azotobacter + 100% N + Foliar spray of PPFM) which was significantly superior over other treatments and found at par with treatment $T_5$ (Seed treatment of PPFM + *Azotobacter* + 75% N + Foliar spray of PPFM). The treatment $T_7$ absolute control was noted least nutrient uptake.

The treatment $T_6$ (Seed treatment of PPFM + Azotobacter + 100% N + Foliar spray of PPFM) was recorded maximum yield i.e. 92.00 q $ha^{-1}$ and found almost at par with treatment $T_5$ (Seed treatment of PPFM + *Azotobacter* + 75% N + Foliar spray of PPFM) i.e. 90.66 q $ha^{-1}$. The treatment $T_7$ (absolute control) noted the least treatment ii.e75.00q $ha^{-1}$. From the result, there is a possibility of saving nitrogen fertilizer to an extent of 25 per cent.

The PPFM bacteria can be mostly used for mitigation of drought and also called as drought tolerance biological measures due to its ability to produce phytohormones under stress condition due to their characteristics pink pigmentation.

# 1. INTRODUCTION

As a member of the solanaceae family, the tomato (*Lycopersicon esculentum L.*) is one of the most widely grown and significant economic crops. India is the top country in the world for producing, consuming, and exporting tomato introduced by the Portuguese in the 17th century, specifically grown in Andhra Pradesh, Karnataka, Tamil Nadu, and Maharashtra, which together with Madhya Pradesh, West Bengal, Punjab, Bihar, and Rajasthan account for 3/4 of the total area. India is the world's top producer of tomato with an annual output of 1.1 million tonnes (Khan and Raj, 2006). Due to its high financial value and high consumption rate, the annual trade of tomato accounts for roughly 17% of the global spice trade (Ahmed *et al.*, 2000) .

Microbes directly aid plant growth through improved nutrient uptake and hormonal stimulation. The cooperative actions of numerous different living organisms in a heterogeneous environment have an impact on plant growth. A field of growing plants is a collection of microbial activity, including soil microorganisms, air microbes, plant surface microbes, and internal plant-colonizing microbes or methylotrophs. A broad diversity of microorganisms lives in the phyllosphere as hosts. Most likely saprophytes, many of the microorganisms that inhabit phylloplanes eat substances that are leached from the leaf. Symbiotic relationships are ones where both bacteria and plants benefit from their contact. These bacteria are phytosymbionts, meaning they feed on plant waste like methanol. Microbes directly aid plant growth through improved nutrient uptake and hormonal stimulation. The cooperative actions of numerous different living organisms in a heterogeneous environment have an impact on plant growth. Microbes in the soil are capable of utilising methanol, a reduced one-carbon molecule generated from plants, as their only source of carbon and energy (Abanda-Nkpwatt *et al.,* 2006 and Sy *et al*., 2005). Methylobacterium spp. is one illustration of this.

Methylobacterium is a group of epiphytic Pink Pigmented Facultative Methylotrophs, or methylotrophs (PPFM). being a member of the class Alpha-proteobacteria. They are Gram-negative aerobic bacteria that can also grow on multi carbon growth substrates, such as $C_2$, $C_3$, and $C_4$ chemicals, as well as $C_1$ substances like formate, formaldehyde, methanol, and methylamine (Lidstorm, 1992). On a mineral medium based on methanol, the carbon source can readily be separated. They are also resilient of intense light and radiation exposure thanks to their distinctive pink coloration, which is caused by carotenoids. These characteristics could explain why they appear in

various ecological systems, including soil, plants, air, water, and even humans (Anesti *et al.,* 2005; Rice *et al.,* 2000).

In nature, PPFM are omnipresent and are often found in soils, on the surface of leaves, and in the rhizosphere of a wide variety of flora (Corpe, 1985; Corpe and Rheem, 1989; Hirano and Upper, 1992; Holland and Polacco, 1994). Field-grown crops typically have more than 80% of the viable PPFMs, with an average of $10^6$ cfu per leaflet, on their leaves. These PPFMs are with an average of $10^6$ cfu per leaflet, on their leaves. These PPFMs are particularly prevalent in the rhizosphere. Physiological characteristics like branching, seedling vigour, root differentiation, and heat and cold tolerance can all be altered by PPFMs. (Freyermuth and colleagues, 1996; Holland, 1997) Through the induction of systemic resistance, they can render plants resistant to diseases. Madhaiyan and others (2004) and crop photosynthesis need to be boosted. (Cervantes and others, 2005) Where plants employ methanol, which has evolved from leaves, as their sole source of energy and carbon, is the most usual niche for synergism between Methylobacterium and phyllosphere. Rotsenko and colleagues, (2001) Consequently, methylobacteria may create phytohormones including cytokinin and auxins, which are known to encourage plant development (Ivanova *et al*., 2001). growth (Koenig *et al.,* 2002). They are also capable of fixing atmospheric nitrogen. (Sy and others, 200 Unlike Rhizobium species, which typically fix molecular nitrogen from the atmosphere through symbiotic relationships with plants, Azotobacter species are free-living, nitrogen-fixing bacteria. Nevertheless, certain Azotobacter species are connected with plants. In the presence of readily available nitrogen sources, such as ammonium ions and nitrates, nitrogen fixation is impeded.

Azotobacter asymbiotically fixes nitrogen in tomatoes, increasing their production (Aryal *et al*., 2016). However, it has been found that combining the use of two or three different beneficial microbes as inoculums works better than a single inoculation (Alagwadi and Gaur, 1998), and this strategy can be utilised to reduce the need for inorganic fertilizer. Based on these methods, an effort is made to identify, characterise, screen, and choose the best PPFM strains for use as bioinoculants with Azotobacter to reduce nitrogen dosages while still promoting growth, yield, and nutrient uptake in tomato. The production of tomatoes can be significantly impacted by efficient phyllosphere and rhizosphere bacteria. The field trial "Studies on Pink Pigmented Facultative Methylotrophs in tomato crop" was conducted Isolation of Pink Pigmented Facultative Methylotrophs (PPFM) from phyllosphere (leaf surface) of any one of the crop like Tomato, chilli , brinjal, Potato and Cauliflower from different locations.

1. Identification and characterization of Pink Pigmented Facultative Methylotrophs on the basis of cultural, morphological, biochemical characters.
2. Assessment of Pink Pigmented Facultative Methylotrophs as a Nitrogen Fixer.
3. To develop the liquid formulation of Pink Pigmented Facultative Methylotrophs. To study the effect of liquid formulation of Pink Pigmented Facultative Methylotrophs on population dynamics of Methylotrophs, plant growth parameters, nutrient uptake and yield in tomato crop **(*Var.* Phule Kesari)**.

# 2. REVIEW OF LITERATURE

An attempt has been made in this chapter to review the work done to studies on Pink Pigmented Facultative Methylotrophs in tomato.

Due to changes in exudates, the structural and functional variety of microorganisms on the plant surface varies by plant species.The Pink pigmented facultative methylotrophs (PPFM) are found all over in the nature, but they are best known for their close relationship with plants. (Lidstrom and Christoserdova, 2002; Lodewyckx *et al*., 2002).

The natural occurrence of various populations of PPFM among various vegetable crops *viz*., tomato, chilli, brinjal, bitter gourd, bhindi, cucumber, cauliflower, radish and mint at flowering stage was found by Anurajan *et al.*(2003). The highest number of PPFM colonies were found among the vegetable crops studied was recorded in tomato (23.72 cfu/$cm^2$ leaf area while the minimum was observed in radish (1.12 cfu/$cm^2$ leaf area).

*Azotobacter* and 50% fertilizer gave highest growth and yield in tomato plant. *Azotobacter* showed promising result in growth enhancement of tomato plant as compared to recommended dose of fertilizer.(Kalpana *et al*., 2019).

## 2.1 Methylotrophs

Methylotrophy as a phenomenon has been known since the late nineteenth century (1904) and during the twentieth century, Methylotrophy directed research activities formed a small field within the field of microbiology.

Patricia *et al.* (1967) isolated the several mixed cultures of methane-oxidizing bacteria from mud and water. Among them culture HR (consisting of two gram-negative rods, one 0.5 x 1.0 μ, the other 0.8 x 2 to 3 μ) was found to be the fastest growing to give the highest yields. Optimal conditions for rapid growth and high cell yields from methane were found to be: 30 C, $NH_4^+$ as nitrogen source and pH 6.5. Requirements for $CO_2$ and $Cu^{++}$ were observed. Under these conditions, generation times of approximately 3 hrs and cell yields from methane between 65 and 70% could be attained. Culture HR can utilize methane, methanol, ethyl alcohol, 1-propanol, *n*-butyl alcohol and glucose, but not propane, for growth. Yeast and beef extracts are inhibitory. Carbon balances demonstrate that few if any products other than cells and $CO_2$ are produced from methane under the growth conditions used.

Whittenbury *et al.* (1970) isolate the *Methylotrophs* bacteria from Mud and water (from ponds, rivers, streams and ditches) and soil samples, obtained from the U.K., the European continent, America and Egypt. Reported that *Methylobacterium* because of their

distinctive colouring, species that can use methanol as their sole source of carbon and energy can be easily separated using methanol mineral salts (MMS) as a selective medium.

Patt *et al.* (1974) isolated and studied the methane utilizing Facultative Methylotrophs and reported the *Methylotrophic* bacteria are common in nature and have been found on a variety of plant species; they constitute a significant part of aerobic and heterotrophic plant species, as well as the aerobic and heterotrophic microflora on the surfaces of juvenile leaves. These bacteria can grow on C1 chemicals such as methanol and methylamine, as well as a wide range of $C_2$, $C_3$ and $C_4$ compounds.

Hanson *et al.* (1976) characterized the *Methylobacterium* as it is a new genus and new species of methane oxidizing bacteria produces the colonies. These bacteria are pink, circular and convex with entire margins. Gram negative cells are found and are normally found singularly with some rosettes. Also, some Methylotrophs are rod shaped ex. *Methylobacterium organophilum* spp. nov., denotes the preference of this organism for organic carbon and energy sources more complex than methane. This bacterium differs from all previously described genera and species of methane- oxidizing bacteria in its ability to utilize a variety of organic substrates with carbon-carbon bonds as sources of carbon and energy.

Green and Bousifield (1982) studied that *Methylobacterium organophilum* strain xx was found to be phenotypically highly similar to several methane non-utilizing, Pink-Pigmented bacteria after researchers analysed 149 strains using 140 biochemical, physiological and morphological parameters. *Methylobacterium organophilum*, a facultatively Methylotrophic bacteria (PPFM), fell into one of two clusters with >70% similarity, which were highly separated from other facultative Methylotrophs and non-Methylotrophic reference strains. As a result, they proposed a new tax on that could be distinguished from the majority of the genera to which they had previously been classified. To house this taxon, the genus *Methylobacterium* was chosen.

Green and Bousifield (1983) reported that *Methylobacterium organophilum* is phenotypically highly similar to the methane-non-utilizing, Pink-Pigmented Facultatively Methylotrophic bacteria and that the latter should be excluded from the various genera to which they have been assigned previously. Therefore, in accordance with an earlier suggestion, the description of the genus *Methylobacterium* be emended to permit the inclusion of methane non utilizing organisms which are otherwise highly similar to the type species. They further proposed that all of the Pink- Pigmented Facultatively

Methylotrophic bacteria which include the species *Pseudomonas rhodos, Pseudomonas radiora* and *Pseudomonas mesophilica* be transferred to *Methylobacterium*.

Bousifield and Green (1985) recategorized the genus *Protomonas* on the point of the priority of the genus *Methylobacterium* over the genus *Protomonas*. *Protomonas* extroquens was renamed as *Methylobacterium extorquens*.

Corpe (1985) reported that *Methylobacterium* strains are generally observed in soils as well as on the surfaces of leaves from a wide range of plants.

Corpe and Rheem (1989) studied the Ecology of the Methylotrophic bacteria on living leaf surfaces and shows that Methylotrophic bacteria are extremely important part of the aerobic, heterotrophic, microflora of young leaf surfaces. Free methanol of endogenous origin, is present in growing leaves. The accessibility of methanol on the leaf surface may allow the Methylotrophs to compete successfully with other heterotrophs that require multicarbon compounds that are also leached from the growing plant surfaces.

Hanson and Hanson (1996) indicates that the Methane-utilizing bacteria (methanotrophs) are a diverse genus of gram-negative bacteria closely linked to other Proteobacteria species. Based on the pathways employed for formaldehyde assimilation, the principal source of cell carbon and other physiological and morphological traits, these bacteria are divided into three groups. Agricultural techniques and other human activities have a significant impact on their natural activities. Recent evidence indicates that naturally occurring, uncultured *methanotrophs* represent new genera. *Methanotrophs* that are capable of oxidizing methane at atmospheric levels exhibit methane oxidation kinetics different from those of *Methanotrophs* available in pure cultures.

Trotsenko *et al.* (2001) reported that the association of *Methylobacterium* species with the bacterium and the host plants appear to have a symbiotic connection.

Jour *et al.* (2004) conclude that *Methylobacterium nodulans sp*. are aerobic, facultatively methylotrophic, legume root-nodule forming and have ability to fix atmospheric nitrogen.

Madhaiyan *et al.* (2005) reported that the phyllosphere is the most prevalent environment for Methylobacterium-Plant synergism, where they use methanol evolved from leaves as their only source of carbon and energy and methylobacteria may make phytohormones like cytokinin and auxins in response.

Jones *et al.* (2007) reported that Pink Pigmented Facultative Methylotrophs have ability to solubilize mineral phosphates.

Manish *et al.* (2016) while working with Methylotrophic bacteria in sustainable agriculture they conclude that Methylotrophic bacteria are well-known for playing a significant role in the biogeochemical cycle in soil ecosystems, ultimately fortifying plants and sustaining agriculture. Methylotrophs also improve air quality by using volatile organic compounds such as dichloromethane, formaldehyde, methanol and formic acid. Additionally, Methylotrophs are involved in phosphorous, nitrogen and carbon cycling and can help reduce global warming. Ahlawat *et al*. (2018) studied that rhizospheric and non-rhizospheric methylotrophs are commonly used as bioinoculants and their use as an alternative to chemical fertilisers is also on the rise. Their link to plant growth can be used to improve sustainable agriculture in environmentally beneficial and cost-effective ways. Plant development is aided by a variety of methods, including phytohormone synthesis, nodulation, nitrogen fixation and nutrient acquisition. Similarly, methylotrophic bacteria provide a biocontrol option as well as nitrogen fixation, phytohormone synthesis, ACC deaminase production and phosphate solubilization. These beneficial methylotrophic bacteria have the potential to improve plant growth and development in sustainable agricultural systems, either as an individual inoculant or as a coinoculant with other plant beneficial microorganisms.

## 2.2 Isolation of Pink Pigmented Facultative Methylotrophs

Bassalik *et al.* (1913) isolated and studied the first *Methylobacterium* strain from earth worm casts and given a name *Bacillus extorquens*.

Basile *et al.* (1969) isolated the Pink Pigmented Facultative Methylotrophic bacterium (PPFMB) belonging to the genus *Methylobacterium* as a covert contaminant from the tissue culture of liwerwort *Scaspania nemorosa*. He reported that it is everywhere in nature and observed in variety of habitant. He also demonstrated that PPFMs can help plants grow faster. PPFMs produce a diffusible material that encourages the growth of *Scapania nemerosa*, a liverwort with which the bacterium is frequently associated, in a tissue culture system. Later he identified this bacteria “fertilizer” as vitamin B12.

Kuono and Ozaki (1975) isolated and studied from a range of soil and water samples, 59 distinct PPFM isolates were obtained.

William *et al.* (1985) reported showed the establishment of Methylotrophic colonies on Methanol-ammonium salts agar surfaces impressed with leaf discs or complete leaves of green plants resulted in a pattern matching the distribution of the organisms on the leaf Pink Pigmented Facultatively Methylotrophic (PPFM) bacteria were the most common Methylotrophs found and identified. PPFM abundance ranged from 0.1 to 500

cfu/cm$^2$ on both dorsal and ventral leaf surfaces of over 50 field and glasshouse collected plant species.

Green *et al.* (1992) discovered that despite the fact that Methylobacterium strains may thrive at temperatures ranging from 50 to 370 degrees Celsius, the temperature range of 25 to 300 degrees Celsius might be used for isolation and subsequent investigations. These organisms are slow growers, taking up to two days at 30°C to develop clearly visible colonies or confluent growth and up to seven days on AMS medium for colonies to reach their maximum size of 1-3 mm in diameter. On glycerol-peptone (GP) agar, growth is sometimes more luxuriant, with a deeper pink colouring.

Green *et al*. (1992) was devised a leaf impression technique for the isolation of PPFM strains from leaf surfaces, with AMS or GP media being advised. Alternatives include homogenising full leaves or embedding them in molten agar, both of which are less effective than the impression process.

Wood *et al.* (1998) isolated and studied A novel Pink-Pigmented Facultative Methylotrophs, *Methylobacterium thiocyanatum spp.* Which is capable of growth on thiocyanate or cyanate as sole nitrogen sources and reported that this organism satisfied all the morphological, biochemical and growth-substrate criteria to be placed in the genus *Methylobacterium* Methanol-grown organisms contained high activities of hydroxyl pyruvate reductase. Showing that the serine pathway was used for Methylotrophic growth. *M. thiocyanatum* was capable to utilize thiocyanate or cyanate as the only source of nitrogen for growth and thiocyanate as the only source of sulfur in the absence of other sulfur compounds. It tolerated high concentrations (at least 50 mM) of thiocyanate or cyanate when these were supplied as nitrogen sources. Growing cultures degraded thiocyanate to produce thiosulfate as a major sulfur end product, apparently with the intermediate formation of volatile sulfur compounds (probably hydrogen sulfide and carbonyl sulfide).

Ivanova *et al.* (2000) noted the existence of an expression of genes controlling the secretion and synthesis of cytokinins by the pink-pigmented facultative methylotroph *Methylobacterium mesophilicum* with the serine pathway and non-pigmented obligate methylotroph *Methylovorus mays* with the ribulose monophosphate pathway of C1 metabolism were shown using the polymerase chain reaction (PCR) and reverse transcription–PCR methods. The way these genes are expressed constitutive. The cytokinin culture-related activity of liquid and its fractions was determined by a biotest with *Amarantus caudatus* L. seedlings. Using enzyme-linked immunosorbent analysis. He

detected zeatin (riboside) in the culture liquid of both bacteria investigated. The data obtained show that the aerobic methylobacteria are phytosymbionts that are able to utilize the single- and polycarbon compounds secreted by symbiotic plants and to synthesize cytokinins.

Ivanova *et al*. (2001) advocated PPFMs invade both the rhizosphere and the phyllosphere, enhancing plant growth by supplying plant growth hormones such as cytokinin's and auxins. Obligately and facultatively methylotrophic bacteria with various C1 metabolic pathways were shown to be able to create auxins, particularly indole-3-acetic acid (IAA), in levels of 3–100 µg/ml. Only methylobacteria with the serine route of C1 metabolism produced indole-3-pyruvic acid and indole-3-acetamide (*Methylobacterium mesophilicum and Aminobacter aminovorans*).The addition of L-tryptophan to the growing medium enhanced auxin production in methylobacteria, but ammonium ions hindered it. Tryptophan decarboxylase and tryptophan side-chain oxidase were absent in the methylobacteria studied. They also contained numerous aminotransferases at the same time. Methylobacteria are thought to produce IAA from indole-3-pyruvic acid.

Benoit *et al*. (2014) isolated strain BJ001T of the genus Methylobacterium was isolated from internal poplar tissues (*Populus deltoidesnigra* DN34) and characterized as a pink pigmented, aerobic, facultatively Methylotrophic bacteria. The strain BJ001T was found to be linked to *M. thiocyanatum*, *M. extorquens*, *M. zatmanii* and *M. rhodesianum* based on phylogenetic analysis. The strain BJ001T, on the other hand, was distinct from these species in its carbon-source consumption pattern, particularly its use of methane as the only source of carbon and energy; this ability is shared by only one other member of the genus *Methylobacterium organophilum*. Furthermore, strain BJ001T is the only member of the genus Methylobacterium that has been characterized as a poplar tree endophyte. The isolate is classified as a unique species, *M. populi spp*., based on its physiological, genotypic and ecological characteristics (type strain, BJ001T = ATCC BAA-705T = NCIMB 13946T).

Jang and Lee (2008) obtained and studied twenty three (23) isolates of Pink Pigmented Facultative Methylotrophic bacteria from soil collected from De La Salle University- Manila University. They were described in terms of morphological and biochemical properties, as well as antimicrobial resistance to specific antimicrobials. All isolates were gram-negative cells rod shaped with sudanophilic cysts. All bacterial isolates showed circular, entire, opaque, raised to convex colonies regardless of the media used. Differences however, in terms of intensity of pink pigmentation and consistency were

observed when the isolates were grown in different media. In terms of biochemical characteristics, all isolates exhibited urease, catalase, amylase and oxidase activities. Variations in terms of their capacity to oxidize different sugars and citrate as carbon and energy sources were observed among the isolates. The temperature for the optimum growth of the bacterial isolates was at 30°C. Some strains however, were observed to grow at $37^0$C and $40^0$C. Based on the phenotypic characteristics observed, the isolates are assigned to the genus *Methylobacterium.*

Rinki *et al.* (2009) obtained and studied a twenty isolates from the Neem leaves that is pink-pigmented facultative methylotrophic bacteria. All isolates exhibited pink to orange- pink pigmentation, entire margin, round colonies with a smooth glistening surface and convex elevation. Most of the colonies were opaque with butyrous consistency. Staining revealed rod to coccobacilli shaped, Gram negative cells, containing poly-β-hydroxybutyrate granules. Biochemical analyses showed that all were catalase positive; majority of them were positive for citrate utilization, urease and oxidase activities but were negative for amylase activity. They can be cultivated on AMS agar with methanol, glycerol peptone agar (GPA) and tryptic soy agar (TSA) with variations in colonial morphology. Based on the observed characteristics, the isolates obtained belong to the genus *Methylobacterium*.

Mizuno *et al.* (2012) isolated Pink Pigmented Facultative Methylotrophs were isolated from various vegetable leaves by homogenizing fresh leaves in ice-cold sterilized water for one minute with an Ace homogenizer at 15, 000 rpm, then serially diluting and plating homogenates onto AMS agar media. On AMS agar medium, all types of vegetable leaves produced pink coloured colonies, however the cfu values found differed between the vegetable crops.

Nalayani *et al.* (2014) isolated fourteen Pink Pigmented Facultative Methylotrophic bacteria belonging to *Methylobacterium* species from the phyllosphere of 10 cotton genotype. In that eight isolates oxidized elemental sulfur while three isolates were able to oxidize both elemental sulfur and thiosulphate under in vitro condition. The PPFMB can be explored as a new inoculant for nutrition of cotton.

Nysanth *et al.* (2018) isolated the *Methylobacterium* spp. are Pink-Pigmented Facultative Methylotrophs (PPFMs) are a type of bacterium that can live on single carbon compounds like formate, formaldehyde and methanol as well as multicarbon compounds with no carbon-carbon bonds. They are found in the phyllosphere and rhizosphere of

plants and have been isolated from Paddy. Methylotrophs are known to increase agricultural output and soil fertility.

## 2.3 Characterization of Pink Pigmented Facultative Methylotrophs

### 2.3.1 Morphological Characterization

Heumann *et al*. (1962) reported that branched or pleomorphic methylotrophs are common in older stationary stage cultures. They have a budding or polar growth morphology. Although some strains are not highly motile, they all have a single polar, subpolar, or lateral flagellum. Sudanophilic inclusions (poly-hydroxybutyrate) and volutin granules are frequently found in cells.

Downs and Harrison (1974) concluded that colonies on glycerol peptone agar range in size from 1 to 3 mm in diameter and range in colour from light pink to bright orange red, whereas colonies on methanol salts agar are uniformly pale pink. The pigment is a carotenoid that is insoluble. Strains grow as a pink surface ring or pellicle in static liquid environments. Patt *et al.* (1976) emended the genus *Methylobacterium*, all strains were rods measuring 0.8 to 1.0 by 1.0 to 8.0 μm in diameter, appearing singly or occasionally branched and pleomorphic motile by single polar, subpolar, or lateral flagella, albeit some were not violently motile. Sudanophilic inclusions (poly-hydroxybutyrate) and volutin granules are frequently found in cells. Despite the fact that many strains are gram variable, representative strains have the multilayered cell wall structure and citrate-synthase type that gram negative bacteria (Patt *et al*., 1974).

Green and Bousifield (1982) discovered that Methylotrophs are gram negative, despite the fact that many strains stain as gramme variable.

### 2.3.2 Biochemical Characterization

Green *et al*. (1992) reported that all *Methylobacterium* catalase and oxidase-positive strains, chemoorganotrophs and facultative Methylotrophs capable of thriving on a wide range of C1 chemicals. The voges proskauer and methyl red tests are negative. Some strains convert nitrate to nitrite, all strains produce urease and some strains have low lipolytic activity. There are no enzymes for β -galactosidase, L-ornithine decarboxylase, L-lysine decarboxylase, or L arginine dihydrolase.

Madhaiyan *et al.* (2002) resulted that Facultative Methylotrophs are aerobic organisms with enzymes such as catalase, oxidase and urease. Only two PPFM strains, PPFM-RL-3 and PPFM-RL-10 were found to be positive in the methyl red and voges proskauer tests. No isolates produced carbinol indole or $H_2S$ or did they create hydrolyzed casein, starch, gelatin, or cellulose and just a few had denitrification capability.

### 2.3.3 Carbon Utilization Tests

Species within the genus *Methylobacterium* are distinguished primarily by the type of substances they use as carbon and energy sources.

Anthony *et al*. (1982) studied the occurrence of PPFM on vegetable crops and reported that Facultative Methylotrophs could utilize different carbon compounds and assimilate C1 compound via serine pathway.

Green and Bousifield (1982) notified that methylamine, trimethylamine, acetate, citrate, L- glutamate, D-glucose, D-xylose, fructose and betaine were among the chemicals reported by the majority (95%) of *Methylobacterium* strains. The results of testing for carbon source utilization by 12 known *Methylobacterium* species revealed that none of the strains investigated appear to consume any of the disaccharides or sugar alcohols.

Urakami and Komagata (1984) reported that some strains may also use Larabinose, Dxylose, D-fucose, D-glucose, D-galactose, D-fructose, L-Aspartate, L-glutamate, adipate, Sebacate, D-tartarate, citrate, saccharte, mono-methylamine, trimethylamine N- oxide, ethanolamine, butylamine, dimethylglycine, and betaine, ammonia, nitrate and urea were shown to be nitrogen sources.

## 2.4 Growth Characteristics of Methylotrophs

On nutrient agar, most *Methylobacterium* strains grow slowly, with the exception of a few. Colonies on agar are 1 to 3 mm in diameter and pale pink to brilliant orange red after seven days of incubation at 30ºC, but colonies on methanol mineral salts (MMS) agar are more homogeneous light pink.

Green *et al*. (1992) reported that all *Methylobacterium* strains thrive best at temperatures between 250 and 300 degrees Celsius. Some strains will grow at or below 510 degrees Celsius, while others will thrive at or over 370 degrees Celsius. Although certain strains can thrive at pH levels ranging from 4.0 to 10.0 growth is best around neutrality. External growth factors are not known to be necessary for *Methylobacterium* strains to thrive and flourish.

Senthilkumar *et al.* (2002) was investigated the compatibility of a pink coloured facultative Methylotrophs. All microorganisms employed as bioinoculants were confirmed to be compatible with *Methylobacterium sp.* Co47. *viz., Rhizobium spp.* COC10, *Azospirillum lipoferum* AZ 204, *Bacillus megatherium* var *phosphatic* PSB1*, Pseudomonas fluorescens* PF1, *Trichoderma viride* TV6 and *Aspergillus awamori* PSF1 except *Trichoderma harzianum* TH1.

Glycerol peptone agar which is also a typical medium for pink coloured facultative Methylotrophs, grew all of the organisms well (PPFMs). On the same way, the PPFMs grew moderately in medium used to produce other bioinoculants. Because these organisms are compatible, PPFMs have the potential to be used as a novel component in the preparation of mixed bioinoculants for a variety of crops.

## 2.5 Development of Liquid Formulation of Pink Pigmented Facultative Methylotrophs

Maurice *et al.* (2001) studied several liquid formulations available today to sustain high viable rhizobial numbers for extended periods of time and physiological changes in aspects such in terms of seed stability and their ability to form nodules in *Rhizobium* that had been kept in commercial liquid formulation for several years.

Paul *et al.* (2002) reported that a liquid inoculants formulation with good field performance characteristics could overcome many problems associated with processing solid carriers. The field performance was evaluated of two phenotype formulation with network of 27 collaborators in sixteen countries to compare liquid formulations to local inoculants products and to sterilized Canadian peat based inoculants. The formulation tested with different additives like PVP, Fe-EDTA, glycerol, trehalose, mannitol in modified YEMA medium revealed that shelf life of inoculants was observed upto 180 days.

Singleton *et al.* (2002) developed liquid formulations of *Rhizobium* by adding various additives in the yeast extract mannitol media and claimed cell numbers of 1 x $10^{10}$ cells/ml in the liquid inoculant to increase the shelf life of bacteria.

Chandra *et al.* (2005) showed that in the case of *Azospirillium* the population came dropped to 105 at six month duration at room temperature where as in liquid, survived up to 2 years and population maintained up to 108/ml. Similarly, *Azotobacter*, KMB, *Rhizobium* inoculant in powder form maintained the shelf life up to 6 months expects PSM which serviced up to 8 months. But in case of liquid formulations, *Azotobacter*, PSM and KMB survived up to two years followed by *Rhizobium*, only for 14 months.

Girisha *et al.* (2006) studied the Liquid inoculant formulation of Cowpea rhizobia prepared with PVP as an osmoprotectant and observed to have higher shelf life than those without PVP amendment.

Vendan and Thangaraju (2006) developed liquid formulation of *Azospirillum brasilense* amended with trehalose (10 mM), glycerol (10 mM) and PVP in $N_2$ free malate broth and reported 108 cells/ml up to 10 months storage under room temperature.

Standardized doses of liquid formulation (10 ml/kg seed, 150 ml/ha seedlings and 300 ml/ha) for seed treatment, seedling root dipping and soil application.

Savalkar *et al*. (2007) studied the endophytic nitrogen fixing bacteria from sugarcane and selection of efficient strains for their mass production as liquid state bioinoculant with formulation by fermentation based biotechnologies, by designing a new common media and formulation of the same with cell protectants and cell growth booster. A quality product formulated with Cell Growth Booster (CGB) and Cell Protectant (CP) with neutral pH, higher shelf life, easy in handling storage and application has been developed. Such type of liquid formulation with CGB and CP will also increase utilization efficiency of Liquid Bioinoculant by stem, leaves and plantlets.

Panlada *et al.* (2007) described that few of the polymers and chemicals which can be used as additives and protectants in liquid inoculants include PVP, methyl cellulose, gum arabica, trehalose, glycerol, sodium alginate, poly ethylene glycol, polyvinyl alcohol and tapioca flour.

Om Prakash *et al*. (2010) monitored the growth and viable count of three rhizobial isolates on YEM, modified YEM containing sucrose, glycerol and YEM containing trehalose were showed viable count up to 120 to 360 days and observed more after 360 days of storage in media amended with gum arabica.

Kumaresan and Reetha (2011) studied survival of *Azospirillum* amended with different chemical additive and they reported that liquid bioinoculant formulation has become the preferred technology to tackle the difficulties that come with it with shorter shelf life, high contamination, poor quality, low field performance and processing solid carrier in carrier based bioinoculant formulation. They evaluated different concentrations of six different chemical amendments like poly-vinyl-pyrrolidone (PVP), glycerol, gum arabica, trehalose, polyethylene glycol (PEG) and polyvinyl alcohol (PVA) for their ability to support growth and promote survival of *Azospirillum brasilense* in N2 free malic acid broth during the storage. Some concentrations of various additives to N2 free malic acid broth promoted higher *Azospirillum* population compared to Azospirillum cells in N2 free malic acid broth alone. Liquid *Azospirillum* bioinoculant formulated with trehalose (10mM) promoted long term survival of *Azospirillum* followed by glycerol (10 mM) gum arabica (0.3%) and PVP (2%) and they supported 108 cells/ml up to 11 months of storage under ambient temperature (28°C to 32°C), whereas PEG (1%), PVA (0.5%) and control (lignite carrier) recorded the same population up to 8 months, 6 months and 5 months

respectively. The results of the present study clearly indicated that the liquid formulation of *Azospirillum* could be used more effectively than the carrier based formulation.

Chandra *et al*. (2012) tested several compounds *viz.* PVP, Fe-EDTA, glycerol, trehalose, glucose, mannitol etc. for their ability to promote survival of bacteria after inoculation. The bacteria of NPK were multiplied in fermenter. Liquid suspension concentrate was formulated from ex-fermenter slurry of each organism. The base material which contained emulsifier, dispersant, cell protectant, moisturizer etc.

## 2.6 Shelf Life of Consortium

Rao *et al.* (2007) formulated liquid inoculants formulation with the media supporting growth of three biofertilizer organism *viz., Rhizobium, Azospirillum* and P- solubilizing *Bacillus megaterium* (PSB) using different concentration of cell protectant like arabinose, trehalose, glycerol, PVP, Fe-EDTA with liquid medium maintaining good titer even after 360 days.

Chandra *et al*. (2009) developed the liquid biofertilizers and introduced the product in field successfully. Critical formulation requirement determined in NPK– Liquid formulation of *Azotobacter, Azospirillum*, phosphate solubilizing microorganisms and potash mobilizing bacteria with about 24 month shelf life. He used various dyes, additives, wetters, stickers, humectants in NPK- Liquid Biofertilizers.

Sharma *et al.* (2010) reported that the research demonstrates from that liquid biofertilizers mainly liquid rhizobia with 2 per cent PVP were discovered to maintain higher CFU count and was very effective for enhancing the soyabean yield particularly in US, Brazil, Europe and Asia.

Pindi and Satyanarayana (2012) stated that liquid formulation facilitates long shelf life with minimum contamination, carrier free activity, handling comfort, storage and transfer confort, easy quality control, enhanced export potential and are preferred by the farmer community as well as manufacturers.

## 2.7 Effect of Methylotrophs on Plant Growth Parameters

The microorganisms and plants have a symbiotic connection in which both of them benefit from each other. A large variety of bacterial species, most of which are found in the rhizosphere of plants, have been investigated and proved to be advantageous to plant development, yield and crop quality. The application of bioinoculants as microbial consortia or Combine inoculation was found to be more effective in increasing crop plant growth and yield.

Schiffmann and Label (1973) reported that nodule senescence occurred as the plant matured because of decreased biosynthesis of leghemoglobin in peanut nodules.

Iruthayathas *et al.* (1983) examined the various strains of winged soybean and bean *Rhizobium* with several strains of *Azospirillum* for combined inoculation in winged bean and soybean under temperate and tropical conditions and observed that Substantial increases in nodulation, $N_2$-fixation, shoot dry matter production and N-gain due to the mixed inoculation were obtained in some combinations of these bacteria.

Alagwadi and Gaur (1988) studied the Combined inoculation of Rhizobium and 'Phosphate-solubilizing *'Pseudomonas striata* or *Bacillus polymyxa* with and without added chemical fertilizer on chickpea yield and nutrient content was studied under greenhouse conditions and concluded that Combined inoculation of Rhizobium and *P. striata* or *B. polymyxa* increased the nodulation and nitrogenase activity, available phosphorus content of the soil, dry matter content, the grain yield and N and P uptake significantly over the uninoculated control. The inoculation effects were more pronounced in the presence of added fertilizers. The possibilities of saving half the dose of N and replacing superphosphate with rock phosphate and inoculation with 'phosphate-solubilizers'.

Weller (1988) and Whipps (2001) reported that plant growth promoting rhizobacteria (PGPR) are a group of bacteria that includes strains from the genera *Azospirillium*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Erwinia*, *Methylobacterium*, *Pseudomonas*, *Rhizobium*, *Serratia* and others.

Holland and Polacco (1992) described that a method of treating plants that involves spraying Pink Pigmented Facultative Methylotrophic (PPFM) bacteria on the plant to increase its productivity. This is related to increasing the productivity of a stressed plant by applying Pink Pigmented Facultative Methylotrophs (PPFMs) to the plant and then spraying it with an aqueous solution containing methanol.

Balamurgan and Gunashekaram (1996) conducted a field trial to evaluate the consequences of combine inoculation of *Rhizobium* strain Tt9 with a *phosphobacterium*. *Bacillus megaterium var*. *Phosphaticum* (PB 1) at various graded levels of phosphorus in groundnut (Co 1). Maximum growth, nodulation, plant dry weight and yield were acquired in treatments which received hundred per cent chemical fertilizer. The combined inoculation of *Rhizobium* and *phosphobacteria* at 50 per cent accord an analogous yield to 100 per cent phosphorus strength with no bacterial inoculation indicating a saving of 50 per cent chemical fertilizer.

Munsanje *et al.* (1996) examined the action of PPFMs on methanol which resulted in a yield rise. Methanol and/or urea treatments were applied to soybean test plots in field trials. The populations of PPFMs on treated and control plants were measured 10 days after spraying. After the plants were harvested, the yields were calculated. The number of PPFMs increased in tandem with the yield increases in each of the treatment regimes. In response to the methanol/urea spray treatments, the PPFM population was doubled, resulting in a 45 percent increase in yield above control plants with normal PPFM populations. Two weeks after spraying, dry weight measurements of the plant were used to calculate yield. The application of methanol resulted in enhanced growth only in the presence of PPFMs, according to the findings. Seed inoculums or seed coatings containing PPFMs could be utilised to improve seed germinability, storability, or vigour.

Singh *et al*. (1996) reported that Nodulation and grain yield of pigeon pea *(Cajanus cajan)* were significantly increased due to *Rhizobium spp*. and *Glomus fasciculatum* (AM) inoculation at time the plants were cultivated in pots of unsterilized soil. It was conceivable to determine the increase in total N uptake of the crop, utilization of fertilizer N and biologically fixed N2 with AM inoculation, which was about equivalent to *Rhizobium spp*. inoculation. More N2 was fixed with a combination of *Rhizobium spp*. and VAM. However, biologically fixed N2 was estimated to be higher in straw than in grain which indicates a difference in translocation and utilization of fixed $N_2$.

Devan and *et al*. (2000) reported increased plant growth, yield and nutrient uptake in pigeon pea in combined inoculation treatment comprising of *Rhizobium, Azospirillum* and *Pseudomonas striata.*

Biswas *et al.* (2000) studied the rhizobial inoculation which influences seedling vigour and yield of rice and reported that *diazotrophic* bacteria assess many growth promoting activities on rice seedling vigor, its carryover effect on straw and grain yield and the persistence of an inoculant strain on rice roots under greenhouse conditions. Growth responses to inoculation exhibited bacterial strain rice variety specificity that were either stimulatory or inhibitory. Growth responses included changes in rates of seedling emergence, radical elongation, height and dry matter, plumule length, cumulative leaf and root areas and grain and straw yields. Most notable were the inoculation responses to *Rhizobium leguminosarum* bv. *trifolii* E11 and *Rhizobium* spp. which revitalizing initial rice development resulting in a carryover outcome of remarkably increased grain and straw yields at maturity, even though their culturable populations on roots diminished to below detectable values at 60 days after planting. These studies indicate that certain strains of no

photosynthetic diazotrophs, including rhizobia, can promote growth and vigor of rice seedlings and this benefit of early seedling development can carry over to significantly increased grain yield at maturity.

Eric Giraud *et al.* (2000) studied bacteria of the Methylobacterium genus make up the fourth rhizobial branch. On the basis of 16S ribosomal DNA analysis, Rhizobia isolated from *Crotalaria* legumes were classified to a new species, "*Methylobacterium nodulans*," within the Methylobacterium genus. They showed that these rhizobia may thrive on methanol in a facultative manner, which is a hallmark of *Methylobacterium spp*. However, "*M. nodulans*" is the only nodulating *Methylobacterium spp*. discovered thus far. *Bradyrhizobium* Nod A, which is responsible for nitrogen fixation, is closely linked to "*M. nodulans*" Nod A, according to phylogenetic sequencing analysis.

Sy *et al.* (2001) and Jackson *et al.* (2006) studied the *Methylotrophs* which act as PGPR and resulted that members of the genus Methylobacterium are among the PGPR that are versatile in nature and ubiquitous on plant surfaces with the ability to dominate the phyllosphere population. They are associated with plants in a variety of ways, including free-living, epiphytic, endophytic and symbiotic. So, because of their relevance in the biosphere and potential commercial applications, methylotrophs have gotten a lot of attention recently.

Reddy *et al.* (2002) deliberated the consequences of Combine inoculation of PPFMs and Rhizobium on groundnut cultivar Co (Gn) 4 and observed that there was significantly increase in plant growth, maximum pod weight, biomass production and yield parameters of groundnut.

Paulraj *et al*. (2002) examined the consequences of microbial consortia *Azophos* (constitute 50% of each *Azospirillum* and phosphate solubilizing bacteria) on growing cardamom seedlings at artificial situation and recorded that the combine inoculation of *Azospirillum* and phosphate solubilizing bacteria as *Azophos* was observed to be superior in enhancing the germination percentage and vigour index of cardamom than single inoculation.

Paulraj *et al.* (2002) have also registered that due to bioinoculation with Methylobacterium, the chlorophyll concentration of cardamom, rubber and coffee varies. By increasing the number of stomata, chlorophyll concentration and malic acid content of sugarcane clone Co 86032, a combined treatment of seed imbibition, soil application and phyllosphere spray boosted photosynthetic activity. (Cervantes-martinez *et al.*, 2004).

Anurajan *et al*. (2003) studied that gibberellic acid is a set of plant growth hormones generated by Methylobacterium that functions by affecting plant morphology by expanding plant tissue, particularly the stem, which increases mineral uptake and thus influences the chlorophyll content, soluble sugar and protein content of plants.

Madhaiyan *et al.* (2004b) taken pot culture trial on soybean, results suggested that the shoot length, root length, plant biomass and nodulation efficiency of soybean seeds treated with PPFM strain *Methylobacterium spp*. (Sb 34) increased.

Madhaiyan *et al.* (2005) recorded that cotton plant height, plant dry weight, boll number, boll weight and kapas production all rose significantly when PPFMs were applied as a foliar spray. They also discovered that inoculating sugarcane plants with PPFMs resulted in a considerable increase in plant growth can output and sugar quality.

Thangamani *et al*. (2005) examined that Methylobacteria generates plant growth regulators such zeatin and related cytokinin and auxins, which have a major effect on seed germination and seedling growth. Methylobacteria is also positive for urease test and indole synthesis.

Thangamani and Sundaram (2005b) outlined that microbial formulation of PPFM, *Azospirillum spp*., *Azotobacter spp*., phosphate solubilizers and *Arbuscular Mycorrhizae* increased the yield of tomato crop by 62-73 per cent over absolute control.

Thangamani and Sundaram (2005c) observed that seedling dip of tomato seedlings in microbial consortia boosted the growth and yield parameters of tomato hybrids compared to single inoculation and absolute control.

Madhaiyan *et al.* (2006) outlined that the Combine inoculation of *Methylobacterium spp*. with *Rhizobium spp*. also remarkably enhanced plant development, nodulation and yield aspects in groundnut contrast to single inoculation of *Rhizobium spp*. and *Methylobacterium spp*.

Lee *et al.* (2006) investigated the actions of three plant-growth promoting, N2 fixing *Methylobacterium spp*. CBMB20, *Enterobacter spp*. CBMB30 and *Burkholderia spp*. CBMB40 *Methylobacterium spp*. CBMB40 *Methylobacterium spp*. CBMB40 *Methylobacterium spp.* CBMB40 *Methylobacterium spp*. Rice seedlings treated with Methylotrophic strains had better seed germination, seedling vigour index (SVI) and biomass.

Madhaiyan *et al.* (2006b) had taken Cotton field trials and pot culture studies revealed that foliar spraying with 30% methanol (or) PPFMs improved plant height, leaf area, boll number and boll dry weight resulting in an increase in seed cotton yield over

control. Foliar spraying of PPFMs boosted sugarcane plant height and leaf area, resulting in a 9.8% increase in cane production over control. Methanol (or) PPFMs raised total cytokinin levels in cotton and sugarcane.

Madhaiyan *et al.* (2009b) the researchers looked examined the effects of co-inoculating *Methylobacterium oryzae* with nitrogen-fixing *Azospirillum brasilense* or a phosphatesolubilizing bacterium *Burkholderia pyrrocinia* on tomato, red pepper and rice growth and nutrient uptake. In comparison to absolute control plants, seed inoculation and soil/foliar application of the bacterial strains alone or under dual inoculation increased plant development in terms of shoot or root length and increased nutrient uptake.

Radha *et al.* (2009) outlined the treatments that got PPFM spray had considerably higher plant height, number of leaves, shoot dry weight and root dry weight than the uninoculated control. Kim *et al*. (2010) made similar observations with red pepper while dealing with *M. oryzae* strains (CBMB20 and CBMB110). When compared to the uninoculated control, the Methylobacterium strain treated tomato and red pepper seeds had a higher germination percentage and root length. CBMB20 treatment resulted in a 39.4 percent increase in root length compared to the control, whereas CBMB110 treatment resulted in a 61.3 percent increase in root length compared to the control.

Meenakshi and Savalgi (2009) revealed that treatment with both seed inoculation and foliar spray of *Methylobacterium* had high chlorophyll content. Madhaiyan *et al*. (2004a) found that rice cultivar Co-47 that received Methylobacterium had better photosynthetic activity, which they attributed to increased chlorophyll concentration, maleic acid level and the number of stomata.

Anitha *et al*. (2010) from the 8 PPFM strains he isolate the PPFM-SOY (isolated from soybean leaves) and the germination of heat-treated soybean, maize and paddy seeds was strongly impacted by PPFM-GN (isolated from groundnut leaves). It was also discovered that soybean seedlings treated with PPFM-GN old culture filtrate had a higher seedling vigour index (5456) than control seedlings (1987).

Meena *et al.* (2012) concludes that the cell free culture filtrates of PPFM strains improved wheat (*Triticum aestivum*) seed germination, with *Methylobacterium spp*. (NC4) achieving the greatest germination rates of 98.3% and *Methylobacterium spp*. (NC4) achieving the highest seedling length and vigour (NC28).

Sheela *et al*. (2013) investigated the influence of various Methylotrophs on *Coleus forskohlii* growth and yield under pot culture settings. *Methylobacterium* inoculation

enhanced plant height, shoot biomass, chlorophyll content, stem girth, leaf area and tuber production.

Jeyajothi *et al*. (2014) examined recently the use of PPFM and *Pseudomonas* foliar sprays in combination with a biofertilizer increased the microbial population in the soil, allowing nutrients to be more easily accessed by the plants.

Nalayani *et al.* (2014) Microbial consortia containing different strains of Bacillus, *Pseudomonas* and *Azospirillum* with PPFM and foliar spray of PPFM utilized for seeds and soil with recommended N and P fertilizers showed that PPFMs should be utilized as a potential bioinoculant to increase yield from cotton plants.

Sivakumar *et al.* (2017) conducted pot culture experiment in tomato with foliar application of various PGR and PPFM (1%), PPFM (2%) and PPFM (3%) under drought condition created based on field capacity of soil the analysis revealed that the PPFM and PGR could be effectively improving drought tolerance capacity of tomato crop under drought.

Raghavendra and Santhosh (2019) showed that efficient strains of PPFM was enhanced the plant development characters such as height of plant, tillers/hill, number of leaves/hill, root length, root dry weight, shoot dry weight and SPAD value chlorophyll was recorded in direct seeded rice.

Santosh and Sreenivasa (2020) reported that use of native pink pigmented facultative methylotrophs enhanced plant height, dry matter and chlorophyll content at different growth stages.

# 3. MATERIAL AND METHODS

The present investigation was undertaken on "Studies on Pink Pigmented Facultative Methylotrophs in tomato (*Lycopersicon esculentum*.)". The experiment was carried out under field as well as laboratory conditions during the year 2020-2021. Field experiments were laid out at the Instructional Farm of Post Graduate Institute, MPKV, Rahuri. Whereas, laboratory experiment was performed at Post Graduate Laboratory, Department of Plant Pathology and Agricultural Microbiology, MPKV., Rahuri. The materials utilized and methods acquired to conduct the research experiments are described in this chapter.

## 3.1 Materials

### 3.1.1 Experimental Site

The field trial was taken at PGI instructional Farm, Department of Plant Pathology and Agricultural Microbiology, MPKV, Rahuri during *Summer* season 2022.

### 3.1.2 Seeds

The seedling of tomato(*Lycopersicon esculentum*.) variety **Phule kesari** obtained from AICRP on Vegetables Improvement Project Central Campus, M.P.K.V. Rahuri Dist. Ahmednagar.

### 3.1.3 Glassware's

Standard Borosil brand glassware's *viz.,* Petri plates, conical flask, measuring Cylinders, Spirit jar, volumetric flasks, microscopic glass slides, test tubes, Pipettes, cover slips.

### 3.1.4 Equipment's

The laboratory equipment's used were autoclave, incubator, laminar air flow cabinet, refrigerator, research microscope, camera, electronic top pan balance, needle, hot air oven, spirit lamp, shaker was used.

### 3.1.5 Cultural Media

In the laboratory, Ammonium Mineral Salts (AMS) medium (Whittenbury *et al.*, 1970) was utilized for isolation of the organisms. The same medium was also utilized for maintaining pure culture of isolated *Methylotroph* strains. The composition of Ammonium Mineral Salts (AMS) medium is given below

| Contents | $gL^{-1}$ |
|---|---|
| $K_2HPO_4$ | 0.7 |
| $KH_2PO_4$ | 0.54 |
| $MgSO_4.7H_2O$ | 1.0 |
| $CaCl_2.2H_2O$ | 0.2 |
| $FeSO_4.7H_2O$ | 0.004 |
| $NH_4Cl.7H_2O$ | 0.5 |
| $ZnSO_4.7H_2O$ | 0.0001 |
| $MnCl_4.4H_2O$ | 0.00003 |
| $H_3BO_3$ | 0.0003 |
| $CoCl_2.6H_2O$ | 0.0002 |
| $CuCl_2.2H_2O$ | 0.00001 |
| $NiCl_2.6H_2O$ | 0.00002 |
| $Na_2MoO_4.2H_2O$ | 0.00006 |
| Agar | 15.0 |
| | Final pH = 6.8 ± 0.2 at 25°C |

### 3.1.6 Miscellaneous Material

Brown paper bags, weight box, labels, polythene, paper bags, cotton wool, mercuric chloride ($HgCl_2$) blotting papers, glass marking pencils, permanent markers, sterile water, test tube stand, rubber band etc. were used as and when required during the present investigation.

## 3.2 Methodology

### 3.2.1 Isolation and Purification of Pink Pigmented Facultative Methylotrophs (PPFMs) :

The parameters such as cultural and morphological were studied for identification of PPFM with respective media. All the methods for staining reaction were followed as per method laid down in manual of microbiological methods.

The Ammonium Mineral Salts (AMS) medium (Whittenbury *et al*., 1970) is an only Methylotroph isolation medium. The AMS media was autoclaved at 121°C for 15 minutes and then cooled to 50°C. After sterilization and before pouring media onto Petri plates, a filter sterilized vitamin solution (Colby and Zatman, 1973) was added, along with 0.5 percent (v/v) methanol. The medium was adjusted to a pH of 7.0.

#### 3.2.1.1 Collection of samples :

The leaf samples were collected from Tomato (*Lycopersicon esculentum* L.) plants grown in area of the AICRP on Vegetables Improvement Project, PGI farm, M.P.K.V., Rahuri and also from nearby villages of Rahuri Tehsil. The samples were conveyed to the lab in aseptic polythene bags and kept at 4º degrees Celsius.

**3.2.1.2 Isolation techniques :**

**Leaf imprinting technique**

The upper and lower surfaces of leaf samples were placed individually on the solidified AMS agar medium in order to make an impression of it. The leaves were then removed and the plates were incubated for 5 to 7 days at 30°C.

## 3.3 Morphological Characterization of PPFM

After cultivating PPFM bacterial isolates on AMS agar medium for one week at 30°C, the colony morphology was studied. The cell size, cell shape, motility studies and gram reaction of each isolate were also investigated.

### 3.3.1 Cell Size

The cell size of the 72-hour-grown facultative Methylotroph isolates was measured using an ocular and stage micrometer by microscope.

### 3.3.2 Cell Shape (Becking, 1974)

The purified cultures at log stage were observed microscopically for the cell morphological characters.

### 3.3.3 Motility Studies

The bacterial motility of the 72-hour-grown PPFM isolates was examined microscopically using a hollow slide.

### 3.3.4 Gram Staining (Rangaswami and Bagyaraj, 1993)

A tiny film of bacteria from one day old culture was made on a washed slide, fixed by gently heating and gram stained and observed under microscope with oil immersion technique.

## 3.4 Biochemical Characterization

### 3.4.1 Oxidase Test

The PPFM isolates were streaked on to trypticase soy agar medium and cultured for 48 hours at 37°C inverted. Following the incubation period, 2-3 drops of P-amino dimethyl aniline oxalate solution were applied to the streaked area and the plates were inspected for a colour change from pink to maroon, then purple, within 30 seconds, indicating a favorable reaction.

### 3.4.2 Catalase Activity

A loopful of FM isolates cultured on AMS agar slant for 24 hours was transferred to a glass test tube containing 0.5 ml distilled water and well mixed with 0.5 ml 3 percent hydrogen peroxide solution and the effervescence was observed.

### 3.4.3 Urease Test

The urease test was carried out on 5 ml of urea broth in test tubes with phenol red (pH 6.8) as the pH indicator. The cultures were inoculated and incubated for 24 hours in a sterilised urea broth. The appearance of red signifies a favorable response to the test.

### 3.4.4 Casein Hydrolysis

On skim milk agar plates, the PPFM isolates were streaked and incubated at room temperature. The existence of clear zones surrounding the colonies on the plates was tested for hydrolysis of casein and was regarded a favorable reaction.

### 3.4.5 Starch Hydrolysis

The PPFM isolates were streaked on 2 percent insoluble starch nutrient agar plates and cultured at room temperature. The presence of clear zones surrounding the colonies was detected on the plates after flooding with iodine solution and this was deemed a favorable result.

### 3.4.6 Citrate Utilization Test

The PPFM isolates were placed in test tubes with Simmons citrate agar medium and cultured at 35°C for 48 hours. The only carbon and energy source in Simmons citrate agar is citrate. Growth and a shift in colour from green to blue as a result of the pH change suggested a favourable reaction.

### 3.4.7 Carbon Source Utilization Test

The following carbon compounds were substituted for methanol at 0.5 percent level in AMS liquid medium such as glucose, D- Galactose, dichloromethane, chloroform and glycerol inoculated with one percent (v/v) standard inoculum (109 cfu $ml^{-1}$) and incubated in an incubator shaker (150 rpm) at 30°C for 5 days and compared to a negative control containing no added carbon Because cultures on some carbon compounds grow slowly, a considerable incubation period was required.

## 3.5 Studies on Efficiency of PPFM Isolates According to the Production of Beneficial Growth Parameters

### 3.5.1 Nitrogen Estimation

The 100 mL of the N-free malate medium was dispensed and autoclaved into a 250 mL conical flask. After that, each flask received one ml of culture inoculum that had been aged for 24 hours. The flasks were incubated for seven days at 37°C.

The culture was homogenized after 7 days of incubation and 10 ml was digested using 5 ml of concentrated $H_2SO_4$ and 0.2 g digestion catalyst mixture $K_2SO_4$: $CuSO_4$: selenium (100:10:1). With distilled water, the volume was increased to 10 ml after cooling.

Later, ten milliliters of the aliquot were transferred to the distillation unit at Micro-kjeldalh's. The material was distilled after mixing it with 20 mL of 40% NaOH. The ammonia was trapped in a 4% boric acid mixed indicator (Bromocresol green 0.066 g and methyl red 0.033 g in 100 ml methanol) until the solution turned green. It was titrated against 0.05 N $H_2SO_4$ to evaluate the total nitrogen content of the culture, which was expressed as mg N fixed per gram of malate.

$$\text{Per cent N} = \frac{\text{Titrate value x 0.014 x N of } H_2SO_4 \text{ x Vol. made}}{\text{Sample used}} \text{ x 100}$$

### 3.6 Liquid Formulation

The obtained efficient PPFM bacteria were formulated for mass production as liquid consortium by comparing the selective media for these isolates and appropriate Medium 4 was designed. The suitable medium was again formulated by using different concentration of cell protectants like arabinose, trehalose, glycerol, PVP (Poly-Vinyl-Pyrrolidone) and Fe-EDTA at different pH levels. And was devised as $LM_1$, $LM_2$, $LM_3$, $LM_4$ and $LM_5$. These media were tested and contrast to development by transferring in a flask 1 per cent inoculum of PPFM bacteria and incubated on rotary shaker at 110 rpm for 72 hrs.

After incubation a loopful culture was streaked on sterilized respective selective media plates. The plates were kept for incubation at 28 ± $1^0$c and observed for growth by comparing cfu count on each devised media, which shows maximum cfu of PPFM bacteria was observed and selected as standard appropriate PPFM consortium medium. The luxuriant growth of PPFM bacteria was observed on the $LM_4$ medium as a consortium medium.

### 3.7 Shelf Life of Consortium

Shelf life study was done after the bacterial consortium preparation by taking periodical cell count of bacteria at monthly duration achieved through successive dilution and plating method on suitable selective media.

The procedure carried out is given below:-

1. One ml of sample of consortium was suspended in 9 ml of sterilized water blank.
2. Serial dilution was made from $10^{-1}$ to $10^{-6}$. One ml aliquot of dilutions from $10^{-3}$ to $10^{-6}$ was transferred to sterilized Petri plates of respective selective medium separately.

3. For count of bacteria, respective selective media were used. The sterilized medium before solidification ($45^0$c) was put into each Petri plates and assorted the contents in plates by rotating the plates gently.
4. After solidification, plates were kept inverted at 28±2 $^0$c in BOD incubator for 4 -7 days. All of the plates were examined for the appearance of bacterial colonies.
5. The population of bacteria was estimated by counting the number of colonies of the respective organism per plate by a colony counter and computing the average number of particular groups per ml of consortium by using the following formula.

$$\text{Cfu /ml of consortium} = \frac{\text{Average plate count}}{\text{Sample}} \times \text{Dilution factor}$$

### 3.8 Assessment of Efficient Strains of PPFM under Field Condition

The field trial was carried out to analyses the consequences of promising isolate of PPFM selected based on artificial studies and its consequences on growth Parameters and yield of tomato.

#### 3.8.1 Location of Experimental Field

The field trial was carried out during *summer* season of 2021-22 at the Instructional Farm of Post Graduate Institute, M.P.K.V., Rahuria.

#### 3.8.2 Soil

The experimental soil was medium black with clay texture with more than 60 cm depth with uniform and leveled topography. The soil was neutral in reaction. A composite soil sample (to depth of 0-30 cm) was drawn from the experimental area before sowing and was analyzed for chemical properties and the fertility status of soil.

#### 3.8.3 Climate

Geographically Research Farm of Central Campus, Post Graduate Institute MPKV., Rahuri located in Ahmednagar district. This area comes under arid to semi-arid zone (Scarcity tract) with an annual rainfall of 500-750 mm in two peak values, one in the month of July and on other in the month of September. The average rainfall is 500mm. The rainfall was erratic and distributed unevenly in rainy days.

The mean temperature maximum and minimum for the year was ranges from $30^0$ to $49^0$C and $7^0$ to $24.2^0$C.

### 3.9 Experimental Details

The experimental details are given below

| | | | |
|---|---|---|---|
| 1. | Name of the crop | : | Tomato (*Lycopersicon esculentum*) |
| 2. | Variety | : | Phule Kesari |
| 3. | Season | : | Summer-2022 |
| 4. | Experimental Design | : | Randomized Block Design (RBD) |
| 5. | No. of Replications | : | 03 |
| 6. | No. of Treatments | : | 07 |
| 7. | Total Experimental Plots | : | 21 |
| 8. | Spacing | : | 60 cm x 45 cm |
| 9. | Plot size | : | Gross = 3.60 m x 4.50 m |
| | | | Net = 2.40 m x 2.70 m |
| 10. | Seed rate | : | 1.5 - 2.0 kg $ha^{-1}$ |
| 11. | FYM | : | 10 to 11 t $ha^{-1}$ |
| 12. | Fertilizer dose | : | 100:50:50 kg $ha^{-1}$ $N:P_2O_5:K_2O$ |

### 3.9.1 Details of Treatment

The experiment consists of 7 treatments combination consisting graded level of fertilizer with Biofertilizer (*Azotobacter*) and inoculant of PPFM bacteria with 3 replications.

The detail of treatments as below:

**Table 3.1 Treatments details and symbols used for the experiment**

| Sr. No. | Treatments | Symbol |
|---|---|---|
| 1. | 100% N only without seed treatment of *Azotobacter* and PPFM and foliar spray of PPFM | $T_1$ |
| 2. | Seed treatment with *Azotobacter* (MPKV strain) and PPFM + Foliar spray of PPFM only | $T_2$ |
| 3. | Seed treatment with *Azotobacter* (MPKV strain) and PPFM + 25% N + Foliar spray of PPFM | $T_3$ |
| 4. | Seed treatment with *Azotobacter* (MPKV strain) and PPFM + 50% N + Foliar spray of PPFM | $T_4$ |
| 5. | Seed treatment with *Azotobacter* (MPKV strain) and PPFM + 75% N + Foliar spray of PPFM | $T_5$ |
| 6. | Seed treatment with *Azotobacter* (MPKV strain) and PPFM + 100% N+ Foliar spray of PPFM | $T_6$ |
| 7. | Absolute control | $T_7$ |

### 3.9.2 Replications

Treatments were replicated three times.

### 3.9.3 Plan and Layout of the Experiment

Design : RBD

Plot size : Gross = 3.60 x 4.5 $m^2$

Net = 2.40 x 2.7 $m^2$

Replications : Three

Treatments : Seven

## 3.10 Cultural Practices :

### 3.10.1 Preparation of Experimental Plot

The land was deep ploughed and harrowed and experimental layout was prepared as ridges and furrow having gross plot size 3.60× 4.50 $m^2$ as per number of replication

### 3.10.2 Fertilizer Application

As per the treatment details the recommended nutrient doses were applied in each replication through of urea, SSP and MOP of the time of planting.

### 3.10.3 Inoculation to the Carrier

The inoculum of *Azotobacter* was produced by inoculating 72 hrs old log stage cultures of *Azotobacter* (MPKV strain) in 100 ml of YEMA broth and *Methylobacterium* in 100 ml of AMS broth (Whittenbury *et al.*, 1970). For five to six days, the flasks were put on a shaker at 28±2°C. In the case of single inoculation, the culture broth was mixed with pre-sterilized lignite powder at a rate of 30 ml per 100 g carrier in the case of combination inoculation, 15 ml of *Azotobacter* strain and 15 ml of *Methylobacterium* isolates were added per 100 g carrier. The treatments as well as other combinations of treatments are listed below.

### 3.10.4 Seed and Sowing

For sowing of tomato variety *Phule kesari* was used having seed rate 750 g $ha^{-1}$. Seeds were sown by dibbling method with spacing of 60 x 45 cm.

Before transplanting seedlings were treated with *Methylobacterium* strain and *Azotobacter* (MPKV strain) @ 500 g $ha^{-1}$ as per treatment schedule. Optimum soil moisture was maintained during the trial stage period by continuous irrigation. Requisite plant protection management practices were applied as per advocated practices.

### 3.10.5 Phyllosphere Spray of PPFM Isolates

The *Methylobacterium* cultures (1 x $10^9$ CFU/ml of culture) were diluted to 1:100 and sprayed at 25 ml/plant at the time of flowering stage.

### 3.10.6 Aftercare of Crop

Three hand weeding's were done at different intervals during the crop growth period to keep the crop free from weeds. Irrigations were given as per crop requirement. All other cultural practices as recommended in Package of Practice for solanaceous vegetable crops were followed from time to time to ensure a good crop stand.

### 3.10.7 Harvesting

The tomato fruits were harvested when they were medium size fruit appeared and done 2 to 3 fresh fruit harvest to assess the green tomato fruit yield and after complete maturing dry yield of was also assessed.

### 3.10.8 Observations

Without causing any damage to the root system the tomato plants were drawn out from at flowering stage and harvesting stage and their root and shoot system was isolated and submitted to the following observations.

**a. Plant height**

The five plants were randomly chosen from each net plot and plant height were recorded in cm from ground level of stem to the base of last opened leaf at flowering and harvesting stage.

**b. Number of leaves**

The green fleshy and completely opened leaves of randomly selected five plant from each net plot were counted at flowering and harvesting stage.

**c. Fresh weight (g plant$^{-1}$)**

The green sample were collected at flowering and harvesting stage randomly from each plot and the fresh weight of the plant were noted immediately after separating it and showed as gram per plant (g plant$^{-1}$).

**d. Dry weight (g plant$^{-1}$)**

The green sample were collected at flowering and harvesting stage randomly from each plot. The plants were promptly cleaned with tap water, 0.1 N HCl and twice distilled water after being uprooted. The plant samples were air dried for two days before being placed in a hot air oven at 65-70°C to achieve a constant weight. Later the dry matter weight was reported.

$$\text{Dry matter content (\%)} = \frac{\text{(Weight of sample) - (Dry weight of sample)}}{\text{Fresh weight of sample}} \times 100$$

**e. Number of branches**

Total five plants were selected randomly from each net plot and number of branches per plant was recorded at both flowering and harvesting stage.

**f. Number of fruits**

The green matured fruits of randomly selected five plants from net plot were counted their average was demonstrated as number of fruits per plant at 70 and 90 DAT.

**g. Length of root**

The five plants were randomly uprooted from each net plot and root length were measured in cm from ground level of root to the tip of root at flowering and harvesting stage.

**h. Fruit yield (g/plant)**

All medium sized fresh nearly ripened fruits in the field were harvested by 3 to 4 picking . The average fruit Yield (kg) per net plot per picking was measured. From the net plot, the fruit yield per hector was calculated.

### 3.11 Nutrient Uptake Studies

The oven-dried plant samples were ground into a fine powder and utilised to calculate nitrogen and phosphorus. The leaf samples were collected from AICRP on vegetable improvement project ,experimental research farm of PGI,MPKV Rahuri. From leaves (Dorsal and Ventral side) Pink Pigmented Facultative Methylotrophs,(PPFM) were isolated on AMS medium.

**Formulae**

$$\text{Uptake of NPK (kg ha}^{-1}\text{)} = \frac{\text{Concentration of N\% P\% K\% x dry matter (kg ha}^{-1}\text{)}}{100}$$

### 3.11.1 Estimation of Nitrogen from Plant Sample

At the harvesting stage, the total nitrogen content of the plant sample was determined using Micro-digestion kjeldhal's and distillation method. 500 mg of oven dried finely ground samples were digested with five ml of concentrated $H_2SO_4$ in the presence of a 200 mg catalyst combination for the analysis (containing potassium sulphate, copper sulphate and selenium in 100:10:1 ratio). On a Micro- kjeldhal digestion device, the samples were digested until a clear solution was formed. The digest was allowed to cool before being diluted with distilled water. In a semi micro kjeldhal distillation apparatus, the digested samples were distilled after adding 20 ml of 40% NaOH to make the digest alkaline. The ammonia produced was absorbed in a 4% boric acid solution and titrated

against 0.05 N $H_2SO_4$. The titrate results were converted to mg of nitrogen and per cent nitrogen was computed using a standard of 1 mg of nitrogen per five ml solution of ammonium sulphate.

### 3.11.2 Estimation of Phosphorus

The estimation of total phosphorus concentration of tomato plant tissue was done at harvesting stage of crop by Vandomolybdate yellow colour method using colour intensity at wavelength470 wavelength on spectrophotometer.

### 3.11.3 Estimation of Potassium

The assessment of total potassium strength of tomato plant tissue was done at harvesting stage of crop by flame photometer method.

## 3.12 Statistical Analysis

The data was statistically analyzed by following the standard method for analysis of variance. The standard error for treatment and critical difference at 5 per cent of significance were worked out as suggested by Panse and Sukhatme, 1985.

# 4. RESULTS AND DISCUSSION

By gathering samples from the A.I.C.R.P. on Vegetables Improvement Project Experimental Research Farm of PGI M.P.K.V., Rahuri, and neighboring villages of Rahuri Tehsil, the Pink Pigmented Facultative Methylotrophs (PPFM) were isolated as per table 4.1.

Studies on pink pigmented facultative methylotrophs (PPFM) in tomato (*Lycopersicon esculentum L.*) were conducted as part of the current inquiry at the Department of Plant Pathology and Agricultural Microbiology, PGI, MPKV Rahuri, both in the lab and out in the field. In this study, PPFMs were isolated, evaluated, purified, and morphologically and biochemically characterized. A consortium of highly effective PPFMs bacteria were also used, and liquid formulation was standardized. In order to assess the impact of different nitrogenous fertilizer levels on the growth and nutrient uptake of tomato, the liquid consortia of PPFMs were used in the field investigation. Below are the specifics of the investigation's findings and a discussion of them.

**Table 4.1 Isolation of different isolates of PPFM from different crops**

| Sr. No. | Place | Source | Crop | Isolates obtained |
|---|---|---|---|---|
| 1. | AICRP on Vegetable Improvement Project, MPKV, Rahuri | Phyllosphere | Chilli | $PPFM_1$ |
| 2. | PGI farm, Department of Plant Pathology and Agril. Microbiology, MPKV, Rahuri | Phyllosphere | Tomato | $PPFM_2$ |
| 3. | PGI farm Department of Irrigation and Water Management, MPKV Rahuri. | Phyllosphere | Potato | $PPFM_3$ |
| 4. | PGI farm, Department of Agril. Entomology, MPKV, Rahuri | Phyllosphere | Brinjal | $PPFM_4$ |
| 5. | Village – Digraj | Phyllosphere | Cauliflower | $PPFM_5$ |
| 6. | Village – Wambhori | Phyllosphere | Cowpea | $PPFM_6$ |
| 7. | Village – Musalwadi | Phyllosphere | Maize | $PPFM_7$ |
| 8. | Village-Babhulgaon | Phyllosphere | Chilli | $PPFM_8$ |

The acronym PPFM stands for Pink Pigmented Facultative Methylotroph.

Eight isolates in total were collected, and they were given the PPFM series coding. Based on their analysis on AMS agar medium with methanol as the only carbon source, these isolates were tentatively classified as Pink Pigmented Facultative Methylotrophs.

Because of their pigmentation, Methylobacterium species can only utilised methanol as a source of carbon and energy, and because they can be easily isolated on

methanol mineral salts (MMS) or ammonium mineral salts (AMS) as a selective medium. Additionally, a variety of $C_2$, $C_3$, and $C_4$ compounds, as well as $C_1$ substances including methanol and methylamine, can support the growth of these bacteria. These results concur with those of Patt et al. and Whittenbury et al. (1970). (1974).

Facultative methylotrophs can exploit a heterogeneity of carbon molecules and absorb $C_1$ via the serine route. They can also develop at the expense of reduced carbon compounds that include one or more carbon compounds but no carbon-carbon bonds, according to Anthony's findings (1982).

## 4.2 Identification of Pink Pigmented Facultative Methylotrophs

Different morphological characteristics, such as size, shape, gram reaction, colony colour, etc., as well as numerous biochemical parameters, such as oxidase test, urease test, indole generation, casein hydrolysis, carbon consumption, etc., were looked at in order to identify PPFM isolates. Table 4.2 and Table 4.3, respectively, present the results.

### 4.2.1 Morphological Characterization of PPFM Isolates

Table 4.2 provides the findings with reference to the morphological characterisation of PPFM isolates. All PPFM isolates had rod-shaped, motile, and gram-negative cells. All of the isolates' cell sizes vary in terms of dimension. The measured range was between 0.6 and 0.9 x 1.5 m. Pigmentation PPFM isolates. All PPFM isolates had rod-shaped, motile, and gram- negative cells. All of the isolates' cell sizes vary in terms of dimension. The measured range was between 0.6 and 0.9 x 1.5 m. Pigmentation caused the PPFM bacteria's cells to appear pink, light pink, or dark pink in colour. Heumann (1962), Downs and Harrison (1974), Green and Bousifield, and others made comparable discoveries (1982).

**Table 4.2 Morphological characterization of Methylotrophs**

| Sr. No. | Isolates | Cell size (µm) | Cell shape | Gram Reaction | Pigmentation | Cell Motility |
|---|---|---|---|---|---|---|
| 1. | $PPFM_1$ | 0.8 x 1.3 | Rod | Negative | Light pink | Motile |
| 2. | $PPFM_2$ | 0.9 x 1.2 | Rod | Negative | Light pink | Motile |
| 3. | $PPFM_3$ | 0.8 x 1.2 | Rod | Negative | Light pink | Motile |
| 4. | $PPFM_4$ | 0.9 x 1.5 | Rod | Negative | Light pink | Motile |
| 5. | $PPFM_5$ | 0.7 x 1.1 | Rod | Negative | Light pink | Motile |
| 6. | $PPFM_6$ | 0.7 x 1.6 | Rod | Negative | Light pink | Motile |
| 7. | $PPFM_7$ | 0.6 x 1.5 | Rod | Negative | Dark Pink | Motile |
| 8. | $PPFM_8$ | 0.6 x 1.3 | Rod | Negative | Dark Pink | Motile |

### 4.2.2 Biochemical Characterization of PPFM Isolates

The isolates' genus was determined using the biochemical assays. The eight isolates underwent a variety of significant biochemical measures. According to tests for oxidase, urease, catalase activity, and citrate utilization, these isolates had favorable results. A small number of isolates also passed the test to produce indole. No isolate tested positive for casein hydrolysis, the MR and VP tests, or starch hydrolysis out of the total isolates.

The same result was noticed by Thangamani (2005), while Hansen et al. (1976) noted that all of the isolates discovered had positive findings for the tests for indole synthesis, oxidase activity, catalase activity, citrate utilization, and urease activity. All of the isolated samples tested negative for the production of $H_2S$, casein or gelatin hydrolysis, cellulose degradation, lipolytic activity, MR and VP tests, and nitrate reduction. Methylobacterium nodulans ORS-2060 and NPFM-OS-03, NPFM-OS-04, and NPFM-Co-01 all tested positive for starch hydrolysis.

**Table 4.3 Biochemical characterization of Methylotrophs**

| Sr. No. | Isolates | Oxidase Test | Urease Test | MR and VP | Casein hydrolysis | Starch hydrolysis | Citrate utilization test | Catalase activity | Indole production test |
|---|---|---|---|---|---|---|---|---|---|
| 1. | $PPFM_1$ | + | + | - | - | - | + | + | + |
| 2. | $PPFM_2$ | + | + | - | - | - | + | + | + |
| 3. | $PPFM_3$ | + | + | - | - | - | + | + | + |
| 4. | $PPFM_4$ | + | + | - | - | - | + | + | - |
| 5. | $PPFM_5$ | + | + | - | - | - | + | + | - |
| 6. | $PPFM_6$ | + | + | - | - | - | + | + | - |
| 7. | $PPFM_7$ | + | + | - | - | + | + | + | - |
| 8. | $PPFM_8$ | + | + | - | - | - | + | + | - |

### 4.2.3 Carbon Source Utilization Test

The PPFMs bacterium may thrive on a wide variety of multicarbon compounds. The isolated organisms are listed in table 4.4 after their use of various carbon compounds was determined. A few isolates in this test showed negative findings when exposed to chloroform and glycerol, while most showed positive results when exposed to glucose, D-galactose, dichloromethane, and other substances.

PPFM can grow on a range of multicarbon substrates in addition to C1 compounds. The findings are comparable to those of Green and Bousifield (1982) and Green (1992).The three effective isolates PPFM1, PPFM2, and PPFM3 were chosen for further research based on the indole synthesis test and growth on multicarbon compounds, among other factors, such as morphological, cultural, and biochemical characteristics.

**Table 4.4 Ability of isolate to use different carbon source**

| Sr. No. | Isolates | Glucose | D-galactose | Dichloro-Methane | Chloroform | Glycerol |
|---|---|---|---|---|---|---|
| 1 | $PPFM_1$ | + | + | + | + | + |
| 2 | $PPFM_2$ | + | + | + | + | + |
| 3 | $PPFM_3$ | + | + | + | + | + |
| 4 | $PPFM_4$ | + | + | + | _ | + |
| 5 | $PPFM_5$ | + | + | + | _ | _ |
| 6 | $PPFM_6$ | + | + | + | + | _ |
| 7 | $PPFM_7$ | + | + | + | _ | + |
| 8 | $PPFM_8$ | + | + | + | _ | _ |

## 4.3 Nitrogen Fixation by Pink Pigmented Facultative Methylotroph Isolates

According to the goal of the current inquiry, the isolates PPFM1, PPFM2, and PPFM3 that were chosen based on the indole synthesis test were further evaluated for their capacity to fix nitrogen. Following PPFM1 (1.097 mg N/g of ingested malate), which had the highest nitrogen fixation, was PPFM2 (0.989 mg N/g of consumed malate). The least amount of nitrogen could be fixed (0.81 mg N/g of ingested malate) in PPFM3.

Fazilah *et al*. (2020) investigated the four Methylobacterium species, including *Methylobacterium aeroletum and Methylobacterium radiotolerance*, that were isolated from palm oil and made a similar observation. The ability of Methylobacterium spp. Isolates to fix nitrogen was assessed using a qualitative approach on two different media. The results showed that all of the isolates were capable of fixing nitrogen.

**Table 4.5 Nitrogen fixation by Pink Pigmented Facultative Methylotroph isolates**

| Sr. No. | Isolates | Nitrogen (mg/g of malate medium) |
|---|---|---|
| 1. | $PPFM_1$ | 1.097 |
| 2. | $PPFM_2$ | 0.989 |
| 3. | $PPFM_3$ | 0.816 |

PPFM1 was chosen for additional investigation based on its great efficiency in fixing nitrogen**.**

## 4.4 Formulation

### 4.4.1 Standardization of Medium

By varying the unique selective media for this isolate listed in Table No. 4.6, the most effective PPFM bacteria were prepared for their mass production as liquid consortia based on their capacity to fix nitrogen. The appropriate medium was chosen by preparing several combinations while taking into account common ingredients, their strength, pH level, etc. It was determined that Medium 4 was the best medium for PPFM development.

**Table 4.6 Formulation of different media for standardization of medium**

| Sr. No. | Ingredients | Medium 1 | Medium 2 | Medium 3 | Medium 4 | Medium 5 |
|---|---|---|---|---|---|---|
| 1. | $K_2HPO_4$ | 0.5 g | 1.5 g | 1 g | 0.7 g | 2 g |
| 2. | $KH_2PO_4$ | 0.5 g | 0.5 g | 0.5 g | 0.54 g | 0.5 g |
| 3. | $MgSO_4.7H_2O$ | 1.5 g | 1.75 g | 2 g | 1.0 g | 2.5 g |
| 4. | $CaCl_2.2H_2O$ | 0.1 g | 0.2 g | 0.3 g | 0.2 g | 0.25 g |
| 5. | $FeSO_4.7H_2O$ | 0.004 g | 0.004 g | 0.004 g | 0.004 g | 0.004 g |
| 6. | $NH_4Cl.7H_2O$ | 0.5 g | 0.5 g | 0.5 g | 0.5 g | 0.5 g |
| 7. | $ZnSO_4.7H_2O$ | 0.1 mg | 0.1 mg | 0.1 mg | 0.1 mg | 0.1 mg |
| 8. | $MnCl_4.4H_2O$ | 0.03 mg | 0.03 mg | 0.03 mg | 0.03 mg | 0.03 mg |
| 9. | $H_3BO_3$ | 0.3 mg | 0.3 mg | 0.3 mg | 0.3 mg | 0.3 mg |
| 10. | $CaCl_2.6H_2O$ | 0.2 mg | 0.2 mg | 0.2 mg | 0.2 mg | 0.2 mg |
| 11. | $CuCl_2.2H_2O$ | 0.01 mg | 0.01 mg | 0.01 mg | 0.01 mg | 0.01 mg |
| 12. | $NiCl_2.6H_2O$ | 0.02 mg | 0.02 mg | 0.02 mg | 0.02 mg | 0.02 mg |
| 13. | $Na_2MoO_4.2H_2O$ | 0.06 mg | 0.06 mg | 0.06 mg | 0.06 mg | 0.06 mg |
| 14. | Agar | 30.0 g | 30.0 g | 30.0 g | 30.0 g | 30.0 g |
| 15. | Distilled water | 1 L | 1 L | 1 L | 1 L | 1 L |
| 16. | pH | 5.5 | 5.7 | 5.9 | 6.2 | 6.5 |
| 17. | Methanol | 0.5 % | 0.5 % | 0.5 % | 0.5 % | 0.5 % |

The outcomes were consistent with Green's recommendations from 1992, who recommended utilising AMS media for the isolation and growth of the PPFM genus from leaf surfaces using the leaf imprint technique. Rinke Kumar *et al*. (2009), Mizuno *et al*., used the specialised medium known as AMS for the isolation of PPFM (2012).

### 4.4.2 Liquid Formulation

The most effective isolate, PPFM1, was selected for the development of a liquid consortium on a suitable standardized medium 4 based on its capacity to fix nitrogen. The liquid medium LM1, LM2, LM3, LM4 and LM5 was created by adding various concentrations of cell protectants such as arabinose, trehalose, glycerol, PVP (Poly- Vinyl-pyrrolidine) and Fe-EDTA in varied concentrations at various pH levels.

A 1% inoculum of PPFM bacteria was added to the flask containing the liquid media after this media had been tested. This flask was incubated on a rotary shaker for 72 hours at 150 rpm. After incubation, a loopful culture was streaked onto a selective medium plate that had been sterilized. All of the plates were incubated at 28 20C to record the observation of PPFM growth. By varying the cfu count on each medium, the medium with the highest cfu count was chosen as the ideal standard liquid methylotrophs consortium media.

On LM4 medium, the PPFM bacteria's profuse cell count was determined and they were selected for consortium preparation. Indistinguishable liquid formulations of diverse microbes were also created by Santosh *et al*. (2015) utilising effective strains chosen for their ability to fix nitrogen. Cell protectants were used in various combinations and at the right concentrations. To this broth during the media preparation, glycerol (0.5%), polyethylene glycol (0.5%), polyvinyl pyrrolidine (PVP) (0.5%), gum Arabic (0.5%), and sodium alginate (0.1%) were all added. The prepared media was inoculated with 1.0 ml of mother culture that had been produced over night and then incubated at a temperature of 28 ±2 ºC in a BOD incubator.

Vedan and Thangaraju (2006) for an Azospirillum formulation, Om Prakash (2010) for rhizobial isolates, and Chandra *et al.* (2012) for a liquid NPK formulation containing trehalose (10 mM), sucrose, PVP (2%) and glycerol (10 mM) in an N-free malic acid broth, as well as PVP, Fe-EDTA, glycerol, mannitol, etc. These bioformulations have a shelf life of 1-2 years due to the use of base materials such emulsifiers, dispersants, cell protectants, and moisturizers**.**

### 4.4.3 Shelf Life of Consortium

For the creation of the liquid consortium, the PPFM1 isolate was chosen based on its ability to fix nitrogen. High density polyethylene (HDPE) bottles that had undergone UV sterilization were used to package the prepared liquid bioformulation. The inoculants that had been created were kept at room temperature. The shelf life of liquid consortium was measured by keeping track of consecutive counts at monthly intervals, and the PPFM bacteria's optimal cell count was finished for 360 days. After storage, the highest cell count was seen 180 days in advance of a gradual decline up to 360 days later. Table 4.8 provided an overview of these findings.

These findings were in line with those of Santosh et al. (2015), who recommended that liquid bioformulations be created and filled into 100 ml UV-sterilized high density polyethylene bottles. Formulated cultures were kept in a BOD incubator at a temperature of 28 ± 2 ºC, and their shelf life was monitored using a standard plate count at monthly intervals for up to 180 days following storage.

Additionally, Chandra (2009) established indistinguishable results. He created a liquid bio-fertilizer and determined an NPK-Liquid formulation of Azotobacter, Azospirillum, phosphate-solubilizing microorganisms, and potash-mobilizing bacteria with a shelf life of roughly 24 months. It was discovered that liquid bio-fertilizers, particularly

liquid Rhizobium with 2% PVP, maintained a higher cfu count. Long shelf life with little contamination, carrier-free activity, handling, storage, and ease of transportation are all made feasible by the liquid formulation. Those that worked on the liquid microbial consortium in 2012 were Pindi and Satyanarayana.

**Table 4.7 Standardization of liquid formulation**

| Sr. No. | Ingredient | $LM_1$ | $LM_2$ | $LM_3$ | $LM_4$ | $LM_5$ |
|---|---|---|---|---|---|---|
| 1. | Std. medium | Medium 4 | Medium 4 | Medium 4 | Medium 4 | Medium 4 |
| 2. | Trehalose | 0.7g | 1.2g | 1.5g | 1.9g | 2.1g |
| 3. | PVP | 2.5g | 5g | 7.5 | 10g | 12.5g |
| 4. | Glycerol | 4ml | 6ml | 8ml | 10 ml | 12ml |
| 5. | Fe-EDTA | 0.05g | 0.07g | 0.09g | 0.13g | 0.11g |
| 6. | Arabinose | 0.2g | 0.4g | 0.6g | 0.8g | 0.10g |
| 7. | Distilled water | 1L | 1L | 1L | 1L | 1L |
| 8. | PH | 6.4 | 6.7 | 6.9 | 7.2 | 7.4 |

**Note :-** LM - Liquid medium

**Table 4.8 Shelf life of PPFM bacterial liquid formulation**

| Month | cfu×$10^9$ per ml of PPFM bacterial formulation |
|---|---|
| $1^{st}$ | 6.98 |
| $2^{nd}$ | 11.56 |
| $3^{rd}$ | 13.33 |
| $4^{th}$ | 15.32 |
| $5^{th}$ | 16.89 |
| $6^{th}$ | 19.02 |
| $7^{th}$ | 13.89 |
| $8^{th}$ | 7.68 |
| $9^{th}$ | 4.23 |
| $10^{th}$ | 1.56 |
| $11^{th}$ | 0.016 |
| $12^{th}$ | 0.07 |

## 4.5 Field Experiment

Studies on Pink Pigmented Facultative Methylotrophs in Tomato (*Lycopersicon esculentum* L.) were conducted in the summer of 2021 to examine the combined effects of PPFMs and Azotobacter on various growth parameters, including plant height, number of leaves, root length, fresh weight, dry weight, number of branches, number of fruits, yield,

nutrient uptake in tomato, and population dynamics of PPFMs recorded. This chapter includes a statistical analysis and discussion of the field trial's specifics.

The results revealed that combined inoculations of Azotobacter and PGPR were superior to single inoculations of Azotobacter in that they greatly enhanced the number of branches, plant height, fresh and dry weight of plants, root length, number of fruits, as well as fruit yield and nutrient uptake. Khan *et al.* (2012) in tomato, Jagadeesh and Uppar (2008) in tomato, and Yadav *et al.* (2011) in papaya established this type of results.

### 4.5.1 Plant Height

The information regarding plant height (cm), which was recorded during the tomato plant's blossoming and harvesting stages, is listed in table 4.9 and illustrated graphically in fig. 1. The data showed that different treatment combinations of PPFM, Azotobacter, and nitrogen dose are to blame for the variation in tomato plant height.

**Table 4.9 Combined effect of seed treatment of Pink Pigmented Facultative Methylotrophs and *Azotobacter* and foliar spray of PPFM bacteria under various nitrogen doses on plant height (cm) at flowering and harvesting stage of Tomato**

| Tr. No. | Treatment | Plant height (cm) | |
|---|---|---|---|
| | | Flowering stage | Harvesting stage |
| $T_1$ | 100% N only without seed treatment of *Azotobacter* and PPFM and foliar spray of PPFM | 54.44 | 69.80 |
| $T_2$ | Seed treatment of *Azotobacter* + PPFM + Foliar spray of PPFM only. | 48.97 | 63.69 |
| $T_3$ | Seed treatment of PPFM + *Azotobacter* + 25 % N + Foliar spray of PPFM | 51.64 | 65.93 |
| $T_4$ | Seed treatment of PPFM + *Azotobacter* + 50% N + Foliar spray of PPFM | 53.06 | 67.60 |
| $T_5$ | Seed treatment of PPFM + *Azotobacter* +75% N + Foliar spray of PPFM | 55.80 | 71.73 |
| $T_6$ | Seed treatment PPFM+ *Azotobacter* + 100% N + Foliar spray of PPFM. | 57.20 | 73.40 |
| $T_7$ | Absolute control | 47.55 | 61.45 |
| | SE m ± | 0.46 | 0.72 |
| | C.D. at 5% | 1.42 | 2.24 |

The tallest plants were produced by treatment T6, which is significantly more successful than other treatments and produces plants that measure 57.20 cm at blooming and 73.40 cm at harvest.

The results from treatments T6 (57.20 cm at flowering and 73.40 cm at harvesting stage) and T5 (55.80 cm at flowering and 71.73 cm at harvesting) were statistically equivalent to those from treatments T1, T4, T3, T2, and T7. The connection produced significant outcomes. The minimal plant height for treatment T7 (absolute control) was found to be 47.55 cm during flowering and 61.45 cm during harvest.

The plant may have grown higher as a result of foliar spraying and seed treatment with PPFM bacteria, according to the findings of the current experiment. The PPFM bacteria, which also serves as a plant growth hormone and PGPR, is responsible for supplying auxin and cytokinin, two essential growth regulators, to plants. Since cytokinin's main job is cell differentiation, all growth parameters show an upward trend in comparison to absolute control. Reddy et al. (2002) reported that integrate inoculation was superior to single inoculation in peanuts, and Jadhav and Dekhane (2014) reported the same for tomatoes. Similar findings in terms of plant height, seed germination, root length, seedling length, seed vigour index, etc. were made by Subhaswaraj et al. (2017) in the tomato plant and by Reddy et al. (2002) in the groundnut plant.

### 4.5.2 Length of Root

The observations regarding to length of root (cm) was noted at flowering and harvesting stage of Tomato are outlined in Table No. 4.10 and graphically presented in Fig. 2. From the observations it was revealed that the variation in length of root of Tomato is because of various treatment combination of PPFM, *Azotobacter* and nitrogen dose.

The maximum length of root was noted in treatment $T_6$ (Seed treatment with PPFM + *Azotobacter* + 100% N + Foliar spray of PPFM) i.e. (14.00 cm at flowering and 22.33 cm at harvesting stage) which is significantly dominant over other treatments.

The results noted by treatment $T_6$ (14.00 cm at flowering and 22.33 cm at harvesting stage) and $T_5$ (14.00 cm at flowering and 22.33 cm at harvesting) were found statistically at par to each other followed by the treatments $T_1$, $T_4$, $T_3$, $T_2$ and $T_7$. The result of interaction were found to be significant. Whereas, smallest height was found at treatment $T_7$ (Absolute Control) i.e. 7.66 cm at flowering and 16.66 cm at harvesting stage).

The various treatment wise result of length of root showed that combined effect *Azotobacter* and PPFM with various nitrogen doses influenced the root length as compared to absolute control. It is advocated that PPFM bacteria produces auxin and cytokinin

Santosh *et al.* (2019) and enhances the plant growth parameters. Similar kind of suggestion was given by Madhaiyan *et al* (2004). Therefore, the given data is matched.

**Table 4.10 Combined effect of seed treatment of Pink Pigmented Facultative Methylotrophs and *Azotobacter* and foliar spray of PPFM bacteria under various nitrogen doses on length of root (cm) at flowering and harvesting stage of Tomato**

| Tr. No. | Treatment | Length of root (cm) | |
|---|---|---|---|
| | | At flowering stage | At harvesting stage |
| $T_1$ | 100% N only without seed treatment of *Azotobacter* and PPFM and foliar spray of PPFM | 12.33 | 22.00 |
| $T_2$ | Seed treatment of *Azotobacter* + PPFM + Foliar spray of PPFM only. | 6.33 | 16.33 |
| $T_3$ | Seed treatment of PPFM + *Azotobacter* + 25 % N + Foliar spray of PPFM | 10.33 | 19.33 |
| $T_4$ | Seed treatment of PPFM + *Azotobacter* + 50% N + Foliar spray of PPFM | 11.66 | 21.00 |
| $T_5$ | Seed treatment of PPFM + *Azotobacter* +75% N + Foliar spray of PPFM | 14.00 | 22.33 |
| $T_6$ | Seed treatment PPFM+ *Azotobacter* + 100% N + Foliar spray of PPFM. | 14.33 | 23.00 |
| $T_7$ | Absolute control | 7.66 | 16.66 |
| | SE m ± | 0.9 | 0.7 |
| | C.D. at 5% | 2.78 | 2.1 |

### 4.5.3 Number of Leaves

The data regarding to number of leaves was recorded at flowering and harvesting stage of tomato are presented in Table No. 4.11 and graphically presented in Fig. 3. From the observations it was observed that the difference in number of leaves in tomato is because of various treatment combination of PPFM, *Azotobacter* and nitrogen dose.

The data noted at flowering and harvesting stage from all the observations the highest number of leaves was found in treatment $T_6$ (Seed treatment with PPFM + *Azotobacter*+ 100% N + Foliar spray of PPFM) i.e. (93.13 leaves plant$^{-1}$ at flowering and 199.60 leaves plant$^{-1}$ at harvesting stage) which is significantly dominant over other treatments. In general treatment $T_6$ was noted maximum number of leaves whereas treatment $T_7$ (Absolute control) was recorded least number of leaves.

The results noted by treatment $T_6$ (93.13 leaves plant$^{-1}$ at flowering and 199.6 leaves plant$^{-1}$ at harvesting stage) and $T_5$ (91.4 leaves per plant at flowering and 196.9

leaves per plant at harvesting) were observed statistically at par to each other followed by the treatments $T_1$, $T_4$, $T_3$, $T_2$ and $T_7$. The result of interaction was found to be significant. Whereas, smallest number of leaves was found at treatment $T_7$ (Absolute Control) i.e. (75.26 leaves per plant at flowering and 181.65 leaves per plant at harvesting stage).

**Table 4.11 Combined effect of seed treatment of Pink Pigmented Facultative Methylotrophs and *Azotobacter* and foliar spray of PPFM bacteria under various nitrogen doses on number of leaves at flowering and harvesting stage of tomato**

| Tr. No. | Treatment | No. of leaves plant$^{-1}$ | |
|---|---|---|---|
| | | At flowering stage | At harvesting stage |
| $T_1$ | 100% N only without seed treatment of *Azotobacter* and PPFM and foliar spray of PPFM | 88.2 | 193.86 |
| $T_2$ | Seed treatment of *Azotobacter* + PPFM + Foliar spray of PPFM only. | 77.86 | 184.71 |
| $T_3$ | Seed treatment of PPFM + *Azotobacter* + 25 % N + Foliar spray of PPFM | 80.13 | 187.76 |
| $T_4$ | Seed treatment of PPFM + *Azotobacter* + 50% N + Foliar spray of PPFM | 85 | 190.81 |
| $T_5$ | Seed treatment of PPFM + *Azotobacter* +75% N + Foliar spray of PPFM | 91.4 | 196.9 |
| $T_6$ | Seed treatment PPFM+ *Azotobacter* + 100% N + Foliar spray of PPFM. | 93.13 | 199.6 |
| $T_7$ | Absolute control | 75.26 | 181.65 |
| | SE m ± | 1.02 | 0.99 |
| | C.D. at 5% | 2.20 | 3.06 |

The result of number of leaves per plant were in conformity with the findings of Radha *et al*(2009) he recorded the observations at 30 DAS and 60 DAS and found that significant increase in number of leaves with integrate inoculation of PPFM with *Rhizobium* in Soybean. Similar kind of suggestion was also given by Sivakumar *et al.* (2018) by foliar spray of PPFM at various concentrations in Tomato induces ability towards drought tolerant and enhances the plant growth parameters. The current results are matched with the earlier results.

### 4.5.4 Fresh Weight of Tomato

The data regarding fresh weight of tomato was done at flowering and harvesting stage of tomato are put up in table 4.12 and graphically presented in fig. 4. From the

observations it was examined that the difference in fresh weight of tomato is because of various treatment combination of PPFM, *Azotobacter* and nitrogen dose.

At flowering and harvesting stage of tomato crop it was observed that when we maximize dose of nitrogen along with seed treatment of PPFM and *Azotobacter* and also the foliar spray of PPFM there was noteworthy enhancement in fresh weight of tomato. The data noted at flowering and harvesting stage among all the observations the maximum fresh weight was found in treatment $T_6$ (Seed treatment with PPFM + *Azotobacter* + 100% N + Foliar spray of PPFM) i.e. (164.66 g plant$^{-1}$at flowering and 247.33 g plant$^{-1}$ at harvesting stage) which is significantly superior over other treatments. In general treatment $T_6$ was noted down maximum fresh weight whereas treatment $T_7$ (Absolute control) was recorded least fresh weight.

**Table 4.12 Combined effect of seed treatment of Pink Pigmented Facultative Methylotrophs and *Azotobacter* and foliar spray of PPFM bacteria under various nitrogen doses on fresh weight of tomato(g plant$^{-1}$) at flowering and harvesting stage of tomato**

| Tr. No. | Treatment | Fresh weight (gm plant$^{-1}$) | |
|---|---|---|---|
| | | At flowering stage | At harvesting stage |
| $T_1$ | 100% N only without seed treatment of *Azotobacter* and PPFM and foliar spray of PPFM | 162.33 | 243.33 |
| $T_2$ | Seed treatment of *Azotobacter* + PPFM + Foliar spray of PPFM only. | 148.00 | 207.00 |
| $T_3$ | Seed treatment of PPFM + *Azotobacter* + 25 % N + Foliar spray of PPFM | 155.33 | 235.66 |
| $T_4$ | Seed treatment of PPFM + *Azotobacter* + 50% N + Foliar spray of PPFM | 157.33 | 238.00 |
| $T_5$ | Seed treatment of PPFM + *Azotobacter* +75% N + Foliar spray of PPFM | 163.6 | 245.66 |
| $T_6$ | Seed treatment PPFM+ *Azotobacter* + 100% N + Foliar spray of PPFM. | 164.66 | 247.33 |
| $T_7$ | Absolute control | 143.33 | 204.66 |
| | SE m ± | 2.15 | 2.0 |
| | C.D. at 5% | 6.6 | 6.2 |

The results noted by treatment $T_6$ (164.66 g plant$^{-1}$ at flowering and 247.33 g plant$^{-1}$ at harvesting stage) and $T_5$ (163.6 g plant$^{-1}$ at flowering and 245.66 g plant$^{-1}$ at harvesting) were found statistically at par to each other followed by the treatments $T_{1,}$ $T_{4,}$ $T_{3,}$ $T_2$ and $T_{7.}$ The result of interaction was found to be significant. Whereas, smallest fresh

weight was found at treatment $T_7$ (absolute Control) i.e. (143.33 g plant$^{-1}$ at flowering and 204.66 g plant$^{-1}$ at harvesting stage).

The result of fresh weight of tomato were also in conformity with the result obtained by Madhaiyan *et al.*(2005) that foliar spray of PPFM enhances the fresh weight of plants significantly in Cotton plants. Also, the results were found in conformity with Reddy *et al*. (2002) he studied the integrated effect of PPFM and *Rhizobium* on Groundnut and recorded that increase in plant development, biomass production and yield of Groundnut. So the results are matched with earlier findings.

### 4.5.5 Number of Branches per Plant

The data regarding to number of branches per plant in tomato was examined at flowering and harvesting stage of tomato are outlined in table 4.14 and graphically presented in Fig. 6. From the observations recorded it was examined that the variation in number of branches in tomato is due to various treatment combination of PPFM, *Azotobacter* and nitrogen dose.

**Table 4.14 Combined effect of seed treatment of Pink Pigmented Facultative Methylotrophs and *Azotobacter* and foliar spray of PPFM bacteria under various nitrogen doses on number of branches per plant in tomato at flowering and harvesting stage of tomato**

| Tr. No. | Treatment | No. of branches plant$^{-1}$ | |
|---|---|---|---|
| | | At flowering stage | At harvesting stage |
| $T_1$ | 100% N only without seed treatment of *Azotobacter* and PPFM and foliar spray of PPFM | 6.33 | 10.00 |
| $T_2$ | Seed treatment of *Azotobacter* + PPFM + Foliar spray of PPFM only. | 4.00 | 7.00 |
| $T_3$ | Seed treatment of PPFM + *Azotobacter* + 25 % N + Foliar spray of PPFM | 4.66 | 7.66 |
| $T_4$ | Seed treatment of PPFM + *Azotobacter* + 50% N + Foliar spray of PPFM | 6.33 | 8.66 |
| $T_5$ | Seed treatment of PPFM + *Azotobacter* +75% N + Foliar spray of PPFM | 7.33 | 11.33 |
| $T_6$ | Seed treatment PPFM+ *Azotobacter* + 100% N + Foliar spray of PPFM. | 7.66 | 13.33 |
| $T_7$ | Absolute control | 4.00 | 7.00 |
| | SE m ± | 0.9 | 1.4 |
| | C.D. at 5% | 2.8 | 4.3 |

The maximum number of branches was found in treatment $T_6$ (Seed treatment with PPFM + *Azotobacter* + 100% N + Foliar spray of PPFM) i.e. (7.66 branches plant$^{-1}$ at

flowering and 13.33 branches plant$^{-1}$ at harvesting stage) which was significantly dominant over other treatments. Generally, it was examined that the treatment $T_6$ was found higher number of branches and treatment $T_7$ was found least number of branches this effect was due to various treatment combination of PPFM and *Azotobacter* under various nitrogen doses.

The results noted by treatment $T_6$ (7.66 branches plant$^{-1}$at flowering and 13.33 branches plant$^{-1}$ at harvesting stage) and $T_5$ (7.33 branches plant$^{-1}$ at flowering and 11.33 branches plant$^{-1}$ at harvesting stage of plant growth) were found statistically at par to each other which were significantly dominant over treatments $T_1$, $T_4$, $T_3$, $T_2$ and $T_7$. The result of interaction was found to be significant. Whereas lesser number of branches plant$^{-1}$ was found at treatment $T_7$ (Absolute Control) i.e. (4.00 branches plant$^{-1}$at flowering and 7.00 branches plant$^{-1}$ at harvesting stage of plant growth).

The result obtained for number of branches plant$^{-1}$ were in agreement with the similar result founded by Khan *et al.* (2012) in tomato. The results are also found in conformity with Kanitkar *et al*. (2020) who was found that integrated inoculation of *Azotobacter* with other favourable biological agent enhances the different growth parameters in tomato as an example number of branches per plant, plant height, number of fruits per plant and fruit yield etc.

### 4.5.6 Number of Fruits per Plant

The data regarding to number of fruits per plant in tomato was recorded at harvesting stage of tomato are outlined in table 4.15 and graphically presented in fig. 7. From the observation recorded it was observed that the variation in number of fruits plant$^{-1}$ of tomato was due to difference in treatment combination of PPFM, *Azotobacter* and various nitrogen doses.

At harvesting stage of tomato crop it was inspected that when we maximize dose of nitrogen along with seed treatment of PPFM and *Azotobacter* and also the foliar spray of PPFM there was noteworthy enhancement in number of fruits per plants in tomato . The data noted at flowering and harvesting stage. Among all the treatments the highest number of fruits was found in treatment $T_6$ (Seed treatment with PPFM + *Azotobacter* + 100% N + Foliar spray of PPFM) i.e. (48.66) which was significantly superior over other treatments. In general treatment $T_6$ was noted down maximum number of fruits per plant whereas treatment $T_7$ (Absolute control) was recorded least number of fruits per plant.

**Table 4.15 Combined effect of seed treatment of Pink Pigmented Facultative Methylotrophs and *Azotobacter* and foliar spray of PPFM bacteria under various nitrogen doses on number of fruits per plant in tomato at harvesting stage of tomato**

| Tr. No. | Treatments | No of fruits plant$^{-1}$ |
|---|---|---|
| $T_1$ | 100% N only without seed treatment of *Azotobacter* and PPFM and foliar spray of PPFM | 46.33 |
| $T_2$ | Seed treatment of *Azotobacter* + PPFM + Foliar spray of PPFM only. | 36.00 |
| $T_3$ | Seed treatment of PPFM + *Azotobacter* + 25 % N + Foliar spray of PPFM | 41.66 |
| $T_4$ | Seed treatment of PPFM + *Azotobacter* + 50% N + Foliar spray of PPFM | 45.66 |
| $T_5$ | Seed treatment of PPFM + *Azotobacter* +75% N + Foliar spray of PPFM | 47.33 |
| $T_6$ | Seed treatment PPFM+ *Azotobacter* + 100% N + Foliar spray of PPFM. | 48.66 |
| $T_7$ | Absolute control | 35.66 |
| | SE m ± | 1.9 |
| | C.D. at 5% | 5.9 |

The results noted by treatment $T_6$ (48.66) and $T_5$ (47.33) were found statistically at par to each other followed by the treatments $T_1$, $T_4$, $T_3$, $T_2$ and $T_7$. The results of interaction were found to be significant. Whereas, smallest fresh weight was found at treatment $T_7$ (Absolute Control) i.e. (35.66).

The results found accordance with the result obtained by Kanitkar *et al.* (2020) and also found correspondence result with Nanthakumar and Veeraragavatham (1999) in brinjal and by Trimurtulu *et al*. (2011) in tomato.

### 4.5.7 Yield

The observations noted for yield of tomato per plot and calculated for one hectare are reported in table 4.16 and graphically presented in fig.8. From the observations noted it was examined that the variation in yield of tomato was due to various treatment combination of PPFM, *Azotobacter* and various nitrogen dose.

Among all the data the maximum yield was reported in treatment $T_6$ (Seed treatment with PPFM + *Azotobacter* + 100% N + Foliar spray of PPFM) i.e. (93.44 q ha$^{-1}$) which is significantly dominant over other treatments. In general treatment $T_6$ was noted down maximum yield whereas treatment $T_7$ (Absolute control) was recorded lesser yield.

The results noted by treatment $T_6$ (92.00 q $ha^{-1}$) and $T_5$ (90.66 q $ha^{-1}$) were found statistically at par to each other followed by the treatments $T_1$ (88.66 q $ha^{-1}$), $T_4$ (87.33 q $ha^{-1}$), $T_3$ (83.00 q $ha^{-1}$), $T_2$ (80.33q $ha^{-1}$) and $T_7$ (75.00 q $ha^{-1}$). The results of interaction were found to be significant. Whereas, minimum yield was found at treatment $T_7$ (Absolute Control) i.e. (75.00 q $ha^{-1}$).

**Table 4.16 Combined effect of seed treatment of Pink Pigmented Facultative Methylotrophs and *Azotobacter* and foliar spray of PPFM bacteria under various nitrogen doses on yield in tomato**

| Tr. No. | Treatments | Yield (q $ha^{-1}$) | % increase over control |
|---|---|---|---|
| $T_1$ | 100% N only without seed treatment of *Azotobacter* and PPFM and foliar spray of PPFM | 88.66 | 15.90 |
| $T_2$ | Seed treatmemt of *Azotobacter* + PPFM + Foliar spray of PPFM only. | 80.33 | 4.72 |
| $T_3$ | Seed treatment of PPFM + *Azotobacter* + 25 % N + Foliar spray of PPFM | 83.00 | 9.45 |
| $T_4$ | Seed treatment of PPFM + *Azotobacter* + 50% N + Foliar spray of PPFM | 87.33 | 14.24 |
| $T_5$ | Seed treatment of PPFM + *Azotobacter* +75% N + Foliar spray of PPFM | 90.66 | 17.80 |
| $T_6$ | Seed treatment PPFM+ *Azotobacter* + 100% N + Foliar spray of PPFM. | 92.00 | 19.33 |
| $T_7$ | Absolute control | 75.00 | 0 |
| | SE m ± | 1.3 | - |
| | C.D. at 5% | 5.9 | - |

Enhancement in yield characters due to combined inoculation of PGPRs as advocated by Patil *et al.* (1992), Belimov *et al.* (1995), Alagwadi and Gaur, (1988) and Pratibha *et al.* (1995). Also similar results were obtained by Santosh *etal*. (2019) that native PPFM also produces yield attributing properties and enhances the yield of tomato crop. The given results was in conformity with Nagaraju and Mohankumar (2010).Correspondence result were also advocated by Sivakumar *et al*. (2018) that Pink Pigmented Facultative Methylotrophs increases the yield in Tomato under drought condition.

This results are similar to the beneficial aspects of Pink Pigmented *Methylobacterium* sp. as a potent bio-fertilizer for increasing crop production in soybean (Long, 2000), maize, blackgram, groundnut, sugarcane, rice, cotton, sunhemp (Madhaiyan, 2002), tomato (Anu Rajan, 2003 and Thangamani and Sundaram, 2005).

### 4.5.8 Nutrient Uptake in Tomato

Nutrient uptake in tomato remarkably varied because of different treatment of Azotobacter, PPFM and various nitrogen doses. The observations regarding to total nitrogen, phosphorous and potassium uptake kg $ha^{-1}$ was outlined in table 4.17 and figure 9, 10 and 11 respectively. Total nitrogen, phosphorous and potassium uptake was recorded at harvesting stage of tomato crop by standard protocol and data obtained are as below.

**a) Nitrogen uptake**

The maximum nitrogen uptake was recorded at treatment $T_6$ (Seed treatment with PPFM + Azotobacter + 100% N + Foliar spray of PPFM) was 85.66 kg $ha^{-1}$ was statistically at par to treatment $T_5$ i.e. (85.66 kg $ha^{-1}$) followed by treatment $T_1$ (84.33 kg $ha^{-1}$), $T_4$ (82.33 kg $ha^{-1}$), $T_3$ (79.66 kg $ha^{-1}$), $T_2$ (76.00 kg $ha^{-1}$) and $T_7$ (69.33 kg $ha^{-1}$). The lowest nitrogen uptake was noted in treatment $T_7$ (Absolute control) i.e. 69.33 kg $ha^{-1}$. Treatment $T_6$ was found dominant over other treatments.

**b) Phosphorous uptake**

The Phosphorous uptake in tomato was differed due to various treatment combination of PPFM, *Azotobacter* under graded doses of nitrogen. The data of phosphorous uptake of tomato was outlined in table 4.17.

The highest P uptake was obtained at treatment $T_6$ (Seed treatment with PPFM + *Azotobacter* + 100% N + Foliar spray of PPFM) was (46.66 kg $ha^{-1}$) was statistically at par to treatment $T_5$ i.e. (43.33 kg $ha^{-1}$) followed by treatment $T_1$ (40.66 kg $ha^{-1}$), $T_4$ (39.00 kg $ha^{-1}$), $T_3$ (37.33 kg $ha^{-1}$), $T_2$ (33.33 kg $ha^{-1}$) and $T_7$ (30.33 kg $ha^{-1}$). The lowest phosphorous uptake was noted in treatment $T_7$ (Absolute control) i.e. 30.33 kg $ha^{-1}$.Treatment $T_6$ (46.66 kg $ha^{-1}$) was found dominant over treatment $T_7$ (30.33 kg $ha^{-1}$).

**c) Potassium uptake**

The effect of seed treatment of PPFM, *Azotobacter* along with foliar spray of PPFM with various doses of nitrogen in tomato had impact on potassium uptake also it showed variation with different treatment combination. The result depicted in Table 4.17 reported that the combined effect of seed treatment of PPFM, *Azotobacter* along with 100% nitrogen dose including foliar spray of PPFM gave significantly higher potassium uptake over other treatments.

The highest K uptake was recorded at treatment $T_6$ (Seed treatment with PPFM + *Azotobacter*+ 100% N + Foliar spray of PPFM) was (52.00 kg $ha^{-1}$) was statistically at par to treatment $T_5$ i.e. (47.66 kg $ha^{-1}$) followed by treatment $T_1$ (45.66 kg $ha^{-1}$), $T_4$ (45.33 kg $ha^{-1}$), $T_3$ (43.00 kg $ha^{-1}$), $T_2$ (41.33 kg $ha^{-1}$) and $T_7$ (39.33 kg $ha^{-1}$). The least potassium

uptake was noted in treatment $T_7$ (Absolute control) i.e. 39.33 kg $ha^{-1}$.Treatment $T_6$ (52.00 kg $ha^{-1}$) was found dominant over treatment$T_7$ (39.33 kg $ha^{-1}$).

The enhancement in nutrient uptake in tomato by various treatment combination of PPFM, *Azotobacter* and foliar spray of PPFM with graded doses of nitrogen were also documented by several workers earlier such as Dhopavakar *etal.*(2021) who shown that effect of different biofertilizers and various level of N and P fertilizers and evaluated the consumption of nutrients and found that group inoculation achieve better than individual inoculation in tomato (*Lycopersicon esculentum*).

**Table 4.17 Combined effect of seed treatment of Pink Pigmented Facultative Methylotrophs and *Azotobacter* and foliar spray of PPFM bacteria under various nitrogen doses on nutrient uptake in tomato**

| Tr. No. | Treatments | Nutrient uptake (kg $ha^{-1}$) | | |
|---|---|---|---|---|
| | | N | P | K |
| $T_1$ | 100% N only without seed treatment of *Azotobacter* and PPFM and foliar spray of PPFM. | 84.33 | 40.66 | 45.66 |
| $T_2$ | Seed treatmemt of *Azotobacter* + PPFM + Foliar spray of PPFM only. | 76.00 | 33.33 | 41.33 |
| $T_3$ | Seed treatment of PPFM + *Azotobacter* + 25 % N + Foliar spray of PPFM. | 79.66 | 37.33 | 43.00 |
| $T_4$ | Seed treatment of PPFM + *Azotobacter* + 50% N + Foliar spray of PPFM. | 82.33 | 39.00 | 45.33 |
| $T_5$ | Seed treatment of PPFM + *Azotobacter* +75% N + Foliar spray of PPFM. | 85.66 | 43.33 | 47.66 |
| $T_6$ | Seed treatment PPFM+ *Azotobacter* + 100% N + Foliar spray of PPFM. | 89.33 | 46.66 | 52.00 |
| $T_7$ | Absolute control. | 69.33 | 30.33 | 39.33 |
| | SE m ± | 1.8 | 1.8 | 1.7 |
| | C.D. at 5% | 5.5 | 5.5 | 5.3 |

The result of nutrient uptake was also found in conformity with Patil and Narayana (2017) that they documented that various treatment combination of *Azotobacter* and *Azospirillum* along with other biofertilizers including different level of N, P, K and recorded the nutrient uptake in Gherkin (*Cucumis anguria* L.). So, the outcomes recorded for nutrient uptake are matched with the previous results.

### 4.5.10 Population Count of PPFM

The bacterial count of Pink Pigmented Facultative Methylotrophs on phyllosphere and rhizosphere was calculated at two different growth stages of tomato first at flowering and second at harvesting stage. It was outlined in table 4.18 and depicted in fig. 12 and 13

respectively. The consequences noted depicted that the bacterial population were found abundant in phyllosphere as compared to rhizosphere. It was recorded that at flowering stage observed greater number of bacteria as contrast to harvesting stage. Bacterial population was estimated by standard dilution and plating method.

**a) Population Count of PPFM at Phyllosphere**

The bacterial population of Pink Pigmented Facultative Methylotroph at phyllosphere was noted at flowering and harvesting stage of tomato by the standard dilution and plating method. The observations recorded are highlighted in table 4.18 and in fig. 12.

The population of *Methylobacterium* at phyllospheric region was significantly influenced by inoculation of *Methylobacterium*. The phyllospheric region found maximum number of bacterial population than rhizospheric region. The maximum microbial population was recorded in treatment T6 (Seed treatment with *Azotobacter* and PPFM +100% N + Foliar spray of PPFM) i.e $10^6.00 \times 10^6$ cfu $g^{-1}$ of leaf fresh weight at flowering and 40.00 x $10^6$ cfu $g^{-1}$ of leaf fresh weight at harvesting stage it was followed by treatments $T_5$, $T_1$, $T_4$, $T_3$, $T_2$ and $T_7$. And minimum bacterial population was examined in treatment $T_7$ (Absolute control) i.e. 97.21 $\times 10^6$ cfu $g^{-1}$ of leaf fresh weight at flowering and 34.86 x $10^6$ cfu $g^{-1}$ of leaf fresh weight at harvesting stage.

From the result obtained it was clearly pointed out that the population of *Methylobacterium* was found in greater amount at flowering stage as compared to harvesting stage.

**b) Population Count of PPFM at Rhizosphere**

The bacterial population of Pink Pigmented Facultative Methylotroph in rhizospheric region was recorded at flowering and harvesting stage of tomato by the standard dilution and plating method. The data acquired is noted down in Table 4.18 and in Fig. 13.

The population of *Methylobacterium* at rhizospheric region was significantly influenced by *Methylobacterium* inoculation. The maximum microbial population was founded in treatment T6 (Seed treatment with *Azotobacter* and PPFM +100% N + Foliar spray of PPFM) i.e. 39.22 x $10^3$ cfu $g^{-1}$ dry weight of soil and 13.98 $\times$ $10^3$ cfu $g^{-1}$ at harvesting stage. Followed by treatments $T_5$, $T_1$, $T_4$, $T_3$, $T_2$ and least bacterial population was reported in treatment $T_7$ (Absolute control) i.e., 30.22 x $10^3$ cfu $g^{-1}$of dry weight of soil at flowering and 10.48 x $10^3$ cfu $g^{-1}$ dry weight of soil at harvesting stage.

The population count of PPFM bacteria in phyllosphere and rhizosphere was examined at flowering and harvesting stage. The highest bacterial population was noted by treatment $T_6$ (Seed treatment with and PPFM and *Azotobacter* + 100% N + Foliar spray of PPFM.) was highest. Whereas, population count of uninoculated absolute control treatment was least. Also, maximum population was recorded at flowering stage than harvesting stage.

In the current investigation the phyllosphere spraying of PPFM significantly influenced the phyllosphere and rhizosphere *Methylobacterium* population. This was mostly due to growth hormone production by *Methylobacterium sp*. mainly greater cytokinin formation in apical plan t tissues and rhizosphere soil. The similar investigation was also recorded by Raghavendra *et al.* (2019) phyllosphere spraying of PPFMs remarkably persuaded the *Methylobacterium* population in the phyllosphere of direct seeded rice crop by producing phytohormones and enhances plant growth parameters also.

**Table 4.18 Combined effect of seed treatment of Pink Pigmented Facultative Methylotrophs and *Azotobacter* and foliar spray of PPFM bacteria under various nitrogen doses on population count of PPFM in tomato**

| Tr. No. | Treatment | Phyllosphere (x $10^6$ cfu $g^{-1}$ fresh weight of leaf ) | | Rhizosphere (x $10^3$ cfu $g^{-1}$ fresh weight of Soil ) | |
|---|---|---|---|---|---|
| | | Flowering Stage | Harvesting stage | Flowering stage | Harvesting stage |
| $T_1$ | 100% N only without seed treatment of *Azotobacter* and PPFM and foliar spray of PPFM | 82.33 | 17.99 | 19.87 | 7.23 |
| $T_2$ | Seed treatment of *Azotobacter*+ PPFM + Foliar spray of PPFM only. | 84.23 | 24.00 | 23.23 | 9.32 |
| $T_3$ | Seed treatment of PPFM + *Azotobacter* + 25 % N + Foliar spray of PPFM | 91.22 | 26.97 | 26.33 | 9.86 |
| $T_4$ | Seed treatment of PPFM + *Azotobacter*+ 50% N + Foliar spray of PPFM | 94.26 | 28.00 | 28.97 | 11.21 |
| $T_5$ | Seed treatment of PPFM + *Azotobacter* +75% N + Foliar spray of PPFM | 102.33 | 35.38 | 34.98 | 13.25 |
| $T_6$ | Seed treatment PPFM+ *Azotobacter* + 100% N + Foliar spray of PPFM. | 106.00 | 40.00 | 39.22 | 13.98 |
| $T_7$ | Absolute control | 97.21 | 34.86 | 30.22 | 10.48 |

# 5. SUMMARY AND CONCLUSION

The current study entitled “Studies on Pink Pigmented Facultative Methylotrophs in tomato (*Lycopersicon esculentum*.),” was conducted in laboratory as well as a field experiment during the summer season of (2022) at the Department of Plant Pathology and Agricultural Microbiology, Post Graduate Institute Mahatma Phule Krishi Vidyapeeth, Rahuri, with the goal of isolating, characterizing and studying their effect on growth characteristics of tomato under field condition.

The samples of leaves of different crops such as tomato, chilli, brinjal, and potato etc. were collected from different region of M.P.K.V., Rahuri and nearby villages of Rahuri Tehsil. After collection of samples the PPFM bacteria were isolated from different crops on AMS agar media by leaf imprinting technique and cultured on AMS agar media with methanol as the sole carbon and energy source.

Morphologically the cells of PPFM were found be rod shaped, motile and gram reaction was found to be negative for all isolates. Cell size of bacteria was recorded in the range of 0.6×1.3µm to 0.9×1.5µm dimension and shows light pink to dark pink in color. Biochemical test was carried out to identify the PPFM bacteria. All the isolates shown positive result towards oxidase test, urease test, catalase activity and citrate utilization test. On the basis of result obtained from various morphological and biochemical parameters it was confirmed that isolates bacteria were Pink Pigmented Facultative Methylotrophic bacteria. As per the result of indole production test only three isolates i.e., $PPFM_{1,}$ $PPFM_2$ and $PPFM_3$ were chosen for further analysis.

The three isolates chosen as per the result of indole production test i.e. $PPFM_{1,}$ $PPFM_2$ and $PPFM_3$were analyzed for nitrogen fixation ability by using N- free malate medium. The maximum nitrogen fixation was recorded by $PPFM_1$ isolate i. e. 1.097 mg N $g^{-1}$ of malate medium. It was found that PPFM bacteria have inferior ability towards nitrogen fixation.

For preparation of liquid consortia of PPFM media was standardized as per the procedure on the basis of characteristics pink pigmentation medium 4 was observed most suitable medium for PPFM growth. On the basis of nitrogen fixation capacity, the most efficient isolate that is $PPFM_1$ was chosen for the preparation of liquid consortium on suitable standardized medium 4 as per protocol. For preparation of liquid consortia at different pH various cell protectant such as arabinose, trehalose, glycerol, PVP and

Fe-EDTA and prepared liquid medium $LM_1$, $LM_2$, $LM_3$, $LM_4$ and $LM_5$. The maximum cell count was noted on $LM_4$ medium and chosen for consortium preparation.

After formulation of liquid consortia, the shelf of PPFM formulation stored in UV sterilized high density polyethylene (HDPE) bottles were investigated for shelf life of consortium. Cell count was taken for monthly duration consecutively for upto 360 days. The maximum cell count was recorded at 180 days after storage and thereafter gently decreases up to 360 days.

The field trial was carried out in summer (2021) to assess the combined effect of PPFM and *Azotobacter* and various treatment combination on various plant growth parameters in Chilli like plant height, number of leaves, root length, fresh weight and dry weight, number of branches, number of fruits, yield, nutrient uptake in chilli and population dynamics of PPFMs recorded at flowering and harvesting stage under graded level of nitrogen in field condition.

Due to various combination of treatment of PPFM along with *Azotobacter* under various level of nitrogen all growth parameters of chilli were influenced by the treatment $T_6$ (Seed treatment of PPFM + *Azotobacter* + 100% N + Foliar spray of PPFM) which was significantly dominant over rest of the treatments and it was at par with treatment $T_5$ (Seed treatment of PPFM + *Azotobacter* + 75% N + Foliar spray of PPFM)

Total nutrient uptake were also influenced by by application of treatment $T_6$ (Seed treatment of PPFM + Azotobacter + 100% N + Foliar spray of PPFM) which was significantly superior over other treatments and found at par with treatment $T_5$ (Seed treatment of PPFM + *Azotobacter* + 75% N + Foliar spray of PPFM). The treatment $T_7$ absolute control was noted least nutrient uptake.

The treatment $T_6$ (Seed treatment of PPFM + Azotobacter + 100% N + Foliar spray of PPFM) was recorded maximum yield i.e. 93.44 q $ha^{-1}$ and found almost at par with treatment $T_5$ (Seed treatment of PPFM + *Azotobacter*+ 75% N + Foliar spray of PPFM) i.e. 92.24 q $ha^{-1}$. The treatment $T_7$ (absolute control) noted the least treatment i.e. 78.30 q $ha^{-1}$. From the result, there is a possibility of saving nitrogen fertilizer to an extent of 25 per cent.

At flowering and harvesting stage of plant growth the population count of PPFM was recorded from *phyllosphere* and *rhizosphere.* Population count of PPFM found significantly higher in treatment $T_6$ (Seed treatment of PPFM + Azotobacter + 100% N + Foliar spray of PPFM) and it was at par with treatment $T_5$ (Seed treatment of PPFM +

*Azotobacter* + 75% N + Foliar spray of PPFM). The uninoculated treatment noted the least PPFMs population.

**Conclusion:**

1. The combined effect of seed treatment of PPFM and Azotobacter along with foliar spray of PPFM and various level of nitrogen doses found significantly superior results in various plant growth parameters such as plant height, length of root, number of leaves, fresh weight and dry weight, number of branches, number of fruits, yield and nutrient uptake.
2. The treatment $T_6$ (Seed treatment of PPFM + Azotobacter + 100% N + Foliar spray of PPFM) recorded maximum yield i.e. 93.44 q $ha^{-1}$ and found almost at par with treatment $T_5$ (Seed treatment of PPFM + *Azotobacter* + 75% N + Foliar spray of PPFM) i.e. 92.24 q $ha^{-1}$. The treatment $T_7$ (absolute control) noted the least treatment i.e. 78.30 q $ha^{-1}$. From the result, there is a possibility of saving nitrogen fertilizer to an extent of 25 per cent.
3. In future PPFM bacteria can be mostly used for drought tolerance mitigation and biological measures due to its ability to produce phytohormones under stress condition due to their characteristic's pink pigmentation.

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# 7. APPENDIX

**Appendix-I: Composition of different growth media/indicator/reagent used**

| **Ammonium mineral salt medium (AMS) (Whittenbury *et al*., 1970)** | |
|---|---|
| $NH_4Cl$ | 0.50 g |
| $K_2HPO_4$ | 0.70 g |
| $KH_2PO_4$ | 0.54 g |
| $MgSO_4.7H_2O$ | 1.00 g |
| $CaCl_2.2H_2O$ | 0.20 g |
| $FeSO_4.7H_2O$ | 4.00 µg |
| $ZnSO_4.7H_2O$ | 100 µg |
| $MnCl_4.4H_2O$ | 30.0µg |
| $H_3BO_3$ | 300.0 µg |
| $CoCl_2.6H_2O$ | 200 µg |
| $CuCl_2.2H_2O$ | 10.0 µg |
| $NiCl_2.6H_2O$ | 20 µg |
| $Na_2MoO_4.2H_2O$ | 60.0 µg |
| Agar | 15.0 g |
| Distilled water | 1000 ml |
| pH | 6.8 |
| Methanol | 0.5 % |
| **N-free malate medium** | |
| Malic acid | 5.0 g |
| Dipotassium hydrogen orthophosphate | 0.5 g |
| Magnesium sulphate | 0.2 g |
| Sodium chloride | 0.1 g |
| Calcium Chloride | 2.0 g |
| Fe- EDTA (1.64% w/v aqueous) | 4.0 ml |
| Trace element solution | 2.0 ml |
| Bromothymol blue (0.5% alcoholic solution) | 2.0 ml |
| Vitamin solution | 1.0 ml |
| Potassium hydroxide | 4.0 g |
| Agar | 1.75 g |
| Distilled water | 1000 ml |
| pH | 6.8 |
| **Vitamin solution** | |
| Biotin | 10.0 mg |
| Pyridoxin | 20.0 mg |
| Distilled water | 1000 ml |
| **Simmon's citrate agar** | |
| Sodium citrate | 0.2 g |
| Magnesium sulphate | 0.02 g |
| Ammonium dihydrogen phosphate | 0.1 g |
| Dipotassium hydrogen phosphate | 0.1 g |
| Sodium chloride | 0.5 g |

| | |
|---|---|
| Bromothymol blue | 0.008 g |
| Agar | 2.0 g |
| Distilled water | 100.0 ml |
| pH | 6.8 |
| **Trypticase soy agar medium (Seeley *et al.*,1991)** | |
| Tryptone | 17.0 g |
| Soyatone | 3.0 g |
| Glucose | 2.50 g |
| Agar | 18.0 g |
| Distilled water | 1000 ml |
| Skim milk agar | |
| Skim milk powder | 100 g |
| Peptone | 5.0 g |
| Agar | 15.0 g |
| pH | 7.2 |

# 8. VITAE

**MISS. NAKADE SUCHITA SHASHIKANT**

Candidate for the degree of

**MASTER OF SCIENCE (AGRICULTURE)**

**IN**

**AGRICULTURAL MICROBIOLOGY**

**2023**

| | | | |
|---|---|---|---|
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