Agric. Chem. Biotechnol. **43**(2), 104-108(2000)

Plant Growth-promoting Activity of *Acremonium strictum* MJN1 Isolated from Roots of *Panax ginseng*

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Received April 11, 2000

The plant growth-promoting activity of *Acremonium strictum* MJN1 isolated from roots of *Panax ginseng* was explored. The mycelial extract of *A. strictum* MJN1 enhanced the rice seedling growth by 14.5 and 9.0% in the dried weight of shoots and roots, and the growth of red pepper by 54 and 85% in the top length and the dried weight in pot experiments, respectively. The plant growth-promoting substances in the mycelial extract of *Acremonium strictum* MJN1 were identified as D-adenosine and glycerol. Both commercial D-adenosine and glycerol also promoted significantly the rice seedling growth but, unlike the mycelial extract of *A. strictum* MJN1, hardly affected the yields of plants grown in pots or field. Therefore, it is possible that other plant growth-promoting substances are produced by *A. strictum* MJN1. However, this study shows that *A. strictum* MJN1 has a great potential as a bio-fertilizer.

Key words: *plant growth-promoting activity,* Acremonium strictum, Panax ginseng, *D-adenosine.*

Microorganisms constituting the rhizosphere community have attracted particular attention in studying the soil ecosystem of cultivated land due to their potential to influence plant growth and crop yields[1-3]. The rhizosphere microorganisms include symbionts (*Rhizobium*, certain actinomycetes, and mycorrhizal fungi) and free living saprophytes that increase the availability of nutrients to plants or plant growth substances [4,5]. They are also known to benefit plants by suppressing the growth of deleterious rhizosphere microorganisms[6]. Likewise, rhizosphere fungi benefit land plants by promoting their growth[7,8], and ceratin plant growth-promoting fungi (PGPF) also possess properties antagonistic towards plant pathogens[9,10]. The production of metabolites with phytohormonal activities by soilborne fungi implicates that rhizosphere fungi influence plant metabolism through specific metabolites[11-14]. In some cases, PGPF isolated from plant rhizosphere showed ability to induce systemic resistance to soilborne disease, which supported the notion that soilborne fungi could induce re-programming of the host metabolism[15]. Therefore, PGPF have a great potential not only as a bio-fertilizer but also as a bio-control system. However, the molecular mechanisms underlying mutualism between land plants and PGPF have not yet been well understood except for the highly specialized case of arbuscular mycorrhizal fungi[16].

Here we present the identification and characterization of the PGPF isolated from roots of *Panax ginseng* C. A. Meyer. *A. strictum* MJN1 accumulates plant growth-promoting metabolites in mycelium. The plant growth-promoting substances were identified, and their relevant effects on the growth of plants were examined.

Materials and Methods

Identification of a rhizosphere fungus. The fungal strain generously provided by M. Bibikova (Moscow, Russia) was originally isolated from roots of *P. ginseng* C. A. Meyer. The identification was done through KCTC (Korean Collection For Type Cultures).

Culture conditions and preparation of mycelial extract. *A. strictum* MJN1 was cultured in PD (potato dextrose) broth with inoculation from fungal discs containing 7 to 10-day-old cultures on PD agar. The inoculated PD broth was incubated at 23-25°C for 14 days with shaking at a speed of 150 rpm. The mycelial mat was collected through filtration using a cheese cloth, washed in sterile water, and dried on a filter paper at room temperature for 2-3 days. The dried mycelial mat was extracted by incubating in 20 ml of 70% (v/v) aqueous ethanol per gram of mycelium for 2-3 days. The extraction filtrate was made through filtration with a cheese cloth and used to measure plant growth-promoting activity after serial dilutions in sterile H_2O. For the mass-culture of *A. strictum* MJN1, a rich medium (1.5% glycerol, 0.6% peptone, 1.5% sucrose, 0.5%

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Abbreviations: PD, potato dextrose; PGPF, plant growth-promoting fungi.

NaCl, 0.5% KCl, 0.0015% $FeSO_4$, 0.002% $CuSO_4$, 0.03% KH_2PO_4, 0.005% $MgSO_4$, 210^{-5}% $MnSO_4$, pH 7.0) was used, and the culture was incubated for 14 days at 25°C with shaking at a speed of 150 rpm.

Evaluation of the effect of mycelial extract on the rice seedling growth. Rice seeds were surface-sterilized for 2 min using 0.5% NaOCl and placed on a filter paper soaked with serial dilutions of mycelial extract. After 5 days at 25°C in a dark chamber, the number of germinated rice seedlings was counted. The germinated seedlings were collected, and shoot and root were separated and dried at 80-100°C to a constant weight for measuring the dried weight. The reported values are averages of three replicates of 100 seeds each. Therefore, the value was based upon 300 seeds per each treatment.

Pot experiments. Polyvinylcarbonate pots (24.5×12 cm) filled with 700 g of dried potting soil (Nongwoo Co., Suwon, Korea, pH 6.5) were sown with red pepper seeds (Youngwoong, No. VP-Hy-227; Nongwoo Co.) at a depth of about 0.5 cm and placed in a growth chamber (15 h-light period, 5000 Lux at 27°C; 9 h-dark period, at 25°C; an average relative humidity of 75%). The pots sown with red pepper seeds were watered every 2 days with tap water, and 50 ml of water-diluted mycelial extract was sprayed on leafs of red pepper plants once a week for three weeks after the four leaf-stage (approximately 30 days after sowing). In all experiments, pots were prepared in duplicates. Twenty days after the final treatment, the plants were harvested, and the top length and dried weight were measured. The drying condition was same as above. The reported values of top length and dried weight were averages of duplicates of 6 plants each. Therefore, each value was based upon 12 plants per treatment.

Chromatographic analysis. The mycelial extract of 70% aqueous ethanol was applied onto preparative TLC (Merch, precoated silica gel $60F_{254}$; n-butanol/methanol/water = 4 : 4 : 3 (v/v/v); visualization, 254 nm). The bands were cut out based on indexation of UV-absorption and eluted with ethanol. The extract was concentrated by evaporation under reduced pressure at 45°C. The concentrate was dissolved in equal volume of ethanol used for original mycelial extraction for use in rice seed germination experiments. The fraction with Rf 0.8 (Fr-1) was further fractionated with TLC (ethylacetate/n-butanol/formic acid/water = 5 : 3 : 1 : 1 (v/v/v/v); visualization, 254 nm). Fractionations were tested for plant growth-promoting activity using a seedling growth assay.

The fraction with Rf 0.7 (Fr-2) was chromatographed using a silica column. The column was eluted with increasing concentrations of methanol in chloroform. Eluted fractions were analyzed through TLC (n-butanol/ethyl-acetate/methanol/water = 3 : 4 : 2 : 1) with visualization by 5% H_2SO_4 in ethanol and were subjected to a rice seedling growth assay.

Instrumental analysis. The separated fraction of Fr-1 through TLC was subjected to HPLC (Nacalai, C-18 column; 5% methanol and 0.01% trifluoroacetic acid at a flow rate of 1 ml per min; diode array detection). For the further analysis of Fr-2, an electron spray ionization (ESI) mass spectroscopy and an electron ionization mass spectrometry (EI-MS) were carried out on a VG quatro mass spectrometer (VG analytical, UK) and a VG70-VSEQ mass spectrometer, respectively. Each residue's structure of Fr-2 was determined through ^{13}C, ^{1}H-NMR experiments using a JNM-LA 300 (Jeol).

Adenosine deaminase treatment. Adenosine deaminase (EC 3.5.4.4., Sigma catalogue no. A1155) that specifically deaminates 9-β-D(-)adenosine to inosine was incubated with 1 μg of adenosine in 100 mM potassium phosphate buffer. One unit of enzyme was used at 25°C. The resulting reaction mixtures were separated with the same HPLC procedure used for the purification of Fr-1.

Results and Discussion

Plant growth-promoting activity of a rhizosphere fungus. We explored the plant growth-promoting activity of the rhizosphere fungus isolated from roots of *P. ginseng* C. A. Meyer by examining the effects on the rice seedling growth and on the growth of red pepper in pot experiments. The mycelial extract from the fungal isolate showed significant growth-promoting activity (Table 1). Supple-

Table 1. The plant growth-promoting effect of the mycelial extract of *A. strictum* MJN1 on the rice seedling growth.

Concentration[1]	Germination		Dried shoot weight		Dried root weight	
	n[2]	%	mg	%	mg	%
Control	79.3	100.0	184.3	100.0	184.4	100.0
10^{-1}	64.7	81.6	102.9	55.8	94.1	51.0
10^{-2}	75.7	95.5	178.6	96.9	167.6	90.9
10^{-3}	79.7	100.5	211.0[3]	114.5	200.6	108.8
10^{-4}	77.3	97.5	176.4	95.7	184.9	100.3
10^{-5}	74.7	94.2	181.7	98.6	162.9	88.3

[1]Mycelial extract (1 g mycelial mat/20 ml 70% EtOH) was diluted in sterile H_2O.

[2]n is the average number of germinated seeds

[3]The value is significant at $p<0.05$, when compared using a least significant difference (LSD) test.

Table 2. The plant growth-promoting effect of the mycelial extract of *A. strictum* MJN1 on the growth of red pepper in pot experiments.

Concentration[1]	Dried weight(g)		Top length(cm)	
	Mean	%	Mean	%
Control	0.07	100	9.80	100
10^{-3}	0.11	157	11.75[3]	120
10^{-4}	0.15[2]	181	13.25[3]	135

[1]Mycelial extract (1 g mycelial mat/20 ml 70% EtOH) was diluted in sterile H_2O.

[2,3]The values are significant at $p<0.05$ and $p<0.01$, respectively when compared using a least significant difference (LSD) test.

mentation of the extract at a 10^{-3} dilution resulted in 14 and 9% increase of shoot and root weight, respectively. However, no significant promoting effect was observed for germination at any concentration. The extracts affected the yields of shoot and root weight in a negative manner at high concentrations. For example, 50% decrease was observed when mycelial extract was used at a 10^{-1} dilution. This growth inhibition is unlikely due to an inhibitor(s) because purified fractions of mycelial extracts also showed low growth-promoting effect at high concentrations (see below). Therefore, the inhibitory effect at high concentrations and promoting effect at certain concentration implicate the involvement of a plant growth regulator-like substance(s) derived from the fungal metabolites.

For pot experiments with red pepper, the extract was applied at the concentration that showed the promoting effect on rice seedling growth. Consistent with the promoting activity observed in rice seedling growth, the yields of red pepper plants increased by 40 to 80% when the mycelial extract was supplemented at a 10^{-4} dilution but showed the relatively low increase when supplemented at the higher concentration of 10^{-3} dilution (Table 2).

Identification of PGPF. The fungal isolate was identified as *A. strictum* by its morphological characteristics and named as *A. strictum* MJN1. Colonies reached 1.5-2.5 cm in diameter in 10 days at 20°C on malt extract agar, were creamy to pinkish, and were usually moist and smooth (Fig. 1A). Conidia were cylindrical with the size of 3.3-5.5×0.9-1.8 μm and were hyaline in color. It did not form

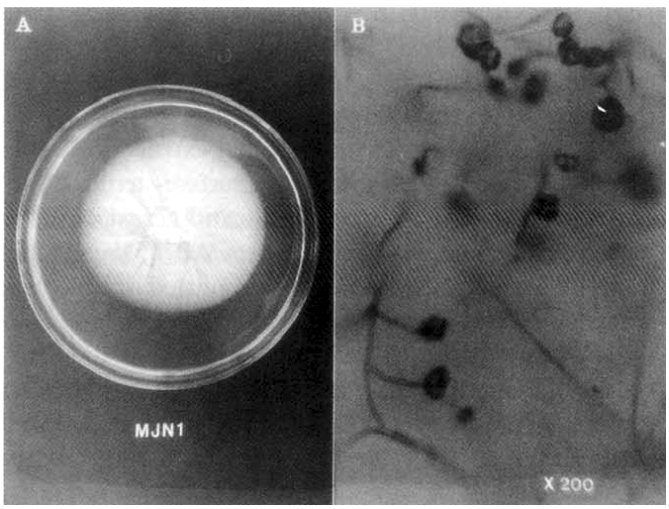

Fig. 1. Morphological characteristics of *A. strictum* MJN1 isolated from *P. ginseng*. (A) *A. strictum* MJN1 was cultivated at 25 for 10 days on malt extract agar (Difco) and photographed. (B) Hyphal bundle with numerous phialides and conidial heads (magnification, 200).

chlamydospores or sclerotia, and ascomata were absent. Simple phialides rose from submerged or slightly fasciculated aerial hyphae and had a thin wall. Conidia had rounded ends or a symmetrical fusiform, were generally one-celled, and hardly stained in aniline blue. Phialides were usually not proliferating, and conidiophores were not verticillate (Fig. 1B).

Identification of the metabolites responsible for the plant growth-promoting activity. The plant growth-promoting activity of *A. strictum* MJN1 mycelial extract was further explored after fractionation through TLC. The bands of Rf 0.8 and 0.7 showed significant growth-promoting activity in rice seedling growth (Table 3). The band eluent of Rf 0.8 (Fr-1) increased dried root weight up to 150%, dried shoot weight to 112%, and germination to 119% at a 10^{-3} dilution. The band eluent of Rf 0.7 (Fr-2) promoted both shoot and root growth up to 130-140%. In pot experiments with red pepper, whereas Fr-1 showed no significant effect, a significant growth-promoting activity was observed with supplementation of Fr-2 at a 10^{-3} dilution (Table 4). The difference of two fractions in the growth of red pepper implicates that each Fr-1 and Fr-2 contained distinctive

Table 3. The plant growth-promoting effect of TLC fractions of the mycelial extract of *A. strictum* MJN1 on the rice seedling growth.

Concen-tration[1]	Fr-1						Fr-2					
	Germination		Dried shoot weight		Dried root weight		Germination		Dried shoot weight		Dried root weight	
	n	%	mg	%	mg	%	n	%	mg	%	mg	%
Control	74.7	100.0	36.5	100.0	34.0	100.0	70.3	100.0	27.6	100.0	25.6	100.0
10^{-3}	89.0[2]	119.0	40.9	112.1	52.4[2]	154.1	78.7	111.9	36.8[2]	133.3	35.7[2]	139.5
10^{-4}	90.3[2]	120.9	43.3	118.6	46.9[2]	137.9	71.0	101.0	31.3	113.4	32.8	128.1

[1]Fractions dissolved in the original volume of mycelial extracts were diluted in sterile H_2O.

[2]The values are significant at $p<0.05$, when compared using a least significant difference (LSD) test.

Table 4. The plant growth-promoting effect of TLC fractions of the mycelial extract of *A. strictum* MJN1 on the growth of red pepper in pot experiments.

Concentration[1]	Fr-1				Fr-2			
	Dried weight (g)		Top length(cm)		Dried weight(g)		Top length(cm)	
	Mean	%	Mean	%	Mean	%	Mean	%
Control	0.080	100	9.770	100	0.080	100	9.770	100
10^{-3}	0.097	117	10.301	106	0.154[2]	186	13.230[2]	135
10^{-4}	0.087	105	10.110	104	0.106[2]	128	11.530[2]	118

[1]Fractions dissolved in the original volume of mycelial extracts were diluted in sterile H_2O.
[2]The values are significant at $p<0.01$, when compared using a least significant difference (LSD) test.

Table 5. The plant growth-promoting effect of the active substance, D-adenosine isolated from *A. strictum* MJN1 on the rice seedling growth.

Concentration[1]	Germination		Dried shoot weight t		Dried root weight	
	mean	%	mean	%	mean	%
control	29.0	100.0	48.8	100.0	37.1	100.0
10^{-5}	28.7	99.0	52.7	108.0	39.9	107.5
10^{-6}	29.7	102.4	58.1[2]	119.1	49.4[2]	133.2
10^{-7}	29.3	101.0	53.2	109.0	41.8	112.9

[1]Dilutions are relative to the original mycelial extract concentration (1 g mycelial mat/20 ml 70% EtOH).
[2]The values are significant at $p<0.05$, when compared using a least significant difference (LSD) test.

substance(s) that affects the plant growth. Based on these observations, we suggest that *A. strictum* MJN1 produces substances affecting plant growth in a positive manner and at least two metabolites are responsible for the observed plant growth-promoting activity.

The subsequent chromatographic purification of Fr-1 was carried out through preparative HPLC (Fig. 2). The substance(s) with 9.8 min of retention time promoted rice seedling growth to a significant extent (Table 5) and increased the dried weight of shoots and roots up to 120 and 130% at a 10^{-6} dilution, respectively. Treatment at higher or lower dilutions than 10^{-6} resulted in weaker but still significant promoting effects. The substance had a maximum UV-absorption at 260 nm and showed the same characteristics as adenosine in the mass spectrometry analysis (EI-MS m/z (abundance) = 267 ($[M]^{+1}$, 3.8), 237 (7.8), 220 (1.5), 178 (40.5), 164 (89.5), 148 (9.9), 135 (100.0), 119 (7.7), 108 (31.8), 73 (11.4); HRMS m/z = Found 267.0965; Cald. for $C_{10}H_{13}N_5O_4$, 267.0968). The substance was also undistinguishable from commercially available adenosine (Sigma) in HPLC analysis. The isolated substance was completely converted to inosine by adenosine deaminase, suggesting that it is D-adenosine (data not shown). The final isolation yield of D-adenosine was 0.29 mg per liter of the culture. There is no precedent report that D-adenosine has plant growth-promoting activity. However, the unusual isomer, L-adenosine was shown to act as a second messenger of triacotanol, a well-known plant growth-promoting substance[17-19]. The treatment of adenosine deaminase indicated that L-enantiomer was not included in the isolated adenosine, at least to an extent detectable in our analysis condition. Therefore, this is the first report showing that D-adenosine has the plant growth-promoting activity. However, a further research is needed to unveil the

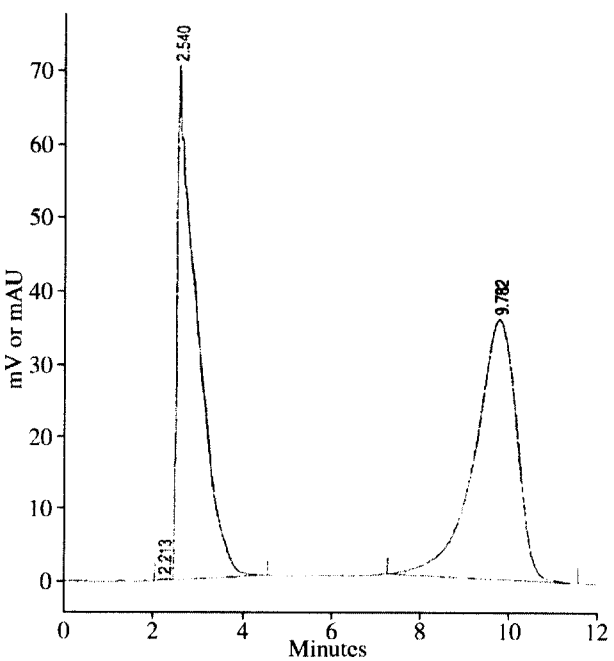

Fig. 2. HPLC chromatogram of the active substance of Fr-1. The active substances partially purified from Fr-1 through TLC (ethylacetate/n-butanol/formic acid/water = 5 : 3 : 1 : 1) were subjected to HPLC using a C18 column (4.6150 mm), and samples were eluted with an isocratic condition of 5% methanol containing 0.01% trifluoroacetic acid (TFA). Optical density was measured at 260 nm. The fraction of 9.782 showed growth promoting effect on rice seeds.

biological significance of D-adenosine, and it is still possible that a substance other than D-adenosine exerted the major promoting roles of Fr-1 but failed to be recovered during fractionation.

The fractionation of Fr-2 contained two major compounds. The isolated substances were indistinguishable from commercial glycerol and glucose in ^{1}H-NMR and ^{13}C-NMR (data not shown). Commercial glycerol also showed promoting effect on the rice seedling growth to an extent comparable to that of Fr-2, which implicates that the active substance in Fr-2 is glycerol. However, glycerol at 0.5 ppm solution increased the top length of red pepper and cucumber only at the early growth stage without any significant enhancement at the harvest, whereas Fr-2 exerted a significant yield enhancement. This result suggests that Fr-2 contains growth-promoting substance(s) other than glycerol. Unlike glycerol, glucose showed no effect on the rice seedling growth (data not shown).

In the rhizosphere of plants, a variety of colonizing or non-colonizing microorganisms resides and interacts with plants, and this affects the farming yield. However, the complex systems involved in these ecological systems have frustrated the research effort toward understanding the detailed mechanism by which microorganisms affect the plant growth. In this study, we found that simple metabolites, D-adenosine and glycerol produced by *A. strictum,* played significant roles in plant growth regulation. However, the possibilities that other substances may contribute to plant growth-promoting activity cannot be excluded. It has been reported that *A. roseum* I 4267 produces acremoauxin A, a novel auxin derivative[14]. Therefore, *A. strictum* MJN1 may produce unidentified phytohormones. It is also possible that the substances that are available to plant roots upon the lysis of rhizosphere microorganisms affect the growth of plants. However, our results show that *A. strictum* MJN1 produces the plant growth-promoting substances and thus has a great potental as a bio-fertilizer.

Acknowledgments. This work was supported by the MAFF-SGRP (Ministry of Agriculture, Forestry and Fisheries-Special Grants Research Program). We thank M. Bibikova for kindly providing *A. strictum.*

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