Korean J. Soil Sci. Fert. **40**(4), 249-254(2007)

Article

Optimization of the Inoculation Dose of Plant-Growth Promoting Bacteria *Azospirillum brasilense* Strain CW903 Assessed by Tomato, Red Pepper and Rice under Greenhouse Condition

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Inoculation dose of agriculturally important microbes is an important criterion that decides the establishment and hence their effects on plant growth. Effects of the inoculation dose of *Azospirillum brasilense* strain CW903 on the growth and nutrient absorption of three different crops, tomato, rice and red pepper were assessed under green house condition. Three different concentrations of *A. brasilense* strain CW903 (10^5, 10^6 and 10^8 cfu mL^{-1}) were applied through seed treatment and through the soil near the root zone (1 mL per plant) at 20 and 30 days after sowing. Positive effects on the growth of tomato, rice and red pepper were found at 10^6 and 10^8 cfu mL^{-1} inoculation doses of *A. brasilense* strain CW903. The inoculation dose of 10^8 cfu mL^{-1} of *A. brasilense* strain CW903 recorded the best effects on growth parameters like shoot and root length and the absorption of important nutrients.

Key words : *Azospirillum brasilense,* Nutrient absorption, Plant-growth promoting bacteria, Red pepper, Rice, Tomato

Introduction

Azospirillum is a free-living, plant-growth promoting bacterium, capable of affecting growth and yield of numerous plant species, many of agronomic and ecological significance. They are isolated from the rhizosphere of many grasses and cereals and even from ornamental crops and their roles on plant growth and yield have been well established (James, 2000; Bashan et al., 2004; Gadagi et al., 2004). Apart as a plant-growth promoting bacteria (PGPB), they are also used in environmental problems such as soil erosion, bioremediation of water etc (reviewed in Bashan et al., 2004). Though, *Azospirillum* originally considered to be a nitrogen fixing bacteria, their contribution to biological nitrogen fixation has been questioned and in recent decades the stimulatory effect of *Azospirillumon* plants has been attributed to several factors such as production of phytohormones, facilitating the nutrient uptake etc. The additive hypothesis states that more than one mechanism that operates simultaneously or in succession when induced in appropriate environmental conditions, results in the observed changes in plant growth due to *Azospirillum* inoculation (Bashan and Levanony,1990). Despite intensive studies on physiology and molecular biology of these bacteria, the exact mode of action of the bacteria on plants remains yet unclear.

Numerous factors govern the successful establishment of the introduced bacterium in the rhizosphere. The application dose of the inoculum is animportant factor that governs the survivability and establishment of the introduced bacteria that in turn play a role on their plant growth promotion. The inoculation dose for other PGPB has been studied and their effects have been demonstrated (Zheng and He, 1994; Bai et al.,2002). Though numerous studies document the beneficial effects of *Azospirillum,* the optimization of inoculum dose for *Azospirillum* is still lagging behind.

Our experiment was designed to study the *Azospirillum* brasilense strain CW903 dose effects on different plants under pot culture conditions. The effects of *A. brasilense* strain CW903 inoculation on plant growth and nutrient uptake were also measured at different inoculation dose.

Received : 25 April 2007 Accepted : 5 June 2007
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Materials and Methods

Bacterial strains and culture conditions *Azospirillum* brasilense strain CW903 originally isolated from Taro rhizosphere(Kim et al., 2005) was cultured on Nfb medium supplemented with 1g NH_4Cl per litre (Baldani and Dobereiner, 1980). Nfb medium contained (g L^{-1}) DL-Malic acid, 5.0 K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $CaCl_2 \cdot 2H_2O$, 0.02; 2 mL of minor element solution (stock solution of trace metals per 1000 mLconsists of $CuSO_4 \cdot 5H_2O$, 0.4; $ZnSO_4 \cdot 7H_2O$, 0.12; H_3BO_3, 1.4; $Na_2MoO_4 \cdot 2H_2O$, 1.0; $MnSO_4 \cdot H_2O$, 1.5), 2 mL of bromothymol blue solution (0.5% dissolve in 0.2 N KOH), 4 mL of 1.64% Fe-EDTA, 1 mL of vitamin solution (stock solution of vitamin per 100 mL consists of biotin, 10 mg; and pyridoxol·HCl, 20 mg). The pH was adjusted to 6.8 with 2 N KOH, and the medium was autoclaved at 121°C for 20 min.

Seed, bacterial inocula and plant growth conditions Pot culture experiments under green house conditions were carried out to test the effects of inoculation of the nitrogen fixing *A. brasilense* strain CW903 at different concentrations. Sterilization of seeds was achieved through incubation in 70% ethanol, 1 min; 2% NaOCl for 30 sec followed by 5-10 washing steps using sterile distilled water. For the preparation of bacterial inocula, the culture was proliferated in Nfb broth containing NH_4Cl to mid-exponential growth phase (OD_{600} = 1.2). The cell pellets obtained by centrifuging at 10,000 × g (4°C) for 10 min were washed and resuspended in 30 mM $MgSO_4$ to different dilutions such that they contained 6.65×10^5, 1.8×10^6, and 4.8×10^8 cfu mL^{-1}. The population was estimated by serial dilution and plating of the bacterial aliquots on Nfb media. The seeds were kept imbibed in the bacterial suspension for 6 h at room temperature under shaking (60 rpm). For control, the seeds suspended in 30 mM $MgSO_4$ were used.

Surface disinfected tomato, red pepper and rice seeds either inoculated with bacteria or untreated were sown in plastic pots (top diameter, 100 mm; bottom diameter, 75 mm; height, 85 mm) filled with approximately 250 g of air-dried Wonjo-Mix bed soil (Nong-Kyung Co., Ltd, Jincheon-gun, Chungbuk, Republic of Korea). It contains 65-75% cocoa peat, 15-20% zeolite, 10-15% perlite and macronutrient (mg L^{-1}) NH_4-N, 150-200; NO_3-N, 100-150; available P_2O_5, 200-300; pH 5.7 to 7.0; 40±5% moisture content; and 30±5 kPa water holding capacity. The pots were held in racks (20 pots per rack)and grown under green house conditions and watered regularly. The treatments were arranged in a completely randomized design with fourreplications per treatment. Bacteria was applied twice at 20 and 30 days after sowing (DAS) through soil inoculation at 1 mL of suspended liquid culture near the root zone. The crop was uprooted at 45 DAS and nutrient analysis was carried out after recording the growth parameters.

Nutrient analysis and population estimation Nitrogen (N) concentration in the plant materials was measured after digestion with sulphuric acid and potassium sulphate using the Kjeldahl Auto1030 analyzer. Concentration of P in roots and shoots was measured according to Jackson (1973) using ammonium metavanadate reagent. Standards were prepared with potassium dihydrogen phosphate (Sigma, USA). The uptake of other nutrients by the plants was measured through inductively coupled plasma optical emission spectroscopy (ICP-OES, Optima 5300DV, Perkin Elmer, USA) analysis after digesting the sample (500 mg)with perchloric acid, sulphuric acid and distilled water (10:1:2) in a hot plate. After digestion, the samples were filtered twice through filter paper (No. 6, Advantec Toyo), the volume was made 100 mL in volumetric flask and a 10 mL sample was used for analysis. Quality Control Standard 21 stock solution (100 μg mL^{-1} of 5% HNO_3/tr.Tart./tr.HF) from PerkinElmer Life and Analytical Sciences (710 Bridge Port Avenue, Shelton, CT 06484, USA) was used. The working solutions were prepared from primary stock solution using deionized distilled water.

The most probable number (MPN) method was used to estimate the *Azospirillum* population in the rhizosphere soil samples. Ten gram of soil was suspended in 90Lof sterile distilled water and serially diluted. Aliquots of 1 ml were inoculated to test tubes containing Nfb semisolid media. There were three replicates for each dilution. Seven days after inoculation at 30°C, the vials were analyzed for the presence or absence of a characteristic pellicle formed on semi-solid N-free media.

Statistical analysis Standard curves were generated by regression analysis (Excel, Microsoft) for every enzyme assay. The data were subjected to analysis of variance and testing of means by Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$ using SAS, Version 9.1

(SAS Institute Inc., 2001).

Results and Discussion

Since its discovery in the mid 1970s *Azospirillum* has consistently proven to be a very promising PGPB. In several developing and developed countries, *Azospirillumis* used as the bacterial inoculant of choice, alone or together with other PGPB and vesicular arbuscular mycorrhizal (VAM) fungi, for many crops (Bashan et al., 2004). Besides, *Azospirillum* also proves to be a promising inoculant to mitigate the stress conditions (Barassi et al.,2006). The inoculation dose is an important factor in agricultural application of microbial inoculants. In this study, the PGPB *Azospirillum brasilense* strain CW903 (Kim et al., 2005) was used to investigate the optimal dose of inoculation tested with three different crops. The *A. brasilensestrain* CW903 increased red pepper, tomato, and rice shoot and root length in a dose-dependent manner with an apparent maximum efficiency for bacterial concentration around 10^8 cfu mL^{-1}. In red pepper, application of *A. brasilensestrain* CW903 applied at different concentrations increased significantly the shoot length when compared to uninoculated control, while it had no effect on root length. The rootto shoot ratio increased at all the concentrations and the highest effect was recorded at an inoculation dose of 10^8 cfu mL^{-1} (Table 1). In tomato, the inoculation doses of 10^6 and 10^8 cfu mL^{-1} cell density significantly increased all the growth parameters measured. Although, no significant differences were observed with in these two inoculation doses, the higher the inoculation dose the greater was the effect observed in shoot, root lengths, stem girth and root to shoot ratio. The stem girth recorded an increase of 6.78 to 20% over the control in the bacterial inoculations (Table 2). In rice, no such significant effects on the root or shoot length could be observed with the inoculation of *A. brasilensestrain* CW903 at different inoculation doses though the bacterial inoculation increased the root and shoot length. However, the root to shoot ratio increased in the bacterial inoculations with the highest obtained at an inoculation dose of 10^5 cfu mL^{-1} (Table 3). As from the results discussed above, it can be observed that inoculation of *A. brasilense* strain CW903 had a growth promoting effect on all the three crops tested. The growth promoting effect was dose dependent and an inoculation dose of 10^8 cfu mL^{-1} was found to be the optimal dose for it recorded the

Table 1. Effects of different inoculation dose *Azospirillum brasilensestrain* CW903 on the shoot and root length and root to shoot ratio of red pepper.†

Treatment	Shoot length	Root length	Root/shoot ratio ǂ
	cm	cm	
Control	12.19b	16.70	0.435c
CW903 (6.65×10^5 cfu mL^{-1})	14.98a	16.86	0.483b
CW903 (1.8×10^6 cfu mL^{-1})	14.60a	16.92	0.491b
CW903 (4.8×10^8 cfu mL^{-1})	15.36a	18.51	0.502a
LSD (P=0.05)	1.23	NS	0.01

† Data are means four replications and observed at 45 days after sowing. In the same column, significant differences according to LSD at P= 0.05 levels are indicated by different letters.

ǂ The shoot to root ratio was obtained dividing shoot fresh weight by root fresh weight.

Table 2. Effects of different inoculation dose of *Azospirillum brasilense* strain CW903 on the growth parameters of tomato.†

Treatment	Shoot length	Root length	Stem girth	Root/shoot ratio
	cm	cm	mm	
Control	62.68b	24.56b	8.70b	0.15b
CW903 (6.65×10^5 cfu mL^{-1})	63.93b	26.42ba	9.29ba	0.18a
CW903 (1.8×10^6 cfu mL^{-1})	74.13a	27.45a	9.19a	0.18a
CW903 (4.8×10^8 cfu mL^{-1})	75.05a	27.20a	10.44a	0.19a
LSD (P=0.05)	9.17	2.60	1.48	0.016

† Data are means four replications obtained at 45 days after sowing. In the same column, significant differences according to LSD at P= 0.05 levels are indicated by different letters.

Table 3. Effects of different inoculation dose of *Azospirillum brasilense* strain CW903 on the shoot and root length and root to shoot ratio of rice.†

Treatment	Shoot length	Root length	Root/shoot ratio †
	cm	cm	
Control	23.78	12.59	0.275c
CW903 (6.65×10^5 cfu mL^{-1})	27.55	12.75	0.429a
CW903 (1.8×10^6 cfu mL^{-1})	30.29	13.22	0.391b
CW903 (4.8×10^8 cfu mL^{-1})	28.21	13.31	0.405b
LSD (P=0.05)	NS	NS	0.016

† Data are means four replications obtained at 45 days after sowing. In the same column, significant differences according to LSD at P= 0.05 levels are indicated by different letters.

higher values for most of the parameters tested. Such an optimal concentration on plant growth particularly on root modifications has often been observed for other PGPB-plant interactions (Harari et al., 1988; Larcher et al., 2003).

The effects of *A. brasilense strain* CW903 varied on the nutrient uptake of the crops studied. In red pepper, the uptake of primary nutrients N, P, and K and secondary nutrients like Ca and Mg increased significantly with bacterial inoculation. However, the effects on different nutrients varied among the inoculation doses used (Table 4). For most nutrients, the inoculation dose 10^8 cfu mL^{-1} recorded the highest nutrient concentration in red pepper and similarly the dose 10^5 cfu mL^{-1} showed no effect when compared to control (Table 4). *A. brasilense* strain CW903 inoculation to rice recorded similar effects on the nutrient uptake in rice. Inoculation at a dose 10^8 cfu mL^{-1} recorded the highest concentration or the uptake of N, P, K and other nutrients with the exception of Cu (Table 5). In tomato the results recorded a different trend. For tomato shoots, in most cases and particularly for N, P, and K the inoculation of *A. brasilense* strain CW903 produced no enhancements and sometimes lower than the uninoculated control (Table 6). However, the concentration of P, K and other micro nutrients except Fe, increased with the bacterial inoculation though it varied among the inoculation doses. For most of the nutrients analyzed, the inoculation dose 10^6 cfu mL^{-1} proved to be superior to the other concentrations (Table 6). It can be inferred from the above results, in general the inoculation of *A. brasilense* strain CW903 enhanced the nutrient uptake and the effects varied with the type of crops

Table 4. Effects of *Azospirillum brasilense* CW903 inoculation on nutrient uptake in red pepper.†

Treatment	N	P	K	Ca	Mg	Mn	Zn	Cu	Fe
	%	mg kg^{-1}							
Control	3.98b	557.17c	57060d	14519b	6826c	179.11b	100.38c	4.42b	463.8c
CW903 (6.65×10^5 cfu mL^{-1})	4.67a	586.52b	66214b	13416b	8596b	178.41b	344.56a	463.8c	865.8b
CW903 (1.8×10^6 cfu mL^{-1})	4.90a	614.29a	64455c	17131a	8623b	195.26a	265.20b	4.60b	934.6b
CW903 (4.8×10^8 cfu mL^{-1})	5.04a	627.14a	70539a	17644a	9063a	200.83a	271.93b	865.8b	1012.5a
LSD (P=0.05)	0.48	24.69	1688.5	1574	389.92	6.41	31.87	6.09a	74.69

† Data are means four replications and in the same column, significant differences according to LSD at P= 0.05 levels are indicated by different letters.

Table 5. Effects of *Azospirillum brasilense* CW903 inoculation on nutrient uptake in rice.†

Treatment	N	P	K	Ca	Mg	Mn	Zn	Cu	Fe
	%	mg kg^{-1}							
Control	1.67b	496.39c	23570c	2306b	2704b	402.68b	112.34c	6.89ba	793.65cb
CW903 (6.65×10^5 cfu mL^{-1})	2.24a	543.82b	28064b	2572b	3472a	422.13b	114.98c	5.86bc	848.26b
CW903 (1.8×10^6 cfu mL^{-1})	2.05a	582.62a	33542a	3490a	3189a	618.44a	189.73b	7.34a	757.53c
CW903 (4.8×10^8 cfu mL^{-1})	2.07a	558.63ba	32477a	3710a	3120a	717.99a	246.22a	5.34c	942.78a
LSD (P=0.05)	0.26	28.18	2282.7	635.34	395.31	131.49	19.98	1.105	88.29

† Data are means four replications and in the same column, significant differences according to LSD at P= 0.05 levels are indicated by different letters.

Table 6. Effects of Azospirillum brasilenseCW903 inoculation on nutrient uptake in shoot and root of tomato.†

Treatment	N	P	K	Ca	Mg	Mn	Zn	Cu	Fe
	%	----------	----------	----------	---------- mg kg^{-1} ----------	----------	----------	----------	----------
Shoot									
Control	1.67	589.3a	60561a	9866b	4691a	206.05b	115.37a	8.38ba	128.93c
CW903 (6.65×10^5 cfu mL^{-1})	1.72	495.6c	52925b	8697b	3699c	196.35c	103.42b	8.19b	154.453b
CW903 (1.8×10^6 cfu mL^{-1})	1.71	545.2ba	53691b	13610a	4566a	130.59d	90.82c	8.70a	156.63ba
CW903 (4.8×10^8 cfu mL^{-1})	1.87	514.2bc	48262d	10942b	4286b	227.87a	87.68c	8.41ba	169.293a
LSD (P=0.05)	NS	48.73	3186.3	2668.1	241.43	9.099	6.93	0.449	13.16
Root									
Control	1.50	539.8b	23327b	6768c	2953b	432.66b	172.25b	5.86c	1127.48
CW903 (6.65×10^5 cfu mL^{-1})	1.64	614.2a	32302a	7057b	2652b	486.16a	272.37a	8.45ba	1290.18
CW903 (1.8×10^6 cfu mL^{-1})	1.49	619.1a	29999a	7618a	3623a	250.33c	279.27a	8.18b	1146.29
CW903 (4.8×10^8 cfu mL^{-1})	1.54	620.4a	26438ba	6923cb	2856b	404.16b	171.61b	8.76a	1363.79
LSD (P=0.05)	NS	25.22	5894.8	256.66	347.1	45.6	19.30	0.34	NS

† Data are means four replications and in the same column, significant differences according to LSD at P= 0.05 levels are indicated by different letters.

studied. For tomato, because of the bulkiness of the plant material the nutrients were analyzed separately for roots and shoots and if it were done for the whole plant as that for rice and red pepper the results could be different. Hence, it would not be appropriate to conclude that inoculation of *A. brasilense* strain CW903 is not beneficial for tomato. In recent decades, numerous studies have pointed out that plant growth promotion by *Azospirillum* would be due to a multitude of effects operating simultaneously or in succession and the most common explanation is the production of phytohormones that alter the metabolism and morphology of the plant yielding better mineral and water absorption (Bashan et al., 2004). In this study also, the plant growth promotion by *A. brasilense* strainCW903 can be attributed to the increased nutrient uptake. Alternatively, the possibility of plant-growth promotion due to a concerted action of other mechanisms that were not considered in this study could not be ruled out.

For any observed beneficial effect on plants, colonization by the inoculated bacteria is an important factor. The inappropriate root colonization usually resulted in marginal or no effects on plant growth (Benizri et al., 2001). *Azospirillum* is mostly considered as a rhizosphere soil dweller and hence in this study we estimated the population of *Azospirillum* in the rhizosphere soil samples by a MPN technique. The presence or absence of the characteristic pellicle formed on semi-solid N-free media was selected as a criterion. In all the crop samples, the bacterial population at an inoculation dose of 10^8 cfu mL^{-1} recorded the highest value (Table 7). The possible explanation of the enhancement of the observed parameters at this particular inoculation dose is that increased colonization or the presence of more number of bacteria in this condition.

Conclusion

It can be inferred from this study that inoculation of *A.*

Table 7. Population of *Azospirillum* in the rhizosphere soil of tomato, red pepper and rice.†

Treatment	Population of *Azospirillum*		
	Tomato	Rice	Red pepper
	----------	log cfu g^{-1} rhizosphere soil	----------
Control	3.43b	2.86c	3.46c
CW903 (6.65×10^5 cfu mL^{-1})	3.62b	3.86b	3.72b
CW903 (1.8×10^6 cfu mL^{-1})	4.32a	3.96ba	4.32a
CW903 (4.8×10^8 cfu mL^{-1})	4.46a	4.08a	4.46a
LSD (P=0.05)	0.299	0.179	0.171

† Data are means four replications after 45 days after sowing. In the same column, significant differences according to LSD at P= 0.05 levels are indicated by different letters.

brasilense strain CW903 prove beneficial for the crop plants studied increasing the nutrient absorption and the growth parameters. The effective inoculation dose for this strain through seed and soil was in the range of 10^6-10^8 cfu mL^{-1}. The best plant growth and nutrient absorption was found when CW903 10^8 cfu mL^{-1} of *A. brasilense* strain was applied through seed treatment and through the soil (1 mL per plant) at 20 and 30 days after sowing near the root zone.

Acknowledgements

This work was supported by the research grant from Chungbuk National University 2006. The authors also thank Soo-Jae Park and Sook-Jin Kim for their help and assistance in conducting the pot culture experiments and for analyzing the samples by ICP-OES.

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온실조건에서 토마토, 고추, 벼를 이용한 식물생장촉진 미생물 *Azospirillum brasilense* CW903 접종의 최적 조건 평가

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식물생장촉진 미생물을 농업적으로 활용하는데 있어서 미생물의 접종 수준이 근권에서의 원활한 정착과 식물의 생장촉진 효과를 결정하는데 중요한 기준이 된다. *Azospirillum brasilense* CW903균주 10^5, 10^6 및 10^8 cfu mL^{-1} 현탁액에 토마토, 벼, 고추의 종자를 침지시킨 후 포트에 파종하여 온실에서 재배하였으며 파종 후 20일과 30일에 1 mL씩 근권토양에 접종하여 각 식물의 생장과 양분흡수를 조사하였다. *Azospirillum brasilense* CW903 균주 10^6 및 10^8 cfu mL^{-1} 현탁액을 처리하였을 때 식물생장 촉진효과가 나타났으며, 10^8 cfu mL^{-1} 현탁액을 처리하였을 각 식물의 줄기와 뿌리의 생장 및 양분흡수가 가장 우수한 것으로 나타났다.
