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Gibberellin-Producing Endophytic Fungi Isolated from *Monochoria vaginalis*

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The role of endophytic fungi in plant growth and development is well documented. However, endophytic fungi with growth promotion capacity have never been isolated from weeds previously. In the current study, we isolated 8 fungal endophytes from the roots of *Monochoria vaginalis*, a serious weed of rice paddy in Korea. These isolates were screened on Waito-C, in order to identify plant growth promoting metabolites. Two fungal isolates (M5.A & M1.5) significantly promoted the plant height and shoot length of Waito-C during preliminary screening experiments. The culture filtrates (CFs) of M5.A and M1.5 also promoted the shoot length of *Echinocloa crusgalli*. Gibberellins (GAs) analysis of the CFs of M5.A and M1.5 showed that these endophytic fungi secrete higher quantities of GAs as compared with wild-type *G. fujikuroi* KCCM12329. The CF of M5.A contained bioactive GAs (GA_3, 2.8 ng/ml; GA_4, 2.6 ng/ml, and GA_7, 6.68 ng/ml) in conjunction with physiologically inactive GA_9 (1.61 ng/ml) and GA_{24} (0.18 ng/ml). The CF of M1.5 contained physiologically active GAs (GA_3, 1.64 ng/ml; GA_4, 1.37 ng/ml and GA_7, 6.29 ng/ml) in conjunction with physiologically inactive GA_9 (3.44 ng/ml), GA_{12} (0.3 ng/ml), and GA_{24} (0.59 ng/ml). M5.A and M1.5 were identified as new strains of *Penicillium* sp. and *Aspergillus* sp., respectively, based on their 18S rDNA sequence homology and phylogenetic analysis.

Keywords: *Penicillium* sp., *Aspergillus* sp., gibberellins, endophytic fungi, *Monochoria vaginalis*, growth promotion

The plant growth regulation potential of fungal endophytes is partly due to the endophytes' production of phytohormones such as indole-3-acetic acid (IAA), cytokinins, and other plant growth promoting substances [30], and/or partly owing to the fact that endophytes could have enhanced the hosts' uptake of nutritional elements such as nitrogen and phosphorus [14]. The endophytic fungi also confer benefits to host plants, including tolerance to herbivory, heat, salt, disease, and drought [15].

Penicillium citrinum and *Aspergillus fumigatus* have been reported as fungal endophytes that promoted plant growth by secreting gibberellins (GAs) in the rhizosphere of their hosts [11]. *Penicillium* sp. produce 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. Their industrial uses include formation of Roquefort and Camembert cheeses, salami-sausages starter culture; antibacterial penicillin; and antifungal griseofulvin [1], [6]. On the other hand, *Aspergillus* is an opportunistic pathogen as well as a major allergen [7]. Some species can cause infection in humans and other animals. Other species are important in commercial microbial fermentations and produce natural products that can be used in the development of medications to treat human ailments. *A. niger* is a major source of citric acid, as it accounts for over 99% of global citric acid production. The plant growth promoting potential of *Aspergillus* is well known, as it produces substantial amounts of phosphatases [25]. The favorable affects of *A. fumigatus* on the growth and mineral nutrition of mung bean and clusterbean are also well documented [24].

GAs are diterpenoid plant hormones, first detected in the 1920s from culture filtrate (CF) of *G. fujikuroi*, a known pathogen of rice plants [18]. GAs appear to be involved in every aspect of plant growth and development, but their most typical property involves the enhancement of stem growth [17]. GAs may modify the sex expression of flowers, induce the parthenocarpic development of the

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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 [16]. Currently, 136 GAs have been identified, and 12 fungi, pathogenic and nonpathogenic, associated with plants and/or soil, have been reported as GAs producers [13, 27]. Recently, new strains of *C. sphaerospermum* and *P. citrinum* have also been reported as being GAs producers [8, 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 secondary metabolites [30]. The fungal endophytes may be used as ecofriendly biofertilizers, as there is increasing concern about the excessive use of fertilizers in agricultural fields and their subsequent negative impact on the environment. The fungal endophytes may be a good option, as they cause no damage to host plants. In the current study, we isolated growth promoting fungal endophytes from *Monochoria vaginalis*. *M. vaginalis* is a serious weed of rice paddy worldwide. It is reported as the worst weed of Southeast Asia, after *Echinocloa colonum* [28]. This paper reports a unique study, as there are no previous reports on the isolation of plant growth promoting fungi from weed species. The aim of the current study was to investigate weed-inhabiting potential fungal endophytes that can be used as an environmentally friendly biofertilizer in the future.

Materials and Methods

Isolation of Fungal Endophytes

We isolated endophytic fungi from the roots of *M. vaginalis*, which were collected from cultivated rice fields. The root samples were then suspended in Tween 80 solution (2–3 drops in 50 ml of distilled water) and placed in a shaking incubator set at 120 rpm for 5 min at room temperature. The roots were then rinsed in distilled water to remove any residual Tween 80 (detergent). The cleaned samples were surface sterilized by suspending them in 50 ml of 1% perchloric acid, and than placed on a shaking incubator (120 rpm for 5 min) [11]. The roots were washed with autoclaved distilled water, dried between a layer of sterile filter paper, cut into pieces (0.5 cm each), cultured on Hagem medium plates, and incubated at 25°C until the emergence of fungal cells [11]. The Hagem medium plates were supplemented with 80 μg/ml streptomycin, in order to avoid bacterial growth in the medium. In order to obtain true endophytic fungi, the effectiveness of the surface sterilization procedure was tested by the imprinting technique [11], and sterilized root pieces were placed on Hagem medium plates. The absence of any microbial growth on the imprinted media plates after 4–7 days of incubation confirmed the effectiveness of surface sterilization [11]. Pure fungi cultures were isolated, and grown on potato dextrose agar (PDA) medium plates and slants; the slants were used for storage purpose. For GA production, the fungal isolates were incubated at 30°C and 120 rpm for 7 days in Czapek broth medium, containing 1% glucose and peptone [10]. The wild-type strain of *G. fujikuroi* KCCM12329, provided by the Korean Culture Center of Microorganisms, was used as the positive control for GA production.

Bioassay on Waito-C and *E. crusgalli*

The CFs obtained from fungal isolates were bioassayed on Waito-C and *E. crusgalli* seedlings, in order to identify fungal inoculums with highest plant growth promoting capacity. Waito-C is a GA-deficient rice mutant, with blocked GA metabolism [8]. Seeds of Waito-C were surface sterilized with 1% sodium hypochlorite (NaOCl) for 15 min and then 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. Two Waito-C sprouts were transplanted in each glass tube (50 ml), which already contained 20 ml of 0.8% water-agar medium. The plants were grown in a controlled environmental chamber with a 16-h 30°C day (light intensity of 1,000 μmol $m^{-2}s^{-1}$) and 8-h 20°C night regime. 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 plant heights and shoot lengths were observed 7 days after the application and compared with Waito-C seedlings, which had been treated either with distilled water (negative control) or Czapek medium (positive control).

In a separate experiment, seeds of *E. crusgalli* were surface sterilized with 1% NaOCl for 15 min, washed thoroughly, and then sown in an autoclaved soil, under greenhouse conditions (30±2°C). Fungal isolates M5.A and M1.5 were applied to the seedlings, since they had caused maximum plant height and shoot length promotion of Waito-C. Twenty μl of M5.A and M1.5 CFs were applied to 1-week-old *E. crusgalli* seedlings and the resultant shoot lengths were observed after 1 week of such treatment.

Extraction and Quantification of GAs

GAs were extracted and quantified by following an established protocol [12]. CFs of M5.A, M1.5, and *G. fujikuroi* were chromatographed on a 3.9×300 m Bondapak C_{18} column (Waters Corp., Milford, MA, USA) 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, USA). 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, USA). 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

Table 1. Identification of GAs present in the CF of fungal isolate CC-8, using GC/MS–SIM.

HPLC fraction	GAs	KRI[a]	Source	*m/z* (%, relative intensity of base peak)[b]
13~15	GA_3	2,692	Sample	504(100) 208(45) 347(10)
			Standard	506(100) 210(42) 349(8)
34~35	GA_4	2,506	Sample	284(100) 225(80) 289(70)
			Standard	286(100) 227(78) 291(66)
34~35	GA_7	2,525	Sample	222(100) 223(93) 356(66)
			Standard	224(100) 225(80) 356(54)
37~38	GA_9	2,305	Sample	298(100) 270(78) 227(48)
			Standard	300(100) 272(77) 229(50)
42~ 44	GA_{12}	2,335	Sample	300(100) 240(31) 328(21)
			Standard	302(100) 242(28) 330(20)
36~37	GA_{24}	2,444	Sample	314(100) 226(89) 286(77)
			Standard	316(100) 228(85) 288(76)

[a]KRI, Kovats retention indices.
[b]Identified as methyl ester trimethylsilyl ether derivatives by comparison with reference spectra and KRI data.

supplemented [$^2H_2$] GA internal standards (obtained from Prof. Lewis N. Mander, Australian National University, Canberra, Australia) and the fungal GAs were monitored simultaneously. The retention time was determined using hydrocarbon standards to calculate the KRI (Kovats retention index) value (Table 1), and the GA quantification was based on the peak area ratios of non-deuterated (extracted) GAs to deuterated GAs.

Genomic DNA Extraction and Fungal Identification

Fungal isolate was identified by sequencing the internal transcribed spacer (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 BLASTn 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, and the neighbor-joining tree was generated using the same software [23].

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, USA).

RESULTS

Bioassay of Endophytic Fungi on Waito-C and *E. crusgalli*

The CFs of 8 endophytic fungi were screened on Waito-C for their plant growth promotion capacity. Seven fungal isolates promoted rice growth, whereas M2.1 inhibited it. Two fungal isolates, M5.A and M1.5 significantly promoted plant height (17.7 and 16.8 cm) and shoot length (7.96 and 7.8 cm) respectively, as compared with Czapek (14.2 and 5.67 cm) and distilled water (13.6 and 4.87 cm) treatments (Fig. 1).

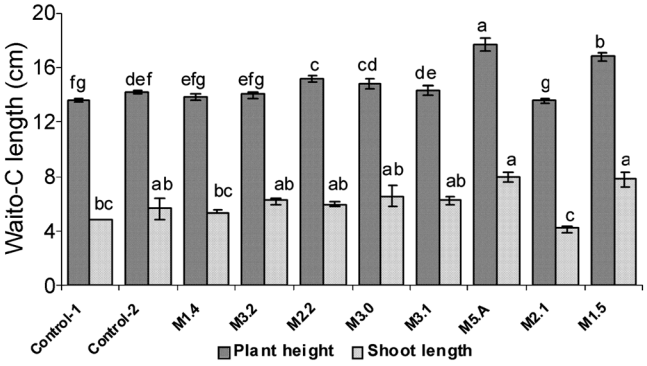

Fig. 1. Effects of fungal CFs (10 µl) on the plant height and shoot length of Waito-C seedlings (n=15) after 7 days of incubation. Data bars having a common letter(s) are not significantly different at the 5% level by DMRT. Error bars show standard deviations. Control-1 stands for distilled water; Control-2 stands for Czapek medium.

The CFs (20 µl each) of M5.A and M1.5 were applied on *E. crusgalli*, and it was observed that the shoot length of *E. crusgalli* was significantly improved by the CFs of these fungal isolates. Shoot lengths of 7.5 cm and 6.7 cm were observed for M5.A- and M1.5-treated plants, respectively, which were significantly higher than the control treatments (Fig. 2).

GAs Analysis

GA analysis of CF of M5.A showed the presence of bioactive GA_3 (2.8 ng/ml), GA_4 (2.6 ng/ml), and GA_7, (6.68 ng/ml) in conjunction with physiologically inactive GA_9 (1.61 ng/ml) and GA_{24} (0.18 ng/ml). The CF of M1.5 contained physiologically active GA_3 (1.64 ng/ml), GA_4 (1.37 ng/ml) and GA_7 (6.29 ng/ml) along with inactive GA_9 (3.44 ng/ml), GA_{12} (0.3 ng/ml) and GA_{24} (0.59 ng/ml). The CFs of fungal isolates M5.A and M1.5 contained

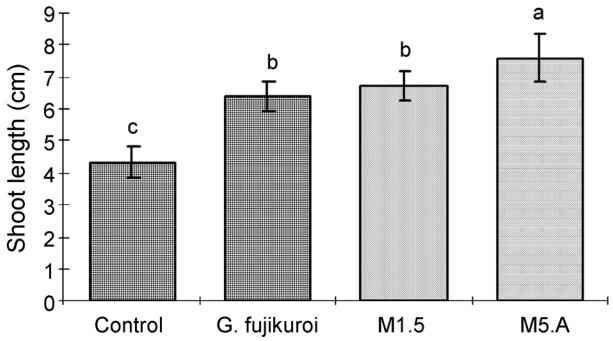

Fig. 2. Effects of CFs (20 µl) of M1.5 and M5.A on the shoot length of *E. crusgalli* sprouts (n=25) after 7 days of incubation. Data bars having a common letter(s) are not significantly different at the 5% level by DMRT. Error bars show standard deviations.

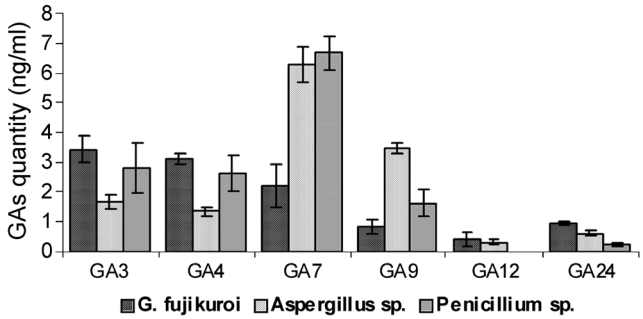

Fig. 3. Quantities of various GAs secreted by fungal isolates M5.A and M1.5 and *G. fujikuroi* were added to Czapek broth medium (40 ml) and incubated at 30°C, 120 rpm for 7 days.
GAs secreted in the medium were analyzed by GC/MS SIM. Error bars show standard deviations.

higher amounts of GA_7 and GA_9 than wild-type *G. fujikuroi* during the current investigations (Fig. 3).

Identification of Fungal Isolates M5.A and M1.5

The phylogenetic analysis of fungal isolates M5.A and M1.5 were carried out by the neighbor-joining method. A consensus tree was constructed for M5.A from 19 (18 references and 1 clone) and for M1.5 from 12 (11 references and 1 clone) aligned ITS sequences with 1K bootstrap replications. The strains were selected through BLAST search showing the maximum sequence homology percentage and query coverage, and lowest E values. Fungal isolates M5.A and M1.5 showed 99% sequence homology with *Penicillium* and *Aspergillus* species, respectively. During the phylogenetic analysis, fungal isolate M5.A formed a clade with *P. pinophilum*, whereas M1.5 formed a clade with *Aspergillus* species (Fig. 4). Therefore, on the basis of sequence homology and phylogenetic analyses, isolate M5.A was identified as a new strain of *Penicillium* species, whereas isolate M1.5 was identified as a new strain of *Aspergillus* species. The 18S rDNA sequences of both fungal isolates were submitted to NCBI GenBank. The NCBI GenBank allotted Accession No. GU270552 for the new strain of *Penicillium* sp. and Accession No. GU270551 for the new strain of *Aspergillus* sp.

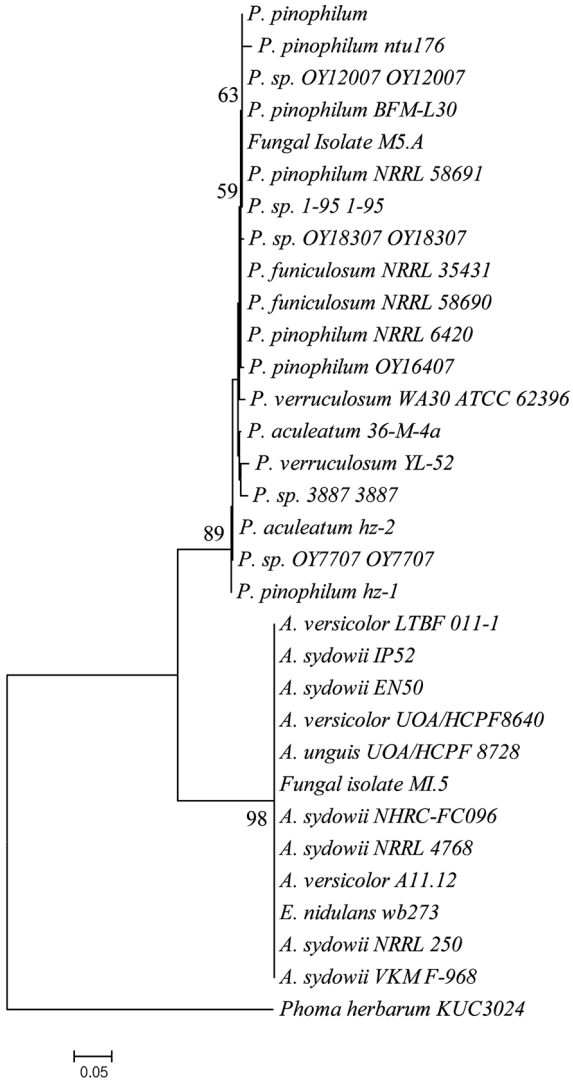

Fig. 4. Phylogenetic tree constructed by the neighbor-joining method using the 18S rDNA sequence (ITS region) of two fungal isolates (M5.A and M1.5) and related fungi.
Fungal isolate M5.A formed a clade with *P. pinophilum* (63% bootstrap), which identified fungal isolate M5.A as a new strain of *Penicillium*, whereas fungal isolate M1.5 formed a clade (98% bootstrap) with the rest of the *Aspergillus* species, which identified fungal isolate M1.5 as a new strain of *Aspergillus* sp. *Phoma herbarum* KUC3024 was taken as an outgroup.

DISCUSSION

Many weeds form worthy resources in diverse areas of human interest. They are used traditionally as food for both humans and animals, and continue to be used as valuable therapeutic plants. There is considerable interest in obtaining pharmaceuticals from many taxa occupying disturbed habitats. It was demonstrated that weed species form a substantially higher proportion of source plants in pharmacopoeias than would be expected from their proportion in the general flora [20]. Previously, weeds were never investigated for their reservoir of plant growth promoting endophytic fungi (PGPEF). PGPEF are known plant symbionts, although the GA production and plant growth promotion capacity of this group is not explored yet [9]. This article reports the isolation of PGPEF from the roots of *M. vaginalis*, and investigated their growth promoting capacity by screening fungal CFs on Waito-C rice and *E. crusgalli*. Waito-C is a GA-deficient rice mutant, which lacks GA 3β-hydroxylase (3-oxidase), and thus blocks conversion of GA_{20} to bioactive GA_1 [4]. *E. crusgalli* was used for bioassay experiment as

seeds of *M. vaginalis* failed to germinate. These experiments were carried out, as screening of microbial CFs for the presence of secondary metabolites is an established method for the identification of biologically active compounds [9]. It is also well established that the microbial extracts will continue to be an efficient source of novel secondary metabolites [3].

The plant growth promoting fungi (PGPF) are associated with plant roots and secrete a number of secondary metabolites including GAs in the rhizosphere [9]. GAs-producing fungi reported previously were mainly plant pathogens identified by the visible shoot elongation symptom of the disease caused by the secreted GAs. Recently, besides the orchid-associated *F. proliferatum* strain ET1 [26], more and more hidden endophytes, mainly isolates from roots of apparently healthy plants, are being discovered as GA producers. For instance, *Penicillium commune* and 10 so-far undetermined endophytic fungal species were isolated from roots of healthy *Sesamum indicum* [2]. Recently, *Penicillium citrinum* has been reported to promote growth of the dune plants by secretion of bioactive GAs in the rhizosphere [11]. GAs-producing *Phoma herbarum*, and *Chrysosporium pseudomerdarium* have been reported from soybean [10]. These endophytic fungal strains significantly promoted growth of their respective host plants. The growth promoting ability of these fungi might be an advantage for plants under environmental stress condition such as those grown under stressful conditions.

GA secretion by PGPF was previously reported by several researchers [8, 27], which shows the importance of GA-producing fungi in plant growth and development, especially under nutrient deficient conditions. In the present study, we report the GA production potential of two new strains of *Penicillium* sp. and *Aspergillus* sp., which demonstrates the favorable role of these two fungal strains in promoting growth of plants. Both fungal strains contained physiologically active GA_3, GA_4, and GA_7, although their concentrations were higher in M5.A than in M1.5, and subsequently growth of M5.A was more vigorous than M1.5. The concentrations of GA_9 and GA_{24} were higher in M1.5, but as these GAs are inactive, they play no direct role in plant growth. *G. fujikuroi* was selected as a positive control during GAs analysis of selected fungal strains, as *G. fujikuroi* is well known for its GAs secretion capacity and produces GAs in industrially viable quantities [23]. The GC/MS SIM was used for the analysis of GAs present in the CFs of M5.A and M1.5. 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 interesting and unexpected new knowledge regarding a particular extract [5].

The study of the morphological characteristics of a fungus provides valuable information for identification, although recently, it has been mostly replaced with molecular and phylogenetic approaches. 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 [22]. 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. Phylogenetic analysis is also crucial in molecular identification, since BLAST search alone cannot overcome the possibilities of statistical errors. The neighbor-joining tree with bootstrapping aided us in the identification of fungal isolates M5.A and M1.5. On the basis of sequence homology and phylogenetic analysis, isolates M5.A and M1.5 were thus identified as new strains of *Penicillium* sp. and *Aspergillus* sp. respectively. As the PGPEF were isolated from a weed species, the current study further strengthens the favorable role of weeds in human welfare and reasserts their importance in the ecosystem. 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 these PGPEF.

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