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Isolation of Gibberellins-Producing Fungi from the Root of Several *Sesamum indicum* Plants

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Abstract Gibberellins (GAs) play important roles in plant growth and development. Fifty-four fungi were isolated from the roots of 4 kinds of *Sesamum indicum* plants, and the production of GAs was spectrophotometrically examined. The number of GA-producing fungi was two strains from *S. indicum*, four strains from Gold *S. indicum*, and five strains from Brown *S. indicum*. Eleven fungi with GAs-producing activity were incubated for seven days in 40 ml of Czapek's liquid medium at 25°C and 120 rpm, and the amount of each GA in the medium was measured by gas chromatography-mass spectrometery (GC-MS). *Penicillium commune* KNU5379 produced more GA_3, GA_4, and GA_7 than *Gibberella fujikuroi*, *Fusarium proliferatum*, and *Neurospora crassa* which are known as GAs-producing fungi. GAs-producing activity of the *P. commune* KNU5379 was shown to produce 71.69 ng of GA_1, 252.42 ng of GA_3, 612.00 ng of GA_4, 259.00 ng of GA_7, and 202.69 ng of GA_9 in 25 ml of liquid medium. Bioassay of culture fluid of GA-producing fungi was also performed on rice sprout.

Key words: Gibberellin, *Gibberella fujikuroi*, *Fusarium proliferatum*, *Sesamum indicum*

Gibberellins (GAs) are tetracyclic diterpenoid compounds playing an important role in the regulation of plant growth and development, such as stem growth, cell division and extension, seed germination, induction of hydrolytic enzymes during seed germination, flower induction, and fruit setting [33]. They are produced in green plant, fungi, and bacteria [38]. The overgrowth symptoms of plant are typical effects of GA, which led to the discovery of GAs early this century by Japanese phytopathologists, who found that "bakanae" disease of rice was caused by the GA-producing ascomycete *G. fujikuroi* [21]. So far, 130 GAs have been identified in higher plant, fungi, and bacteria, but only a few of them are biologically active (e.g., GA_1, GA_3, GA_4, and GA_7) [12]. Furthermore, some other GAs with low or no activity in the known bioassays may play a role at a specific developmental stage: For instance, little is known about the role of GAs during seed development, even though they reach their highest concentrations in immature seeds of many plant species [10].

The biosynthesis of GA in higher plants can be divided into three stages: (1) biosynthesis of *ent*-kaurene in proplastid, (2) conversion of *ent*-kaurene to GA12 via microsomal cytochrome P450 monooxygenases, and (3) formation of C_{20} and C_{19} GAs in the cytoplasm [30]. Gibberellins are secondary metabolites, which are important biotechnological products and produced commercially by fungi for the agricultural and horticultural industry [4]. For example, most seedless table grapes are now grown with the application of GA_3. The rind of citrus fruit typically softens at maturity, and is subject to injury by pests and environmental factors which adversely affect the appearance of otherwise marketable fruit. By inhibiting senescence, GAs maintain the rind in better condition. Control of russet may be achieved by the application of GAs in apples such as "golden delicious." A variety of ornamental plants can be induced to flower either earlier than usual or in off-seasons. Sporadic flowering in some plants is often a problem with plant breeders, but may be ameliorated with GA application [28].

Here, we describe the isolation of new potent GAs-producing fungi from the root of *S. indicum* plants, determination of GA-producing activity by spectrophotometric method, and quantitative analysis of the kinds of GAs produced by fungi with gas chromatography and mass spectrometry (GC-MS).

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Fig. 1. A photograph of *S. indicum* plants. The root of this plant was used for isolation of GA-producing fungi.

MATERIALS AND METHODS

Collection of Sample

The roots of young species of Gold, Brown, Super, and *S. indicum* plants (Fig. 1) were collected from the Chilgok Rural Development Administration. Wild-type strain of *G. fujikuroi*, *F. proliferatum*, and *N. crassa* were provided by the Korean Culture Center of Microorganisms.

Isolation of Fungi

The roots of plants were washed with water to thoroughly remove soil. Those were treated with Tween 80 for 5 min and washed with distilled water. The roots were submerged in perchloric acid (1%) of bleaching solution for 5 min, repeatedly shaken two times, and finally washed with autoclaved distilled water for three times. Roots were cut with scissors into 1.5 cm piecemeal and thoroughly dried on sterile filter papers. They were then placed on the Hagem medium containing streptomycin to prevent the growth of bacteria, and single cell isolation was carried out [2, 41].

Media and Cultural Conditions

Isolated fungi were cultured on the Hagem medium with 0.5% glucose, 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% NH_4Cl, 0.1% $FeCl_3$, 80 ppm of streptomycin, and 1.5% agar at pH 5.6. For the production of GAs, the isolated fungi were cultured in 40 ml of Czapek's liquid medium with 1% glucose, 1% Bacto-peptone, 0.1% KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.001% $FeSO_4 \cdot 7H_2O$, at pH 7.3±0.2, 25°C, and 120 rpm for seven days.

GA Measurement

GAs-producing fungi were prescreened spectrophotometrically by the determination of GAs [15].

Extraction of Endogenous Gibberellins

Gibberellins were extracted by the established procedure [23]. The culture solution was acidified to pH 2.5 with 6 N HCl. Deuterated (20 ng each of [17,17-$^2H_2$] GA_1, GA_3, GA_4, GA_7, and GA_9) internal standards were added to the culture fluid, and it was partitioned 3 times with equal volumes of ethyl acetate. The aqueous phase was discarded. The ethyl acetate fraction (which contains free GAs) was evaporated, and the residue was dissolved in 60% methanol and its pH was adjusted to 8.3 with 2 N NH_4OH. The sample was applied to a C18 column (90–130 μm, 60 Å pore size, Altech) that was prewashed with 100% methanol. The eluent was evaporated in a rotary evaporator and then dried onto 3 g of celite. Celite was loaded onto a 5 g SiO_2 column (ICN silica 32–100, aktiv 60 Å) and then eluted with 80 ml of elution solution (ethyl acetate:hexane=95:5). The eluate was evaporated under vacuum and dissolved in phosphate buffer (pH 8.0). The pH was adjusted with 2 N NaOH to approximately 8–9. The sample was partitioned 3 times with phosphate buffer. PVPP (polyvinyl polypyrrolidine) was added to phosphate buffer fractions and the mixture was shaken for 1 h. After shaking, the mixture was filtered and adjusted to pH 2.5 with 6 N HCl. The solution was partitioned 3 times with ethyl acetate, and the ethyl acetate fractions were evaporated, and diluted in 100% methanol for further fractionation on reversed-phase high-performance liquid chromatography (HPLC).

High-Performance Liquid Chromatography (HPLC)

The GAs were chromatographed on a 3.9×300 mm μ BondaPak HPLC C18 column (Waters) and eluted at 1.5 ml min^{-1} with the following gradient: 0 to 5 min, isocratic 28% methanol (MeOH) in 1% aqueous acetic acid; 5 to 35 min, linear gradient from 28 to 86% MeOH; 35 to 36 min, 86 to 100% MeOH; 36 to 40 min, isocratic 100% MeOH. A 1.5 ml of each fractions was collected up to 50 min. Small aliquots (15 μl) from each fraction were taken, and radioactivity was measured with liquid scintillation spectrometry (Beckman, LS 1801) to determine accurate retention times of each GA, based on the elution of ^{3}H-GA standards. The fractions were dried on a Savant Speedvac and were combined according to the retention times of ^{3}H-GA standards and previously determined retention times of the labeled (deuterated) GA standards.

GC-MS Selected Ion Monitoring

Each dried GA fraction was redissolved in 100% methanol, transferred to a 1 ml reaction vial, and dried under N_2 at 40°C. The sample was solubilized in 35 μl of methanol, and the GA methyl ester was prepared with ethereal diazomethane. The sample was dried under N_2, redissolved in methanol, and methylated once more. The sample was solubilized in 35 μl of pyridine and silylated for 30 min at 65°C with the same amount of *N,D*-Bis (trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% TMCS (Pierce Chemical Co.). The sample was then reduced to dryness with N_2 and

solubilized in anhydrous dichloromethane. One μl of each sample was injected onto the column of a 30 m, 0.25 mm (i.d.), 0.25 μm film thickness HP-1 capillary column (J & W). The GC (Hewlett Packard 6890N) oven temperature was programmed for a 1 min hold at 60°C, then to rise at 15°C min^{-1} to 200°C followed by 5°C min^{-1} to 285°C. Helium carrier gas was maintained at a head pressure of 30 kPa. The GC was directly interfaced to a Mass Selective Detector with an interface and source temperature of 280°C, an ionizing voltage of 70 eV and a dwell time of 100 min.

Quantification of Endogenous GAs

Collection and analysis of the GC-MS data was accomplished with a GC-MS (Hewlett Packard 5973 Network Mass Selective Detector). Three major ions of the supplemented [$^2H_2$]GA internal standards (obtained from Prof. Lewis N. Mander, Australian National University, Canberra, Australia) and the endogenous GA were monitored simultaneously. Retention time was determined by using the hydrocarbon standards to calculate the KRI value. Quantification of gibberellin was based on the peak area ratios of endogenous (nondeuterated sample) to deuterated GAs, after correcting for any contribution from the deuterated standard to nondeuterated GA. The endogenous concentrations of GA_1, GA_3, GA_4, GA_7, and GA_9 were calculated from the peak area ratios of 506/508, 504/506, 284/286, 222/224, and 298/300, respectively.

Identification of Fungi

The sequences of ITS region were used to identify the GA-producing fungi [19, 20, 22, 24, 37].

Table 1. Isolation of GAs-producing fungi from Gold, Brown, Super, and *S. indicum* plants.

Source of plant	No. of isolated fungi	No. of GAs-producing fungi
Sesamum indicum	11	2
Super *Sesamum indicum*	15	0
Gold *Sesamum indicum*	14	4
Brown *Sesamum indicum*	14	5

Bioassay

The surface of Waito-c rice seeds was sterilized in "spotac" solution for 1 day and treated with growth inhibitor "Uniconazol" (20 ppm) after washing clearly with distilled water. Surface sterilized Waito-c rice seeds were placed on the water agar (0.8%). The GA-producing fungi were incubated for 7 days in fluids of fungi grown in Czapek's liquid medium in the dark at 30°C. The plant heights were measured with a scale. The statistical analysis system (SAS) program was used to determine average values and standard deviation.

RESULTS

Isolation of Fungi from *Sesamum indicum* Species

Fifty-four fungi were isolated from the roots of Gold, Brown, Super, and *S. indicum* plants: Eleven strains were isolated from *S. indicum*, fifteen strains from Super *S. indicum*, fourteen strains from Gold *S. indicum*, and fourteen strains from Brown *S. indicum* (Table 1).

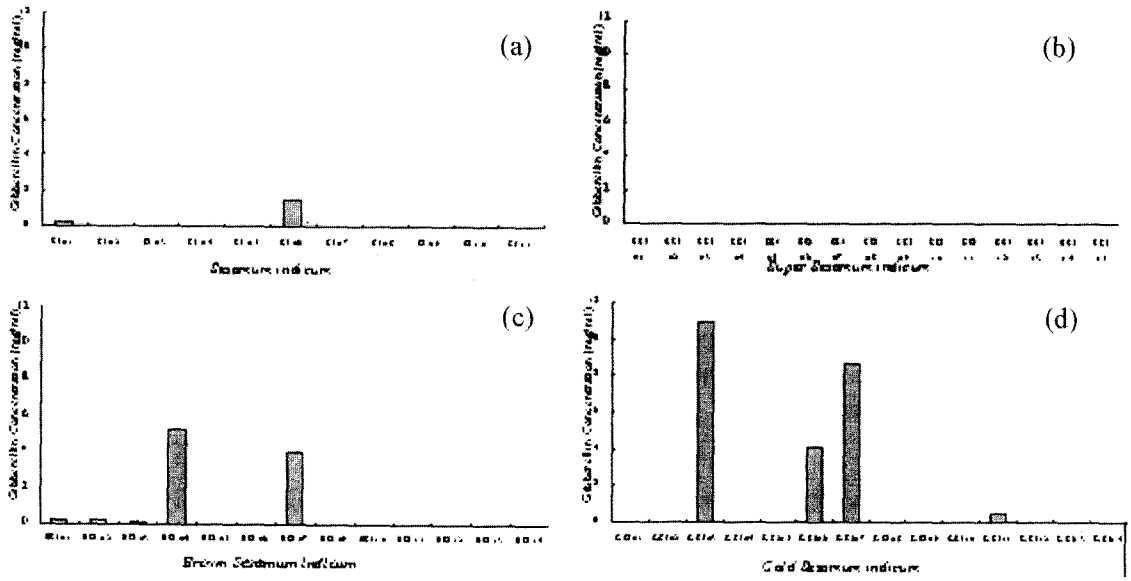

Fig. 2. Isolation of fungi from the roots of Gold, Brown, Super, and *S. indicum* plants. The isolated fungi were tested for their GA_3 production.
a) *S. indicum*, b) Super *S. indicum*, c) Gold *S. indicum*, and d) Brown *S. indicum*.

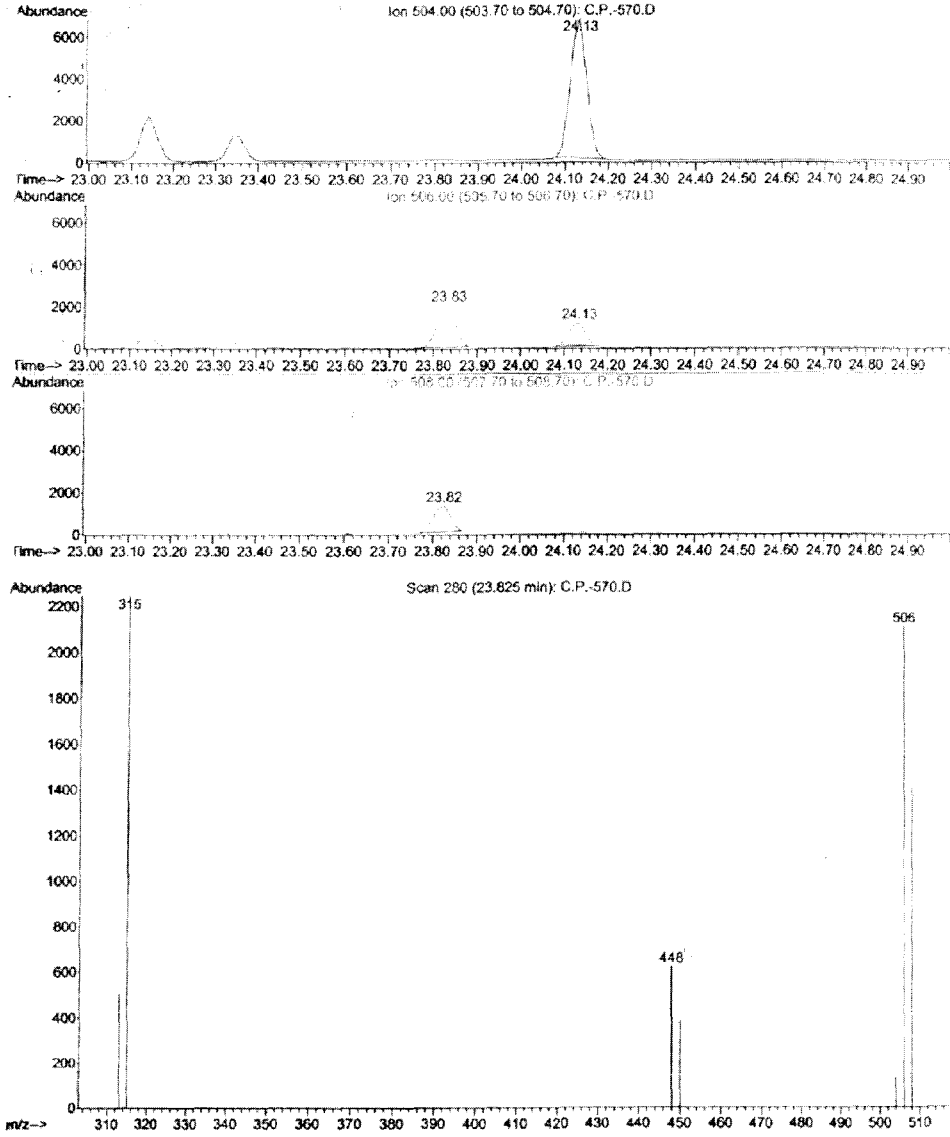

Fig. 3. GC-MS analysis of culture filtrates of the BSI03.

Quantification of gibberellin was based on the peak area ratios of endogenous to deuterated GAs, after correcting for any contribution from the deuterated standard to nondeuterated GA. The endogenous concentrations of GA_1 and GA_3 were calculated from the peak area ratios of 506/508 and 504/506 respectively. Gas chromatographic and mass spectrometric (GC-MS) analyses of culture BSI03.

Screening of Fungi Which Produce GAs

Fifty-four isolates of fungi from the roots of Gold, Brown, Super, and *S. indicum* plants were tested for their activity for GAs production. Eleven fungi were identified to produce GAs: The number of fungi producing GAs was two from *S. indicum*, four from Gold *S. indicum*, and five from Brown *S. indicum* (Table 1). GAs-production was examined by a spectrophotometric method [15] (Fig. 2).

GAs Analysis

As mentioned above, eleven fungi producing GAs were isolated from the roots of Gold, Brown, Super, and *S. indicum* plants (Fig. 2), and their activity for GAs production was analyzed by gas chromatography and mass spectrometry (GC-MS) (Fig. 3). Five kinds of GAs tested were biologically active: GA_1, GA_3, GA_4, GA_7, and GA_9 (Table 2). The fungal strain BSI03 showed the highest activity for GAs production: 71.69 ng of GA_1, 252.42 ng of GA_3, 612 ng of GA_4, 259 ng of GA_7, and 202.69 ng of GA_9 in 25 ml of culture medium. The GAs productivity of the fungal strain BSI03 was compared with those of *G. fujikuroi*, *F. proliferatum*, and *N. crassa*. The production of GA_3, GA_4, and GA_7 from BSI03 was higher than the above 3 kinds of fungal strains known as GAs producer. It was shown that the production of GAs from *G. fujikuroi* was GA_1 173.75 ng; GA_3 180.69 ng; GA_4 580.13 ng; GA_7

Table 2. GAs production from the isolated strain, determined by gas chromatography and mass spectrometry (GC-MS). Eleven fungi with GAs-production activity were incubated for 7 days in 40 ml of Czapek's liquid medium at 25°C and 120 rpm to determine the amount of GAs, and the amount of each GAs in the medium was analyzed.

Source of plant	Sample No.	GA_1	GA_3	GA_4	GA_7	GA_9
		ng/25 ml				
S. indicum	SI01	0.29	3.12	2.33	1.17	N.D
	SI06	0.21	3.88	3.34	3.68	N.D
Gold *S. indicum*	GSI03	0.39	12.01	3.49	N.D	N.D
	GSI06	0.39	8.56	1.98	N.D	N.D
	GSI07	ND	N.D	4.83	N.D	N.D
	GSI11	1.38	26.00	5.12	3.94	N.D
Brown *S. indicum*	BSI01	6.75	47.04	60.81	48.92	3.44
	BSI02	15.06	100.06	161.12	86.91	10.86
	BSI03	71.69	252.42	612.00	259.00	202.69
	BSI04	1.30	6.54	N.D	N.D	N.D
	BSI07	0.38	2.24	2.75	1.66	N.D
	GF[1]	173.75	180.69	580.13	203.50	503.43
	FP[2]	105.42	200.06	571.36	217.53	175.22
	NC[3]	12.6	103.28	165.78	95.38	18.16

ND: not determined.

1: *G. fujikuroi*; 2: *F. proliferatum*; 3: *N. crassa*.

203.50 ng, and GA_9 503.43 ng of 25 ml culture medium under the same culture condition (Table 2).

Identification of Fungi

The ITS region of the fungal strain BSI03 was cloned and sequenced. The sequence of ITS regions revealed that the strain BSI03 had 100% similarity to *P. commune*. Therefore, the strain BSI03 was identified as *P. commune* KNU5379 (Fig. 4).

Bioassay

The bioassay of culture fluid from GAs-producing fungi was carried out. The plant tested was Waito-c rice. The GAs-producing fungi were cultured for 7 days in the fluids of fungi, grown in Czapek's liquid medium, in the dark at 30°C. Two μl of cultured fluid were added to the branch point of 2-leaf rice sprout. The *P. commune* KNU5379 showed better GAs-producing activity than any other fungal strains isolated in this study (Table 3).

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  1 GTAGGTGAAC CTGCGGAAGG ATCATTACCG AGTGAGGGCC CTCTGGGTCC AACCTCCCAC 60
 61 CCGTGTTTAT TTTACCTTGT TGCTTCGGCG GGCCCGCCTT AACTGGCCGC CGGGGGGCTC 120
121 ACGCCCCCGG GCCCGCGCCC GCCGAAGACA CCCTCGAACT CTGTCTGAAG ATTGAAGTCT 180
181 GAGTGAAAAT ATAAATTATT TAAAACTTTC AACAACGGAT CTCTTGGTTC CGGCATCGAT 240
241 GAAGAACGCA GCGAAATGCG ATACGTAATG TGAATTGCAA ATTCAGTGAA TCATCGAGTC 300
301 TTTGAACGCA CATTGCGCCC CCTGGTATTC CGGGGGGCAT GCCTGTCCGA GCGTCATTGC 360
361 TGCCCTCAAG CCCGGCTTGT GTGTTGGGCC CCGTCCTCCG ATTT 404
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Fig. 4. Nucleotide sequences of the ITS region of *P. commune* KNU5379.

Table 3. Bioassay of cultural fluids of GA-producing fungi with rice sprout. The GA-producing fungi were incubated for 7 days in Czapek's liquid medium in the dark at 30°C. The culture fluid of fungi was added to Waito-c rice seeds. The plant heights were measured with a scale.

Sample No.	Average	S.D.
Control	4.92	0.22
SI01	4.88	0.26
SI06	5.04	0.21
GSI03	4.82	0.28
GSI06	5.06	0.50
GSI07	5.26	0.23
GSI11	4.92	0.08
BSI01	5.32	0.31
BSI02	5.30	0.16
P. commune KNU5379	5.48	0.26
BSI04	4.86	0.53
BSI07	5.10	0.39
G. fujikuroi	6.16	0.27

Discussion

This paper describes the GAs-producing fungi isolated from the roots of *S. indicum* plants. Over the last 50 years, there have been many researches on the GA production. GAs consist of a number of large families of tetracyclic deterpenoid compounds, some of which are bioactive growth regulators which control diverse developmental processes such as seed germination, stem elongation, leaf expansion, trichome development, and fruit development [30]. Because of these properties and their commercial values, interests on the GAs over the last half-century stimulated many studies of their biosynthesis [27, 39], catabolism [12, 21, 43], and the development of an optimal production process, involving optimization of culture conditions [38].

Sixty-two strains of fungi were isolated from the roots of Gold, Brown, Super, and *S. indicum* plants which were tested for their GAs production. The fungal strain BSI03 showed the highest productivity of GAs. Spectrophotometric method was used to screen GA-producing fungi, but this result did not agree with gas chromatographic and mass spectrometric (GC-MS) analyses. The fungal strain BSI03 was identified as *P. commune* KNU5379, based on the sequence analysis of the ITS region.

GAs are also produced by many other fungi, such as *G. fujikuroi* [39], *Sphaceloma manihoticola* [34], *N. crassa* [17], *Phaeosphaeria* sp. *L.* 487 [16], and so on. *P. commune* KNU5379 has not yet been reported as a good GAs producer, but it showed good productivity of GA_3, GA_4, and GA_7, compared with any other GAs-producing fungi such as *G. fujikuroi*, *F. proliferatum*, and *N. crassa*. The absolute productivity of *P. commune* KNU5379 was somewhat

lower than that of other fungi known as GAs producers, because the optimal condition for GAs-production by *P. commune* KNU5379 was not determined yet. The relative productivity of *P. commune* KNU5379 was higher than that of *G. fujikuroi*, especially in the case of GA_3, GA_4, and GA_7, but was lower than *G. fujikuroi* in the case of GA_1 and GA_9. The bioassay result indicates that GA_1 may be more important than any other bioactive GAs in rice, because *G. fujikuroi* showed higher growth promoting activity in spite of lower contents of GA_3, GA_4, and GA_7, except GA_1. The result shows the importance of phytohormone analysis, and interrelationships between plants and microorganisms may help explain the beneficial effects of fungi to the host plant [11]. The important factors for the production of GAs are nitrogen and carbon sources as nutrient, culture period, temperature, pH, and agitation as environmental factors. The quantities and qualities of GAs are affected by nitrogen source [38], and it is known that the production of GAs is inhibited also by nitrogen source [3]. Wild-type *G. fujikuroi* IMI58289 and some other microbes did not produce GAs until the nitrogen source was exhausted from the medium [3, 6]. It was reported that the production of GAs starts immediately after the depletion of nitrogen in the medium [38]. The high concentration of glucose also inhibits GAs production, and the optimal fermentation condition of GAs is a period of 6-8 days at pH 4.0 [5]. The amount of GAs production by *P. commune* KNU5379 was somewhat lower than that of the productivity of other microbes reported earlier, because optimal conditions for the production of GAs by *P. commune* KNU5379 have not yet been determined. So, the optimal fermentation conditions of GAs by *P. commune* KNU5379 remains still to be studied.

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