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Phoma herbarum as a New Gibberellin-Producing and Plant Growth-Promoting Fungus

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Endophytic fungi are known for the production of valuable metabolites, but information on the gibberellin production capacity of this group is limited. We isolated 9 endophytic fungi from the roots of salt-stressed soybean plants and screened them on waito-c rice, in order to identify plant growth promoting fungal strains. The fungal isolate TK-2-4 gave maximum plant length (20.35 cm) promotion in comparison with wild-type *Gibberella fujikuroi* (19.5 cm). In a separate experiment, bioassay of TK-2-4 promoted plant length and biomass of soybean cultivar Taegwangkong. The TK-2-4 culture filtrate was analyzed for the presence of gibberellins, and it was found that all physiologically active gibberellins, especially GA_4 and GA_7, were present in higher amounts (GA_1, 0.11 ng/ml; GA_3, 2.91 ng/ml; GA_4, 3.21 ng/ml; and GA_7, 1.4 ng/ml) in conjunction with physiologically inactive GA_9 (0.05 ng/ml), GA_{12} (0.23 ng/ml), GA_{15} (0.42 ng/ml), GA_{19} (0.53 ng/ml), and GA_{20} (0.06 ng/ml). The fungal isolate TK-2-4 was later identified as a new strain of *Phoma herbarum*, through the phylogenetic analysis of 28S rDNA sequence.

Keywords: *Phoma herbarum*, gibberellin production, endophytic fungi, soybean, growth promotion

Endophytic fungi have been shown to confer benefits to host plants, including tolerance to herbivory, heat, salt, disease, and drought, as well as an increased biomass, both above and below the ground [17, 33]. Endophytic colonization may also improve the ecological adaptability of the host by enhancing its tolerance to biotic and abiotic stresses [22]. *Phoma herbarum* Westend. (Fungi imperfecti) is a ubiquitous saprophyte and toxigenic pathogen to plants and animals [31], including humans under some occasions [26]. *P. herbarum* possesses strong adaptability to diverse environments including salty and chilly surroundings [35, 31]. *P. herbarum* is also a versatile producer of many natural products with potent activities [2, 5, 6, 14, 15, 23, 30, 35, 36].

Gibberellins (GAs) are diterpenoid plant hormones, first detected in the 1930s from culture filtrates of *Gibberella fujikuroi*, a known pathogen of rice plants [20]. GAs appear to be involved in every aspect of plant growth and development, but their most typical (and spectacular) property involves the enhancement of stem growth [19]. GAs may modify the sex expression of flowers, induce the parthenocarpic development of fruit, and delay senescence. They obviate the need for exposure to red light in the germination of seeds and spores, and the need for vernalization in the growth of bulbs and tubers. They are associated with the breaking of winter dormancy and stimulate the formation of hydrolytic enzymes in germinating cereal grain [18]. Currently, 136 GAs have been identified, and 12 fungi, pathogenic and nonpathogenic, associated with plants and/or soil have been reported as GA producers [10, 16, 28]. A new strain of *Penicillium citrinum* had also been reported as a GA producer [11]. However, there are no reports regarding the GA production capacity of *Phoma herbarum*.

There is increasing concern about the excessive use of fertilizer and pesticides in agricultural fields and their subsequent negative impact on crops and the environment. The aim of the present study was to select potential fungal inoculums for plant growth promotion in order to avoid the excess use of fertilizer and pesticides in agricultural fields.

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MATERIALS AND METHODS

Sample Collection and Isolation of Endophytic Fungi

We collected roots of famous soybean cultivar Taegwangkong, which had already been subjected to two weeks of 100 mM NaCl-induced salt stress under green-house conditions. The root samples were washed with a Tween 80 solution (detergent), and surface sterilized with 1% perchloric acid solution. The roots were then cut into 0.5 cm pieces, cultured on Hagem media plates, and incubated at 25°C until fungal cells emerged [29]. Pure fungi cultures were isolated and stored on potato dextrose agar (PDA) slants.

Fungal Strains, Culture Medium, and Growth Conditions

The screening and isolation of the plant root fungi was carried out on Hagem minimal medium plates, supplemented with 80 μg/ml streptomycin [34]. PDA plates and slants were used for storage, whereas the Czapek broth medium, containing 1% glucose and peptone was used for GA production [7] by incubating the fungal isolate at 30°C and at 120 rpm for 7 days. The wild-type strain of *Gibberella fujikuroi* was provided by the Korean Culture Center of Microorganisms (KCCM), which was used as a control during the experiment.

Bioassay on Waito-C Rice and Soybean

The culture filtrate of fungal isolates was bioassayed on waito-c rice sprouts in order to identify the presence of plant growth promoting metabolites. The seeds of waito-c rice were surface sterilized and treated with 20 μg/ml uniconazol for 24 h, in order to check the GA biosynthesis. The treated seeds were washed thoroughly and soaked in autoclaved distilled H_2O for germination. The young seedlings were transplanted in glass tubes containing a 0.8% water–agar medium and kept in a growth chamber. Forty ml of culture fluid was centrifuged at 5,000 ×*g* at 4°C for 15 min and the resulting pellet and supernatant were immediately stored at −70°C and later lyophilized (ISE Bondiro Freeze dryer). The lyophilized supernatant was mixed with 1 ml of autoclaved distilled water and 10 μl of supernatant solution was applied on the apical meristem of rice seedlings at the 2-leaf stage [11]. The shoot and plant lengths were observed 7 days after the application and compared with waito-c rice seedlings, that had been treated either with distilled water or *G. fujikuroi* culture filtrate.

With regards the soybean bioassay, the Hwangkeumkong seeds were surface sterilized with 5% NaClO for 15 min and then washed with distilled water. Seeds were sown in an autoclaved perlite, and 20 ml of the Hoagland solution [9] was applied at germination time. Fungal isolate TK-2-4 was selected since it exhibited the best results in the rice screening experiment, and 5 ml of fungal supernatant of TK-2-4 was applied to soybean seedlings at the 2-leaf stage. The plant length, shoot length, plant fresh weight, and shoot fresh weight were measured after one week of the culture filtrate application.

Extraction and Quantification of Gibberellins

Gibberellins were extracted from the culture filtrate of TK-2-4 by following an established protocol [13]. GAs were chromatographed on a 3.9×300 m Bondapak, C18 column (Waters Corp., Milford, MA, U.S.A.) 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. Forty-eight fractions of 1.5 ml each were collected. The fractions were then prepared for gas chromatography/mass spectrometry (GC/MS) with selected ion monitoring (SIM) (6890N network GC system, and 5973 network mass selective detector; Agilent Technologies, Palo Alto, CA, U.S.A.). For each GA, 1 μl of sample was injected in a 30 m×0.25 mm i.d., 0.25 μm film thickness DB-1 capillary column (J & W Scientific Co., Folsom, CA, U.S.A.). The GC 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 ms. Full-scan mode (the first trial) and three major ions of the supplemented [$^2H_2$] GAs internal standards (obtained from Prof. Lewis N. Mander, Australian National University, Canberra, Australia) and the fungal gibberellins were monitored simultaneously. The retention time was determined using hydrocarbon standards to calculate the KRI (Kovats Retention Index) value, whereas the GAs quantification was based on the peak area ratios of nondeuterated (extracted) GAs to deuterated GAs.

Genomic DNA Extraction and Fungal Identification

Genomic DNA isolation and PCR were performed according to an established protocol [11]. Universal primers for the internal transcribed spacer (ITS) region (ITS1/4) as well as D1/D2 region primers of 28S rDNA, LR0R(F) (ACCCGCTGAACTTAAGC) (http://www.botany.duke.edu/fungi/mycolab/primers.htm), and TW13(R) (GGTC CGTGTTTCAAGACG) [27], were used to identify the fungal isolate. The BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST/) was used to look for nucleotide sequence homology. The sequences obtained were then aligned by ClustalW using MEGA version 4 software [25], and the neighbor-joining tree was generated using the same software. The bootstrap replications (1K) were used as a statistical support for the nodes in the phylogenetic tree.

Statistical Analysis

The data were analyzed statistically for standard deviation by using MS-Excel. The mean values were compared, using the Duncan's multiple range test (DMRT) at $P<0.05$ (ANOVA SAS release 9.1; SAS, Cary, NC, U.S.A.).

RESULTS

Screening of Endophytic Fungi for Plant Growth Promotion

The culture filtrates of nine endophytic fungi were screened for plant growth promoting metabolites by applying them on waito-c rice. Eight fungal isolates promoted growth of waito-c rice, whereas one isolate inhibited it. The fungal isolate TK-2-4 significantly promoted whole plant length (20.35 cm) and shoot length (9.51 cm) as compared with control (19.05/8.65 cm) and *G. fujikuroi* (16.3/6.0 cm), respectively (Fig. 1).

Bioassay of Fungal Isolate TK-2-4 on Soybean

The culture filtrate of isolate TK-2-4 was bioassayed on its host soybean cultivar Taegwangkong. It was found that

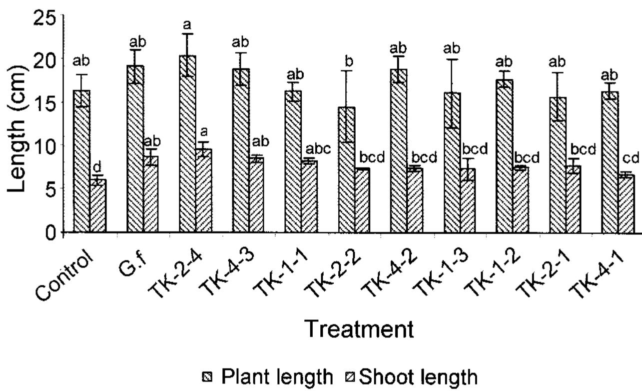

Fig. 1. Effect of fungal culture filtrates (10 µl) on length of waito-c rice seedlings after 7 days of incubation.
Data bars having a common letter(s) are not significantly different at the 5% level by DMRT. Error bars show standard deviations.

plant length, shoot length, plant fresh weight, and shoot fresh weight were promoted with such application as compared with control. The plant length (31.3 cm) and shoot length (19.3 cm) were higher than the control and Czapek-treated plants (Fig. 2). The plant fresh weight (2.56 gm) and shoot fresh weight (1.83 gm) were also enhanced by the fungal culture filtrate as compared with control and Czapek-treated plants (Fig. 3).

Analysis of TK-2-4 Culture Filtrate for Gibberellins

Gibberellins analysis showed the presence of GA_1 (0.11 ng/ml), GA_3 (2.91 ng/ml), GA_4 (3.21 ng/ml), GA_7 (1.4 ng/ml), GA_9 (0.05 ng/ml), GA_{12} (0.23 ng/ml), GA_{15} (0.42 ng/ml), GA_{19} (0.53 ng/ml), and GA_{20} (0.06 ng/ml) in the culture filtrate of TK-2-4. Among them, GA_1, GA_3, GA_4, and GA_7 are physiologically active GAs. Fungal isolate TK-2-4 produced significantly higher amounts of GA_4 and GA_7 than wild-type *G. fujikuroi* during the current investigation (Fig. 4).

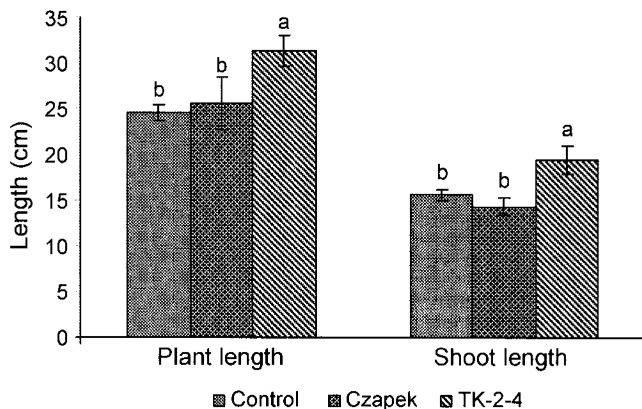

Fig. 2. Effect of culture filtrate of TK-2-4 on plant and shoot lengths of soybean seedlings.
Data bars having a common letter(s) are not significantly different at the 5% level by DMRT. Error bars show standard deviations.

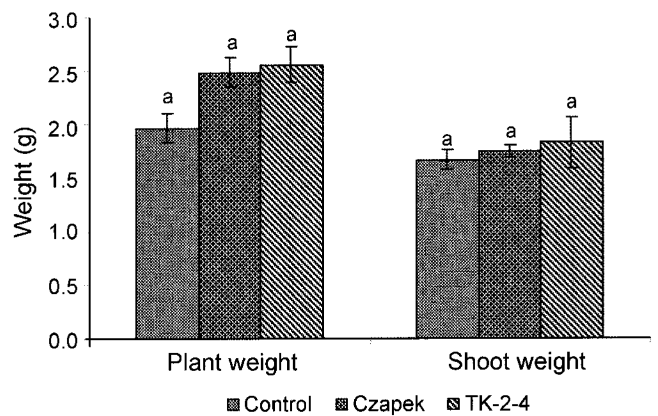

Fig. 3. Effect of culture filtrate of TK-2-4 on plant and shoot weights of soybean seedlings.
Data bars having a common letter(s) are not significantly different at the 5% level by DMRT. Error bars show standard deviations.

Molecular and Phylogenetic Identification of TK-2-4

The phylogenetic analysis of fungal isolate TK-2-4 was carried out by the neighbor-joining (NJ) method. A consensus tree was constructed from 21 (20 references and 1 clone) aligned ITS as well as partial 28S rDNA 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 out group. ITS region sequencing, phylogenetic tree construction, and bootstrapping failed to yield good identification results. For the D1/D2 region sequence of 28S rDNA, BLAST search showed that fungal isolate TK-2-4 has 99% sequence homology with *Phoma herbarum*. In the dendrogram, fungal isolate TK-2-4 formed a clade (100% bootstrap support) with *Phoma herbarum* strains (Fig. 5). On the basis of sequence homology and phylogenetic analysis, isolate TK-2-4 was thus identified as a new strain of *Phoma herbarum*. The

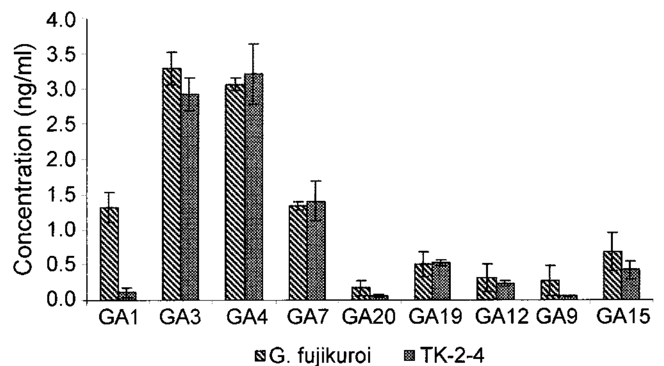

Fig. 4. Levels of various gibberellin produced by fungal isolate TK-2-4 and *G. fujikuroi*.
Of the 4 bioactive gibberellins, GA_4 and GA_7 were produced in higher amounts by TK-2-4 in comparison with *G. fujikuroi*. Error bars show standard deviations.

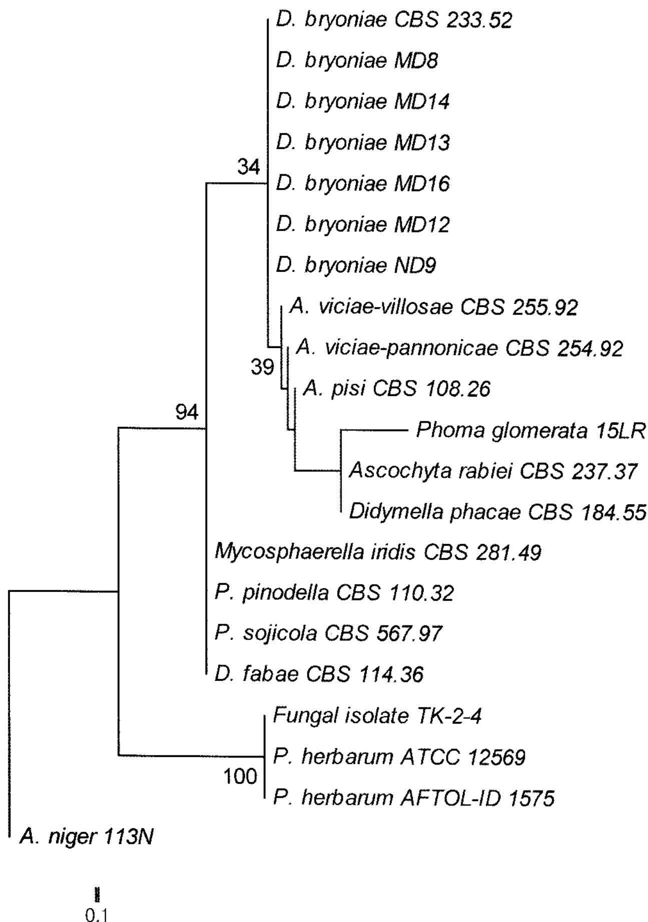

Fig. 5. Phylogenetic tree constructed by the neighbor-joining method using the 28S rDNA sequence (D1/D2 domains) of *Phoma herbarum* and related fungi.

TK-2-4 formed a clade (100% bootstrap support) with two strains of *Phoma herbarum*, which identify TK-2-4 as a new strain of *Phoma herbarum*. *A. niger* was taken as an out group.

28S rDNA sequence was submitted to NCBI GenBank and was designated Accession No. FJ808679.

DISCUSSION

Fungal endophytes are well-known plant symbionts, although information on the gibberellins production and plant growth promotion capacity of this group is limited. Therefore, we investigated the fungal endophytes of soybean plant for their exogenous GA production capacity, as gibberellins are vital for plant growth and development. The culture filtrates of isolated fungi were thus screened for GA production, as screening of microbial culture filtrates for the presence of secondary metabolites is an established method for the identification of biologically active molecules [8]. The microbial extracts had been and will continue to be an efficient source of novel secondary metabolites [1]. The culture filtrate of fungal isolate TK-2-4 significantly enhanced growth of waito-c rice and was thus analyzed for the presence of gibberellins.

The usage of water–agar growth media for rice helped in the determination of the sole effect of the culture filtrate on rice seedling growth, as the water–agar media was free of nutrients. We used waito-c rice for the screening experiment, as waito-c is a dwarf and GA-deficient rice cultivar, owing to the blocked C13 hydroxylation pathway of GA biosynthesis. Our current screening results are in agreement with a previous report [11]. The culture filtrate of TK-2-4 was also bioassayed on soybean, as we wanted to investigate the effect of TK-2-4 on its host plant. It was observed that the growth attributes of soybean were promoted by such an application. Almost similar experimental arrangement and results were demonstrated in a previous study carried out on an endophytic fungus [11]. The soybean plants were grown in perlite growth medium, under controlled growth chamber conditions, in order to provide controlled growth conditions to the plants. Perlite is frequently used as a substrate for soilless culture since it is a natural material (eco-friendly), inorganic, and therefore is physically stable with a neutral pH. It is also sterile and free of pest, pathogens, and weed seeds.

The plant growth promoting fungi (PGPF) are associated with plant roots and they secrete a number of secondary metabolites including gibberellins in the rhizosphere. Gibberellin secretion by PGPF was reported by several researchers [10, 11, 16, 28], which showed the importance of PGPF in plant growth and development, especially under nutrient deficient conditions. In the present study, we report the ability of *Phoma herbarum* to produce 9 different gibberellins that also include bioactive GA_1, GA_3, GA_4, and GA_7. The bioactive GA_4 and GA_7 amounts were higher in TK-2-4 culture filtrate than those of wild-type *G. fujikuroi*, which demonstrated the favorable role of *Phoma herbarum* in promoting growth of host plants. In an earlier study, two new strains of *Phoma glomerata* were isolated from the roots of *Campanula punctata* and *Viola partinii*, respectively. Both of these strains produce bioactive gibberellins, which confirms the potential and importance of *Phoma* species in growth promotion of their respective host plants [21]. The GC/MS SIM technique was used for the analysis of GAs in the culture filtrate of TK-2-4. 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 provides an interesting and unexpected new knowledge regarding a particular extract [4].

Although sequencing of internal transcribed spacer (ITS) regions has recently allowed researchers to gain an immense amount of knowledge in regard to fungal identification [12, 24], the results usually fail to produce good phylogenetic comparisons at the species level and

thus make fungal identification at the species level difficult. However, the 5' end of the large subunit (LSU) contains variable regions D1 and D2 of about 600 bps, which are important in identification of closely related species [3, 32]. In the current study, the ITS region failed to properly identify our fungal isolate, whereas good sequence homology and phylogenetic tree were obtained for the D1/D2 region of the 28S rDNA gene. On the basis of sequence homology and phylogenetic analysis results, isolate TK-2-4 was identified as a new strain of *Phoma herbarum*. The phylogenetic tree construction, in conjunction with BLAST searching, has gained immense importance among researchers. This method of relating the isolate in question with those exhibiting a maximum DNA sequence homology helps to overcome the possibility of errors by conducting statistical analyses, such as bootstrap tests.

Our current study reports valuable information on the gibberellins-producing capacity of an endophytic strain of *Phoma herbarum*. It also highlights the importance of fungi in growth promotion of their host plants, which may also help in avoiding excessive use of fertilizers and pesticides in agricultural fields. Further study is suggested on the identification and characterization of the GA-encoding gene cluster and the development of optimized GA-producing media for *Phoma herbarum*.

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