

## GENETIC AND METABOLIC DIVERSITY OF PINK-PIGMENTED FACULTATIVE METHYLOTROPHS IN PHYLLOSHERE OF TROPICAL PLANTS

D. Balachandar; P. Raja; SP. Sundaram\*

Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

Submitted: June 19, 2007; Returned to authors for corrections: October 10, 2007; Approved: November 16, 2007.

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### ABSTRACT

Diversity of Pink-Pigmented Facultative Methylobacteria (PPFMs) in phyllosphere of cotton, maize and sunflower was determined based on differential carbon-substrate utilization profile and Random Amplified Polymorphic DNA data. Results indicate that six diversified groups of PPFMs are found in these crops. Sunflower and maize phyllosphere harbor four different groups of methylobacteria while cotton has only two groups.

**Key words:** diversity; *Methylobacterium*; phyllosphere; PPFM; RAPD

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Pink-Pigmented Facultative Methylobacteria (PPFMs) mostly belonging to the genus *Methylobacterium* are phylogenetically diverse and use one-carbon compounds such as formate, formaldehyde, methanol and as well as a wide range of multi-carbon substrates as sole source of carbon and energy (5). They belong to a subgroup of *Proteobacteria*, order Rhizobiales and family Methylobacteriaceae. The unique feature of PPFMs is their ability to oxidize methanol, a methylobacterial property based on the presence of methanol dehydrogenase (*mxhF*) gene (2). Specifically, the genus *Methylobacterium* comprises 23 species isolated from a wide range of environments (4). While, PPFM bacteria on plant surfaces are benefited by methanol produced by plants (26), these bacteria secrete a variety of auxins (11) and cytokinins (15) that are utilized by the plants leading to an increased plant growth and yield. PPFMs have been reported to increase plant growth, germination, vigor index, yield and systemic resistance (16). Members of PPFM are ubiquitous in nature and found in a variety of habitats including phyllosphere, rhizosphere, root nodules, dust, freshwater, drinking water, lake sediments, etc (3,5). *Methylobacterium* spp. are common leaf epiphytes representing an important bacterial population on leaves (7) and are persistently present in the phyllosphere of numerous plants (8). Even though most of the PPFM species are reported to colonize plants of all regions, till now, no study has been undertaken on the diversity of PPFMs

within and among the plant species. Thus, it gains significance to investigate the occurrence and distribution of natural populations of PPFMs and their dynamics in relation to different host plants.

Random amplified polymorphic DNA (RAPD) is a powerful molecular fingerprinting technique, which is highly discriminative and allows distinction even between closely related strains (18,28). Methylobacteria could be discriminated at species level as they differ in range of carbon compounds utilizing pattern (27). In this study, we have explored the genetic and metabolic diversity of PPFM bacteria in the phyllosphere of cotton, maize and sunflower to reveal the heterogeneity of PPFMs within a single plant species and across different plant species using RAPD and differential carbon-substrate utilization profiling.

The PPFMs were isolated using 0.5% (v/v) filter-sterilized methanol supplemented Ammonium Mineral Salts (AMS) medium (8) from the phyllosphere of cotton (cultivar MCU12), maize (cultivar Co2) and sunflower (cultivar Co3) exclusively raised for this study in the experimental plots of Tamil Nadu Agricultural University, Coimbatore, India. Ten plants of each crop were selected randomly at 30 days after sowing and 5<sup>th</sup> and 7<sup>th</sup> leaves of main stem were collected in sterile bags and PPFMs were isolated by leaf-imprinting technique as previously described (8). PPFM isolates were grown in methanol

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\*Corresponding Author. Mailing address: Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore 641 003, Tamil Nadu, India. Tel.: (+91) 422 6611294. E-mail: professorsundaram@yahoo.com

supplemented AMS medium for 7 days at 30°C and genomic DNA was extracted by hexadecyltrimethyl ammonium bromide (CTAB) method as described by Ivanova *et al.* (10). The integrity and concentration of purified DNA was determined by agarose gel electrophoresis (24) and DNA concentration was adjusted to 20 ng/μL for PCR amplification.

The *mxaf* gene in the isolates was detected with specific primers mxaf1003 (5'GCG GCACCAACTGGG GCTGGT 3') and mxar1561 (5'GGG CAG CAT GAA GGG CTC CC3') (19). PCR reactions were carried out in Eppendorf master cycler, Germany in 40 μL containing 50 ng genomic DNA, 0.2 mM dNTP mixture, 1 μM primers, 2.5 mM MgCl<sub>2</sub> and 2 U *Taq* DNA polymerase (Bangalore Genei, India) under the following conditions: 95°C for 1 min followed by 35 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 1 min and final extension at 72°C for 5 min. PCR products were separated by electrophoresis on 1.5% agarose gels stained with ethidium bromide and documented in Alphaimager TM1200 documentation and analysis system.

Carbon substrate utilization profile of PPFM isolates was analyzed as described previously (13) in AMS medium substituted with different carbon compounds at 0.5% (w/v) as presented in Table 1. The carbon substrate utilization pattern of the isolates were compared with that of standard species and grouped based on the similarity (27, 29). RAPD analysis was performed in 30 PPFM isolates using twenty different 10-base primers [OPM 1 (5'GTTGGTGGCT3'); OPM2 (ACAACGCCTC); OPM3 (GGGGGATGAG); OPM4 (GGCGGTTGTC); OPM5 (GGGAACGTGT); OPM6 (CTGGGCAACT); OPM7 (CCGT GACTCA); OPM8 (TCTGTTCCCC); OPM9 (GTCTTGCGGA); OPM10 (TCTGGCGCAC); OPQ1 (GGGACGATGG); OPQ2 (TCTGTCGGTC); OPQ3 (GGTCACCTCA); OPQ4 (AGTGC GCTGA); OPQ5 (CCGCGTCTTG); OPQ6 (GAGCGCCTTG); OPQ7 (CCCCGATGGT); OPQ8 (CTCCAGCGGA); OPQ9 (GG CTAACCGA) and OPQ 10 (TGTGCCCGAA)] as described by Guthrie *et al.*, 1990 (6). The resulting polymorphic differences were analyzed using the program NTSYS-PC2 by estimating the distance of PPFM isolates using Jaccard's Similarity Coefficient (12) and inferring the topology of the tree by the method of Clustering UPGMA (20). The indices of diversity, richness and evenness were calculated using the number of isolates belonging to each group based on carbon utilization pattern and RAPD profile. Diversity was calculated using the Shannon index:  $H' = -\sum^s [(n_i/n) \ln(n_i/n)]$ , where  $n_i$  is the number of isolates in each group and  $n$  is the number of isolates in all groups (25). For richness, the Margalef index was used:  $R = S-1/\ln(n)$ , where  $S$  in this case is the number of groups and  $n$  is the number of isolates in all groups (17). The Pielou index was used as a measure of evenness:  $E = H'/\ln(S)$ , where  $H'$  is the Shannon index and  $S$  is the number of groups (23).

A total of sixty leaf samples, twenty each from cotton, maize and sunflower were analyzed for the diversity of PPFMs using differential carbon-substrate utilization pattern and RAPD-PCR

**Table 1.** Differential carbon-substrate utilization pattern among the representative PPFM isolates in phyllosphere of tropical plants.

| Carbon source <sup>a</sup> | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | M1 | M2 | M3 | M4 | M5 | M6 | M7 | M8 | M9 | M10 | M11 | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 | S10 |   |   |   |   |
|----------------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|-----|-----|----|----|----|----|----|----|----|----|----|-----|---|---|---|---|
| Methylamine                | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | +   | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | + |   |   |   |
| Acetate                    | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | +   | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | + |   |   |   |
| Citrate                    | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | +   | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | + | + |   |   |
| L-Glutamate                | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | +   | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | + | + |   |   |
| D-Glucose                  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | +   | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | + | + | + |   |
| D-Xylose                   | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | +   | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | + | + | + |   |
| Fructose                   | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | +   | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | + | + | + |   |
| Betaine                    | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | +   | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | + | + | + | + |
| Thiocyanate                | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | +   | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | + | + | + | + |
| Tartarate                  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | +   | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | + | + | + | + |
| Arabinose                  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | +   | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | + | + | + | + |
| Dimethylamine              | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | +   | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | + | + | + | + |
| Trimethylamine             | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | +   | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | + | + | + | + |
| Cyanide                    | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | +   | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | + | + | + | + |
| Nutrient broth             | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | +   | +  | +  | +  | +  | +  | +  | +  | +  | +  | +   | + | + | + | + |

<sup>a</sup> The carbon compounds were substituted for methanol in AMS liquid medium at 0.5% (w/v) level; C1 to C9, Cotton isolates; M1 to M11, Maize isolates; S1 to S10, Sunflower isolates; +, Growth; -, no growth.

profiling. A total of 62 PPFMs isolated from three different crops' phyllosphere by leaf-impression technique were further authenticated for methylo-trophy by PCR amplification of partial *mxoF* gene using genomic DNA of the isolates. The results confirmed the ability of all the isolates to amplify about 550 bp sized partial *mxoF* gene.

The growth of PPFM isolates was recorded as presence or absence in AMS medium substituted with different carbon substrates and presented as Table 1. Carbon-substrate utilization profile of the isolates was exactly coincided with already reported species of *Methylobacterium*. When comparing the carbon-substrate utilization profile of isolates with type strains of plant associated *Methylobacterium*, 62 isolates were clustered to six different groups (Table 2). Out of 62 isolates, 30 isolates (48.37%)

had similar profile of *Methylobacterium thiocyanatum*; 10 isolates (16.13%) as *M. aminovorans*; 10 isolates (16.13%) as *M. suomiense*; 4 isolate each of *M. extorquens*, *M. fujisawaense* and *M. zatmanii* (27,29). Among the three tropical crop plants studied, sunflower and maize phyllosphere harbor four different groups of PPFMs, while cotton had only two types (Table 2).

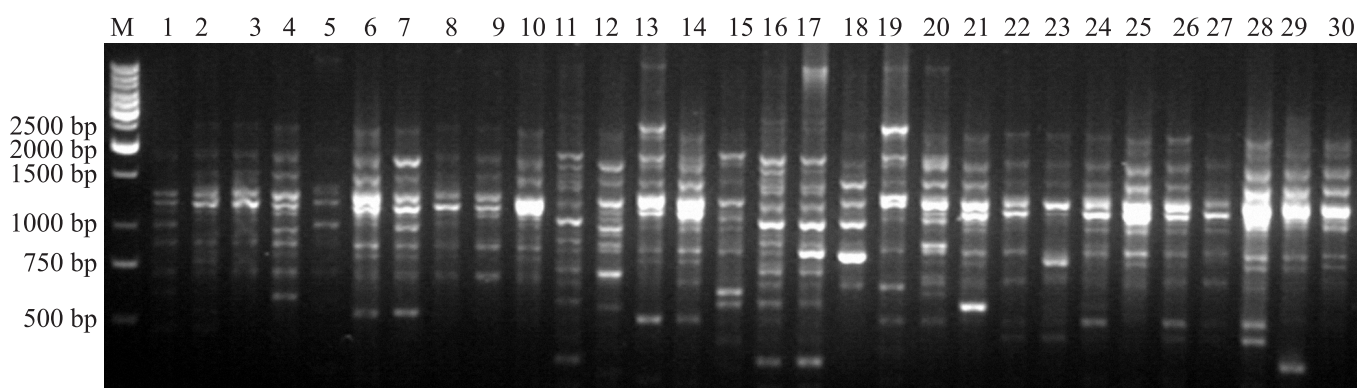
Nearly 50% of the isolates (30 isolates) of PPFMs representing six different carbon-substrate utilization profiling and three different crop plants were analyzed for RAPD banding pattern. Among the twenty primers screened, seven RAPD primers (OPQ1, OPQ2, OPQ6, OPQ10, OPM3, OPM7 and OPM10) able to produce polymorphic amplicons were used for grouping the isolates. The representative banding pattern of primer OPQ10 was presented as Fig. 1. Dendrogram of combining metabolic

**Table 2.** Occurrence and distribution of PPFM isolates from phyllosphere of three different crop plants based on carbon utilization profiling.

| Crop      | Total number of isolates <sup>a</sup> | Representative isolates selected for RAPD | PPFM Species showing similar C-profile <sup>b</sup> |
|-----------|---------------------------------------|---|---|
| Cotton    | 18                                    | C1, C2, C3, C4, C6, C7, C8, C9            | <i>M. thiocyanatum</i>                              |
|           | 2                                     | C5  | <i>M. extorquens</i>                                |
| Maize     | 8                                     | M1, M7, M8, M9                            | <i>M. thiocyanatum</i>                              |
|           | 8                                     | M4, M5, M10, M11                          | <i>M. aminovorans</i>                               |
|           | 4                                     | M2, M6                                    | <i>M. fujisawaense</i>                              |
|           | 2                                     | M3  | <i>M. extorquens</i>                                |
| Sunflower | 10                                    | S2, S3, S7, S8, S6                        | <i>M. suomiense</i>                                 |
|           | 4                                     | S9, S10                                   | <i>M. zatmanii</i>                                  |
|           | 2                                     | S4  | <i>M. aminovorans</i>                               |
|           | 4                                     | S5, S1                                    | <i>M. thiocyanatum</i>                              |

<sup>a</sup> The number of isolates showing similar carbon substrate utilization profile;

<sup>b</sup> Carbon substrate utilization profile compared with key data of Jourand *et al.* (13) and VanAken *et al.* (27).

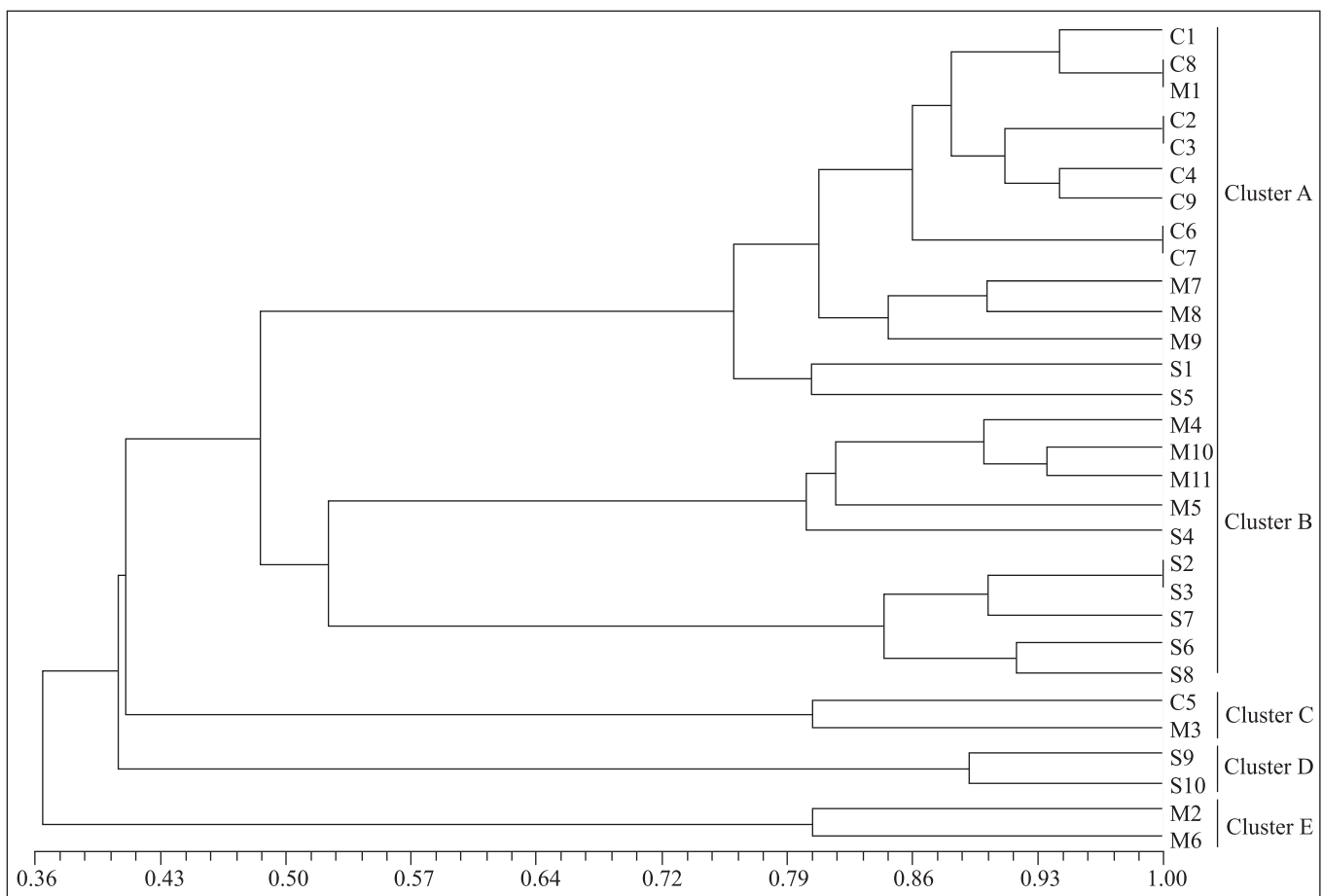


**Figure 1.** RAPD pattern of representative PPFM isolates from phyllosphere of three different crops generated by primer OPQ10. M - Marker DNA ladder (1 kbp); Lane 1 to 9 - Cotton isolates (C1-C9); Lane 10 to 20 - Maize isolates (M1-M11); Lane 21 to 30 - Sunflower isolates (S1-S10).

diversity data (presence or absence of growth in different carbon-substrates) and RAPD banding pattern (binary matrix recording presence or absence of band) of PPFM isolates revealed that the PPFM isolates are grouped into 5 different clusters (Fig. 2). Cluster A comprises of fourteen isolates (8 from cotton; 4 from maize and 2 from sunflower); cluster B comprises of 10 isolates (4 from maize and 6 from sunflower); cluster C includes one cotton isolate (C5) and one maize isolate (M3); cluster D with two sunflower isolates (S9, S10) and cluster E with two maize isolates (M2, M6). With reference to crop species, most of cotton isolates except C5 formed one cluster (A), maize isolates are present in clusters A, B, C and E, whereas the sunflower isolates are distributed in clusters A, B and D. The combining of both carbon-substrate utilization pattern and RAPD banding pattern increased the resolution of divergence of PPFM isolates, when comparing the individual data sets.

To quantify the PPFMs' diversity, the combined sets of carbon substrate utilization profile and RAPD banding pattern

data were subjected to statistical analysis. Shannon index of diversity ( $H'$ ) which refers the number of species and evenness of the species was very low in cotton (0.32) compared to maize (1.26) and sunflower (1.22). Margalef index ( $R$ ) specifically refers the species richness in PPFM community, which was also reported very low in cotton (1.67) and high in maize and sunflower (3.67 and 3.66 respectively). Pielou index referring the evenness of the species in a community was also recorded to be very low in cotton (0.469). All the three diversity indices calculated for PPFM isolates in phyllosphere of these crop plants revealed that cotton leaves had narrow diversity while maize and sunflower with widely diversified PPFMs. On the basis of genetic and metabolic studies of the isolates, significant diversity was found among the PPFM isolates in phyllosphere of these crops. Great genotypic and metabolic diversity of PPFMs in phyllosphere revealed by this study is the first kind of report in tropical crop plants. Higher degree of PPFMs' diversity in sunflower and maize leaves and low in cotton leaves reported



**Figure 2.** Dendrogram showing relatedness of 30 PPFM isolates based on Jaccard's similarity coefficient calculated using combined data of RAPD-PCR banding pattern and carbon-substrate utilization profile of isolates.

in this study may be due to their differential emission of large variety of volatile compounds including methanol through leaves, which are to be studied thoroughly about the relationship of leaf emitting metabolites with PPFMs' diversity (21).

Recent findings have demonstrated that degree of plant – PPFMs association varies as symbiotic (13), epiphytic (22) and as endophytic (14). These bacteria on phyllosphere produce cytokinins, auxins and vitamin B12 and stimulate the plant development (1). However, the over all nature of their relationship with plant is yet understood poorly and the biological significance of these bacterial species is still unknown. Under these circumstance, the diversity studies of PPFMs gain importance to understand the plant interactions. We have demonstrated the genotypic and metabolic diversity and community structure of PPFMs in different tropical plants' phyllosphere. Findings of this study indicate in depth molecular studies are needed to understand the diversity of methylobacteria in phyllosphere, as the leave metabolites influence the colonization (9). Further, detailed studies are necessary to explore the complexity of interaction between these PPFMs and host plants wherein a multitude of synergistic and antagonistic interactions are operative in the phyllosphere.

## RESUMO

### Diversidade metabólica e genética de microrganismos metilotróficos facultativos pigmentados na filosfera de plantas tropicais

A diversidade de microrganismos metilotróficos facultativos pigmentados (PPFMs) na filosfera de algodão, milho e girassol foi determinada baseada no perfil diferencial de utilização de substratos de carbono e em dados de RAPD. Os resultados indicaram a existência de seis grupos diferentes de PPFMs nessas plantas. As filosferas de girassol e milho apresentaram quatro grupos diferentes de metilobactérias enquanto a de algodão apresentou apenas dois grupos.

**Palavras-chave:** diversidade, *Methylobacterium*, filosfera, PPFM, RAPD

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