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RESEARCH ARTICLE

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Genetic Diversity of *Epicoccum nigrum* and its Effects on *Fusarium graminearum*

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ABSTRACT

Epicoccum nigrum is a saprophytic or endophytic fungus that is found worldwide. Because of the antagonist effects of *E. nigrum* on many plant pathogens, current studies on *E. nigrum* have focused on the development of biological control agents and the utilization of its various metabolites. In this study, *E. nigrum* was collected from a wheat field, and its genetic diversity was analyzed. Phylogenetic analyses identified 63 isolates of *E. nigrum* divided into seven groups, indicating a wide genetic diversity. Isolates antagonized the wheat pathogen *Fusarium graminearum*, and reduced disease symptoms caused by *F. graminearum* in wheat coleoptiles. Moreover, pretreatment of wheat coleoptiles with *E. nigrum* induced the upregulation of pathogen-related (PR) genes, PR1, PR2, PR3, PR5, PR9, and PR10 in wheat coleoptiles responding to *F. graminearum* invasion. Overall, this study indicates that *E. nigrum* isolates can be used as biological pathogen inhibitors applied in wheat fields.

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Antagonism; *Epiccocum nigrum*; *Fusarium graminearum*; genetic diversity

1. Introduction

Epicoccum nigrum is a saprophytic and endophytic ascomycete fungus that is found worldwide and exerts antagonistic effects on various plant pathogens (i.e., *Sclerotinia sclerotiorum*, *Pythium irregulare*, and *Monilinia* spp.) [1–4]. Because of the antagonist effects of *E. nigrum*, the development of biological control agents from *E. nigrum* and the utilization of various metabolites generated by *E. nigrum* is well underway [5,6]. Similar to other prevalent fungal genera, *E. nigrum* is usually isolated from the inner tissues of several plants (i.e., sugarcane, peach, and wheat) [6–8].

Fusarium graminearum (teleomorph *Gibberella zeae*) also has a high frequency of wheat colonization [9,10]. It is a pathogen that causes Fusarium head blight (FHB) in wheat and results in serious damage to crop production worldwide [11,12]. Cereals with FHB are usually contaminated with mycotoxins, such as zearalenone and trichothecenes that result in mycotoxicosis in humans [13,14]. Previous *in vitro* studies have shown that endophytic *E. nigrum* can inhibit the growth and mycotoxin production of *F. graminearum* [2,15,16]. However, there is still insufficient on researches whether *E. nigrum* effectively prevents *F. graminearum* infection in host plants, as well as on the diversity of *E. nigrum* populations.

Many studies have shown that endophytic fungi have plant growth promoting (PGP) traits that can improve plant growth and production. *Fusarium petersiae* and *Penicillium chrysogenum* isolated from *Cheilanthes vellea* produce high levels of indole-3-acetic acid (IAA) and siderophore that promote wheat growth [17]. *Pestalotiopsis microspore* is derived from important medicinal plants and induces pathogen resistance in tomato plants while also improving fruit weight [18]. *Epiccocum nigrum* is also considered to have PGP properties and the treatment of *E. nigrum* can improve the emergence rate of sorghum seeds, increase shoot and root length, and increase the weight of sorghum [19]. However, interactions between *E. nigrum* and host plants are not well studied. Genetic diversity is also not well studied and there is no clear standard for its classification [1]. Therefore, it is necessary to investigate the morphological, genetic, and biological characteristics of *E. nigrum* to investigate its genetic diversity and species classification.

The objectives of this study were to analyze the genetic diversity of *E. nigrum* in a Korean wheat field and to identify wheat isolates that carry antagonistic activity against *F. graminearum*. More specifically, we tested the PGP effects of *E. nigrum* to determine whether it has the possibility of being epiphytic or endogenous as well as whether it can

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affect plant growth. This study showed the antagonist effects of *E. nigrum* against *F. graminearum* in wheat plants and the influence of *E. nigrum* on wheat immunity.

2. Materials and methods

2.1. Isolation of fungal isolates from a wheat field

Pentachloronitrobenzene (PCNB) medium [13] was used to collect *E. nigrum* from a wheat field at Dong-A Research Farm in Gimhae, Korea (35.29°N, 127.75°E) as previously described [20]. Plates with PCNB medium were placed at both wheat height and ground level for 30 min. The plates were then incubated at 25 °C for 2 days, and fungal isolates with the colony morphology of *E. nigrum* were transferred to a complete medium (CM). Sample collection was carried out in both 2019 and 2020. The isolates used in this experiment were stored in 20% glycerol at −80 °C.

2.2. Identification of fungal isolates

Previously described primers for highly conserved fungal rRNA genes (i.e., ITS1 and ITS4) were used to amplify fungal DNA [21,22]. Amplified PCR products were confirmed using electrophoresis and were sequenced at Macrogen (Seoul, Korea). After manually checking the obtained sequences and correcting any errors, the nucleotide sequences were assembled using the SeqMan (DNASTAR, Madison, WI, USA) program. Finally, a basic local alignment searching tool (i.e., BLASTN) was used to identify assembled nucleotide sequences at the National Center for Biotechnology Information. Primers used in this study are listed in Table 1.

Table 1. Primers used in this study.

Primer name	Sequence (5′→3′)
For identification [23,24]	
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC
EF_F	GCYCCYGGHCAYCGTGAYTTYAT
EF_R	ATGACACCRACRGCRACRGTYTG
LSU_F	ACCCGCTGAACTTAAGC
LSU_R	CGCCAGTTCTGCTTACC
SSU_F	GTAGTCATATGCTTGTCTC
SSU_R	CTTCCGTCAATTCCTTTAAG
For qRT-PCR [25]	
β-actin_F	AAATCTGGCATCACACTTTCTAC
β-actin_R	GTCTCAAACATAATCTGGGTCATC
PR1_F	CTGGAGCACGAAGCTGCAG
PR1_R	CGAGTGCTGGAGCTTGCAGT
PR2_F	CTCGACATCGGTAACGACCAG
PR2_R	GCGGCGATGTACTTGATGTTC
PR3_F	AGAGATAAGCAAGGCCACGTC
PR3_R	GGTTGCTCACCAGGTCCTTC
PR5_F	ACAGCTACGCCAAGGACGAC
PR5_R	CGCGTCCTAATCTAAGGGCAG
PR9_F	GAGATTCCACAGATGCAAACGAG
PR9_R	GGAGGCCCTTGTTTCTGAATG
PR10_F	TTAAACCAGCACGAGAAACATCAG
PR10_R	ATCCTCCCTCGATTATTCTCACG

2.3. Observation of colony and conidia morphology

The mycelial growth of fungal isolates was measured on potato dextrose agar (PDA) after cultivation for 7 days at 25 °C in the dark. For conidia production, each isolate was inoculated onto CM agar and was incubated under near-ultraviolet light (i.e., 20 W and 50 lux) for 7 days at 25 °C. Mycelia were subsequently gathered using 10 ml of distilled water (DW) and were filtered using sterilized gauze. Conidia were then observed using an Olympus BX50 microscope (Olympus, Tokyo, Japan).

2.4. Phylogenetic analyses

Phylogenetic analyses of *E. nigrum* isolates were performed using an internal transcribed spacer (ITS), the large (LSU) and small subunit (SSU) of rDNA, and translation elongation factor 1 alpha (EFα) [23]. *Fusarium graminearum* and *Petrakia echinata* (GenBank Accession No. KR047057.1 and MN310550.1) were used as outgroup taxa. Sequences of the ITS, LSU, SSU, and EFα regions, as well as concatenated sequences, were aligned using the MEGA X software; a phylogram was generated using MEGA X, based on the maximum likelihood (ML) method. Support for branches was evaluated using 1000 bootstrap values [26,27].

2.5. Detached leaf assay

Wheat seeds were soaked in 70% ethanol for 1 min soaked in 5% sodium hypochlorite for 3 min and were washed twice with DW. Sterilized wheat seeds were germinated at 25 °C for 1 day, and were transferred to the growth chamber with 25 °C, 60% moisture, and 12 h each of light and darkness. After 14-day cultivation, wheat leaves were cut to ~8 cm, and the front of the leaves was placed on a 1% agar medium containing 0.5 mM benzimidazole [28]. A 1 mm incision was made at the center of the wheat leaf, and a 5 mm mycelium fragment was placed in the cut. The wheat leaves were then placed in the growth chamber and mycelium fragments were removed after 3 days. Finally, the Fiji (http://fiji.sc/) program was used to measure the lesion area of wheat leaves after an additional 3-day incubation. *Fusarium graminearum* and *Fusarium oxysporum* were used as positive and negative controls, respectively.

2.6. Determination of the in vitro antagonistic effects of E. nigrum against F. graminearum

The antagonistic effects of *E. nigrum* isolate on *F. graminearum* were tested on CM agar. Isolates were inoculated in the center of CM agar and were cultured for 4 days at 25 °C. *Fusarium graminearum* was then inoculated at the edge of CM agar and was cultured at 25 °C. Mycelial growth of *F. graminearum* was measured at 7 days.

2.7. Antagonistic effects of E. nigrum against F. graminearum in wheat

Each isolate was inoculated into wheat coleoptiles and seeds [29,30]. For coleoptile inoculation, sterilized wheat seeds were cultivated at 25 °C for 1 day. Germinated wheat seeds were then transferred to the growth chamber at 25 °C for 12 h each of light and darkness. After 24 h, 1 mm of the tops of wheat coleoptiles was cut off, and 1 µl of the conidia suspensions (1×10^6 conidia/ml in 0.01% Tween-20) of each isolate was inoculated. After an additional 1 day of incubation, 1 mm from the top of the treated coleoptile was removed and reinoculated with 1 µl of the *F. graminearum* conidia suspension (1×10^6 conidia/ml in 0.01% Tween-20). Coleoptiles were then cultured in the growth chamber for 7 days.

For *F. graminearum* inoculation on wheat seeds, 5 g of sterilized wheat seeds were immersed in *E. nigrum* conidia suspension (1×10^6 conidia/ml in 0.01% Tween-20) and shaken using a rocking shaker at 50 rpm for 24 h in room temperature. Wheat seeds were placed in the growth chamber for 2 days at 25 °C under 12 h each of light and darkness. The top 1 mm of wheat coleoptiles were then removed and 1 µl of *F. graminearum* conidia suspension (1×10^6 conidia/ml in 0.01% Tween-20) was inoculated. Coleoptiles were then placed in the growth chamber for 7 days and disease lesions on the stems were measured.

2.8. Quantitative real-time PCR (qRT-PCR)

To validate differently expressed genes related to pathogen-related (PR) proteins, qRT-PCR was performed. The top 1 mm of 2-day-old wheat coleoptiles were cut off, inoculated with 1 µl of *E. nigrum* conidia suspension (1×10^6 conidia/ml in 0.01% Tween-20), and then cultured in a growth chamber for 48 h. Coleoptiles were subsequently harvested at 0, 4, 8, 12, 24, and 48 h after inoculation and were ground under liquid nitrogen. Total RNA was extracted from each sample using the Easy-spin Total RNA Extraction Kit (iNtRON Biotechnology, Seongnam, Korea), in accordance with manufacturer's protocol. cDNA was generated using ReverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Synthesized cDNA was diluted to 100 ng/µl, of which 2 µl was used for qRT-PCR. Relative transcription levels were normalized using an internal reference gene, β-actin [31]. In addition, 1 µl of the *F. graminearum* conidia suspension (1×10^6 conidia/ml in 0.01% Tween-20) was inoculated into wheat coleoptiles 24 h after *E. nigrum* was inoculated.

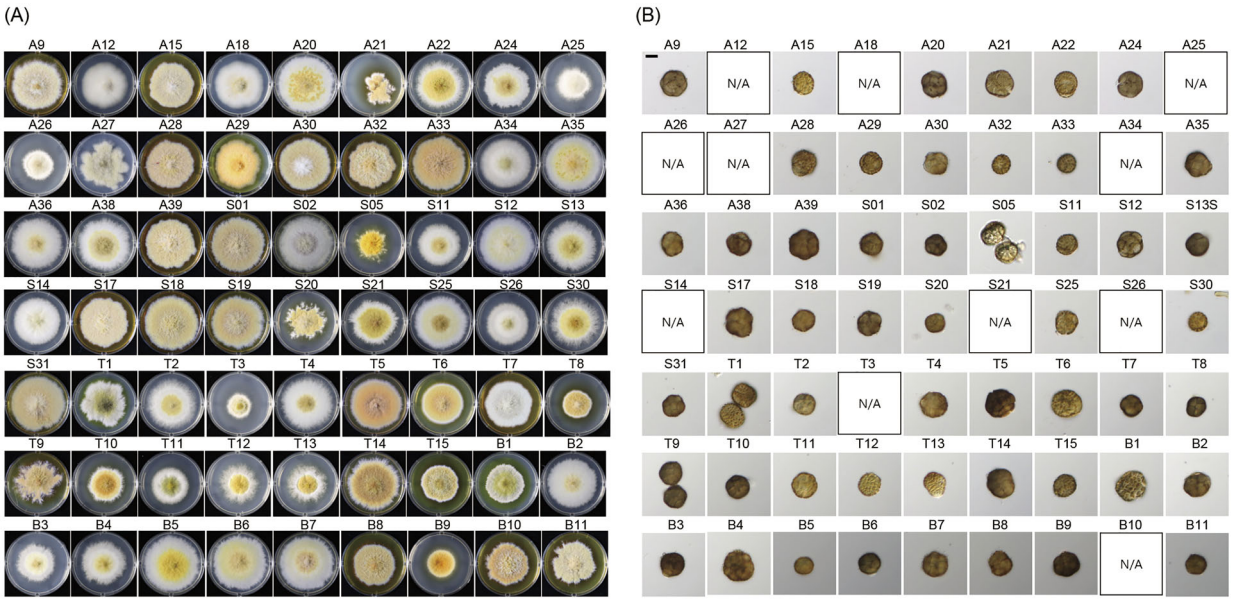

Figure 1. Colonial and conidial morphology of *Epicoccum nigrum* isolates. (A) Isolates were cultivated on PDA for 7 days at 25 °C; (B) Isolates were incubated on CM under near UV (20 W and 50 lux) for 7 days at 25 °C. Scale bar = 10 µm.

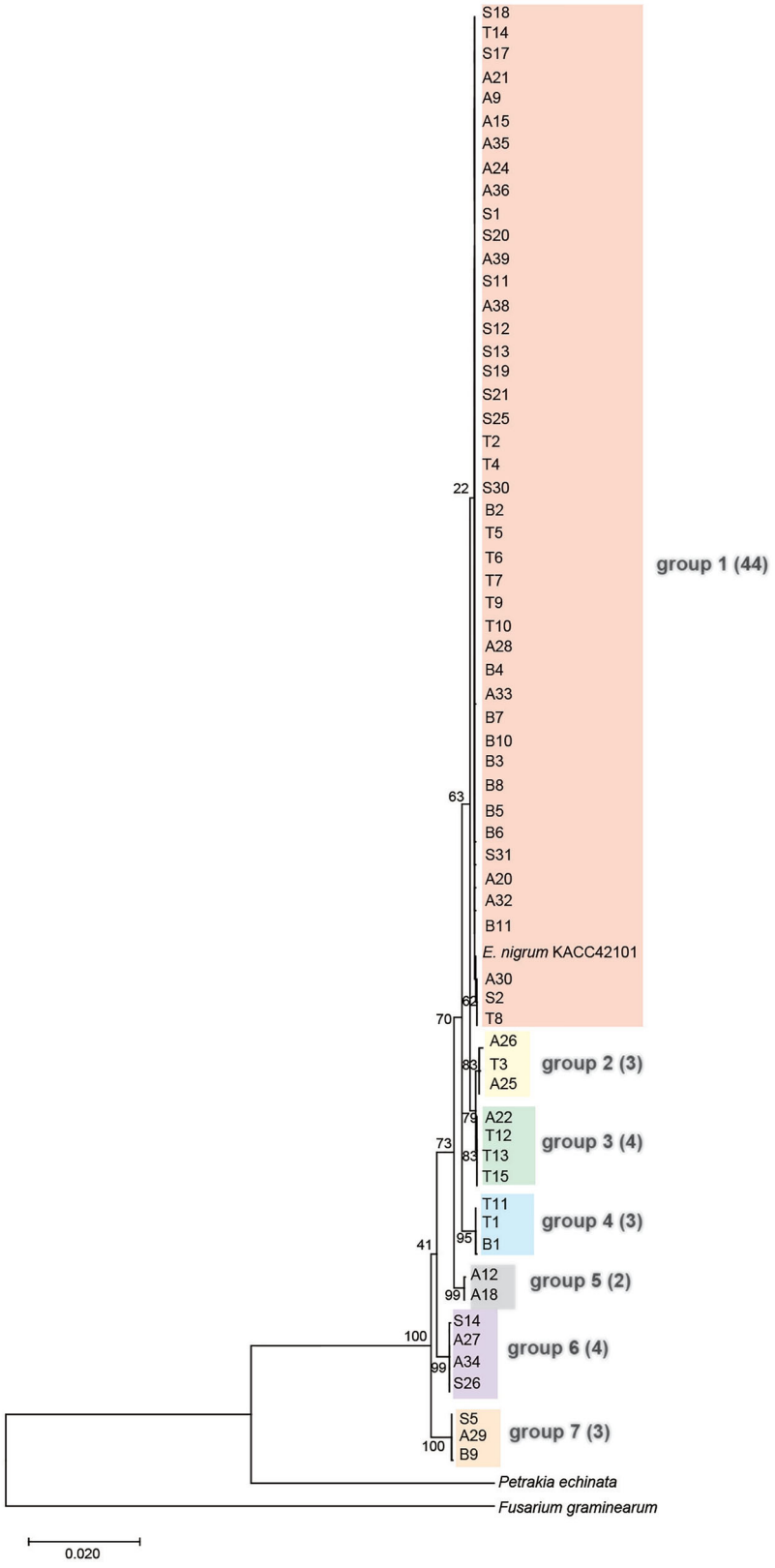

Figure 2. Phylogenetic analysis of *Epicoccum nigrum* isolates. The phylogenetic tree was inferred using concatenated ITS, LSU, SSU, and EFα sequences of 63 isolates. Sequences were aligned using Clustal W in MEGA X, which was used to perform 1000 bootstrap phylogenetic analyses using maximum likelihood.

2.9. Production of IAA

Potato dextrose broth (PDB), supplemented with 5 mM L-tryptophan, was used to test the IAA production of each isolate [32]. Inoculated flasks were incubated at 200 rpm for 5 days at 25 °C. After incubation, 1 ml of the culture medium was centrifuged at 13,000 rpm for 10 min; 100 µl of the supernatant was combined with 200 µl of Salkowski's reagent

(i.e., 1 ml of 0.5 M $FeCl_3$ and 49 ml of 35% perchloric acid) and incubated for 20 min at room temperature. IAA production was determined using a spectrophotometer at 530 nm. *Colletotrichum gloeosporioides* [33] and *F. graminearum* were used as controls.

3. Results

3.1. Identification of fungal isolates

A total of 63 strains were identified as *E. nigrum*. The isolates had white or yellow-brown colonies on PDA, and 31 of them showed yellow or dark pigments on PDA (Figure 1(A)). *Epiccocum nigrum* generally produces dark brown spherical conidia, which are difficult to induce under normal culture conditions [1,34]. In CM and under near-UV, 52 of the 63 isolates produced conidia successfully, whereas 11 isolates did not produce conidia at all (Figure 1(B)).

3.2. Phylogenetic analyses

Phylogenetic analyses were performed on the ITS, LSU, SSU, and EFα regions with a length of 528, 969, 1004, and 858 bp, respectively, as well as the outgroup sequences with the same length as each region. Although most strains of isolates were divided into a similar pattern, there were certain differences among analyses of four different rDNA regions (Figures S1–S4). To compromise these differences, we conducted an additional combined phylogenetic analysis, and finally, the isolates were divided into seven groups (Figure 2).

3.3. Antagonist effects of E. nigrum isolates on F. graminearum

Before confirming the antagonist effects of *E. nigrum*, we first confirmed the virulence of *E. nigrum* isolates in wheat, which showed that isolates did not cause disease in wheat (Figure 3). In the dual culture of *E. nigrum* and *F. graminearum*, the isolates

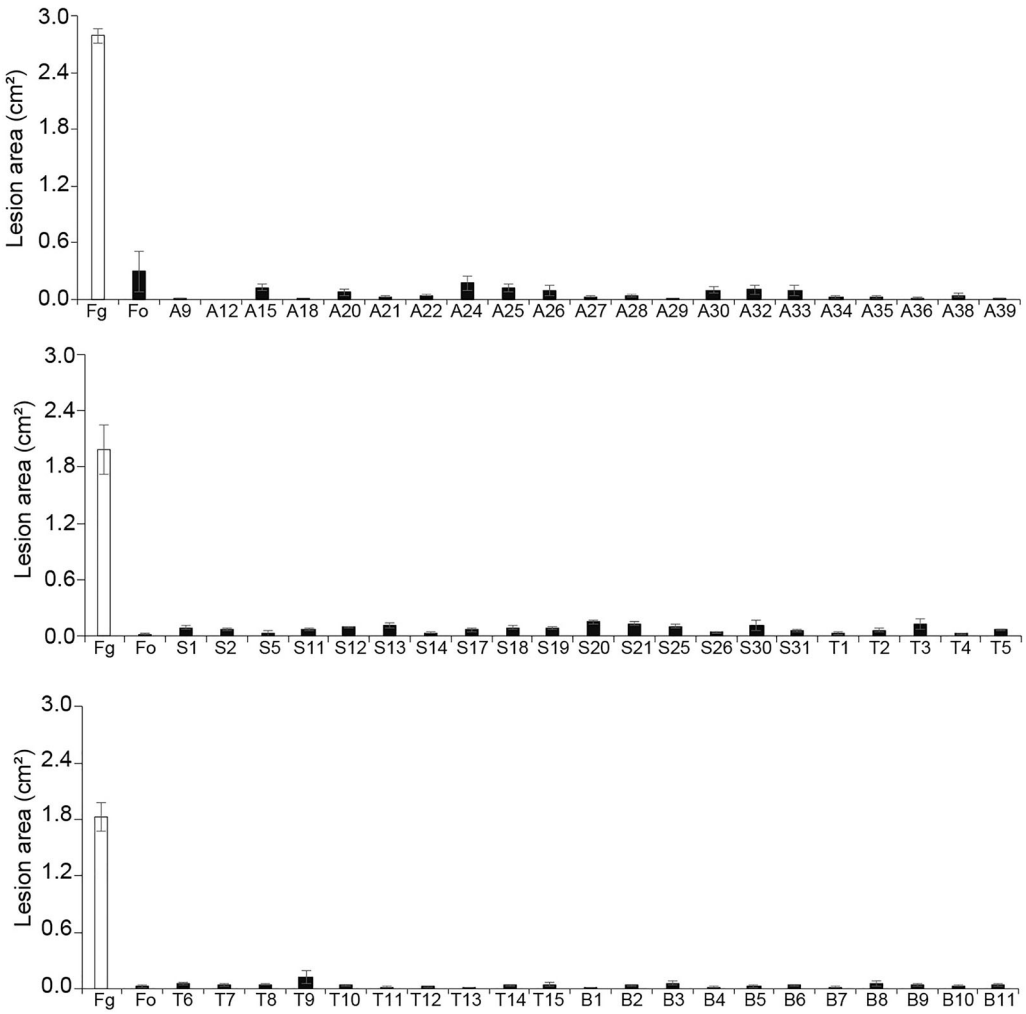

Figure 3. Pathogenicity of *Epicoccum nigrum* isolates on wheat leaves. Mycelia plugs of each strain were placed on the middle of wheat leaves and were cultivated for 3 days at 25 °C. The plugs were then removed and wheat leaves were cultivated for another 3 days. Lesion areas were measured using the Fiji program. The error bar represents a standard deviation from five replicates. Fg, *Fusarium graminearum*; Fo, *F. oxysporum*.

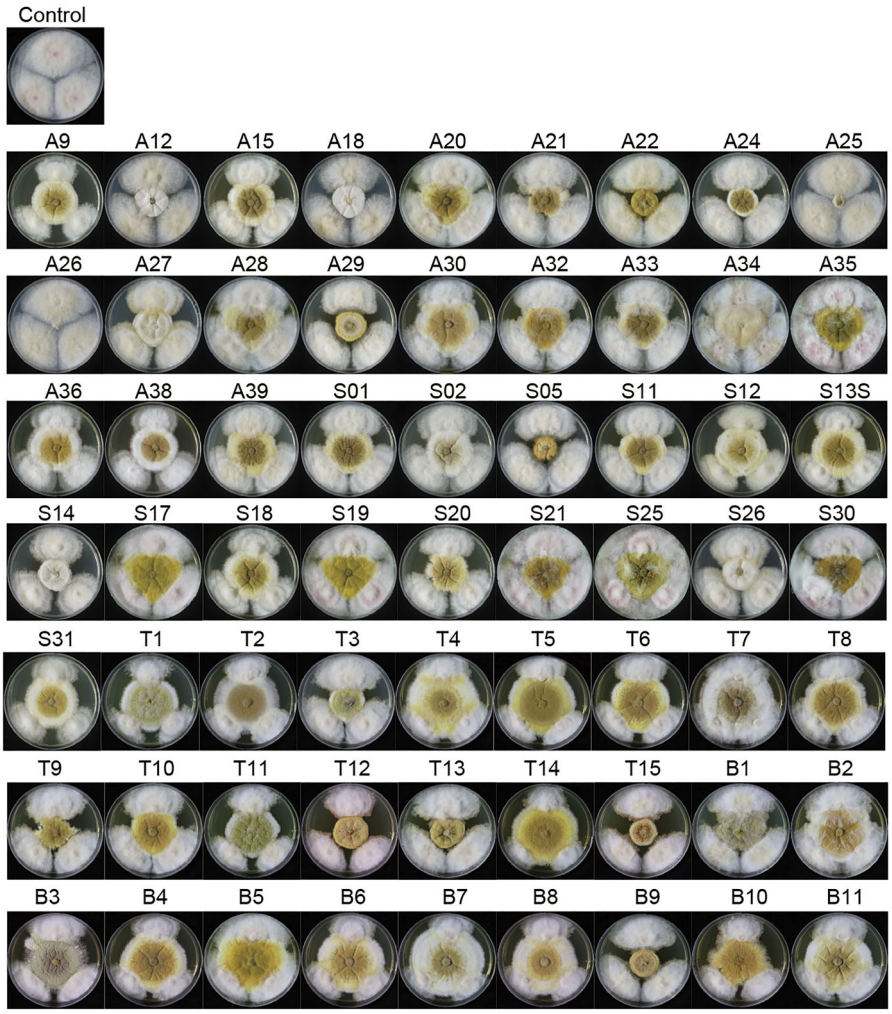

Figure 4. Antagonistic activity of *Epicoccum nigrum* isolates against *Fusarium graminearum*. Each isolate was inoculated in the center of the CM and was cultured for 4 days at 25 °C. *Fusarium graminearum* was then inoculated at the edge of the CM and cultured for another 7 days.

showed antagonism to *F. graminearum*, excluding certain strains (i.e., A20, A25, A26, A28, A34, A35, S17, S19, S21, S25, and S30) (Figure 4). When wheat was treated with isolates, the lengths of lesions were significantly shorter than those in the control that was not treated with *E. nigrum*. Strains A33, A39, and S11 showed the strongest inhibition in the experiment (Figure 5).

3.4. Effects of E. nigrum on PR gene expression

We inoculated the coleoptiles with isolates of A33 and A39 that showed high antagonistic effects on *F. graminearum* in the coleoptile test and inoculated with *F. graminearum* after 24 h to confirm the differential expression of PR genes in wheat coleoptiles. Results of the qRT-PCR showed that PR genes in wheat coleoptiles inoculated with only *E. nigrum* were not differentially transcribed with the control group that was not inoculated with *E. nigrum*. However, the transcription of PR genes in coleoptiles treated with *E. nigrum* was upregulated 24 h after *F. graminearum* inoculation, as compared to the control groups without *E. nigrum* inoculation (Figure 6).

3.5. IAA production of isolates

All 63 isolates produced IAA (Figure 7) and most produced 21-41 mg/l IAA. None of those produced more IAA than *C. gloeosporioides* and *F. graminearum* also produced higher IAA than most *E. nigrum* isolates tested in this study (Table 2).

4. Discussion

Epicoccum nigrum is distributed in different types of soils and host plants. Although *E. nigrum* has been described as a weak plant pathogen in a range of plants, the species is considered a saprophytic fungus and can exhibit an endophytic lifestyle [7,35]. *Epiccocum nigrum* has also been used as a biocontrol agent against certain pathogens in peaches, nectarines, sunflowers, and other plants [36–39].

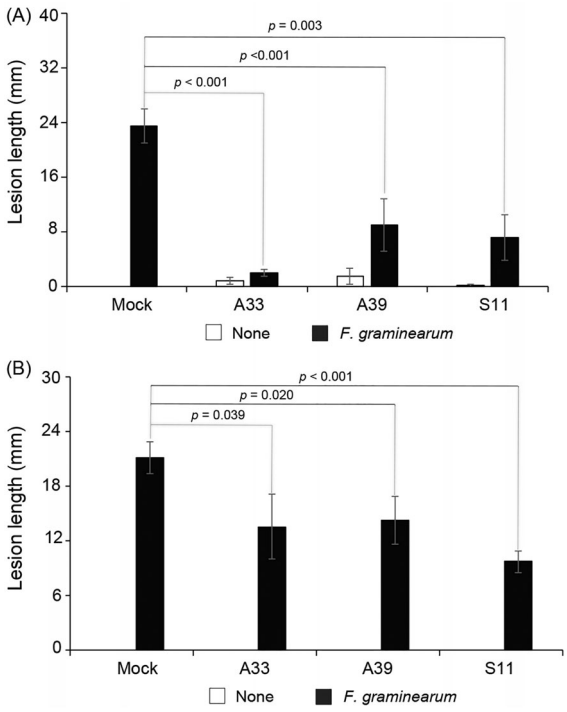

Figure 5. Disease control activity of *Epicoccum nigrum* isolates against *Fusarium graminearum* in wheat coleoptiles. (A) Tops of the 2-day-old wheat coleoptiles (i.e., 1 mm) were cut off and conidia suspensions of each isolate was injected. Coleoptiles were cultured in the growth chamber at 25 °C for 24 h, after which the tops of coleoptiles were removed again and the conidia suspension of *F. graminearum* was inoculated. Lesion length was measured 24 h after *F. graminearum* inoculation; (B) Surface sterilized wheat seeds were immersed in conidia suspensions of each isolate and were shaken using a rocking shaker for 24 h. Wheat seeds were then transferred into the growth chamber and were cultured for 2 days. The top 1 mm of wheat coleoptiles were removed and the conidia suspension of *F. graminearum* was inoculated. Lesion length was measured 24 h after *F. graminearum* inoculation. Error bar represents standard error and statistical analyses were performed via *t*-test.

Although *E. nigrum* was only collected at a single location in this study, the isolates showed diverse morphological differences and were divided into multiple groups. The isolates belonging to groups 2–7 showed similar mycelial growth and conidial morphology and isolates belonging to group 1 showed diverse morphological characteristics, which might indicate that group 1 can be further divided into more groups; these results suggest that *E. nigrum* has high genetic diversity.

Previous studies on *E. nigrum* primarily focused on its biological control properties. This fungus produces antimicrobial agents such as 2-methyl-3-nonyl prodiginine and Bis (2-ethylhexyl) phthalate against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* [40]. Additionally, *E. nigrum* isolated from maize can inhibit the mycelial growth of *F. graminearum*, and also reduce the yield of trichothecenes and zearalenone produced by *F. graminearum* in maize [16]. Similarly, most *E. nigrum* isolates collected during this study showed antagonistic effects against *F. graminearum* (Figure 4). Moreover, the pretreatment of wheat seeds with isolated strains resulted in the inhibition of disease progression by *F. graminearum* (Figure 5).

Plants resist pathogen infection through a series of defense mechanisms, such as pattern-triggered immunity and effector-triggered immunity [41,42]. Effector-triggered immunity is caused by effector proteins that are secreted by pathogenic fungi and these effector proteins are key components of fungal virulence in plants [43,44]. The efficiency of PR proteins in plant–fungal pathogen interactions has been widely recognized and there is a growing list of

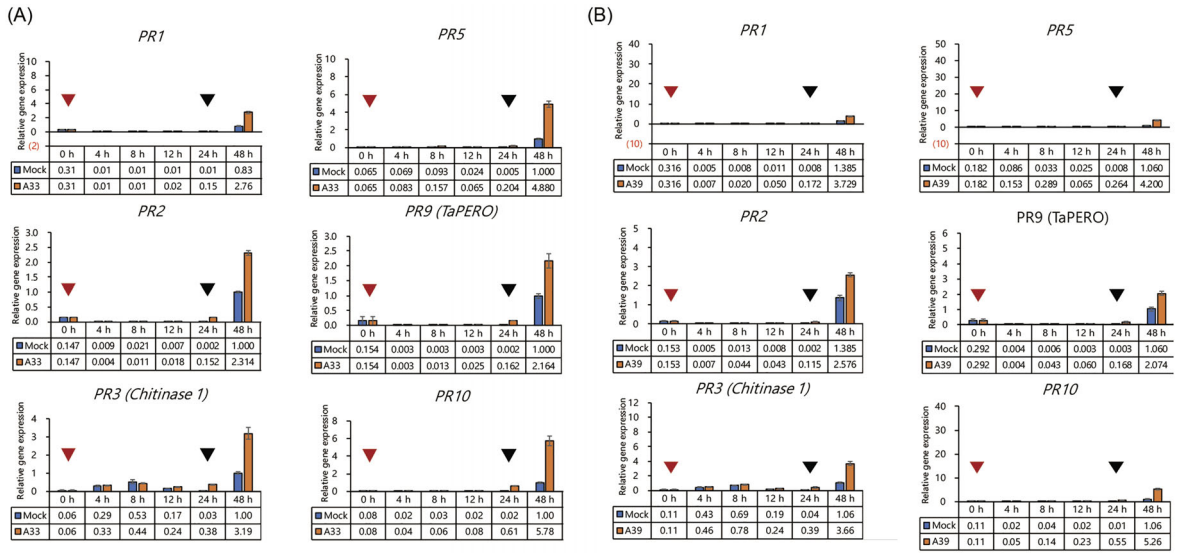

Figure 6. Effect of *Epicoccum nigrum* isolates on wheat PR gene expression. Tops of the 2-day-old wheat coleoptiles were cut off and the conidia suspensions of *E. nigrum* A33 (A) and *E. nigrum* A39 (B) were injected into them. Coleoptiles were then cultured at 25 °C in the growth chamber for 24 h. The tops of the coleoptiles were removed again and the conidia suspension of *Fusarium graminearum* was inoculated. Coleoptile were then cultured at 25 °C for another 24 h. The red arrow represents the injection time point of the isolate and the black arrow represents the inoculation time point of *F. graminearum*. Gene expression levels were normalized to β-actin gene expression.

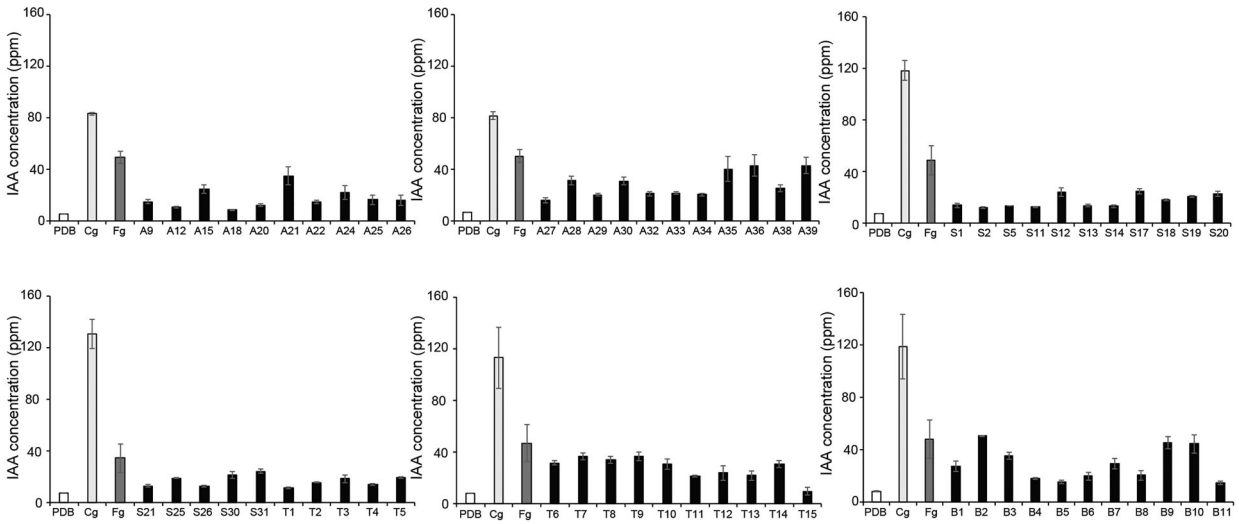

Figure 7. IAA production of *Epicoccum nigrum* isolates. Each isolate was inoculated in PDB supplied with 5 mM L-tryptophan and was cultivated for 5 days at 25 °C. Following this, 1 ml of each culture medium was used to determine the production of IAA using a spectrophotometer at 530 mm. Error bar represents the standard deviation from three replicates. Fg, *Fusarium graminearum*; Cg, *C. gloeosporioides*.

Table 2. IAA production of isolates.

IAA production (mg/l)	Isolate code	No. of *E. nigrum* isolates
1–11	A18, T15, A12	3
11–21	T1, S2, A20, S11, S26, S21, S5, S14, S13, S1, T4, A22, B11, A9, B5, T2, A26, A27, A25, S18, B4, T3, S25, T5, B6, A29, A34, B8, S19, T11, A32, S30	32
21–31	A33, T13, A24, S20, T12, S12, S31, S17, A15, A38, B1, B7, T10, T14, A30	15
31–41	A28, T6, T8, A21, B3, T9, T7, A35	8
41–51	*F. graminearum*, A36, A39, B10, B9, B2	5
>51	*C. gloeosporioides*	0

pathogen-effector proteins that directly interact with PR proteins during infection [45,46]. The expression levels of PR genes were also verified in this study. Consistent with the result that *E. nigrum* isolates had no pathogenicity toward wheat leaves (Figure 3), *E. nigrum* treatment to wheat coleoptiles did not trigger the transcription of PR genes (Figure 6). However, pretreatment of *E. nigrum* to wheat coleoptiles resulted in faster transcription of PR genes responding to *F. graminearum* inoculation (Figure 6), suggesting that endogenous *E. nigrum* may interact with host plants and induce upregulation of PR gene expression to protect against *F. graminearum* (Figure 5(B)).

In conclusion, this study suggests that *E. nigrum* collected from wheat fields has high genetic diversity. All isolates showed *F. graminearum* inhibition in the medium, and isolates (i.e., A33, A39, and S11) showed strong *F. graminearum* inhibition in the host plants. This study also showed that pretreatment of *E. nigrum* could induce the upregulation of PR gene expression in host plants responding to *F. graminearum* infection. Similar to the results of previous studies [19], the isolates in this study were also found to produce IAA that exerted positive promoting effects on plant growth (Figure 7). This study not only reveals the genetic diversity of *E. nigrum* in a wheat field but also provides a new strategy for protecting wheat from *F. graminearum* infection. Future studies should focus on determining the mechanisms by which endogenous *E. nigrum* induces the upregulation of PR gene expression in host plants.

Disclosure statement

No potential conflict of interest was reported by the authors.

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