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ORIGINAL ARTICLE

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Regulation of ethylene levels in canola (*Brassica campestris*) by 1-aminocyclopropane-1-carboxylate deaminase-containing *Methylobacterium fujisawaense*

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Abstract We report the presence of ACC deaminase in *Methylobacterium fujisawaense* and its lowering of ethylene levels and promotion of root elongation in canola seedlings under gnotobiotic conditions. To test a part of the previous model proposed for ACC deaminase producing bacteria with *Methylobacterium*, ACC levels and various enzyme activities were monitored in canola. Lower amounts of ACC were present in the tissues of seeds treated with *M. fujisawaense* strains than in control seeds treated with $MgSO_4$. Though the increased activities of ACC synthase in the tissue extracts of the treated seedlings might be due to bacterial indole-3-acetic acid, the amount of ACC was reduced due to bacterial ACC deaminase activity. The activities of ACC oxidase, the enzyme catalyzing conversion of ACC to ethylene remained lower in *M. fujisawaense* treated seedlings. This consequently lowered the ethylene in plants and prevented ethylene inhibition of root elongation. Our results collectively suggest that *Methylobacterium* commonly found in soils, as well as on the surfaces of leaves, seeds, and in the rhizosphere of a wide variety of plants could be better exploited to promote plant growth.

Keywords ACC deaminase · Ethylene · Indole acetic acid · *Methylobacterium*

Abbreviations ACC: 1-aminocyclopropane-1-carboxylate · ACO: ACC oxidase · ACS: ACC synthase · AMS: Ammonium mineral salts · AVG: L-α-(2-aminoethoxyvinyl) glycine hydrochloride · CFU: Colony-forming units · IAA: Indole-3-acetic acid · iPA: Isopentenyladenosine · MTA: 5′-methylthioadenosine · PGPR: Plant growth promoting rhizobacteria · PLP: Pyridoxal phosphate · PPFMs: Pink-pigmented facultative methylotrophic bacteria · PVPP: Polyvinylpolypyrrolidone · SAM: *S*-adenosyl methionine · *t*-ZR: *trans*-Zeatin riboside

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Introduction

The gaseous hydrocarbon ethylene, most popularly associated with ripening, plays a major role regulating seed germination, seedling growth, leaf and petal abscission, organ senescence, stress, and pathogen responses throughout the entire life of the plant (Schaller and Kieber 2002). Interestingly, ethylene can promote and inhibit growth depending on the cell type and plant species (Lehman et al. 1996). Low levels of ethylene appear to enhance root extension; higher levels of ethylene, produced by fast growing roots, can lead to inhibition of root elongation (Mattoo and Suttle 1991; Ma et al. 1998). In general, shoot and root elongation is normally inhibited by ethylene (Abeles et al. 1992) and high levels of ethylene are known to cause proliferation of small lateral roots (Mayak et al. 1999). Ethylene promotes rooting only at a narrow range of concentrations and the role of ethylene in rhizogenesis is still not been clearly understood.

Glick et al. (1998) suggested that plant growth promoting rhizobacteria (PGPR) can stimulate plant growth by lowering the ethylene levels. In ethylene synthesis, methionine is converted to *S*-adenosyl methionine (SAM), 1-aminocyclopropane-1-carboxylate (ACC), and ethylene in three consecutive reactions catalyzed by the enzymes SAM-synthetase, ACC-synthase (ACS), and ACC-oxidase (ACO), respectively (Bleecker and Kende 2000). It was postulated that much of the ACC produced by this latter reaction may be exuded from seeds or plant roots along with other small molecules normally present in seed or root exudate (Bayliss et al. 1997). The ACC in the exudate may be taken up by bacteria and subsequently be hydrolyzed by the enzyme, ACC deaminase to ammonia and α-ketobutyrate. In order to maintain the equilibrium between internal and

external ACC levels, more ACC is exuded by the plant and drawn away from the ethylene synthesis pathway (Glick et al. 1998; Penrose and Glick 2001); this mechanism effectively reduces the amount of ethylene evolved by the plant. Thus, the ability to promote root elongation by PGPR is a direct consequence of the presence of ACC deaminase and more and more experimental evidence indicates that ACC deaminase is one of the key mechanisms by which rhizobacteria promote the growth of plants, especially root elongation.

The presence of ACC deaminase has been studied in various plant growth promoting bacteria like *Enterobacter cloacae* (Penrose and Glick 2001), *Rhizobium* (Ma et al. 2003), and *Pseudomonas*, *Variovorax*, *Alcaligenes*, *Bacillus* (Belimov et al. 2001), etc. The ACC deaminase containing bacteria may be found on leaves and flowers as well as on seeds and roots, and the model based on the interaction of bacteria with seeds and roots is likely applicable to the entire plant (Glick 2004). Bacteria of the genus *Methylobacterium* (PPFMs, pink-pigmented facultative methylotrophic bacteria) are strict aerobic, Gram-negative rods, able to grow on C_1 compounds (Green 1992; Trotsenko et al. 2001). Several aspects of plant growth promotion by *Methylobacterium* have also been investigated, such as the production of urease enzyme (Holland and Polacco 1992), stimulation of seed germination and promotion of root growth and morphology (Holland 1997; Freyermuth et al. 1996), and induced systemic resistance (Madhaiyan et al. 2004).

In this study, we surveyed *Methylobacterium* strains for ACC deaminase activity and the various steps involved in lowering the ethylene levels as proposed in the model for plant growth promotion by ACC deaminase containing PGPR. Three of the five *Methylobacterium* strains were found to possess ACC deaminase activity and were further considered for growth pouch assays and for testing plant growth promoting traits in canola. Though previous studies present a detailed scenario of the steps involved in the process of lowering ethylene by bacteria containing ACC deaminase, the role of indole-3-acetic acid (IAA) of microbial origin in reducing the ethylene levels requires more study. So we also made an attempt to know the role of IAA produced by *Methylobacterium* inoculation on the enzyme activities for ethylene production.

Materials and methods

Bacterial strains and growth conditions

The *Methylobacterium* strains used are listed in Table 1. The isolates from rice (stem, leaf, and rhizosphere soil) were obtained previously by plating the aliquots onto ammonium mineral salts (AMS) minimal medium, with 0.5% methanol as the sole carbon source, as described by Holland and Polacco (1992). The 16S rDNA gene sequence analysis of the isolated bacterial strains placed them under *Methylobacterium*. The *Methylobacterium fujisawaense* type strain KACC10744 (DSM 5686^T) was obtained from the Korean Agricultural Culture Collection (KACC), Suwon, Republic of Korea. The ACC deaminase knockout mutant *E. cloacae* UW4/*AcdS*$^-$ was kindly provided by Bernard R. Glick, Department of Biology, University of Waterloo, Ontario, Canada and was cultured in tryptic soy broth (Difco, Laboratories, Detroit, MI, USA) containing 15 μg tetracycline ml^{-1} at 30°C.

ACC deaminase assay

The isolates were checked for their ability to utilize ACC as a N source by assessing their growth in DF minimal salts medium (Dworkin and Foster 1958) supplemented with 3 mM ACC. ACC deaminase activity of the isolates was measured using a spectrophotometer that measured the absorbance at 540 nm, the α-ketobutyrate produced from the enzymatic cleavage of ACC as described previously by Shah et al. (1998), and a modified procedure of Honma and Shimomura (1978). The α-ketobutyrate produced was estimated by comparing the absorbance at 540 nm of a sample to a standard curve of α-ketobutyrate (Sigma-Aldrich Co., St Louis, MO, USA) ranging between 0.1 and 1.0 mmol. A stock solution of α-ketobutyrate was prepared in 0.1 M Tris–HCl (pH 8.5) and stored at 4°C. To measure specific activity of the cultures protein estimation was carried out according to Lowry method (Lowry et al. 1951).

Plant growth promoting characteristics of *Methylobacterium* strains

The production of indoles in the culture supernatants of the bacteria was measured spectrophotometrically at 530 nm after incubation of the bacteria for 5 days on liquid minimal medium (composition in g l^{-1}: 2 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 0.025 g $MgSO_4 \cdot 7H_2O$, and 0.002 g $FeSO_4 \cdot 7H_2O$, pH 7.2) supplemented with 0.5% methanol (v/v) and with L-tryptophan (100 mg l^{-1}). The reaction consisted 2 ml of cell free suspension to which 100 μl of 10 mM orthophosphoric acid added and 4 ml of Salkowski's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) followed by incubation at room temperature for 25 min. The assay was calibrated by generating a standard curve for samples containing IAA. The culture extracts for analyzing cytokinins were prepared according to Koenig et al. (2002) and the immunoassays were performed as described later to determine the amounts of the two cytokinins, isopentenyladenosine (iPA) and *trans*-zeatin riboside (*t*-ZR), present in the culture supernatants.

Ethylene production by seedlings treated with ACC deaminase producing *Methylobacterium* strains

Surface sterilized canola (*Brassica campestris*) seeds (Hungnong seeds, Seminis Korea Inc., Republic of

Table 1 Details of *Methylobacterium* strains used.[a] The strains, their sources, appearance of the colonies on agar plates, their reaction towards Gram-staining are given

Strain	Location	Crops/cultivar	Parts	Colony morphology	Gram staining	Accession number	Taxon
CBMB20	Cheongwon, Chungbuk	Rice/Nampeoung	Stem	Reddish pink	–	AY683045	*Methylobacterium fujisawaense* (99%)[b]
CBMB110	Yeongnam, Milyang	Rice/Il-Mi	Leaf	Reddish pink	–	AY683046	*M. fujisawaense* (99%)
CBMB120	Cheongwon, Chungbuk	Rice/Dong-Jin	Soil	Pink	–	AY683047	*Methylobacterium extorquens* (99%)
CBMB130	Cheongwon, Chungbuk	Rice/Dong-Jin	Soil	Pink	–	AY683048	*M. extorquens* (99%)
Mf^T	–	–	–	Pink	–	AJ250801	*M. fujisawaense* DSM 5686^T

[a]Isolates from rice (*Oryza sativa* L.) identified according to their published 16S rDNA sequences (NCBI) and best hits with percent values in brackets are given; [T]*M. fujisawaense* type strain obtained from Korean Agricultural Culture Collection (KACC, Suwon, Republic of Korea)
[b]According to sequence identities (%) at NCBI

Korea) were imbibed in 0.03 M $MgSO_4$ (control) or a bacterial suspension ($A_{600\ nm} = 0.15$) for 2 h. Thirty seeds were placed in 120-ml vials on sterile filter paper before 2 ml of water was added. The ethylene production was measured on day 7, after removing the excess water. The vials were capped with a rubber septum and following 4-h incubation, 1 ml of headspace was sampled from each vial and the ethylene content measured in a gas chromatograph (GC) (DS 6200, Donam Instruments Inc., Republic of Korea) packed with Poropak-Q column at 70°C, equipped with a flame ionization detector. The carrier gas was N_2 at the flow rate of 60 ml min^{-1} and the combustion gas was H_2 at the flow rate of 50 ml min^{-1} with the combustion-supporting gas air at the flow rate of 500 ml min^{-1}. The capacity of the column for ethylene trapping was checked in a model experiment in which air containing 0.2 nmol ethylene l^{-1} was passed through the column for 60 min; under these conditions, 100% of the ethylene was trapped.

Gnotobiotic root elongation assay

Gnotobiotic root elongation assay was performed with canola to study effects of seed treatment with ACC deaminase producing methylobacteria. The bacterial strains were grown aerobically to late log phase in liquid AMS medium and then transferred to DF salts medium containing ACC (3.0 mM) as the sole source of nitrogen. The culture conditions and the procedure for gnotobiotic growth pouch assay followed Penrose and Glick (2003). Surface sterilized canola seeds were incubated for 1 h at room temperature with the appropriate treatment: sterile 0.03 M $MgSO_4$ (used as a negative control), 10^{-4} M L-α-(2-aminoethoxyvinyl) glycine hydrochloride (AVG) (Sigma, USA) dissolved in 0.03 M $MgSO_4$ (used as a positive control), or bacterial suspensions in sterile 0.03 M $MgSO_4$ (absorbance of 0.15 at 600 nm). *E. cloacae* UW4 *AcdS*$^-$ is a mutant strain that lacks ACC deaminase activity and was used as a negative control in this study. The root lengths of the canola seedlings were measured following 5 days of growth at 20 ± 1°C beginning with a cycle of 12 h of dark followed by 12 h of light (18 μmol m^{-2} s^{-2}). The seedlings were assayed for ACC levels, in vitro ACO, ACS activity, and for immunoassays estimating IAA and cytokinins.

Measurement of ACC level in plant tissue

Plant tissues frozen in liquid nitrogen, stored at −20°C were used. One gram of frozen tissue was extracted in 5 ml 80% methanol containing butylated hydroxytoluene as antioxidant (BHT, 2 mg l^{-1}) and incubated at room temperature for 45 min. Samples were centrifuged at 2,000*g* at 20°C for 15 min, the pellet resuspended in 4 ml methanol and centrifuged. The combined supernatants were evaporated to dryness under vacuum. Residues were resuspended in 2 ml distilled water and ACC levels were determined by the method of Wachter et al. (1999) using the protocol of Lizada and Yang (1979). In brief, the upper aqueous phase (0.5 ml) obtained by extracting with dichloromethane was mixed with 0.1 ml $HgCl_2$ (80 mM) in test tubes and sealed with rubber septa. 0.2 ml NaOCl solution (40 parts NaOH, 80 parts 12.5% NaOCl solution, 30 parts distilled water) was injected into the tubes, shaken, and incubated for 8 min. One milliliter of the gaseous portion was removed and assayed for ethylene with a GC as described above. The efficiency of ACC oxidation, on average 60%, was estimated by analyzing replicate samples containing internal standards of ACC.

In vitro ACO and ACS activity

The protein extracts for measuring the in vitro ACO activity were prepared according to Petruzzelli et al. (2000). The frozen tissues were pulverized in liquid nitrogen and homogenized in 2 ml g^{-1} of extraction

buffer consisting of 100 mM Tris–HCl (pH 7.2), 10% (w/v) glycerol, and 30 mM sodium ascorbate. The homogenate was centrifuged at 28,000*g* for 15 min at 4°C. The supernatant was used for the in vitro ACO assays (Malerba et al. 1995). The enzyme activity was assayed at 30°C for 15 min in 10-ml screwcap tubes fitted with a Teflon-coated septum containing 1.5 ml of supernatant, 50 μM $FeSO_4$, 1 mM ACC, and 5% (v/v) CO_2. At the end of this time period, the quantity of ethylene released into the headspace was determined by GC.

Enzyme extracts for the ACS activity were obtained by homogenizing a 1 g sample of pulverized tissues in 4 ml of 100 mM Na phosphate (pH 9.0) containing 5 μM pyridoxal phosphate (PLP), 4 mM 2-mercaptoethanol (2-ME), 1 mM EDTA, and 10% glycerol in the presence of 1 g polyvinylpolypyrrolidone (PVPP). The precipitate obtained between 35 and 75% saturation after ammonium-sulfate fractionation was resuspended in 2.5 ml of a solution containing 100 mM Na phosphate (pH 7.8), 5 μM PLP, 0.5 mM 2-ME, and 10% glycerol. The preparation of the enzymes was carried out at 4°C (Satoh et al. 1997). Protein content was determined according to the method of Lowry et al. (1951) with BSA as a standard. ACS activity was assayed by incubating a 100-μl enzyme solution (1.29-mg protein) with 100 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)–KOH (pH 8.5), 5 μM PLP, 100 μM *S*-adenosyl-L-methionine, and test chemicals at given concentrations in a total volume of 400 μl. After incubation for 15 min at 30°C, the amount of ACC produced was determined as described above.

Immunoanalysis of IAA and cytokinins in plant tissues

The IAA and cytokinins concentrations in plant extracts were determined using immunoassay by enzyme linked immunosorbant assay (ELISA). Extracts were prepared by homogenizing the seedlings (1 g) with TBS buffer (per liter, 3.03 g Trizma base, 5.84 g NaCl, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.2 g sodium azide, pH 7.2) in a ratio of 1:4 (w/v) in sterile 50 ml centrifuged tubes. Supernatants of the extracts (collected by centrifuging the extract at 3,885*g* for 5 min, and recentrifuging at 3,885*g* for 3 min) were used for immunoassays. All cytokinins and IAA were purchased from Sigma, and ELISA test kits were from AGDIA (AGDIA Inc., Indiana, USA). ELISA tests were performed according to kit instructions. Stock solutions of IAA and cytokinins (10 mM) were prepared in absolute methanol and stored at 4°C. Dilutions were made with sterile AMS media as diluent to build the standard curves. Samples were diluted as needed to obtain accurate estimates within the ranges of standard concentrations. The absorbance was read at 405 nm using an ELISA plate reader (BIO-RAD Model 550, Japan).

Statistical analysis

All experiments were repeated twice with three replicates each and the extractions were conducted in triplicate. Standard curves were generated by regression analysis (Excel, Microsoft) for every enzyme assay. The data were subjected to analysis of variance and testing of means by Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$ using SAS, Version 8.2 (SAS 2001).

Results

Identification and plant growth promoting characteristics of *Methylobacterium* strains

The characteristics and the results of taxonomic identification of the *Methylobacterium* strains are given in Table 1. The strains were identified up to the species level based on 16S rRNA gene sequence similarity (99%) and it was established that the studied bacteria belonged to *M. fujisawaense* and *M. extorquens*. The *Methylobacterium* strains varied in their ability to utilize ACC and significant differences could be observed in the ACC deaminase activity of cell free extracts. No ACC deaminase activity was detected in cell free extracts of the *M. extorquens* strains CBMB120 and CBMB130. The

Table 2 ACC deaminase activities, IAA and cytokinins production of the *Methylobacterium* strains. IAA in the culture supernatants grown in minimal medium was estimated with (Trp^+) and without (Trp^-) the supplementation of L-tryptophan at 100 mg l^{-1}. Cytokinins present in the culture extracts were determined according to ELISA immunoassay tests. Data are presented as mean ± SE of three replicates

Strain	ACC deaminase activity (nmol α-ketobutyrate mg^{-1} of protein h^{-1})	IAA production (μg ml^{-1} of culture)		Cytokinins recovered (ng l^{-1} of culture)		
		Trp^+	Trp^-	iPA	*t*-ZR	Total
CBMB20	94.48 ± 6.83	2.33 ± 0.11	1.72 ± 0.08	47.01 ± 0.45	32.92 ± 1.43	79.92 ± 0.98
CBMB110	24.74 ± 4.12	4.03 ± 0.20	1.07 ± 0.05	41.87 ± 1.26	26.23 ± 1.24	68.10 ± 2.05
CBMB120	–	7.04 ± 0.26	0.61 ± 0.04	38.85 ± 0.93	15.93 ± 0.46[a]	54.78 ± 0.63[a]
CBMB130	–	8.29 ± 0.49	0.51 ± 0.02	40.09 ± 0.43	16.05 ± 0.36[a]	56.14 ± 0.76[a]
Mf[T]	5.23 ± 0.58	9.04 ± 0.33	1.91 ± 0.03	37.19 ± 0.42	17.06 ± 0.23[a]	58.12 ± 0.26[a]
LSD ($P \leq 0.05$)	15.06	0.57	0.08	1.95	3.02	3.88

[a]The treatments are not significantly different from each other at 5% threshold (LSD). [T]*M. fujisawaense* type strain

activities were higher in the extracts of *M. fujisawaense* strains, CBMB20 and CBMB110, than in the type strain *M. fujisawaense* producing 94.48, 24.74, and 5.23 nmol α-ketobutyrate mg^{-1} of protein h^{-1}, respectively (Table 2). Other properties of *Methylobacterium*, which could be considered characteristics that are of concern in plant PGPR interactions, were studied. The quantitative analysis with the Salkowski reagent of the culture liquids of methylobacteria grown in defined medium with L-tryptophan and incubated for 5 days produced significantly different amounts of IAA ($P = 0.05$). The five *Methylobacterium* strains accumulated the phytohormone in amounts ranging from 2.33 to 9.04 μg ml^{-1} in the presence of L-tryptophan (Table 2). When L-tryptophan was not supplemented to the culturing medium, the production of IAA was in the range of 0.51–1.91 μg ml^{-1}. Immunoassays using ELISA kits were performed to determine whether the *Methylobacterium* produced cytokinins. The cytokinins *t*-ZR and iPA were present at detectable and replicable levels in all of the cultures tested. *t*-ZR was present in smaller quantities than iPA (Table 2). The levels of cytokinins recovered from the cultures were variable and the strains CBMB20 and CBMB110 produced higher amounts of cytokinins compared to the other strains. Since the strains CBMB120 and CBMB130 showed no growth on ACC containing media, we could not proceed with these strains and selected only CBMB20 and CBMB110 for the gnotobiotic assays.

Gnotobiotic root elongation assay

The germination percentage of the *Methylobacterium* treated seeds was comparatively greater when compared to control. CBMB20 recorded the highest percentage of germination (96.67) followed by CBMB110 (91.67%). But the germination percentage in *E. cloacae* UW4/*AcdS*$^-$ and AVG treated seeds showed significantly lower values compared to control (Table 3). Following 5 days in gnotobiotic growth pouches, canola roots from seeds treated with *Methylobacterium* strains CBMB20 and CBMB110 showed significant increases in root length when compared with either the control or the *AcdS*$^-$ knockout mutant treated seeds. The average root length of the *M. fujisawaense* strains CBMB20, CBMB110 treated plants were 8.50 and 7.67 cm, respectively, when compared with those treated with *E. cloacae* UW4/*AcdS*$^-$ (4.98 cm) and the untreated seeds, i.e. control (4.76 cm) (Table 3). The roots of the *M. fujisawaense* strains including reference strain treated canola were, on average, nearly twice as long as either untreated plants or plants treated with the mutant strain. Seeds treated with AVG, an ethylene synthesis inhibitor, produced an increase of 30.88% in root length compared to control.

Monitoring ethylene production

Measurements of ethylene emission by GC detected considerable amounts of ethylene emitted, both from treated and untreated plants from 0.204 and 2.40 nmol g^{-1} FW h^{-1}. *Methylobacterium* treated canola seedlings emitted lower levels of ethylene when compared to the untreated seedlings (Fig. 1a). The ethylene emitted from the seedlings treated with a suspension of *M. fujisawaense* strains CBMB20 and CBMB110 was equivalent to that from seedlings treated with the ethylene inhibitor AVG and that from *E. cloacae* UW4/*AcdS*$^-$, a negative ACC mutant, is equal to that of untreated canola seedlings.

Enzyme activities regulating ethylene production

ACC, the immediate precursor of ethylene was quantified in the tissues of 8-day-old canola seedlings and compared between the treated and untreated seeds, showed significant differences ($P = 0.05$). The ACC accumulated in the plant tissues was significantly reduced in treatments with *M. fujisawaense* strains, CBMB20, CBMB110, and *M. fujisawaense* type strain with a reduction of 37.38, 61.50, and 13.61% respec-

Table 3 Effects of *Methylobacterium* inoculation on the root length of canola under gnotobiotic conditions. Seeds that failed to germinate on day 2 were marked and the roots developed from these seeds were not measured. Data are represented as mean ± SE

Treatment[a]	Germination (%)	Root length (cm)[b]	% Increase over control in root length
Control	86.67 ± 0.79	4.76 ± 0.31	
CBMB 20	96.67 ± 2.67	8.50 ± 0.47	78.56
CBMB110	91.67 ± 1.73	7.67 ± 0.27	61.13
AVG	78.33 ± 3.93[c]	6.23 ± 0.16[c]	30.88
UW4/*AcdS*$^-$	80.00 ± 1.89[c]	4.98 ± 0.37	4.62
Mf^T	88.33 ± 3.46	6.47 ± 0.32[c]	35.62
LSD ($P \leq 0.05$)	4.52	0.41	

[a]Treatment abbreviations: control, 0.03 M $MgSO_4$; *M. fujisawaense* strains CBMB20 and CBMB110; AVG, 0.0001 M L-α-(2-aminoethoxyvinyl)glycine hydrochloride; *Enterobacter cloacae* UW4/*AcdS*$^-$ and *Mf*^{T}, *M. fujisawaense* type strain

[b]Mean root lengths of 5-day-old canola seedlings assessed by means of the root elongation assay from measurements of 60 seedlings (6 seeds/growth pouch; 10 growth pouches/repetition; two repetitions)

[c]The treatments are not significantly different from each other at 5% threshold (LSD)

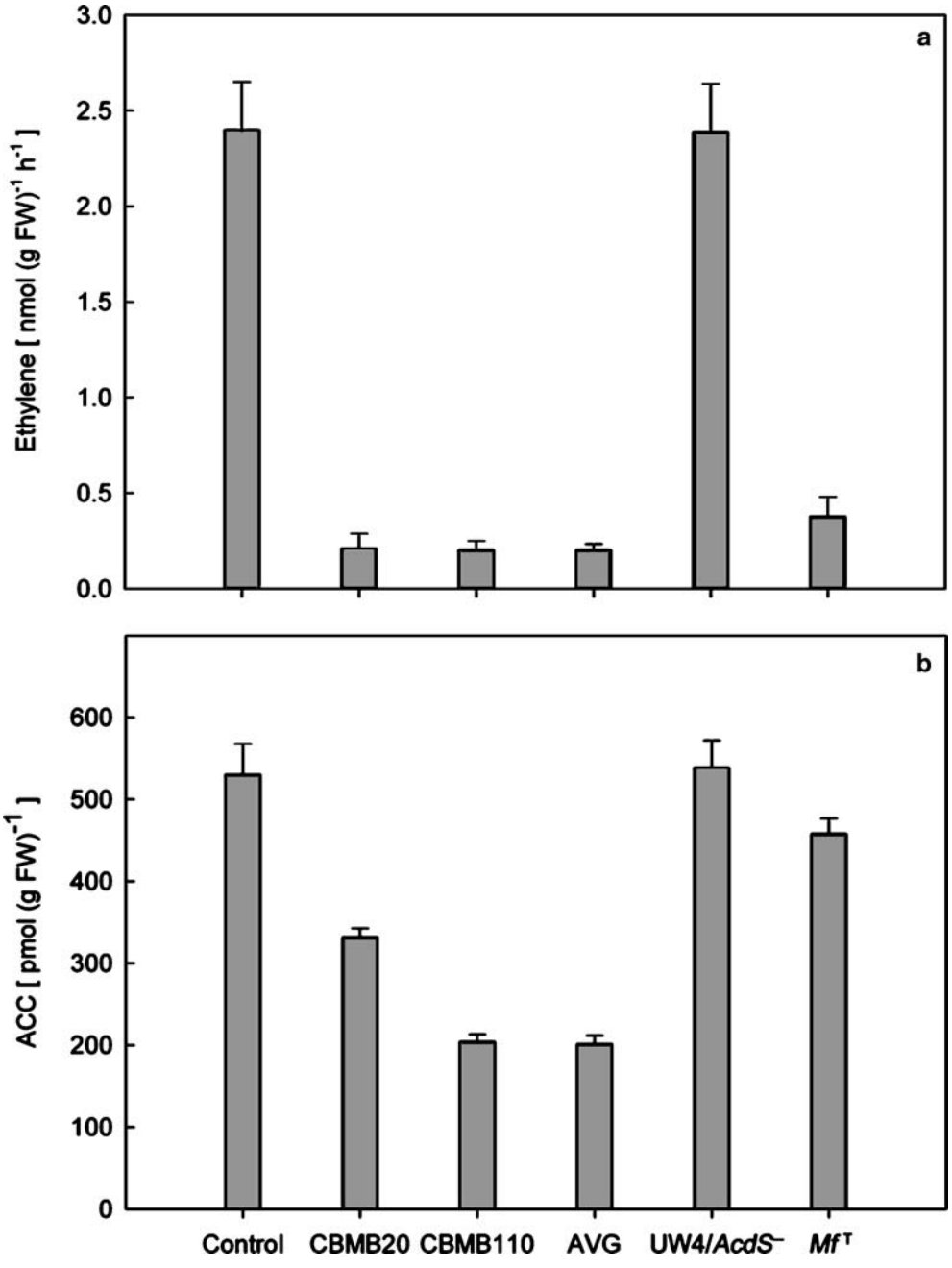

Fig. 1 Effect of *Methylobacterium* inoculation on ethylene emissions (**a**) and ACC accumulations (**b**) in canola. For ethylene emission measurements, seedlings were grown on vials for 7 days, sealed, and following 4-h incubation, ethylene in the headspace sample was measured on a GC. Each value represents the mean ± SE, $n=3$. AVG included as a positive control and the UW4/*AcdS*$^{-}$ as a negative control. ^{T}*Methylobacterium fujisawaense* type strain

tively, when compared to control. Seedlings from canola seeds treated with the ethylene inhibitor, AVG, showed the lowest level of ACC, with 62.01% reduction. But those treated with the ACC negative mutant *E. cloacae* UW4/*AcdS*$^{-}$ showed equal amounts of ACC as that of control (Fig. 1b).

The activity of ACS, the enzyme catalyzing the conversion of SAM to ACC could be measured on a GC by measuring the amount of ACC. The in vitro ACS activity measured from the extracts remained significantly higher in the *Methylobacterium* treated seedlings (Fig. 2a). The enzyme activity of ACO, which converts ACC into ethylene could be measured in vivo, i.e. ethylene production of intact tissues incubated with a saturating ACC concentration, and in vitro, i.e. ethylene production in assays with protein extracts (Fluhr and Mattoo 1996). The in vitro ACO activity measured in canola seedlings treated with *Methylobacterium* strains decreased when compared with the untreated seedlings (Fig. 2b). In contrast the seedlings treated with *E. cloacae* UW4/*AcdS*$^{-}$ had higher amounts of ACO, since this bacterial strain lacked the ACC deaminase gene.

IAA and cytokinins in plant tissues

IAA and cytokinins in plant extracts were detected using ELISA test immunoassays. The amount of IAA and cytokinins increased with bacterial treatments compared to AVG treatment which inhibits ethylene production.

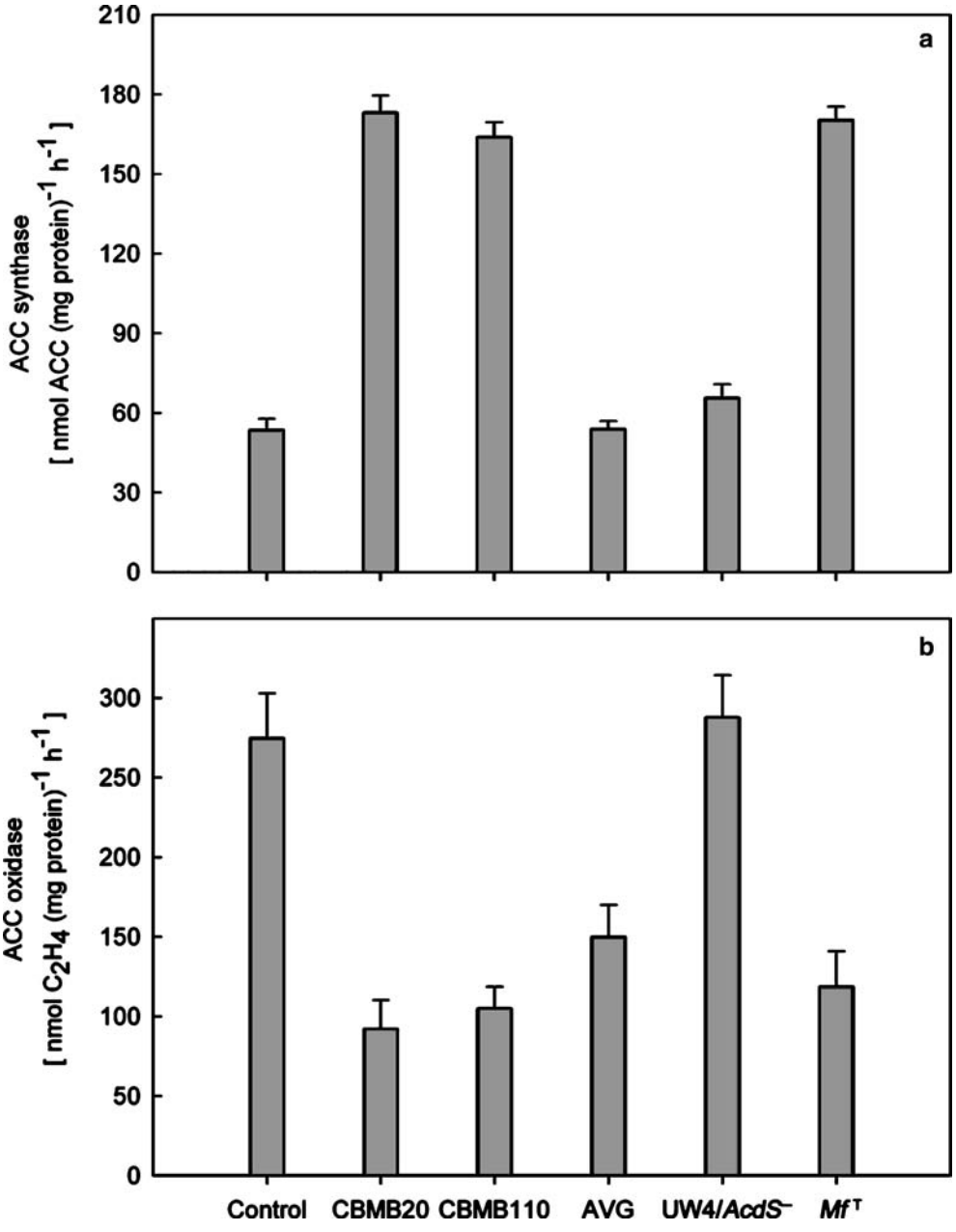

Fig. 2 Effects of *Methylobacterium* inoculation on the in vitro activity of ACC synthase (**a**) and ACC oxidase (**b**) obtained from canola seedlings. ACC synthase activity was determined from the amount of ACC formed with 100 µM *S*-adenosyl-L-methionine as a substrate. ACC oxidase was determined by supplying 1 mM ACC as a substrate. Each value represents the mean ± SE, $n = 3$. ^{T}*M. fujisawaense* type strain

The IAA concentrations varied from 32.21 to 83.36 pmol g^{-1} FW with significant differences between the treatments, and in *Methylobacterium* treated plants it ranged from 50.15 to 83.18 pmol g^{-1} FW (Fig. 3a). The total cytokinins (iPA + *t*-ZR) in bacterial treatments varied from 1.18 to 2.50 nmol g^{-1} FW with variations within them. Maximum amount of cytokinins could be seen in the extracts of plants treated with *M. fujisawaense* strains CBMB20 and CBMB110 (Fig. 3b).

Discussion

The *Methylobacterium* strains used in this study produced considerable amounts of IAA and cytokinins. The phytohormones produced by *Methylobacterium* stimulate seed germination and plant development (Madhaiyan et al. 2005a, b) and the cytokinins produced by them are physiologically meaningful to the host plants or alternatively, they induce greater cytokinins in the host (Butler et al. 2000). But out of the five strains used, only three strains utilized ACC revealing the presence of ACC deaminase and also possessed considerable ACC deaminase activity. Similar results were also obtained in *Rhizobium* where only five of the 13 strains surveyed for ACC deaminase activity were positive (Ma et al. 2003).

Under the gnotobiotic growth pouch assay, the *Methylobacterium* treated seedlings had longer roots and also emitted lower ethylene when compared to control or the seedlings treated with *E. cloacae* UW4/*AcdS*$^-$. The inoculation of three ACC deaminase containing

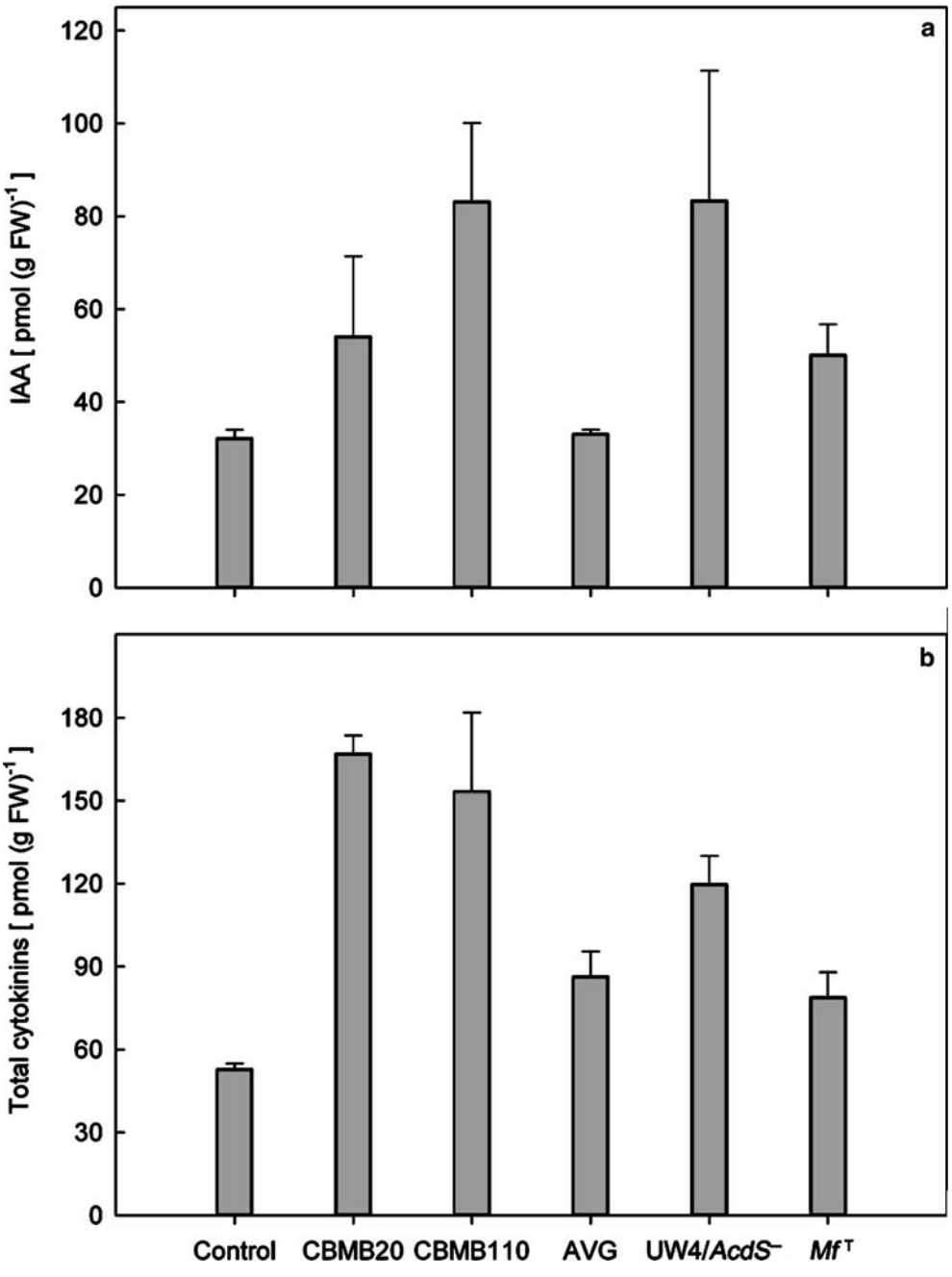

Fig. 3 IAA (**a**) and total cytokinins (**b**) (iPA and *t*-ZR) levels in canola plants treated with *Methylobacterium* strains. Seedlings were grown for 10 days in 12 h light/12 h dark at 20 ± 1°C in a growth pouch, after which the whole seedlings were collected. Each value represents the mean ± SE, $n = 3$. $^{T}M.$ *fujisawaense* type strain

Bacillus strains stimulated root elongation in canola seedlings under gnotobiotic conditions (Ghosh et al. 2003) whereas significant reduction could be noted in the length of canola roots between seeds treated with the wild type bacterium and those treated with *E. cloacae* UW4/*AcdS*$^{-}$ mutant (Li et al. 2000). When the PGPR contains the enzyme ACC deaminase, the bacterial cells act as a sink for ACC, the immediate biosynthetic precursor of ethylene thereby lowering plant ethylene levels and decreasing the negative effects of various environmental stresses (Stearns et al. 2005). Treatment with AVG also resulted in increased root lengths and the promotive effects may result from a reduction in ethylene concentration or inhibition of ethylene action (Khalafalla and Hattori 2000).

Ethylene action and biosynthesis is regulated by different hormones with auxin stimulating its production by promoting the de novo synthesis of ACC synthase and ACC and cytokinins and brassinosteroid (BR) increasing auxin-induced ethylene production (Yi et al. 1999; Swarup et al. 2002). The IAA and cytokinins concentrations in the extracts of canola increased with *Methylobacterium* inoculations in this study. An increase of ACS activity was observed in the treated plants which might be a direct consequence of increased IAA and cytokinins. This is in accordance to the model of

Glick et al. (1998) suggesting that microbial binding to the surface of seed or root exudates taken up by the plant together with endogenous plant IAA can induce ACC synthase which converts SAM to ACC.

But ACC formed from methionine, by the action of ACS remained lower in the seedlings treated with ACC deaminase producing *Methylobacterium* strains. It was postulated that much of the ACC produced by the action of ACS may be exuded from the seeds or plants (Bayliss et al. 1997) and might be taken up by the bacteria and subsequently hydrolyzed by enzyme ACC deaminase to ammonia and α-ketobutyrate. Supportingly, in our study the ACC present in the plants treated with *E. cloacae* UW4/*AcdS*$^-$, a negative mutant of ACC deaminase remained higher than in those treated with ACC deaminase producing *Methylobacterium*. With reference to the *Methylobacterium* strains used the *M. fujisawaense* that recorded lower ACC deaminase activity than the other two strains, resulted in higher ethylene emissions and the ACC in the plant tissues also remained higher. Also the ACC in the AVG treated plants remained lower, since the conversion of methionine to ACC is strongly inhibited by AVG, an inhibitor of pyridoxal phosphate mediated reactions (Adams and Yang 1979). The ACO activity remained lower in the plants treated with *Methylobacterium* strains, when compared to the untreated seeds or treatment with AVG and *E. cloacae* UW4/*AcdS*$^-$. Since ACC is the substrate for ACO, reductions in the substrate level would lead to reduced activities of ACO subsequently reducing the amount of plant ethylene, the product of ACC oxidation.

This is the first report on the existence and prevalence of ACC deaminase in *Methylobacterium* spp. providing evidence for promotion of root elongation by *Methylobacterium* as a consequence of reducing ethylene levels in the plant. The results obtained here suggests that plant growth promotion by the ACC utilizing

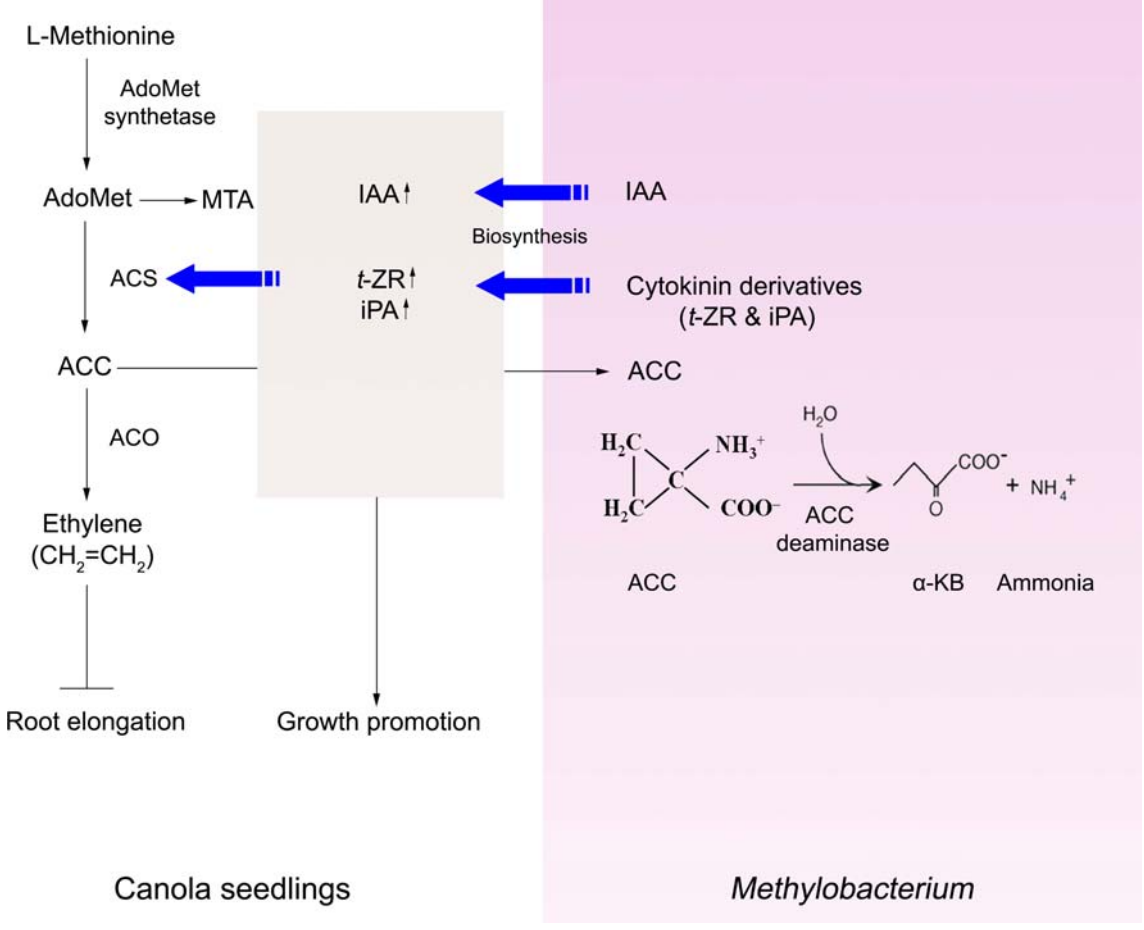

Fig. 4 Hypothetical model illustrating the steps involved in lowering of plant ethylene levels and other activities of *Methylobacterium* in plant growth promotion (modified from Glick et al. 1998). The model is subdivided into two phases: the ethylene biosynthetic pathway in plants and the ACC breakdown in *Methylobacterium* resulting in reduced ethylene in plants. The *arrows* indicate a chemical or physical step in the mechanism, the *block arrows* indicating increases in the concentration of compounds and the symbol ⊥ indicates inhibition of root elongation by ethylene. *Methylobacterium* results in increased plant hormones and also cleaves ACC, the immediate precursor of ethylene preventing ethylene inhibition of root elongation in canola seedlings. *ACC* 1-aminocyclopropane-1-carboxylic acid, *ACO* ACC oxidase, *ACS* ACC synthase, *AdoMet S*-Adenosyl-L-methionine, *IAA* indole-3-acetic acid, *iPA* isopentenyladenosine, *MTA* 5′-methylthioadenosine, *t-ZR trans*-Zeatin riboside

Methylobacterium is due to the reduction of plant ethylene through the utilization of ammonia cleaved from ACC by its ACC deaminase according to the hypothesis made by Glick et al. (1998). From our results, we proposed a modification of the model of Glick et al. (1998) for how *Methylobacterium* promotes plant growth (Fig. 4). Inoculation of canola with *Methylobacterium* strains increases the IAA and cytokinin concentration of the plants resulting in increased ACS activity. If the PGPR strain contains no ACC deaminase, ACC levels in the seedlings are unaltered and ethylene is still produced which inhibits root elongation. If the PGPR strain contains ACC deaminase, ACC levels in the plant drop reducing ethylene levels and releasing root growth from the inhibitory effects of this hormone. Also the ubiquitous nature and the ability to effectively utilize methanol, the metabolic waste product of plants provides additional benefits in the use of *Methylobacterium* as a plant growth promoting bacteria.

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