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Chrysosporium pseudomerdarium Produces Gibberellins and Promotes Plant Growth

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We isolated 10 endophytic fungi from the roots of drought stressed soybean cultivar Hwangkeumkong and bioassyed on waito-c rice and soybean seedlings, in order to identify plant growth-promoting fungi. The fungal isolate D-2-1 provided the best result for plant height and biomass promotion as compared to wild type *Gibberella fujikuroi*. The D-2-1 culture filtrate (CF) was analyzed for the presence of gibberellins (GAs) and it was observed that all physiologically active GAs, especially gibberellic acid, were present in higher amounts (GA_1, 0.24 ng/ml; GA_3, 8.99 ng/ml; GA_4, 2.58 ng/ml and GA_7, 1.39 ng/ml) in conjunction with physiologically inactive GA_5, GA_9, GA_{15}, GA_{19}, and GA_{24}. The fungal isolate D-2-1 was identified as a new strain of *Chrysosporium pseudomerdarium* through phylogenetic analysis of 18S rDNA sequence. Plant growth promotion and GAs production capacity of genus *Chrysosporium* have been reported for the first time in this study.

***Keywords*: *Chrysosporium pseudomerdarium*, gibberellin production, endophytic fungi, soybean, plant growth**

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 (Waller *et al*., 2005; Márquez *et al*., 2007). Endophytic colonization may also improve the ecological adaptability of the host plants by enhancing tolerance to a-biotic stresses (Schulz and Boyle, 2005). The genus *Chrysosporium* gained an increased attention in recent times for its potential to degrade keratin and its activity in soil and water sediments of polluted and fresh water sites. Perhaps some species of *Chrysosporium* may be utilized for recycling of keratinous waste in soil and as indicators to water pollution, which would certainly promote a congenial environment. Some of the metabolites secreted by *Chrysosporium*, particularly enzymes, and antimicrobials are gaining the attention of pharmaceutical industry (Kushwaha, 2000). The wide distribution of *Chrysosporium* is attributed to its antagonistic potential and ability to produce enzymes and other extracellular metabolites (Nigam, 1993).

Gibberellins (GAs) is a family of diterpenoid plant hormones, first detected in the 1920s from CF of *Gibberella fujikuroi*, a known pathogen of rice (Ogas, 2000). GAs appear to be involved in every aspect of plant growth and development, but their most typical (and spectacular) property is 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. GAs 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. GAs are associated with the breaking of winter dormancy and stimulate the formation of hydrolytic enzymes during seed germination (Martin, 1983). Currently 136 GAs have been identified; while 7 fungi associated with plants and/or soil have been reported as GA producers (MacMillan, 2002). Other reports suggested that GAs had been produced by 12 different fungi isolated from different sources (Kawaide, 2006; Vandenbussche *et al*., 2007). Recently, 4 strains of endophytic fungi were reported as GAs producers (Khan *et al*., 2008, 2009a, 2009b; Hamayun *et al*., 2009). However, there is no previous report on the GA production of any member of genus *Chrysosporium*.

There are increasing concerns regarding the excessive use of fertilizers in agricultural fields as well as their subsequent negative impact on environment. The use of fungi for plant growth promotion, while reducing the inputs of fertilizer may possess great potential for crops, especially vegetables, which respond very well to fungal inoculation. Considering the importance of fungi in plant growth and development, we isolated GAs producing fungi from soybean and investigated their possible role in plant growth and development.

Materials and Methods

Isolation of fungal endophytes from soybean

Endophytic fungi were isolated from the roots of soybean cultivar Hwangkeumkong, subjected to two weeks of drought

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stress induced by 16% polyethylene glycol (PEG), under green-house conditions. The roots 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 (Vazquez *et al.*, 2000). The pure cultures of fungi were isolated and stored on PDA (Potato dextrose agar, Scharlau Chemie S.A., Spain) plates and slants.

Fungal strains, culture medium, and growth conditions

The screening and isolation of fungi from soybean roots were carried out on Hagem minimal medium plates supplemented with 80 μg/ml streptomycin (Yamada *et al.*, 2001). PDA plates and slants were used for fungal storage, while Czapek broth medium containing 1% glucose and peptone was used for GA production (Hasan, 2002), by incubating the fungal isolate at 30°C and 120 rpm for 7 days. A wild type strain of *Gibberella fujikuroi* was used as a control during the experiment. This strain of *G. fujikuroi* had been provided by the Korean Culture Center of Microorganisms (KCCM).

Bioassay on waito-c and soybean

The CFs obtained from fungal isolates were bioassayed on waito-c sprouts for the presence of plant growth promoting metabolites. Waito-c is a dwarf rice mutant with reduced GAs production. Seeds of waito-c were surface sterilized and treated with 20 μg/ml uniconazol for 24 h, in order to check GAs biosynthesis. The treated seeds were then washed thoroughly and soaked in autoclaved distilled water (DW) for germination. The rice seedlings were transplanted in 0.8% water-agar medium, contained in glass tubes. The rice seedlings were grown in a controlled environment chamber with day (16 h 30°C) and night cycle (8 h 20°C) at light intensity of 1,000 $\mu mol/m^2/s^1$. Forty mililiter of culture suspension of each fungus was centrifuged for 15 min at 5,000×g and 4°C, and the resulting pellets and supernatants were immediately stored at -70°C and later lyophilized (ISE Bondiro Freeze dryer). The lyophilized supernatant was mixed with 1 ml of autoclaved DW and 10 μl of supernatant solution was applied on the apical meristem of each rice seedlings at two-leaf stage (Khan *et al.*, 2008). The whole plant length and shoot length were observed after 7 days of CF application and compared with waito-c seedlings, which had been treated either with distilled water (negative control) or *G. fujikuroi* (positive control).

In a separate bioassay experiment, seeds of Hwangkeumkong were surface sterilized with 5% NaClO for 15 min and washed thoroughly with autoclaved DW. Seeds were sown in autoclaved perlite and 20 ml of Hoagland solution was applied to each sprout at germination time (Hoagland and Arnon, 1950). Fungal isolate D-2-1 was tested for growth promotion of host soybean plant, since it had induced maximum stem elongation in waito-c during the screening experiment. A 5 ml of D-2-1 supernatant was applied to soybean seedlings at 2-leaf stage. The plant length, shoot length, plant fresh weight and shoot fresh weight attributes were measured after 7 days of CF treatment.

Extraction and quantification of gibberellins

To analyze the presence of gibberellins (GAs) in the CF of fungal isolate D-2-1, GAs were extracted following the protocol of Lee *et al.* (1998). The extracted GAs were subjected to reverse-phase C18-HPLC. The GAs were chromatographed on a 3.9×300 m Bondapak, C18 column (Waters Corp., Milford, 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 chromatograph/mass spectrometer (GC/MS) with selected ion monitoring (SIM) (6890N network GC system, Agilent Technologies, 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., USA). The GC oven temperature was programmed at 60°C for 1 min hold, then a rise to 200°C at 15°C per min, and then to 285°C at 5°C per min. Helium was used as a carrier gas and its pressure was maintained at 30 kPa. The GC was di-

Table 1. Screening of fungal isolates for their plant growth promoting capacity

Fungal isolates	Plant height (cm/plant)	Shoot length (cm/plant)	Increment (cm/plant)	Growth status
Control	16.3^{c} ± 1.8	6.0^{cd} ± 0.5	0.0	NA
G. fujikuroi	19.0^{a} ± 1.9	8.6^{a} ± 0.9	2.6	Promoted
D-5-2	17.3^{b} ± 1.2	8.7^{a} ± 1.6	2.7	Promoted
D-2-2	15.7^{cd} ± 1.7	7.9^{ab} ± 0.9	1.9	Promoted
D-2-1	19.1^{a} ± 2.3	9.1^{a} ± 0.6	3.1	Promoted
D-4-2	17.5^{b} ± 1.3	6.9^{bc} ± 0.4	0.9	Promoted
D-4-1	14.1^{e} ± 1.4	6.9^{bc} ± 0.3	0.9	Promoted
D-1-2	12.1^{f} ± 0.07	6.4^{cd} ± 0.2	0.4	Promoted
D-2-3	15.1^{cd} ± 3.0	6.3^{cd} ± 0.07	0.3	Promoted
D-5-3	17.4^{b} ± 0.2	5.7^{cd} ± 0.7	-0.2	Inhibited
D-5-1	11.0^{g} ± 2.0	5.5^{d} ± 0.8	-0.5	Inhibited
D-3-2	11.8^{fg} ± 0.7	6.7^{bcd} ± 0.9	0.7	Promoted

In a column, treatment means having a common letter(s) are not significantly different at the 5% level by DMRT. The experiment was repeated twice and each time consisted of three replicates.

rectly 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 (5973 Network Mass Selective Detector, Agilent Technologies, USA). Full scan mode (first trial) and three major ions of the 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, while the quantification of GAs was based on peak area ratios of non-deuterated (extracted) GAs to deuterated GAs.

Genomic DNA extraction and gel electrophoresis

An efficient method for the isolation of genomic DNA from fungal isolates was developed, since the usual CTAB extraction method and mycelial grinding had caused DNA shearing. A rich mycelial culture was obtained by growing the fungus in Czapek broth medium for 7 days and then lyophilizing it for 24 h. A 0.5 g of the lyophilized sample was broken carefully in 2 ml eppendorf tube, with a blunt end spatula or glass rod and a double volume of lyses buffer (20 mM Tris-HCl; pH 8, 10 mM EDTA, 1% SDS) containing 1% of 2-mercaptoethanol was added. The mixture was vortexed briefly (30 sec) to obtain homogeneity and incubated for 2 h in a water bath at 55°C. A 250 µl/ml of pre-heated 4% CTAB extraction buffer was added to lysed cells mixture and incubated at 65°C for 1 h. The mixture was cooled and a double volume of chloroform was added to it, and mixed gently by inversion for 10 min, then centrifuged at 12,000×g for 10 min and the upper layer was transferred carefully with a micropipette to 1.5 ml eppendorf tubes. An equal volume of cooled iso-propanol was added and mixed gently by inversion until coiled strand of nucleic acid (DNA and RNA) become visible. The condensed nucleic acid was spooled out, washed with ice-cooled 70% ethanol, air dried and suspended in 200 µl of autoclaved deionized DW. A 10 µl of RNase A was then added and incubated at 37°C for 2 h. Chloroform extraction and iso-propanol precipitation of cleaned DNA was carried out as already mentioned. The precipitated DNA in iso-propanol was incubated overnight in a refrigerator at -20°C, harvested next day at 12,000×g for 10 min, air dried and then suspended in 50 µl of autoclaved deionized DW. An agarose gel-electrophoresis was carried out to check purity and quantity of the isolated DNA. A 0.7% agarose gel in 50 ml of 0.5% TE buffer was loaded with 1 µl of DNA sample and run for 25 min against *Hin*dIII λ DNA ladder. After staining the gel with ethidium bromide solution for 15 min, the DNA bands were checked and recorded.

Molecular and phylogenetic identification

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'. A 25 µl of PCR mixture contained 2.5 µl of dNTPs and *Ex* Taq buffer, 2 µl of each primer, 0.5 µl of DNA sample and 0.2 µl of *Ex* Taq polymerase. The remaining volume was adjusted with 15.3 µl of autoclaved deionized DW. The reaction cycle comprised 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 resulting product was gene cleaned using a Nucleogen gene clean kit, ligated in T-vector using TaKaRa Perfect T-cloning kit, and then inserted into DHα *E. coli* mutant cells (RBC) by overnight incubation (37°C). The 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 compare the sequence homology of nucleotide of 18S ITS (1/4) region of fungi. The closely related sequences obtained were aligned through CLUSTAL W using MEGA version 4 software (Tamura *et al.*, 2007), and the maximum parsimony tree was constructed 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 was analyzed statistically for standard deviation by using MS-EXCEL. The mean values were compared, using Duncan's multiple range test (DMRT) at $P<0.05$ (ANOVA SAS release 9.1; SAS, USA).

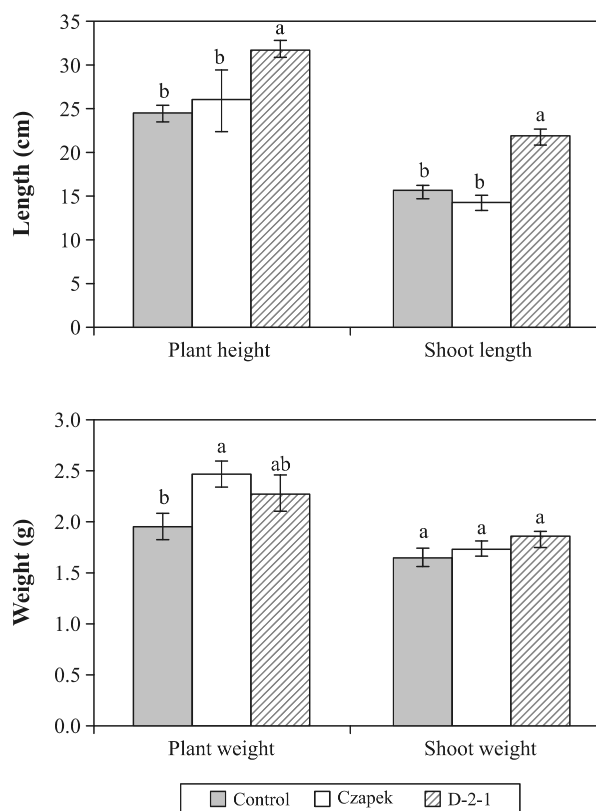

Fig. 1. Effect of CF of D-2-1 on plant height and plant weight of soybean seedlings. Data bars having a common letter(s) are not significantly different at the 5% level by DMRT. Error bars show standard deviations. The experiment consisted of 3 replicates.

Results and Discussion

Bioassay of D-2-1 on waito-c

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), as microbial extracts had been and will continue to be an efficient source of novel secondary metabolites (Cragg *et al*., 1997). During current study, 10 endophytic fungi were isolated from the roots of a drought stressed soybean cultivar Hwangkeumkong, and bioassayed them on waito-c for finding their role in plant growth and development. The results showed that 8 fungal isolates promoted growth of waito-c, while 2 inhibited it (Table 1). The fungal isolate D-2-1 gave maximum growth promotion and was thus selected for further investigation. We used nutrient free water-agar media in current experiment, which aided in the determination of the sole effect of fungal CF on the growth of rice seedlings. We selected waito-c for screening experiment, which is a dwarf rice mutant, with blocked GA biosynthesis pathway and thus a significantly reduced GA biosynthesis. Use of waito-c thus ensured that the plant growth enhancement was mostly induced by GAs present in the external medium i.e. fungal CF. Current findings confirmed previous reports of Khan *et al*. (2008) and Hamayun *et al*. (2009), which also reported an increase in waito-c shoot length when the plants were treated with fungal CF.

Bioassay of D-2-1 on soybean

The CF of D-2-1 was also bioassayed on host soybean plants and it was observed that all growth attributes studied were promoted with fungal CF application. The plant height was significantly promoted (31.85 cm) as compared to control and Czapek treated plants. The shoot length was also enhanced (21.8 cm) compared to control treatments. The fresh weight attributes were also enhanced by the fungal CF as compared to control treatments (Fig. 1). Soybean was grown in perlite under growth chamber condition, in order to study the effect of fungi on host plants under controlled environmental conditions. Perlite provided a semi-hydroponic medium for soybean growth, as it is eco-friendly, inorganic and therefore physically stable with a neutral pH. It is sterile, free from pests, pathogens and weeds seeds, lightweight, safe and easy to handle. Our current findings about fungus-host interaction are in agreement with earlier reports on the subject (Khan *et al*., 2009a).

Gibberellins quantification

The analysis of D-2-1 CF showed the presence of 9 different gibberellins, which also included bioactive GA_1, GA_3, GA_4, and GA_7. The level of GA_3 produced by the D-2-1 was much higher (8.99 ng/ml) as compared to 3.12 ng/ml produced by the wild type *G. fujikuroi*. The difference in other bioactive gibberellins content of D-2-1 and *G. fujikuroi* was not significant (Fig. 2). We analyzed the presence of gibberellins, in CF of isolate D-2-1 through GC/MS SIM. 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 the accumulation of interesting and unexpected new knowledge regarding a particular extract (Franck *et al*., 2005).

Identification and phylogenetic analysis

Morphological characteristics of a fungus provide valuable information for identification, but traditional identification techniques were mostly replaced with newly introduced molecular and phylogenetic approaches. Genomic DNA sequencing is an objective, reproducible and rapid technique for fungal identification, and is thus gaining immense importance. Sequencing of internal transcribed spacer (ITS) regions has gained an immense importance in fungal identification in recent times due to the presence of highly preserved 5.8S gene that helps in the identification at a higher taxonomic level, while ITS1 and ITS2 are useful for the identification at a lower taxonomic level (Kim and Lee, 2000; Lee *et al*., 2001; Sugita and Nishikava, 2003). In current study, the phylogenetic analysis of the 17 (16 references and 1 clone) aligned ITS1 sequences were conducted with 1K bootstrap replications. These strains were selected through BLAST search showing maximum sequence homology percentage and query coverage, and lowest E value, while *S. cerevisiae* was used as an outgroup. A distance tree using

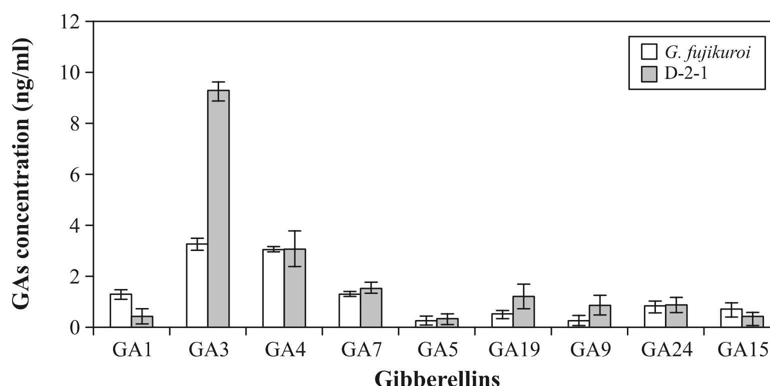

Fig. 2. Production of various GAs by D-2-1 and *G. fujikuroi*. Of 4 bioactive GAs i.e. GA_1, GA_3, GA_4, and GA_7, only GA_1 was produced in lower amounts by D-2-1 compared to *G. fujikuroi*. Error bars show standard deviations. GAs analysis was repeated three times.

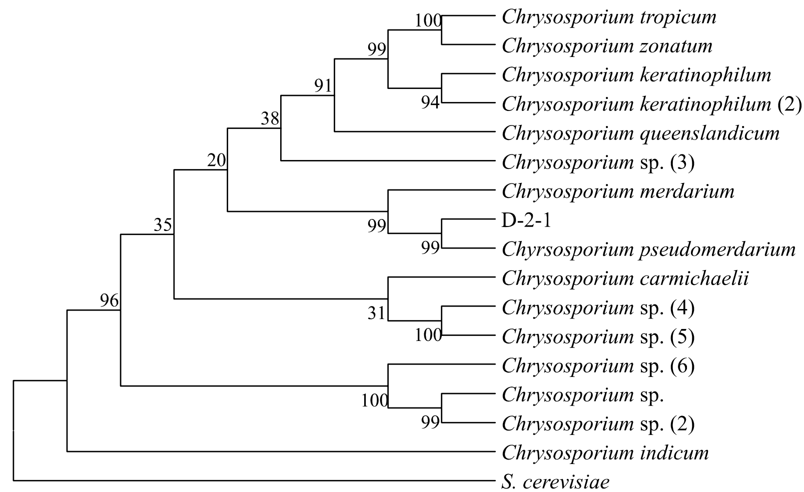

Fig. 3. Identification of fungal isolate D-2-1 by phylogenetic analysis. Maximum parsimony distance tree with 1K replications of bootstrap test was constructed using 17 taxa (16 reference sequences and 1 clone). D-2-1 formed a subclade with *C. pseudomerdarium* with a bootstrap support of 100%, which identify D-2-1 as a new strain of *C. pseudomerdarium*.

maximum parsimony method was constructed in which D-2-1 isolate formed a subclade with a *C. pseudomerdarium* strain (100% bootstrap support), which showed 99% sequence homology with D-2-1. On the basis of sequence homology and phylogenetic analysis results, fungal isolate D-2-1 was thus identified as a new strain of *C. pseudomerdarium*. The 18S rDNA sequence of this new fungal strain was submitted to the GenBank database and was given accession no. EU 823311. The fungal isolate D-2-1 was thus identified as *C. pseudomerdarium* IJL01 (Lab name) (Fig. 3). Phylogentic analysis was carried out as phylogenetic tree construction along with BLAST search has been gaining immense importance. This method of relating the isolate in question with those showing a maximum DNA sequence homology helps to overcome possibilities of errors by conducting statistical analyses, such as bootstrap tests.

Conclusion

Endophytic fungi are well known plant symbionts, although information on their gibberellins production and plant growth promotion is limited. Our current study reports valuable information on the gibberellins producing potential of an endophytic fungus. It also highlights the importance of using *C. pseudomerdarium* for plant growth and development under drought stress environment. However, further study is suggested on the characterization of GA encoding gene cluster and the development of media for optimized GAs production of *C. pseudomerdarium* IJL01.

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