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Article

Effect of Inoculation of *Methylobacterium oryzae* on the Growth of Red Pepper at Different Organic Fertilizer Levels

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Plant growth promoting ability of *Methylobacterium oryzae* CBMB20 was evaluated under different levels of organic fertilizer application on red pepper plants in a pot experiment. Oil cake as an organic N fertilizer was applied at the rates of 70, 85, 100 and 120% of the conventional recommended level. Each treatment was further treated with or without *M. oryzae* CBMB20 inoculation. The recommended amount of compost for red pepper was added in all the treatments. Results revealed that plant height, dry biomass and fruit yield were enhanced in increasing order as the rate of fertilization increased. Overall plant growth was improved due to the inoculation of *M. oryzae* CBMB20 and red pepper fruit yield was also increased by 10-35% in the plants inoculated with *M. oryzae* CBMB20 at different rates of organic fert1izer application. Total methylotrophic bacterial population in rhizosphere soil measured at the time of harvest was significantly higher in *M. oryzae* CBMB20 inoculated treatments. The growth promoting effect of *M. oryzae* CBMB20 found in red pepper could be due to the effective colonization of the bacteria in the rhizosphere and its ability of enhancing nutrient availability and producing plant growth hormones. With the plant growth promoting effect of *M. oryzae* CBMB20, the rate of organic fertilizer application can be reduced without any significant decreases in biomass production and yield of red pepper.

Key words: *Methylobacterium oryzae*, Plant growth promotion, Organic fertilizer, Red pepper

Introduction

The use of chemical fertilizers to enhance soil fertility and crop productivity has often negatively affected the complex system of the biogeochemical cycles (Steinshamn et al., 2004). Fertilizer use has caused leaching and run-off of nutrients, especially N and P, leading to environmental degradation (Gyaneshwar et al., 2002). Important reasons for these problems are low use efficiency and the continuous long-term use of fertilizers. Despite the negative environmental effects, the total amount of fertilizers used worldwide is projected to increase with the growing world population due to the need to produce more food through intensive agriculture that requires large quantities of fertilizer (Vitousek et al., 1997; Frink et al., 1999). Although the population has been growing and available land for agriculture has been shrinking, intensive agriculture that involves heavy and continuous use of fertilizers has ensured high crop productivity. Therefore the challenge in modern agriculture is to continue agricultural productivity in a way that minimizes harmful environmental effects of fertilizers.

Recently there has been a resurgence of interest in environmentally friendly sustainable agricultural practices (Edwards, 1989). Utilization of organic fertilizers in combination with beneficial microorganisms instead of chemical fertilizers are known to improve plant growth through supply of plant nutrients and help to sustain environmental health and soil productivity. Microbial inoculants are promising components for integrated solutions to agro-environmental problems caused by the excessive application of chemical or organic fertilizers. Using the plant growth promoting microbes, application level of fertilizer can be significantly reduced. Inoculants,

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possessing the capacity to promote plant growth, enhance nutrient availability and uptake, and support the health of plants (Dobbelaere et al., 2001; Vessey, 2003; Kloepper et al., 2004; Weller, 2007; Madhaiyan et al., 2004, 2009, 2010; Adesemoye et al., 2008).

A plant growth promoting bacterial strain from genera *Methylobacterium* has been reported to have many plant growth promoting abilities including N fixation, P solublization, production of plant growth promoters, and biological disease control (Madhaiyan et al., 2004, 2007, 2009; Ryu et al., 2006). Apart from several plant growth promoting attributes, isolates from *Methylobacterium* genera are able to demonstrate mutual synergistic effects with other groups of bacteria and arbuscular mycorrhizal fungi on various crops and improve growth and nutrient uptake (Madhaiyan et al., 2010; Kim et al., 2010).

However, not much is known about the interaction between microbial inoculation and soil fertility or fertilizer application level in their plant growth promoting effects. In general, the beneficial effects of microbial inoculation are more evident in certain levels of soil fertility. However, under the conditions of sufficient or deficient nutrient supply, the plant growth promoting effect of microbial inoculation is minimal or absent. The lower microbial activities under low soil fertility cannot provide any sufficient growth promoting effects. And the effect of microbial inoculation is very difficult to be observed in soils where fertility including nutrient supply is high enough for plant growth.

The aim of this work was to evaluate the effect of inoculation of plant growth promoting *Methylobacterium oryzae* CBMB20 with different levels of organic fertilizer on the growth and yield improvement of red pepper plant under pot trial conditions.

Materials and Methods

Soil The soil used for this study was collected from an experimental field of Chungbuk Agricultural Research Institute. Some of the characteristics of the soil are shown in Table 1. Soil pH was measured in 1:5 water suspensions using a glass electrode, and electrical conductivity (EC) was determined in 1:5 water extracts using a conductivity meter (Check mate 90, Corning, NY, USA). Organic matter was analyzed by Tyurin method. Available phosphorus was extracted using Lancaster procedure and analyzed by UV/Vis spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). Exchangeable cations were extracted with 1 M ammonium acetate (pH 7.0) after the soil was pre-washed with glycol-ethanol, and analyzed using an inductively coupled plasma optical emission spectrometer (ICP-OES, Optima 5300 DV, Perkin Elmer, USA). Cation exchange capacity was measured using ammonium acetate method. The detail analytical procedures were followed the standard methods of the Rural Development Administration (NAIST, 1988).

Bacterial strain and inoculum culture *Methylobacterium oryzae* strain CBMB20 is a pink-pigmented facultative methylotrophic bacteria isolated from rice tissues, and its various beneficial traits related to plant growth promotion was previously studied (Madhaiyan et al.,2007). For inoculum preparation, a single colony of *M. oryzae* CBMB20 grown on ammonium mineral salt (AMS) agar amended with 0.5% sodium succinate was transferred to 25 mL of AMS broth and incubated at 30°C on a shaker (120 rpm) for 72 h. After 72 h of incubation, 2.5 mL the culture (1 x 10^8 cells mL^{-1}) was transferred to 250 mL fresh AMS broth and allowed to grow for another 72 h.

Plant growth promotion experiment For evaluating the effect of *M. oryzae* CBMB20 inoculation on the red pepper plant growth under various organic fertilizer levels, a pot experiment was conducted in a greenhouse of Chungbuk Agricultural Research Institute. Plastic pots (bottom diameter 25 cm, top diameter 28 cm, height 30 cm) were filled with 15 kg soil. The treatments of organic fertilizer consisted of 4 sets (70, 85, 100 and 120%). In the 100% organic fertilizer treatment set, oil cake (N:P:K ratio 4:2.1:1 with 70% organic matter content) was applied at the rate of 2.54 g kg^{-1} soil and K was amended with 0.042 g K_2O kg^{-1}

Table 1. Chemical properties of soil used in the study.

pH (1:5)	EC	Organic C	Available P_2O_5	Exchangeable cation K	Exchangeable cation Ca	Exchangeable cation Mg	Exchangeable cation Na	CEC
	dS m^{-1}	g kg^{-1}	mg kg^{-1}	------------	------------ $cmol_c$ kg^{-1}	------------	------------	
7.8	0.9	17.2	287	0.3	8.3	1.5	0.2	9.4

soil with fertilizer KCl. Organic fertilizer application rate was determined based on the recommended basal chemical fertilizer application rate for red pepper (N:P:K=12.2: 6.4:6.1, kg per 10a). For 70, 85 and 120% sets, fertilizer application rate was calculated accordingly based on the 100% set. Each set was further treated with or without *M. oryzae* CBMB20 inoculation. Compost prepared with 30% saw dust, 40% cow dung, 10% pig dung, 10% chicken dung and 10% rice bran was added at the rate of 14.93 g kg^{-1} soil in all the treatments. Thirty day old red pepper (*Capsicum annuum* L. cv. Daetong) seedlings grown in the nursery were transplanted to pots. At the time of transplanting, 20 mL of *M. oryzae* CBMB20 inoculum was introduced into the soil around roots of red pepper plant. During the experiment plants were re-inoculated with *M. oryzae* CBMB20 at 11, 25, 39, 53, 67, 84 and 95 days after transplant. Twenty mL of the inoculum was applied on the soil surface and enough water was applied to facilitate the flow of bacterial inoculum down to the root zone soil. Four pots per treatment, each with a single plant, were arranged in a completely randomized design. Plant height was measured at 20, 40, 60, 80 and 113 days after transplant. Root and shoot dry weights were recorded at 113 days after transplant. Red pepper fruits were collected three times at 58, 87 and 113 days after transplant and the total fruit dry weight was recorded.

Enumeration of methylotrophic bacterial population in rhizosphere soil Rhizosphere soil samples, tightly adhered to the roots, were collected at 113 days after transplant the red pepper plants. Ammonium mineral salt agar (with 0.5% methanol) was used for the determination of total methylotrophic bacterial population. Ten g of rhizosphere soil was added to 90 mL of sterile distilled water, and shaken for 30 min at 150 rpm at 28°C. Ten fold serial dilutions of the respective suspensions were then plated onto AMS agar amended with 10 μg mL^{-1}filter sterilized cycloheximide to inhibit fungal growth. The plates were then incubated at 28°C for 3-10 days and after which bacterial colonies were counted. Bacterial polpulation was expressed as log cfu g^{-1} of soil.

Statistical analyses Significant differences among the treatments were calculated by Duncan’s multiple range tests using SAS software, version 9.1 (SAS, Cary, NC).

Results

Effect of *M. oryzae* CBMB20 inoculation on plant height of red pepper at different organic fertilizer levels In the uninoculated treatments, plant height increase was observed with increasing rate of fertilizer application, and in most observations a significant increase of plant height was found in the treatments of 100 and 120% fertilizer level as compared to 70 or 85% fertilizer level (Table 2). The differences in plant height between fertilizer level 100 and 120% were mostly insignificant.

With the inoculation of *M. oryzae* CBMB20 at different

Table 2. Effect of *Methylobacterium oryzae* CBMB20 inoculation on red pepper plant height at different organic fertilizer levels.

Fertilizer level	*M.oryzae* CBMB20 inoculation	Plant height†				
		20DAP‡	40DAP	60DAP	80DAP	113DAP
		------------------------------- cm -------------------------------				
70%	Uninoculated	32.2 ± 1.9ab	38.1 ± 2.3a	43.9 ± 0.8a	46.3 ± 1.5a	53.8 ± 2.1a
	Inoculated	30.5 ± 0.9a	42.9 ± 2.3b	48.0 ± 1.5b	51.8 ± 0.8c	59.9 ± 0.8bc
85%	Uninoculated	33.7 ± 0.6bc	43.6 ± 1.8b	45.9 ± 2.1ab	48.5 ± 2.2bc	57.0 ± 2.6ab
	Inoculated	33.6 ± 2.3bc	46.7 ± 1.7c	51.3 ± 0.9bc	57.2 ± 0.9c	62.8 ± 2.3c
100%	Uninoculated	34.5 ± 2.2bc	49.4 ± 1.4d	50.0 ± 0.3b	56.2 ± 3.5c	63.1 ± 1.1cd
	Inoculated	35.1 ± 1.2c	52.0 ± 0.4de	53.4 ± 0.6c	58.2 ± 3.1c	66.8 ± 1.0e
120%	Uninoculated	34.6 ± 1.7bc	50.0 ± 1.2de	52.2 ± 2.1c	56.2 ± 2.2c	61.3 ± 0.6bc
	Inoculated	35.1 ± 1.9c	52.6 ± 1.9e	56.3 ± 1.0d	59.8 ± 3.1c	65.9 ± 2.8de

†Each value represents the mean of 4 replicates ± standard deviation and means within the column with the same letter are not significantly different by Duncan’s multiple range test at $p = 0.05$.

‡DAP, days after transplant.

levels of organic fertilizer application plant height of red pepper was increased in all observations except in 20 days after transplant (Table 2). Inoculation of *M. oryzae* CBMB20 increased the plant height by 5.2-12.7% at all fertilizer levels as compared to their respective uninoculated controls, and most of the increases in inoculation treatments were significant. The highest plant height was observed in the treatment of *M. oryzae* CBMB20 inoculation with 120% organic fertilizer application. But the differences between the inoculation treatments of 100 and 120% organic fertilizer application were mostly not significant.

Effect of *M. oryzae* CBMB20 inoculation on dry biomass of red pepper at different organic fertilizer levels The effect of *M. oryzae* CBMB20 inoculation on the red pepper dry biomass accumulation in shoot, root, fruits and total plant at different organic fertilizer levels was observed (Table 3). At 70% organic fertilizer treatment set 58, 11, 19, 34%; at 85% set 11, 13, 26, 19%; at 100% treatment set 22, 21, 26, 24%, at 120% treatment set 18, 19, 10, 14% increase shoot, root, fruit and whole plant dry biomass accumulation of red pepper, respectively, were observed in uninoculated treatments (Table 3). Biomass of red pepper plant increased with the increasing application of organic fertilizer, but the differences between the treatments of 100 and 120% organic fertilizer application were not significant.

Inoculation of *M. oryzae* CBMB20 increased the plant biomass in all organic fertilizer levels as compared to the respective uninoculated controls. The total plant biomass in *M. oryzae* CBMB20 inoculated treatments were significantly higher than those found in the respective uninoculated controls. The highest plant biomass accumulation was observed in the treatments of *M. oryzae* CBMB20 inoculation with 100 or 120% organic fertilizer applications.

Colonization of methylotrophic bacterial population in the red pepper rhizosphere In *M. oryzae* CBMB20 inoculated sets total methylotrophic bacterial populations in the red pepper rhizosphere soils were in the range from 2.51 to 2.60 log cfu g^{-1} soil, whereas in uninoculated treatment sets the populations were less than 1.01 log cfu g $soil^{-1}$ (Table 4). The difference in total heterotrophic methylotrophic bacterial population between inoculated and uninoculated treatments were significant in all organic fertilizer levels. However, the differences in the bacterial population among the treatments of different organic fertilizer levels were not significant both in inoculated and uninoculated treatments.

Discussion

Organic matterin soil plays an important role to achieve sustainable agricultural production. It possesses many beneficial effects on the physical, chemical and biological characteristics of soil. The organic degradable refuse of plant and animal origin provides a good source of nutrients to improve soil productivity. In the present study level of organic fertilizer significantly enhanced red pepper plant

Table 3. Effect of *Methylobacterium oryzae* CBMB20 inoculation on red pepper plant biomass at different organic fertilizer levels.

Fertilizer level	*M. oryzae* CBMB20	Dry weight†			
		Shoot	Root	Fruit	Total plant
		---------- g ----------			
70%	Uninoculated	7.2 ± 2.3a	1.8 ± 0.5a	9.8 ± 1.1a	18.8 ± 3.1a
	Inoculated	11.4 ± 1.1bc	2.0 ± 0.2ab	11.7 ± 0.8ab	25.1 ± 1.9bc
85%	Uninoculated	9.6 ± 2.5ab	2.3 ± 0.4bc	9.9 ± 0.7a	21.7 ± 2.7ab
	Inoculated	10.7 ± 1.6b	2.6 ± 0.3cd	12.5 ± 1.5b	25.8 ± 2.7c
100%	Uninoculated	13.5 ± 1.6cd	2.9 ± 0.1de	13.8 ± 1.4cd	30.2 ± 1.4d
	Inoculated	16.5 ± 0.6ef	3.5 ± 0.2f	17.4 ± 0.9f	37.4 ± 1.4e
120%	Uninoculated	15.4 ± 1.8de	2.7 ± 0.3cd	14.7 ± 1.7de	32.8 ± 3.5d
	Inoculated	18.1 ± 1.0f	3.2 ± 0.3ef	16.2 ± 1.4ef	37.5 ± 1.3e

†Biomass data were collected at the time of harvest (113 days after transplant), each value represents the mean of 4 replicates ± standard deviation. Means within the column with the same letter are not significantly different by Duncan's multiple range test at $p = 0.05$.

Table 4. Effect of organic fertilizer level on the methylotrophic bacterial population in the red pepper rhizosphere.

Fertilizer level	*M. oryzae* CBMB20 inoculation	Methylotrophic bacterial population†
		log cfu g^{-1} soil
70%	Uninoculated	0.71 ± 0.21ab
	Inoculated	2.53 ± 0.06c
85%	Uninoculated	1.01 ± 0.08b
	Inoculated	2.60 ± 0.08c
100%	Uninoculated	0.93 ± 0.25b
	Inoculated	2.54 ± 0.08c
120%	Uninoculated	0.53 ± 0.01a
	Inoculated	2.51 ± 0.01c

†Bacterial populations were measured at the time of harvest (113 days after transplant), andeach value represents the mean of 4 replicates ± standard deviation. Means within the column with the same letter are not significantly different by Duncan's multiple range test at $p = 0.05$.

height and dry biomass in increasing order as the amount of fertilizer level increased i.e. 70, 85, 100 and 120 % (Table 2 and 3). The increase in red pepper plant growth with the level of increasing fertilizer applicationwas in agreement of the work carried out by Kang et al. (2004) who reported the effect of fertilizer concentration and irrigation method on growth and fruiting of ornamental pepper (*Capsicum annuum* L.). Roy et al. (2010) has reported that the organic amendments of soil increased the nutrient supply and plant productivity at different magnitude depending on the quality of residue used and its mode of application.

Increased organic fertilizer application level with inoculation of *M. oryzae* CBMB20 significantly improved red pepper plant growth. Over all 10-26% increase was observed in the red pepper fruit yield and 14-34% total plant dry biomass due to the inoculation of *M. oryzae* CBMB20 among different levels of organic fertilizer treatments. The effect of *M. oryzae* CBMB20 inoculation on the red pepper plant growth was not much remarkable at the low fertilizer level (70 and 85%). And at this level of nutrient supply the plant growth promoting activity of *M. oryzae* CBMB20 can not be fully manifested. Considering the no significant effect of *M. oryzae* CBMB20 inoculation in biomass accumulation at fertilization levels of 100 and 120%, under nutrient-rich condition in the rhizoshpere the contributions of plant growth promoting bacteria can be diminished.

Earlier Ryu et al., (2006) reported that inoculation of red pepper seeds with *Methylobacterium* strains showed a significant increase in root length when compared with either the uninoculated control or *M. extorquens* miaA$^-$ knockout mutant-treated seeds. The treatment with *Methylobacterium* sp. CBMB20 produced significant increases in the accumulation of IAA and the cytokinins t-ZR and DHZR in the red pepper extracts (Ryu et al., 2006). Recently, inoculation effect of *M. oryzae* CBMB20 on crop growth and nutrient uptake of tomato, red pepper and rice alone and in coinoculation with nitrogen-fixing *Azospirillum brasilense* CW903 or a phosphate solubilizing bacterium *Burkholderia pyrrocinia* CBPB-HOD has been reported by Madhaiyan et al. (2010). Seed inoculation and soil/foliar application of the bacterial strains alone or under dual inoculation increased plant growth in terms of shoot or root length and increased nutrient uptake of plants studied compared to uninoculated control plants. Co-inoculation of *M. oryzae* CBMB20 with *A. brasilense* CW903 or *B. pyrrocinia* CBPB-HOD improved the N and P concentration of plants, although the results varied among the plant species tested (Madhaiyan et al., 2010). Methylobacterium promoted plant growth possibly by producing phytohormones and stimulating germination (Madhaiyan et al.,2004; Omer et al., 2004). Recently the presence of enzyme 1-aminocyclopropane-1-carboxylate (ACC)-deaminase containing Methylobacterium, which is involved in lowering ethylene levels and promoting root elongation in canola, has been reported (Madhaiyan et al., 2006). Therefore the plant growth promoting effect of *M. oryzae* CBMB20 observed in this study could be due to its capacity of enhancing nutrient availability and producing plant growth regulators.

The effect of microbial inoculants on their activity in the rhizosphere is decisive for maximizing plant growth promoting capacity. The soil microbial community in the rhizosphere plays a key role in plant nutrition and thus in plant growth (Kohler et al., 2007). Hence we measured the total methylobacterial population in the rhizosphere soil in all different levels of fertilizer treatments with and without *M. oryzae* CBMB20 inoculation. In the *M. oryzae* CBMB20 inoculated rhizosphere soil the total methylobacterial populations were significantly higher than those foundin uninoculated soil. No significant differences among the different levels of fertilizer and both with or without inoculation of *M. oryzae* CBMB20 were found. This result indicates that the inoculated *M. oryzae* CBMB20 can be effectively colonized in the rhizosphere soil of red pepper plants and that even the lowest level of organic fertilizer application might be sufficient to support the colonization of *M. oryzae* CBMB20. The better growth of red pepper plants observed in the treatments of *M. oryzae* CBMB20 inoculation could be due to the growth promoting effects of this rhizobacteria as previously reported by Ryu et al. (2006) and Kim et al. (2010). Although bacterial population was not different among the fertilizer treatments, the effect of *M. oryzae* CBMB20 inoculation on biomass accumulation of red pepper plant was more prominent at 100% fertilizer level.

Considering the biomass data of red pepper plant presented in Table 3, the optimal application level of organic fertilizer could be 100% of the conventional recommended level where the maximum biomass accumulation was found. The differences in biomass data between the treatments of 100 and 120% organic fertilizer application were not significant. Also the biomass data of red pepper plant inoculated with *M. oryzae* CBMB20 were not significantly different between 100 and 120% fertilizer treatments. The biomass accumulation observed in the treatment of 100% organic fertilizer and *M. oryzae* CBMB20 inoculation was significantly higher than that found in the treatment of 120% organic fertilizer only. These results suggest that the plant growth promoting effect of *M. oryzae* CBMB20 can be maximized in the 100% level of fertilizer application, and further growth promoting effect of *M. oryzae* CBMB20 can not be expected with more fertilizer application than 100% level. With the plant growth promoting effect of *M. oryzae* CBMB20 the application level of fertilizer can be reduced without any significant decreases in biomass production and yield of red pepper.

Conclusions

Experimental results from the presented study clearly demonstrated that the inoculation of *M. oryzae* CBMB20 improved the growth and yield ofred pepper plant at different levels of organic fertilizers. This is the preliminary step towards the exploitation of *M. oryzae* CBMB20 for improving the growth and yield of red pepper plant under different fertilizer managements. Based on the plant growth promoting abilities of *M. oryzae* CBMB20 under different fertilizer management practices, this strain can be utilized as plant growth promoting bioinoculant at large scale field trials.

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다양한 유기질비료 수준에서 *Methylobacterium oryzae* CBMB20의 처리에 따른 고추의 생육 평가

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다양한 유기질비료 수준에서 *Methylobacterium oryzae* CBMB20의 처리가 고추의 생육촉진에 미치는 영향을 조사하였다. 유기질비료 권장시비수준의 70, 85, 100, 120% 시용구에서 시비량이 증가함에 따라 고추의 신장, 건물중 및 수량에서 유의성 있는 증가를 확인하였다. *M. oryzae* CBMB20의 접종 처리에서 고추의 생장이 증가하였으며, 고추의 수량 또한 각 유기질비료 수준별로 10~35% 증가하였다. 수확기에 조사한 근권토양의 메탄올자화세균의 밀도는 *M. oryzae* CBMB20 처리구에서 2.51~2.63 log CFU g^{-1} soil 이었으며, 미처리구에서는 1.0 log CFU g^{-1} soil 이하로 나타났다. 따라서 접종한 *M. oryzae* CBMB20은 고추 작물 근권토양에 효과적으로 군집하였으며, 식물생장촉진 호르몬의 분비 등의 작용을 통한 작물생장촉진효과를 발휘한 것으로 판단된다. *M. oryzae* CBMB20을 접종하지 않은 경우에는 고추의 최대 생장과 수량이 유기질 비료 권장시비 수준 120%에서 나타났으나, *M. oryzae* CBMB20을 접종한 경우에는 최대 생장과 수량이 권장시비 수준에서 나타났다. 이러한 결과는 식물생장촉진미생물의 접종을 통하여 비료 사용량의 절감 가능성을 제시하는 것이며, 식물생장 촉진 미생물 제제로서 *M. oryzae* CBMB20의 활용이 가능하리라 사료된다.