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Full Length Research Paper

Pathogenic variation and molecular characterization of *Fusarium* species isolated from wilted sesame in China

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Twenty-five isolates of *Fusarium* species were obtained from wilted sesame (*Sesamum indicum* L.) grown on 25 farms from 22 regions of China. These were identified as *Fusarium oxysporum* (20 isolates) and other *Fusarium* spp. (5 isolates) by polymerase chain reaction and sequencing of the internal transcribed spacer region of fungal ribosomal RNA genes. Molecular characterization of *Fusarium* isolates was performed using amplified fragment length polymorphism (AFLP) analysis of genomic DNA. The 25 isolates were grouped into two types (type I for *F. oxysporum* and type II for other *Fusarium spp.*). The pathogenicity of the 25 isolates was tested on sesame cultivar 3433. And the *Fusarium* isolates varied in the degree of disease severity they caused; with five *F. oxysporum* isolates (02, 08, 11, 20, and 25) were having more aggressive virulence to sesame than the other isolates. The results of AFLP analysis were combined with the pathogenicity data to conduct correlation analysis, and it was found that type I could be divided into 2 subtypes: type IA, with middle to weak pathogenicity, and type IB, with strong pathogenicity. These findings are an important foundation for sesame resistance breeding.

Key words: *Sesamum indicum* L., *Fusarium* wilt, *Fusarium oxysporum*, amplified fragment length polymorphism (AFLP), pathogenicity.

INTRODUCTION

Sesame (*Sesamum indicum* L.) is one of the oldest known oil seed crops, and its use probably dates back to 2130 BC (Weiss, 1983). Nearly all of the world sesame cultivation in the world is found cultivated in developing tropical and subtropical countries (Ashri, 1998) in tropical and subtropical areas. Sesame is subjected susceptible to attack by eight or more economically important fungal diseases (Kolte, 1985) and by 65 species of insects at different growth stages of its growth (Ahuja and Bkhetia, 1995), causing considerable yield losses. Fungal diseases infecting the root, stem root, stem, and foliage are important biotic factors causing losses in sesame yield (Abd-El-Ghany et al., 1974; Pramod et al., 1992; Dinakaran et al., 1994; Bahkali and Moslem, 1996).

In China, sesame is planted mostly in the Yangtze and Huang River valleys. First reported in North America in 1950 (Armstrong and Armstrong, 1950), Sesame *Fusarium* wilt, (caused by *Fusarium oxysporum* f.sp. *sesame* (Fos)), is one of the most important soil borne diseases causing economic losses in sesame in China (Li, 1989; Yang et al., 1992; Zhang et al., 2001). Disease symptoms are partial or total wilting of plants at flowering and podding, purple bands on stems, extending from the base upwards, with browning of the stem tissue in the purple band area and browning or blackening of internal tissue when the main stem or primary branches split. Infected young plants may not show the purple band symptom but have conspicuous internal browning and blackening. Sesame fields infected with *Fusarium* exhibit conspicuous patches of dead plants. And the fungus can survive on infected crop debris in the soil for about three

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years with infection re-occurring from seeds or soil.

Breeding for host resistance is the most effective, efficient, and environmentally friendly method to control *Fusarium* wilt. Examples of this include non-opening varieties, which are less susceptible to the disease. Several studies (Gaikwad and Pachpande, 1992; Xiao et al., 1992; Raghuwanshi et al., 1995; El-Bramawy, 1997, 2003, 2006; El-Shakhess, 1998; El-Shazly et al., 1999; Ammar et al., 2004; Silme and Çagirgan, 2010; El-Shakhess et al., 2007) carried out, have screened for screening the resistance to *F. oxysporum* wilt. However to our knowledge, no report concerning the breeding for resistance of *F. oxysporum* on sesame in China has been published to date.

In the present study, *Fusarium* species were isolated from wilted sesame in order to identify them and to determine their pathogenicity on sesame material. We also report here the utility of a DNA-based method, polymerase chain reaction (PCR) and sequencing of the internal transcribed spacer (ITS) region along with amplified fragment length polymorphism (AFLP) analysis of genomic DNA to differentiate and analyze the diversity of *Fusarium* species isolated from sesame.

MATERIALS AND METHODS

Isolation, morphology, and identification of the causal fungi

Wilted sesame plants were collected from 25 farms in 22 regions (Table 1) along the Yangtze and Huang River valleys in the summer of 2009. The plants were washed under running tap water; then, sections of diseased parts tissues were surface sterilized in 70% ethanol for 30 s followed by 30 s in 0.1% (v/v) $HgCl_2$, rinsed in sterile distilled water, and air-dried on sterile filter paper. These sections were then cut into 5 × 2 mm pieces, placed on potato dextrose agar (PDA) medium, and incubated for 4 to 5 days at 28°C. Once *Fusarium*-like colonies were observed, the hyphal tips of the colonies were transferred to new PDA medium. Pure cultures of the isolates were obtained using a single-spore culture technique and representative isolates were maintained on PDA slants. Macroscopic and microscopic characteristics of the pure cultures were studied on PDA cultures, and the species were identified using illustrated keys (Leslie and Summerell, 2006).

Genomic DNA extraction of DNA

Each *Fusarium* isolate was grown on PDA with a layer of sterile glassine for 3 to 4 days at 28°C in the dark; then, the harvested mycelium was lyophilized. Genomic *Fusarium* DNA was isolated from the lyophilized mycelium in the presence of CTAB buffer (2% (w/v) celyltrimethyl ammonium bromide, 2% (w/v) polyvinylpyrrolidone, 10 mM Tris-HCl (pH 8.0), 25 mM EDTA, and 2.0 M NaCl) then extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). DNA was then precipitated by adding two volumes of absolute ethanol and pelleted by centrifugation for 15 min at 12,000 rpm. The pellet was washed with 70% ethanol, air-dried, and re-suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)). RNA was degraded by treatment with RNase A (50 µg/ml) for 30 min at 37°C. DNA concentration and purity were spectrophotometrically measured using a Biomate 3 DNA/RNA calculator (Thermo, Germany).

Molecular identification of the ITS region

The ITS region was amplified using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers. PCR was carried out with a total volume of 50 µl containing 5 µl 10× Taq polymerase buffer (Fermentas) and 2 units of Taq polymerase supplemented with 1 µl 10 mM dNTPs, 5 µl 25 mM $MgCl_2$, 1 µl 0.025 mM of each primer, and 10 ng genomic DNA. PCR was performed with the following conditions: initial denaturation at 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min with a final extension of 10 min at 72°C. The amplified PCR product was purified and sequenced by NanJin Genscript Company and then used to query the NCBI BLAST database, the results of which were used as out groups for phylogenetic analysis.

AFLP analysis

Amplified fragment length polymorphism (AFLP) analysis was performed as described by Vos et al. (1995) and modified by Mayek-Perez et al. (2001). For template preparation, all reaction mixtures contained 10 mM Tris-HAc pH 7.5, 10 mM $MgAc_2$, 50 mM KAc, 5 mM DTT, and 50 ng/µl BSA. In the first step, 200 ng of isolated genomic DNA were digested in a total volume of 10 µl at 37°C for 3 h and 65°C for 0.5 h with 3 U of EcoRI (TaKaRa, Shuzo, Japan) and 3 U of MseI (New England Biolabs, Frankfurt, Germany). Next, ligation of adaptors was performed with 2.5 µl of a solution containing 2.5 pmol of EcoRI adaptor, 25 pmol of MseI adaptor, 0.75 U of T4 DNA ligase (MBI Fermentas, St. Leonrot, France) for 12 h at 15°C in a total volume of 12.5 µl. This mixture was diluted 20-fold with double-distilled (dd) H_2O, and 2.5 µl were used as template for the first PCR amplification. PCR was performed as described by Mayek-Perez et al. (2001) in a total volume of 10 µl. The oligonucleotide primers used for the pre-amplification step were:

EcoRI + A 5′-AGACTGCGTACCAATTCA-3′
MseI + A 5′-GACGATGAGTCCTGAGTAAA-3′

The pre-amplification product was diluted 50-fold with ddH_2O; 2.5 µl of this dilution were used as a template for the second selective amplification step using two selective nucleotides, A plus A, T, G, or C.

Pathogenicity tests

The pathogenicity of 28 25 isolates from 22 regions was tested on sesame cv. 3433. Pathogenicity testing was carried out at the seedling stage as described by Qureshi et al. (2003) with some modifications. Healthy and plump sesame seeds were sterilized with 0.1% mercuric chloride (corrosive sublimate) for 4 to 5 min and rinsed three times with sterile water. Twenty seeds were placed on 2.0% water-agar (w/v) medium with a piece of sterile filter paper along the medium fringe in a 60 mm (diameter) × 95 mm (height) sterile jar. Next each jar was inoculated with 10 mm (diameter) fungus clump from the fringe of 6-day-old culture colonies of each *Fusarium* isolate (grown on PDA in 9 cm plates at 30□ C in the dark) was inoculated in the middle of the 2.0% water-agar (w/v) medium The inoculums was placed in the middle of the jar. Each treatment of 20 seeds, arranged in one jar, was replicated four times in a randomized block design. Jars were incubated at 30□ C in the dark and 1 ml of sterile water was added to each jar to promote seed germination. Pathogenicity was recorded 10 days after seed inoculation using the following severity assessment key: 0 = healthy seedling; 1 = r ≤ 1/3 (r = root areas browning in the seedling; 2 = 1/3 < r ≤ 2/3; 3 = 2/3 < r ≤ 1; 4 = r = 1, 0 < s ≤ 1/2 (s =

Table 1. Fungal isolates of *Fusarium* species isolated collected from wilted sesame collected from different geographical regions in China.

Isolate	Geographical origin (field)	Species	Disease severity index	Geographical origin (field)
01	JinXian (JiangXi)	*Fusarium* sp. *141GP/S*	0.40	JinXian (JiangXi)
02	WuXue (HuBei)	*Fusarium oxysporum*	3.63	WuXue (HuBei)
03	HuangMei (HuBei)	*Fusarium oxysporum*	2.50	HuangMei (HuBei)
04	YangXin (HuBei)	*Fusarium oxysporum*	1.79	YangXin (HuBei)
05	JianLi (HuBei)	*Fusarium* sp. *124DZ/F*	0.15	JianLi (HuBei)
06	JingZhou (HuBei)	*Fusarium oxysporum*	2.99	JingZhou (HuBei)
07	WuHan (HuBei)	*Fusarium oxysporum*	0.91	WuHan (HuBei)
08	WuHan (HuBei)	*Fusarium oxysporum*	3.93	WuHan (HuBei)
09	YangLuo (HuBei)	*Fusarium oxysporum*	0.15	YangLuo (HuBei)
10	HuangPi (HuBei)	*Fusarium oxysporum*	0.96	HuangPi (HuBei)
11	XinZhou (HuBei)	*Fusarium oxysporum*	3.59	XinZhou (HuBei)
12	YiCheng (HuBei)	*Fusarium oxysporum*	2.10	YiCheng (HuBei)
13	XiangFan (HuBei)	*Fusarium solani*	0.08	XiangFan (HuBei)
14	ZaoYang (HuBei)	*Fusarium oxysporum*	2.95	ZaoYang (HuBei)
15	HeFei (AnHui)	*Fusarium solani*	0.80	HeFei (AnHui)
16	FuYang (AnHui)	*Fusarium oxysporum*	0.14	FuYang (AnHui)
17	HuangChuan (HeNan)	*Fusarium oxysporum*	1.11	HuangChuan (HeNan)
18	PingYu (HeNan)	*Fusarium oxysporum*	2.99	PingYu (HeNan)
19	PingYu (HeNan)	*Fusarium oxysporum*	2.58	PingYu (HeNan)
20	PingYu (HeNan)	*Fusarium oxysporum*	3.50	PingYu (HeNan)
21	ZhuMaDian (HeNan)	*Fusarium oxysporum*	1.31	ZhuMaDian (HeNan)
22	NanYang (HeNan)	*Fusarium proliferatum*	0.34	NanYang (HeNan)
23	LuoHe (HeNan)	*Fusarium oxysporum*	0.55	LuoHe (HeNan)
24	LiaoYang (LiaoNing)	*Fusarium oxysporum*	2.57	LiaoYang (LiaoNing)
25	LiaoYang (LiaoNing)	*Fusarium oxysporum*	3.12	LiaoYang (LiaoNing)

shoot areas browning of the shoot in the seedling); 5 = r = 1, 1/2 < s ≤ 1. The disease severity index was calculated as: Disease severity index = ∑ (disease severity scale × number of plants at each severity scale) / total number of emerged seeds sown.

Data analysis

All pathogenicity tests were conducted in a completely randomized design. All data from each of the repeated experiments were analyzed separately and subjected to analysis of variance (ANOVA).

For AFLP analysis, the presence and absence of each band was determined and designated 1 or 0, respectively. The genetic similarity between individuals was estimated using the simple matching coefficient (Hair et al., 1992; Sokal and Michener, 1958). The similarity matrix generated was then used to produce a UPGMA dendrogram using NTSYSpc-2.2 (Hair et al., 1992).

RESULTS

Isolation, morphology, and identification of the causal fungi

Twenty-five strains of *Fusarium* spp. were isolated from the wilted sesame plants collected from the 22 regions described in Table 1. The *Fusarium* isolates produced white to pale violet colonies on PDA with aerial mycelia and had a cottony or somewhat ropey texture. The color of the undersurface of the colonies varied among the isolates from pink or light violet to dark violet or dark magenta (Figure 1a, b). Microconidia were formed in false heads on short monophialides (Figure 1c). Uni- or bicellular and ovoid to ellipsoid microconidia were abundant (Figure 1d). Canoe-shaped macroconidia contained three to five septa and had a long apical cell and a foot-shaped basal cell (Figure 1e). Chlamydospores were mostly single cells but were sometimes seen as short chains in two-week-old cultures (Figure 1f). On some PDA cultures, macroconidia were produced from orange sporodochia.

Molecular identification of the ITS region

The ITS region was amplified using ITS1 and ITS4 primers for the 25 isolates, and the amplified PCR products were purified and sequenced. The sequences were aligned with results from a query of the BLAST database (Table 1). Most of 25 isolates belonged to the species *F. oxysporum*, two isolates belonged to *Fusarium solani*, one isolate was *Fusarium proliferatum*, one was *Fusarium sp. 141GP/S*, and one was *Fusarium sp. 124DZ/F* (Table 1).

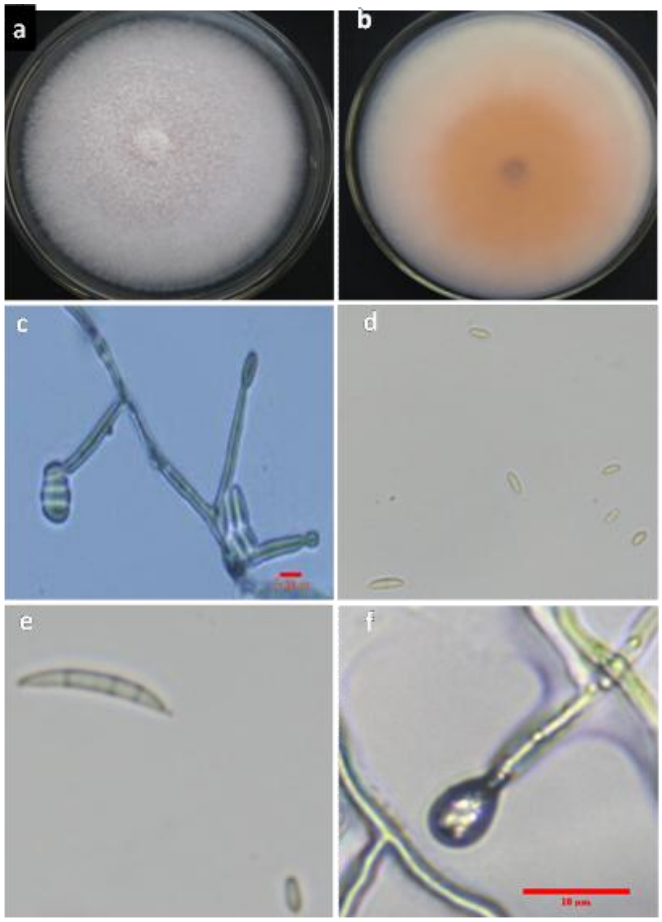

Figure 1. Colony characteristics of *Fusarium oxysporum* f.sp. *sesame* conidiospore and chlamydospore morphology cultivated on PDA medium for seven days. (a) front of the colony; (b) back of the colony; (c) microconidium and conidiophore; (d) microconidium; (e) macroconidium; (f) chlamydospore.

AFLP analysis

Based on AFLP clustering analysis, the 25 isolates were divided into 2 types with a genetic similarity coefficient of 0.69 (Figure 2). Type I included 20 isolates, all of *F. oxysporum*, and type II included five isolates belonging to other species of *Fusarium*. Correlation analysis was done based on the AFLP data in combination with the pathogenicity data (Table 1). The results indicated that type I could again be divided into two subtypes (IA and IB). Most of the isolates from of the type IA had showed middle to weak pathogenicity, with only two isolates (No.20 and No.25) exhibiting strong pathogenicity. All isolates of the type IB isolates had exhibited strong pathogenicity and their disease severity indices (DSI) were higher than any isolate from type IA. The five isolates from of the type II all had showed weak pathogenicity. In summary there was a correlation between the results of AFLP clustering analysis and isolate pathogenicity.

Pathogenicity tests

THE 25 isolates were divided into three groups according to types by pathogenicity test (Table 1): group I had weak pathogenicity (12 isolates, DSI < 1.5); type group II had moderate pathogenicity (8 isolates, 1.5 ≤ DSI < 3.0); and type group III had strong pathogenicity (5 isolates, 3.0 ≤ DSI). All strong pathogenicity isolates belonged to *F. oxysporum* with, on the other hands all other *Fusarium spp*. exhibiting weak pathogenicity.

DISCUSSION

Wilted sesame samples were collected from the main sesame producing areas in China and the pathogens isolated from these samples were scored based on pathogenicity. A total of 25 isolates were obtained from 25 farms within 22 regions. They were scored and arranged based on their pathogenicity. The ITS region sequences amplified from the fungal ribosomal RNA genes of each isolate were aligned and used as an out group in phylogenetic analysis (data not shown). AFLP clustering analysis and pathogenicity tests on sesame cultivar 3433 were performed. We found that most of the collected *Fusarium* samples belonged to *F. oxysporum*, all of which had middle to strong pathogenicity, consistent this finding is coincide with published works (Bahkali and Moslem, 1996; El-Bramawy, 2006). We also found It was evident that AFLPs could be used to differentiate between species of *Fusarium* and that there was a correlation between the results of AFLP clustering analysis and pathogenicity data.

This method was used for testing pathogenicity of *Fusarium* Spp during this investigation is suitable for identifying different pathogenicity levels in the sesame shoot phase and was is distinct from tests of pathogenicity in adult plants (Assigbestse et al., 1994; Khanzada et al., 2004; Pratt et al., 1998; AI-Ghamdi et al., 2001). In contrast to methods involving adult period pathogenicity tests, in which difficult inoculation techniques and precise temperature and humidity controls over long periods are required, this method was is less difficult to conduct, of short duration, and showed good reproducibility and high reliability. The pathogens from different farms and different regions had prodigious differences in pathogenicity, indicating that wilted sesame pathologies in China are differentiated based on pathogen. Pathogens from different farms within the same region had different pathogenicities, therefore the pathogens from the same region or physiological race had prodigious differentiation, making it difficult to prevent or cure sesame wilt caused by *Fusarium*. All pathogenic isolates of *Fusarium* showed different degrees of pathogenicity including those which were collected from the same farm.

The main pathogen causing sesame wilt was *F. oxysporum*; however, we identified five other *Fusarium*

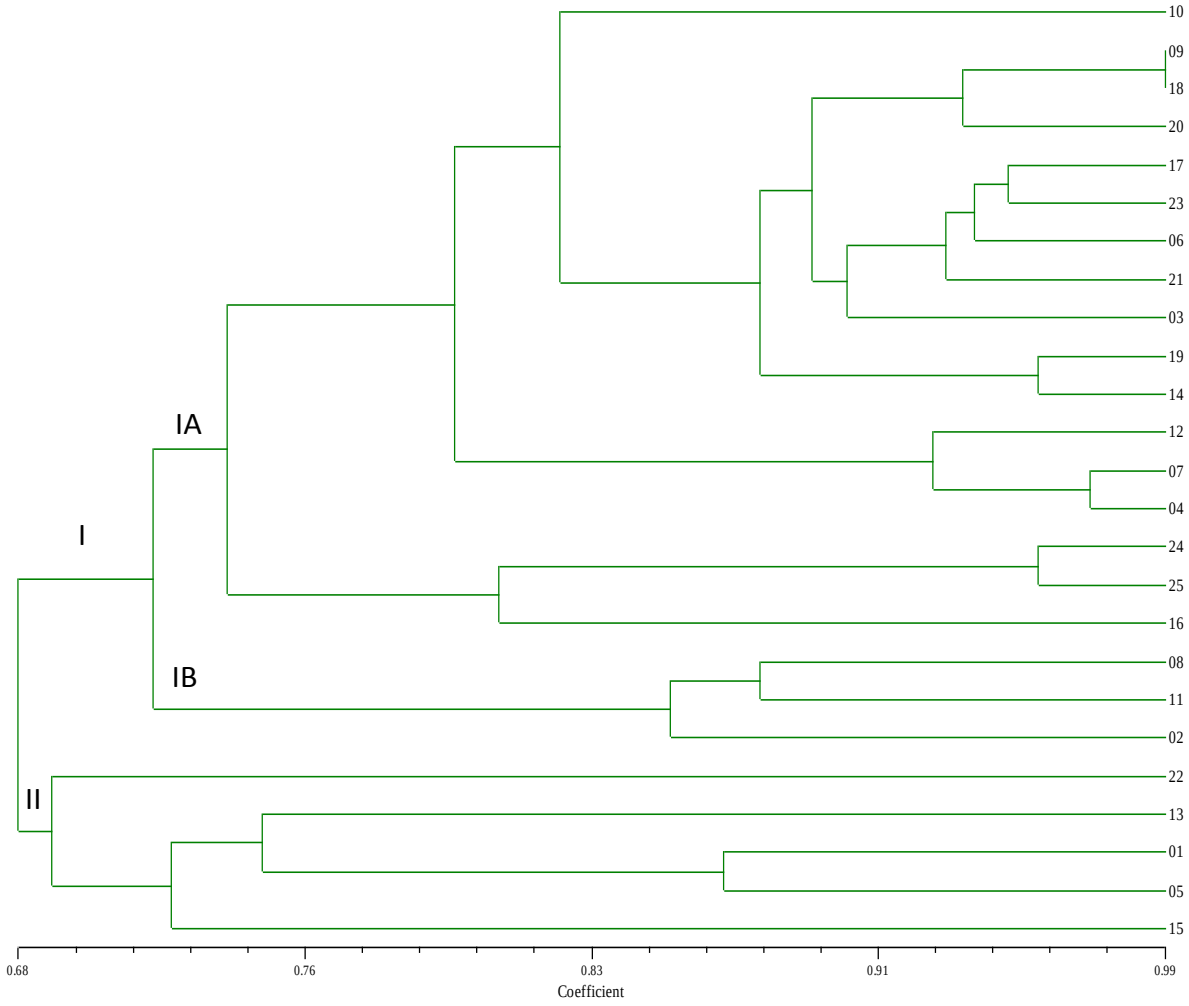

Figure 2. Result of AFLP cluster analysis.

spp. from wilted sesame plants. These five isolates had less pathogenicity on sesame cultivar 3433 than did *F. oxysporum*. We found it was clear, that these five isolates and the other *F. oxysporum* isolates had obvious differences in genetic distance by AFLP clustering analysis and the five isolates had less pathogenicity on sesame cultivar 3433 than did *F. oxysporum*. All this suggests that the five isolates were perhaps not the true pathogens but arrived after the true pathogenic organism and were present on the dead tissues of the wilted sesame plants having arrived after the true pathogenic organism. Since the wilted plants we collected belonged to different sesame varieties, verification of pathogenicity of the isolates should be carried out on different sesame cultivars.

Through this research we determined that pathogens of wilted sesame plants from different regions were showed distinct at the molecular and pathogenicity levels. We also obtained many fungal strains from wilted sesame from different regions in China. Based on these results, therefore, highly resistant or highly vulnerable sesame varieties can be found determined by conducting pathogenicity testing on different sesame plants. This work lays a foundation that would be of great help for other molecular level studies of sesame resistance breeding.

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