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Article

Regulation of Ethylene Emission in Tomato (*Lycopersicon esculentum* Mill.) and Red Pepper (*Capsicum annuum* L.) Inoculated with ACC Deaminase Producing *Methylobacterium* spp.

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Improvement of plant growth by Methylotrophic bacteria can be influenced through alterations in growth modulating enzymes or hormones, especially by decreasing ethylene levels enzymatically by 1-aminocyclopropane-1-carboxylate (ACC) deaminase or by production of indole-3-acetic acid (IAA). In this study, the effect of seven strains of *Methylobacterium* on seedling ethylene emission of tomato and red pepper plants was evaluated under greenhouse condition. Ethylene emission was lowest in *Methylobacterium oryzae* CBMB20 inoculated tomato plants and CBMB110 inoculated red pepper plants at 47 days after sowing (DAS). However, at 58 DAS all inoculated plants showed almost similar pattern of ethylene emission. *Methylobacterium* inoculated tomato and red pepper plants showed significantly less ethylene emission compared to control. Our results demonstrated that *Methylobacterium* spp. inoculation promotes plant growth due to the reduction of ethylene emission and therefore can be potentially used in sustainable agriculture production systems.

Key words: *Methylobacterium* spp., tomato, red pepper, ACC deaminase, ethylene emission

Introduction

For transplanting economically important seedlings, the major field of concern is the production of seedlings that can overcome the adverse effects of biotic and abiotic stresses which could lead to premature damages during the tender stage of plant development (Sonesson 1994; Suzanne 1998; Greenwood et al. 2007). Techniques have already been adopted for the rapid growth of healthy seedlings, which include seed priming, external application of phytohormones, and proper manuring, (Van Iersel. 1999; Stamps 2000; Scoggins et al. 2002). Moreover, Plant growth-promoting bacteria-based bioinoculant application for healthy seedling development adds value to an integrated crop production technique.

Ethylene, which is most popularly associated with ripening, plays a major role in 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 while 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).

Methylotrophic bacteria are able to grow on one-carbon compounds such as methanol or formaldehyde as sole carbon source and most of them are members of the genus *Methylobacterium,* pink-pigmented facultative methylotrophs (PPFMs) (Green 1992). The beneficial effect of PPFMs application on plant growth is determined by the efficiency of their colonization. Several studies have reported rhizosphere and intercellular colonization of plant tissues by *Methylobacterium* species (Sy et al. 2005; Poonguzhali et al. 2008). The association of *Methylobacterium* species with plant seems to be symbiotic in which the bacteria utilize methanol, the waste metabolite from plants and in turn produce compounds

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that promote plant growth. The plant growth promotion effects of *Methylobacterium* have attracted increasing interest in recent years.

The *Methylobacterium* strains used in this study were isolated from rice (Madhaiyan et al. 2006). Production of plant growth regulators like indole-3-acetic acid, cytokinins and ACC deaminase which is involved in modulation of ethylene levels in plants and plant growth promotion by these *Methylobacterium* strains under gnotobiotic condition have been investigated previously (Madhaiyan et al. 2006, 2007). Similarly, Hong et al. (2009) reported the inoculation with these *Methylobacterium* strains enhanced healthy seedling development in various crops. In this study, inoculation of *Methylobacterium* strains significantly enhanced root elongation (5.4 to 32.6%) and dry biomass accumulation (9.7 to 78.3%) in tomato seedlings (Hong et al. 2009). Moreover, *Methylobacterium* strains with IAA and ACC deaminase, have characteristic effects on different aspects of growth of red pepper and tomato seedlings which is comparable or better than exogenous applications of synthetic IAA under gnotobiotic and greenhouse conditions (Deka Boruah et al. 2010).

In previous studies, *Methylobacterium* strains were found to possess ACC deaminase activity and tested for their potential in plant growth promotion traits in various crops (Deka Boruah et al. 2010; Hong 2009; Madhaiyan et al. 2006, 2007; Ryu et al. 2006). This current study evaluated the inoculation effect of seven strains of ACC deaminase producing *Methylobacterium* on seedling ethylene emission of tomato and red pepper plants under greenhouse condition.

Materials and Methods

Bacterial strains The *Methylobacterium* strains used in this study are listed in Table 1. Stock cultures were stored at -80℃ in 50% glycerol. 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).

Greenhouse bioassay The effect of inoculation of seven *Methylobacterium* strains (CBMB12, CBMB15, CBMB20, CBMB27, CBMB31, CBMB35, CBMB110) on ethylene emission of plants were evaluated under greenhouse condition. Bacterial cells of 72-h-old cultures of *Methylobacterium* strains were harvested by centrifugation at 10,000 g for 10 min at 4℃, washed twice and suspended in 0.03 M $MgSO_4$. Homogenous bacterial suspensions were adjusted to O.D. 600 nm = 1.0 (10^8 c.f.u. ml^{-1}). Surface sterilized seeds (first with 70% ethanol for 1 min followed by 2% sodium hypochlorite for 1 min) were immersed in bacterial cell suspensions prepared as mentioned above, for 4 h at 60 rpm.

Bacterized tomato and red pepper seeds were sown in seedling trays (50 holes $tray^{-1}$ and one seed $hole^{-1}$) containing Biosangto-Mix bed soil [Heung Nong Co., Ltd, Incheon, Gyeonggi-do, Republic of Korea; it contains 65-70% coco peat, 15-20% peat moss, 8-10% perlite and macronutrient (mg L^{-1}) NH_4-N, 80-100; NO_3-N, 150-200; available P_2O_5, 230-330; K_2O, 80-120; pH 5.5 to 6.5; moisture content 50-60% and water

Table 1. *Methylobacterium* strains used in this study.

Strains	Name of the bacteria	NCBI Accession numbers	Plant growth promoting characters		References
			IAA†	ACC deaminse‡	
CBMB12	*Methylobacterium* sp.	EF126740	++	++	Unpublished data
CBMB15	*Methylobacterium* sp.	EF126745	+	++	
CBMB31	*Methylobacterium* sp.	EF126747	++	++	
CBMB35	*Methylobacterium* sp.	EF126741	+	++	
CBMB20	*Methylobacterium oryzae*	AY683045	+	++	Madhaiyan et al. 2006
CBMB27	*Methylobacterium phyllosphaerae*	EF126746	+	++	Unpublished data
CBMB110	*Methylobacterium oryzae*	AY683046	+	++	Madhaiyan et al. 2006;

†+ = <5.0 μg ml^{-1}, ++ = >5.0 μg ml^{-1} but less then <10.0 μg ml^{-1}; ‡++ = >50 nmol α-keto butyrate mg^{-1} protein h^{-1} but less then <100 α-keto butyrate mg^{-1} protein h^{-1}.

holding capacity 35-40%] and incubated in a growth chamber (DS 54 GLP, DASOL Scientific Co., Ltd., Korea) at 25℃ with 14/10 h day/night photoperiod.

Twenty one day old tomato and red pepper seedlings were transplanted into 300 ml plastic pots containing Biosangto-Mix bed soil fertilized with 10 ml of Hoagland's nutrient solution. After transplanting, pots were transferred to the greenhouse and plants were allowed to grow under natural illumination. *Methylobacterium* spp. (O.D. 600 nm = 1.0, 10^8 cfu ml^{-1}) inoculation was performed with a hand-held pneumatic sprayer until run-off and applied to the soil (10 ml) weekly after transplanting.

Effect of *Methylobacterium* spp. inoculation on the ethylene emission of tomato and red pepper For ethylene analysis, harvested tomato and red pepper leaves and whole plants were placed in 120 ml and 500 ml vials, respectively. One ml sample of the headspace was injected into a Gas Chromatograph (dsCHROM 6200, Donam Instruments Inc., Republic of Korea) packed with Poropak-Q column at 70℃ and equipped with a flame ionization detector. The amount of ethylene emission was expressed as nmol of ethylene h^{-1} g dry $weight^{-1}$ by comparing to a standard curve generated with pure ethylene (Praxair, Praxair Korea Co., Ltd).

Statistical analysis Data were subjected to analysis of variance (ANOVA). Significance at $P \leqq 0.05$ was tested by least significant difference (LSD) using SAS package, Version 9.1 (SAS, 2009).

Results

Effect of *Methylobacterium* spp. inoculation on the ethylene emission of plant leaves under greenhouse condition The influence of *Methylobcterium* spp. inoculation on the ethylene emission of tomato and red pepper seedlings was studied under greenhouse conditions. The ethylene emission of plant leaves at 47 days after sowing (DAS) is given in Fig. 1. In general, ethylene emission in ACC deaminase producing *Methylobacterium* inoculated plant leaves remained low compared to control. The reduction in ethylene emission in *Methylobacterium* spp. pretreated tomato leaves compared to control ranged from 7.3 to 40.0%.

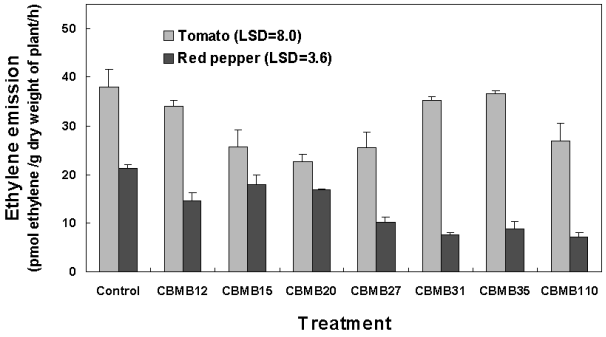

Fig. 1. Effect of *Methylobacterium* spp. inoculation on ethylene emission of plant leaves under greenhouse condition (47 DAS). Data are means of three replicates per treatment. Error bars represent standard error means of treatment values. Significance at $P \leqq 0.05$ was tested by least significant difference (LSD).

The lowest ethylene emission of 22.9 pmol of ethylene h^{-1} g dry $weight^{-1}$ was recorded in tomato leaves inoculated with CBMB20. Inoculation with *Methylobacterium* spp. CBMB12, CBMB31 and CBMB35 recorded reduced ethylene emissions of 34.0, 35.3 and 36.7 pmol of ethylene h^{-1} g dry $weight^{-1}$, however, these were not significantly different to control. Similarly, reduced ethylene emissions were also observed in ACC deaminase producing *Methylobacterium* strains pretreated red pepper leaves as depicted in Fig. 1. *Methylobacterium* strains pretreatment reduced ethylene emission in red pepper leaves by 15.5 to 65.7% compared to control. The lowest ethylene emission of 7.3 and 7.7 pmol of ethylene h^{-1} g dry $weight^{-1}$ was recorded in red pepper leaves pretreated with CBMB110 and CBMB31 strains. Except for *Methylobacterium* strain CBMB15 inoculation which recorded 18.0 pmol of ethylene h^{-1} g dry $weight^{-1}$ inoculation with *Methylobacterium* strains significantly reduced ethylene emission in red pepper leaves.

Effect of *Methylobacterium* spp. inoculation on the ethylene emission of whole plants under greenhouse condition Effects of *Methylobacterium* strains on tomato and red pepper growth are shown in Fig. 2, 3. Compared to control, in all *Methylobacterium* inoculated treatments significant increase in plant growth were observed. The influence of ACC deaminase producing *Methylobacterium* strains on the ethylene emission of red pepper and tomato plants at 58 DAS is presented in Fig 4. Inoculation with *Methylobacterium* strains significantly ($P \leqq 0.05$) reduced ethylene emission in tomato and red pepper plants compared to control.

Fig. 2. Comparison of effect of inoculation with *Methylobacterium* strains on seedling development of tomato under greenhouse condition.

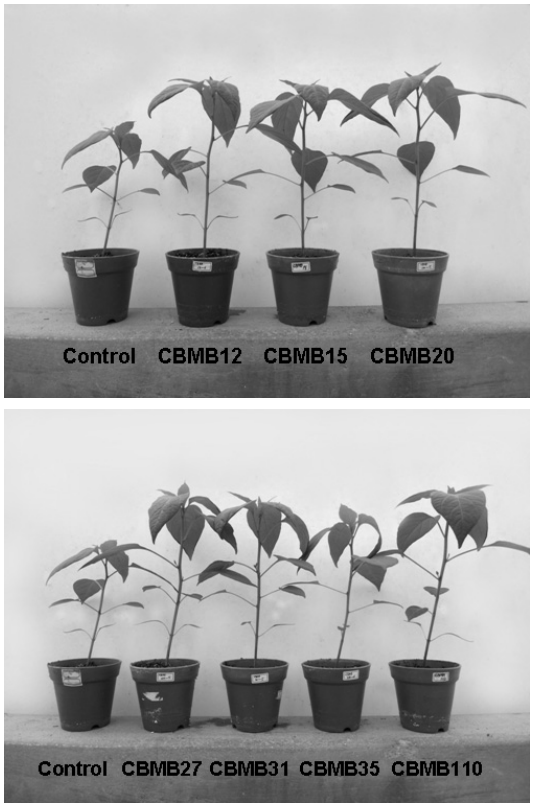

Fig. 3. Comparison of effect of inoculation with *Methylobacterium* strains on seedling development of red pepper under greenhouse condition.

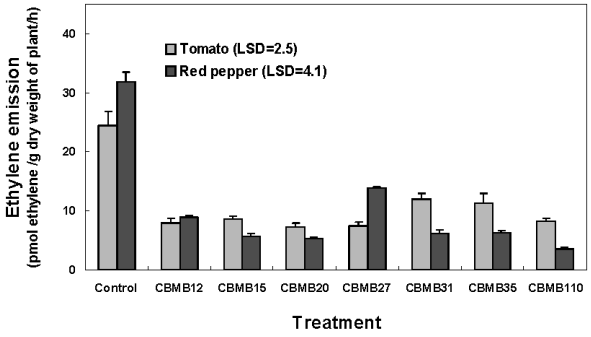

Fig. 4. Effect of *Methylobacterium* spp. inoculation on ethylene emission of plants under greenhouse condition (58 DAS). Data are means of three replicates per treatment. Error bars represent standard error means of treatment values. Significance at $P \leqq 0.05$ was tested by least significant difference (LSD).

Interestingly, the reduction in ethylene emission is more pronounced in whole tomato and red pepper plants samples compared to leaf samples. Ethylene emission was reduced by 51.0 to 70.2% in whole tomato plants as influenced by *Methylobacterium* spp. pretreatment compared to control. The lowest ethylene emission of 7.3 pmol of ethylene h^{-1} g dry $weight^{-1}$ was recorded in tomato plants inoculated with CBMB20. In red pepper plants, *Methylobacterium* inoculation reduced ethylene emission by 56.3 to 88.9% compared to control. The lowest ethylene emission of 3.5 pmol of ethylene h^{-1} g dry $weight^{-1}$ was recorded in red pepper plants inoculated with CBMB110.

Discussion

Healthy seedling emergence is an important prerequisite for the growth and development of vegetable crops. However, various factors hinder crop growth during the seedling stage especially due to the prepriming ability of weed seeds (Baskin and Baskin 1989; Koger et al. 2004; Christopher et al. 2006; Main et al. 2006). Therefore, the assurance of a healthy seedling in crop production starts from radicle emergence. This is particularly true for transplanted and bedding plants where sufficient root-shoot ratio is important for higher rate of survival and better field performance (Zandstra and Liptay 1999).

In this study, the effect of *Methylobacterium* strains inoculation on ethylene emission of crops was investigated under greenhouse condition. Three of *Methylobacterium* strains (CBMB20, CBMB27 and CBMB110) have been

previously evaluated for their plant growth promoting properties (Madhaiyan et al. 2006; Lee et al. 2006; Madhaiyan et al. 2009). All the tested *Methylobacterium* strains produced indole-3-acetic acid and ACC deaminase activity. Ryu et al. (2006) reported the synthesis of plant growth substances by *Methylobacterium* spp. Lee at al. (2006) also reported the effect of methylotrophic bacteria on the physiological enhancement of early rice seedling growth.

In the present study, plant leaves and whole plants treated with ACC deaminase producing *Methylobacterium* strains showed reduced ethylene emissions. ACC deaminase is mostly responsible for controlling stress ethylene level of plants, thereby, influencing root elongation (Glick et al. 1998; Ma et al. 1998). The plausible reason may be due to the ACC deaminase in *Methylobacterim*, which cleaves the ACC into α-ketobutyrate and ammonia (Glick et al. 1998; Madhaiyan et al. 2006). This mechanism effectively reduces the amount of ethylene evolved by the plant. Thus, the ability to promote plant growth by plant growth promoting rhizobacteria (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 (Madhaiyan et al. 2006). In a previous study, the effect of ACC deaminase producing *Methylobacterium* on ethylene levels and root elongation of *Brassica campestris* was reported by Madhaiyan et al. (2006). All these studies also corroborate with the present findings. Moreover, 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).

The greenhouse study conducted showed that inoculation with *Methylobacterium* significantly reduced ethylene emission in tomato and red pepper. Healthy seedling development resulting from inoculation of *Methylobacterium* may be due to the cumulative activity of the plant growth promoting characteristics of the strains tested. More extensive field studies on the inoculation of *Methylobacterium* can lead to the development of an efficient bioinoculum for improved seedling development of transplanted horticultural crops and bedding plants.

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