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RESEARCH ARTICLE

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Characterization of endophytic plant growth promoting traits of *Methylobacterium* sp. isolated from banana (*Musa* sp.)

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ABSTRACT : A total of 26 endophytic *Methylobacterium* sp. strains were obtained from surface sterilized leaves of two banana cultivar (Robusta and Nattu poovan) and the strains were tested for its ability to fix atmospheric nitrogen, EPS and IAA production for promoting banana tree growth. The result of the present study demonstrated that endophytic population ranged from 4.03 and 4.14 log cfu per gram of leaf tissue. Among these four isolates were chosen based on colony morphology and their distinct pigmentation. All the selected four isolates were able to grow in nitrogen free methanol mineral salt medium. The synthesis of indole-3-acetic acid (IAA) in the presence of L-tryptophan was detected in all the isolates tested. The isolate FM2 (*Methylobacterium* sp.) produced the highest amount of IAA (13.01µg ml^{-1}) in medium supplemented with L-tryptophan and was able to synthesize IAA in the absence of L-tryptophan. The maximum amount of ASP and WSP was recorded in FM3 (21.73 µg ml^{-1}) and FM1 (157.79 µg ml^{-1}), respectively. They were tentatively identified at species level based on carbon utilization test. The classified strains were also screened for methanol dehydrogenase (*mxaF* gene sequencing) using specific primers and obtained 555 bp PCR product. So the *Methylobacterium* sp. strains analyzed here had a promising potential for developing as a plant growth promoting bacteria for sustainable agriculture.

KEY WORDS : *Musa* sp., Nitrogen fixation, Indole-3-acetic acid, Exopolysaccharide, *Methylobacterium* sp.

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INTRODUCTION

The phyllosphere is the largest habitat for a wide diversity of micro-organisms. The predominant micro-organisms present in the leaf phyllosphere are bacteria and filamentous fungi. Phyllosphere bacteria normally found on the leaf surfaces of plants as epiphytes and in the interior of plant tissues as endophytes (Monier and Lindow, 2004). Endophytes are micro-organisms that do not visibly affect the host plant but can be isolated from surface-sterilized plant tissue or the inner parts of plants (Compant *et al*., 2005).

The density of endophytic population may vary from

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10^2 to 10^9 (Overbeek and Elsas, 2008) and depends on many factors, which include the plant cultivar (genotype), interaction with other organisms and other environmental factors (Hallmann *et al*., 1997). The interaction between host plants and endophytic bacterial mechanism is not well understood. However, endophytic bacteria profound to have beneficial effects on their host plant (Ulrich *et al*., 2008). These beneficial effects include plant growth promotion and biological control of phytopathogens to increase plant fitness (Hardoim *et al*., 2008).

Bacteria belonging to the genus *Methylobacterium*, commonly known as pink-pigmented facultative methylotrophic bacteria (PPFMs), which are ubiquitous on leaf surfaces and potentially dominating the phyllosphere bacterial community normally occur as rhizosphere inhabitants and endophytes when isolated on selective media (Corpe and Rheem, 1989). They are aerobic, Gram-negative bacteria and they can able to grow on a wide range of multi-carbon substrates, they also have the capability to grow on one carbon compounds such as formate, formaldehyde or methanol as the sole carbon and energy source.

Methylobacterium sp. normally consume plant waste products like methanol and produce metabolites which is useful for plant development (Holland, 1997). Several beneficial aspects have been investigated, such as the production of urease (Holland and Polacco, 1992), stimulation of seed germination and plant growth (Madhaiyan *et al*., 2004), cytokinin production (Koenig *et al*., 2002), auxin synthesis (Omer *et al*., 2004), vitamin B12 (Basile *et al*.,1985) and ACC deaminase (Madhaiyan *et al*., 2006).

The endophytic bacteria have the potential to perform nitrogen fixation, mainly in non-leguminous plants, has been the objective of most studies (Teixeira *et al*., 2007). These bacteria are an alternative supplement to nitrogen fertilizers. Species of the genera *Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae, Azotobacter, Beijerinckia, Methylobacterium* and *Azospirillum* have been found to have nitrogen fixing ability in diverse cultures (Cavalcante *et al*., 2007). Nitrogen fixation and plant growth promotion by plant growth promoting bacteria are important criteria for an effective biofertilizer.

Drought stress is one of the major agricultural problems which leads to limiting agricultural productivity in most of the arid and semi arid regions of the world. This form of abiotic stress affects the plant water relations at both the cellular and whole plant level, causing both specific and non-specific reactions and damage. Bacteria can able to thrive under stress conditions, due to their exopolysaccharide (EPS) production, which protects micro-organisms from water stress by enhancing water retention and regulating the diffusion of organic carbon sources (Chenu and Robertson, 1996). EPS also helps micro-organisms to irreversibly attach and colonize the roots due to involvement of a network of fibrillar material that permanently connects the bacteria to the root surface (Bashan *et al.*, 2004).

Banana is one of the most important fruit crop of the world, especially in tropical countries and it is often called as 'apple of paradise'. It requires high amounts of chemical fertilizers for commercial cultivation, which is costly and can be hazardous to the environment, when used excessively. With the increasing awareness about the economic and environmental consequences of use of chemical fertilizers it is important to explore region specific microbial strain which can be used as a potential plant growth promoter to get higher yield. The use of indigenous micro-organisms is an effective way to acclimatize natural conditions and enhance the plant-microbe interactions (Verma *et al*., 2013). Plant growth promoting rhizobacteria (PGPR) could be used for growth promotion, nutrient uptake and some time as an alternative source of N-fertilizer of non-leguminous crops.

The aim of present study was to isolate and characterize endophytic methylobacteria from banana plants and to screen their beneficial traits to adopt as bioinoculant.

EXPERIMENTAL METHODS

Sample collection and bacterial isolation :

The endophytic bacteria used in the present study were previously isolated from the leaves of the banana tree cultivar ''Robusta and Nattu poovan'' from banana germplasm collection Tamil Nadu Agricultural University, Coimbatore. The leaves were collected from superior portion of banana plants in the vegetative phase. The banana leaves were surface sterilized in 70 per cent ethanol for 1 min, followed by immersion in 2 per cent

sodium hypochlorite (NaClO) for 2 min. The fragments were subsequently washed two times in sterile distilled water and they were triturated in sterile phosphate-buffered saline (PBS) under constant agitation. Appropriate dilutions were subsequently plated onto Ammonium mineral salt medium (AMS) medium (Whittenbury *et al*., 1970) supplemented with filter-sterilized cycloheximide (10 µg ml^{-1}) and methanol (0.5% v/v). The plates were incubated at 30ºC for 7 days and the number of colony forming units (CFU) was determined to estimate the population density of pink-pigmented bacteria.

Carbon utilization test :

Carbon utilization profiling were tested for Endophytic *Methylobacterium* sp. isolates was determined as described by Green and Bousfield (1982).

Identification of methanol dehydrogenase (*mxaF*) gene by PCR :

The presence of (*mxaF*), required for methanol utilization gene, were amplified from DNA extracts using specific primers (McDonald and Murrell, 1997). PCR products were analysed in 2 per cent agarose gel.

Growth on nitrogen-limited medium :

The N_2 fixing ability of the bacterial isolates were studied using nitrogen free methanol mineral salt medium. The bacterial cultures were inoculated and incubated at 30^0C for 5-7 days and the bacterial growth on the medium indicated positive result. The growth measurement was taken in a UV VIS spectrophotometer (cary 50 Bio,Varian) at 600nm and expressed as- no growth(<0.10 OD); + (0.1 to 0.3); ++(0.3 to 0.5).

Quantitative analysis of indole compounds :

Auxin, indole-3-acetic acid (IAA) produced by the cultures was estimated by growing the isolates in AMS medium supplemented with L-tryptophan as precursor of IAA (Gordon and Weber, 1951). The isolates were incubated in 100 ml of AMS broth supplemented with 0.1 per cent (w/v) L-tryptophan and incubated at 30ºC for 7 days. The supernatant of the culture fluid was obtained by centrifuge at 4ºC for 15 min at 8000 rpm. Then, Salkowski colouring reagent (35% $HClO_4$ 50 ml, 0.5M $FeCl_3$ 1 ml) and the supernatant were mixed in the ratio of 2:1 and left in the dark for 30 min and the absorbance was measured at 540 nm. The IAA concentration in the culture was estimated based on the IAA standard curve.

Polysaccharide production :

Water soluble polysaccharide :

Seventy two hours grown culture was used for estimation of water soluble and alkali stable polysaccharide (Sutherland and Wilkinson, 1971). Cultures were centrifuged at 5000×g for 15 mins. The cell pellet was set aside for the analysis of alkali stable fraction of polysaccharide. To 20 ml of the supernatant, an equal volume of 90 per cent ethanol was added, mixed well and placed at 4°C overnight to precipitate the water soluble portion of polysaccharide. Then it was centrifuged at 7000×g for 15 mins. The pellet was dissolved in one per cent acetic acid and the polysaccharide content was estimated by anthrone reagent method. Five ml of the anthrone reagent was pippetted out into the tubes and 1.0 ml of the sample was added and the mixture was shaken. The tubes were cooled and heated exactly for 10 mins on a boiling water bath. Then the tubes were cooled rapidly under tap water. The absorbance was measured in a calorimeter at 620 nm. The quantity of polysaccharide content present in the sample was determined by referring the standard graph prepared for glucose and expressed as mg per g of glucose released per g dry weight of the cells (Nelson, 1944).

Alkali stable polysaccharide :

The harvested cell pellet was suspended in 5 ml of distilled water and added with 5 ml of 0.1N KOH and then it was boiled for 30 min and cooled down to room temperature. The suspension was centrifuged at 5000×g for 15 mins and the supernatant was retained and neutralized with 0.1N HCl for the estimation of alkali stable polysaccharide by anthrone reagent method as aforementioned in water soluble polysaccharide.

Statistical analysis :

All the data were subjected to statistical analysis with software, Microsoft Excel for Windows 2007 add-in with XLSTAT version 2010.5.05 (XLSTAT, 2010). Statistically significant differences were analyzed using Duncan's multiple range test (DMRT).

EXPERIMENTAL RESULTS AND ANALYSIS

The results obtained from the present investigation as well as relevant discussion have been summarized under the following heads :

Isolation and characterization of endophytic *Methylobacterium* sp. :

The density of endophytic populations was found to be 4.03 and 4.17 log cfu per gram of leaf tissue. Selection was performed on the basis of size, shape, colour and elevation of colonies. Altogether 4 morphologically distinct colonies were selected from numerous colonies and named it as FM 1, FM 2, FM 3 and FM 4. All PPFMs strains tested were, respectively rod shaped occuring singly or occasionally in rosettes. The selected colonies showing circular in shape and their pigmentation looking whitish pink to pink in colour (Table 1 and 3). Several studies have been conducted on the association of *Methylobacterium* sp. with many plant systems. Chanprame *et al*. (1996) detected *Methylobacterium mesophilicum* on the leaf surface of a wide range of plant species as a frequently contaminating bacterium in plant tissue culture. Dourado *et al*. (2012) found that most of the strain isolated from contaminated oil and other samples were similiar to *M. fujis awaense, M. radiotolerans* and *M. oryzae* species. In the present study, the results were correlated with the above findings and we furnished the *Methylobacterium* sp. density in Table 1 and it was found to be 4.03 and 4.17 log cfu per g of leaf tissue.

Carbon utilization test :

In the present study 12 carbon compounds were tested and presented in Table 2. All 4 strains were able to utilize D-glucose, fructose, tartarate, glutamate, trimethylamine, citrate and betaine were tested. Lactose and arabinose was the preferred carbon source for three *Methylobacterium* sp. strains except FM 3. None of the strains tested could grow in methylamine and dimethylamine. 2 strains FM 2 and FM 4 could utilize ethanol while FM1 and FM 3 could not.

Based on the carbon utilization the isolates were classified into three groups. Two isolates *viz.,* FM 1 and FM 2 came under group I utilizing glucose, fructose, lactose, arabinose, tartarate, glutamate, trimethylamine, betaine and which were tentatively identified as

Table 1: Isolation and identification of endophytic *Methylobacterium* sp. in banana cultivar

Banana variety	Area	Age of plant	Genome constitution	Population (Log cfu g^{-1} of leaf tissue)
Robusta	Coimbatore (TNAU)	9 months	AAA	4.03 (±0.10)
Nattu poovan	Coimbatore (TNAU)	10 months	AAB	4.14 (±0.05)

Table 2 : Carbon utilization profiling by different *Methylobacterium* sp.

Isolates	1	2	3	4	5	6	7	8	9	10	11	12
FM 1	++	+	+	++	+	+	+	-	-	+	++	-
FM 2	++	++	+	+	+	+	+	-	-	+	++	++
FM 3	++	++	-	-	+	++	+	-	-	+	++	-
FM 4	++	++	-	++	++	++	+	-	-	+	++	++

++ indicates good growth; + indicates moderate growth; - indicates no growth; 1- Glucose; 2 - Fructose; 3 -Lactose; 4 -Arabinose; 5 -Tartarate; 6 -Glutamate; 7 - Citrate; 8 - Methylamine; 9 - Dimethylamine; 10 - Trimethylamine; 11 - Betaine; 12 - Ethanol

Table 3 : Production of IAA , polysaccharide and fixation ability by selected FM strains

Isolates	Growth (A_{600nm})	IAA (µg ml^{-1})		EPS (µg ml^{-1})		Colony morphology			
		Trp^+	Trp^-	WSP	ASP	Shape	Elevation	surface	Pigment production
FM 1	+	8.47 (±0.35)^{c}	5.17 (±0.38)^{ab}	157.79 (±10.15)^{a}	16.91 (±1.25)^{b}	Circular	Low convex	Blend	Whitish pink
FM 2	+	13.01 (±0.30)^{a}	4.59 (±0.29)^{b}	150.73 (±4.79)^{a}	11.76 (±0.82)^{c}	Circular	Low convex	Blend	Light pink
FM 3	+	10.28 (±0.18)^{b}	5.83 (±0.17)^{a}	150.47 (±5.04)^{a}	21.73 (±0.90)^{a}	Circular	Convex	Blend	Pink
FM 4	+	2.31 (±0.04)^{d}	0.97 (±0.17)^{c}	139.43 (±7.20)^{a}	13.85 (±0.87)^{bc}	Circular	Convex	Blend	Pink

Each value represents mean ±S.E. of three replicates. In the same column, significant differences according to LSD at P=0.05 levels are indicated by different letters. + indicates presence; - indicates absence; WSP-Water soluble polysaccharide; ASP- Alkali stable polysaccharide; IAA- Indole acetic acid; EPS- Exopolysaccharide; Trp^+ -with tryptophan; Trp^- without tryptophan.

Methylobacterium salsuginis. One isolate FM 4 came under group II rather than utilizing the above mentioned carbon sources except lactose which were tentatively identified as *Methylobacterium rhodesianum.* Remaining one isolate FM 3 came under group III utilizing all the 7 carbon sources except ethanol and lactose, which was tentatively identified as *M. radiotolerans*. Green and Bousfield (1982) have already established the classification pattern of *Methylobacterium* at species level based on the C utilization. The results on the variability to the utilization of carbon compounds by the FMs isolates lead to place them into three groups as per the finding of Green and Bousfield (1982). This experiment could results in the identification of 4 species of *Methylobacterium* namely *M. salsuginis*, *M. rhodesianum* and *M. radiotolerans.*

PCR screening of endophytic *Methylobacterium* sp. for the presence of *mxaF* gene :

The *mxaF* gene specific primers amplified a single fragment of 555bp from four *Methylobacterium* sp. strains (Plate 1). Molecular study namely PCR amplification of *mxaF* was conducted to find out the proof for placing, previously unidentified methylotrophic strains at the species level. This *mxaF* gene from four methylobacterial strains including *Methylobacterium extorquens* AM1 have previously been cloned and sequenced (Anderson *et al.,* 1990). In this study *mxaF* gene specific primers amplify a single fragment of 555 bp from four endophytic *Methylobacterium* sp. isolates.

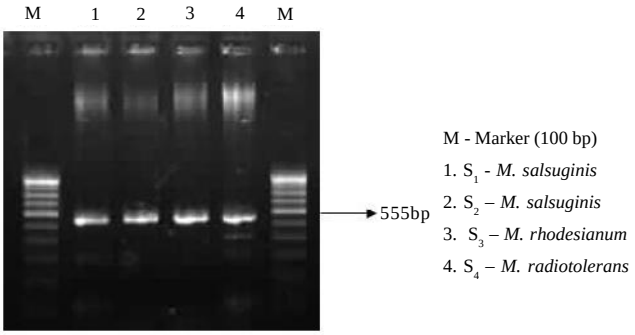

Plate 1: Amplification of methanol dehydrogenase (*mxaF*) gene of endophytic *Methylobacterium* sp. strain by PCR

Growth of *Methylobacterium* sp. isolates for nitrogen-fixing ability :

Isolates obtained from banana leaves were tested for the ability to grow in N-free MMS medium as qualitative evidence of the atmospheric nitrogen fixation (Table 3). All the four isolates recorded positive growth in N- Free medium. Biological nitrogen fixation could have been the only means to meet the nitrogen requirement of the bacterium to grow on N-free MMS medium, as the medium had no organic or inorganic sources of nitrogen. Our results are similar to those reported for presence of a functional *nifH* gene in a non-nodulating *Methylobacterium* sp. which does not belong to *M. nodulans* (Raja *et al*., 2006).

***In vitro* synthesis of indole acetic acid (IAA) by endophytic *Methylobacterium* sp. :**

The growth hormone indole acetic acid production ability of the selected *Methylobacterium* sp. strains were tested both in the presence and absence of tryptophan and results are presented in Table 3. In general IAA were produced by the methylotrophic strains irrespective of the presence or absence of precursor tryptophan. Among the *Methylobacterium* sp. strains, FM2 recorded maximum amount of IAA production (13.01 µg ml^{-1}) followed by FM 3 (10.28 µg ml^{-1}) in the presence of tryptophan. While in the absence of tryptophan, the maximum and minimum IAA was recorded in FM3 and FM4 (5.83 µg ml^{-1}) and (0.97 µg ml^{-1}), respectively. Facultative methylotrophic bacteria were selected from banana cultivar which has the ability to produce IAA isolated from the phyllosphere. Previous studies indicated that the free living PPFM produces cytokinin compounds like trans zeatin, zeatin riboside and trans zeatin riboside and the auxins like IAA (Fall, 1996).

Exopolysaccharide production :

Four isolates were tested for the production of WSP and ASP in AMS medium and the results are reported in Table 3. The amount of ASP production varied from 21.73 µg ml^{-1} of culture liquid in FM 3 to 11.76 µg ml^{-1} of culture liquid in FM 2 in AMS medium. Among the PPFMs, maximum WSP production in AMS medium was recorded in case of FM 1 (157.79 µg ml^{-1}) and minimum in FM 4 (139.43 µg ml^{-1}). EPS production in bacteria occurs as a response to the stress (Robertson and Firestone, 1992). A role for EPS material in the protection of *A. brasilense* Sp245 cells against dessication was suggested by Konnova *et al*. (2001). In the present study, the results are similar to the aforementioned reports.

Conclusion :

In conclusion, from the above findings these endophytic bacteria could be used as effective bioinoculant for sustainable agricultural production. However, greenhouse and field investigations are necessary to confirm this potentiality.

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