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ORIGINAL PAPER

Epiphytic pink-pigmented methylotrophic bacteria enhance germination and seedling growth of wheat (*Triticum aestivum*) by producing phytohormone

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Abstract Methylotrophic bacteria were isolated from the phyllosphere of different crop plants such as sugarcane, pigeonpea, mustard, potato and radish. The methylotrophic isolates were differentiated based on growth characteristics and colony morphology on methanol supplemented ammonium mineral salts medium. Amplification of the *mxaF* gene helped in the identification of the methylotrophic isolates as belonging to the genus *Methylobacterium.* Cell-free culture filtrates of these strains enhanced seed germination of wheat (*Triticum aestivum*) with highest values of 98.3% observed using *Methylobacterium* sp. (NC4). Highest values of seedling length and vigour were recorded with *Methylobacterium* sp. (NC28). HPLC analysis of production by bacterial strains ranged from 1.09 to 9.89 $\mu g\ ml^{-1}$ of cytokinins in the culture filtrate. Such cytokinin producing beneficial methylotrophs can be useful in developing bio-inoculants through co-inoculation of pink-pigmented facultative methylotrophs with other compatible bacterial strains, for improving plant growth and productivity, in an environment-friendly manner.

Keywords Phyllosphere · PPFM · Methylobacteria · *mxaF* · Cytokinin · Seed germination

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Introduction

Pink-pigmented facultative methylotrophs (PPFMs) are phylogenetically diverse proteobacteria with the ability to use C-1 compounds such as formate, formaldehyde and methanol as sole source of carbon and energy (Green and Bousifield 1982). They are ubiquitous in nature, inhabiting a variety of habitats including phyllosphere, root nodules, dust, freshwater, drinking water and lake sediments (Green and Bousifield 1982; Corpe and Rheem 1989). The genus *Methylobacterium* is among the commonly recorded leaf epiphytes and represent an abundant and stable members of the phyllosphere community of a wide range of crop plants (Hirano and Upper 1991; Holland and Polacco 1994; Wellner et al. 2011). These bacteria are phytosymbionts that consume waste products such as methanol produced by the plants (Sy et al. 2005; Gourion et al. 2006; Abanda-Nkpwatt et al. 2006) and synthesize a variety of metabolites useful for the plants including phytohormones (Ivanova et al. 2001; Koenig et al. 2002) that promote plant growth and

yield. By producing plant growth regulators, these organisms are reported to influence seed germination and seedling growth (Dileepkumar and Dube 1992; Holland and Polacco 1992; Holland 1997; Omer et al. 2004). PPFMs possess the ability to alter various physiological traits such as branching, seedling vigour, root differentiation and tolerance to heat and cold (Freyermuth et al. 1996; Holland 1997). They can induce systemic resistance in plants to minimize adverse effects of pathogenic microorganisms (Madhaiyan et al. 2004) and increase the photosynthetic activity in crops (Cervantes et al. 2005).

The importance of *Methylobacterium* in the phyllosphere of plants is well recognized (Knief et al. 2010; Kowalchuk et al. 2010; Wellner et al. 2011). Methanol dehydrogenase (MDH) is a key enzyme to oxidize methanol to formaldehyde, the intermediate of both assimilative and dissimilative metabolism in methylotrophs (Hanson and Hanson 1996; McDonald and Murrell 1997). *mxaF,* a highly conserved functional gene encoding the α-subunit of methanol dehydrogenase was used as a marker for the characterization, identification of methylotrophs and their diversity (McDonald and Murrell 1997; Henckel et al. 1999; Horz et al. 2001). Heyer et al. (2002) observed that the phylogeny of *mxaF* genes and suggested that horizontal transfer of this gene may have occurred across type II MOB (methane oxidising bacteria), and 16S rDNA sequences based phylogeny needs to be utilized for species level identification. Wellner et al. (2011) utilized cultivation dependent and DGGE analysis to understand the diversity of phyllosphere bacteria, with emphasis on *Methylobacterium* spp.

Cytokinins are adenine derived phytohormones that stimulate physiological processes in plants (Srivastava 2002) which are usually produced by phytosymbiotic methylotrophic bacteria inhabiting plant phyllosphere (Holland 1997; Lee et al. 2005). Using a variety of biochemical and cytological methods, it has been confirmed that members of the genus *Methylobacterium* when maintained in liquid culture, produce cytokinins and secrete them into the medium (Kutschera 2007). India is one of the wheat producing and consuming country in the world. To explore the significance of these phytosymbionts on crop plants, we have isolated and identified a number of methylotrophic bacteria from the phyllosphere of different crop plants and evaluated the impact of their cell-free secretions on the seed germination and growth of wheat (*Triticum aestivum*), followed by quantitative analyses of cytokinins.

Materials and methods

Sample collection

Leaf samples from five different tropical crop plants namely, pigeonpea (*Cajanus cajan*), sugarcane (*Sachharum officinarum*), mustard (*Brassica juncea*), potato (*Solanum tuberosum*), radish (*Raphanus sativus*) were collected from agricultural fields in Mau, India (26.01°N; 83.28°E) with the help of forceps and transported to the laboratory in ice bags (4°C). Leaf samples were immediately subjected to the isolation of bacterial population using enrichment media.

Isolation of methylotrophic bacteria

Plant leaves (3 g) were agitated at 150 rpm at 30°C for 2 h in 500 ml Erlenmeyer flasks containing 25 g of glass beads (0.1 cm dia.) and 50 ml of phosphate buffer saline (PBS, containing (gl^{-1}) Na_2HPO_4 1.44; KH_2PO_4 0.24; KCl 0.20; NaCl 8.0; pH 7.4). After agitation, appropriate dilutions of the flask contents were plated onto ammonium mineral salt (AMS) medium (Zahra et al. 2004) (per liter composition; 0.7 g K_2HPO_4, 0.54 g KH_2PO_4, 1 g $MgSO_4 7H_2O$, 0.2 g $CaCl_2 2H_2O$, 4 mg $FeSO_4 7H_2O$, 0.5 g NH_4Cl, 100 g $ZnSO_4 7H_2O$, 30 g $MnCl_2 4H_2O$, 300 g H_3BO_3, 200 g $CoCl_2 6H_2O$, 10 g $CuCl_2 2H_2O$, 20 g $NiCl_2 6H_2O$, 60 g $Na_2MoO_4 2H_2O$, 20 g agar) supplemented with methanol (0.5%; v/v) and cycloheximide (0.1%; 30 mg/ml). Pates were incubated at 30°C for 4−6 days (Kuklinsky et al. 2004). Single, well isolated and differentiated colonies from the enrichment media were transferred on the medium slants and cultures were maintained as glycerol stocks.

Genomic DNA isolation and *mxaF* gene amplification

Cell pellets from the bacterial cultures grown in AMS broth (1.5 ml) for six days were suspended in 0.5 ml SET buffer (75 mM NaCl, 25 mM EDTA and 20 mM Tris) with 10 μl of lysozyme (10 mg ml^{-1}). Genomic DNA of the bacterial cultures was isolated as described by (Pospiech and Neumann 1995). The

integrity and concentration of purified DNA was determined on agarose gel electrophoresis. The total extracted genomic DNA was dissolved in sterile distilled water to obtain a final concentration of 20 ng/µl. The presence of the *mxaF* in the bacterial isolates was authenticated by the partial amplification of the gene using specific primers (Olivier et al. 2005). The forward primer *mxaF*-1003 (5′-GCGGCACCAACTGGGGCTGGT-3′) and reverse *mxaR*-1561 (5′-GGGCAGCATGAAGGGCTCCC-3′) of 550 bp were used (McDonald and Murrell 1997). The amplification was carried out in 100 µl aliquots by mixing 50–90 ng template DNA with the PCR buffer (10×); 100 µM (each) dATP, dCTP, dTTP and dGTP; primers (100 ng each) and 1.0 U *Taq* polymerase. PCR amplification was performed in a thermocycler (BioRad PTC0220) using following conditions: initial denaturation at 94°C for 5 min, 35 cycles consisting of 95°C for 1 min (denaturation), 52°C for 1 min (annealing), 72°C for 1 min (primer extension) and final extension 72°C for 5 min. PCR products were separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide and documented in Alpha Imager TM1200 analysis system.

RFLP analysis of *mxaF* replicons

After *mxaF* gene amplification, the products (1 µg) were digested with endonuclease *Hae*III (Bangalore Genei, India) at 37°C overnight and restriction products were resolved on 6% Polyacrylamide gel. The ethidium bromide stained gel banding pattern was obtained and analyzed by Alpha Imager EC documentation system. Different phylotypes or operational taxonomic units were obtained by similarity and clustering analysis using NTSYSpc-2.02e software from *mxaF*-RFLP pattern. Similarity among the isolates was calculated by Jaccard's coefficient (Jaccard 1912) and dendrogram was constructed using UPGMA method (Nei and Li 1979). PCR products of the representative isolates were purified and sequenced using ABI 3130*xl* automated genetic analyser (Applied Biosystem, UK).

BLAST search and phylogenetic analysis

The partial *mxaF* gene sequences of the isolated strains were compared with those available in the databases. Identification was confirmed using *mxaF* sequence similarity of ≥97% with those of type strains in the GenBank. Sequence alignment and comparison was performed using the multiple sequence alignment program CLUSTAL W2 (Thompson et al. 1994). Minor modifications were done manually on the basis of conserved domains and columns containing more than 50% gaps were removed. The phylogenetic tree was constructed on the aligned datasets using neighbor joining (NJ) (Saitou and Nei 1987) method in MEGA 4.0.2 (Tamura et al. 2007). Bootstrap analysis was performed as described by (Felsenstein 1981) on 1,000 random samples taken from the multiple alignments.

Accession numbers

The sequences were submitted to GenBank and accession numbers were assigned for 16 representative isolates from HQ221357 to HQ221372.

Extraction of cell-free bacterial secretion

Cell-free culture filtrates belonging to 16 representative isolates (after 6 days of growth) were obtained after centrifugation at 10,000×*g* for 15 min. The culture filtrates were fractionated with equal volumes of ethyl acetate thrice and the upper organic layer was subject to dryness under vacuum. The extract was dissolved in 1 ml methanol which was finally used for seed germination assay and HPLC analysis.

Seed germination assay

Wheat seeds (var. PBW 343) used in the seed germination assay was obtained from Directorate of Seed Research, Mau, India. Seed germination studies were carried out as described by (Tiwari et al. 2011). Surface sterilized wheat seeds were immersed in 0.525% NaOCl solution for 15 min followed by subsequent washing with sterilized distilled water. Dried seeds (100) were imbibed in sterile water (2 ml) containing 200 µl extract of different bacterial isolates for 30 min and then dried under air. Seeds soaked in sterile water alone served as control. Seeds placed in Petri plates containing 0.7% water agar were incubated at 25°C in the dark. Three replications were maintained for each treatment. Seed germination, seed vigour index and shoot and root length were recorded in treatments and control (Naik and Sreenivasa 2009).

Table 1 Sampling details of methylotrophs from Mau district of Uttar Pradesh, India

Plant phyllosphere	Location (Mau, India) (26°01′08″N; 83°28′56″E)	Log CFU g^{-1} leaf samples[a]	PPFMs selected	Isolate number
Sugarcane (*Sacchaum officinarum*)	Mahrabandha	6.55	11	NC1–NC11
Pigeonpea (*Cajanus cajan*)	Harpur	6.31	9	NC12–NC20
Mustard (*Brassica juncea*)	Harpur	6.47	11	NC21–NC31
Potato (*Solanum tuberosum*)	Mahrabandha	5.00	8	NC32–NC39
Radish (*Raphanus sativus*)	Salahabad	5.69	10	NC40–NC49

[a] Log values of the colony forming units

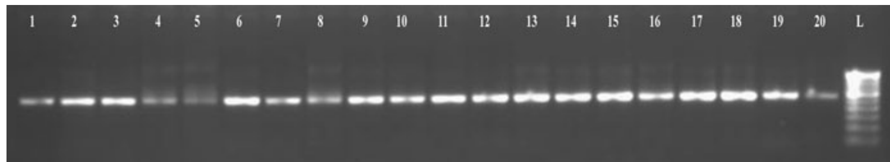

Fig. 1 *mxaF* gene amplification (550 bp) of PPFM isolates (NC1–NC20)

HPLC analysis for cytokinin

High performance liquid chromatography (HPLC) of ethyl acetate fractions from bacterial isolates was performed with the HPLC system (Waters, USA) equipped with binary Waters 515 reciprocating pumps, a variable photodiode array (PDA) detector (Waters 2996), system controller with Waters® Empower™ software for data integration and analysis. Reverse phase liquid chromatographic analysis of the samples was carried out in isocratic conditions with RP-C-18 column (250 × 4.6 mm id, 5 μm particle size) at 25 ± 1°C. Analysis conditions included injection volume 10 μl, flow rate 1 ml/min of the mobile phase methanol: 1.0% acetic acid in water (60:40, v/v) and detection at 254 and 280 nm for cytokinin. Samples were subjected to membrane filtration through 0.45 μm membrane filter prior to injection in the sample loop. HPLC grade solvents and chemicals were purchased from Sigma, USA. Qualitative characterization of the compounds in the sample was done by comparing retention time (R*t*) and co-injection while quantitative analysis was performed by comparing peak areas of the standard chemical.

Statistical analysis

The data obtained on seed germination and HPLC analysis were subjected to analysis of variance (ANOVA) following Duncan's multiple range test with the software SPSS for windows 8.0.0. Differences were considered to be significant at the 95% confidence level. On the basis of RFLP pattern, diversity indices were calculated using BioToolKit320.

Results

From the phyllosphere of five different crop plants, a total of 49 bacterial isolates were isolated on AMS medium. The number of bacterial colonies in the phyllosphere ranged from 10 × 10^4 to 356 × 10^4 cfu (Table 1). The highest numbers of colony forming units (cfu) were obtained from the phyllosphere of the mustard (log cfu 6.477) and sugarcane plants (log cfu 6.551). Pink-pigmented bacteria were selected on the basis of different colony morphotypes on specific AMS media.

mxaF gene amplification

Genomic DNA from all 49 bacterial isolates was used as template for the detection of *mxaF* gene fragments which were amplified using PCR. Among these, twenty isolates showed amplification and partial sequences of 550 bp which authenticated them as *Methylobacterium* (Fig. 1). RFLP analysis of all 49 isolates using *Hae*III (Fig. 2) resulted in 16 distinct

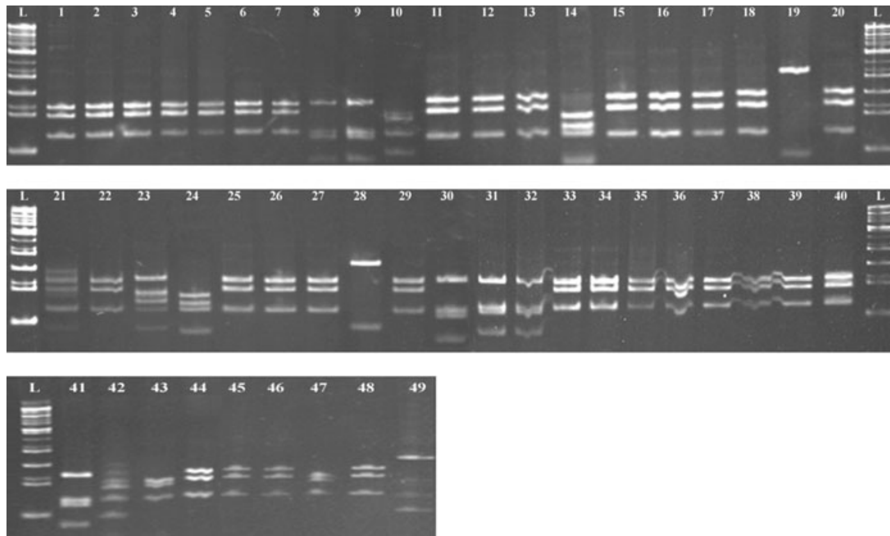

Fig. 2 PAGE analysis of *Hae*III restricted *mxaF* gene (Isolates from NC1 to NC49)

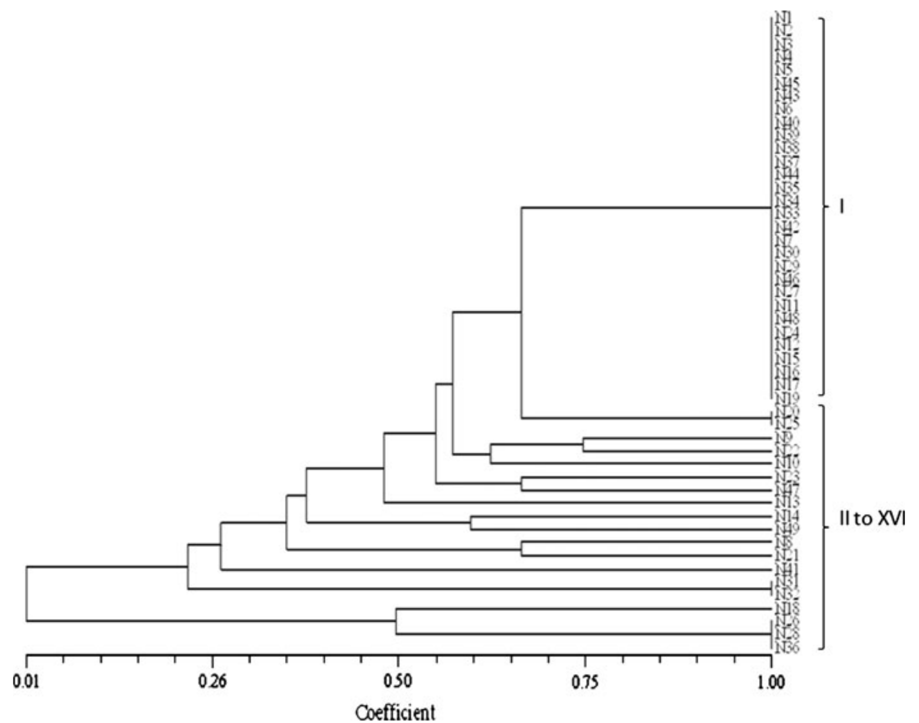

Fig. 3 Clustering of the methylotrophic bacteria based on PAGE profile using UPGMA method

groups of *mxaF* consisting bacteria (Fig. 3). Group I was the largest group (30 isolates). The representatives of each group (total 16 isolates) were sequenced for further identification.

Phylogenetic analysis based on *mxaF* gene sequence (Fig. 4) revealed that the representative isolates belonged to α-proteobacteria and presumptively identified them as belonging to genus *Methylobacterium* (Table 2). Out of the 49 isolates from different crop plants, 4 methylotrophic bacteria were identified from each of the samples from sugarcane, pigeonpea and mustard leaves, 1 from potato and 3 from radish leaves.

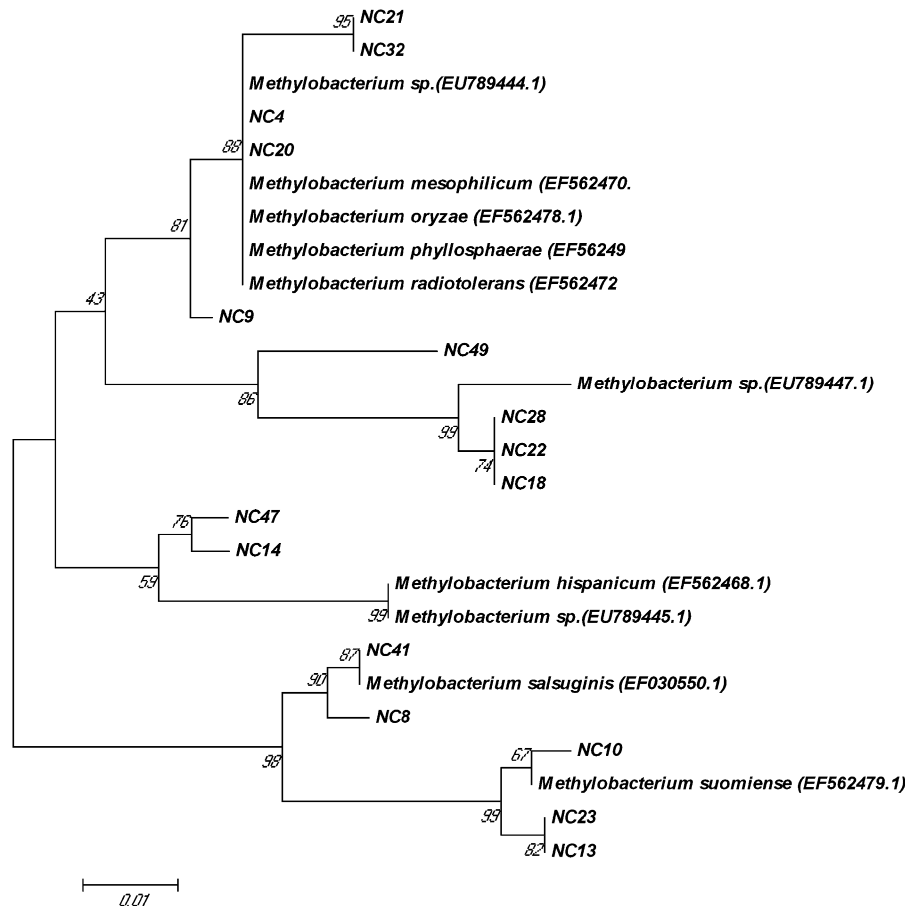

Fig. 4 Phylogenetic tree based on the *mxaF* gene sequences of methylotroph isolates using neighbor-joining method. The data of all genera obtained, are from GenBank database. The *numbers* on the tree indicates the percentages of bootstrap sampling derived from 1,000 replications. *Bar* inferred nucleotide substitutions per nucleotide

Effect on seed germination percentage, seed vigour and length

Treatment with ethyl acetate extract of cell-free culture filtrates of 16 representative members of *Methylobacterium* enhanced wheat seed germination and led to stimulation of seedling growth (Table 3). Among the treatments, the cell free filtrate of *Methylobacterium* sp. (NC4) recorded highest percent germination (98.3%), while lowest values (85%) were recorded with that of *Methylobacterium* sp. (NC49) as compared to control (80%). An enhancement of 5–18% enhancement in percent germination was recorded. Treatment with the extract of a sugarcane isolate *Methylobacterium* sp. (NC4) for 6 days, brought about the highest enhancement in root length (5.13 cm) in wheat seedlings, followed by (5.10 cm) by *Methylobacterium* sp. (NC13), a pigeonpea isolate. In terms of shoot length, wheat seedlings treated with *Methylobacterium* sp. (NC20) isolated from pigeonpea recorded the highest values of 5.73 cm (Table 3). The highest seed vigour index of 1022.3 was recorded with *Methylobacterium* sp. (NC4) which was two fold higher as compared to control (547.6). All other cell free filtrates also resulted in 20–40% higher seed vigour index, over control (Table 3; Fig. 5).

Cytokinin content and wheat seedling length

The highest levels of cytokinins (9.89 μg ml^{-1}) in the cell-free culture filtrate was recorded in the cell free filtrates of *Methylobacterium* sp. (NC4), an isolate from sugarcane plant leaf phyllosphere followed by *Methylobacterium* sp. (NC13) (8.12 μg ml^{-1}) in pigeonpea isolate (Table 2). *Methylobacterium* sp. (NC18), a pigeonpea isolates showed least cytokinin production (1.09 μg ml^{-1}). The effect of ethyl acetate extract of methylotrophic bacterial culture filtrates on seedling length of wheat was linearly correlated with their respective cytokinin content, in most cases (Fig. 6).

Table 2 Cytokinin estimation by high pressure liquid chromatography (NC1–NC11 sugarcane, NC12–NC20 pigeonpea, NC21–NC31 mustard, NC32–NC39 potato, NC40–NC49 radish)

Source	Isolate name and strain number	Amount of cytokinin produced (μg ml^{-1})*	GenBank accession number
Sugarcane	*Methylobacterium* sp. NC4	9.89 a	HQ221357
Sugarcane	*Methylobacterium* sp. NC 8	3.36 d	HQ221358
Sugarcane	*Methylobacteriu* sp. NC9	2.50 ij	HQ221359
Sugarcane	*Methylobacterium* sp. NC10	1.54 l	HQ221360
Pigeonpea	*Methylobacterium* sp. NC13	8.12 b	HQ221361
Pigeonpea	*Methylobacterium* sp. NC14	3.36 jk	HQ221362
Pigeonpea	*Methylobacterium* sp. NC18	1.09 m	HQ221363
Pigeonpea	*Methylobacterium* sp. NC20	2.08 gh	HQ221364
Mustard	*Methylobacterium* sp. NC21	2.23 d	HQ221365
Mustard	*Methylobacterium* sp. NC22	3.93 h	HQ221366
Mustard	*Methylobacterium* sp. NC23	4.74 e	HQ221367
Mustard	*Methylobacterium* sp. NC28	5.17 c	HQ221368
Potato	*Methylobacterium* sp. NC32	2.22 g	HQ221369
Radish	*Methylobacterium* sp. NC41	2.43 f	HQ221370
Radish	*Methylobacterium* sp. NC47	1.27 kl	HQ221371
Radish	*Methylobacterium* sp. NC49	1.80 i	HQ221372
	SEM±	0.065172	
	CD ($P = 0.05$)	0.187737	
	CV %	3.7	

* *Values* in *same column* followed by a *different letter* are significantly different ($\alpha = 0.05$), using Duncan's multiple range test

Discussion

Aerobic methylobacteria are a physiologically and taxonomically diverse group of bacteria with prominent plant growth promoting attributes (Ivanova et al. 2001; Wellner et al. 2011), especially as a result of their abundance on the leaf phyllosphere (Knief et al. 2010). In the present investigation, a number of pink pigmented methylotrophs were isolated from different crop plants, including sugarcane, mustard, pigeonpea, potato and radish. Based on cultivation dependent approach (isolation on AMS media supplemented with methanol) and amplification of the *mxaF* gene, all the isolates characterized in this study were identified as belonging to the genus *Methylobacterium*. This genus includes organisms which are known to be facultative methylotrophs capable of utilizing methanol and some other C_1 compounds, as well as a wide range of multicarbon substrates as their sole carbon and energy source (Green 1992). The gene *mxaF*, a functional gene encoding the α-subunit of methanol dehydrogenase can be used as a marker for confirming the functionality methylotrophs (McDonald and Murrell 1997; Henckel et al. 1999; Horz et al. 2001) and aided in segregation of the 49 isolates into 16 groups in our study. Among the collection of isolates, the bacterial communities inhabiting mustard and sugarcane leaves were found to be the most diverse in terms of their morphotypes and phylogenetic analyses using *mxaF*.

Methylotrophic bacteria are reported to influence the seed germination and growth of many crop plants (Abanda-Nkpwatt et al. 2006; Madhaiyan et al. 2004, 2005, 2006; Radha et al. 2009; Soumya et al. 2011) by producing plant growth regulators like cytokinins and indole acetic acid (Ivanova et al. 2001). Our results illustrated the promise of the isolates, which enhanced the percent germination of wheat seeds by 5–18% over control, besides enhancing vigour index by 33–70%. In comparison to control, extracts also caused early germination of the wheat seeds (data not shown). Proliferation of roots and shoots of treated seeds was also enhanced and in comparison to control and the difference of enhancement ranged from 17.9% in *Methylobacterium* sp. (NC 8) to 39.6% in *Methylobacterium* sp. (NC28) extract treated seeds. The methylotrophic bacteria secreted significant amount of cytokinin in their culture media as is evident from HPLC analysis. The amount of cytokinin produced by the strain NC4 (9.89 μg ml^{-1}) isolated from the

Table 3 Effect of bacterial cell-free culture filtrate extract on root and shoot length, seed vigour and germination

Treatment	Root length (cm)	Shoot length (cm)	Germination[b] %	Seed vigour index[c]
Control	3.23 f[a]	3.53 j	80.0 ± 2.00	547.6
NC4	5.13 ab	5.27 ef	98.3 ± 0.58	1022.3
NC8	3.57 e	4.67 h	94.3 ± 0.58	777.0
NC9	4.53 c	4.33 i	95.0 ± 2.00	841.7
NC10	4.53 c	4.33 i	97.3 ± 0.58	879.8
NC13	5.10 a	4.20 i	96.0 ± 1.00	920.7
NC14	5.00 b	4.93 gh	88.3 ± 2.08	876.8
NC18	4.10 d	5.40 def	97.0 ± 0.00	940.5
NC20	4.07 d	5.60 bcd	97.7 ± 1.15	954.4
NC21	4.20 d	4.90 gh	97.0 ± 1.00	900.9
NC22	4.50 c	5.53 cde	97.2 ± 1.00	982.9
NC23	4.53 c	5.90 b	96.3 ± 1.15	1025.3
NC28	5.00 b	6.20 a	95.7 ± 1.15	1105.4
NC32	4.20 d	4.83 gh	90.7 ± 1.53	819.0
NC41	4.47 c	5.73 bc	89.7 ± 1.53	914.9
NC47	4.10 d	5.30 def	96.3 ± 2.08	924.0
NC49	5.00 b	5.13 fg	85.0 ± 2.00	861.0
SEM±	0.06	0.10	–	
CD ($P = 0.05$)	0.17	0.30	–	
CV	2.4	3.5	–	

[a] *Values* in *same column* followed by a *different letters* are significantly different ($\alpha = 0.05$) in Duncan's multiple range test

[b] Germination percentage per 100 seeds, number of seeds germinated/number of seeds sown × 100

[c] Vigour index, germination percentage × seedling length

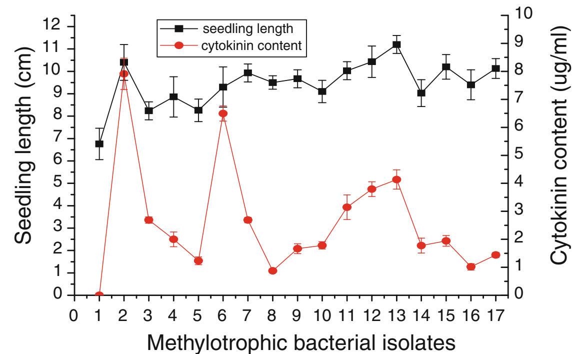

Fig. 6 Effect of ethyl acetate extract of cell-free culture filtrate on wheat seedling length and cytokinin produced by the respective methylotrophic bacterial isolates

pigeonpea was significantly higher in comparison to earlier reported data for *Bacillus subtilis* (1.2 mgl^{-1}, Arkhipova et al. 2007), *Azotobacter vinelandii* (0.75 μgl^{-1}, Taller and Wong 1989), *Azotobacter paspali* (20 μgl^{-1}) and *A. vinelandii* (50 μgl^{-1}) (Baera and Brown 1974). The level of cytokinin production by the bacterial strains showed positive effect on the wheat seedling growth, a parameter of seedling health but, in certain cases the same positive effect was not observed. This may be speculated to be due to the presence of certain other phytohormones produced by these methylobacteria, the studies for which are in progress. To conclude, we have demonstrated the presence of plant growth promoting methylotrophic bacterial community on phyllosphere

Fig. 5 Effect of PPFMs cell extracts on seed germination of wheat (*Triticum aestivum* variety PBW343)

of different crop plants. It would be interesting to analyse their relationships with other groups of methylotrophs using 16S rDNA analyses. The beneficial methylotrophic bacterial community present in the phyllosphere needs to be evaluated as inoculants, not only for plant growth promotion, but also for their utility in biocontrol against foliar diseases.

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