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Azospirillum spp. from Crop Roots: A Promoter of Plant Growth

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Abstract
Nitrogen-fixing bacteria were isolated from the roots of several gramineous and non-gramineous crops in Japan, Thailand and the Philippines. All the isolates, being incapable of utilizing glucose as a sole carbon source, were identified as a species similar to Azospirillum brasilense. Some of those isolates clearly exhibited a growth-promoting effect on roots and shoots in response to inoculation to the roots of their respective host plants.

Discipline: Soils, fertilizers and plant nutrition
Additional key words: inoculation, maize, nitrogenase, rice, spinach

Introduction

The most common nutrient definitely affecting the production of crops is nitrogen. Most of the nitrogen input into soils is provided in the form of recycled inorganic compounds, such as nitrates and ammonia, which are derived from the excretion and the decomposition of organisms. Transfer of the nitrogen from atmosphere to bioshere takes place by nitrogen fixation. There are three ways for the nitrogen fixation: 1) spontaneous fixation in the atmosphere by electrical discharge, 2) industrial fixation by Haber-Bosch process, 3) biological nitrogen-fixation by prokaryotic microorganisms. The most important biological nitrogen-fixation is symbiotic systems in legume-*Rhizobium*, non-legume tree-*Frankia*, and *Azolla-Anabaena*.

In the other plants, there are no symbiotic nitrogen fixing systems. However, a number of nitrogen-fixing bacteria exist in the root surface and rhizosphere of gramineous and non-gramineous crops as well; they can fix nitrogen by associative symbiosis under microaerobic conditions[2]. *Azospirillum* is one of the well-known nitrogen fixing soil bacteria, which was isolated first from the root surface of forage grasses and some cereals[4]. It is confirmed that *Azospirillum* promotes the growth of its host plants by producing plant hormones such as gibberellin and cytokinin-like substances[17]. *Azospirillum* accelerating the growth of host plants has also been isolated from the roots of other plants, including non-gramineous crops[6,7].

In Japan, various nitrogen-fixing bacteria have been isolated from the roots of rice plants: one of them is identified as *Krebsiella oxytoca*[9,14]. It is recognized that nitrogen-fixing ability in rice plants by the association of these bacteria varies among the rice varieties and that it is generally higher in indica than in japonica[16]. Nitrogen-fixing bacteria were also isolated from the roots of various gramineous plants and these isolates were identified as *A. brasilense* or its similar bacteria[12]. Gamo and Toriyama (1989)[6], Gamo and Ahn (1990)[7] attempted to isolate nitrogen-fixing bacteria from the roots of some selected gramineous and non-gramineous crops in Japan, the Philippines and Thailand. The present paper describes characterization of those isolates with special emphasis placed on their effect on plant-growth promotion.

Isolation and collection of *Azospirillum*

Root samples of gramineous crops were collected from Tokachi, Tsukuba, Nagano and Okinawa in Japan, the Philippines and Thailand. Collections were also made from non-gramineous crops grown in Tsukuba and Kukizaki both in Ibaraki, Japan.

Azospirillum was isolated after the direction of Krieg and Döbereiner (1984)[11], using semisolid nitrogen-free malate (NFb) medium. After enrichment of nitrogen-fixing bacteria forming a pelicle (Plate 1) in the NFb medium, the growth was streaked onto plates with a solidified NFb medium containing an yeast extract and Congo Red, whereby typical small, dense, single colonies stained as scarlet color were subcultured to the semisolid NFb medium. Rod shaped and motile bacteria were identified as *Azospirillum* spp. and preserved in the semisolid NFb medium at 10°C with monthly transfers. For long-term preservation, TSS broth[18] was used to store it at −80°C. Long-term maintenance of *Azospirillum* requires a careful treatment.

1) Gramineous crops

In the course of isoslation work, 95 isolates exhibiting a high nitrogen-fixing ability in the NFb medium were collected from the roots of several cereals and wild grasses. However, it was difficult to isolate *Azospirillum* from the root samples collected in Tokachi (Hokkaido) and Tsukuba (Ibaraki), and in a few samples from Okinawa and the Philippines. Acetylene reduction ability (ARA) of the isolates in free living state is shown in Table 1. A high ARA is observed in the isolates from the roots of sugarcane in the Philippines and of rice in Thailand.

Some characteristics of those isolates examined were shown in Table 2. Most of the isolates cannot use glucose as a sole carbon source: they were identified as *A. brasilense* or closely related bacteria. However, these isolates did not have the same components of antigens as the type culture of *A. brasilense* (ATCC 29707) based on Ouchterony agar gel immunodiffusion. Two isolates yielded dark pink colonies forming carotenoid. Most of the isolates were capable of reducing NO_3^- to NO_2^-, while two isolates had no ability to reduce NO_3^-. Forty-five percent of the isolates displayed no nitrite reductase activity.

2) Non-gramineous crops

Azospirillum was isolated from the roots of five vegetable crops: i.e. spinach (*Spinacia oleracea*), Chinese cabbage (*Brassica chinensis*), Chinese

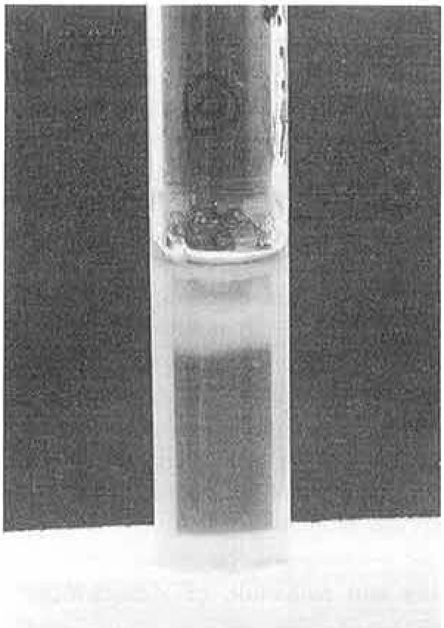

Plate 1. Growth of *Azospirillum* spp. forming a pelicle under the surface of a semisolid NFb medium

Table 1. Nitrogen fixation (acetylene reduction) of *Azospirillum* spp. in free-living state (NFb medium)[6]

Host plants isolated	No. of isolates	ARA (nmoles $C_2H_4hr^{-1}$/tube)	
		Mean	Max.
Nagano			
Maize	6	208.0	256.7
Okinawa			
Setaria	3	110.7	156.5
Rice	1	221.6	
Sugarcane	11	182.1	247.9
Buffel grass	3	21.6	37.1
Philippines			
Rice	8	202.6	236.2
Sugarcane	13	256.4	418.8
Wild sugarcane	8	174.9	257.3
Sorghum	7	84.1	112.6
Maize	11	218.9	251.1
Napier grass	2	246.3	272.4
Thailand			
Buffel grass	5	226.0	250.0
Rice	3	213.4	304.8
Maize	12	216.5	293.4
A. brasilense (ATCC)	1	279.7	

mustard (*Brassica rapa* var. *pervidis*), soybean (*Glycine max*), and cucumber (*Cucumis sativus*), grown in the soils of Tsukuba and Kukizaki both in Ibaraki, Japan. As shown in Table 3, all the isolates were identified as a species similar to *Azospirillum brasilense*: they were incapable of utilizing glucose as a sole carbon source, possessing nitrate reductase (Nr) and nitrite reductase (Nir) activities except for no Nir activity in case of the isolates from soybean. However, the ARA values in all the isolates were slightly lower than those in the type culture of *A. brasilense*.

Responses of the host plants to the isolate inoculations

1) Gramineous crops

In order to clarify growth-promoting effects of *Azospirillum*, all the isolates from the gramineous plants were inoculated to the roots of the following three cereal crops; rice (*Oryza sativa* cv. Nipponbare), wheat (*Triticum aestivum* cv. Norin 61) and maize (*Zea mays* cv. Daiheigen).

The experimental result indicated that no signifi-

Table 2. Colony color, utilization of glucose and reductase activity in *Azospirillum* spp. isolated from several plants in different countries[6)]

Host plants isolated	No. of isolates examined	Red/pink colony[a)]	Utilization of glucose[a)]	Reductase[a)]	
				Nitrate (Nr^+)	Nitrite (Nir^+)
Nagano					
Maize	6	0	0	6	6
Okinawa					
Setaria	3	0	1	3	3
Rice	1	1	0	1	1
Sugarcane	11	0	0	11	0
Buffel grass	3	0	3	3	1
Philippines					
Rice	8	0	0	8	0
Sugarcane	13	1	0	13	4
Wild sugarcane	8	2	0	8	8
Sorghum	7	0	0	7	4
Maize	12	1	2	10	10
Napier grass	2	0	0	2	0
Thailand					
Buffel grass	6	0	0	6	2
Rice	3	1	0	3	1
Maize	12	1	0	12	12

a): Number of isolates.

Table 3. Nitrogen fixation, nitrate and nitrite reductions, and glucose utilization of *Azospirillum* spp. isolated from five vegetable crops[7)]

Code no.	Host plant	No. of isolates	ARA (nmole C_2H_4 hr^{-1})		Use of glucose	Reduction of	
			Mean	Max.		Nitrate	Nitrite
1	Spinach	6	122.4	144.4	−[a)]	+	+
2	Chinese cabbage	8	155.1	185.7	−	+	+
4	Soybean	4	127.1	137.2	−	+	−
6	Cucumber	5	167.2	219.3	−	+	+
8	Komatsuna	4	202.0	228.1	−	+	+
	A. brasilense (ATCC 29145)	−	278.1		−	+	+

a): − ; 100% of the isolates are negative, + ; 100% of the isolates are positive.

Table 4. Effect of inoculation of *Azospirillum* spp. isolated from Okinawa, the Philippines and Thailand on growth and dry weight of maize[a)6)]

Inoculant	No. of isolates tested[e)]	Plant height (cm)		Root dry weight (g plant^{-1})		Shoot dry weight (g plant^{-1})	
		Mean	Max.	Mean	Max.	Mean	Max.
Okinawa[b)]							
Non-inoculated	3	45.7	50.0	0.70	0.77	1.90	2.58
A. spp. (Setaria)	4	42.8	46.5	0.62	0.73	2.48	2.87
A. spp. (Rice)	3	44.2	49.5	0.60	0.70	2.31	2.80
A. spp. (Sugarcane)	10	45.7	54.0	0.87	1.10	2.49	2.93
A. spp. (Buffel grass)	1	40.0		0.78		2.04	
A. lipoferum	1	39.0		0.86		2.62	
A. brasilense	1	40.5		0.83		2.20	
Philippines[c)]							
Non-inoculated	3	38.1	39.0	0.60	0.67	1.82	1.92
A. spp. (Rice)	8	40.1	46.9	0.69	0.80	1.94	2.29
A. spp. (Sugarcane)	12	43.8	56.2	0.65	0.92	1.87	2.24
A. spp. (Wild sugarcane)	7	39.7	46.5	0.65	0.73	1.55	1.71
A. spp. (Sorghum)	7	41.2	54.5	0.64	0.77	2.04	2.53
A. spp. (Maize)	13	44.0	55.0	0.69	0.79	1.80	2.10
A. spp. (Napier grass)	3	39.0	46.2	0.65	0.79	2.01	2.62
A. lipoferum	1	26.5		0.52		1.26	
A. brasilense	1	26.0		0.65		1.30	
Thailand[d)]							
Non-inoculated	6	32.5	35.0	0.61	0.81	1.58	1.82
A. spp. (Rice)	3	35.5	36.7	0.68	0.75	1.65	1.84
A. spp. (Maize)	13	34.3	40.0	0.62	0.81	1.55	1.79
A. spp. (Buffel grass)	6	34.0	37.7	0.74	1.04	1.64	1.85
A. amazonense	2	38.5	39.0	0.95	1.17	2.22	2.35

a): Cultivar inoculated; Dentcorn (cv: Daiheigen).

b): Inoculation experiment of *Azospirillum* spp. isolated from Okinawa required a 60-day period of cultivation from May 28, 1987 in a greenhouse at 25–28°C.

c): Inoculation experiment of *Azospirillum* spp. isolated from the Philippines required a 45-day period of cultivation from March 23, 1988 in a greenhouse at 25–28°C.

d): Inoculation experiment of *Azospirillum* spp. isolated from Thailand required a 40-day period of cultivation from March 23, 1988 in a greenhouse at 25–28°C.

e): Number of duplications.

cant effects were seen in rice and wheat plants, while the isolates from the roots of sugarcane, sorghum, maize and buffel grass exhibited promoting effects on the growth of roots and shoots in maize. The inoculation of those isolates increased the length and number of roots in general, and plant height and weight as well in some isolates (Table 4 and Plate 2). On the basis of the growth rates in the inoculation experiment, approximately 20 isolates were selected for further tests aiming at practical use in future.

2) Vegetable crops

Azospirillum spp. isolated from five vegetable crops, including spinach, Chinese cabbage, cucumber, Chinese mustard and soybean, were inoculated to the roots of their host plants. The results are presented in Tables 5 and 6. Significant impacts in promoting plant growth were observed in spinach: roots of the inoculated spinach were considerably longer than those of the uninoculated plants (Plate 3), and plant height and leaf weight were also higher in the inoculated plots.

In other three vegetable crops, i.e. Chinese cabbage, cucumber and Chinese mustard, some effects were also observed in the growth of leaves (Table 5), whereas no increase took place in root length. In Chinese cabbage, a slight increase in root and

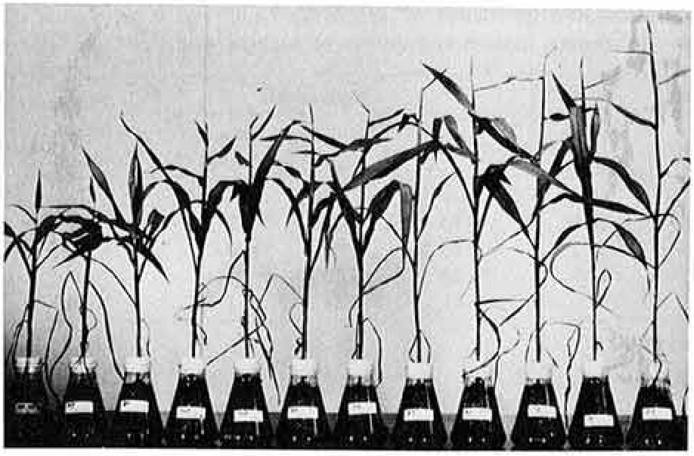

Plate 2. Effect of inoculation of *Azospirillum* spp. isolated from grass roots on shoot development of maize (cv: Daiheigen) The 4th and 5th plants from the left: non-inoculated, and the others: inoculated with *Azospirillum* spp.

Table 5. Effects of *Azospirillum* inoculation on the growth of four vegetable crops[a) 7)]

Host plant for inoculation	Isolate inoculated[b)]	No. of plant	Fresh wt. of root (g)	Plant height (cm)		Fresh wt. of leaves (g)	
				Mean	Max.	Mean	Max.
Spinach	Non-inoculated	4	0.36	11.1	15	3.25	4.3
	A. sp. (1–2)	4	0.37	16.1	19	7.26	10.8
	A. sp. (1–5)	5	0.43	19.9	24	7.63	9.1
	A. sp. (1–6)	5	0.39	19.2	25	8.49	17.4
Chinese cabbage	Non-inoculated	3	1.8	17.3	18	11.3	16.6
	A. sp. (2–1)	3	2.3	17.7	21	14.0	17.0
	A. sp. (2–5)	3	2.3	20.7	23	16.6	21.8
	A. sp. (2–6)	3	1.4	18.0	19	14.1	19.6
Cucumber	Non-inoculated	2	7.0	39.0	42	20.7	22.0
	A. sp. (6–1)	2	7.1	40.0	42	27.2	28.4
	A. sp. (6–3)	2	6.6	42.0	43	25.6	25.8
	A. sp. (6–5)	2	6.6	40.0	43	25.2	29.4
Chinese mustard	Non-inoculated	9	0.13	20.2	24	6.57	10.0
	A. sp. (8–2)	5	0.21	22.0	24	9.72	15.0
	A. sp. (8–3)	5	0.25	22.6	25	7.32	9.2

a): Observed in 45 days after planting.
b): See Table 3. Data obtained for other isolates are omitted.

leaf developments was seen in a few cases. In cucumber, the total fresh weight of stems and leaves was remarkably increased as compared to the uninoculated plants. However, no stimulation was observed in root development in cucumber. In the case of inoculation of the isolates from Chinese mustard, significant stimulation of root and plant growth was observed.

Dual inoculation of the *Azospirillum* spp. isolated from the roots of soybean and *Bradyrhizobium japonicum* (USDA 110) was undertaken in soybean (Table 6). It did not stimulate the growth of soybean plants and even suppressed the nodulation and nitrogen fixation[15].

Table 6. Effects of dual inoculation of *Azospirillum* spp. and *B. japonicum* (USDA 110) on the nitrogen fixation and growth of soybean plant[a) 7)]

Isolate inoculated[b)]	ARA (μmole C_2H_4 hr^{-1})		Nodule			Plant height (cm)	Fresh wt.	
	Plant	Nodule (g)	Number	Total wt. (g)	Mean wt. (mg)		Root (g)	Shoot (g)
Non-inoculated	4.14	25.6	37	0.162	4.4	47	2.58	8.8
A. sp. (4-1)	1.88	26.9	30	0.070	2.3	44	2.47	8.0
A. sp. (4-2)	2.20	22.2	33	0.099	3.0	49	2.65	9.8
A. sp. (4-3)	4.12	28.5	22	0.139	6.3	44	2.48	7.1
A. sp. (4-4)	4.04	25.6	41	0.142	3.5	42	2.04	6.7

a): Mean values of two plants.
b): See Table 3.

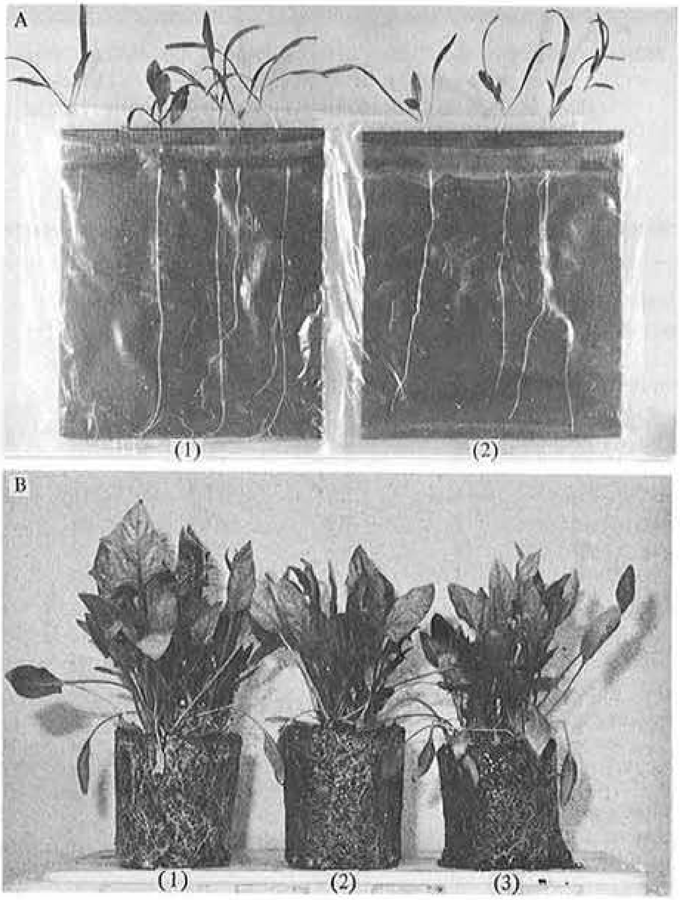

Plate 3. Effect of inoculation of *Azospirillum* spp. isolated from the roots of spinach on the growth of their host plants
A: Spinach showing a difference in root length between inoculated (1) and control (2).
B: Spinach grown in soils containing vermiculite and leaf mold with inoculation of isolates, (1) and (2), and without inoculation (3).

Discussion

The root surface and rhizosphere of some crops, including not only gramineous but also non-gramineous crops, provide various types of microorganisms useful for plant growth and nutrition intake: one of them is *Azospirillum*. It fixes atmospheric dinitrogen in microaerobic condition on the root surface or inside of the root cortex of various plants and stimulates the root development by producing plant hormones. *Azospirillum* is widely distributed, covering a range from tropical to cold climate regions with higher frequencies in the tropics[5,8]. However, there is a variation in its population density: a survey indicated that Tokachi and Tsukuba in Japan are sparsely populated. The isolates of *Azospirillum* spp. collected under the present study are expected to be useful for bacterization in plant growth[13]. In the case of dual inoculation of *Azospirillum* and *B. japonicum*, inoculation of the former bacteria should be done after the nodule formation in soybean roots, because it generally inhibits the nodulation.

The nodulation in the roots of rice, wheat and oilseed rape were recently succeeded by the inoculation of rhizobia with or without treatment of both cellulase and pectolyase or 2,4-D[1,2,3,10]. However, the resulting nodules have a low nitrogen-fixing ability at present. Such a low ability in the nodulated nonleguminous crops could be improved in future through the development of DNA recombination techniques.

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