J. Microbiol. Biotechnol. (2009), **19**(6), 560–565
doi: 10.4014/jmb.0809.520
First published online 28 November 2008

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Gibberellin Production and Plant Growth Enhancement by Newly Isolated Strain of *Scolecobasidium tshawytschae*

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Received: September 11, 2008 / Revised: October 4, 2008 / Accepted: October 9, 2008

We isolated nine endophytic fungi from the roots of salt-stressed soybean cultivar Daewonkong and screened them for growth-promoting secondary metabolites. Of all fungal isolates, P-4-3 induced maximum growth promotion of waito-c rice and soybean. Analysis of the culture filtrate of P-4-3 showed the presence of physiologically active gibberellins GA_1, GA_3, GA_4, and GA_7, along with physiologically inactive GA_{15} and GA_{24}. The plant growth promotion and gibberellin-producing capacity of P-4-3 was much higher than wild-type *Gibberella fujikuroi*, which was taken as the control during the present study. The fungal isolate P-4-3 was identified as a new strain of *Scolecobasidium tshawytschae* through the morphological characteristics and phylogenetic analysis of 18S rDNA sequence. Gibberellins production and plant growth promoting ability of genus *Scolecobasidium* was reported for the first time in the present study.

Keywords: *Scolecobasidium tshawytschae*, gibberellins, plant growth, endophytic fungi, 18S rDNA, waito-c rice

Endophytic fungi of roots have been found colonizing plants, and have usually been defined as those growing asymptomatically within the tissues of their host plants, excluding pathogenic fungi and mycorrhizae [17]. Since this relationship is subjected to change and getting replaced by other members according to environmental conditions and host requirement, such fungal members can be defined as those located within apparently healthy, functional root tissues at the moment of sample collection. Unlike mycorrhizal fungi, endophytes reside entirely within host tissues and emerge during host senescence. Endophytes have been shown to confer fitness benefits to host plants including tolerance to herbivory, heat, salt, disease, and drought, and increased below- and above-ground biomass [2]. Endophytic colonization may also improve the ecological adaptability of the host by enhancing tolerance to biotic and abiotic stresses [23]. *Scolecobasidium tshawytschae* is a slow-growing fungus with worldwide distribution and has been isolated from soil, desert soil, salt marshes, plants, compost, decaying leaves, and fish aquarium water. It is the cause of fish mycosis. There have not been any reports of human infections.

Endophytic fungi are very little known for the production of growth stimulating hormones, especially gibberellins (GAs). Gibberellins are a family of diterpenoid plant harmones, first detected in 1930s from culture filtrates of *Gibberella fujikuroi*, a known pathogen of rice plants [26]. GAs appear to be involved in every aspect of plant growth and development, but their most spectacular property is the enhancement of stem growth [20]. Currently 136 GAs have been identified, and 7 fungi, pathogenic and nonpathogenic, associated with plants and/or soil have been reported as GA producers [16]. Until now, these hormones had been reported in 12 different fungi from different sources [12, 27], and a strain of *Fusarium proliferatum* had also been reported as a GA producer [21]. However, there are no reports on GA production by genus *Scolecobasidium* so far.

The importance of GA-producing fungal symbionts in plant growth and development cannot be overlooked, especially under stress conditions. During the present study, we isolated endophytic fungi from salt-stressed soybean plants and investigated their role on the growth and development of soybean. Furthermore, the GA-producing capacity of the fungal isolate with maximum growth promotion was also ascertained.

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MATERIALS AND METHODS

Isolation of Endophytic Fungi from Soybean

We collected roots from a salt-stressed (100 mM NaCl) soybean cv. Daewonkong grown in perlite. The root samples were washed with water, treated with Tween 80 solution, and surface-sterilized using perchloric acid (1%) solution. The surface-sterilized roots were then cut into 0.5 cm pieces in a laminar hood, cultured on Hagem media plates supplemented with 80 ppm Streptomycine [19, 29], and incubated at 25°C until emergence of fungi from inside of root pieces [1, 28]. The isolated pure cultures of root fungi were stored on PDA slants. Czapek broth medium containing 1% glucose and peptone was used for GA production [10] by incubating the strain at 30°C and 120 rpm for 7 days. We used wild-type *Gibberella fujikuroi* as the control during the experiment.

Screening of Fungal Culture Filtrate for Plant Growth Promotion on Waito-C Rice

The culture filtrates of endophytic fungi were screened on waito-c rice seedlings for the presence of plant growth-promoting metabolites. For this purpose, the lyophilized supernatants of each fungal isolate were mixed with 1 ml of autoclaved distilled water. Seeds of waito-c rice were surface-sterilized [18] and treated with 20 ppm uniconazol for 24 h, in order to further minimize GA biosynthesis. The treated seeds were washed thoroughly and soaked in autoclaved distilled water until radical emergence. The young seedlings were transplanted in glass tubes containing 0.8% water-agar medium and grown in a growth chamber. Ten μl of supernatant solution of fungal culture filtrate was applied on the apical meristem of rice seedlings at the 2-leaf stage. The shoot and plant lengths were observed after a week of culture filtrate application and compared with waito-c rice seedlings treated either with distilled water or culture filtrate of *G. fujikuroi*.

Bioassay on Soybean

Seeds of soybean cv. Hwangkeumkong were surface-sterilized with 5% NaClO for 15 min and then washed with distilled water. Seeds were sown in autoclaved perlite and 20 ml of Hoagland solution was applied at germination time. Fungal isolate P-4-3 was selected on the basis of best results in the rice screening experiment, and 5 ml of fungal supernatant of P-4-3 was applied to soybean seedlings at the 2-leaf stage. Plant length, shoot length, plant fresh weight, and shoot fresh weight were observed after one week of culture filtrate application.

Extraction and Quantification of Gibberellins

Fungal GAs were extracted from culture filtrates after 7 days of incubation according to established protocol [15]. Quantification of GAs was accomplished with GC-MS SIM. The three major ions of the supplemented [$^2H_2$] GA internal standards (obtained from Prof. Lewis N. Mander, Australian National University, Australia) and extracted GAs were monitored simultaneously. The retention time was determined using hydrocarbon standards to calculate the KRI value, and the GAs quantification was based on peak area ratios of non-deuterated (extracted) GAs to deuterated GAs.

Identification of Fungal Isolate P-4-3 Using Traditional Techniques

Subcultures on PDA and Hagem agar plates were examined periodically and identified on sporulation, both macroscopically and microscopically. Hyphae were mounted on slides and examined after staining with lactophenol.

Genomic DNA Extraction from Mycelia and Gel Electrophoresis

An efficient method was developed for the isolation of genomic DNA from endophytic fungi, because the usual CTAB extraction method and mycelial grinding was causing DNA shearing. Rich mycelial culture was obtained by growing the fungus in Czapek culture broth (supplemented with 1% glucose and peptone) for 7 days on a rotary shaking incubator (120 rpm and 28°C), and lyophilized for 24 h. The lyophilized sample (0.5 g) was broken carefully in a 2-ml Eppendorf, with a blunt-end spatula or glass rod. A double volume of lysis buffer (20 mM Tris-HCl, pH 8.0; 10 mM EDTA; 1% SDS) containing 1% of 2-mercaptoethanol was added. The mixture was vortexed briefly (30 sec) to obtain homogeneity and left to incubate for 2 h in a water bath set at 55°C. Pre-heated 4% CTAB extraction buffer (250 μm/ml) was added to the lysed cell mixture and incubated further at 65°C for 1 h. Chloroform extraction followed by isopropanol precipitation yielded a condensed strand of nucleic acid, which was cleaned from RNA using 10 μl of RNase A for 2 h of incubation at 37°C. The isolated DNA was suspended in 50 μl of autoclaved deionized distilled water and tested for purity and quantity by agarose gel electrophoresis.

Molecular and Phylogenetic Identification

The fungal isolate was identified by sequencing the internal transcribed region (ITS) of 18S rDNA, using universal primers ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). The PCR mixture (25 μl) contained 2.5 μl of dNTPs and Ex-*Taq* buffer, 2 μl of each primer, 0.5 μl of DNA

Table 1. Screening of fungal isolates for their growth promotion of waito-c rice.

Fungal isolates	Plant height (cm/plant)	Shoot length (cm/shoot)	Increase (cm/shoot)	Growth status
Control	16.3±1.8	6.0±0.5	0.0	NA
G. fujikuroi	19.0±1.9	8.6±0.9	2.6	Promoted
P-2-4	18.3±2.4	8.9±0.3	2.9	Promoted
P-4-3	19.4±1.8	9.5±0.4	3.5	Promoted
P-1-1	16.2±1.1	8.2±0.3	2.2	Promoted
P-2-2	14.5±4.1	7.3±0.07	1.3	Promoted
P-4-2	18.8±0.2	7.3±0.3	1.3	Promoted
P-1-3	16.0±3.8	7.3±1.2	1.3	Promoted
P-1-2	17.7±0.9	7.5±0.2	1.5	Promoted
P-2-1	15.6±2.7	7.7±0.8	1.7	Promoted
P-4-1	16.3±0.9	6.7±0.2	0.7	Promoted

sample, and 0.2 μl of Ex-*Taq* polymerase. The rest volume was adjusted with 15.3 μl of autoclaved deionized distilled water. The reaction cycle consisted of 2 min of initial denaturation at 95°C, followed by 35 cycles of 30 sec denaturation time (95°C), 60 sec of annealing (55°C), and 30 sec of extension (72°C), and a final extension time of 5 min at 72°C. The resultant product was gene cleaned using a Nucleogen gene clean kit, ligated in T-vector using a Takara Perfect T-cloning kit, and then inserted into DHα *E. coli* mutant cells (RBC) by overnight incubation (37°C). Transformed cells were selected, grown overnight (37°C) in LB broth, and their plasmids were extracted using a SolGent Plasmid mini-prep kit, which were later sequenced. The BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST/) was used to look for nucleotide sequence homology for the 18S ITS (1/4) region for the fungi. The obtained sequences were aligned by Clustal W using MEGA version 4 software [25], and the neighbor-joining tree was generated using the same software. Bootstrap replications (1K) were used for a statistical support for the nodes in the phylogenetic tree.

RESULTS

Screening of Endophytic Fungi for Their Plant Growth-Promoting Capacity

The culture filtrates of nine endophytic fungi were screened for plant growth-promoting metabolites by applying them on waito-c rice. All fungal isolates promoted growth of waito-c rice seedlings (Table 1). The fungal isolate P-4-3 produced best growth promotion as compared with the control and was thus selected for further investigation.

Bioassay of P-4-3 on Soybean

We also bioassayed the P-4-3 culture filtrate on soybean cultivar Hwangkeumkong and found that the growth attributes of plant height, shoot length, plant fresh weight, and shoot fresh weight were promoted with such application. The

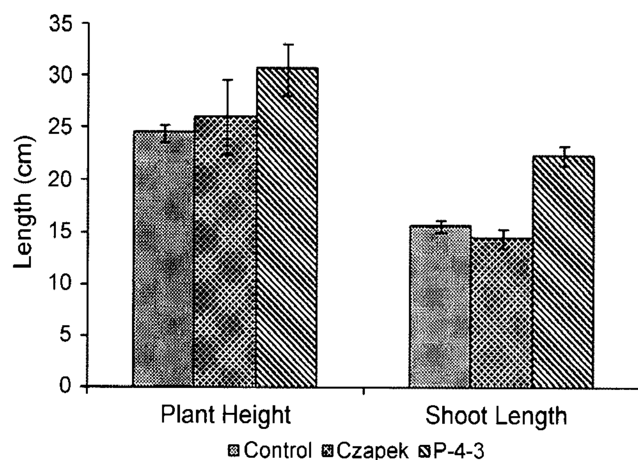

Fig. 1. Effect of P-4-3 culture filtrate on plant height and shoot length of soybean seedlings.

P-4-3 culture filtrate-treated seedlings showed increased lengths over those by wild-type *G. fujikuroi*, indicating possibility of higher GA production. Error bars indicate standard deviations.

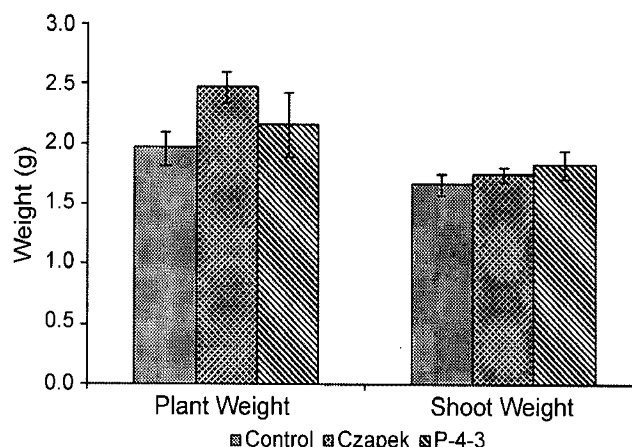

Fig. 2. Effect of P-4-3 culture filtrate on plant and shoot weights of soybean seedlings.

P-4-3 culture filtrate-treated seedlings showed increased plant weight over control. Error bars show standard deviations.

plant height (30.60 cm) and shoot length (22.35 cm) were much higher than the control and Czapek-treated plants (Fig. 1). The fresh weight parameters were also insignificantly enhanced by fungal culture filtrate as compared with the control (Fig. 2).

Analysis of Culture Filtrate of P-4-3 for Presence of Gibberellins

Gibberellins analysis showed the presence of GA_1 (0.3 ng/ml), GA_3 (17.84 ng/ml), GA_4 (18.58 ng/ml), GA_7 (8.95 ng/ml), GA_{15} (0.45 ng/ml), and GA_{24} (1.07 ng/ml) in the culture filtrate of P-4-3. Among them, GA_1, GA_3, GA_4, and GA_7 are physiologically active GAs. Fungal isolate P-4-3 produced many folds higher amounts of GA_3, GA_4, and GA_7 than wild-type *G. fujikuroi* during the current investigation (Fig. 3).

Morphological Identification of P-4-3

Macroscopic observation showed that the fungus is a slow-growing, slightly domed/ raised/ heaped, velvety, olivaceous

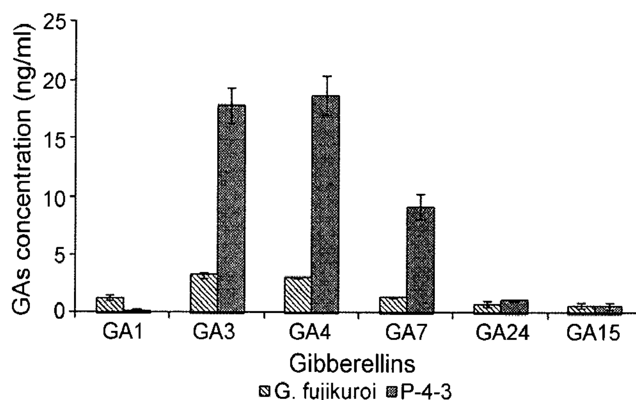

Fig. 3. Gibberellins content of fungal isolate P-4-3 and wild-type *G. fujikuroi*.

The GC-MS SIM analysis of culture filtrate extracts from P-4-3 showed the presence of all four bioactive GAs. Error bars indicate standard deviations.

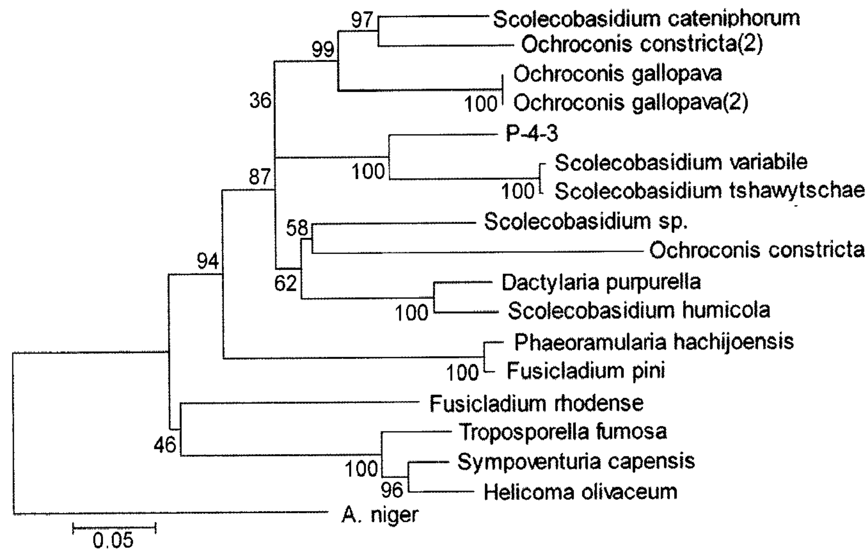

Fig. 4. Phylogenetic analysis of fungal isolate P-4-3.
For the identification of fungal isolate P-4-3, a distance tree was constructed using the NJ method and 1,000 bootstrap replications. Total 18 taxa were selected (17 reference and one clone) for this purpose through extensive BLAST search. The isolate P-4-3 formed a clade (100% bootstrap support) with subclade of *S. variable* and *S. tshawytschae*. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method. Phylogenetic analyses were conducted using MEGA4 software.

to dark brown single colony, attaining a size of about 17 mm radius after 10 days of incubation on PDA plates, at 30°C. The microscopic study showed that 4-celled, oval conidia produced sympodially, often attached to conidiophores by thread-like denticles; the fungus lacked a known sexual stage. The morphological characteristics of fungal isolate P-4-3 designate it as a strain of *Scolecobasidium tshawytschae.*

Molecular and Phylogenetic Identification of P-4-3

The phylogenetic analysis of fungal isolate P-4-3 was carried out by using the neighbor joining (NJ) method and the distance tree constructed from 18 (17 references and 1 clone) aligned ITS1 sequences with 1,000 bootstrap replications. These strains were selected through BLAST search, showing maximum sequence homology percentage and query coverage, and lowest E values. *A. niger* was used as the outgroup. In the dendrogram, fungal isolate P-4-3 formed a subclade with a strain of *Scolecobasidium tshawytschae* (100% bootstrap support) (Fig. 4). On the basis of sequence homology and phylogenetic analysis, isolate P-4-3 was thus identified as a new strain of *Scolecobasidium tshawytschae*. The ITS1 sequence was submitted to NCBI GenBank and was given the accession no. EU823314. The fungal isolate was named *Scolecobasidium tshawytschae* IJL03 (Lab name).

DISCUSSION

Endophytic fungi play a vital role in the growth of their host plants. In the current study, we determined the presence of plant growth-promoting metabolites in culture filtrates of nine endophytic fungal isolates through a screening experiment on waito-c rice. Use of rice plants for the screening experiment is better, as they can easily grow under controlled and sterilized conditions, hydroponically, using autoclaved water-agar media. Since this medium is devoid of any nutrient, the sole effect of culture filtrate can easily be estimated. Waito-c rice is a known dwarf rice cultivar with reduced GA biosynthesis. Treatment of its seeds with uniconazol, a GA biosynthesis retardant, further suppresses GA production by blocking the GA biosynthesis pathway. Shoot elongation of these seedlings can thus effectively be related to an activity of plant growth-promoting secondary metabolites from fungal culture filtrates applied [3, 21]. Microbial extracts had been and will continue to be a productive source of biologically active compounds. Screening for microbial secondary metabolites is an established method to identify novel biologically active molecules [6, 11]. The plant growth-promoting activity of fungal isolate P-4-3 was reconfirmed by conducting a bioassay experiment on Hwangkeumkong, a high-yielding soybean cultivar of Korea. Application of the fungal isolate greatly promoted the plant height of soybean, thus indicating a positive relationship with the host plant. Perlite was used as the growth medium owing to its sterility, being free from pests, pathogens, and weeds seeds. Perlite has a high porosity and its intercellular structure renders it an appropriate growing medium with improved aeration and drainage, reducing over-watering and under-watering losses. Current results are in line with previous reports on shoot length promotion through fungal culture filtrate treatment [22, 23].

GA analysis of the culture filtrates of P-4-3 and *G. fujikuroi* showed that the bioactive GA production

capacity of P-4-3 was many folds higher than wild-type *G. Fujikuroi*, which narrates the significance of this newly isolated fungal strain. Gibberellins quantification by GC-MS SIM is well established and the most reliable technique in ongoing investigations worldwide [4, 5, 22, 23]. The major advantage of GC-MS is its unbiased character. In comparison with non-MS detection-based chromatographic techniques (HPLC-DAD, GC-FID), where only compounds targeted by a special analytical protocol are found, GC-MS analysis can result in interesting and unexpected new knowledge about a particular extract [7]. Quantitative analysis is done by acquiring compound-specific molecular ions in selected ion monitoring (SIM) to increase the signal-to-noise ratio (SNR) of the MS experiment [9].

Study of morphological characteristics of a fungus provides valuable information for identification, although recently, it is mostly replaced with molecular and phylogenetic approaches. DNA sequence analysis methods are objective, reproducible, and rapid means of identification, and thus gaining importance. Many rDNA genes are highly conserved for members of the same taxonomic group, and therefore are used extensively for identification. These are named ITS (internal transcribed spacer), IGS (intergenic spacer), and D1/D2 (domains 1 and 2). Of these, ITS (I and II) had been employed more, thus using ITS genes for fungal identification has become a common practice. Use of IGS and D1/D2, along with actin-encoding genes, is also gaining importance nowadays and is providing additional data for inter- and intra-specific level identification [13, 14, 24]. We used the 5.8S gene and flanking ITS1/4 regions for fungal identification. It is because the highly conserved 5.8S gene is suitable for higher taxanomic level analysis, whereas the highly variable ITS regions are useful for analysis at lower taxanomic levels. Constructing a phylogenetic tree is crucial in molecular identification, since BLAST search alone cannot overcome possibilities of statistical errors. Bootstrap consensus is applied to the constructed tree so as to read maximum sequence replications. The neighbor-joining tree with bootstrapping gave us a clear picture for identifying fungal isolate P-4-3. Maximum sequence identity was obtained with *Scolecobasidium tshawytschae* (99%), where *Aspergillus niger* was taken as the outgroup. Although many phylogenetic trees exist and are used for analysis, the neighbor-joining method has been designated a most reliable tree construction method-especially when dealing with closely related strains under varying rates of evolution [8, 30].

Acknowledgment

This study was supported by the Technology Development Program for Agriculture and Forestry, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

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