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Article

Effect of *Azospirillum brasilense* and *Methylobacterium oryzae* Inoculation on Growth of Red Pepper (*Capsicum annuum* L.)

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Plant growth-promoting effects of rhizobacterial inoculation obtained in pot experiments cannot always be dependably reproduced in fields. In this study, we investigated the effect of inoculation with *Azospirillum brasilense* and *Methylobacterium oryzae*, which have displayed growth promoting effects in several pot experiments, on growth and fruit yield of red pepper under field condition in a plastic-film house. Four rows spaced 90 cm apart were prepared after application of compost (10 Mg ha^{-1}), and red pepper seedlings (*Capsicum annum* L., Nocgwang) were transplanted in each row with 40-cm space. Experimental treatments were consisted of *A. brasilense* CW903 inoculation, *M. oryzae* CBMB20 inoculation, and uninoculated control. Twelve plots, 10 plants per plot, were allotted to the three treatments with four replicates in a completely randomized design. At the time of transplanting, 50 mL of each inoculum (1×10^{8} cells mL^{-1}) was introduced into root zone soil of each plant, and re-inoculated at 7 and 14 days after transplant. Plant growth and fruit yield were measured during the experiment. Both *A. brasilense* CW903 and *M. oryzae* CBMB20 could not promote growth of red pepper plants. All growth parameters measured were not significantly different among treatments. There were large variations in fruit yield recorded on plot basis, and no statistically significant differences were found among treatments. The failure to demonstrate the expected plant growth promoting effect of the inoculants is possibly due to various environmental factors, including weather and soil characteristics, reducing the possibility to express the potential of the inoculated bacterial strains.

Key words: *Azospirillum brasilense*, *Methylobacterium oryzae*, Plant growth promotion, Red pepper

Introduction

Plant growth in agricultural soils is influenced by various biotic and abiotic factors. Microorganisms that colonize the rhizosphere exert beneficial effects ranging from direct influence mechanisms to an indirect effect on plant growth. Thus, soil bacteria that inhabit plant roots and benefit plants by providing growth promotion are termed plant growth-promoting rhizobacteria (Bashan and Holguin 1997). Various rhizobacteria were reported to have many plant growth promoting abilities including N fixation, P solublization, production of plant growth hormones, GAs, cytokinins and auxins, and biological disease control (Madhaiyan et al., 2004, 2006a, 2007, 2009; Omer et al., 2004; Ryu et al., 2006).

Various species of bacteria including *Azotobacter*, *Azospirillum*, *Bacillus*, *Pseudomonas*, *Rhizobium*, and *Methylobacterium* have been isolated and characterized in terms of their plant growth promotion abilities (Dobbelaere et al., 2002; Gray and Smith, 2005; Nautiyal et al., 2006; Poonguzhali et al., 2008; Ryu et al., 2006). While appropriate application of chemical fertilizers is essential in modern farming systems, deleterious environmental impacts of fertilization should be minimized. Using plant growth promoting microbes, possessing the versatile plant-beneficial traits, fertilizer application can be reduced (Adesemoye et al., 2008; Ahemad and Khan, 2011; Dobbelaere et al., 2002; Kloepper et al., 2004; Madhaiyan et al., 2004, 2009, 2010; Vessey, 2003; Weller, 2007). Various bacterial species have been successfully used in inoculation trials in pot experiments under greenhouse condition (Bashan and Holguin, 1997; Masheshawari, 2011). However, commercial application of plant growth promoting microbes on a large scale is still very limited, and the main obstacle is the unpredictability and inconsistency of field results (Bashan and Holguin, 1997).

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Recently we isolated bacterial species of *Methylobacterium* and *Azospirillum* from rice and taro, respectively, and their plant growth promoting abilities were confirmed in laboratory and greenhouse experiments (Chauhan et al., 2010; Kim et al., 2005; Madhaiyan et al., 2004, 2007, 2010; Ryu et al., 2006). *Methylobacterium* spp. are a group of bacteria known as pink pigmented facultative methylotrophs (Austin and Goodfellow, 1979), and they were reported to distribute ubiquitously in the plant phyllosphere and rhizosphere (Corpe and Basile, 1982). *Methylobacterium* improves plant growth through the production of the enzyme urease or phytohormone IAA and cytokinins, lowering ethylene levels in plants, production of siderophores and protection against pathogens (Idris et al., 2004; Madhaiyan et al., 2004, 2006a, 2006b; Omer et al., 2004). *Azospirillum brasilense* is a well studied bacterium found in the rhizosphere of various crop plants originally defined as a nitrogen fixer but later known for its production of the plant growth hormone IAA (Bashan and Holguin, 1997; Bashan et al., 2004; Okon et al., 1995). However, most of these informations correspond to experiments performed under controlled conditions.

Therefore, before applications in a large scale of real farming systems, it is necessary to confirm the reproducibility of plant growth-promoting potential of rhizobacteria in field conditions. And much more information is needed to explain the success or failure in plant growth and yield response upon bacterial inoculation. In this study, we investigated the effect of rhizosphere inoculation with *A. brasilense* and *M. oryzae*, which have displayed growth promoting effects in several pot experiments, on the growth and fruit yield of red pepper plant under a reduced fertilization level in the field of plastic-film house.

Materials and Methods

Bacterial strains and inoculum culture *Methylobacterium oryzae* CBMB20 (Madhaiyan et al., 2006a, 2007) and *Azospirillum brasilense* CW903 (Kim et al., 2005) were used in this study. The strains were cultured in ammonium mineral salts minimal broth with 0.5% methanol for 4 days (*M. oryzae* CBMB20), and in nitrogen free malic acid broth supplemented with 1 g NH_4Cl per liter for 48 h (*A. brasilense* CW903) at 30°C under shaking.

Plant culture with bacterial inoculation Plant growth experiment was conducted in a plastic-film house at Experimental Farm of Daegu University. Characteristics of the house soil are shown in Table 1. The experimental field plots were prepared after application of compost (10 Mg ha^{-1}) two weeks before transplanting of red pepper seedlings. Four rows spaced 90 cm apart were prepared in the plastic-film house (6.5 m × 35 m). Thirty five-day-old red pepper seedlings (*Capsicum annum* L., Nocgwang) from a commercial nursery were transplanted on May 4, 2011 in each raw with 40-cm space.

Treatments of *M. oryzae* CBMB20 inoculation, *A. brasilense* CW903 inoculation, and uninoculated control were included in the experiment. Twelve plots, 10 plants in each plot, were allotted to the three treatments with four replicates in a completely randomized design. At the time of transplanting, 50 mL of inoculum (1×10^8 cells mL^{-1}) was introduced into the root zone soil of each red pepper plant, and plants were re-inoculated at 7 and 14 days after transplant. 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.

Plant height, number of leaves or stems, and length of the largest leaf were recorded at 20, 40, 50, 60, and 70 days after transplant. Fruits larger than 8 cm were harvested at 40, 50, 60, 70, and 80 days after transplant, and total number and fresh weight of the fruits were recorded. Four plants were collected in each plot at 80 days after transplant, and fresh weights of shoot and root were recorded.

Table 1. Some physicochemical characteristics of the soil used in experiment.

pH	EC_e	Organic matter	Total N	Avail. P_2O_5	Exch. Cation			CEC	Texture
					Ca	Mg	K		
(1:5)	dS m^{-1}	g kg^{-1}	g kg^{-1}	mg kg^{-1}	---------------- $cmol_c$ kg^{-1} ----------------				
6.7	3.2	26	1.7	390	5.8	2.6	1.4	11.5	Sandy clay

Statistical analyses Significant differences among the treatments were calculated by Duncan's multiple range tests using SigmaStat version 2.0 (Systat Software, San Jose, CA).

Results and Discussion

Although plant growth promoting abilities of *Methylobacterium* and *Azospirillum* strains were found in several laboratory and pot experiments, these plant responses upon the rhizobacterial inoculation should be confirmed under field conditions before large-scale commercial applications. The effects of *A. brasilense* CW903 and *M. oryzae* CBMB20 on growth of red pepper plants were investigated in a plastic-film house culture experiment.

Growth parameters measured five times during the experiment are presented in Table 2. Both strains of *A. brasilense* CW903 and *M. oryzae* CBMB20 could not promote growth of red pepper plant. All growth parameters measured were not significantly different among the treatments. At 80 days after transplant, mean fresh biomass of red pepper plants treated with *A. brasilense* CW903 or *M. oryzae* CBMB20 was not significantly different compared to the biomass observed in non-inoculated control plants (Table 3). And plant growth promoting effects of the two bacterial strains were also not significantly different. Fruit yield of

Table 2. Effect of inoculation of *Azospirillum brasilense* CW903 and *Methylobacterium oryzae* CBMB20 on red pepper plant growth. Values (mean ± SE) in the same column of each measuring date followed by the same letter are not significantly different at *P*=0.05 (Duncan's multiple range test).

Date	Treatment	Plant height	Number of leaves	Number of stems	Length of the largest leaf
		cm			cm
20 DAT	Control	39.5 ± 1.85a	30.9 ± 4.15a	-	7.7 ± 0.42a
	A. brasilense	40.2 ± 0.81a	30.5 ± 2.51a	-	7.8 ± 0.49a
	M. oryzae	40.2 ± 3.51a	29.5 ± 3.45a	-	7.8 ± 0.68a
40 DAT	Control	75.8 ± 3.09a	-	82.2 ± 10.0a	12.5 ± 0.73a
	A. brasilense	76.5 ± 1.93a	-	82.5 ± 10.3a	11.8 ± 0.39a
	M. oryzae	77.4 ± 2.52a	-	91.8 ± 7.75a	12.8 ± 0.62a
50 DAT	Control	84.9 ± 4.69a	-	102.2 ± 22.9a	13.8 ± 0.84a
	A. brasilense	81.1 ± 1.60a	-	83.9 ± 10.5a	12.3 ± 0.33a
	M. oryzae	82.3 ± 2.64a	-	99.0 ± 12.9a	12.8 ± 0.62a
60 DAT	Control	104.5 ± 4.51a	-	139.8 ± 4.67a	13.6 ± 0.44a
	A. brasilense	102.0 ± 1.29a	-	140.1 ± 10.3a	12.9 ± 0.33a
	M. oryzae	102.9 ± 3.64a	-	137.6 ± 2.87a	13.2 ± 0.29a
70 DAT	Control	122.4 ± 2.95a	-	133.3 ± 10.7a	14.2 ± 0.49a
	A. brasilense	118.4 ± 1.56a	-	120.4 ± 2.58a	13.2 ± 0.33a
	M. oryzae	120.4 ± 3.40a	-	133.7 ± 7.72a	13.8 ± 0.52a

Table 3. Effect of inoculation of *Azospirillum brasilense* CW903 and *Methylobacterium oryzae* CBMB20 on the biomass of red pepper plant. Four red pepper plants were collected in each plot at 80 days after transplant, and fresh weights of shoot and root were recorded. Values (mean ± SE) in the same column followed by the same letter are not significantly different at *P*=0.05 (Duncan's multiple range test).

Treatment	Shoot	Root
	g plant^{-1}	g plant^{-1}
Control	479.8 ± 61.28a	47.3 ± 4.99a
A. brasilense CW903	466.5 ± 76.15a	47.8 ± 4.79a
M. oryzae CBMB20	480.5 ± 91.26a	48.0 ± 5.89a
$LSD_{0.05}$	123.5	8.4

red pepper plant was estimated by measuring total number and fresh weight of the fruits (>8 cm long) harvested in each plot 5 times from 40 to 80 days after transplant (Table 4). There were large variations in total number and fresh weight of fruits harvested and measured on plot basis, and any statistically significant differences were not found among treatments. These results indicate that the positive plant growth-promoting potential of the inoculants expressed in pot experiments cannot always be reproduced under field conditions.

As demonstrated in numerous worldwide trials, we have already found positive effects of *A. brasilense* CW903 and *M. oryzae* CBMB20 inoculation on plant growth in the previous laboratory and pot experiments with wheat, rice, red pepper, tomato, canola, and groundnut (Madhaiyan et al., 2004; Madhaiyan et al., 2006a; Madhaiyan et al., 2006b; Madhaiyan et al., 2007; Madhaiyan et al., 2010; Ryu et al., 2006). Plant growth promoting effect of *A. brasilense* could be attributed to several mechanisms including enhancement of root development, production of growth regulators and nitrogen fixation (Bashan and Holguin, 1997; Kim et al., 2005). *Methylobacterium oryzae* can produce phytohormones like cytokinins and auxins (Madhaiyan et al., 2005), fix atmospheric N (Jourand et al., 2004; Raja et al., 2006), regulate the ethylene level in rhizosphere by producing the enzyme ACC deaminase (Chinnadurai et al., 2009; Madhaiyan et al., 2006a), and stimulate resistance against pathogens (Madhaiyan et al., 2006b).

Most of this information corresponds to experiments performed using small number of plants grown in pots under controlled conditions. Although the positive responses of crop plants to inoculation of *A. brasilense* CW903 and *M. oryzae* CBMB20 were clearly demonstrated under controlled conditions (Kim et al., 2011; Madhaiyan et al., 2010; Ryu et al., 2006), in this study conducted under field condition, inoculations of *A. brasilense* CW903 and *M. oryzae* CBMB20 were not effective on promotion of the growth and fruit yield of red pepper plant. Bashan et al. (2004) suggested that the good positive plant growth-promoting effects obtained under pot experiments cannot always be dependably reproduced under field conditions. Recently, Naiman et al. (2009) also reported that inoculation of wheat with *A. brasilense* and *P. fluorescens* could not significantly increase the aerial biomass production and grain yield in a field experiment. This inconsistency restricts further development of plant growth promoting rhizobacteria as a commercial inoculum on large scale crop cultivations (Bashan and Holguin, 1997; Maheshwari, 2011). However, the factors or conditions responsible for the inconsistent performance of plant growth promoting rhizobacteria in pot and field trials have not yet been clearly identified.

In this study, we did not investigate the ecological and environmental factors that determine the survival and activity of inoculated rhizobacteria in the plant rhizosphere. The failure to demonstrate the expected plant growth-promoting effects of bacterial inoculants under field condition is possibly due to the considerable competition between native and added bacteria which reducing the possibility to express the potential of the inoculated strains. Also the variability in the performance of plant growth promoting rhizobacteria is possibly due to the various environmental factors, including weather and soil characteristics, which may affect their growth and effects on plant. Generally, the most pronounced effect of inoculation is observed at low to intermediate soil fertility levels (Dobbelaere et al., 2002; Saubidet et al., 2002; Tien et al., 1979;

Table 4. Comparison of fruit yield of red pepper plants inoculated with *A. brasilense* CW903 and *M. oryzae* CBMB20. At 40, 50, 60, 70, and 80 days after transplant, fruits larger than 8 cm were harvested and total number and fresh weight of the fruits were recorded. Values (mean ± SE) in the same column followed by the same letter are not significantly different at *P*=0.05 (Duncan's multiple range test).

Treatment	Number of fruit	Weight of fruit
	ea plot^{-1}	g plot^{-1}
Control	337 ± 61a	2788 ± 737a
A. brasilense CW903	318 ± 87a	2670 ± 1165a
M. oryzae CBMB20	351 ± 83a	2823 ± 1108a
$LSD_{0.05}$	124.2	1633.3

Zimmer and Bothe, 1988).

With inoculation of growth-promoting rhizobacteria, the rapid establishment of root system by increasing root branching, root length, and/or the amount of root hairs, and this is advantageous for young seedlings in obtaining water and nutrients from their environment, thereby enhancing their chances for survival and growth (Ryu et al., 2006). This probably could be a reason for the more pronounced growth-promoting effect of bacterial inoculation at the early growth stages of plants (Dobbelaere et al., 2002; Madhaiyan et al., 2010; Tien et al., 1979). However, in this study root biomass measured at 80 days after transplant was not significantly different among the treatments (Table 3), and the shoot growth and fruit yield of red pepper not affected by the inoculation could be associated with this negative effect of inoculation on root growth. It is known that a rapid development of root system with bacterial inoculation would not always be associated with the growth or yield of plants in relatively fertile soils (Bashan and Holguin, 1997). In this study, red pepper plant was grown in the plastic-film house previously used in potato cultivation with sole application of commercial compost at the rate of 10 Mg ha^{-1}. Since the red pepper plants in control plots did not show any specific nutrient deficiencies, soil fertility was probably not quite below the optimal level for red pepper. Therefore, the beneficial effects of bacterial inoculations for red pepper plants in utilization of nutrients may not be expected, and this is probably associated with the no significant growth-promoting effects of the inoculations with *A. brasilense* CW903 and *M. oryzae* CBMB20 found in this experiment.

Nitrogen fixation is another important mechanism proposed to explain the promotion of plant growth by N-fixing rhizobacteria including *Azospirillum* spp.; regularly the contribution is smaller than 5% (Bashan and Holguin, 1997). Saubidet et al. (2002) reported that the increase of NO_3^- observed in wheat plants inoculated with *Azospirillum* spp. could not be explained by N fixation, and only by NO_3^- uptake from the soil. The bacterial strains *A. brasilense* CW903 and *M. oryzae* CBMB20 used in this experiment are known to have the ability to fix atmospheric N, but it is still not clearly displayed how much N can be supplied to the host plants by the bacterial N fixation. And the beneficial effect of N-fixation with inoculation of plant growth-promoting rhizobacteria also could be negligible for plants grown in relatively fertile soils.

Yet, there is no definite agreement on exactly how the bacteria can effect plant growth, and other factors responsible for the failure in plant growth stimulation with bacterial inoculation under field conditions cannot be further discussed here. One of the challenges of using plant growth-promoting rhizobacteria is obviously natural variation. Although the possibility to express the plant growth-promoting potential of rhizobacteria would be considerably restricted under relatively well managed growth environments, it is difficult to predict how an organism may respond when placed in the field. For commercial utilization of plant growth-promoting rhizobacteria as the potential tools for sustainable agriculture, various parameters critical in displaying the growth or yield promotion ability of the beneficial bacterial inoculants should be further investigated.

Conclusion

Various mechanisms through which the plant growth-promoting rhizobacteria affect the plant have been proposed, and growth-promoting effects of various bacterial strains were confirmed from many laboratory and pot trials. However, it is true that the positive effects of bacterial inoculations obtained under controlled environments cannot always be dependably reproduced under field conditions. Some parameters, such as the number of bacterial cells and the inoculation method, have been proposed to be critical for successful inoculation. But the factors or conditions responsible for the success or failure in plant growth and yield response upon inoculation have not yet been clearly identified. For better understanding of the basic features of the bacteria-root interaction and the parameters critical to obtain successful inoculation should be further investigated before large-scale field application with reliable efficacy.

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