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ORIGINAL PAPER

Growth promotion of cucumber by pure cultures of gibberellin-producing *Phoma* sp. GAH7

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Abstract The beneficial effects of plant growth promoting fungi (PGPF) on plant growth and development are well documented. However, limited information is available on gibberellin (GA) production capacity of PGPF of endophytic origin. In current study, 11 fungal endophytes were isolated from cucumber roots and then screened on Waito-C rice, in order to identify plant growth promoting fungal strains. The fungal isolate GAH7 provided the maximum shoot length (11.3 cm) in comparison to control treatment (7.8 cm). In a separate experiment, bioassay of GAH7 significantly promoted growth attributes of cucumber. The GAH7 culture filtrate (CF) was found to contain physiologically active gibberellins in higher concentrations (GA_1, 0.81 ng/ml; GA_3, 4.34 ng/ml and GA_4, 9.31 ng/ml) in conjunction with physiologically inactive GA_9 (0.74 ng/ml), GA_{15} (0.97 ng/ml), GA_{19} (1.67 ng/ml) and GA_{20} (0.46 ng/ml). Isolate GAH7 produced higher amounts of GA_3, GA_4, GA_9 and GA_{19} than wild type *Fusarium fujikuroi*, which was used as control for GA production. Gibberellins were analyzed through gas chromatograph/mass spectrometer (GC/MS) with selected ion monitoring (SIM). The fungal isolate GAH7 was later identified as a new strain of *Phoma* on the basis of sequence homology (99%) and phylogenetic analysis of 18S rDNA sequence.

Keywords *Phoma* sp. · Gibberellins · Endophytic fungi · Cucumber · Growth promotion

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Introduction

The beneficial role of certain rhizospheric fungi in plant growth promotion has been reported by many researchers (Hamayun et al. 2009a; Khan et al. 2009b). Plants infected with an endophyte often grow faster than non-infected ones (Cheplick et al. 1989). This growth promoting effect is at least in part due to the endophyte's production of phytohormones, such as indole-3-acetic acid (IAA), cytokines and other plant growth-promoting substances (Zou and Tan 1999) and partly owing to the fact that endophytes could have enhanced the hosts' uptake of nutritional elements such as nitrogen (Reis et al. 2000) and phosphorus (Malinowski and Belesky 1999). The endophytic fungi have also been shown to confer benefits to host plants, including tolerance to herbivory, heat, salt, disease, and drought (Marquez et al. 2007; Waller et al. 2005). *Phoma* (fungi imperfecti) is a cosmopolitan, dematiaceous filamentous fungus that inhabits the soil and plant material. *Phoma* is a ubiquitous saprophyte and toxigenic pathogen to plants and animals (Vishniac 1996) including human beings under some occasions (Taskinen et al. 1997).

Gibberellins (GAs) are diterpenoid plant hormones, first detected in the 1920s from culture filtrates of *Gibberella*

fujikuroi, a known pathogen of rice plants (Ogas 2000). GAs appear to be involved in plant growth and development, but their most typical (and spectacular) property involves the enhancement of stem growth (Nishijima et al. 1995). 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 vernalisation 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 (Martin 1983). Currently 136 GAs have been identified, while 12 fungi, pathogenic and non-pathogenic, associated with plants have been reported as GA producers (MacMillan 2002; Kawaide 2006; Vandenbussche et al. 2007). A new strain of *Penicillium citrinum* had also been reported as a GA producer (Khan et al. 2008).

Literature on gibberellins production by endophytic fungi is still limited, although fungal endophytes are already reported as rich sources of valuable secondary metabolites. They may be used as bio-fertilizers, as there is an increasing concern about the excessive use of fertilizer in agricultural fields and their subsequent negative impact on plant and environment. The aim of the current study was to select potential fungal inoculums for plant growth promotion in order to avoid the excessive use of fertilizer in the agriculture industry.

Materials and Methods

Sample collection and isolation of fungal strains

Endophytic fungi were isolated from the roots of cucumber (*Cucumis sativus* L.), grown under green-house conditions. Cucumber plants were grown in 5.5 l pots, containing sandy loam soil. Six cucumber plants were randomly selected and harvested after 4 weeks of germination and their roots were screened for the presence of endophytic fungi. The fine root pieces were thoroughly washed with tap water and the dirt was thus removed. These roots 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. Tween 80 was used as detergent and was removed from cucumber roots by washing them with distilled water. The cleaned samples were surface sterilized by suspending them in 50 ml of 1% perchloric acid, and placed in shaking incubator (120 rpm for 5 min). The roots were then washed with autoclaved distilled water, dried between sterilized filter papers and cut into 0.5 cm pieces. These pieces were cultured on Hagem media plates and incubated at 25°C till the emergence of fungal cells (Khan et al. 2009a). The effectiveness of surface sterilization was determined by imprinting sterilized root pieces on Hagem media plates. Absence of any microbial growth on imprinted plates after 4–7 days of incubation were considered enough for effective surface sterilization of roots (Khan et al. 2008). The Hagem minimal medium plates were supplemented with 80 μg/ml streptomycin (Yamada et al. 2001). Pure fungi culture was isolated, grown on potato dextrose agar (PDA) media plates and slants (Khan et al. 2008). The PDA slants were used for storage purpose. Czapek broth medium, containing 1% glucose and peptone, was used for GA production (Hasan 2002) 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 bioassay and GA production, was provided by the Korean Culture Center of Microorganisms (Hamayun et al. 2009b).

Bioassay on waito-c and cucumber

The culture filtrates (CFs) of isolated fungi were bioassayed on waito-c rice sprouts in order to identify their plant growth promoting capacity. The seeds of waito-c were surface sterilized with 1% perchloric acid and treated with 20 μg/ml uniconazol for 24 h, in order to check the GA biosynthesis. The treated seeds were then washed thoroughly and soaked in autoclaved distilled H_2O for germination. Two waito-c seedlings were transplanted in each glass tube (50 ml), which already contained 20 ml of 0.8% water-agar medium. The glass tubes were transferred to a controlled growth chamber with a 16-h 30°C day and 8-h 20°C night regime and light intensity of 1,000 μmol $m^{-2}s^{-1}$ (Jang et al. 2008).

For bioassay experiment, the fungal isolates were grown in flasks containing Czapek medium. The flasks were placed on shaking incubator for 7 days at 30°C and 120 rpm. The fungal cultures (40 ml each) were then centrifuged at 5,000*g* at 4°C for 15 min and the resulting pellets and supernatants were immediately stored at −70°C and 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 (Hamayun et al. 2009a; Khan et al. 2009b). 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).

In regards to the cucumber bioassay, the seeds were surface sterilized with 5% NaClO for 15 min and then washed with distilled water. Seeds were sown in an autoclaved horticulture soil, under green house condition (30 ± 2°C). The horticulture soil contained peat moss (13–18%), perlite (7–11%), coco-peat (63–68%) and zeolite (6–8%), while the macro-nutrients were present as follows: NH_4^+ ~ 90 mg/l;

NO_3^- ~205 mg/l; P_2O_5 ~350 mg/l and K_2O ~100 mg/l. Fungal isolate GAH7 was selected for application, since it had caused maximum stem length promotion of waito-c rice. Two week old cucumber seedlings were treated with CF (10 ml) of GAH7 and the growth attributes i.e. plant length, shoot length, plant fresh weight and plant dry weight were recorded after 14 days of treatment. The growth promotion caused by CF of isolate GAH7 was compared with plants treated with same amounts of *F. fujikuroi*, Czapek medium and distilled water.

Extraction and quantification of gibberellins

Gibberellins were extracted from the CF of GAH7 by following an established protocol (Lee et al. 1998). GAs were chromatographed on a 3.9 × 300 mm Bondapak, C_{18} column (Waters Corp., Milford, MA, USA) and eluted at 1.5 ml/min with the following gradient: 0–5 min, isocratic 28% MeOH in 1% aqueous acetic acid; 5–35 min, linear gradient from 28 to 86% MeOH; 35–36 min, 86–100% MeOH; 36–40 min, isocratic 100% MeOH. Forty-eight fractions of 1.5 ml each were collected. The fractions were then prepared for gas chromatograph/mass spectrometer (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, 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, while the GAs quantification was based on the peak area ratios of non-deuterated (extracted) GAs to deuterated GAs.

Genomic DNA extraction and fungal identification

Genomic DNA isolation and PCR was performed according to an established protocol (Khan et al. 2009b). 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 (Tamura et al. 2007) and the maximum parsimony tree was generated using the same software. The bootstrap replications (1 K) were used as a statistical support for the nodes in the phylogenetic tree.

Statistical analysis

The data was 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

Screening of fungal isolates for plant growth promotion

The CFs of eleven endophytic fungi were screened for plant growth promotion by applying them on waito-c rice. Nine fungal isolates promoted growth of waito-c rice while two isolates inhibited it. The fungal isolate GAH7 significantly promoted shoot length (11.3 cm) as compared to Czapek (9.05 cm) and distilled water (7.9 cm), respectively (Fig. 1).

Bioassay of fungal isolate GAH7 on cucumber

The CF (10 ml) of isolate GAH7 significantly increased growth attributes of cucumber seedlings as compared to Czapek and distilled water treated plants. The plant length

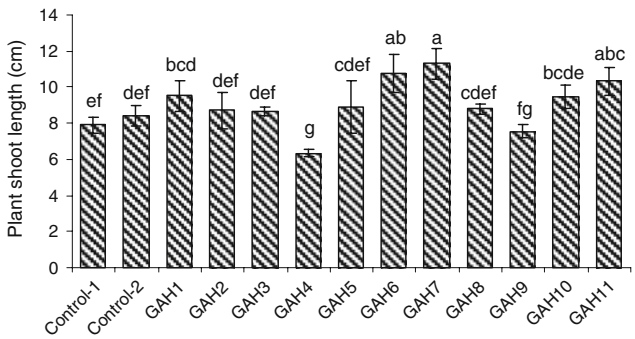

Fig. 1 Effect of fungal CFs (10 µl) on shoot length of waito-c seedlings after 7 days of incubation. *Data bars* having a *common letter(s)* are not significantly different at the 5% level by DMRT. Error bars show standard deviations. Control-1 stands for distilled water; control-2 stands for Czapek medium

(35.71 cm), shoot length (18.06 cm), plant fresh weight (4.98 g), and plant dry weight (0.35 g) were much higher than the Czapek medium and distilled water applied plants. However, the growth promotion capacity of isolate GAH7 was almost similar to that of *F. fujikuroi* (Table 1).

Analysis of CF of GAH7 for gibberellins

GA analysis of CF of isolate GAH7 showed the presence of bioactive GAs (GA_1, 0.81 ng/ml; GA_3, 4.34 ng/ml and GA_4, 9.31 ng/ml), in conjunction with physiologically inactive GA_9 (0.74 ng/ml), GA_{15} (0.97 ng/ml), GA_{19} (1.67 ng/ml) and GA_{20} (0.46 ng/ml). Fungal isolate GAH7 produced higher amounts of GA_3, GA_4, GA_9 and GA_{19} than wild type *F. fujikuroi* during current investigations (Fig. 2).

Identification of fungal isolate GAH7

The phylogenetic analysis of fungal isolate GAH7 was carried out by maximum parsimony (MP) method. Consensus tree was constructed from 19 (18 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. *A. bisporus* was used as out group. BLAST search showed that fungal isolate GAH7 has 99% sequence homology with *Phoma* sp. In the dendrogram, fungal isolate GAH7 formed a clade (58% bootstrap support) with *Phoma glomerata* (Fig. 3). On the basis of sequence homology and phylogenetic analysis, isolate GAH7 was thus identified as a new strain of *Phoma* sp. The 18S rDNA sequence was submitted to NCBI GenBank and was given accession no. FJ950743.

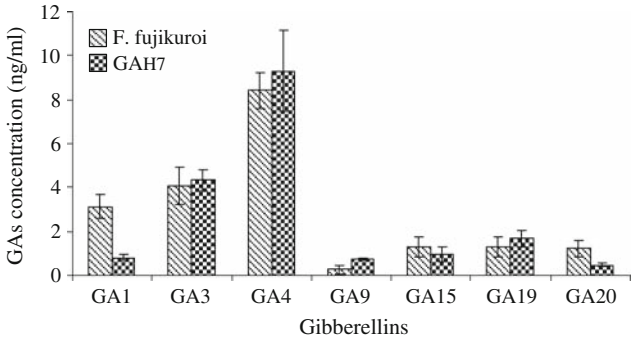

Fig. 2 Levels of various gibberellins secreted by fungal isolate GAH7 and *F. fujikuroi*. *Error bars* show standard deviations

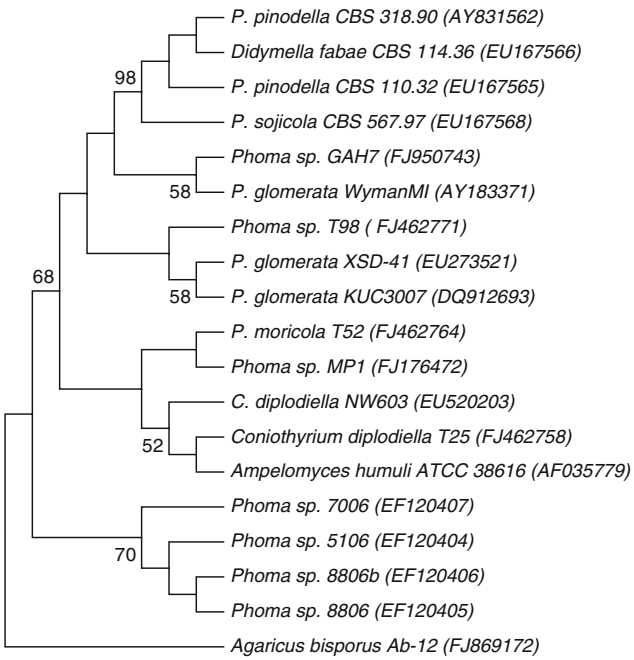

Fig. 3 Phylogenetic tree constructed by the maximum parsimony method using the 18S rDNA sequence (ITS region) of *Phoma* sp. GAH7 and related fungi. GAH7 formed a clad (58% bootstrap support) with *Phoma glomerata*, which identify fungal isolate GAH7 as a new strain of *Phoma* species. *Agaricus bisporous* was taken as an out group

Discussion

Endophytism represents a new area of research based on the benefits of mutualistic interactions between host crops and non pathogenic fungi. The advantages conferred by endophytic fungi include their ability to promote plant growth and tolerance to abiotic and biotic stresses. As such, the practical applications of endophytes as potential sources of bioorganic nutrients and as biocontrol agents can significantly improve yields in an environmentally sound

Table 1 Influence of CF of *Phoma* sp. GAH7 on growth attributes of cucumber

Treatments	Plant length (cm/plant)	Shoot length (cm/plant)	Plant FW (g/plant)	Plant DW (g/plant)
Control (distilled H_2O)	25.34 ± 1.43[c]	15.08 ± 0.81[b]	3.13 ± 0.04[c]	0.22 ± 0.03[b]
Czapek medium	30.4 ± 1.25[b]	16.15 ± 0.68[b]	3.71 ± 0.05[b]	0.26 ± 0.02[b]
F. fujikuroi	36.87 ± 0.96[a]	17.47 ± 0.83[a]	4.22 ± 0.19[a]	0.34 ± 0.06[a]
Phoma sp. GAH7	35.71 ± 0.71[a]	18.06 ± 0.34[a]	4.98 ± 0.12[a]	0.35 ± 0.04[a]

In a column, treatment means having a common letter(s) are not significantly different at the 5% level by DMRT. *FW* stands for fresh weight; *DW* stands for dry weight

way (Diene and Narisawa 2009). In current study, we isolated fungal endophytes from cucumber plant and investigated their growth promotion capacity. The CF of fungal isolate with maximum shoot elongation potential was further bioassayed on cucumber plant, which promoted growth attributes of cucumber with respect to control treatments. The fungal isolate GAH7 was later checked for GA production, as screening of microbial CFs for the presence of secondary metabolites is an established method for the identification of biologically active molecules (Higgs et al. 2001). The microbial extracts had been and will continue to be an efficient source of novel secondary metabolites (Cragg et al. 1997).

The usage of water-agar growth media for rice helped in the determination of the sole effect of CF on rice seedling growth, as 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, due to blocked C_{13} hydroxylation pathway of GA biosynthesis. Our current screening results are in agreement with previous reports (Hamayun et al. 2009a; Khan et al. 2008). The CF of GAH7 was also bioassayed on cucumber, as we wanted to investigate the effect of GAH7 on its host plant. It was observed that growth attributes of cucumber was significantly promoted by such an application. Our present investigation confirmed an earlier report on growth promotion of host plant by an endophytic fungus (Hamayun et al. 2009b).

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 (Kawaide 2006; Vandenbussche et al. 2007), which showed the importance of PGPF in plant growth and development, especially under nutrient deficient conditions. In present study, we reported the ability of *Phoma* sp. to produce 7 different gibberellins that also included bioactive GA_1, GA_3, and GA_4. The bioactive GA_3 and GA_4 amounts were higher in CF of GAH7 than those of wild type *F. fujikuroi*, which demonstrated the favorable role of *Phoma* sp. in promoting growth of host plants. The fungus *F. fujikuroi* was selected as a positive control for GA production as it corresponds to the mating group C of the *Gibberella fujikuroi* species complex (O'Donnell et al. 1998), is the only organism capable of excreting GAs in industrially viable quantities (Takahashi et al. 1991).

Molecular and phylogenetic approaches are employed for the identification of fungi recently. 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. ITS (I and II) had been employed more for fungal identification, although IGS and D1/D2, along with actin encoding genes is also gaining importance nowadays as they provide additional information on inter- and intra-specific identification (Kim and Lee 2000; Lee et al. 2001; Sugita and Nishikawa 2003). We used 5.8S gene and flanking ITS1/4 regions for identification of fungal isolate GAH7. It is because highly conserved 5.8S gene is suitable for higher taxonomic level analysis while highly variable ITS regions are useful for analysis at lower taxonomic levels. The phylogenetic tree construction is also crucial in molecular identification, since BLAST search alone cannot overcome the possibilities of statistical errors. Bootstrap consensus technique is applied in-order to read maximum sequence replications. On the basis of sequence homology and phylogenetic analysis results, isolate GAH7 was identified as a new strain of *Phoma* sp.

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

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