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Article

Effect of Methylotrophic Bacteria in Seedling Development of Some Crops under Gnotobiotic Condition

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Healthy seedling generation is the major concern in overcoming adverse effects of biotic and abiotic stresses during tender stage of development in vegetables and horticultural crops. Because of this, priority is given to research leading to the generation of healthy seedlings in crops subjected to transplanting and bedding. In this study, growth pouch experiments were conducted to determine the effect of inoculation of six different strains of *Methylobacterium* sp. namely, *M. oryzae* CBMB20, *M. phyllosphaerae* CBMB27, *M. suomiense* CBMB120, and *Methylobacterium* strains CBMB12, CBMB15 and CBMB17 on the seedling development of the vegetable crops cabbage, Chinese cabbage and cucumber; and horticultural crops tomato and red pepper. Crops treated with the test strains generally showed higher seedling dry matte accumulation compared to the control. Significantly higher accumulation was exhibited by CBMB12, CBMB17, and CBMB20 in cabbage, as well as for CBMB27 and CBMB120 on tomato and Chinese cabbage, respectively. Furthermore, all the strains promoted root elongation in cucumber and tomato seedlings while in Chinese cabbage and red pepper, root elongation was observed with CBMB120 and CBMB12 inoculation, respectively. Large scale nursery study is needed to develop a thorough protocol for healthy seedling development with the use of these strains.

Key words: *Methylobacterium* sp., Inoculation effect, Seedling, Vegetable crops, Horticultural crops, Biological activity

Introduction

Use of plant growth promoting bacteria (PGPB) is considered a major research area for sustainable crop production in the 21st century because of their multiple beneficial effects to plants (Kloepper et al., 1980; Deka Boruah et al., 2003; Kennedy et al., 2004). Several PGPB groups have been identified and recognized for instance for their potential use as biofertilizers. Among these are *Azospirillum* (Baldani et al., 1987), *Bacillus* (Astrid et al., 2003; Orhan et al., 2006; Ali et al., 2009), *Pseudomonads* (Deka Boruah et al., 2003), and *Methylobacterium* (Madhaiyan et al., 2006; Poonguzhali et al., 2008). Recently, attention has been turned to members of the genus *Methylobacterium* for their potential use as plant inoculant for sustained crop production. Species of the genus *Methylobacterium* are known to be ubiquitous in nature, maintain intimate associations with plants (Holland and Polacco, 1994; Lidstrom and Chistoserdova, 2002; Sy et al., 2005) and promote plant growth in different stages of development. Studies have reported an increase in the percentage of seed germination, seed vigor, and dry matter accumulation (Madhaiyan et al., 2004; Madhaiyan et al., 2005; Lee et al., 2006) with inoculation of strains of *Methylobacterium*. Wide spread quorum sensing (Poonguzhali et al., 2008), and appreciably high ability to colonize the roots, stem and leaves (Sy et al., 2005; Poonguzhali et al., 2008) of members of this group was also noted. The possible mechanism by which *Methylobacterium* promotes plant growth includes production of phytohormones, such as indole 3 acetic acid (IAA), cytokinin or vitamins (Basile et al., 1985; Koenig et al., 2002, Lidstrom and Chistoserdova 2002), enhancement of nitrogen metabolism in plants by bacterial urease (Holland and Polacco, 1992), formation of root nodules in legumes (Sy et al., 2001), nitrate reductase activity and 1 amino cyclopropane 1 carboxylate deaminase activity (Madhaiyan et al., 2006). A more thorough investigation of the effects of *Methylobacterium* sp. on the different growth stages of plants is needed however, to document its pattern of

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application.

For economically important transplant seedlings, the major field of concern is the production of seedlings that can overcome the adverse effects of biotic and abiotic stresses that leads to premature damages during this tender stage of plant development (Sonesson 1994; Suzanne, 1998; Greenwood et al., 2007). Techniques have been adopted for the rapid growth of healthy seedlings, including seed priming, external application of phytohormones, and proper manuring, among others. (Van Iersel, 1999; Stamps 2000; Scoggins et al., 2002). Further investigation is however needed to develop a more efficient formulation to promote early seed germination and healthy seedling development. This study reports on the effect of inoculation of six strains of genus *Methylobacterium* spp. on root growth and seedling development of the vegetable crops, cucumber, cabbage, and Chinese cabbage and the horticultural crops, tomato and red pepper.

Materials and Methods

Bacterial strains, media and inocrum preparation The *Methylobacterium* strains used in this study are listed in Table 1. The strains possess more than one plant growth promoting characteristics. Stock cultures were stored at -80℃ in 50% glycerol. All the strains were maintained in ammonium mineral salt (AMS) media.

Bacterial inocula for seed bacterization were prepared by growing the strains in AMS broth for 72 h. Cell pellets were collected by centrifugation (10,000 rpm, 10 min, 4 ℃) and washed twice in distilled water. Bacteria, in sterile distilled water suspension (OD_{600}-0.8) were used for seed inoculation.

Test plants Cabbage (*Brassica oleracea* var. capitata), Chinese cabbage (*Brassica rapa*), cucumber (*Cucumis sativus*), tomato (*Lycopersicon esculentum* Mill) and red pepper (*Capsicum annum* L) were used as test plants.

Preparation of gnotobiotic growth pouch Growth pouches (CY9 seed germination pouch, Mega International) were prepared for gnotobiotic assays to check root elongation. This was done by adding 20 mL of distilled water to each growth pouch, wrapping them in aluminum foil in groups of 10 in an upright position to prevent water loss and then autoclaving these at 121℃ for 15 min (Figure 1).

Seed sterilization Seeds of the test plants were surface sterilized before treatment with the bacterial suspension. These were first washed with sterile distilled water twice before treatment with 70% ethanol for 1 min. This was followed by treatment with sodium hypochlorite solution depending on the type of seeds used viz., for tomato and red pepper - 2% sodium hypochlorite solution for 30 second; lettuce- 2.5% sodium hypochlorite solution for 6 min; Chinese cabbage, canola and cucumber- 1% sodium hypochlorite

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

Strain ID	Name of the bacteria	NCBI accession No.	Biological character					References
			IAA†	Cytokinin‡	Quorum sensing§	Nitrate reductase¶	ACCD∫	
CBMB20	Methylobacterium oryzae	AY683045	+	++	-	++	++	Madhaiyan et al., 2004; Madhaiyan et al., 2006; Poonguzhali et al., 2007; Lee et al., 2006
CBMB27	M. phyllosphaerae	EF126746	+	ND	ND	ND	++	Madhaiyan et al., 2006 Madhaiyan et al., 2009
CBMB120	M. suomiense	AY683047	++	++	+++	ND	-	Poonguzhali et al., 2007
CBMB12	Methylobacterium	EF126740	+	ND	ND	ND	++	Madhaiyan et al., (un published data)
CBMB15	Methylobacterium	EF126745	+	ND	ND	ND	++	Madhaiyan et al., (un published data)
CBMB17	Methylobacterium	EF126752	+	ND	ND	ND	++	Madhaiyan et al., (un published data)

+ = Present, - = absent, ND= not detected; †+ = < 5.0 °·g ml 1, ++ = >5 μg ml^{-1} but less than <10 μg ml^{-1}; ‡++ = >50 ng L^{-1} but <100 ng L^{-1}
§++= > 150 ng; ¶++= > 150 nmol C_2H_4 h^{-1} mg^{-1} protein but <200 nmol C_2H_4 h^{-1} mg^{-1} protein;
∫++= >50 nmol α-keto butyrate mg^{-1} protein h^{-1} but <100 nmol α-keto butyrate mg^{-1} protein h^{-1}.

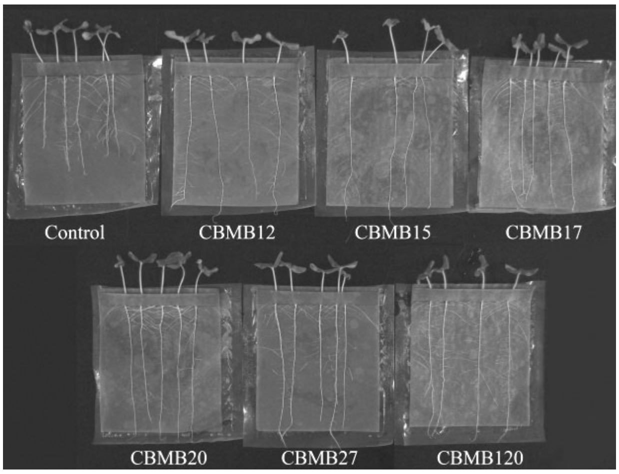

Fig. 1. Comparison of effects of inoculation with *Methylobacterium* strains on seedling development of cucumber in gnotobiotic growth pouch.

solution for 10 min. Finally, the treated seeds were thoroughly rinsed more than five times with sterile distilled water.

Seed bacterization Surface sterilized seeds were then immersed in the bacterial cell suspension, prepared as mentioned above for 4 h at 60 rpm. After decanting the suspension, the dried seeds were transferred to growth pouches (six seeds per pouch), with six replications per treatment. Two empty pouches were placed at the ends of each rack so that plants at the end of a rack are not subjected to extremes of light or air circulation. The racks were placed in a plastic tray containing sterile distilled water. The pouches were then incubated in a growth chamber at 20℃, with 12 h day night photoperiod.

Measurement of seedling development The effect of bacterial treatment on root elongation was determined after 14 days from seed germination by measuring the length from the base of the plants to root tip. Total dry matter accumulation was measured after drying the seedling in an oven at 70℃ until a constant weight was obtained.

Statistics analysis Data generated were normalised, and mean standard error (SE), analysis of variance and least significant difference (LSD) were calculated using SAS Version 9.1 (SAS, 2004).

Results

Effect of *Methylobacterium* strains on root elongation The effect of the six *Methylobacterium* strains on root elongation of the tested vegetable and horticultural crops is presented in Table 2. Root elongation was observed to be significantly higher with the inoculation of *Methylobacterium* CBMB15, *M. phyllosphaerae* CBMB27, and *M. oryzae* CBMB20 in cucumber compared to that of the control. This was also observed with *Methylobacterium* CBMB17 and *M. suomiense* CBMB120 in cabbage and with *Methylobacterium* CBMB12 and *M. oryzae* CBMB20 in tomato seedlings. In addition, cucumber, cabbage and tomato seedlings inoculated with all *Methylobacterium* strains showed greater root length. *Methylobacterium* CBMB15 produced the highest root length in cucumber (22% higher than control) while the lowest (5.42% higher than control) was obtained for *Methylobacterium* strain CBMB12. Similarly, the highest percentage of root elongation recorded among the treated seedlings was found for CBMB17 in cabbage and CBMB12 in tomato, at 100% and 32.6%, respectively. For the same plant species, the lowest (16.21%) root elongation activity was shown by CBMB15. For Chinese cabbage, a 2.04% increment was shown by *Methylobacterium* CMBM120. None of the other bacterial strains was found to significantly promote root elongation compared to the

Table 2. Effect of inoculation of different *Methylobacterium* strains on root length of test crops.

Name of the strains	Length (cm)				
	Cucumber	Cabbage	Chinese cabbage	Red pepper	Tomato
Control	12.9±3.3c	3.7±1.5c	9.8±1.9a	12.7±3.6ab	9.2±1.4c
CBMB12	13.6±3.7bc	5.4±1.8bc	7.6±2.5b	13.8±3.5a	12.2±2.6a
CBMB15	15.8±1.1a	4.3±2.3bc	8.3±2.8ab	10.8±3.9b	11.1±3.0abc
CBMB17	14.8±1.3abc	7.4±2.5a	8.5±2.3ab	10.8±2.5b	9.7±2.8bc
CBMB20	15.0±1.9ab	5.1±2.8bc	8.8±2.9ab	11.1±2.3b	10.9±1.9ab
CBMB27	15.6±1.5a	4.8±2.1bc	8.6±2.1ab	11.9±3.2b	10.0±3.8bc
CBMB120	14.4±2.8abc	6.2±2.5ab	10.0±2.4a	11.7±2.6b	10.0±4.2bc

Value in each column were the average of 15 seedlings, ±1.0= standard deviation of observed mean value; mean value followed by similar letter are not significantly different from each other by LSD test (p<0.05).

control. Similar results were seen for red pepper seedlings, where only *Methylobacterium* CBMB12 promoted root elongation by 8.66% higher than that of the control.

Effect of *Methylobacterium* on dry biomass of seedlings Effect of different *Methylobacterium* strains on dry matter accumulation of the tested crops is summarized in Table 3. All bacterial strains produced appreciably higher dry biomass accumulation in cucumber and tomato seedlings compared to that of the control. The increase in dry biomass in cucumber was at 4.36% to 62.04% while in tomato it was at 9.72% to 39.29%. Among the strains used, *Methylobacterium* CBMB15 showed significantly, the highest dry biomass accumulation while the lowest was recorded in CBMB20 treated cucumber seedlings. In tomatoes, *Methylobacterium* CBMB12 gave the highest dry biomass accumulation and *M. suomiens*e CBMB20 and *Methylobacterium* CBMB15 showed the lowest. On the other hand, all *Methylobacterium* strains, except CBMB12, showed appreciably higher dry biomass production compared to control, with that of *Methylobacterium* CBMB15 (47.69%) and *M. phyllosphaere* CBMB27 (36.04%), showing statistically significant values. Inoculation with *Methylobacterium* strains had no significant influence on cabbage and Chinese cabbage dry biomass production compared to the control.

Discussion

Healthy seedling emergence is portent to a consistent pattern of growth and development in the life cycle of vegetable crops. Moreover, different biotic and abiotic factors pose strains to the growth of the crop during the seedling stage; especially with that of weeds because of its pre priming (Baskin and Baskin, 1989; Koger et al., 2002; Christopher et al., 2006; Main et al., 2006). Therefore, the assurance of a healthy seedling starting from the emergence of radicle is very important. This is particularly true for transplanted and bedding plants where sufficient root shoot ratio is important for higher rate of survival and to ensure a good take off during transplantation (Zandstra and Liptay, 1999). In this study, the effect of six different *Methylobacterium* strains on seedling development of five different crops was investigated. Three of these *Methylobacterium* strains have been previously evaluated for their positive plant growth promoting properties (Madhaiyan et al., 2004; Madhaiyan et al., 2006; Lee et al., 2006; Poonguzhali et al., 2007; Madhaiyan et al., 2009). All the tested *Methylobacterium* strains produced indole-3-acetic acid and except *M. suomiense* CBMB120, all the strains showed 1-aminocyclopropane-1-carboxylase deaminase (ACCD) activity. ACCD is mostly responsible for controlling the plant ethylene level thereby, influencing root elongation (Glick et al. 1998; Ma et al., 1998).

In the present study, inoculation of *M. phyllosphaere* CBMB27, and *M. oryzae* CBMB20 significantly enhanced root elongation in cucumber and strain *Methylobacterium* CBMB15 significantly enhanced root elongation and dry biomass in the same crop. In cabbage, significant enhancement of root elongation was produced by the strains *Methylobacterium* CBMB17 and CBMB12; and in Chinese cabbage, by *Methylobacterium* CMBM120. In tomatoes, inoculated seedlings showed higher root elongation (5.43 - 32.61%) and dry biomass accumulation (9.72 - 78.26%). A contrasting result was found for the strain *Methylobacterium* CBMB12 where root elongation was promoted in red pepper but not the dry biomass. All the other *Methylobacterium* strains inoculated increased the dry biomass but not root length as compared to the

Table 3. Effect of inoculation of different *Methylobacterium* strains on dry biomass accumulation of test crops.

Strain used	Total dry weight (mg)				
	Cucumber	Cabbage	Chinese cabbage	Red pepper	Tomato
Control	6.64±6.63bc	6.89±1.41a	22.48±3.74a	24.30±1.33b	12.24±5.44b
CBMB12	10.07±4.48a	7.30±0.75a	12.96±0.64b	15.78±0.66c	16.07±1.91ab
CBMB15	10.76±2.68a	6.36±1.92a	17.83±1.69b	35.89±4.35a	13.48±1.38ab
CBMB17	7.97±0.81ab	8.00±3.73a	17.68±3.62bc	35.11±3.19ab	14.60±2.99ab
CBMB20	6.93±0.71bc	7.49±1.42a	21.34±5.17a	29.59±4.35ab	13.43±0.22b
CBMB27	7.19±0.62ab	6.54±1.34a	15.68±1.49bc	33.06±0.66a	21.82±6.61a
CBMB120	9.34±1.64ab	6.82±1.65a	21.52±3.58a	27.81±1.33ab	13.43±2.75ab

Value in each column were the average of 5 seedlings, ± 1.0= Standard deviation of observed mean value; mean value followed by similar letter are not significantly different from each other by LSD test ($p<0.05$).

control. From the data, we inferred that root elongation in the tested crops may be due to the activity of ACCD, while enhancement of dry biomass accumulation may be due to the cumulative effects of plant growth promoting activities of the strains used. Effect of ACCD producing *Methylobacterium* on ethylene levels in *Brassica campestris* and root elongation was reported by Madhaiyan et al. (2006). Lee at al. (2006) reported in their study the effect of methylotrophic bacteria on physiological enhancement of early rice seedlings. Ryu et al. (2006) reported on the plant growth substances produced by *Methylobacterium* spp. Results of all these studies collaborate the present findings.

Conclusion

From the gnobiotic study conducted, it was found that inoculation with *Methylobacterium* significantly enhanced healthy seedling development in cucumber, tomato and red pepper. *Methylobacterium* inoculated seedlings produced longer roots, which help in the exploration of a wider soil area for nutrient acquisition in nursery grown seedling plugs. Higher root growth leads to a healthy seedling development. Early seedling development resulting from inoculation of *Methylobacterium* may be due to the cumulative activity of the plant growth promoting characteristics of the strains used. More extensive studies on the inoculation of *Methylobacterium* can lead to the development of efficient bioinoculum for seedling generation in transplanted horticultural crops and bedding plants.

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Methylotrophic bacteria 접종이 작물 유묘 생장에 미치는 영향

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농업유용미생물을 이용한 원예 및 채소작물의 건전 유묘 생산을 위하여 본 연구에서는 growth pouch 실험을 통하여 6가지 식물생육촉진 미생물을 접종하여 작물의 초기 뿌리 생장에 미치는 영향을 살펴 보았다. 본 실험에 사용한 6가지 균주는 *Methylobacterium oryzae* CBMB20, *Methylobacterium phyllosphaerae* CBMB27, *Methylobacterium suomiense* CBMB120, *Methylobacterium* strains CBMB12, CBMB15와 CBMB17이었다. 대조구와 비교했을 CBMB12, CBMB17, 및 CBMB20접종은 상추 초기 뿌리 생육에 유의성있는 효과를 보였고, CBMB102접종은 배추 초기 뿌리 생육에 유의성있는 효과를, CBMB27접종은 토마토의 초기 뿌리 생육에 유의성있는 효과를 보였다. 또한 *Methylobacterium suomiense* CBMB120접종은 오이, 토마토, 배추, 그리고 *Methylobacterium* strain CBMB12접종은 고추의 뿌리 초기 생장을 크게 촉진시켰음을 알 수 있었다. 위의 결과를 통하여 실험한 균주들을 각각의 작물 육묘 포트에 처리할 때에도 유묘의 생장속도를 촉진 시킷수 있음을 예상할 수 있다.