

Natural Occurrence of Pink Pigmented Facultative Methylophilic (PPFM) Bacteria on Phyllosphere of Mulberry (*Morus Indica*)

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Abstract:- Pink Pigmented Facultative Methylophilic (PPFM) Bacteria Of The Genus *Methylobacterium* Are Commonly Found In Association With Plants. These Bacteria Are Characterized By Being Able To Rely On Methanol As A Sole Carbon And Energy Source. The Ppfms Are Highly Hydrophobic And Strongly Adhesive On Plant Surfaces And Has The Ability To Produce Cytokinin And Indole-3 Acetic Acid On The Phyllosphere Region Of Mulberry. In Mulberry (*Morus Indica*) Variety MR2, The Natural Population Of PPFM Occupied Approximately 10 Per Cent Of Total Aerobic Bacteria On Phyllosphere. The Serial Dilution Method Recorded Higher Population Than Leaf Imprinting Method. The PPFM Isolate *Methylobacterium* Spp. Mi7 Shown Good Growth Rate Under *In Vitro* Conditions In Ammonium Mineral Salt Broth. The Culture Entered Into Stationary Phase After 10 Days With One Per Cent Initial Methanol. The Natural Population Of PPFM Varied During The Periods Of September To December And January To April On Mulberry Leaves. Heat And Water Stress During Summer Decreased The Natural Ppfms.

Keywords:- PPFM, *Methylobacterium*, *Phyllosphere Bacteria*, *Mulberry Plant*.

I. INTRODUCTION

Mulberry (*Morus* Spp) Is A Fast Growing Deciduous Wood And Perennial Plant. Mulberry Foliage Is The Only Food For The Silkworm (*Bombyx Mori*) And Is Grown Under Varied Climatic Conditions Ranging From Temperate To Tropical. Mulberry Leaf Is A Major Economic Component In Sericulture Since The Quality And Quantity Of Leaf Produced Per Unit Area Have A Direct Bearing On Silk Production. India Is The Second Largest Producer Of Mulberry Raw Silk (28708 Metric Ton), Next To China Accounting More Than 15 Per Cent Global Raw Silk Production. In Our Country, Favorable Climatic Condition For Mulberry Cultivation Prevails In Five States, *Viz.*, Karnataka, Andhra Pradesh, Tamil Nadu, West Bengal, Jammu Kashmir. These States Occupy 97 Per Cent Of Total Mulberry Cultivation And Contribute 95 Per Cent Of Raw Silk Production In India. Tamil Nadu Is Ranked Fourth Place Among The Silk Producing States Of Our Country. The Annual Silk Production In Tamil Nadu Is Around 1200 MT. The Total Area Under Mulberry Cultivation In Tamil Nadu Is Arounds 9491 Acre (Anonymous, 2015). In Tamil Nadu Major Mulberry Cultivating Regions Are Krishnagiri, Dharmapuri, Erode, Thirupur, Coimbatore, Namakkal, Selam, Thirunelveli And

Theni. In Major Mulberry Cultivating Area Experience Reduce Leaf Yield And Low Quality Leaf During The Summer Season Due To Water And Heat Stress.

Current Soil Management Strategies Are Mainly Dependent On Inorganic Chemical- Based Fertilizers, Which Caused A Serious Threat To Human Health And Environment. The Exploitation Of Beneficial Microbes As A Biofertilizer Has Become Paramount Importance In Agriculture Sector For Their Potential Role In Food Safety And Sustainable Crop Production. The Eco- Friendly Approaches Inspire A Wide Range Of Application Of Useful Microscopic Organisms Led To Improved Nutrient Uptake, Plant Growth And Plant Tolerance To Various Abiotic Stresses (Bhardwaj *Et Al.*, 2014). In Recent Years The Use Of Methylophilic Bacteria That Have The Ability To Colonize Different Habitats, Has Been Suggested For Sustainable Agriculture. Methylophilic Bacteria Are Known To Play A Significant Role In The Biogeochemical Cycle In Soil Ecosystems, Ultimately Fortifying Plants And Sustainable Agriculture. Methylophilic Also Improve Air Quality By Using Volatile Organic Compounds Such As Dichloromethane, Formaldehyde, Methanol And Formic Acid (Manish Kumar *Et Al.*, 2016). Additionally Methylophilic Are Involved In Phosphorous, Nitrogen And Carbon Cycling Can Help Reduce Global Warming. Bacteria Belonging To The Genus *Methylobacterium* Are Characterized By Being Able To Rely On Methanol As A Sole Carbon And Energy Source And By Presenting A More Or Less Intense Pink Reddish Pigmentation (Omer *Et Al.*, 2004). The Ppfms Are Ubiquitous In Nature And Are Frequently Reported On Various Plant Species, Are A Substantial Part Of The Aerobic, Heterotrophic Microflora Of The Surface Of Young Plants. They Are Able To Produce Plant Growth Regulators Such As Cytokinins And Auxin (Nadali *Et Al.*, 2010), Which Affect Plant Growth And Different Physiological Processes. The PPFM Can Also, Induce Systemic Resistance Against Disease (Madhaiyan, 2004) And Degrade A Wide Range Of Highly Toxic Compounds And Tolerate Metals (Jahan *Et Al.*, 2013).

II. NATURAL POPULATION OF BACTERIA AND PPFM ON MULBERRY PHYLLOSHERE

In The Present Study The Population Of Aerobic Bacteria And PPFM Was Determined By Serial Dilution Technique And Leaf Imprinting Method. The Population Was Assessed During The Period Of September To December, 2015. The Bacteria And PPFM Population Were Recorded After 35 Days After Pruning (DAP). Forty Five

Days Old Leaves Recorded Highest Bacterial Population Of 39.75×10^5 CFU G^{-1} Dry Weight Of Leaf In Serial Dilution Method. Lindow *Et Al.* (2003) Reported That Inhabitants Of The Phyllosphere Are Termed Epiphytes And May Consist Of A Variety Of Bacteria, Yeast And Fungi. Bacteria Within Phyllosphere May Include Those That Are Pathogenic To The Plant, But Also Include Non- Pathogenic Organisms Which Often Play Important Role In Influencing The Immediate Environment And Health Of Host Plant And May Also Be Involved In More Global Processes Such As Contributing To The Microbial Community On Phyllosphere Of Vegetable Crops Also Studied (Zhang *Et Al.*, 2010). Fungi And Archaea Are Known To Colonize The Leaves, Bacteria Are Numerically Dominant In The Phyllosphere Environment (Andrews And Harris, 2000). Dwelling Bacteria Can Have Neutral, Negative Or Positive Influences On Their Host Plants By Serving As Pathogens Or Preventing Leaf Colonization By Pathogen (Kishore *Et Al.*, 2005). Microflora Influences The Growth And Induces The Health Of Plants Including Mulberry (Shree And Boraiah, 1987). The PPFM Population Of Mulberry Phyllosphere Ranged From 28.16×10^2 (35 DAP) To 18.75×10^2 (75 DAP) CFU G^{-1} Dry Weight Of Leaf By Serial Dilution And Plating Technique. The Population Increase In Late Spring Indicates Increasingly Suitable Condition For *Methylobacterium* Spp. As The Plants Grow And Leaves Age. The Climatic Conditions Might Also Be A Positive Selective Factor. Natural PPFM Population In Red Clover Was Recorded As 10^6 No. Of Cells G^{-1} FW Of Red Clover Leaves By Omer *Et Al.* (2004). The Mean PPFM Population In Clover Was Reported As $3.18 \log$ CFU G^{-1} FW And In Wheat The Population Was $2.67 \log$ CFU G^{-1} FW Of Leaf. It Is Possible That Different Plants Possess Varying Carrying Capacity For PPFM And The Reason For This Is So Far Unknown. Hirano And Upper (1991) Found That Ppfms, Unlike Other Phyllosphere-Inhabiting Bacteria, Exhibit Population Levels Derived From Single Leaf Sample Similar To The Ones Derived From Bulk Samples. As An Ecological Niche, The Plant Phyllosphere Supports Highly Abundant *Methylobacterium* Species Of 10^4 - 10^7 CFU Per Leaflet (Austin *Et Al.*, 1978). The Bacterial Genus *Methylobacterium* Is A Well-Studied Example Of Pink-Pigmented Facultative Methylophiles (Ppfms) That Belong To The Class A- *Proteobacteria*, And Use Methanol As Sole Carbon And Energy Source. These Bacteria Are Not Considered To Be Passive Passengers On Plant Leaves, But Are Known To Stimulate Seed Germination And Plant Development, And Contribute Towards The Aroma Of Strawberry (Bligh *Et Al.*, 1959). Mizuno *Et Al.* (2012) Reported Commercial Green Perilla, *Perilla Frutescens Viridis* (Makino), Gave The Highest Counts Of Ppfms (2.0 - 4.1×10^7 CFU G^{-1} Of Leaf) Of All The Commercial Vegetable Leaves Tested, Amounting To 15 Per Cent Of Total Microbes On The Leaves. The Authors Analyzed The Green Vegetables For The Ratio Of PPFM To Total Bacteria.

Literature On The Origin Of Phyllosphere Ppfms Abounds, But It Is Still Debatable. Corpe (1985) Argues That The Paucity Of Ppfms In The Air Makes It Unlikely That The Atmosphere Is A Major Contributor Of The Methylophiles Encountered On Plant Leaves. Holland And Polacco (1992) Suggested That Leaf-Inhabiting Ppfms Are Probably Descendants Of Seed-Borne Bacteria Rather Than Bacteria From Air, Soil, Or Other Plants. Madhaiyan (2005) Has Reported That Ppfms Are Transmitted Mostly Through Seeds. On Other Hand, Romanovskaya *Et Al.* (2001) Has Reported That Leaves Were Not Colonized After Seed Bacterization Or Soil Application Of A PPFM Strain, And Were Colonized Only After Direct Application To The Phyllosphere, Suggesting That Natural Leaf Colonization Occurred *Via* Transfer Of Soil Particles. According To Knief *Et Al.* (2010), Factors Specific To The Sites From Which The Plants Species Were Collected, More Than The Plant Species Themselves, Have A Strong Influence On The Composition Of The Phyllosphere *Methylobacterium* Community. It Is Found In The Present Study That Ppfms Were Highly Abundant On Mulberry Leaves And The Ratio Of Ppfms To The Total Bacterial Count Was Also Good, Comparing To The Literature Available On PPFM Population On Phyllosphere.

III. RESULTS

The Leaf Samples Of Mulberry Plant (Variety MR2) Cultivated In The Mulberry Field Of Tamil Nadu Agricultural University, Coimbatore During The Period Of September To December, 2015 Was Used For This Study. The Results Of The Bacterial Population Analyzed By The Serial Dilution And Plating Method And Leaf Imprintment Method Are Presented In Table 1. The Results Indicated That There Is A Significant Difference In Bacterial Population During The Period Of 35th Day To 75th Days After Pruning (DAP).

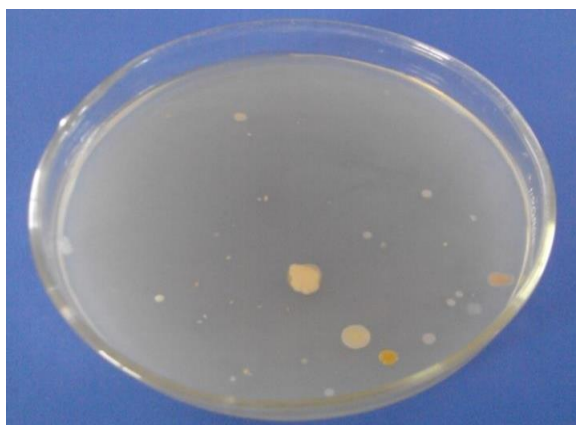
A Low Increase In Population Was Observed Up To 45 DAP And A Decline With Age Of The Plant After 45th DAP. The Population Ranged From 31.25×10^5 To 21.25×10^5 CFU G^{-1} Dry Weight Of Leaf. The Maximum Population Was Recorded On 45th DAP (39.75×10^5 CFU G^{-1} Dry Weight Of Leaf) Followed By 55th DAP (33.16×10^5 CFU G^{-1} Dry Weight Of Leaf).

The Population Of Bacteria As Determined By Leaf Imprintment Method Is Shown In Table 1. The Population Of Bacteria Showed Variation From 41.17 To 26.58 CFU cm^{-2} Of Leaf. The Maximum Population Was Recorded In 45th DAP (51.52 CFU cm^{-2} Of Leaf) Followed By 55th DAP (45.75 CFU cm^{-2} Of Leaf). The Slight Decline In The Population Was Observed From 55th Day To 75th DAP. The Bacterial Communities In Mulberry Phyllosphere Isolated Using Nutrient Agar Media By Both The Method Are Exhibited In Plate 1 And Plate 2. Most Of The Bacterial Colonies Appeared On The Nutrient Agar Plates Were Creamy Coloured And Irregular With Undulate Margin.

| S.No. | Days After Pruning | Population Of Bacteria | |
|--------------|--------------------|--|--|
| | | Serial Dilution And Plating Method (CFU G ⁻¹ Dry Weight Of Leaf) | Leaf Imprinting Method (CFU Cm ⁻² Of Leaf) |
| 1 | 35 | 31.25 X 10 ⁵ | 41.17 |
| 2 | 45 | 39.75 X 10 ⁵ | 51.52 |
| 3 | 55 | 33.16 X 10 ⁵ | 45.75 |
| 4 | 65 | 26.12 X 10 ⁵ | 33.46 |
| 5 | 75 | 21.25 X 10 ⁵ | 26.58 |
| SE D | | 2.68 | 1.37 |
| CD (P= 0.05) | | 5.84 | 2.93 |

Table 1: Occurrence Of Bacteria On Mulberry Phyllosphere

35 Days After Pruning



45 Days After Pruning

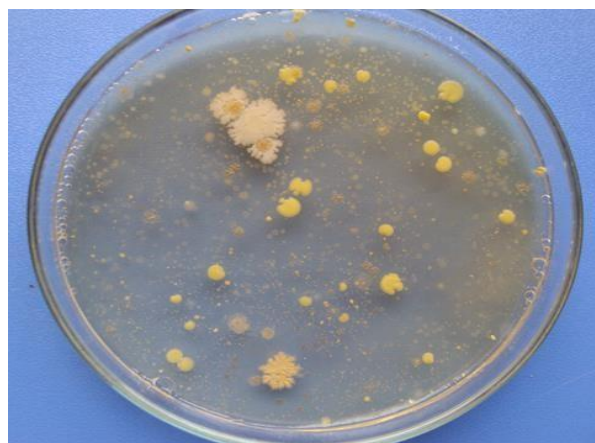


Plate 1: Appearance Of Bacterial Communities Of Mulberry Phyllosphere On Nutrient Agar Plates- Serial Dilution Method

35 Days After Pruning



45 Days After Pruning



Plate 2: Bacterial Flora Of Mulberry Phyllosphere On Nutrient Agar Plates- Leaf Imprintment Method

The PPFM Population In The Phyllosphere Region Of Mulberry Was Analyzed By Serial Dilution Technique And Leaf Imprintment Method On Ammonium Mineral Salt (AMS) Agar Plates And Results Presented In Table 2. In Serial Dilution Technique, Population Sizes Of The PPFM On Mulberry Leaf Showed Variation Ranging From 28.16×10^2 To 18.75×10^2 CFU G⁻¹ Dry Weight Of Leaf. The Maximum Population Was Recorded On 45th DAP (34.20×10^2 CFU G⁻¹ Dry Weight Of Leaf) Followed By 55th DAP (29.51×10^2 CFU G⁻¹ Dry Weight Of Leaf). Statistically There Is A Significant Difference In Population Of PPFM On Mulberry Phyllosphere.

The Population Of PPFM As Determined By Leaf Imprintment Method Showed Variation Ranging From 24.26 To 12.38 CFU Cm⁻² Of Leaf With Age Of Plants. The Maximum Population Was Recorded In 55th DAP (36.25 CFU Cm⁻² Of Leaf) Followed By 45th DAP (31.61 CFU Cm⁻² Of Leaf). Slight Decline In The Population Was Observed From 65th Day To 75th DAP. The Serial Dilution Technique Yielded High Population Of PPFM Compared To Leaf Imprintment Method (Plate 3 And Plate 4). However The Stability Of Bacteria And Natural Population Of PPFM Was Noted On Mulberry Phyllosphere During 35th To 75th DAP.

| S.No. | Days After Pruning | Population Of PPFM | |
|-------------|--------------------|---|---|
| | | Serial Dilution And Plating Method (CFU G ⁻¹ Dry Weight Of Leaf) | Leaf Imprinting Method (CFU Cm ⁻² Of Leaf) |
| 1 | 35 | 28.16×10^2 | 24.26 |
| 2 | 45 | 34.20×10^2 | 31.61 |
| 3 | 55 | 29.51×10^2 | 36.25 |
| 4 | 65 | 23.25×10^2 | 17.27 |
| 5 | 75 | 18.75×10^2 | 12.38 |
| SE D | | 2.14 | 1.24 |
| CD (P=0.05) | | 4.67 | 2.66 |

Table 2: Population Of Naturally Occurring Pink Pigmented Facultative Methylo-trophic (PPFM) Bacteria On Mulberry Phyllosphere

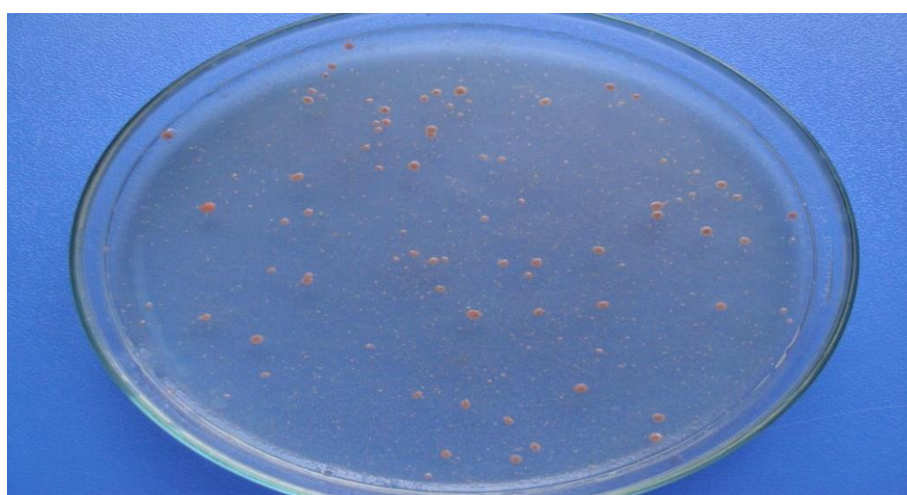


Plate 3: Appearance Of PPFM Communities Of Mulberry Phyllosphere On AMS Agar Plates-Serial Dilution Method

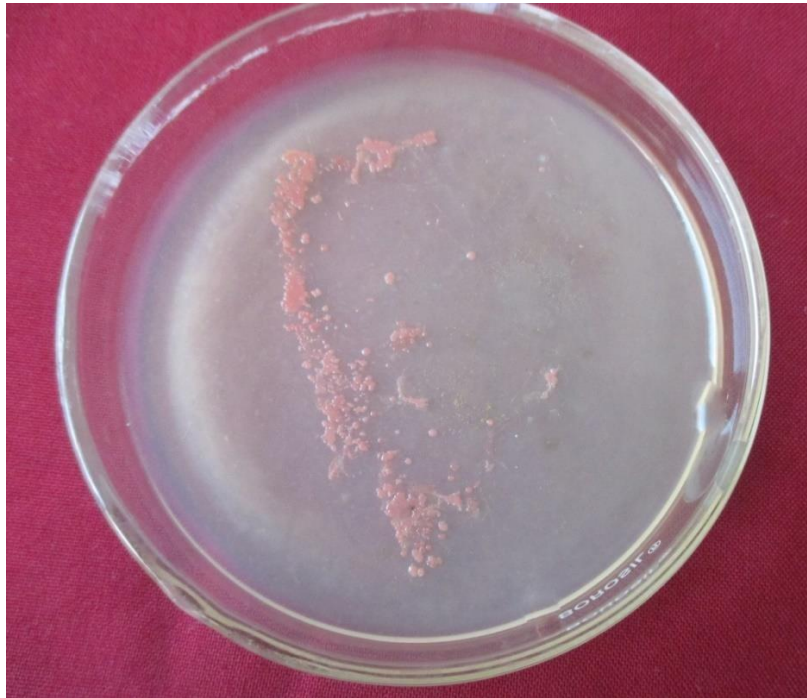


Plate 4: PPFM Communities Of Mulberry Phyllosphere On AMS Agar Plates- LeafImprintment Method

IV. CONCLUSION

Methylotrophic Bacteria Are Known To Play A Significant Role In Sustainable Agriculture. *Methylobacterium* Sp. As Bioinoculant Has Been Found To Promote Crop Growth. The *Methylobacterium* Sp. Mi7 Isolate Used In The Present Study Was Found To Improve The Mulberry Crop Growth, When Used On Phyllosphere. In Mulberry Phyllosphere Bacteria Were Present In Large Numbers With Great Diversity. The Natural Occurrence Of PPFM In Mulberry Phyllosphere Analyzed By Serial Dilution And Plating Technique Shown The Variation During The Periods Of September To December And January To April On Mulberry Phyllosphere. The Maximum Population Of PPFM Occurred During 45th Day After Pruning. The Total Population Of PPFM Was 34.20×10^2 CFU G⁻¹ Dry Weight Of Leaf. Thus The Natural Occurrence Of *Methylobacterium* On Mulberry Phyllosphere Was Found.

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