Isolation and evaluation of inoculation effect of *Azospirillum* sp. on growth, colonization and nutrient uptake of crops under green house condition

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Abstract
Nitrogen-fixing bacteria were isolated from the rhizosphere of different crops in Korea and were tested for their acetylene reduction activity (ARA). A total of 13 isolates were screened and were identified to belong to the reference strains of *Azospirillum.* The isolates were categorized into 2 groups- *A. brasilense and A. lipoferum* based on their sugar and biotin requirement. Among them *A. brasilense* was dominant and *A. brasilense* strains CW301 and CW903 showed the highest ARA. β-galactosidase activity verified through *pLA-lacZ* tagging confirmed their ability to colonize wheat root. Further investigation were made to verify the efficiency of *A. brasilense* CW903 to enhance crop growth and nutrient uptake of red pepper, tomato and rice under green house condition. Except for root lengths of red pepper and rice and root-shoot ratio of tomato, the inoculation of *A. brasilense* CW903 significantly improved other growth parameters ($P \le 0.05$). Except for P, K, Mg and Zn in tomato, inoculation of *A. brasilense* CW903 appreciably enhanced the accumulation of other nutrients in rice and red peper. The consistent improvement in growth and nutrient uptake in non host crops suggest the potential of the *A. brasilense* CW903 for large scale field application.

Key Words
Plant growth-promoting rhizobacteria (PGPR), Nitrogen-fixing bacteria *, Azospirillum.* Acetylene reduction activity, β-galactosidase activity

Introduction
Azospirillum spp. isolated from various geographical regions of the world are the best-characterized genus of plant growth-promoting rhizobacteria (PGPR). They are known to associate with the roots of wheat, tropical grasses, maize, and other cereals (Okon and Hadar 1987; Oh *et al.* 1999). Members of the genus *Azospirillum* are gram-negative to gram-variable, curved-rod shape, motile, oxidase positive and exhibit acetylene-reduction activity (ARA) under micro-aerophilic conditions. *Azospirillum* spp. have been identified mainly as rhizosphere bacteria and its colonization of the rhizosphere has been studied extensively along with reporter gene fusion (Pereg-Gerk *et al.* 2000; Burdman *et al.* 1997; Holguin *et al.* 1999; Steenhoudt and Vanderleyden 2000). Recently, co-inoculation of *Azospirillum* sp. with *Methylobacterium* enhanced nutrient uptake by different crops as reported by Madhaiyan *et al*. (2009). The aims of this study were to isolate and identify efficient nitrogen fixing strains of *Azospirillum* spp. from the roots of various plants from Chungbuk province, South Korea, and to study their ability to colonize through *lacZ* fusions. Inoculation potential of strain *A. brasilense* CW903 on growth and nutrient uptake of rice, red pepper and tomato was also evaluated.

Methods
Isolation and characterization of N-fixing isolates
Screening of N-fixing organisms from the rhizosphere of different crops was carried out by the enrichment culture technique using semisolid malate medium (NFB) and further characterized by gram staining, glucose assimilation and biotin requirement (Baldani and Dobereiner 1980; Tarrand *et al.* 1978).

Acetylene-reduction assay
The acetylene-reduction assay (ARA) was performed on free-living cultures as well as on cultures in association with wheat plants. Ethylene formation was measured using a Varian model 3700 Gas chromatograph. The protein concentration was determined by Lowry method.

Sequencing of bacterial 16s rDNA and conjugation for reporter gene
Amplification for 16S rDNA was performed according to Devereux and Willis (1995) using primer 27F (5′-AGAGTTTGATCCTGG CTCAG-3′,), 1512R primer (5′- CGGCTACCTTGTTACGACT-3′). The amplified product was sequenced and compared with NCBI data base. On the basis of 16s rDNA comparison, five strains of *Azospirillum*, including reference strains, were conjugated with *Escherichia coli* S17.1 carrying the

plasmid *pLA-lacZ* to study colonization (Simon *et al*. 1983). Transconjugants were selected on minimal lactate medium supplemented with ammonium chloride (1 g/L) and tetracycline (5 µg/ml).

Effect of A. brasilense CW903 on plant colonization, plant-growth and nutrient uptake
In situ colonization study was verified in wheat seedlings according to Zeeman *et al*. (1992) in a growth chamber with a day-night cycle of 14 h (27 °C) and 10 h (18 °C) and β-galactosidase activity were determined 10 d after inoculation. Efficiency of *A. brasilense* CW903 on plant growth and nutrient uptake in red pepper, tomato and rice were evaluated under green house condition by seed bacterization. Bacterization of surface sterilized seeds was performed by imbibing the seeds in *A. brasilense* CW903 cell suspension (A600=0.5) for 6 h at 60 rpm. Seeds treated with sterile distilled water alone was considered as control. Seeds either inoculated with bacteria or untreated were sown in plastic pots (100 mm w x 75mm d x 85mm h) filled with approximately 250 g of air-dried wonjo mixed bed soil. The pots were held in racks (20 pots per rack) and grown under green house condition and watered regularly. Growth parameters such as shoot length, root length and root-shoot ratio were recorded 45 days after planting.
Total nitrogen was determined by a Kjeldahl Autoanalyzer Model 1030. Macronutrients (Ca, K, P and Mg) and micronutrients (Zn, Mn and Fe) absorbed by the plants were analysed using ICP-OES after acid digestion. Data were subjected to mean standard error (SE) and inoculation effect of *Azospirillum* on growth and nutrient uptakes were subjected to paired comparison.

Results

A total of 13 strains isolated from the rhizosphere of different plants grown in different regions of Korea were able to fix nitrogen (Table 1). These strains were isolated from a variety of plants including rice, wheat, Sudan grass, onions, and several dicotyledonous plants indicating that these nitrogen-fixing

Table 1. Characteristics of the N2-fixing strains isolated from the rhizosphere of different field-grown plants

Strains	Host plant	Gram reaction	Oxidase reaction	Potato infusion	C and N utilization		Group [a]
					Malic acid	Biotin	
Azospirillum brasilense Sp7[a]	-	-	+	Pink	+	-	I
Azospirillum brasilense CW5	Tobacco	-	+	Pink	+	-	I
Azospirillum brasilense CW202	Rice	-	+	Pink	+	-	I
Azospirillum brasilense CW301	Wheat	-	+	Pink	+	-	I
Azospirillum brasilense CW307	Wheat	-	+	Pink	+	-	I
Azospirillum brasilense CW406	Soybean	-	+	Pink	+	-	I
Azospirillum brasilense CW705	Rice	-	+	Pink	+	-	I
Azospirillum brasilense CW716	Onion	-	+	Pink	+	-	I
Azospirillum brasilense CW805	Onion	-	+	Pink	+	-	I
Azospirillum brasilense CW903	Taro	-	+	Pink	+	-	I
Azospirillum brasilense CW1401	Soybean	-	+	Pink	+	-	I
Azospirillum brasilense CW1402	Rice	-	+	Pink	+	-	I
Azospirillum brasilense CW1502	Apple	-	+	Pink	+	-	I
Azospirillum brasilense 687[b]	-	-	+	Pink	+	+	II
Azospirillum brasilense CW1503	Sudan grass	-	+	Pink	+	+	II

[a]The groupings are based on the similarities to the reference strains [a]*A. brasilense* Sp7 and [b]*A. lipoferum* 687.

bacteria are inhabitants of the rhizosphere of many plant species. All the isolated strains were gram-negative, oxidase positive, Twelve strains showed characteristics of *A. brasilense* Sp7 and one strain (CW1503) showed characteristics of *A. lipoferum* 687. Among them, *A. brasilense* was found to be the dominant species on plant roots. There was wide variation in nitrogenase activity among the different isolates (Figure 1). *A. brasilense* CW301 and *A brasilense* CW903, which are associated with wheat roots and taro, had the highest ARA activity. The strains that showed the highest nitrogenase activity in wheat roots viz., *A. brasilense* CW301, *A. brasilense* CW903, *A. lipoferum* CW1503 were selected and their identifications were confirmed by 16S rDNA sequencing. 16 srDNA squencing confirmed 98% to 99% homology with *A. brasilense, A. lipoferum*, respectively, and were assigned the GenBank accession numbers AY518780, AY518777, AY518779, respectively. Further colonization studies with *A. brasilense* CW301, *A. brasilense* CW903 and *A. lipoferum* CW1503 showed considerable β-galactosidase activity under aerobic growth. Transconjugant of *A. brasilense* CW301 exhibited the highest activity followed by *A. lipoferum* CW1503 (data not shown).

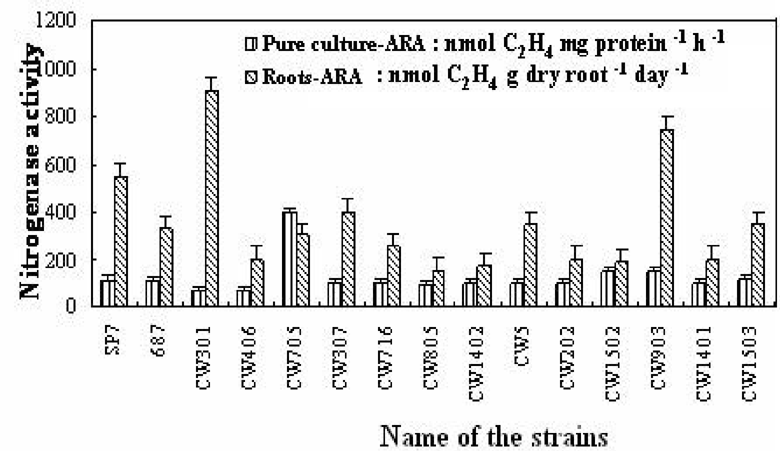

Figure 1. Nitrogenase activity of the isolates in semi-solid nitrogen free (NFB) medium and in association with the roots of wheat seedlings. Reference strains *Azospirillum brasilense* SP7 and *A. lipoferum* 687. Error bars represent standard deviation of observed values.

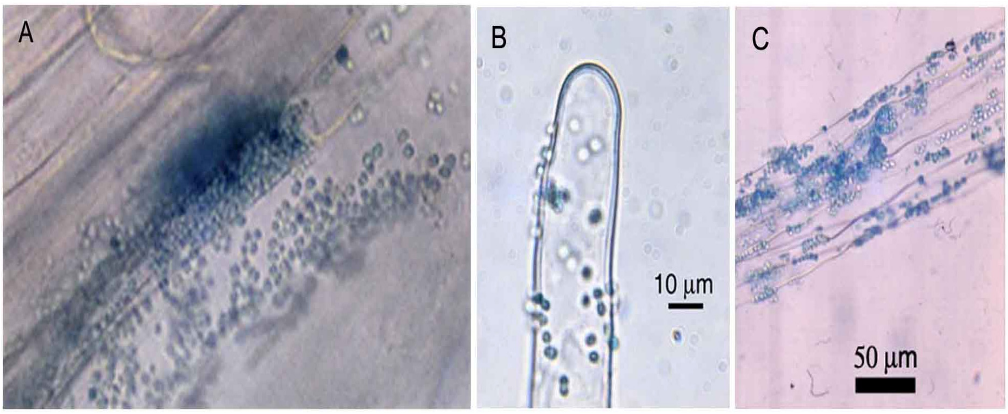

Figure 2. In situ detection of β-galactosidase activity on root segments of wheat plants grown after treatments of *Azospirillum brasilense* CW903. All samples were stained after 10 days of inoculation and X-gal staining was performed on five or six 1 cm root segments excised from the same plant starting from the tip. (A) root inoculated with *A. brasilense* CW903 (X60), (B) Root inoculated with *A. brasilense* (X100); (C) Root inoculated with *A. brasilense* SP7 (X40)

Examination of segments of wheat roots stained with X-Gal readily enabled the visualization of Azospirillum cells bearing *pLA-lacZ* plasmids (Figure 2). This confirms the colonization and multiplication ability of the strains along the root surface of the inoculated plants.

Effect of Azospirillum brasilense CW903 on growth and nutrient uptake of red pepper, rice and tomato

Except for root lengths of red pepper and rice and root-shoot ratio of tomato, treatment of *A. brasilense* CW903 significantly improved the growth of red peper, tomato and rice ($P \leq 0.05$) (Table 2). There was 18.7 – 26% increase in shoot length of red pepper, tomato and rice and 5.7 – 10.8% in root length of tomato.

Table 2. Effect of *Azospirillum brasilense* CW903 on the shoot, root length and root to shoot ratio of three different crops

Name of the plant	Treatments	Length (cm)		Root/shoot ratio
		Shoot	Root	
Red pepper	Control	12.19	16.70	0.435
	CW903	15.36*	18.51	0.502**
Tomato	Control	62.68	24.56	0.15
	CW903	75.05**	27.20**	0.19
Rice	Control	23.78	12.59	0.27
	CW903	28.21*	13.31	0.41*

The data are mean of four replications; *significant differences according to paired comparison.

Except for P, K, Mg and Zn in tomato, inoculation of *A. brasilense* CW903 appreciably enhanced the accumulation of total nutrients (Table 3). The total increment of the uptake of macro-and micronutrients by red pepper, tomato and rice were 12.0-26.7% N, 11.0-60.7% Ca, 4.8 – 77.5% Mn and 19.0 – 119.6% Fe.

Table 3. Effect of treatments of *Azospirillum brasilense* CW903 on total macro- and micronutrient content of different crops.

Name of The plant	Treatments	Macronutrient (mg/g dry plant biomass)					Micronutrient (g/g dry plant biomass)		
		N	P	K	Ca	Mg	Mn	Zn	Fe
Red pepper	Control	3.98	0.56	57.06	14.51	6.82	0.17	0.10	0.46
	CW903	5.04*	0.63	70.53*	17.64	9.06*	0.20	0.34*	1.01*
Tomato	Control	1.67	0.58	60.56	9.86	4.69	0.21	0.12	0.13
	CW903	1.87	0.53	48.26	10.94*	4.28	0.22	0.08	0.17
Rice	Control	1.67	0.49	23.57	2.31	2.70	0.40	0.11	0.79
	CW903	2.07*	0.56	32.48*	3.71*	3.12*	0.71*	0.25	0.94*

The data are mean of four replications; *significant differences according to paired comparison.

Conclusion

Out of the 13 isolates, the strains *A. brasilense* CW301 and CW 903 showed highest ARA activity indicating that these strains may be suitable for inoculating wheat and other crops. β-galactosidase activity confirms that the strains are able to colonize introduced plants. Further inoculation of *A. brasilense* CW 903 on three crop plants prove the potential of this strain to enhance crop growth and nutrient uptake under green house conditions. The consistent improvement in growth and nutrient uptake of red pepper and rice plants by *A. brasilense* CW903 suggest the potential of this strain for large scale field application.

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