Aspergillus niger CSR3 regulates plant endogenous hormones and secondary metabolites by producing gibberellins and indoleacetic acid

Lubna a, Sajjad Asa b, Muhammad Hamayun a, Humaira Gul a, In-Jung Lee c and Anwar Hussain a

aDepartment of Botany, Abdul Wali Khan University Mardan, Mardan, Pakistan; bChair of Oman’s Medicinal Plants & Marine Natural Products, University of Nizwa, Nizwa, Oman; cSchool of Applied Biosciences, Kyungpook National University, Daegu, Republic of Korea

ABSTRACT
In this study, an endophytic fungus, Aspergillus niger CSR3, was isolated from Cannabis sativa. The culture filtrate (CF) was initially screened for growth-promoting activities such as the presence of siderophores, phosphate solubilization, and the production of indole acetic acid (IAA) and gibberellins and was further assayed for its ability to promote the growth of mutant waito-C rice. Nearly all plant growth attributes examined (root-shoot length, biomass, and chlorophyll content) were significantly enhanced by treatment with CSR3. This growth promotion action was due to the presence of various types of gibberellins (GAs) and IAA in the endophyte CF. Moreover, the presence of GA pathway genes (P50-1, P450-3, P450-4, ggs2, and des) was confirmed by means of semi-quantitative RT-PCR. Finally, the application of CSR3 spore suspension with uniconazole and yucasin on maize seedlings revealed that, similar to exogenous IAA and GAs, CSR3 has the potential to alleviate the inhibitory effect of these inhibitors.

Introduction
Plant endophytic fungi are those fungi that may spend their lifecycle partly or completely in an inter- and/or intra-cellular mode in the host plant tissues without causing symptoms of infection in the host (Tan and Zou 2001; Rodriguez et al. 2009). It is estimated that there are more than one million endophytic fungi surviving in nature and such fungi have been found in every plant species studied (Strobel et al. 2004; Verma et al. 2009). Endophytic fungi are the main source of natural bioactive compounds that have potential applications in different fields like agriculture, medicine, and the food industry (Strobel et al. 2004; Verma et al. 2009). Several endophytes have been examined for their ability to produce bioactive products, similar to that produced by their host plants (Zhao et al. 2010). Symbiotic endophytic fungi have been reported to promote plant growth in a number of crops and also promote organic rice cultivation (Anegeard et al. 2010; Wijesooriya and Deshapriya 2016).

Recently, it has been reported that endophytic fungi can produce phytohormones, particularly gibberellins (GAs), that enhance crop growth and alleviate the harmful effects of abiotic stresses (Khan et al. 2011). Gibberellins are tetracyclic diterpenoid acids that regulate various plant developmental and physiological processes including seed germination, seedling development, stem and leaf growth, floral initiation, and flower and fruit growth (Pharis and King 1985; Crozier 2000; King and Evans 2003; Davies 2010). Gibberellins also promote other physiological processes in plants such as root growth and root hair development, and inhibit floral bud differentiation in woody angiosperms. Besides, they regulate vegetative and reproductive bud dormancy, and also delay senescence in many organs in a variety of plant species (Fulcheri et al. 1993; Reinoso et al. 2002). Several endophytic fungi have been reported to produce indole acetic acid (IAA) (Ansari et al. 2013; Waqas et al. 2014). Similar to gibberellic acid (GA), IAA promotes several developmental processes in plants such as root development and axillary bud and flower formation and improves many other processes in plant, and is indispensable for plant growth and development (Reinhardt et al. 2000). IAA is a valuable metabolite and is required from embryogenesis to senescence in plant growth. Advancement in the field of biotechnology has led to an awareness of the significance of many fungi owing to their use in industry and their ability to produce phytohormones. It has been reported that hormones such as IAA and ABA are synthesized in both plants and fungi (Lopez-Carbonell et al. 1994; UNyayar et al. 1997).

Endophytic fungi isolated from different types of plants have been examined; several fungal strains such as Fusarium oxysporum, Aspergillus niger, A. flavus, Penicillium cyclopium, P. funiculosum, P. corylophilum, and Rhizopus stolonifer are capable of producing phyto-stimulatory hormones like gibberellic acid (GA) and indole acetic acid (IAA) (Hasan 2002; Waqas et al. 2012). Besides the ability to produce hormones, these endophytic fungi have been gaining importance due to their role in the solubilization of minerals such as phosphate, zinc, and potassium. Plant growth-promoting fungi (PGPF) enhance host growth by producing different enzymes; enabling phosphate solubilization (Malla et al. 2004; Wakelin et al. 2004) and siderophore production (Costa and Loper 1994); or protecting host plants from pathogens (Ramamoorthy et al. 2001). Endophytic fungi are potential sources of phytochemicals, a property that has significance for both medicinal and agrochemical use. These phytochemicals include phenols and flavonoids, and may be responsible for antioxidant properties of the host plants.
The huge diversity of antioxidant compounds produced by endophytic fungi might protect their host plants from different abiotic stresses (Herrara-Carillo et al. 2009; Torres et al. 2009). Several studies support this claim that plants associated with endophytic fungi have increased production of antioxidant compounds like flavonoids and other phenolic antioxidants (Richardson et al. 1992; Harper et al. 2003; Huang et al. 2007).

In the present study, A. niger CSR3, a fungal endophyte, was isolated from the roots of a medicinal plant, Cannabis sativa, with high phosphorus-solubilizing ability and siderophore production. We assumed that this endophyte might be a source of phytohormones that could facilitate plant growth and alleviate the uniconazole and yucasin inhibition. To test this hypothesis, we analyzed the phytohormone production capability of CSR3 in vitro. We report that the culture filtrate (CF) of strain CSR3 produces phytohormones that significantly improve the growth attributes of waito-C rice.

**Material and methods**

**Isolation of endophytic fungi**

Healthy plants were collected from the district Swat, Khyber Pakhtunkhwa, and were washed thoroughly in running tap water to remove soil. After proper washing, the plant parts were cut into 0.5–1 cm segments. The plant material was treated with 75% ethanol for 3 min followed by immersion in 1–13% Sodium hypochlorite (NaOCl) and again in 75% ethanol for 30 s. Later the segments were rinsed three times with sterile distilled water. The plant pieces were blotted on sterile blotting paper. The efficiency of surface sterilization procedure was ascertained for every segment of tissue following the imprint method of Schulz et al. (1993). In each Petri plate 4–5 segments were placed on Hagem minimal media supplemented with streptomycin (80 ppm), to prevent bacterial growth. To assess the efficiency of surface sterilization, the sterilized roots were also placed on separate Hagem plates. Petri plates were incubated at 30°C for about seven days. After the incubation period, the growing endophyte was inoculated in a fresh set of Petri dishes containing potato dextrose agar (PDA) media to obtain pure cultures, which were stored in a refrigerator for further use.

**Screening of endophytic fungi for IAA production**

Fungal isolate CSR3 was initially screened for IAA production using the colorimetric method (Ullah et al. 2013). The isolated endophyte was grown on Czapek broth medium in a shaking incubator for seven days at 30°C and 120 rpm. After seven days, the samples were filtered and the IAA concentration of the CF was determined by adding 1 mL of Salowski reagent to 2 mL of CF, followed by incubation for 30 min in the dark. Strains positive for IAA production were selected for further study.

**Screening endophytes on GA-deficient waito-C dwarf rice**

Dwarf phenotype waito-C rice with suppressed GA biosynthesis was used to examine CF of the isolated endophytic fungi for GA. First, seeds were surface sterilized with 2.5% sodium hypochlorite for 30 min, then washed three times with distilled water and incubated for 24 h with 20 mg/L of uniconazole to check for GA biosynthesis. Seeds were treated with uniconazole to arrest GA biosynthesis, and to check the effects of CSR3 on mutant rice. The waito-C seeds were pre-germinated and transferred to autoclaved pots containing horticulture soil that had the following nutrient composition of peat moss (10–15%), perlite (35–40%), coco peat (45–50%), zeolite (6–8%), and ~0.09 mg/g, ~0.205 mg/g, P2O5 ~0.35 mg/g, and K2O ~0.1 mg/g to prepare the microbe-free condition (Khan et al. 2011; Asaf et al. 2017). When the seedlings were at the two-leaf stage, 20 μL of the endophyte CF was applied to the tip of the seedlings. After 15 days, chlorophyll content (SPAD-502; Minolta, Tokyo, Japan), root and shoot length, root and shoot fresh weight, and dry biomass were calculated. Subsequently, the plants were harvested and stored in liquid nitrogen for further phytohormonal analysis.

**Determination of phosphate solubilization and siderophore production**

Fungal isolates were inoculated in Pikovskaya (PVK) agar medium (Pikovskaya 1948) by following the method described by Iman (2008). Formation of a clear zone around the colony was an indicator of phosphate solubilizing ability of the fungus and phosphate solubilization index (SI) was determined by using the following formula: ratio of the total diameter (colony + halo zone) and the colony diameter (Premono et al. 1996). Furthermore, the strain was assayed for siderophore activity on CAS agar medium (Vellore 2001). Fungal strains positive for the production of siderophores produce an orange halo around the fungal growth.

**Determination of IAA content by GC/MS**

The CF of CSR3 was analyzed by GC/MS to assess IAA content. For this experiment CSR3 was cultured in 50 mL Czapek broth provided with 0.5 mg/mL of L-tryptophan and incubated for 7 days at 28°C in a shaking incubator at 200 rpm. Cultures were filtered to obtain the CF, which was then acidified (pH 2.8) using 1 N HCl and 40 μL mL-1 [D5]-IAA was added as the IAA internal standard. The acidified CF was quantified by GC–MS/SIM (6890 N network GC system and 5973 network mass selective detector; Agilent Technologies, Palo Alto, CA, USA) as described by Ullah et al. (2013).

**Extraction and quantification of gibberellin in culture broth**

GA from the CF was extracted and quantified as described earlier (Lee et al. 1998). After 7 days of incubation, the CF was fractionated by preparative high-performance liquid chromatography equipped with a reverse-phase C18 column. The GA-containing fractions were collected and analyzed by gas chromatography–mass spectrometry (GC–MS) with selected ion monitoring (SIM) option. After the GC–MS data had been collected and analyzed, three major ions of the supplemented [2H2] GA internal standard and GA were monitored. To calculate the Kovats retention index value, standards were used to determine the retention time, and peak area ratios between non-deuterated and deuterated GA were used to quantify the GA (Lee et al. 1998). Each sample was replicated three times and the experiment was repeated thrice to ensure reproducibility.
Chromatography and GC/MS SIM for endogenous GA quantification

Three replications for each treatment were used to analyze the endogenous GAs of freeze-dried inoculated and non-inoculated *waito-C* rice plants. GA extraction and quantification was carried out as previously described (Lee et al. 1998). GC/MS SIM was performed to determine GAs in the extract. This experiment was performed three times and each treatment was replicated thrice.

Chromatography and GC/MS SIM for endogenous hormonal quantification

To study plant endogenous ABA and JA content, freeze-dried *waito-C* rice plants both inoculated and non-inoculated were used, with three replicates for each treatment. For JA extraction and quantification, the protocol of McCloud and Baldwin was used (McCloud and Baldwin 1997). The extracts were analyzed by GC–MS (6890 N network GC system and 5973 network mass selective detector; Agilent Technologies). Furthermore, we monitored the fragmented ion with a m/z value of 1/4 83 amu, corresponding to the base peaks of JA and [9, 10-2H2]-9, 10-dihydro-JA.

The endogenous ABA extraction and quantification was done according to the method described previously (Qi et al. 1998). Briefly, the extracts were dried and methylated by adding diazomethane. Analyses were done using a GC–MS SIM (5973 network mass selective detector and 6890N network GC system, and Agilent Technologies, Palo Alto, CA, USA). For quantification, the Lab-Base (Thermo Quest, Manchester, UK) data system software was used to monitor responses to ions with m/z values of 162 and 190 for Me-ABA, and 166 and 194 for Me-[2H6]-ABA.

Endophyte identification and phylogenetic analysis

Genomic DNA from the fungal mycelia was extracted with a DNeasy plant mini kit (QIAGEN, Valencia, CA, USA). For fungal endophyte identification, the internal transcribed regions (ITS) and the D1–D2 region of the large-subunit RNA gene were amplified by PCR, sequenced, and compared with sequences in public databases using the BLAST algorithm. The fungus-specific primers ITS1 (5′-TCCGTAGGT-GAACTCTGCGG-3′) and ITS2 (5′-GCTGGTTCTTCTATC-GATGC-3′) were used to amplify the ITS1 region, while the D1–D2 region was amplified by primers NL1 (5′-GATAT-CAATAGCGGGAGAAGG-3′) and NL4 (5′-GTCC GTTGTTCAGACG-3′) (Park et al. 2002). The closely related sequences in public databases were aligned through CLUSTAL W using MEGA version 6.06 software (Tamura et al. 2013). The neighbor-joining (NJ) protocols embedded in MEGA 6 were used to construct phylogenetic trees. The bootstrap replications (1 K) were used as a statistical support for the nodes in the phylogenetic tree.

RNA isolation and semi-quantitative reverse transcriptase (RT) PCR

RNA was extracted from the lyophilized mycelium of endophytic fungi by using a modified protocol (Chan et al. 2004). In brief, fungal mycelium (1.0 g) was pulverized in an ice-cold mortar and pestle under liquid nitrogen. The ground mycelia were immediately transferred to RNA-free falcon tubes containing extraction buffer (0.25 M NaCl, 0.05 M Tris–HCl, pH 7.5, 20 mM EDTA, 1% [w/v] sodium dodecyl sulfate, and 4% [w/v] PVP). The resulting RNA was used as template to synthesize cDNA using a DiaStar RT kit (SolGent, Korea), according to the manufacturer’s standard protocol. To validate GA and IAA biosynthesis in the endophytes, GA and IAA biosynthesis genes were selected and their expression was measured using semi-quantitative RT–PCR (PCRMax Alpha Cycler, UK). A 2 × F-Star Taq PCR Master mix (BioFACT™, Korea) along with 10 nM of gene-specific primers and 100 ng of template cDNA in a final volume of 20 µl was used as reaction mixture. Actin was used as an internal control and the experiment was repeated three times.

Maize treatment with CSR3, exogenous hormones, and their inhibitors

Stock solutions of uniconazole, yucasin, IAA, and GA3 were prepared at the following concentrations: uniconazole, 1000 ppm; yucasin, 100 mM; GA3 and IAA 100 mM each. Uniconazole and yucasin were dissolved in methanol and DMSO, respectively, while, IAA and GA3 solutions were dissolved in EtOH. Further dilutions were prepared from the stock solutions, i.e. uniconazole, 50 ppm and yucain, 50 µM. The maize seeds were surface sterilized as mentioned previously. The seeds were allowed to germinate. The germinated maize seeds were transferred to 450 mL pots (containing three seedlings per pot) filled with autoclaved soil. These pots were divided into six sets each set receiving one of the six treatments. Upon reaching the two-leaf stage, 10 mL of yucasin (50 µM) or uniconazole (50 ppm) was sprayed on maize seedlings two times for 14 days. Fungal spore suspension (10 mL) containing approximately 1000 spores was also sprayed on maize seedlings as a sole treatment, as well as a co-treatment with uniconazole and yucasin, respectively. Distilled water was applied to control plants. The plant growth characteristic, chlorophyll content (SPAD-502; Minolta, Tokyo, Japan), root and shoot length, and root and shoot biomass (fresh and dry) were measured after two weeks of treatment.

Flavonoid estimation in plants

For colorimetric assay of total flavonoid content, 0.1 mL of 10% aluminium chloride and 0.1 mL of potassium acetate (1M) were added to be 0.5 mL of sample. The volume of the mixture was brought to 5 mL by adding 4.3 mL of 80% methanol (Eom et al. 2007). The reaction mixture was vortexed and OD at 415 nm was recorded using a spectrophotometer (PerkinElmer lambda 25). Total flavonoid content present in the sample was calculated from the OD values. Methanol was used as a blank.

Estimation of total phenolic content

For the determination of total phenolic content, 250 mg of healthy leaves were ground with 2 mL of 80% ethanol, and centrifuged for 30 min at 10,000 rpm (Lister and Wilson 2001). The supernatant was dried at 40°C under reduced pressure and dissolved again in 2 mL of distilled water. The suspension was diluted 10 times for a working standard. To this 0.5 mL Folin-Ciocalteau reagent (1 N) was added.
Further, to this mixture, 2 mL of 20% Na₂CO₃ solution was added, shaken well, and subsequently heated on a water bath for 1–2 min at 40°C. The optical density of the reaction mixture upon cooling to room temperature was recorded at 650 nm using a spectrophotometer, and normalized against the blank solution (prepared by excluding extract). Total phenolic content (mg/gm) was evaluated in catechol equivalent after comparing with a standard curve prepared from distilled catechol.

**Estimation of total sugar**

For this, 0.5 g maize leaf tissue was ground in 80% methanol. From this extract 0.5 mL was taken and the volume was made up to 10 mL with distilled water and centrifuged at 3000 rpm for 10 min. About 0.5 mL of supernatant was aliquoted and 1 mL of 80% phenol was added and incubated for 10 min. After incubation, 5 mL of concentrated sulfuric acid was further added and incubated for an additional four hours. Absorbance of this solution was recorded at 420 nm.

**Statistical analysis**

The data were statistically analyzed using SPSS software to estimate the standard deviation and standard error for the data. Means were compared using Duncan’s multiple range test at the \( P < .05 \) level. A \( t \)-test was also used to compare the means.

**Results**

**Isolation of endophytic fungi**

Eleven fungal endophytes were isolated from root of *C. sativa*. Among these endophytes the CSR3 strain was found to have the highest colonization frequency (Table S1). The isolated strains were initially differentiated on the basis of morphological characters. Our selected strain showed rapid growth on the PDA plate initially, formed white to yellowish mycelia, and then quickly turned black as the conidia developed (Figure S1(A,B)). The phosphate solubilization activity on PVK agar medium (Figure S1(C)) indicated that CSR3 can solubilize phosphate and the SI of the CSR3 was 3.13 after 7 days. Similarly, siderophore activity was detected by siderophore production assay on CAS medium and CAS reaction rate was 3.8–5.6 mm/day (Figure S1(D)). Furthermore, endogenous IAA was detected from the isolated strain by colorimetric assay using Salkowski reagent. Therefore, on the basis of these growth-promoting characteristics the CSR3 strain was selected for further study.

**Screening bioassay on waito-C rice seedlings**

To test the growth-stimulating potential and gibberellin production, experiments were carried out on the mutant rice cultivar *Waito-C* (GA-deficient) (Waqas et al. 2014). The CSR3 cultural filtrate treatment significantly increased all growth attributes of *Waito-C* rice when compared to control plants (Figure 1). *Waito-C* rice in association with endophytic fungi showed significant increase in root shoot length and both fresh and dry weights (Table 1). Similarly, other growth parameters including chlorophyll content were also significantly promoted by CSR3 when compared to the control plants.

**Endophyte identification and phylogenetic analysis**

To identify the CSR3 strain and to infer its phylogenetic position, the sequenced ITS region (555 bp, GenBank accession number: MF187478) and D1–D2 from 28S rDNA (999 bp, GenBank accession number: MF187479) were compared to sequences in the NCBI database by BLAST search analysis (http://www.ncbi.nlm.nih.gov/). The results revealed that the CSR3 fungal endophyte exhibited a higher level of ITS sequence identity (99%) and D1–D2 identity

| Shoot length (cm) | Root length (cm) | Fresh weight (mg) | Dry weight (mg) | Chlorophyll content (mg/L) |
|------------------|------------------|------------------|----------------|--------------------------|
| Control          | 102 ± 14.4048    | 53 ± 18.907      | 0.89 ± 0.0424  | 0.584 ± 0.0151           | 230 ± 10 |
| CSR3             | 162 ± 12.54      | 56 ± 4.1833      | 1.426 ± 0.0207 | 0.672 ± 0.0436           | 254.33 ± 5.50 |

Figure 1. Effects of CF from the fungal endophyte *A. niger* CSR3 on the growth characteristic of rice plant (waito-C)
(100%) to A. niger. The NJ was employed to construct a phylogenetic tree for ITS and D1–D2 with MEGA 6, after sequence alignment with Clustal W (version 7.222) (Katoh and Standley 2013) with default parameters. The results revealed that on the basis of both ITS1 and D1–D2 regions, CSR3 forms a single clade with A. niger species, supported by a relatively strong bootstrap value of 100% and 99%, respectively (Figure 2). On the basis of morphology and phylogenetic relationship, strain CSR3 was identified as a member of A. niger.

Quantitation of GA, IAA, and ABA in the culture of CSR3

The CSR3 strain was grown at 30°C and 120 rpm for 10 days and the CF was tested for phytohormones such as GA, IAA, and ABA. These hormones were determined by GC/MS SIM. Both active and non-active GAs were detected in the CF of the CSR3 strain (Figure 3(A)). Physiologically active GA includes GA$_1$ (0.002241 ± 0.000127), GA$_3$ (0.0067 ± 0.000283), GA$_4$ (0.007536 ± 0.004458), and GA$_7$ (0.2446 ± 0.032527), which were identified in the CF. Inactive types of GA present in the CF were GA$_8$ (0.006709 ± 0.000297), GA$_9$ (0.006125 ± 0.001329), GA$_12$ (0.031151 ± 0.000127), GA$_20$ (0.002086 ± 0.000129), and GA$_24$ (0.007856 ± 0.000129). The CSR3 was also found to produce IAA via both tryptophan-dependent and tryptophan-independent pathways. CSR3 produced higher quantities of IAA with tryptophan addition to the growth medium (0.873 ± 0.029 μg mL$^{-1}$) than the amount of IAA produced (0.185 ± 0.234 μg mL$^{-1}$) in the absence of this precursor (Figure 3(B)). Along with this growth-promoting hormone, the selected strain also produced significant amounts (3.5 ± 0.029 μg mL$^{-1}$) of stress hormone ABA (Figure 3(B)).

Expression of GA biosynthesis genes in CSR3

GA biosynthesis genes’ expression was determined by subjecting the isolated RNA of the CSR3 strain to semi-quantitative RT-PCR analysis and by using GA-biosynthesis

Figure 2. Molecular phylogenetic analysis of fungal strain A. niger CSR3 from ITS region (A) and D1–D2 region of LSU rDNAs (B) using NJ method. Numbers above the branches are the bootstrap values of Penicillium species which were selected as outgroup. Green and brown dots represent the position of A. niger CSR3.

Figure 3. (A) GA and (B) IAA production by A. niger CSR3 (A). The fungal CF was centrifuged, and 100 mL of the CF was analyzed for the presence of GAs and IAA using their respective protocols. The bar indicates standard deviation between replicates.
specific-gene primers (Table S2). Expressions of these genes in *F. fujikuroi* were used as a positive control (Figure 4). Actin was used as an internal control. The results revealed that all GA biosynthesis genes, i.e. *P450-1*, *P450-3*, *P450-4*, *ggs2*, and *des*, were expressed both in CSR3 and the positive control *G. fujikuroi* (Figure 4).

**Effects of symbiotic association on host plant hormones ABA, GA, and JA**

Endogenous phytohormones such as GA, ABA, and JA levels in *Waito-C* rice seedlings inoculated with CSR3 and without CSR3 treatments were determined. A significant increase was observed in the endogenous phytohormone levels in *Waito-C* rice associated with the CSR3 strain. This association shows positive effects on endogenous GA in plants (Figure 5(A)). All 10 types of GA (*GA1*, *GA3*, *GA4*, *GA7*, *GA8*, *GA12*, *GA15*, *GA20*, and *GA24*) were found to increase several fold than the control, but bioactive *GA7* (28.2643) and *GA12* (34.23) had the most drastic response to fungal inoculation (Figure 5(A)). Endogenous levels of ABA and JA were also determined in both, *Waito-C* rice seedlings associated with CSR3 and not associated with CSR3 (Figure 5(B)). JA level was reduced by endophytic fungi while the ABA level was elevated when compared to the control.

**Effect of uniconazole on chlorophyll, sugars, flavonoids, and phenolics of maize seedlings**

Application of uniconazole negatively affects all plant growth attributes as well as secondary metabolites content. Plants treated with uniconazole showed significant decrease in root and shoot length, fresh dry weight chlorophyll, flavonoids, and phenolic contents (Figure 6). However, the effect of uniconazole on sugar content was different, and slightly increased the sugar content (Figure S2). On the other hand, exogenous application of GA3 significantly increased seedling growth and chlorophyll, flavonoid, and phenolic contents of plants. Similar results were observed in CSR3 treatments in association with maize seedlings, which enhanced all these parameters as compared to control. Application of both CSR3 and GA3 in maize seedlings exposed to uniconazole stress enabled them to produce higher amounts of than secondary metabolites than did the control.

**Effect of yucasin on chlorophyll, sugars, flavonoids, and phenolics**

Application of yucasin on maize seedling significantly affects seedling growth, chlorophyll, and phenol contents as compared to the control (Figure 7). Plant flavonoids and sugars were reduced by yucasin treatment (Figure S3). On the other hand, application of exogenous IAA and CSR3 increased the amount of phenol and flavonoids when compared to the control. Furthermore, in case of sugars and flavonoids, the CSR3 strain had an even more positive effect than seen with exogenous IAA (Figure S3). Yucasin treatment in combination with IAA and CSR3 did not influence these parameters (Figure S3).

**Alleviating the negative effect of uniconazole on maize seedling growth by CSR3**

The effects of exogenous GA3, uniconazole, and CSR3 strain were evaluated on different growth attributes of maize seedlings. Maize seedlings treated with GA3 and CSR3 showed higher growth rate than the control plants (Figure 6). In contrast, the uniconazole application significantly reduced all plant growth attributes, especially root and shoot length as...
compared to GA₃, CSR3, and control plants (Figures 6 and S1). However, co-application of GA₃ and CSR3 with an inhibitor significantly reversed its inhibitory effect, enhanced root and shoot length, and fresh dry weight of maize (Figure 6). A similar trend was also observed for total chlorophyll, flavonoid, phenolics, and sugars where the adverse effect of uniconazole was mitigated by CSR3 treatment (Figure 2).

**Alleviating the negative effect of yucasin on maize seedling growth by CSR3**

To check the growth-promoting ability and to alleviate the inhibitory effects of yucasin, CSR3 was applied to the plants after treating them with an auxin inhibitor, yucasin (IAA inhibitor). The results revealed that yucasin significantly reduced plant root and shoot length, fresh and dry weights, chlorophyll, sugar, flavonoid, and phenolic contents; all were lower compared to control plants and sole applications of IAA and CSR3 (Figures 8 and S2). Yucasin-imposed growth inhibition was alleviated by treating those maize seedlings with exogenous IAA. Similar results were observed in CSR3 treatments, where it was found to nullify the inhibitory effect of yucasin, and enhance seedling growth.

**Discussion**

In the coming decades, increased focus would be on harmless and environmentally friendly techniques, such as the use of beneficial microorganisms in sustainable crop production (Nina et al. 2014). Large numbers of beneficial endophytic fungi have previously been investigated, with the goal of identifying candidate organisms that have the ability to enhance host plant vigour and performance (Hamilton et al. 2012; Hamilton and Bauerle 2012). For instance, endophytic fungi have been reported to have the ability to produce a wide range of biologically active secondary metabolites (Gunatilaka 2006; Aly et al. 2010; Debbab et al. 2012).

In the present study, we isolated 11 endophytic fungi from the roots of *C. sativa*, of which CSR3 was selected for further study, based on initial screening for IAA, phosphate solubilization, and siderophore production. All these properties are important from an agriculture point of view. Siderophores play important roles as a biocontrol agent and in suppression of diseases that damage host plants (Kumari et al. 2013). Phosphorus is also an important macroelement that is essential for the growth of plants. In order to reduce reliance on synthetic fertilizers, the use of phosphate-solubilizing endophytic fungal species as biofertilizers could be a successful approach (Rahi et al. 2010). The endophytic fungus *A. niger* CSR3 was identified on the basis of morphological
characteristics, sequence of ITS, and D1–D2 regions. In the past, several fungal endophytes were identified using 18S internal transcribed spacer (ITS) and D1–D2 regions of the LSU rDNAs (Ghimire et al. 2011; Khan et al. 2015). Similarly, the phylogenetic analysis NJ method revealed the same phylogenetic indicators for the CSR3 using both ITS and D1–D2 regions. These two regions generated phylogenetic trees with the same topologies and placed the CSR3 strain along with A. niger species with high bootstrap values (Figure 2).

Production of phytohormones is a key attribute enabling endophytes to promote plant growth by improving the ability of the host to tolerate and overcome different abiotic and biotic environmental stresses (Ma et al. 2016; Hamayun et al. 2017; Khan et al. 2017; Sandhya et al. 2017). Therefore, to determine the phytohormones i.e. IAA, GA, and ABA in the CSR3 strain, we used an advanced technique, GC/MS SIM, to analyze the CF for plant hormones. Current findings indicate the presence of both IAA and GA in the CF of CSR3. Furthermore, 10 different kinds of GA, including both physiologically active and inactive forms were detected in CSR3 CF. Similar results were previously reported by Hasan (2002), in that various fungal species such as A. flavus, A. niger, F. oxysporum, P. corylophilum, P. cyclopium, P. funiculosum, and R. stolonifera have the ability to produce GA and IAA. The CSR3 produces different kinds of GA in various concentrations lower than some previously reported fungal strains (Waqas et al. 2014; You et al. 2015). However, the concentration in which these hormones were produced was enough to compensate for the deficiency of GAs and IAA in mutant rice and maize seedlings exposed to hormone inhibitors, restoring their normal growth. Previously, production of GAs and IAA by endophytes was linked to culture conditions. For instance, concentration of GAs and IAA varied in the culture of the same fungus grown on various nutrients and pH (Waqas et al. 2014). Furthermore, production of GA by various endophytic fungi has been reported as the most significant characteristic of PGPF (Zhang et al. 2016; Hamayun et al. 2017).

On the basis of plant growth-promoting characteristics and detection of hormones in the CSR3 culture filtrates, we performed screening experiments by using Waito-C rice. Various endophytic fungi act as external factories of phytohormones, and therefore have a positive impact on growth attributes of Waito-C rice (Sturz et al. 2000; Guo et al. 2015; You et al. 2015). In the current study, the interaction of gibberellins produced by CSR3, and rice endogenous gibberellin (GA), abscisic acid (ABA), and jasmonic acid (JA) were investigated. These results revealed that CSR3 significantly increases plant growth attributes as well as regulates the endogenous phytohormones such as GA, ABA, and JA in waito-C rice through phytostimulation. The present investigation corroborated previous studies that reported the
inhibition or promotion of rice shoot growth due to the presence of plant growth stimulatory or inhibitory secondary metabolites in the CF of the fungus (Hamayun et al. 2009). This result is in agreement with the results of Waqas et al. (2012), who reported that endophytic fungi *Phoma gloverata* and *Penicillium sp.* produce gibberellins (GA) and IAA in the CF which significantly enhanced the growth of GA-deficient rice cultivars. After detection of GA by GC/MS SIM analysis, expression of GA biosynthesis genes was determined by using semi-quantitative RT–PCR analysis. Expression of the selected GA biosynthesis genes was compared with the positive control *F. fujikuroi*. Expression of genes *P50-1, P450-4, ggs2*, and *des* in both CSR3 and *F. fujikuroi* suggests the presence of common GA pathways in these two fungi, which include the GA-specific GGPP synthase (*ggs2*), two cytochrome P450 monooxygenase genes (*P450-1* and *P450-4*). *P450-4* encodes 2-kaurene oxidase, which acts upstream of the P450-1 encoding multifunctional monooxygenases in the GA biosynthesis pathway (Tudzynski et al. 2003).

Similar to its positive impact on GAs, CSR3 inducted *Waigo-C* rice and enhanced ABA production. Previous reports have indicated that different groups of endophytic fungi might have caused different effects on the endogenous ABA levels of plants (Mauch-Mani and Mauch 2005). Various outcomes have been obtained previously, in that plants infected with endophytic fungi had higher ABA content (Danneberg et al. 1993). Comparative information was obtained for ABA contents in roots of soybean (*Glycine max*) plants. In plants inoculated with fungi, the ABA content dependably increased compared with ABA contents in non-inoculated control plants (Murakami-Mizukami et al. 1991; Meixner et al. 2005). Similarly, Herrera-Medina et al. (2007) reported that plants associated with endophytic fungi had higher ABA content in leaves and roots, while the ABA content was low in control plants. Hao et al. (2010) also investigated increased ABA production in plants associated with a fungal endophyte. An interaction between ABA and other signaling pathways, for example, JA and sugar, essential for fungal–plant interactions, has been reported previously (Audenaert et al. 2002; León and Sheen 2003). The ABA signaling pathway associates antagonistically with the JA signaling pathway, and *vice versa*, to tweak plant development (Beaudoin et al. 2000; Ghassemian et al. 2000) and plant disease resistance (Anderson et al. 2004).

On the other hand, the endogenous JA levels were reduced in plants treated with CSR3. This reduction of JA in associated dwarf rice indicates the involvement of fungal GA and IAA (Kang et al. 2015; Waqas et al. 2015). Decreased JA biosynthesis under the influence of ectomycorrhizal fungus, *Paxillus involutus*, has been demonstrated previously (Luo et al. 2011). Both GA and JA play antagonistic roles to regulate balance of energy allocation between growth and defence (Yang et al. 2012). The endophyte CSR3 may also reduce JA signaling and levels in a GA-independent pathway. Furthermore, endophyte-induced reduction in JA signaling and level is
important for growth-promoting potential of CSR3 because JA antagonizes the positive effect of GA in many of plant growth aspects (Hou et al. 2010; Yang et al. 2012; Hou et al. 2013)

To evaluate the growth-promoting effect of CSR3 as a GA and IAA producer, its impact was studied on maize seedlings treated with IAA and GA inhibitors. Uniconazole and yucasin were used to suppress the production of GA and auxins by maize seedlings, respectively. These inhibitors are known to suppress the biosynthesis of their target phytohormones in various plants. Uniconazole, a GA biosynthesis inhibitor, is a triazole-type of compound (Rademacher 2000). Uniconazole inhibits P450 ent-kaurene oxidase (CYP701) and catalyzes the oxidation of ent-kaurene to ent-kaurenoic acid in the biosynthesis of GA (Izumi et al. 1985; Burden et al. 1987). It was reported that yucasin mainly targets a plant FMO or FMO like protein, YUCCA (YUC) takes part in biosynthesis of IAA by means of catalyzing the hydroxylation of the amino group of tryptamine (Dai et al. 2013). Tryptophan aminotransferase TAA1 converts tryptophan to IPyA, and YUC changes IPyA to IAA, the main source of auxin in plants. Yucasin is a strong inhibitor of IAA biosynthesis as it is a competitive inhibitor of YUC having greater affinity of binding than IPyA, which is the substrate of YUC (Kakei et al. 2015). Therefore, yucasin was reported to be a strong inhibitor of YUC enzymes in vitro as well as in plants. It is also helpful to identify missing components of auxin biosynthesis and signaling (Dai et al. 2013). It acts as a nutrient and metabolite signaling molecule that stimulates hormone transduction pathways and is also involved in the responses to a number of stresses (Couée et al. 2006). Being an important component of photosynthesis chlorophyll is needed for absorption of sunlight and it is reported that inhibition of chlorophyll biosynthesis can lead to some plants’ growth retardation and also cell death (Hörtstein and Kräutler 2011). On the other hand, exogenous application of GA, and IAA mitigates plants endogenous pool of GA and IAA, respectively, facilitating the associated physiological stress and restoring plant growth and related attributes (Figure 8).

Interestingly, in our results CSR3 application mitigated the inhibitory effect of both yucasin and uniconazol. Furthermore, the growth attributes and secondary metabolite of maize seedlings associated with the endophytic fungi were significantly enhanced and the inhibitory effect of both uniconazol and yucasin was restored. It has previously been shown that exogenous applications of GA-like substance reverses the inhibitory effect of uniconazol in rice plant (Izumi et al. 1984). Additionally, the application of exogenous GA, and IAA mitigates plants endogenous pool of GA and IAA, respectively, facilitating the associated physiological stress and restoring plant growth and related attributes (Nishimura et al. 2014). Our results revealed that CSR3 facilitates the host plant to enhance all growth attributes and mitigated the inhibitory effect caused by uniconazol and yucasin. Therefore, this endophyte may have a potential use in agriculture to alleviate environmental stress and reduce agricultural cost in the future.

Conclusion

In the current study, it was found that the endophytic A. niger CSR3 has best potential for a wide range of applications as it has phosphate solubilizing, siderophore production activity, and produces well-characterized plant growth regulators IAA and GA. Its use as PGPF significantly improved seedling growth due to its phytohormone–producing ability. Furthermore, CSR3 has the ability to colonize rice roots and significantly enhance regulating GA, ABA, and JA contents in Waito-C rice. Furthermore, co-application of CSR3 on maize seedling with uniconazol and yucasin revealed that it can mitigate the inhibitory effect of both inhibitors. Therefore, the selected strain CSR3 is an attractive potential biofertilizer to enhance the growth of important crops.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through Agriculture, Food and Rural Affairs Research Center Support Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) [grant number 716001-7].

References

Aly AH, Debbab A, Kjer J, Proksch P. 2010. Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. Fung Divers. 41(1):1–16.

Anderson JP, Badruzzaufari E, Schenk PM, Manners JM, Desmond OJ, Ehliert C, Maclean DJ, Ebert PR, Kazan K. 2004. Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in arabisopsis. Plant Cell. 16(12):3460–3479.

Angelard C, Colard A, Niculita-Hirzel H, Croll D, Sanders IR. 2010. Segregation in a mycorrhizal fungus alters rice growth and symbio-
sis-specific gene transcription. Curr Biol. 20(13):1216–1221.

Ansari MW, Trivedi DK, Sahoo RK, Gill SS, Tuteja N. 2013. A critical review on fungi mediated plant responses with special emphasis to Piriformospora indica on improved production and protection of crops. Plant Physiol Biochem. 70:403–410.

Asaf S, Khan MA, Khan AL, Waqas M, Shahzad R, Kim A-Y, Kang S-M, Lee F-J. 2017. Bacterial endophytes from arid land plants regulate endogenous hormone content and promote growth in crop plants: an example of Sphingomonas sp. and Serratia marcescens. J Plant Interact. 12(1):31–38.

Audaenart K, De Meyer GB, Höfte MM. 2002. Absciscic acid determines basal susceptibility of tomato to Botrytis cinerea and suppresses salicylic acid-dependent signaling mechanisms. Plant Physiol. 128 (2):491–501.

Beaudoin N, Serizet C, Gosti F, Giraudat J. 2000. Interactions between abscisic acid and ethylene signaling cascades. Plant Cell. 12 (7):1103–1116.

Burden RS, Carter GA, Clark T, Cooke DT, Croker SJ, Deas AH, Hedden P, James CS, Lenton JR. 1987. Comparative activity of the enantio-
mers of triadimenol and paclobutrazol as inhibitors of fungal growth and plant sterol and gibberellin biosynthesis. Pest Manage Sci. 21 (4):253–267.

Chan C-X, Teo S-S, Ho C-L, Othman RY, Phang S-M. 2004. Optimisation of RNA extraction from Gracilaria changii (Gracilariales, Rhodophyta). J Appl Phycol. 16(4):297–301.

Costa JM, Loper JE. 1994. Characterization of siderophore production by the biological control agent Enterobacter cloacae. Mol Plant Microbe Interact. 7(4):440–448.

Couée I, Salomon C, Gouesbet G, El Amrani A. 2006. Involvement of sol-
uble sugars in reactive oxygen species balance and responses to oxi-
dative stress in plants. J Exp Bot. 57(3):449–459.

Crozier A. 2000. Biosynthesis of hormones and elicitor molecules. Biochemistry and molecular biology of plants. Dai X, Mashiguchi K, Chen Q, Kasahara H, Kamiya Y, Ojha S, DuBois J, Ballou D, Zhao Y. 2013. The biochemical mechanism of auxin
biosynthesis by an Arabidopsis YUCCA flavin-containing monoxygenase. J Biol Chem. 288(3):1448–1457.

Danneberg G, Latus C, Zimmer W, Hundeshagen B, Schneider-Poetsch H, Bothe H. 1993. Influence of vesicular-arbuscular mycorrhiza on phytohormone balances in maize (Zea mays L.). J Plant Physiol. 141(1):33–39.

Davies Pj. 2010. The plant hormones: their nature, occurrence, and functions. Plant hormones. Springer; p. 1–15.

Debabb A, Aly AH, Proksch F. 2012. Endophytes and associated marine derived fungi – ecological and chemical perspectives. Fungal Divers. 57(1):45–83.

Eom S-H, Jin C-W, Park H-J, Kim E-H, Chung I-M, Kim M-J, Yu C-Y, Cho D-H. 2007. Far infrared ray irradiation stimulates antioxidant activity in Vitis flexuosa THUNB. berries. Kor J Med Crop Sci. 15(5):319–323.

Fulchieri M, Luncangeli C, Bottini R. 1993. Inoculation with Azospirillum lipoforum affects growth and gibberellin status of corn seedling roots. Plant Cell Physiol. 34(8):1305–1309.

Ghassemian M, Nambar M, Yu H. 2013. Crosstalk between GA and JA signaling. Plant Physiol. 162(3):1107–1116.

Hamayun M, Hussain A, Khan SA, Kim HY, Khan AL, Waqas M, Irshad N, Eom S-H, Jin C-W, Park H-J, Kim E-H, Chung I-M, Kim M-J, Yu C-Y, Lee I-J. 2015. Ameliorative symbiosis of endophyte (Penicillium funiculosum L.) under salt stress; elevated plant growth of Glycine max L. Plant Physiol Biochem. 94(8):852–861.

Hamayun M, Khan SA, Al-Harrasi A, Al-Rawahi A, Lee I-J. 2015. Endophytic fungi: resource for gibberellins and crop abiotic stress resistance. Crit Rev Biotechnol. 35(1):62–74.

King RW, Evans LT. 2003. Gibberellins and flowering of grasses and cereals: proving open the lid of the ‘florigen’ black box. Annu Rev Plant Biol. 54(1):307–338.

Kumari P, Khanna V, Kaur L, Mukihiba B. 2013. Characterization of functionality traits of plant growth promoting rhizobacteria antagonistic to Fusarium oxysporum f. sp. ciceris. Plant Dis Res. 28(1):11–15.

León P, Sheen J. 2003. Sugar and hormone connections. Trends Plant Sci. 8(3):110–116.

Lister E, Wilson P. 2001. Measurement of total phenolics and ABTS assay for antioxidant activity (personal communication). Lincoln: Crop Research Institute; p. 235–239.

Lopez-Carrillo Z, Torres MS, Singh AP, Vorsa N, Gianfagna T, Meyer W, White JF, Jr. 2009. Phenolic, flavonoid and antioxidant profiling for cool-season grasses with and without endophyte. Proceedings of the 18th Annual Riegert's Turfgrass Symposium. 190–196.

Rayhaan M, Shahzad R, Choi K-S, Lee I-J. 2015. Gibberellin-producing Serratia nematodiphila PEI1011 ameliorates low temperature stress in Capsicum annum L. Eur J Soil Biol. 60:85–93.

Kato K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 30(4):772–780.

Mauch-Mani B, Mauch F. 2005. The role of abscisic acid in plant responses to water stress. Crit Rev Plant Sci. 24(4):328–353.

McCloud ES, Baldwin IT. 1997. Herbivory and caterpillar regurgitants amplify the wound-induced increases in jasmonic acid but not nicotine in Nicotiana sylvestris. Planta. 203(4):435–443.

Narasimhan N, Thomas W, Prem S. 2014. Beneficial organisms for nutrient uptake: VFRC report 2014/1, virtual fertilizer research center. Washington, DC: Wageningen Academic Publishers.
