J. Microbiol. Biotechnol. (2005), **15**(4), 809–814

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Fusarium proliferatum KGL0401 as a New Gibberellin-Producing Fungus

RIM, SOON-OK, JIN-HYUNG LEE, WHA-YOUL CHOI, SEON-KAP HWANG, SEOK-JONG SUH, IN-JOONG LEE[1], IN-KOO RHEE[2], AND JONG-GUK KIM*

Department of Microbiology, [1]Department of Agronomy, [2]Department of Agricultural Chemistry, Kyungpook National University, Daegu 702-701, Korea

Received: November 22, 2004
Accepted: December 3, 2004

Abstract Gibberellins (GAs) play an important role in plant growth and development. Fifteen fungi were isolated from *Physalis alkekengi* var. *francheti* plant roots, and among them, four isolates showed GA-production activity. A bioassay using waito-c rice was carried out with the culture fluid of the GA-producing fungi. The GA-producing fungi were cultured for 7 days in Czapek's liquid medium at 30°C, 120 rpm, under dark conditions. The culture broth was concentrated 30-fold and 10 μl of that concentrate was applied to 2-leaf rice sprouts. The height of the rice seedlings treated with the culture fluid of isolate PA08 was 26 cm high, while that of the seedlings treated with the wild-type *Gibberella fujikuroi* was 13 cm high. As such, the plant growth-promoting activity exhibited by isolate PA08 was 2 times stronger than that exhibited by the wild-type *G. fujikuroi*. The amounts of GA_1, GA_3, GA_4, GA_7, GA_9, GA_{20}, and GA_{24} in the medium were measured using gas chromatography-mass spectrometry (GC-MS), and the quantities produced by isolate PA08 were 4.85 ng/ml, 4.79 ng/ml, 17.30 ng/ml, 6.01 ng/ml, 16.61 ng/ml, 0.08 ng/ml, and 17.30 ng/ml, respectively. Isolate PA08 was also identified as *Fusarium proliferatum* KGL0401 by a genetic analysis of the nucleotide sequences of the internal transcribed spacer region of the ribosomal DNA.

Key words: *Fusarium proliferatum* KGL0401, *Physalis alkekengi* var. *francheti*, Gibberellin

GAs are part of the large family of diterpenoid compounds, some of which are bioactive growth regulators that control such diverse developmental processes as seed germination, stem elongation, leaf expansion, trichome development, and flower and fruit development [16]. Yet, their major trait is the enhancement of stem growth.

*Corresponding author
Phone: 82-53-960-5379; Fax: 82-53-955-5522;
E-mail: kimjg@knu.ac.kr

As well as being phytohormones, GAs have also been found in certain fungi and bacteria. To date, a total of 136 GAs have been identified from natural sources [15]. The first report on GAs in 1828 revealed a significant effect on the stem length of rice plants as a result of disease, and the cause of this rice disease was later identified as the fungus *G. fujikuroi* by Hori in 1898. Thereafter, in 1926, Kurosawa showed that the substance produced by *G. fujikuroi* was responsible for the effect of the disease, and the GAs were finally crystallized in 1938 by Yubuta and Sumiki [15]. GAs are secondary metabolites of the fungus *G. fujikuroi*, which is also used as a commercial source for bioactive GAs known as GA_1, GA_3, GA_4, and GA_7 that are produced by the fermentation of *G. fujikuroi* [18].

The industrial production of plant regulators, such as the GAs produced by *G. fujikuroi*, in batch submerged fermentors can help to increase agricultural production. The maximum production of GA_3 has been achieved with a C:N ratio of 36:8 at pH 5. The effect of temperature on the production of GA_3 is significant and the optimal temperature for GA production is 30°C [3].

GAs have many commercially valuable applications. For example, most seedless grapes are grown with GA treatment. Also, since the rind of citrus fruit tenderizes at maturity, GAs are used to inhibit senescence and maintain the rind in a better condition. Meanwhile, GAs are used to induce a variety of ornamental plants to flower earlier than usual or off-season, and the malt production step during the brewing of beer can be reduced by 2–3 days with the addition of 25–500 μg of GA_3 for each kg of barley [15].

Accordingly, the aim of this study was to find a better GA-producing fungi than the wild-type *G. fujikuroi*. The GA-producing activity was determined by gas chromatography, mass spectrometry (GC-MS), and a bioassay. Thus, the isolation of GA-producing fungi from the root of *P. alkekengi* var. *francheti* is described, along with a bioassay

of the culture broth of the isolated strain and identification of the best GA-producing fungi.

MATERIALS AND METHODS

Collection of Samples

The plant roots of *P. alkekengi* var. *francheti* were collected from the Chilgok Rural Development Department, and the wild-type strain of *G. fujikuroi* was provided by the Korean Culture Center of Microorganisms.

Isolation of Fungi

The plant roots of *P. alkekengi* var. *francheti* were thoroughly washed with running water to remove the soil, and then they were treated with Tween 80 for 5 min and washed with distilled water. Thereafter, the roots were submerged in perchloric acid (1%) for 5 min at 120 rpm twice and washed with autoclaved distilled water three times. The roots were then cut with scissors into 1.5-cm pieces and thoroughly dried on sterile filter paper. Finally, the root pieces were placed on Hagem's medium containing streptomycin to prevent any bacteria growth, and single-cell isolation was carried out [1, 26].

Media and Culture Conditions

The isolated fungi were cultured on Hagem's medium (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, 1.5% agar, pH 5.6). Meanwhile, for the production of GAs, the isolated fungi were cultured in 40 ml of Czapek's liquid medium (1% glucose, 1% peptone, 0.05% KCl, 0.05% $MgSO_4{\cdot}7H_2O$, 0.001% $FeSO_4{\cdot}7H_2O$, pH 7.3±0.2) at 30°C, 120 rpm, for 7 days.

GA Measurement

Prescreening of the GA-producing fungi was performed using a spectrophotometric method to determine the GA activity based on the conversion of gibberellic acid to gibberellenic acid [27].

GA Bioassay

The GA-producing fungi were cultured for 7 days in Czapek's liquid medium in the dark at 30°C. The bioassay was then carried out with the culture broth using waito-c rice. The surface of the waito-c rice seeds was first sterilized in a spotac (Aventis cropsciences) solution for 1 day, and then treated with the growth inhibitor Uniconazol (20 ppm). Next, the surface-sterilized waito-c rice seeds were placed on a water agar (0.8%). After 2 days, 10 µl of the lyophilized culture broth concentrated 30-fold was applied to the 2-leaf rice sprouts. The height of the rice seedling was measured with a scale.

Extraction of Endogenous GAs

The extraction of GAs followed the general procedure described by Lee *et al.* [12]. After cultivation, the culture solution was acidified to pH 2.5 using 6 N HCl. Deuterated (20 ng of [17.17-$^2H_2$] GA_1, GA_3, GA_4, and GA_7, respectively) internal standards were added to the culture, which was then partitioned 3 times with equal volumes of ethyl acetate. The aqueous phase was discarded, while the ethyl acetate fraction (containing the free GAs) was evaporated, diluted in 60% methanol, and adjusted to pH 8.3 using 2 N NH_4OH. The sample was then applied to a C18 column (90–130 µm, 60 Å pore size, Altech) that had been prewashed with 100% methanol. The eluent was evaporated in a rotary evaporator and then dried onto 3 g of celite. The celite was then loaded onto a 5 g SiO_2 column (ICN silica 32–100, 60A) and eluted with 80 ml of elution solution (ethyl acetate:hexane=95:5). The resulting eluate was evaporated under a vacuum, dissolved in a phosphate buffer (pH 8.0), and the pH adjusted to approximately 8–9 using 2 N NaOH. The sample was then partitioned 3 times with the phosphate buffer, polyvinylpolypyrrolidone (PVPP) added to the phosphate buffer fractions, and the mixture shaken for 1 h. Thereafter, the mixture was filtrated and adjusted to pH 2.5 using 6 N HCl. The solution was then partitioned 3 times using ethyl acetate and the ethyl acetate fractions evaporated and diluted in 100% methanol for further fractionation using revered-phase HPLC.

High-Performance Liquid Chromatography (HPLC)

The GAs were chromatographed on a 3.9×300 mm µ BondaPak C18 column (Waters) and eluted at 1.5 ml/min with the following gradient: 0 to 5 min, isocratic 28% 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. Up to 50 1.5-ml fractions were collected. Small aliquots (15 µl) were taken from each fraction and the radioactivity measured using liquid scintillation spectrometry (Beckman, LS 1801) to determine an accurate retention time for each GA based on the elution of the ^{3}H-GA standards. The fractions were dried using a Savant Speedvac and the fractions combined according to the retention times of the ^{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 samples were then dissolved in 35 µl of methanol, and the GA methyl ester prepared with ethereal diazomethane. Thereafter, the samples were dried under N_2, redissolved in methanol, and methylated one more time. The samples were dissolved in 35 µl pyridine, and

silylated for 30 min at 65°C using the same amount of N, D-Bis (trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% TMCS (Pierce Chemical Co.). The samples were then reduced to dryness with N_2 and solubilized in anhydrous dichloromethane. One µl of each sample was injected onto a 30 m, 0.25 mm (i.d.), 0.25 µm film thickness HP-1 capillary column (J & W), and the GC (Hewlett Packard 6890N) oven temperature programmed for a 1 min hold at 60°C, followed by an increase of 15°C min^{-1} to 200°C and then 5°C min^{-1} to 285°C. The helium carrier gas was maintained at a head pressure of 30 kPa. The GC was directly interfaced to a Mass Selective Detector based on source temperature of 280°C, ionizing voltage of 70 eV, and dwell time of 100 min.

Quantification of Endogenous GAs

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

Identification of Fungi

The sequences of the ITS region were used to identify the GA-producing fungi. The specific primers, synthesized by Bioneer Co., had the following sequences: ITS-F: 5'-TCC GTA GGT GAA CCT GCG G-3', and ITS-R: 5'-TCC TCC GCT TAT TGA TAT GC-3'.

RESULTS

Isolation of GA-Producing Fungi from *P. alkekengi* var. *francheti*

Fifteen fungi were isolated from the roots of *P. alkekengi* var. *francheti* and examined for their GA-producing activity. GA-production was determined by a spectrophotometric method [27] and four fungi were shown to produce GAs (data not shown).

A bioassay of a culture broth from the four GA-producing fungi was carried out using waito-c rice. The heights of the rice seedlings treated with the culture fluid of isolates PA03, PA05, PA08, and PA15 were 6.3 cm, 8.2 cm, 26 cm, and 8.7 cm, respectively, after 7 days (Fig. 1). Thus, the plant growth-promoting activity exhibited by isolate PA08 was two times stronger than that of the wild-type *G. fujikuroi* (Fig. 1).

GA Analysis

Four GA-producing fungi were isolated from the roots of *P. alkekengi* var. *francheti*. As PA08 exhibited the best growth-promoting activity, it was used for further analysis and its activity as regards GA production analyzed using

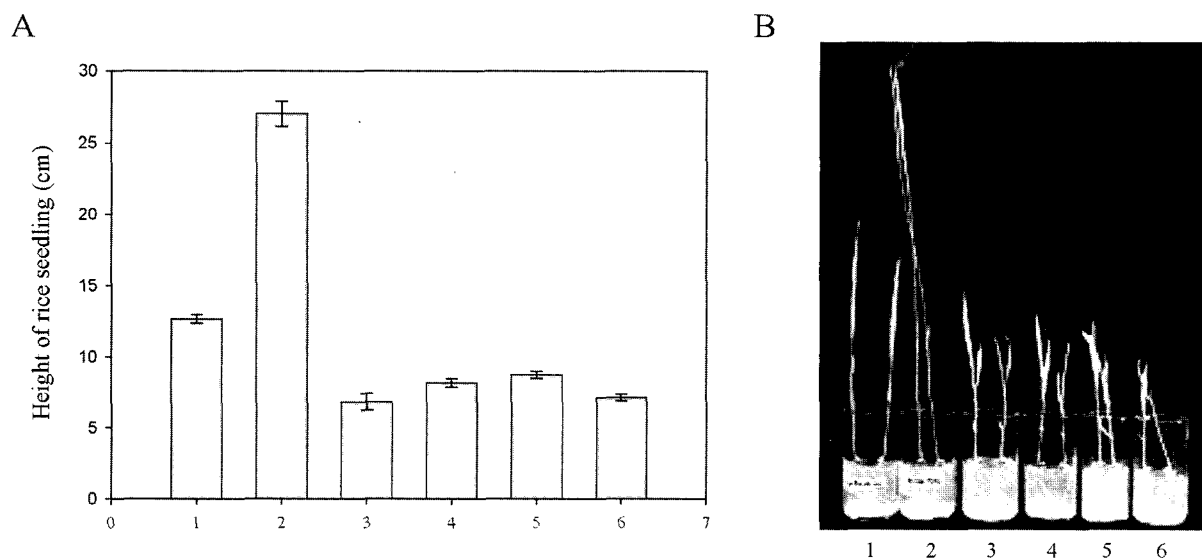

Fig. 1. Effect of culture broth prepared with isolated fungi on waito-c rice.
(A and B) A bioassay of the culture broth from four GA-producing fungi was carried out using waito-c rice. The GA-producing fungi were cultured for 7 days in Czapek's liquid medium at 30°C, 120 rpm, under dark conditions. Two-leaf rice sprouts were treated with 10 µl of the lyophilized culture broth concentrated 30-fold. 1, *G. fujikuroi*; 2, PA08; 3, PA03; 4, PA05; 5, PA15; 6, water.

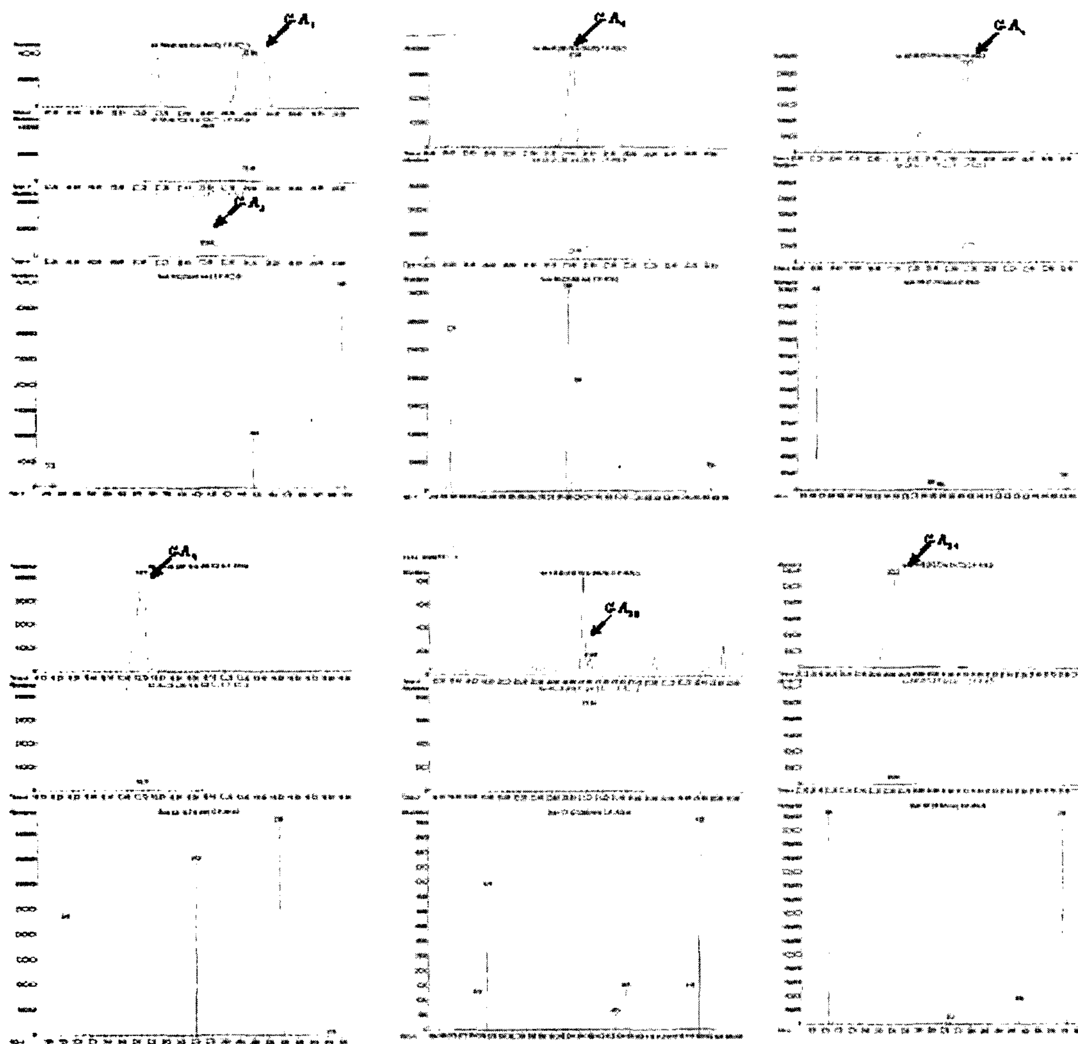

Fig. 2. GC-MS analysis of culture filtrates of PA08.
The quantification of gibberellins was based on the peak area ratios of endogenous to deuterated GAs, after correcting for any contribution from the deuterated standard to non-deuterated GAs.

GC-MS (Fig. 2). The quantities of seven kinds of GA (GA_1, GA_3, GA_4, GA_7, GA_9, GA_{20}, and GA_{24}) from PA08 were measured: GA_1 4.85 ng/ml, GA_3 4.79 ng/ml, GA_4 17.30 ng/ml, GA_7 6.01 ng/ml, GA_9 16.61 ng/ml, GA_{20} 0.08 ng/ml, and GA_{24} 20.92 ng/ml. Meanwhile, the quantities of GAs in the wild-type *G. fujikuroi* under the same conditions were GA_1 4.07 ng/ml, GA_3 4.26 ng/ml, GA_4 13.79 ng/ml, GA_7 0.04 ng/ml, GA_9 11.35 ng/ml, GA_{20} 0.02 ng/ml, GA_{24} 15.18 ng/ml. The particularly bioactive GAs were GA_1, GA_3, GA_4, and GA_7. Strain PA08 showed a higher GA productivity than the wild-type *G. fujikuroi* for all the GAs investigated (Table 1).

Table 1. GA productivity in (ng/ml) of isolate PA08 and *G. fujikuroi*.

Sample no.	GA_1	GA_3	GA_4	GA_7	GA_9	GA_{20}	GA_{24}
PA08	4.85	4.79	17.03	6.01	16.61	0.08	20.92
G. fujikuroi	4.07	4.26	13.79	0.04	11.35	0.05	16.58

PA08 was incubated for 7 days in 40 ml of Czapek's liquid medium at 30°C, 120 rpm, to determine the GA productivity, and the amount of each GA in the medium was analyzed by GC-MS. Under the same conditions, strain PA08 exhibited a higher GA productivity than *G. fujikuroi*.

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  1 GCCCGACGTC GCATGCTCCC GGCCGCCATG GCGGCCGCGG GAATTCGATT TCCTCCGCTT  60
 61 ATTGATATGC TTAAGTTCAG CGGGTATTCC TACCTGATCC GAGGTCAACA TTCAGAAGTT 120
121 GGGGGTTTAA CGGCTTGGCC GCGCCGCGTA CCAGTTGCGA GGGTTTTACT ACTACGCAAT 180
181 GGAAGCTGCA GCGAGACCGC CACTAGATTT CGGGGCCGGC TTGCCGCAAG GGCTCGCCGA 240
241 TCCCCAACAC CAAACCCGGG GGCTTGAGGG TTGAAATGAC GCTCGAACAG GCATGCCCGC 300
301 CAGAATACTG GCGGGCGCAA TGTGCGTTCA AAGATTCGAT GATTCACTGA ATTCTGCAAT 360
361 TCACATTACT TATCGCATTT TGCTGCGTTC TTCATCGATG CCAGAACCAA GAGATCCGTT 420
421 GTTGAAAGTT TTGATTTATT TATGGTTTTA CTCAGAAGTT ACATATAGAA ACAGAGTTTA 480
481 GGGGTCCTCT GGCGGGCCGT CCCGTTTTAC CCGGGAGCGG GCTGATCCGC CGAGGCAACA 540
541 ATTGGTATGT TCACAAGGGT TTGGGAGTTG TAAACTCGGT AATGATCCCT CCGCAGGTTC 600
601 ACCTACCGGA ATCACTAGTG AATTCGCGGC CGCTGCAGGT CGACCATT             660
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Fig. 3. Nucleotide sequences of the ITS region for the strain PA08.
Strain PA08 showed a 99% similarity to *Fusarium proliferatum*.

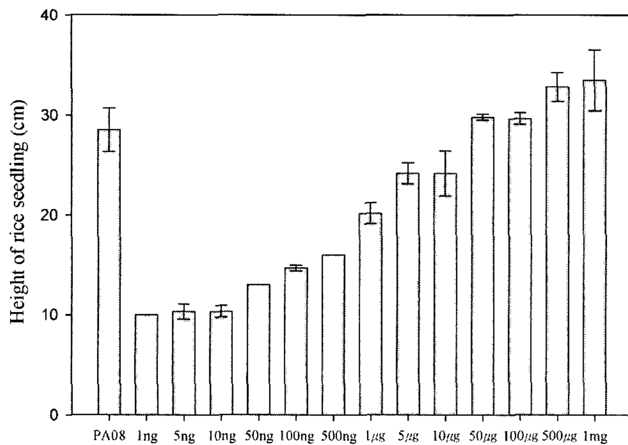

Fig. 4. Comparison of height of waito-c rice treated with various concentrations of GA_3 and culture broth of *F. proliferatum* KGL0401.

The waito-c rice was treated with 10 µl of various concentrations (1 ng to 1 mg) of GA_3, grown at 30°C for 7 days, and the height measured using a scale. PA08: Culture broth of *F. proliferatum* KGL0401.

Identification of Fungal Strain PA08

The ITS region of fungal strain PA08 was cloned and sequenced, and showed a 99% similarity to *Fusarium proliferatum*. Therefore, strain PA08 was named *F. proliferatum* KGL0401 (Fig. 3).

Effect of Culture Fluid on Waito-c

The stems of the waito-c rice grew higher in proportion to increasing the amount of GA_3 (Fig. 4). When comparing the heights of the waito-c rice sprouts treated with the culture broth of *F. proliferatum* KGL0401 and the GA_3-treated waito-c rice sprouts, the heights of the former were nearly equivalent to those of the latter treated with about 40 µg GA_3 (Fig. 4).

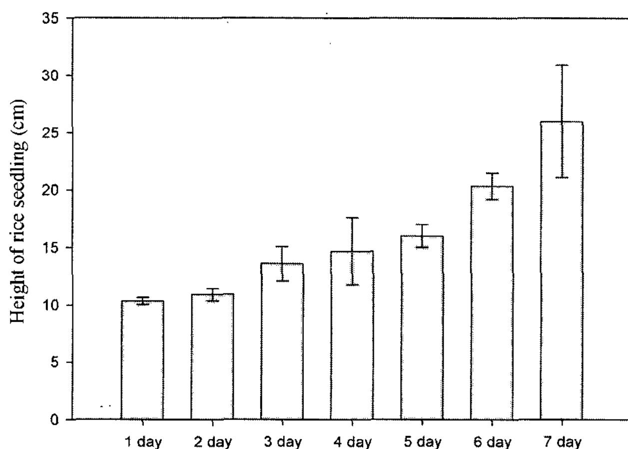

Fig. 5. Time course of height of waito-c rice.

The *F. proliferatum* KGL0401 was cultured for 1 to 7 days, and the culture fluid concentrated 30-fold. Two-leaf rice seedlings were then treated with 10 µl of the concentrated supernatant and cultured for 7 days at 30°C.

Two-leaf rice seedlings were treated with 30-fold concentrations of 10 µl of the supernatant prepared from the culture broth of *F. proliferatum* KGL0401 cultured for 1 to 7 days at 30°C. The quantity of GAs produced by *F. proliferatum* KGL0401 gradually increased as the cultivation continued for 7 days (Fig. 5). As such, the height of the waito-c rice was 26 cm after treatment with the culture fluid of *F. proliferatum* KGL0401 grown for 7 days at 30°C, 120 rpm.

DISCUSSION

GAs are a family of diterpenoid plant hormones produced by the rice pathogen *G. fujikuroi* and a few other fungal genera, such as *Sphaceloma manihoticola*, *Neurospora crassa*, and *Phaeosphaeria* sp. L. 487 [25]. Although it is well known that *G. fujikuroi* is the best GA-producing fungus, the current authors investigated the isolation of GA-producing fungi from the roots of various plants, and 715 kinds of fungi were isolated from the roots of 73 kinds of plant.

Fifteen strains of fungi were isolated from the roots of *P. alkekengi* var. *francheti* and four isolates (PA03, PA05, PA08, and PA15) were identified as having GA production activity based on the Holbrock method (data not shown). A bioassay was carried out using the culture fluid of the four isolated fungi, and strain PA08 exhibited the highest activity with a plant growth-promoting activity that was two times stronger than that of *G. fujikuroi* (Fig. 1). Furthermore, a GC-MS analysis also revealed that fungal strain PA08 had a higher GA productivity than *G. fujikuroi* (Table 1).

Fungal strain PA08 was identified as *F. proliferatum* KGL0401 from a sequence analysis of its ITS region (Fig. 3); however, *F. proliferatum* KGL0401 has not yet been reported as a good GA-producing fungus.

Waito-c rice was treated with 10 µl of various concentrations (1 ng to 1 mg) of GA_3, grown at 30°C for 7 days, and its height measured using a scale. When a bioassay using the culture broth of *F. proliferatum* KGL0401 was carried out on the waito-c rice, the height of the rice was 28.5 cm after 7 days (Fig. 4). Yet the amount of GA_3 in the culture media of *F. proliferatum* KGL0401 was estimated as 143.7 ng/ml by GC-MS analysis. Therefore, it would appear that the growth of the waito-c rice was promoted not only by GA_3, but also by other kinds of GA and metabolic intermediates of GAs contained in the culture fluid of *F. proliferatum* KGL0401. In addition, it was also assumed that the quantity of GAs produced by *F. proliferatum* KGL0401 gradually increased as the cultivation continued for 7 days (Fig. 5).

Several recent reviews have focused on GA biosynthesis and catabolism and the GA response pathway [17]; meanwhile, the highest production of GAs has been obtained using *Fusarium fujikuroi* with a C:N ratio of 36:8, pH 5, and temperature of 30°C [3]. Thus, the optimal conditions for

GA production by *F. proliferatum* KGL0401 needs to be studied. The cloning of GA-biosynthetic genes has already been conducted on *des*, *cps/ks*, *P450-1* to *P450-4*, and *ggs2* in *G. fujikuroi* [27]. Nonetheless, many of the GA-biosynthetic genes in *G. fujikuroi* have still not been studied. However, the present study showed that *F. proliferatum* KGL0401 is superior to wild-type *G. fujikuroi* in GA productivity. Therefore, the cloning and regulation mechanisms of GA biosynthesis in *F. proliferatum* KGL0401 need to be studied.

Acknowledgment

This work was supported by the Biogreen21 program of the Korean Rural Development Department.

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