Morphological and Molecular Identification of *Fusarium ipomoeae* as the Causative Agent of Leaf Spot Disease in Tobacco from China

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**Abstract:** Tobacco (*Nicotiana tabacum* L.), which creates jobs for 33 million people and contributes two trillion dollars’ tax annually, is one of the most important economic plants globally. However, tobacco is seriously threatened by numerous diseases during production. Previously, the field survey of tobacco diseases was conducted in the Guizhou and Guangxi provinces, the two main tobacco-producing areas in China. A serious leaf spot disease, with a 22% to 35% incidence, was observed in farming plants. In order to determine the causal agents, we collected the disease samples and isolated the pathogenic fungi. The pathogen was identified as *Fusarium ipomoeae*, based on the morphological characteristics and phylogenetic analysis. Pathogenicity tests showed that *F. ipomoeae* could induce tobacco leaf spot and blight. To our knowledge, this is the first report worldwide of *F. ipomoeae* causing leaf spots and stems on tobacco. Our study reveals the serious consequences of *F. ipomoeae* on tobacco filed production and provides information for future diagnosis and management of the *Fusarium* disease.

**Keywords:** *Fusarium*; tobacco; morphology; pathogenicity; phylogenetic analysis

1. **Introduction**

Tobacco (*Nicotiana tabacum* L.) is one of the most crucial economic crops all over the world [1]. Globally, according to the estimates of tobacco industry, about 33 million people participate in tobacco planting, product manufacturing, distribution, and retailing [2]. Meanwhile, global revenues from the tobacco industry are estimated at 2 trillion dollars a year [3]. In China, tobacco was planted in more than 14,000 km², and its yield of leafage reached 31.32 million tons in 2011 [4]. Its accounts for more than 39.6% of total global tobacco production [5], in which, farming is especially prevalent in Southwest China [6]. Among the main supplier provinces, Guizhou province produces nearly 30% of the total Chinese tobacco crop and ranks as the second-most tobacco-producing areas [7].

It is well-known that tobacco suffers from various fungal and oomycete pathogens during its whole growing season, such as *Fusarium* spp., *Collectotrichum gloeosporioides*, *Alternaria alternata*, *Botrytis cinerea*, etc. [6,7]. Among them, *Fusarium* spp., which can cause tobacco leaf, root, and stem diseases, have been a serious problem threatening to tobacco production in many countries [8]. The incidence of *Fusarium* spp. has increased considerably in recent years [6–8]. Furthermore, the mycotoxins and secondary metabolites produced by the *Fusarium* species in diseased leaves may be harmful to the health of humans [9]. Therefore, *Fusarium* identification was of particular importance for the effective management of tobacco diseases.

In August 2021, the field survey of tobacco disease was conducted in Zheng’an and Fenggang in Guizhou, Shanglin. The three main tobacco-producing areas in China are
located in Guangxi. Tobacco plants with leaf spots and blights were frequently observed in the farming fields, with a 22 to 35% disease incidence. In order to determine the causal agents, provide information for future diagnosis, and help the management of this disease, the disease samples were collected, and the pathogenic fungi were isolated. Morphology characterization and multi-gene locus phylogenetic analysis were performed. Furthermore, the pathogenicity was also tested following the Koch postulates.

2. Materials and Methods

2.1. Isolation

In August 2021, diseased plants of tobacco were collected from three main producing counties (Zheng’an and Fenggang in Guizhou and Shanglin in Guangxi). For fungal isolation, the diseased tissues were cut into several small segments and placed on potato dextrose agar (PDA, Difcohai) at 25°C in darkness for 3 to 5 days. To obtain pure cultures, hyphal tips from developed colonies were transferred to fresh PDA plates three times [10]. The isolates were inoculated to PDA test tube slants and stored at 4°C [11]. The strain was deposited at Yangtze University, Jingzhou, Hubei, China.

2.2. Morphology

The edge of the colony was cut into 6 mm diameter plates with a sterile punch, and the mycelia plug was transferred to a 90 mm PDA plate and grown for 7 days at 25°C in darkness. Then, the colony morphology was evaluated and visualized. For observing the morphological features of conidia, the marginal hyphae were transferred to carnation leaf-piece agar (CLA) medium (the sterilized carnation leaves were placed in water agar medium) and cultured at 22°C under a light/dark period of 12/12 h [12]. After 7–10 days, conidia and chlamydospores were mounted in sterile water for microscopic observation using a Nikon ECLIPSE Ni–U microscope equipped with a Nikon DS–Ri2 digital camera (Tokyo, Japan) [12].

2.3. DNA Extraction and PCR Amplification

Genomic DNA was extracted from fresh mycelium grown on PDA using a method modified from Cenis [13]. Five loci, including the 5.8S nuclear ribosomal RNA gene with the two flanking internal transcribed spacers (ITS), translation elongation factor 1 alpha (EF–1α), calmodulin (CAM), RNA polymerase largest subunit (RPB1), and RNA polymerase second largest subunit (RPB2) gene regions, were amplified and sequenced. The detail information of corresponding primers were showed as follows: ITS (ITS4: TCCTCCGCTTATTGATATGC; ITS5: GGAAGTAAAAGTCGTAACAAGG) [12], EF–1α (EF1: ATGGGTAAGGARGACAAGAC; EF2: GGARGTACCATSATCATG) [14], CAM (CL1: GARTWCAAGGGCCITTTC; CL2A: TTTITGATCATGAGTTGGAC) [15], RPB1 (Fa: CAYAARGARTCYATGATGGGC; G2R: GTCATYTGDTGDGCGYTCDC) [16], RPB2 (5f2: GAYGAYMGWGTCAHYTYGG; 7cr: CCCATRGGCTTTGTYTRCCCAT) [17]. The PCR programs were set as follows: initial denaturation at 95°C for 90 s, followed by 35 cycles, at 95°C for 30 s, annealing for 30 s, and extension at 72°C for 1 min, as well as terminating with a final extension at 72°C for 10 min. The annealing temperatures were 55°C, 56°C, 55°C, 58°C, and 58°C for ITS, EF–1α, CAM, RPB1, and RPB2, respectively. The PCR products were sent to the company (TSINGKE, Beijing, China) for purification and sequencing.

2.4. Phylogenetic Analysis

The obtained sequences were analyzed by BLASTn (nucleotide blast) searches. The relevant strains (Table 1) were selected both according to BLAST searches and previous references [18]. The five gene sequences were concatenated and edited manually in MEGA v.7.0.26 [19], and the aligned dataset was deposited in TreeBASE. The maximum likelihood [20] and Bayesian inference (BI) methods were used to phylogenetic analysis the ITS, EF–1α, CAM, RPB1, and RPB2 combing sequences for Fusarium incarnatum–equiseti species...
complex (FIESC). ML analysis was performed using RAxML (Randomized Accelerated Maximum Likelihood) v.7.2.8 (A. Stamatakis, Heidelberg, Germany) [21]. The branch support was assessed with 1000 replicates. Bayesian inference (BI) analyses were conducted in MrBayes v.3.2.1 (Huelsenbeck J P and Ronquist F, Rochester, NY, USA) by using the Markov chain Monte Carlo (MCMC) algorithm [22]. Mrmodel test v.2.3 (Posada D and Crandall K A, Oxford, England) [23] was used to determine the best fit evolutionary model (GTR + I + G) using the Akaike information criterion (AIC) parameter. Two MCMC chains were run in the random tree, with a total of 1 million generations, sampling once every 100 generations. When the average standard deviation of the separation frequency was less than 0.01, the first 25% of the samples were discarded, and the operation was stopped. The trees were viewed and edited with Figtree v.1.3.1 [19].

2.5. Pathogenicity Test

Pathogenicity was tested on living tobacco leaves and stems. Healthy tobacco were surface-sterilized in 2% sodium hypochlorite and washed 3 times with sterilized distilled water for 2 min before performing the test [12]. The experiment was repeated three times, with at least three plants for each time. For leaves and stems inoculation, the mycelium block (about 6 mm) cultured on the PDA medium was inoculated to the healthy leaves and base of the stalk and wrapped with absorbent cotton and plastic wrap to keep it moisturized. After inoculation, the plants were cultured in a greenhouse (22 °C, under a light/dark period of 12/12 h). Control plants were inoculated with PDA plugs. Lesion was observed daily, and photographic record for 7 days after inoculation. For root inoculation, fresh mycelium blocks were mixed with rye seeds and cultured at 28 °C for 7 days [1]. Then, 15 culture seeds were mixed into the 5 cm surface soil of each pot and cultured at 22 °C after adding sufficient water. Four days later, the healthy tobacco seedlings at 4-leaf stage were planted into the pot and cultured in greenhouse. Seven days later, the disease symptoms of roots were observed. Seedlings planted in sterile soil were used as controls. The pathogen was re-isolated from the inoculation site using PDA medium. The morphological characteristics and RPB2 sequence were compared with original strains.

2.6. Morphology Characterization Indicated a Fusarium spp.

In total, 77 fungal strains were isolated from those 35 samples. Among them, 68 strains showed similar cultural characters, such as the white colony, with cotton and flocculent aerial mycelium. Thus, they seem to be one species and could be the dominant pathogen for tobacco leaf spot and blight disease. Three represent strains (GZAX 307, GZAX 312, and GZAX 402) were randomly selected from all isolated strains for subsequent research. Observation indicated the three strains had same morphological characteristics. Therefore, the strain GZAX 307 was used for the following microscopy visualization and morphology description.

3. Results

3.1. Serious Leaf Spot and Blight Disease Was Observed on Field Tobacco Plants

The field survey of tobacco disease was conducted in August 2021. During the surveillance, it was normally surrounded by a yellow halo, which appeared on the tobacco leaves, as shown in Figure 1. Surveys indicated a 22 to 35% disease incidence in three counties of Zheng’an and Fenggang in Guizhou province and Shanglin in Guangxi Zhuang Autonomous Region. With the development of the disease, spots were enlarged and concatenated. Severely infected leaves turned out to be blight, then defoliation. In order to determine the causal agents, 35 disease samples were collected from the three counties, and the pathogenic fungi were isolated in the laboratory.
Table 1. Strains used in the phylogenetic analyses and their GenBank accession numbers.

| Species          | Phylogenetic Species | Strain | Host                | Location       | ITS             | EF–1            | CAM             | RPB2             | RPB1             |
|------------------|----------------------|--------|---------------------|----------------|-----------------|-----------------|-----------------|------------------|------------------|
| *F. ipomoeae*    | FIESC 1               | CQ1099 | Rhododendron pulchrum leaf | Jiangsu, China | MK280853        | MK289573        | MK289715        | MK289727         | MK289861         |
|                  |                      | LC7923 | Capsicum majus      | Shandong, China | MK280800        | MK289635        | MK289688        | MK289789         | MK289853         |
|                  |                      | CQ1132 | Vinca major sp.     | Jiangsu, China | MK280854        | MK289574        | MK289716        | MK289728         | MK289862         |
|                  |                      | NRRL 43640 = UTHSC 04–123 | Dog nose | Texas, America | GQ505756        | GQ505667        | GQ505578        | GQ505845         | HM374197         |
|                  |                      | LC12165 = CGMCC3.19496 (T) | Ipomoea aquatica leaf | Fujian, China | MK280832        | MK289599        | MK289704        | MK289752         | MK289859         |
|                  |                      | LC7150 | Bamboo              | Jiangxi, China | MK280818        | MK289627        | MK289678        | MK289781         | MK289852         |
|                  |                      | LC12163 | Hibiscus syriacus | Fujian, China | MK280780        | MK289597        | MK289700        | MK289750         | MK289857         |
|                  |                      | GZAX 402 | Tobacco            | Guizhou, China | OP454871        | OP432881        | OP432880        | OP432883         | OP432882         |
|                  |                      | GZAX 307 | Tobacco            | Guizhou, China | ON961779        | ON982723        | ON982723        | ON962725         | ON962727         |
|                  |                      | GZAX 312 | Tobacco            | Guangxi, China | ON961780        | ON982724        | ON982722        | ON982726         | ON982728         |
| *F. sulawense*   | FIESC 2               | NRRL 36448 = CBS 384.92   | Phaseolus vulgaris seed | Sudan | GQ505741        | GQ505652        | GQ505658        | GQ505828         | GQ505830         |
|                  |                      | NRRL 34002 = ARSEF 4714   | Prosopis n. bicincta on | Costa Rica | JF740925        | JF740833        | ——               | JF741159         | ——               |
|                  |                      | FIESC 9               | Brassa campestris pollen | Hubei, China | MK280802        | MK289584        | MK289697        | MK289739         | MK289799         |
|                  |                      | FIESC 8               | Human eye           | Pennsylvania, America | GQ505747        | GQ505658        | ——               | GQ505883         | HM374181         |
| *F. scirpi*      | FIESC 3               | NRRL 28029 = CDC B–3335 | Phaseolus vulgaris seed | Sudan | GQ505691        | GQ505662        | GQ505514        | GQ505780         | HM374150         |
|                  |                      | FIESC 4               | Brassica campestris pollen | Costa Rica | JF740925        | JF740833        | ——               | JF741159         | ——               |
| *F. arcuatisporum* | FIESC 7             | LC12147 = CGMCC3.19493 (T) | Brassica campestris pollen | Hubei, China | MK280802        | MK289584        | MK289697        | MK289739         | MK289799         |
| *F. sulawense*   | FIESC 10              | NRRL 3214 = FRC R–6054, 7.13 MRC | Unknown                 | Canada | ——               | GQ505499        | GQ505770         | ——               | ——               |
|                  |                      | FIESC 11              | NRRL 36372 = UTHSC 06–638 | Horse            | GQ505676        | GQ505587        | ——               | ——               | ——               |
|                  |                      | FIESC 12              | NRRL 36392 = CBS 259.54 | Unknown plant seedling | GQ505739        | GQ505650        | ——               | ——               | ——               |
|                  |                      | FIESC 13              | NRRL 43635 = UTHSC 06–638 | Horse            | GQ505662        | GQ505573        | ——               | ——               | ——               |
|                  |                      | FIESC 14              | NRRL 26419 = CBS 307.94, BBA 68556 (NT) | Soil  | GQ505688        | GQ505599        | ——               | ——               | ——               |
| *F. irregular*   | FIESC 15              | LC1788 = CGMCC3.19489 (T) | Bamboo              | Guangdong, China | MK280829        | MK289629        | MK289680        | MK289783         | MK289863         |
|                  |                      | NRRL 32864 = FRC R–7245 | Human              | Texas, America  | GQ505702        | GQ505613        | GQ505525        | GQ505791         | HM374160         |
| *F. sulavense*   | FIESC 16 & 17         | NRRL 43730 = CDC 2006734605 | Contact lens | Mississippi, America | GQ505680        | GQ505591        | GQ505769        | ——               | ——               |
| *F. luffae*      | FIESC 18              | LC12167 = CGMCC3.19497 (T) | Luffa aegyptiaca   | Fujian, China  | MK280852        | MK289569        | MK289711        | MK289723         | MK289870         |
| *F. sulavense*   | FIESC 19              | NRRL 43639 = UTHSC 04–135 | Manatepe           | Florida, America | GQ505755        | GQ505666        | GQ505577        | GQ505844         | HM374190         |
| *F. sulavense*   | FIESC 20              | NRRL 36575 = CBS 976.97 | Juniperus chinensis leaf | Hawaii, America | GQ505745        | GQ505656        | ——               | ——               | ——               |
| *F. sulavense*   | FIESC 21              | NRRL 34002 = UTHSC 95–1545 | Human ethmoid sinus | Texas, America  | GQ505715        | GQ505626        | GQ505538        | GQ505804         | HM374165         |
| *F. sulavense*   | FIESC 22              | NRRL 13379 = FRC R–5198, BBA 62200 | Oryza sativa | ——               | ——               | ——               | ——               | ——               | ——               |
Table 1. Cont.

| Species          | Phylogenetic Species | Strain                  | Host              | Location               | ITS            | EF–1           | CAM            | RPB2          | RPB1          |
|------------------|----------------------|-------------------------|-------------------|------------------------|----------------|----------------|----------------|---------------|---------------|
| F. sulawense     | FIESC 24             | NRRL 43297 = W. Elmer 22 | Spartina rhizomes | Connecticut, America  | GQ505746       | GQ505657       | GQ505569       | GQ505835      | ——            |
| F. sulawense     | FIESC 27             | NRRL 20722 = IMI 190455 | Chrysanthemum sp. | Kenya                  | GQ505684       | GQ505595       | GQ505507       | GQ505773      | ——            |
| F. guilinense    | FIESC 21             | LC12160 = CGMCC3.19495 (T) | Musa nana leaf | Guangxi, China         | MK280837       | MK289594       | MK289652       | MK289747      | MK289831      |
| F. sulawense     | FIESC 28             | CBS 430.81 = NRRL 28577 | Grave stone       | Romania                | GQ505692       | GQ505603       | GQ505515       | GQ505781      | ——            |
| F. sulawense     | FIESC 32             | CBS 143596              | Stereum irsutum   | Iran                   | LT970815       | LT97079        | LT970732       | LT970751      | ——            |
| F. nanum         | FIESC 25             | LC12168 = CGMCC3.19498 (T) | Musa nana leaf | Guangxi, China         | GQ505697       | GQ505608       | GQ505520       | GQ505786      | ——            |
| F. hainanense    | FIESC 26             | LC11638 = CGMCC3.19478 (T) | Oriza sp. stem    | Hainan, China          | MK280836       | MK289581       | MK289657       | MK289735      | MK289833      |
| F. citri         | FIESC 29             | LC6896 = CGMCC3.19467 (T) | Citrus reticulata leaf | Hunan, China         | MK280803       | MK289617       | MK289658       | MK289771      | MK289828      |
| F. humuli        | FIESC 33             | CQ1039 = CGMCC3.19374 (T) | Humulus scandens leaf | Jiangsu, China       | MK280845       | MK289570       | MK289712       | MK289724      | MK289840      |
| F. polyphialidicum | ——                  | CBS 961.87             | Plant debris      | South Africa           | GQ505763       | GQ505674       | GQ505585       | GQ505852      | ——            |
The fungal colonies reached 61–62 mm in diam. and were white in color, with cotton flocculent aerial mycelium after 7 days of incubation on PDA (Figure 2A,B). On the surface of the carnation leaves, the sporodochial macroconidia falcate, prominently curved, apical cell papillate to hooked, and basal cell had distinct foot shapes, 4–6 septa, most of which were 5 septa, macroconidia 44–118 × 4–11 μm (Figure 2C,D). Chlamydospores were not observed on PDA; few chlamydospores were produced singly, doubly, or in chains after 2 weeks of CLA under alternating 12 h darkness/12 h fluorescent light at 25 °C (Figure 2E,F). The conidiophores in the sporodochia were variable in length, verticillately branched, and densely packed, with most bearing the apical whorls of three monopodialides, sporodochial phialides subulate to subcylindrical, thin-walled, hyaline (Figure 2G–I). No sexual structures were observed. These characteristics suggest the fungus was Fusarium sp. [24].

3.1. Serious Leaf Spot and Blight Disease Was Observed on Field Tobacco Plants

The field survey of tobacco disease was conducted in August 2021. During the survey, severe blight disease was observed on tobacco leaves, as shown in Figure 1. Surveys indicated a 22 to 35% disease incidence in three counties, and the pathogenic fungi were isolated in the laboratory. In order to determine the causal agents, 35 disease samples were collected from the three counties of Zheng'an and Fenggang in Guizhou province and Shanglin in Guangxi Zhuang Autonomous Region. With the development of the disease, spots were enlarged and the leaves withered. In severe cases, the disease caused defoliation. To identify the causal agent, five gene locus sequences, were performed (take GZAX 307 as an example). Firstly, the obtained sequences were analyzed on BLASTn, and the results showed that GZAX 307, GZAX 312, and GZAX 402 clustered monophyletically with strains of Fusarium sp. [24]. Therefore, based on the morphological and molecular characteristics, the isolates GZAX 307, GZAX 312, and GZAX 402 were identified as Fusarium ipomoeae (Figure 3).

3.2. Phylogenetic Analyses Identified a Fusarium ipomoeae Agent

To further identify the causal agent, the phylogenetic analysis, based on the combination of five gene locus sequences, were performed (take GZAX 307 as an example). Firstly, the obtained sequences were analyzed on BLASTn, and the results showed that over 99% nucleotide sequence identity with members of the FIESC: the ITS sequence showed 100% identity (488/1132 bp) to the F. laceratum strain NRRL 20423; the EF–1α sequence showed...
99.56% identity (684/678 bp) to the *F. ipomoeae* strain NRRL 43640; the RPB2 sequence showed 100% identity (747/839 bp) to the *F. ipomoeae* strain CBS 140909; the RPB1 sequence showed 99.42% identity (1726/1578 bp) to the *F. sulawense* strain LC12173; and the CAM sequence showed 99.28% identity (715/698 bp) to the *F. ipomoeae* strain NRRL 34034. Then, phylogenetic analysis, using concatenated sequences of ITS, EF1–α, RPB2, RPB1, and CAM, showed that GZAX 307, GZAX 312, and GZAX 402 clustered monophyletically with strains of *F. ipomoeae* (the relevant strains are shown in Table 1). Therefore, based on the morphological and molecular characteristics, the isolates GZAX 307, GZAX 312, and GZAX 402 were identified as *F. ipomoeae* (Figure 3).

3.3. *F. ipomoeae* Showed Pathogenicity on Leaf and Stem

To complete the Koch postulates, the strains GZAX 307, GZAX 312, and GZAX 402 were inoculated on the leaves and stems, as well as the roots. The specific morphology and pathogenicity statistics are shown in Table 2. The pathogenicity results were consistent;
therefore, the specific description took GZAX 307 as an example. The results of pathogenicity were the same, and strain 307 was taken as an example to show the symptoms. About 7 days post inoculation, white hyphae crawled on the leaves from the inoculation site; meanwhile, small brown spots appeared around the hyphae (Figure 4A,B). The lesion diameter can reach 42.6 ± 3.02 mm. The pathogen was re-isolated from the inoculated sites and further validated as the same fungus through morphological and phylogenetic analyses. Light brown spots appeared at the inoculation site of tobacco stem after 7 days (Figure 4C). After inoculation, no obvious pathogenicity symptoms occurred in the roots of the tobacco, compared with the control (Figure 4D,E).

Table 2. Morphology characterization and pathogenicity of *F. ipomoeae*.

| Species (Strain) | Location                           | Colonies (mm) | Conidia | Pathogenicity on Tobacco Leaf (mm) | On Stem (mm) | On Root (mm) |
|-----------------|------------------------------------|---------------|---------|-----------------------------------|--------------|--------------|
|                 |                                    |               | Body (µm) | Septa | Wounded | Unwounded | Wounded | Unwounded |
| *F. ipomoeae*   | Zheng’an in Guizhou province       | 61.5 ± 0.5    | 44–118 × 4–11 | 4–6   | 37 ± 2 | 34 ± 3    | 18 ± 2 | 12 ± 6     | —          |
| (GZAX 307)      |                                    |               |          | Septa | Wounded | Unwounded | Wounded | Unwounded |
| *F. ipomoeae*   | Shanglin in Guangxi Zhuang          | 62 ± 1        | 47–123 × 2–9 | 4–6   | 35 ± 1 | 32 ± 2    | 20 ± 4 | 11 ± 5     | —          |
| (GZAX 312)      | autonomous region                   |               |          | Septa | Wounded | Unwounded | Wounded | Unwounded |
| *F. ipomoeae*   | Fenggang in Guizhou province       | 62.5 ± 1      | 42–120 × 4–14 | 4–6   | 33 ± 4 | 34 ± 3    | 17 ± 2 | 10 ± 5     | —          |
| (GZAX 402)      |                                    |               |          | Septa | Wounded | Unwounded | Wounded | Unwounded |

Figure 4. Pathogenicity on tobacco (A,B) leaves, (C) stems, and (D) roots. (E) The roots of control plant.
4. Discussion

Dried and fermented tobacco leaves are raw resources for tobacco industry and product manufacturing [2]. Thus, owning to its important commercial values, this annual, leafy, solanaceous plant was planted globally; therefore, it creates jobs, increases incomes, maintains tax revenues, and sustains trade surpluses [2]. However, the field production of this cash crop is frequently seriously threatened by many fungal pathogens [7,25]. Thus, the safe and sustainable production of tobacco leaves required control of fungal diseases. Additionally, better understanding of the infection of pathogenic fungi is better for the control of the disease.

Previously, in order to know more about the incidence of fungal pathogens in the process of tobacco cultivation, we went to the tobacco-producing areas of Guizhou province and Guangxi Zhuang autonomous region to investigate. During the process of our investigation, we found that the symptoms were irregular brown spots, comprising whitish center, normally surrounded by yellow halo appearing on tobacco leaves. The disease was not deadly, but it seriously affected the quality and greatly reduced the economic value of tobacco. Further investigation revealed that a prevalence of the tobacco leaf spot was 22 to 35% in the field. In order to investigate the pathogen, we collected the samples of this disease from three random sites; through isolation and identification, we found that the dominant pathogen was *Fusarium ipomoeae* (Figures 2 and 3).

Several studies have found that *F. ipomoeae* is associated with plant growth process, which serves as a causal pathogen that significantly affects the quality and quantity of products. Previously, *F. ipomoeae* have been reported to cause leaf spots on peanuts [26] and *Bletilla striata* [27] in China. However, to our knowledge, this is the first report worldwide of *F. ipomoeae* causing leaf spots on tobacco. Compared with the strain found in *Bletilla striata*, the colony and spore morphology in this study was consistent, but there were some differences in the spore sizes. In peanuts, the macroconidia were 4–7 septate, and 3.3–4.5 × 18.5–38.1 µm in size. In *bletilla striata*, the macroconidia were 3–5 septate, and 3.3–4.5 × 18.5–38.1 µm in size. In this study, macroconidia were 4–6 septa, most of which were 5 septa, with the macroconidia 44–118 × 4–11 µm in size. Czapek-Dox agar was used for determining the species strains from peanuts and PDA medium for strains from *Bletilla striata*, which were different from the CLA medium used in this study. However, CLA medium is commonly used to observe the spore morphology of *Fusarium* spp. [28]. The difference in spore size may also be related to host or cultural conditions, and it is necessary to study further.

Pathogenicity assays showed that leaf spot symptoms appeared on the inoculated leaves after 4 days post inoculation in *Bletilla striata* [27], and symptoms similar to those in the field were observed on leaves after 10 days inoculation in peanuts [26]. In the present study, leaf spot symptoms appeared on the inoculated tobacco leaves after one day. The pathogenicity of this pathogen to tobacco leaves was moderate and consistent with the field diseased symptom. A single inoculation site is not large, but when it spreads to the entirety of the tobacco leaves, tobacco production takes a pathogenic blow. Therefore, we should pay close attention to this pathogen in further disease control.

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**Data Availability Statement:** The ITS, EF–1, RPB2, RPB1, and CAM sequences of strain GZAX 307 were deposited under GenBank numbers ON961779, ON982723, ON982725, ON982727, and ON982721, respectively. The ITS, EF–1, RPB2, RPB1, and CAM sequences of strain GZAX 312 were deposited under GenBank numbers ON961780, ON982724, OM982726, ON982728, and ON982722, respectively.

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