Plant growth promontory attributes by 1-aminocyclopropane-1-carboxylate (ACC) deaminase producing *Methylobacterium oryzae* strains isolated from rice

Woo-Jong Yim, P.S. Chauhan, M. Madhaiyan, S. C. Tipayno and **Tong-Min Sa***

Department of Agricultural Chemistry, Chungbuk National University, Cheongju, Chungbuk, 361-763, Republic of Korea.
E-mail: tomsa@chungbuk.ac.kr

Abstract
Species from the pink-pigmented facultative methylotrophic bacteria (PPFMs) genus *Methylobacterium* are versatile in nature and common inhabitants of plants, potentially dominating the phyllosphere population, but are also found in other organs. The consistent success of the *Methylobacterium*-plant association relies on methylotrophy, the ability to utilize the one-carbon compound methanol emitted by plants. Accordingly, the present study investigated the inoculation effects of *Methylobacterium oryzae* strains CBMB20 and CBMB110 on plant growth and accumulation of phytohormone levels of tomato and red pepper under gnotobiotic conditions. Seeds treated with the *Methylobacterium* strains showed a significant increase in root length when compared with the uninoculated control. Extracts of the plant samples were used for indole-3-acetic acid (IAA), trans-zeatin riboside (*t*-ZR), and dihydrozeatin riboside (DHZR) assays by immunoanalysis. The treatment with *M. oryzae* CBMB20 or CBMB110 produced significant increase in accumulation of cytokinins t-ZR and DHZR in red pepper and tomato plant extracts. Greenhouse experiments further confirmed the biomass enhancement and colonization in the red pepper phyllosphere. Therefore, this study proved that the versatility of *Methylobacterium* as a plant-growth promoting bacteria could be better exploited.

Key Words
Methylotrophy, *Methylobacterium*, ACC deaminase, Auxin Production, Cytokinins.

Introduction

Plant interactions with microorganisms are well-documented phenomena. Symbiotic bacteria that inhabit the rhizosphere and form nodules on the root of legumes are able to assimilate atmospheric nitrogen and provide it to the host plant. Rhizosphere bacteria, including members of the genera *Rhizobium* and *Bradyrhizobium* however, are not the only players involved in plant-microbe symbiosis. Many bacteria are present in the plant phyllosphere and there is evidence that these inhabitants have a significant impact on plant growth and development. Among such inhabitants are the pink-pigmented facultative methylotrophic bacteria (PPFMs), which are members of the Genus *Methylobacterium* and are gram-negative alpha-proteobacteria. These plant associated bacteria are easily detected by their pink color and ability to utilize one carbon compounds, such as methanol, as sole carbon and energy source. They are phylogenetically related to both plant-associated bacteria *Agrobacterium* and *Rhizobium* and have more recently been placed in a clade, which includes a *Methylobacterium* strain that is able to symbrotically nodulate and fix nitrogen in legumes (Sy *et al.* 2001). The quantity of IAA produced and sensitivity of the plant tissue also play important role in several functions, such as root elongation and the formation of lateral and adventitious roots. Cytokinins are N6-substituted adenine derivatives that have diverse effects on important physiological functions in plants and whose level can alter the root functions. PGPB plays a role in reducing ethylene in plants via the action of ACC deaminase (ACCd) enzyme (EC 4.1.99.4) that sequesters and hydrolyzes ACC to α-ketobutyrate and ammonia (Glick *et al*. 1998). It was previously proposed that much of the ACC formed in this reaction are exuded from seeds or plant roots along with other small molecules normally present and may be taken up by the bacteria and subsequently hydrolyzed by the ACCd enzyme. This in turn, would lead to more ACC exudation from inside the plant to maintain the equilibrium thus reducing ACC and the amount of ethylene evolved by the plant (Glick *et al*. 1998). IAA secreted by a bacterium may promote root growth directly by stimulating plant cell elongation or cell division or indirectly by influencing bacterial ACCd activity (Patten and Glick 2002). *Methylobacterium* inoculation increased the IAA concentrations of the plants resulting in increased ACS activity. However, the ACC and ethylene concentrations inside the tissues were reduced due to the activity of bacterial ACCd (Madhaiyan *et al*. 2006). In this present study, we are demonstrating the involvement of ACCd producing *Methylobacterium oryzae* strains isolated from rice in plant growth promotion.

Methods

Bacterial Strains and Culture Conditions

The methylotrophic strains *Methylobacterium oryzae* CBMB20 and *Methylobacterium oryzae* CBMB110 were isolated from rice stem and leaf. The methylobacteria were grown for 72-120 h on ammonium mineral salt (AMS) media supplemented with 0.5% methanol and cycloheximide (30 µg/ml).

In vitro estimation of plant growth promoting traits by methylotrophic strains

Methylobacterium strains grown in AMS media were used for the estimation of IAA and cytokinins. The production of IAA by the methylotrophic isolates was determined according to the method by Bric *et al*. (1991). Sterile supernatants of samples taken at 120 h of growth were analyzed for cytokinin production using immunoassays. To determine ACC deaminase activity, the bacterial isolates were grown in 15 ml of LB broth at 30 ºC until they reached the stationary phase after which, cells were collected by centrifugation (at 8000 g). To induce ACC deaminase activity, the cells were resuspended in 7.5 ml of DF minimal salts medium supplemented with 5 mM of ACC as a sole nitrogen source and then incubated for 40 h at 30 °C with shaking (120 rpm). ACC deaminase activity was determined by measuring the production of α-ketobutyrate and expressed as nM of α-ketobutyrate formed min^{-1} mg $protein^{-1}$ (Penrose and Glick, 2003).

Gnotobiotic assays

After surface sterilization, red pepper and tomato seeds were treated with the *Methylobacterium* strains and transferred to growth pouches under aseptic conditions. The culture conditions and the procedure for gnotobiotic growth pouch assay followed that of Penrose and Glick (2003). To check the persistence, treated seeds were transferred to multi-well trays filled with air-dried Wonjo-Mix bed soil and vegetable raising growth medium (Nong-Kyung Co., Ltd, Jincheon-gun, Chungbuk, Republic of Korea) as described by Poonguzhali *et al*. (2008).

IAA and cytokinin in plant extracts

Extracts for IAA and cytokinin assays were prepared by homogenizing the seedlings (1 g) with TBS buffer in a ratio of 1:3 (w/v). Supernatants of the extracts (5000 rpm for 3 min, twice) were used for IAA, trans-zeatin riboside (*t*-ZR), and dihydrozeatin riboside (DHZR) assays. Enzyme-Linked Immunosorbent Assay (ELISA) test kits were used for immunoassays to measure the IAA and cytokinins in the sample. ELISA tests were performed according to kit instructions. The absorbance was read at 405 nm using an ELISA plate reader (BIO-RAD Model 550, Japan).

Results

The *Methylobacterium* strains varied in their ability to utilize ACC and significant differences were observed in the ACC deaminase activity of cell free extracts. CBMB20 and CBMB110 produced 94.5 and 24.7 nmol α-ketobutyrate mg^{-1} of protein h^{-1}, respectively. A quantitative analysis using the Salkowski reagent of the culture liquids of the methylobacteria grown in the defined medium with L-tryptophan and incubated for 5 days produced significantly different amounts of IAA. In the presence of L-tryptophan, the production of IAA by *Methylobacterium* strains CBMB20 and CBMB110 was 2.33 and 4.03 µg/ml, respectively. Immunoassays using ELISA kits were also performed to determine the cytokinins produced by the *Methylobacterium* strains. The cytokinins *t*-ZR, iPA, and DHZR were all present at detectable and replicable levels in the cultures tested, with *t*-ZR present in smaller quantities. The total amount of cytokinins recovered from the cultures varied, but strain CBMB20 produced a significantly higher amount than CBMB110. PPFMs synthesized IAA predominantly by an alternate tryptophan-dependant pathway, through indole-3-pyruvic acid, however, the role of bacterial IAA in plant growth promotion remains undetermined (Ryu *et al*. 2006) (Table 1).

Table 1. ACC deaminase, IAA and cytokinin production of the *M. oryzae* strains isolated from rice

Strains	ACC deaminase activity (nmol α-ketobutyrate mg^{-1} protein h^{-1})	IAA production (µg ml^{-1}culture)		Cytokinin recovered (ng l^{-1}culture)	
		Trp^+	Trp^-	iPA	*t*-ZR
M. oryzae CBMB20	94.48 ± 6.83	2.33 ± 0.11	1.72 ± 0.08	47.01 ± 0.45	32.92 ± 1.43
M. oryzae CBMB110	24.74 ± 4.12	4.03 ± 0.20	1.07 ± 0.05	41.87 ± 1.26	26.23 ± 1.24

The germination percentage and root length of the *Methylobacterium* strain-treated tomato and red pepper seeds were comparatively greater when compared to the uninoculated control. The percentage increase in root length compared to the control was 39.4% when treated with CBMB20, whereas CBMB110 recorded higher increases over the control amounting to 61.3%. These results also matched the results of previous studies with rice and sugarcane crops, where treatment with certain cytokinin-producing *Methylobacterium* strains increased growth (Madhaiyan *et al*. 2005) (Table 2).

Table 2. Effect of *M. oryzae* strains inoculation on the root length of red pepper and tomato under gnotobiotic conditions

Treatment	Root length (cm)	
	Tomato*	Red pepper**
M. oryzae CBMB20	5.81 ± 0.14	8.78 ± 0.17
M. oryzae CBMB110	6.72 ± 0.15	9.78 ± 0.16
Control	4.17 ± 0.12	5.88 ± 0.20

* 15 days old plants; ** 10 days old seedlings.

For the tomato seedlings, no detectable amounts of IAA were found, although the presence of t-ZR and DHZR was recorded. The *Methylobacterium* strains produced significantly higher amounts of t-ZR than the control, with CBMB20 recording the highest, amounting to 0.0125 pmol g^{-1} FW. A similar trend was also seen with DHZR, but the differences were not significant. The effect of *Methylobacterium* inoculation on the plant growth hormones was more prominent in the red pepper seedlings compared to that of the tomato seedlings. The amount of IAA in the treated seedlings significantly differed from that of the control. The cytokinins in the red pepper tissue extract increased with *Methylobacterium* inoculation. The *t*-ZR concentration in the *Methylobacterium*-treated seedlings was significantly increased compared to that of the control and *miaA*$^{-}$ mutant. A similar trend was seen for DHZR, although inversely, CBMB110 produced more at 0.658 pmol g^{-1} FW than CBMB20 at 0.562 pmol g^{-1} FW.

Table 3. Effect of *Methylobacterium oryzae* inoculation on cytokinin concentrations in tomato and red pepper seedlings

Treatment	IAA (pmol g^{-1} FW)	Concentration of Cytokinin (pmol g^{-1} FW)		
		t-ZR	DHZR	Total
Tomato*				
M. oryzae CBMB20	ND	0.0125^{a}	0.475^{ab}	0.4875^{bc}
M. oryzae CBMB110	ND	0.0115^{b}	0.431^{b}	0.4425^{c}
Control	ND	0.0074^{c}	0.468^{ab}	0.4754^{a}
Red pepper**				
M. oryzae CBMB20	61.65^{b}	0.0218^{a}	0.562^{ab}	0.5838^{b}
M. oryzae CBMB110	68.27^{a}	0.0127^{c}	0.658^{a}	0.6707^{a}
Control	60.80^{c}	0.0169^{b}	0.253^{b}	0.2699^{c}

* 15 days old plants; ** 10 days old seedlings; ND – Not determined.

In summary, the total amount of cytokinins in the seedlings greatly varied according to the treatment, with inoculated plants recording significant increases of more than 30% compared to the control (Table 3). *M. oryzae* CBMB20 enhanced the plant biomass and showed phyllosphere colonization using leaf imprinting method in a greenhouse experiment (Figure 1).

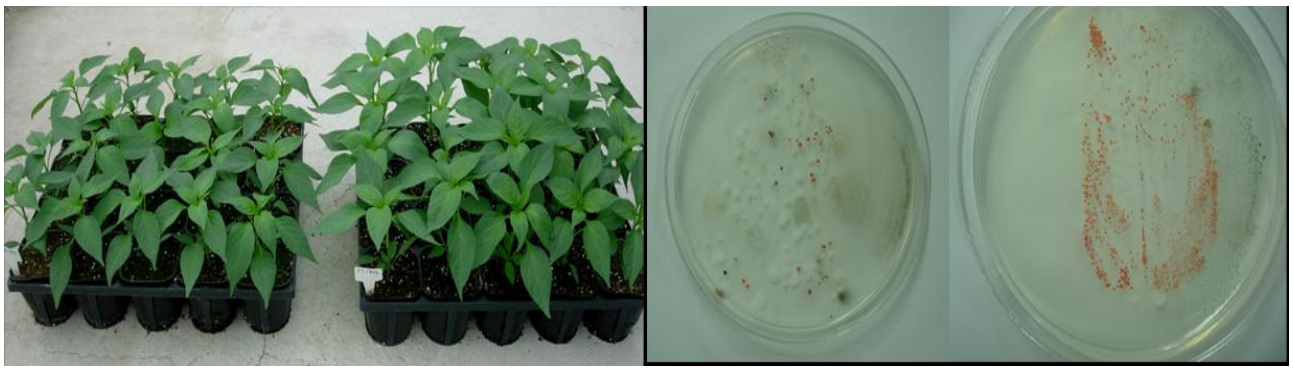

Figure1. Colonization in phyllosphere and plant growth promotion by *M. oryzae* CBMB20 inoculation in red pepper under greenhouse conditions

Conclusion

This present investigation of the inoculation effects of plant-growth promoting methylotrophic bacteria on tomato and red pepper seeds produced satisfactory results, with significant increases in plant growth and plant hormone concentrations over that of the uninoculated control. Therefore, this study proved that the versatility of *Methylobacterium* as plant-growth promoting bacteria could be better exploited.

References

Bric JM, Bostock RM, Silversone SE (1991) Rapid in situ assay for indole acetic acid production by bacteria immobilization on a nitrocellulose membrane. *Applied Environmental Microbiology* **57,** 535–538.

Glick BR, Penrose DM, Li J (1998) A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. *Journal of Theoretical Biology* **190**, 63-68.

Madhaiyan M, Poonguzhali S, Lee HS, Hari K, Sundaram SP, Sa TM (2005) Pink-pigmented facultative methylotrophic bacteria accelerate germination, growth and yield of sugarcane clone Co86032 (*Saccharum officinarum* L.). *Biology Fertility of Soils* **41**, 350–358.

Madhaiyan M, Poonguzhali S, Ryu JH, Sa TM (2006) Regulation of ethylene levels in canola (*Brassica campestris*) by 1-aminocyclopropane- 1-carboxylate deaminase-containing *Methylobacterium fujisawaense*. *Planta* **224,** 268–278

Patten CL, Glick BR (2002) Role of *Pseudomonas putida* indole acetic acid in development of the host plant root system. *Applied Environmental Microbiology* **68,** 3795–3801

Penrose DM, Glick BR (2003) Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiologia Plantarum* **118,** 10–15

Poonguzhali S, Madhaiyan M, Yim W-J, Kim K-A, Sa T-M (2008) Colonization pattern of plant root and leaf surfaces visualized by use of green-fluorescent-marked strain of *Methylobacterium suomiense* and its persistence in rhizosphere. *Applied Microbiology and Biotechnology* **78,** 1033–1043

Ryu J, Madhaiyan M, Poonguzhali S, Yim W, Indiragandhi P, Kim K, Anandham R, Yun J, Kim KH, Sa T (2006) Plant growth substances produced by *Methylobacterium* spp. and their effect on tomato (*Lycopersicon esculentum* L.) and red pepper (*Capsicum annuum* L.) growth. *Journal of Microbiology Biotechnology* **16,** 1622-1628.

Sy A, Giraud E, Jourand P, Garcia N, Willems A, de Lajudie P, Prin Y, Neyra M, Gillis M, other authors (2001) Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. *Journal of Bacteriology* **183**, 214–220