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Production of Auxins and Auxin-like Compounds by Ginseng Growth-promoting Bacterium *Pseudomonas fluorescens* KGPP 207

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High activity of acidic ethylacetate extract from the culture supernatant of ginseng growth-promoting bacterium *Pseudomonas fluorescens* KGPP 207 and its fractions were demonstrated through wheat coleoptile bioassay. The following auxins and auxin-like compounds were identified in these fractions by combined gas chromatography-mass spectrometry: indole-3-acetic acid, indole-3-acetic acid methyl and ethyl ester, indole-3-butyric acid, indole-3-lactic acid and its methyl ester, indole-3-propionic acid, indole-3-pyruvic acid, *p*-hydroxyphenyl acetic acid, *p*-hydroxyphenyl acetic acid methyl and ethyl ester, phenyl acetic acid and its methyl ester. The bacterium KGPP 207 belongs to the strain of *P. fluorescens* which produces plant growth regulators and its beneficial effect on the ginseng growth may be due to the formation of the identified compounds.

Key words: *Pseudomonas fluorescens, auxins, indole-3-acetic acid.*

Numerous soil-inhabiting pseudomonads colonize the surface of seeds and roots, enhancing the plant growth, and have thus been termed as the plant growth-promoting rhizobacteria.[1)] The bacterium *Pseudomonas fluorescens* KGPP 207 isolated from ginseng rhizosphere belongs to this type of pseudomonads and has been revealed to have a beneficial property in promoting the ginseng growth. The weight, length, and diameter of ginseng root were increased by 50.0, 13.2, and 36.2%, respectively, when the second year ginseng roots were inoculated with *P. fluorescens* KGPP 207 in Wagner pots for five months.[2)] It was supposed that various rhizosphere microorganisms affect the growth of plants by the formation of biologically active substances in the region of plant roots.[3)] Some strains of *P. fluorescens*, including Pf-5[4,5)], CHAO[6)], and BL915[7)] produce antifungal metabolites such as pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol, while other strains produce plant hormones[1)] or other plant growth regulators.[8)] In this study, we report on the purification and identification of auxins and auxin-like compounds produced by the ginseng growth-promoting *P. fluorescens* KGPP 207.

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Abbreviations: ABA, abscisic acid; BSTFA, bis(trimethylsilyl)trifluoroacetamide; EtOAc, ethyl acetate; GC-MS, gas chromatography and mass spectrometry; HPAA, *p*-hydroxyphenyl acetic acid; HPAAMe, *p*-hydroxyphenyl acetic acid methyl ester; HPAAEt, *p*-hydroxyphenyl acetic acid ethyl ester; IAA, indole-3-acetic acid; IAAMe, indole-3-acetic acid methyl ester; IAAEt, indole-3-acetic acid ethyl ester; IAld, indole-3-aldehyde; IBA, indole-3-butyric acid; ICA, indole-3-carboxylic acid; ILA, indole-3-lactic acid; IPA, indole-3-propionic acid; IPyA, indole-3-pyruvic acid; PAA, phenyl acetic acid; PAAMe, phenyl acetic acid methyl ester; PDA, potato dextrose agar.

Materials and Methods

Chemicals. Chemicals were purchased from Sigma Chemical Co. unless otherwise noted. Agar and proteose peptone were obtained from Difco Laboratories. Silica gel (70~230 mesh, CC grade) was purchased from Merck and activated at 130°C for more than 5 h prior to use. All other reagents were reagent grade unless otherwise specified.

Bacterium strain and growth conditions. Strain KGPP 207 of *P. fluorescens* was isolated from the soil of productive ginseng field.[9)] The bacterium was grown in a 1-*l* Erlenmeyer flask containing 200 m*l* of King B medium[10)] on a shaker at 28°C for six days.

Extraction. The cells were centrifuged from the culture medium at 22,000 × g for 20 min at 4°C. Supernatant and cells were extracted separately. The antioxidant sodium diethyldithiocarbamate (0.3 g) was added to the 3 *l* supernatant and culture liquid was extracted with 1.5 *l* EtOAc at dark condition. The aqueous layer was then acidified to pH 2.0~2.5 with 50% phosphoric acid and extracted twice with 1.5 *l* EtOAc. The EtOAc extracts were pooled, dried over anhydrous sodium sulfate, and the solvent was evaporated *in vacuo* (sample E-1). The aqueous layer was extracted two times with 1.0 *l* n-butanol. Solvent was evaporated on rotary evaporator under vacuum, and butanol fraction (sample B-1) was obtained as a dark, high density oil.

Fresh cells of *P. fluorescens* were suspended in 0.5 *l* acetone and stirred for 24 h at 4°C in dark condition. The cells were then removed by filtration, and acetone was

evaporated on rotary evaporator under vacuum. Aqueous residue (20 m*l*) was extracted three times with 20 m*l* EtOAc, then acidified to pH 2.0 with 30% phosphoric acid and extracted three times with 20 m*l* EtOAc. EtOAc extracts were pooled, dried over anhydrous sodium sulfate, and the solvent was removed on rotary evaporator (sample C-1).

Antifungal bioassay. The fungicidal activities of test samples (culture broth, E-1, B-1, and C-1) against ginseng pathogens (*Alternaria panax, Rhizoctonia sp., Cylindrocarpon sp., Fusarium sp., Sclerotinia sp.,* and *Phytophtora sp.*) were determined using the paper disc method on PDA in a petri dish. The 25 μ*l* of a sample solution (5~15 mg/0.1 m*l*: about tenfold conc. of each fr. in culture broth) was applied by a syringe to a paper disc (5-mm diameter). After the evaporation of solvent, the paper discs were placed on the agar surface inoculated with each pathogen. All plates were incubated for six days at 25~26°C.

Wheat coleoptile bioassay. Wheat (*Triticum sativum*) seeds were soaked in distilled water for 1 h and placed on a moist filter paper in a plastic box.[11] The seeds were germinated for approximately 96 h at 23~25°C in dark condition. The apical 3-mm segment of each coleoptile was removed, and the next 5-mm segment was excised to be used as a test segment. Test samples were dissolved in 0.01 M dipotassium phosphate-0.005 M citrate buffer(pH 5.6) containing 2% sucrose, at concentrations of 2, 20, and 200 ppm. Solutions of IAA and ABA at concentrations of 10^{-5} and 10^{-6} M were used as the standards and phosphate-citrate buffer as the control. Eight segments were put on the surface of the solution (2 m*l*) in a tube and grown for 16 h in darkness at 22~24°C. After incubation, segments were removed from the tube, placed on a glass plate in a photographic enlarger, and their projected shadow were measured. The mean elongation of segments incubated in the different solutions were compared with that of the control segments in the buffer, and the increased ratio(%) of a sample to control was calculated as follows:

$$\{(Ls-Lb)/(Lc-Lb) - 1\} \times 100$$

Ls: the average length of segments in the sample solution after 16 h incubation
Lb: the average length of segments at the beginning
Lc: the average length of segments in control solution after 16h incubation

Silica gel column chromatography. Chromatographic column (3 cm × 70 cm) was plugged with glass wool, packed with silica gel (suspended in hexane/ethyl acetate, 4/1, v/v), and topped with *ca* 2-cm layer of sea sand. The column was pre-washed by passing 1.0 *l* hexane/ethyl acetate (4/1, v/v). The sample, E-1 (3.2 g), was dissolved in a small volume of ethyl acetate and poured into the column. The column was eluted with hexane-ethyl acetate step gradient from 4 : 1 (v/v), 1 : 1, 1 : 4 to 1 : 9, then, ethyl acetate-acetone from 9 : 1, 4 : 1, 1 : 1, 1 : 4, 1 : 9 to 0 : 1, and finally acetone-methanol from 4 : 1, 2 : 1, 1 : 1 to 0 : 1 (with 1 *l* of each solvent mixture). Fractions of volume, 50 m*l*, were collected and controlled by TLC (TLC aluminum sheets silica gel 60 F_{254}, Merck) in the following solvent systems; (a) chloroform-ethyl acetate-formic acid (35 : 55 : 10, v/v), (b) hexane- ethyl acetate (4 : 1, v/v) and hexane-ethyl acetate (1 : 1, v/v). The separated spots were detected; (a) under UV-detector, and (b) by spraying with 10% vanillin in concentrated sulfuric acid. The identical fractions were combined, solvent was removed in *vacuo*, and 50 combined fractions (F-1~F-50) were collected.

GC-MS conditions. For the determination of the compounds from fractions (F-2, F-9, F-14, F-19, and F-21), a GCQ GC/MS instrument with a Gateway 2000 Chemstation (Finnigan, San Jose, CA, USA) was used. The GC analysis conditions were as follows: fused silica capillary column (0.25 mm i.d. × 30 m, 0.25 μm film thickness) coated with DB-17MS, helium carrier gas flow rate, 90 m*l*/sec; temperature program, isotherm 5 min at 50°C, 50°C to 250°C at 2°C per min, isotherm 120 min; injection port temperature, 250°C. MS analysis conditions were as follows: ionization mode, -70 eV, EI; ion source temperature, 180°C; ion source pressure, $3 \cdot 10^{-5}$ torr; scanning range, 45~500 amu; scanning time, 0.5 sec/decade.

Derivatization. The dry sample (fraction or standard) was taken up in a small volume of methanol or acetone and transferred to a derivatization vial and evaporated under a stream of nitrogen, and dried under vacuum over phosphorus pentaoxide. TMS derivatives were prepared by dissolving the sample in 50~100 μ*l* of acetonitrile and 50~100 μ*l* of BSTFA and then heating at 70°C for 10 min. A sample of the resulting solution was then analyzed by GC-MS.

High performance liquid chromatography. The complete HP1100 series HPLC system (Hewlett Packard) was used for the quantitative analysis of IAA and the related compounds. Analysis was carried out on a Alltech Spherisorb ODS-2 (3 μm, 150 mm × 4.6 mm i.d) main column and Phenomenex Lichrosorb 5 RP-18 (5 μm, 60 mm × 10 mm i.d) guard column. The columns were eluted with water-acetic acid-acetonitrile (76 : 1 : 23 or 70.5 : 0.5 : 29, v/v) for indole derivative analysis or with methanol -50 mM sodium acetate buffer(pH 6.5, 8 : 92, v/v) for PAA analysis at a flow rate 0.6 m*l*/min. Detection was carried out by a photodiode array detector at 254 and 280 nm connected to a fluorometric detector (excitation, 280 nm; emission, 360 nm). The amount of sample injected into the column was 5~10 μl of 50% acetonitrile. IAA and other compounds were quantified by reference to the peak area obtained for respective authentic standards of known concentration.

Results and Discussion

Antifungal activity against ginseng pathogens. *Alternaria panax, Rhizoctonia sp., Cylindrocarpon sp.,*

Table 1. The effect of *P. fluorescens* culture broth on wheat coleoptile elongation in comparison with those of IAA and ABA.

Sample		Wheat coleoptile elongation increased rate (%)
Control broth	1:10	79.2
	1:100	30.6
IAA	10^{-5} M	165.8
	10^{-6} M	159.1
ABA	10^{-5} M	-97.3*
	10^{-6} M	-94.6*

*Growth inhibition

Table 2. The effect of extracts and fractions from *P. fluorescens* on wheat coleoptile elongation.

Sample	Wheat coleoptile elongation increased ratio (%)		
	2 ppm	20 ppm	200 ppm
E-1	3.4	36.9	143.1
B-1	1.8	6.0	14.1
C-1	1.7	43.3	89.3
F-2	14.4	-19.6*	-33.7*
F-9	18.5	59.8	119.1
F-14	38.5	97.5	134.3
F-19	39.5	108.5	143.1
F-21	31.7	81.4	127.3

*Growth inhibition

Fusarium sp., *Sclerotinia sp.*, and *Phytophtora sp.* were used for the initial bioactivity test on PDA with 6-day-old culture broth, ethyl acetate(E-1) and butanol extracts(B-1) of the supernatant, and acetone extract of *P. fluorescens* cells(C-1). Original culture broth and all extracts did not reveal any antifungal activity. This result indicates that *P. fluorescens* KGPP 207 does not belong to the antibiotic producing strains and its beneficial effect on ginseng growth is related with the other function of this microorganism.

Wheat coleoptile bioassay. It is well-known that some *P. fluorescens* strains produce plant hormones[1], therefore wheat coleoptile bioassay was used as a second bioactivity test for auxin production. The culture broth in dilutions of 1 : 10 and 1 : 100 as well as ethyl acetate extract from the supernatant (E-1) have revealed similar effects on wheat coleoptile elongation as that of IAA (Tables 1 and 2). The data indicate that *P. fluorescens* KGPP 207 belongs to the strain which produce plant hormones, because wheat coleoptile biotest is a bioassay specific for auxin production.[12]

Determination of active fractions. Column chromatography on silica gel was used for separation and purification of ethyl acetate extract E-1. All collected fractions (F-1~F-50) were tested through wheat coleoptile bioassay for content of auxins or auxin-like compounds (Table 2, indicates only active fractions). Four fractions (F-9, F-14, F-19, and F-21) have revealed a significant effect on the elongation of wheat coleoptiles comparable with that of IAA. Biologically active compounds containing in these fractions were identified by GC-MS analysis.

Table 3. Molecular weights [M⁺], mass spectra (*m/z*), and gas chromatographic retention times for the compounds produced from the incubation of *P. fluorescens* KGPP 207 in the King B medium.

Compounds	*m/z*		Retention time (min)
	$[M^+]$	fragment ions	
PAAMe	150	149, 92, 91, 89, 77, 65	28.40
PAA	136	92, 91, 89, 77, 65, 51	35.15
HPAAMe	166	108, 107, 79, 78, 77, 51	52.29
HPAAEt	180	108, 107, 78, 77, 51	55.34
HPAA	152	108, 107, 79, 77, 63, 51	59.35
IAAMe	189	131, 130, 128, 103, 102, 77	74.10
IAld	145	144, 117, 116, 115, 89, 63	75.41
IAAEt	203	131, 130, 129, 128, 103, 77	76.38
IAA	175	131, 130, 128, 103, 102, 77	79.23
ICA	161	145, 144, 116, 115, 89, 63	80.00
IPA	189	131, 130, 128, 115, 103, 77	82.25
ILAMe	219	131, 130, 103, 102, 77, 51	84.05
ILA	205	131, 130, 129, 128, 103, 77	85.50
IBA	203	143, 131, 130, 128, 103, 77	86.25
IPyA	203	131, 130, 129, 103, 77, 51	87.35

Identification and quantification of auxins and auxin-like compounds. Combined GC-MS was used for the identification of biologically active compounds in fractions F-2, F-9, F-14, F-19, and F-21. The fractions were analyzed directly or after their derivatization with BSTFA. The compounds identified in the fractions are presented in Tables 3 and 4. The substances were determined by comparison of the mass spectra of their free forms (Table 3) and their TMS derivatives (Table 4) with those of literature data.[13,14] In addition, the compounds identified in the fractions gave peaks having the same retention times as those observed for the authentic standards. The results have confirmed the suggestion that *P. fluorescens* KGPP 207 belongs to plant hormone producing strains of this bacterium because IAA, IBA, PAA, HPAA, and other substances are auxins or auxin-like compounds or their biosynthetic intermediates.[1,15] IAld and ICA are the products of oxidative decarboxylation pathway of IAA metabolism.[16] Methyl and ethyl esters of the identified compounds are considered as the storage forms of these substances, and they were probably involved in the regulation of their biosynthesis.[17]

The compounds are distributed among fractions as follows: F-2, PAAMe; F-9, PAA, HPAAMe, HPAAEt, and HPAA; F-14, IAAMe, IAAEt, and IAld; F-19, IAA, IPA, ILAMe, and ICA; F-21, IBA, ILA, and IPyA. The activities of the fractions in wheat coleoptile bioassay are related with

Table 4. Gas chromatographic retention times(min), molecular ions, base peaks, and other diagnostic ions in the mass spectra of TMS derivatives of compounds produced by *P. fluorescens* KGPP 207.

Compound	Derivative	GC retention time (min)	m/z			
			$[M]^+$	$[M-CH_3]^+$	Base peak	Other fragments
PAAMe	-	28.40	150	-	91	-
PAA	Mono-TMS	32.40	208	193	73	91
HPAAMe	Mono-TMS	49.40	238	223	179	164, 163, 149
HPAA	Di-TMS	51.20	296	281	73	252, 179, 164, 149
IAld	Mono-TMS	71.18	217	202	217	-
IAA	Di-TMS	72.30	319	304	202	276
IAAMe	Mono-TMS	73.00	261	-	202	230
IAAEt	Mono-TMS	75.05	275	-	202	-
ICA	Di-TMS	75.23	305	290	290	246, 216
IPA	Di-TMS	76.35	333	318	202	215
ILA	Tri-TMS	78.13	421	406	202	378, 304
ILAMe	Di-TMS	78.23	363	348	202	348, 304
IBA	Di-TMS	81.50	347	332	202	304, 215
IPyA	Tri-TMS	89.37	419	404	404	348, 314, 260

the content of the above-mentioned substances. Some compounds present in the culture broth were quantitated by HPLC, and their contents were as follows: IAA, 0.5~0.8 mg/*l*; IAAMe, 0.1~0.3 mg/*l*; ILA, 0.2~0.3 mg/*l*; IPyA, 0.4~0.6 mg/*l*; PAA, 1.0~1.2 mg/*l*.

When uninoculated medium was incubated, extracted, separated, and derivatized in an identical manner to the samples of the culture supernatant, and analyzed by GC-MS, no indole compounds other than tryptophan could be detected.

Generally, the application of individual indole compound for promoting ginseng root growth does not give good results, therefore the growth promotion activity of bacterium *P. fluorescens* (strain KGPP 207) on ginseng roots cannot be dependent on only one separate indole compound such as IAA. Its beneficial effect may be due to the production of the specific complex of indole substances and auxin-like compounds.

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