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Isolation of a Gibberellin-producing fungus (*Penicillium* sp. MH7) and Growth Promotion of Crown Daisy (*Chrysanthemum coronarium*)

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Plant growth promoting fungi (PGPF) are well known for the production of useful secondary metabolites. However, limited information is available on the gibberellin (GA) production capacity of PGPF of endophytic origin. In the current study, 15 fungal endophytes were isolated from the roots of Crown daisy, and then screened on Waito-c rice, in order to identify plant growth promoting fungi. The fungal isolate MH7 significantly increased the shoot length (12.1 cm) of Waito-c in comparison with control treatment (7.9 cm). In a separate experiment, the culture filtrate (CF) of MH7 significantly promoted the growth attributes of Crown daisy. The MH7 CF was analyzed for gibberellins and it contained all physiologically active gibberellins (GA_1, 1.37 ng/ml; GA_3, 5.88 ng/ml; GA_4, 8.62 ng/ml; and GA_7, 2.05 ng/ml) in conjunction with physiologically inactive GA_9 (0.83 ng/ml), GA_{12} (0.44 ng/ml), GA_{15} (0.74 ng/ml), GA_{19} (1.16 ng/ml), and GA_{20} (0.98 ng/ml). The CF of MH7 produced higher amounts of GA_3, GA_4, GA_7, GA_9, and GA_{12} than wild-type *Fusarium fujikuroi*, which was used as a control for GA production. The fungal isolate MH7 was later identified as a new strain of *Penicillium* on the basis of its morphological characteristics and phylogenetic analysis of the 18S rDNA sequence.

Keywords: *Penicillium*, gibberellins, endophytic fungi, Crown daisy, growth promotion

The plant growth promoting capacity of fungal endophytes is partly due to the endophytes' production of phytohormones such as indole-3-acetic acid (IAA), cytokines, and other plant growth- promoting substances [32] and/or partly owing to the fact that endophytes could have enhanced the hosts' uptake of nutritional elements such as nitrogen [25] and phosphorus [18]. The endophytic fungi have also been shown to confer benefits to host plants, including tolerance to herbivory, heat, salt, disease, and drought [19, 30]. *Penicillium* is one of the most common fungal genera, found in soil, decaying plant debris, compost piles, and fruit rot. *P. glabrum* has been isolated from diesel fuel. *Penicillium* produced various toxins, such as penicillic acid, peptide nephrotoxin, viomellein, xanthomegin, xanthocillin X, mycophenolic acid, roquefortine C and D, citrinin, penicillin, cyclopiazonic acid, isofumigaclavine A, penitrem A, decumbin, patulin citreoviridin, griseofulvin, verruculogen, ochratoxin, chrysogine, and meleagrin. Industrial uses of *Penicillium* include formation of Roquefort and Camembert cheeses, salami-sausages starter culture, antibacterial antimicrobial penicillin, and antifungal antimicrobial griseofulvin [1, 3, 5].

Gibberellins (GAs) are diterpenoid plant hormones, first detected in the 1920s from culture filtrates of *Gibberella fujikuroi*, a known pathogen of rice plants [23]. 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 [21]. 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 [20]. Currently, 136 GAs have been identified, and 12 fungi, pathogenic and nonpathogenic, associated with plants and/or soil has been reported as GA producers [10, 17, 29]. New strains of *C. sphaerospermum* and *P. citrinum* had also been reported as GA producers [6, 11].

Information on the gibberellins production capacity of fungal endophytes is still limited, although these fungi have already been reported as rich sources of valuable

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secondary metabolites [32]. They may be used as biofertilizers, as there is an increasing concern about the excessive use of fertilizer in agricultural fields and their subsequent negative impact on the environment. The aim of the current study was to select potential fungal inoculums for plant growth promotion in order to avoid the excess use of fertilizer in agricultural fields. For this purpose, endophytic fungi were isolated from the roots of Crown daisy and then screened on Waito-c for the presence of GAs. The GA production potential of the best fungal isolate was analyzed through GC/MS SIM, and the Crown daisy plant was used to evaluate the growth promoting capacity of the selected fungal isolate.

MATERIALS AND METHODS

Isolation of Fungal Endophytes from Crown daisy

Endophytic fungi were isolated from the roots of Crown daisy, which was grown in soil under greenhouse conditions. The root samples were washed with a Tween 80 solution (composed of 70% oleic acid), and surface sterilized with 1% perchloric acid solution. Two drops of Tween 80 were added to 500 ml of distilled water and used for washing seeds. The roots were then cut into 0.5-cm pieces, cultured on Hagem media plates, and incubated at 25°C until fungal cells emerged [11]. The Hagem minimal medium plates were supplemented with 80 µg/ml streptomycin [30, 31]. Pure fungi cultures were isolated, and grown on potato dextrose agar (PDA) medium plates and slants. The PDA slants were used for storage purpose. Czapek broth medium, containing 1% glucose and peptone, was used for GA production [8] by incubating the fungal isolate at 30°C and at 120 rpm for 7 days. The wild-type strain of *Fusarium fujikuroi*, which was used as positive control for GA production, was provided by the Korean Culture Center of Microorganisms (KCCM).

Bioassay on Waito-c and Crown daisy

The CFs of fungal isolates were bioassayed on Waito-c rice sprouts in order to identify their plant growth promoting capacity. Waito-c is a GA-deficient rice mutant, with blocked GA metabolism [6]. 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 CFs 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 (DW), and 10 µl of supernatant solution was applied on the apical meristem of rice seedlings at the two-leaf stage [11]. The shoot lengths were observed 7 days after the application and compared with Waito-c rice seedlings that had been treated either with distilled water (negative control) or Czapek medium (positive control).

With regard to the bioassay experiment, Crown daisy seeds were surface sterilized with 5% NaClO for 15 min and then washed with distilled water. Seeds were sown in an autoclaved soil, under greenhouse condition (30±2°C). Fungal isolate MH7 was selected for application, since it had caused maximum stem length promotion during the screening experiment. Ten ml of MH7 CFs was applied to 2-week-old Crown daisy seedlings, and the growth attributes (*i.e.*, plant length, shoot length, plant fresh weight, plant dry weight) were recorded after 3 weeks of CF treatment (Table 1). The growth promotion caused by the fungal strain was compared with control treatments.

Extraction and Quantification of Gibberellins

Gibberellins were extracted from the CFs of MH7 and *F. fujikuroi* by following an established protocol [16]. GAs were chromatographed on a 3.9×300 m Bondapak, C_{18} 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, and then to rise at 15°C/min to 200°C followed by 5°C/min 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, and 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]. Fungal isolate was identified by sequencing

Table 1. Effect of *Penicillium* sp. MH7 on growth attributes of Crown daisy.

Treatments	Plant length (cm/plant)	Shoot length (cm/plant)	Plant fresh weight (g)	Plant dry weight (g)
Control (distilled H_2O)	17.77^b	7.45^b	0.62^b	0.16^b
Czapek medium (10 ml)	17.9^b	7.79^b	0.68^b	0.18^b
Penicillium sp. MH7 (10 ml)	25.79^a	11.91^a	1.97^a	0.25^a

In a column, treatment means having a common letter(s) are not significantly different at the 5% level by DMRT. The growth promotion was measured after 3 weeks of fungal CF application.

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 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 the MEGA version 4 software [28] 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.

Morphological Identification of Fungal Isolate MH7

Morphological characteristics, such as color, border, texture, presence of pigmentation, and colony size, were recorded for MH7 grown on Czapek yeast agar (CYA) [24]. Analysis of somatic structures using light microscopy (bright-field microscopy) was also performed by preparation of semi-permanent slides.

Statistical Analysis

The data were statistically analyzed for standard deviation, using MS-EXCEL software. 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

CFs of 15 endophytic fungi were screened for plant growth promotion by applying on Waito-c rice. Twelve fungal isolates promoted growth of Waito-c rice, and 3 isolates (MH2, MH8, and MH14) inhibited it. The fungal isolate MH7 significantly promoted the shoot length (12.1 cm) of Waito-c as compared with Czapek (8.4 cm) and distilled water (7.9 cm) treated plants (Fig. 1).

Bioassay on Crown Daisy Seedlings

The CF of isolate MH7 was bioassayed on Crown daisy plant. It was observed that all growth attributes studied were significantly enhanced with MH7 application, as compared with control treatments. The plant length (25.79 cm), shoot length (11.91 cm), plant fresh weight (1.97 g), and plant dry weight (0.25 g) were significantly higher than the control treatments (Table 1).

Analysis of MH7 CFs for Gibberellins

Gibberellins analysis showed the presence of GA_1 (1.37 ng/ml), GA_3 (5.88 ng/ml), GA_4 (8.62 ng/ml), and GA_7 (2.05 ng/ml) in conjunction with physiologically inactive GA_9 (0.83 ng/ml), GA_{12} (0.44 ng/ml), GA_{15} (0.74 ng/ml), GA_{19} (1.16 ng/ml), and GA_{20} (0.98 ng/ml). Fungal isolate MH7 produced higher amounts of GA_3, GA_4, GA_7, GA_9, and GA_{12} than wild-type *F. fujikuroi* during the current investigation (Fig. 2).

Identification of Fungal Isolate MH7

The phylogenetic analysis of fungal isolate MH7 was carried out by the neighbor-joining (NJ) method. A consensus tree was constructed from 27 (26 references and 1 clone) aligned ITS 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. BLAST search showed that fungal isolate MH7 has 99% sequence homology and 98% query coverage with these *Penicillium* species, whereas with *P. citrinum*, MH7 showed 98% sequence homology. In the phylogenetic dendrogram, fungal isolate MH7 formed the same clade with different *Penicillium* species, but a subclade with *P. citrinum* (Fig. 3). On the basis of sequence homology and phylogenetic analysis, isolate MH7 was thus identified as a new strain of *Penicillium* species. The 18S rDNA sequence was submitted to NCBI GenBank and was given an accession number of FJ950741. *Trichoderma viride* was used as the out-group.

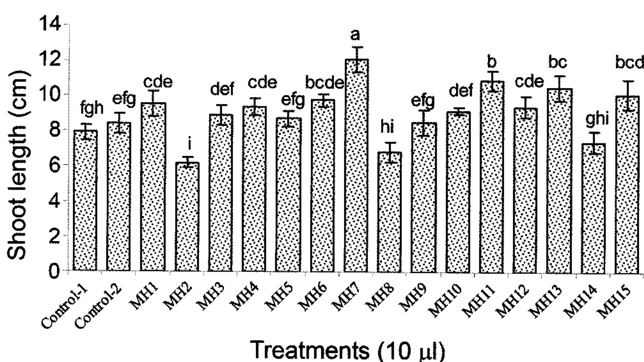

Fig. 1. Effect of fungal CFs (10 μl) on the shoot length of Waito-c rice seedlings after 7 days of incubation.

Data bars having a common letter(s) are not significantly different at the 5% level by DMRT. Error bars show standard deviations. Control-1 stands for distilled water; control-2 stands for Czapek medium.

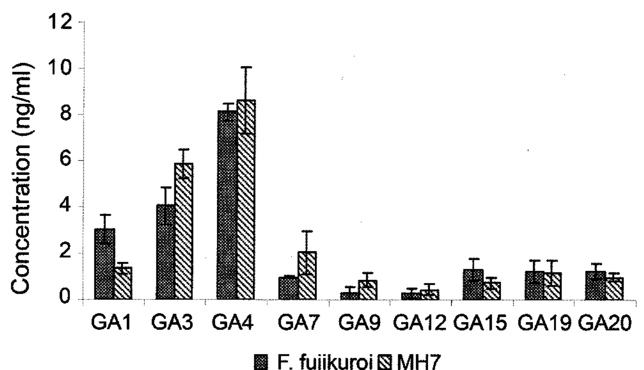

Fig. 2. Levels of various gibberellins secreted by fungal isolate MH7 and *F. fujikuroi*.

Fungal isolate MH7 and *F. fujikuroi* were added to Czapek broth medium (40 ml) and incubated at 30°C with 120 rpm for 7 days. GAs secreted in the medium were analyzed with GC/MS SIM. Error bars show standard deviations.

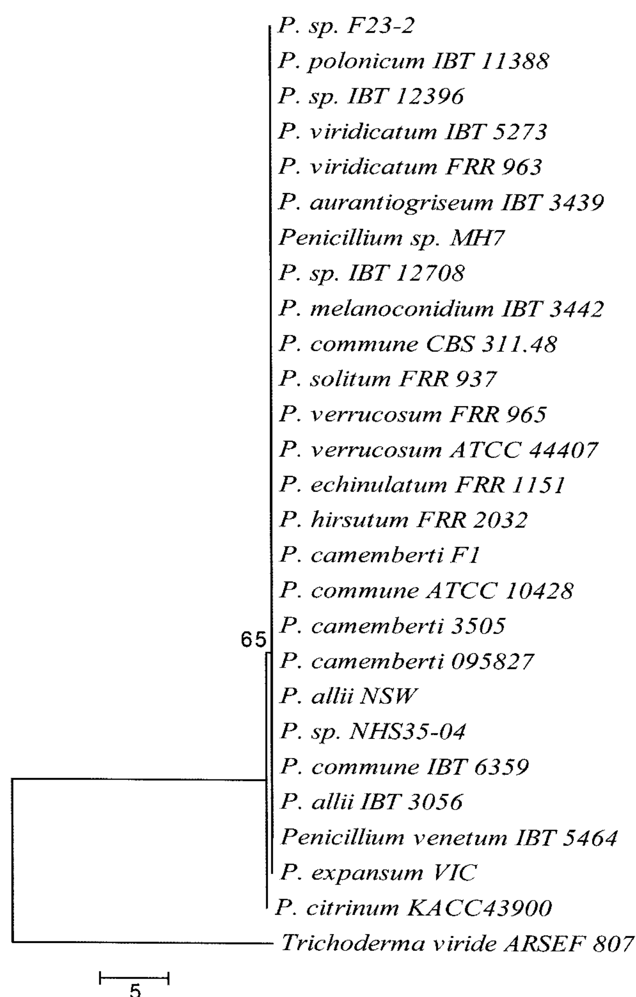

Fig. 3. Phylogenetic tree constructed by the neighbor-joining method using the 18S rDNA sequence (ITS region) of *Penicillium* sp. MH7 and related fungi.

Fungal isolate MH7 formed the same clade with different *Penicillium* species, which identifies fungal isolate MH7 as a new strain of *Penicillium*. *T. viride* was taken as an out-group.

Morphological Identification of MH7

The MH7 colony grown on CYA medium was velvet in texture, and attained a diameter of about 23–27 mm after 7 days of incubation at 28°C. The colony was green in front, pale yellow in reverse, and produced soluble yellow pigments. Penicilli were predominantly terverticillate, with mononematous conidiophores (Fig. 4). Conidia were ellipsoidal to sub-globose, and their walls smooth to slightly rough. The morphological characteristics of fungal isolate MH7 designated it as a new strain of *Penicillium*.

DISCUSSION

Endophytic fungi are well-known plant symbionts, although information on gibberellins production and the plant growth promotion capacity of this group is limited [7]. In the current study, we isolated fungal endophytes from the roots of Crown daisy plant and investigated their growth promoting capacity, as gibberellins are specialized for shoot elongation. The CF with maximum shoot elongation potential exhibited during the screening experiment was analyzed for GA production. The CFs of isolated fungi were screened for growth promotion capacity by applying on Waito-c, as screening of microbial CFs for the presence of secondary metabolites is an established method for the identification of biologically active molecules [7, 9, 13, 14]. The microbial extracts had been and will continue to be an efficient source of novel secondary metabolites [2].

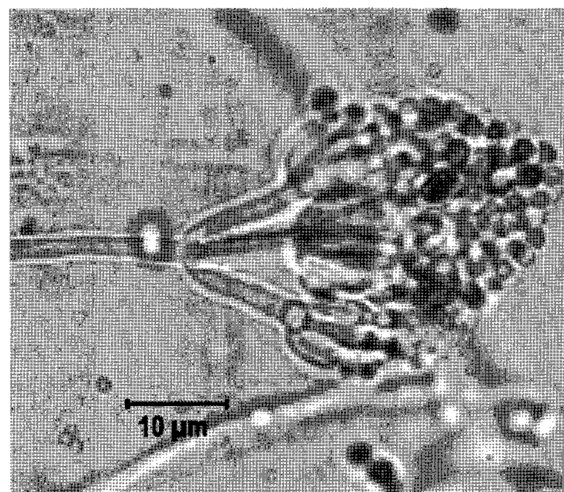

Fig. 4. Conidiophores of isolate MH7 viewed with bright-field microscopy.

The usage of water-agar growth medium for rice helped in the determination of the sole effect of CF on rice seedling growth, as water-agar medium was free of nutrients [12, 13]. We used Waito-c rice for the screening experiment, as Waito-c is a dwarf and GA-deficient rice cultivar, owing to the blocked C_{13} hydroxylation pathway of GA biosynthesis. Our current screening results are in agreement with a previous report [11], which stated that use of Waito-c is a good option for GA screening, as the endogenous level of this rice mutant is very low and an increase in plant length on a comparative basis can be attributed to the exogenously applied GAs. The CFs of MH7 were also bioassayed on Crown daisy, as we wanted to investigate the effect of MH7 on its host plant. It was observed that all growth attributes studied were significantly promoted by such an application. Our present investigation confirmed an earlier report on the growth promotion of soybean plant by an endophytic strain of *Cladosporium sphaerospermum* isolated from soybean [6].

The plant growth promoting fungi (PGPF) are associated with plant roots, and they secrete a number of secondary

metabolites including gibberellins in the rhizosphere [7]. Gibberellin secretion by PGPF was reported by several researchers [10, 29], which showed the importance of GA-producing fungi in plant growth and development, especially under nutrient-deficient conditions. In the present study, we reported the ability of a new strain of *Penicillium*, which produced 9 different gibberellins including bioactive GA_1, GA_3, GA_4, and GA_7. The bioactive GA_3, GA_4, and GA_7 amounts were higher in MH7 CF than those of wild-type *F. fujikuroi*, which again demonstrated the favorable role of *Penicillium* in promoting the growth of host plants. The fungus *Fusarium fujikuroi* was selected as a positive control for GA production as it corresponds to the mating group C of the *Gibberella fujikuroi* species complex [22], and is the only organism capable of excreting GAs in industrially viable quantities [27].

The GC/MS SIM technique was used for the analysis of GAs in the CF of TK-2-4. In previous studies too, GC/MS SIM was used for GA analysis of fungal CFs [6, 7, 11, 12, 13], as GC/MS SIM is an advanced tool for such analysis. 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 provide an interesting and unexpected new knowledge regarding a particular extract [4].

Study of the morphological characteristics of a fungus provides valuable information for identification, although recently, it has mostly been replaced with molecular and phylogenetic approaches, as DNA sequence analysis methods are objective, reproducible, and provide a rapid means of identification. 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 (1 and 2) had been employed more, and 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 intraspecific level identification [14, 15, 26]. 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 taxonomic level analysis, whereas the highly variable ITS regions are useful for analysis at lower taxonomic 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 provides us with a clear picture for identifying fungal isolate MH7. On the basis of morphological characteristics and phylogenetic analysis, isolate MH7 was identified as a new strain of *Penicillium*.

Our current study reports valuable information on the GAs production capacity of a new strain of *Penicillium*. The study also confirmed the importance of endophytic fungi in growth promotion of their host plants. However, further study is suggested on the identification and characterization of the GA-encoding gene cluster and the development of optimized GA-producing media for *Penicillium* sp. MH7.

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