Molecular Identification and Pathogenic Potential of Fungal Mycoflora Associated with Pod Decay in Soybean

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Posted Date: 14 August 2025

doi: 10.20944/preprints202508.1045.v1

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Article

Molecular Identification and Pathogenic Potential of Fungal Mycoflora Associated with Pod Decay in Soybean

Maira Munir [1], Muhammd Naeem [2], Xiaoling Wu [1], Weiying Zeng [3], Zudong Sun [3], Yuze Li [1], Taiwen Yong [1], Feng Yang [1] and Xiaoli Chang [1,*]

[1] College of Agronomy, Sichuan Agricultural University, Chengdu 611130, P.R. China

[2] Institute of crop science, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou 310058, P.R. China

[3] Institute of Economic Crops, Guangxi Academy of Agricultural Science, Nanning, 530007, Guangxi Province, P.R. China

* Correspondence: xl_chang14042@sicau.edu.cn

Abstract:

Seed and pod decay significantly threaten to soybean yield and quality during the fruiting stage worldwide. Although, few previous studies partially explained the occurrence of *Fusarium* species on soybean seeds and pods, However, fungal diversity affecting soybean pods in Sichuan Province China, third-largest soybean cultivation region remains unknown. In this study, we identified pod-infecting fungal communities and evaluated their pathogenic potential on soybean seeds and pods. Using morphological indices and DNA barcode markers, we characterized *Fusarium* species, including *F. verticillioides*, *F. incarnatum*, *F. equiseti*, *F. proliferatum*, *F. fujikuroi*, *F. oxysporum*, *F. chlamydosporum*, and *F. acutatum* through Translation elongation factor gene (*EF1-α*) and RNA polymerases II second largest subunit (*RPB2*) gene analysis. Multi-locus phylogeny assay of Internal transcribed spacer (*rDNA ITS*), β-tubulin (*β-tubulin*), Glyceraldehyde 3-phosphate dehydrogenase (*GADPH*), Chitin Synthase 1 (*CHS-1*), Actin (*ACT*), Beta-tubulin II (*TUB2*) and Calmodulin (*CAL*) distinguished *Colletotrichum* species as *C. truncatum*, *C. karstii*, *C. cliviicola*, *C. plurivorum*, *C. boninense*, and *C. fructicola*. Pathogenicity assays revealed significant damage from *Fusarium* and *Colletotrichum* isolates on soybean pods and seeds, with varying isolation frequencies. Among them, *F. proliferatum*, *F. acutatum*, and *F. verticillioides* caused the most severe symptoms, while *C. fructicola* was most pathogenic, followed by *C. truncatum*, *C. karstii*, *C. cliviicola*, *C. plurivorum*, and *C. boninense*. These findings highlight emerging virulent pathogens responsible for soybean pod decay, paves a valuable foundation for developing resistant cultivars to manage pod associated diseases at the later growth stage of soybean.

Keywords: soybean pods mycoflora; fungal diversity; morpho-molecular phylogeny; pathogenicity assay; disease management

1. Introduction

Soybean (*Glycine max L.*), is known as a globally paramount legume crop cultivated for millennia [1–3]. It serves as a critical source of plant-based protein (40%) and oil (20%) for human nutrition and animal feed, underpinning global food security [4,5]. Nevertheless, seed-borne diseases caused by multiple hazardous pathogens during the reproductive stages inflict severe economic losses through substantial yield reduction, poor seed quality, and compromised marketability [6]. These pathogens, such as *Fusarium*, *Colletotrichum*, *Diaporthe*, *Sclerotinia*, *Cercospora*, *Phytophthora*, have been predominantly reported to infect various soybean organs [7,8]. Among the most detrimental

 doi:10.20944/preprints202508.1045.v1

pathogens, *Fusarium* and *Colletotrichum* stand out due to their widespread prevalence and destructive impact on seed development and viability [9–12].

Fusarium sp. are ubiquitous seed-borne pathogens responsible for complex diseases including root rot, pod blight, seed rot, and sudden death syndrome, that severely diminish germination rates and seedling vigor worldwide [13–16]. Their genetic diversity and adaptability have been well documented, with distinct species exhibiting pronounced pathogenicity across different regions. For instance, *Fusarium proliferatum* displays high aggressiveness in China's Hubei province [12], whereas *F. oxysporum*, *F. equiseti*, and *F. graminearum* are particularly pathogenic in Sichuan province [7,17–21]. Critically, these fungi frequently exist as single entities but as pathogen complexes, greatly complicating disease management strategies and underscoring the urgent need for precise identification at the species level [22–24]. A wide spectrum of *Fusarium* species, such as *F. solani*, *F. oxysporum*, *F. acuminatum*, *F. avenaceum*, *F. cerealis*, *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. proliferatum*, *F. pseudograminearum*, *F. fujikuroi*, *F. asiaticum*, *F. commune*, and *F. verticillioides*, has been associated with soybean isolated from various tissues [25–32]. Numerous studies investigating cultivar resistance, pathogenicity, distribution and incidence rates provide evidences that the colonization of soybean roots by multiple *Fusarium* species is commonplace. However, significant knowledge gaps persist regarding their precise roles and pathogenicity dynamics specifically affecting soybean seeds and pods, as well as the ultimate impacts on seed quality and yield [30,33]. Similarly, *Colletotrichum* sp. ranking among the top 10 most significant plant pathogenic fungi globally, pose a substantial threat. Their exceptionally broad host range (infecting over 3 000 plant species) and capacity for latent infections make effective control exceptionally challenging [34–36]. In soybean, *Colletotrichum* sp. can induce anthracnose across all developmental stages [37], and symptoms manifest as leaf spotting, stem lesions, pod necrosis, and premature defoliation, collectively contributing to considerable yield losses [38]. Crucially, this disease is primarily seed-transmitted, and infected seeds lead to damping-off of seedlings and the development of lesions on cotyledons during the V1 and V2 developmental stages [39]. The genetic diversity within this pathogen complex, along with key epidemiological and biological characteristics of its constituent members, remains poorly characterized, thus necessitating more precise and comprehensive studies [40–44]. Importantly, for both *Fusarium* and *Colletotrichum* pathogens, accurate identification at the species level is the indispensable foundation for developing effective disease management.

Traditional morphological methods often prove inadequate due to overlapping characteristics within species complexes [45,46]. Consequently, molecular approaches, particularly multi-locus phylogenetic analysis targeting conserved genes, such as Internal transcribed spacer (*rDNA ITS*), Translation elongation factor gene (*EF1-α*), RNA polymerases II second largest subunit *(RPB2*) for *Fusarium* isolates, and β-tubulin (*β-tubulin*), Glyceraldehyde 3-phosphate dehydrogenase (*GADPH*), Chitin Synthase 1 (*CHS-1*), Actin (*ACT*), Beta-tubulin II (*TUB2*) and Calmodulin (*CAL*) for *Colletotrichum* isolates [47–49], have become the important standard for robust species delineation and understanding population dynamic; thereby, such precision is vital for tracking the emerging isolates and designing targeted interventions.

Intercropping systems are widely adopted due to their efficient utilization of light resources, improvement of soil structure through microbes, significant reduction of weeds and pests, and higher production under eco-friendly conditions compared to monoculture systems [21]. In Southwest China, particularly Sichuan province, the widespread adoption of maize-soybean relay strip intercropping enhances land productivity (high Land Equivalent Ratio, LER>1.5), soil health, resource use efficiency, disease and pest suppression [50,51]. However, the characteristic high humidity, moderate temperatures, and limited sunlight in this region create a microenvironment highly conducive to fungal proliferation, infection and dispersal [52,53]. Studies confirm that such cool, humid highland conditions significantly shape pathogenic fungal communities and intensify disease pressure, posing a persistent threat to soybean production [54]. Emerging evidence suggests pathogen populations exhibit rapid adaptation, complex genetic diversity, and increased aggressiveness, potentially linked to changing climatic conditions and agricultural practices [55].

 doi:10.20944/preprints202508.1045.v1

Despite this, comprehensive data on the diversity and pathogenicity of seed- and pod- associated fungi, particularly within the distinctive intercropping pattern in Sichuan province, remain limited. Therefore, this study aims to systemically isolate and characterize the mycobiota associated with soybean seeds focusing on *Fusarium* and *Colletotrichum* species based on multi-gene phylogenetic analysis, and additionally, the *in vitro* pathogenicity of typically dominant *Fusarium* and *Colletotrichum* isolates. The findings of the current study offered a promising way to elucidate composition and threat level of major seed pathogens in a critical soybean-growing region with distinctive intercropping cultivation and are expected to provide a useful scientific foundation for typical disease management and soybean resistance breeding.

2. Materials and Methods

2.1. Sampling and Fungal Isolation

A survey was conducted at experimental site to collect soybean pods with discoloration, decay, and the presence of mycelium, under maize-soybean relay strip intercropping from five different soybean cultivating regions in Sichuan province, Southwest China. The fungal pathogens were isolated by washing pod samples under running tap water, followed by drying, excising into small fragments (4-8 mm), and surface sterilization using 1% sodium hypochlorite (w/v) for 1 minute and 75% ethanol (w/v) for 2 minutes. The small fragments were washed thrice, dried on sterile filter paper, and transferred to potato dextrose agar plates (PDA; potato 200 g·L^{-1}, glucose anhydrous 10 g·L^{-1}, and agar 15 g·L^{-1})[25]. The plates were incubated for 7-15 days at 25±2°C in complete darkness, and the fungal isolates were purified by transferring active marginal hyphae onto fresh PDA plates.

2.2. Morphological Identification of Fungal Population

The morphological identification of fungal population was executed by observing colony feature and texture, the length and width of macroconidia, number of septa, conidial shape and size, and growth rate. These fungal isolates were incubated at 25±2°C and colony growth was recorded after two days of incubation, while the growth rate of *Fusarium* species were documented after 7 days of incubation in dark conditions. For *Colletotrichum* species morphological identification, except growth rate (two days of post inoculation) the other cultural variables were distinguished after 15 days of post-incubation. In addition, the general PDA and species-specific CMC (Carboxymethyl-Cellulose 15.0 g·L^{-1}, KH_2PO_4 monobasic 1.0 g·L^{-1}, NH_4NO_3 1.0 g·L^{-1}, yeast extract 1.0 g·L^{-1}, $MgSO_4.7H_2O$ 0.5 g·L^{-1} in distilled water) media were used for the conidial spore production of *Fusarium* species. Similarly, PDA medium was utilized to generate enough spores of *Colletotrichum* species accordingly. The number and size of conidia (n=50) were recorded carefully of each fungal species by observing them under the accessible compound microscope (Eclipse 80i, Nikon, Japan) [56].

2.3. Molecular Identification of Fungal Genera

The 7-day-old mycelium of fungal isolates cultured on PDA plates were scraped with disinfected blades to extract the genomic DNA, following the standard manual of the Rapid Fungi Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China). The quality and quantity of DNA were assessed using a NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, Delaware, USA) after extraction. For each fungal genus, specific primer pairs (listed in Table S1) were selected for PCR amplification. A 50 µL of reaction mixture was prepared, containing 2 µL of each primer, 25 µL of Taq PCR Master mix, 2 µL of DNA from each isolate, and 19 µL of sterilized water. The Peltier Thermal Cycler (S-1000TM, Bio-Bri, China) was used for amplification, and the temperature conditions for different primers are mentioned in Table S1. The amplified products were subjected to electrophoresis on a 1.0% (w/v) agarose gel in 1×TAE buffer, and samples were sequenced using the ABI-PRISM 3730 automatic sequencer (Applied Biosystems, Foster, USA).

2.4. Phylogenetic Analysis

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To evaluate the genetic differences among different fungal genera, molecular evolutionary and phylogenetic analysis were executed. The amplified sequences were trimmed with BioEdit software (developed by Tom Hall; BioEdit free download v.7.0.5.3) and then blasted against multiple databases, including *FUSARIUM*-ID and *Fusarium* MLST for *Fusarium* species [57], and the National Centre for Biotechnology Information (NCBI) for *Colletotrichum* species. Additionally, Clustal X 1.83 was used for alignment of sequences of each constructed tree by removing gaps (missing barcode information) and weighing the characters universally. Phylogenetic trees of combined barcode for *Fusarium* and *Colletotrichum* species were constructed accordingly using MEGA version X with the Neighbor-Joining method, supported by the Tamura–Nei model, respectively [58]. The constructed tree clades were supported by 1000 bootstrap replicates, and resulted sequences were deposited in the NCBI GenBank and TreeBASE (www.treebase.org).

2.5. Pathogenicity Test of Isolated Fungi

For pathogenicity tests, seeds of the soybean cultivar 'Nandou12' and soybean pods were inoculated with spore suspension respectively of each fungal species to fulfil Koch's postulates, following the method described by [59] with minor modifications. Three-prototype isolates of each species were selected randomly to analyses their pathogenic impact on seeds and pods respectively. *Fusarium* spores were produced by adding 3-5 mycelial discs to 20 mL of PDA or CMC medium and then incubated in orbital shaker at 150 r·min^{-1} at 25°C for 7 days. Similarly, *Colletotrichum* isolates spores were obtained by scraping fungal mycelium in deionized water. The final spore concentration was adjusted to 1 × 10^5 spore mL^{-1} with double distilled water (ddH_2O) for inoculation. The seeds and pods were subjected to surface sterilization with 1% NaClO and rinsed three times for 1 minute, followed by air drying under sanitized conditions on double-layered filter paper [60]. Three separate replicates were prepared, each containing 15 seeds per plate and 3 pods for each isolate. The seeds and pods were dipped in spore suspension for 15 minutes to allow diseases development [45]. Seeds treated with ddH_2O served as the negative control, and all plates were incubated in the dark at 25 ± 2°C for 7 days with 70% relative humidity. After incubation, disease severity index (DSI) was assessed as described by [8] with minor modifications. In addition, the percentage of mycelium coverage area (PMC) and seed and pod weight were noted. The DSI and PMC were calculated using the following formula:

$$\boldsymbol{DSI} = \frac{\sum(Severity\ rating\ X\ Seed/pod\ number\ per\ rating)}{(Number\ of\ total\ seeds\ X\ highest\ severity\ rating)} X100$$

$$\boldsymbol{PMC}\ (\%) = \frac{Area\ covered\ by\ mycelium}{Number\ of\ total\ seed/pod\ surface\ area} \times 100$$

2.6. Isolation Frequency Analysis of Fungal Isolates

The isolation frequency of fungal genera especially *Fusarium* and *Colletotrichum* isolates were calculated by dividing the number of times a specific fungus is isolated by the total number of fungal isolates obtained in the study, then multiplying by 100 to express the result as a percentage. The isolation frequency was deliberated using the following formula:

$$\boldsymbol{Isolation\ frequency}\ (\%) = \frac{\boldsymbol{Number\ of\ isolates\ per\ species}}{\boldsymbol{Total\ number\ of\ isolates}} \times \mathbf{100}$$

2.7. Data Process and Analysis

The recorded data was processed through Microsoft office excel 2016 (Microsoft Corporation, Redmond, Washington, USA). The DSI and PMC average values were calculated from independent triplicates in pathogenicity tests. In addition, seed and pod weight were subsequentially recorded for each representative isolates of *Fusarium* and *Colletotrichum* species. The statistical analysis was

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performed by applying the Duncan's multiple range test in IBM SPSS Statistics 20 (IBM Corp., Armonk, N.Y., USA) to underpin the significant differences (P = 0.05).

3. Results

3.1. Identification of Fungal Species Associated with Intercropped Soybean Pods

In the present study, soybean pods (n=182) were collected from soybean under maize-soybean strip intercropping pattern, and total 132 isolates were obtained. Upon morphological characteristics including colonial color and texture of colonies and mycelium, these isolates were primarily clustered into ten groups (Figure 1A). Furthermore, *rDNA ITS* fragments were amplified and sequenced, thus BLASTn analysis revealed these isolate groups represented ten distinct genera, including *Fusarium* sp., *Colletotrichum* sp., *Phomopsis*/*Diaporthe* sp., *Bipolaris* sp., *Nigrospora* sp., *Graphium* sp., *Clonostachys* sp., *Nadulisporium* sp., *Alternaria* sp., and *Boeremia* sp. Phylogenetic analysis showed that different fungal genera were clearly separated but clustered with their corresponding reference isolates (Figure 1B, Table S2).

To analyze the isolation frequency of these fungal genera associated with soybean pods, we found that *Fusarium* sp. (32.57%) was frequently isolated from intercropped soybean pods, followed by *Colletotrichum* sp. (28.03%), *Phomopsis*/*Diaporthe* sp. (12.12%), *Bipolaris* sp. (12.12%). *Nigrospora* sp. (4.54%), *Graphium* sp. (3.03%), *Clonostachys* sp. (3.03%), and *Nadulisporium* sp. (1.51%). Both *Alternaria* sp. and *Boeremia* sp. had the lowest isolation frequency accounting for 0.75% (Figure 1C). Thus, fungal communities associated with soybean pods in Sichuan province are well-characterized, and *Fusarium* and *Colletotrichum* were the dominant genera among them.

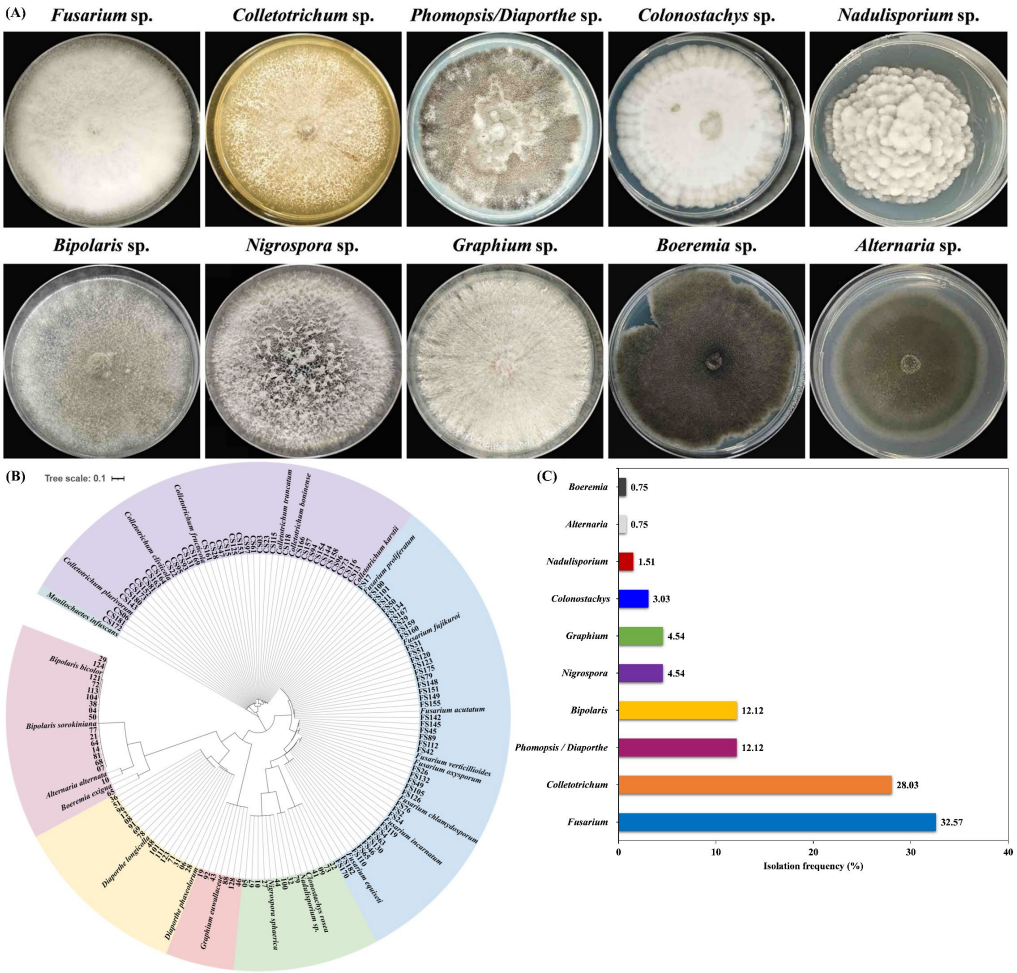

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Figure 1. The obtained mycoflora infecting soybean pods based on morphological features and sequence analysis of *rDNA ITS* fragments. (**A**) Colonial morphology of fungal isolates, (**B**) Phylogenetic tree of fungal isolates constructed using *rDNA* ITS. (**C**) The isolation frequency of fungal isolates associated with intercropped soybean pods. The colonies were observed after 7 days of incubation on PDA. The phylogenetic tree including 132 isolates and 25 reference isolates, and an outgroup *Monilochaetes infuscans* was constructed by MEGA X, and branches showing values >70 was excluded. Bootstrap support values were calculated from 1000 replications. *Monilochaetes infuscans* was used as outgroup.

3.2. Identification of Fusarium Species Associated with Soybean Pods

To further verify *Fusarium species*, morphological features of colonies and conidial spores of different *Fusarium* isolates were observed as shown in Figure 2A and Table 1. After seven days of incubation, *Fusarium* isolates displayed a pronounced variation in colony colors, which ranged from filthy white, dark violet and light violet to light pink. The mycelia also exhibited sparse and fluffy white mycelia, dense white or white-purple mixtures. Almost all *Fusarium* isolates produced pointed and sickle-shaped macroconidia. Based on conidial morphology, size and colony pigmentation, total 43 *Fusarium* isolates clustered into eight morphological groups.

For molecular validation, the *EF1-α* and *RPB2* genes were amplified, sequenced, and blasted against the *Fusarium* MLST and *FUSARIUM*-ID databases. Sequence similarity analysis identified eight distinct *Fusarium* species: *F. verticillioides*, *F. incarnatum*, *F. equiseti*, *F. proliferatum*, *F. fujikuroi*, *F. oxysporum*, *F. chlamydosporum*, and *F. acutatum*. For phylogenetic analysis, maximum-likelihood tree was constructed using combined *EF1-α* and *RPB2* genes. The trees included 43 *Fusarium* isolates from this study and 17 reference isolates and *Nactriaceae* sp. (JF740999.1) serving as an outgroup (Table S3). Phylogenetic analysis clearly resolved the taxonomic relationships and genetic distances among the *Fusarium* species (Figure 2B). All eight species formed distinct clades, except within two species complexes: the *F. incarnatum-equiseti* complex (FEIC) and the *F. proliferatum-fujikuroi* complex (FPFC). These species complexes grouped in the same major clade but formed well-supported distinct subclades. Bootstrap support values exceeded 92% for all species and species complex branches. The generated sequences were deposited in GenBank, and accession numbers are provided in Table S4.

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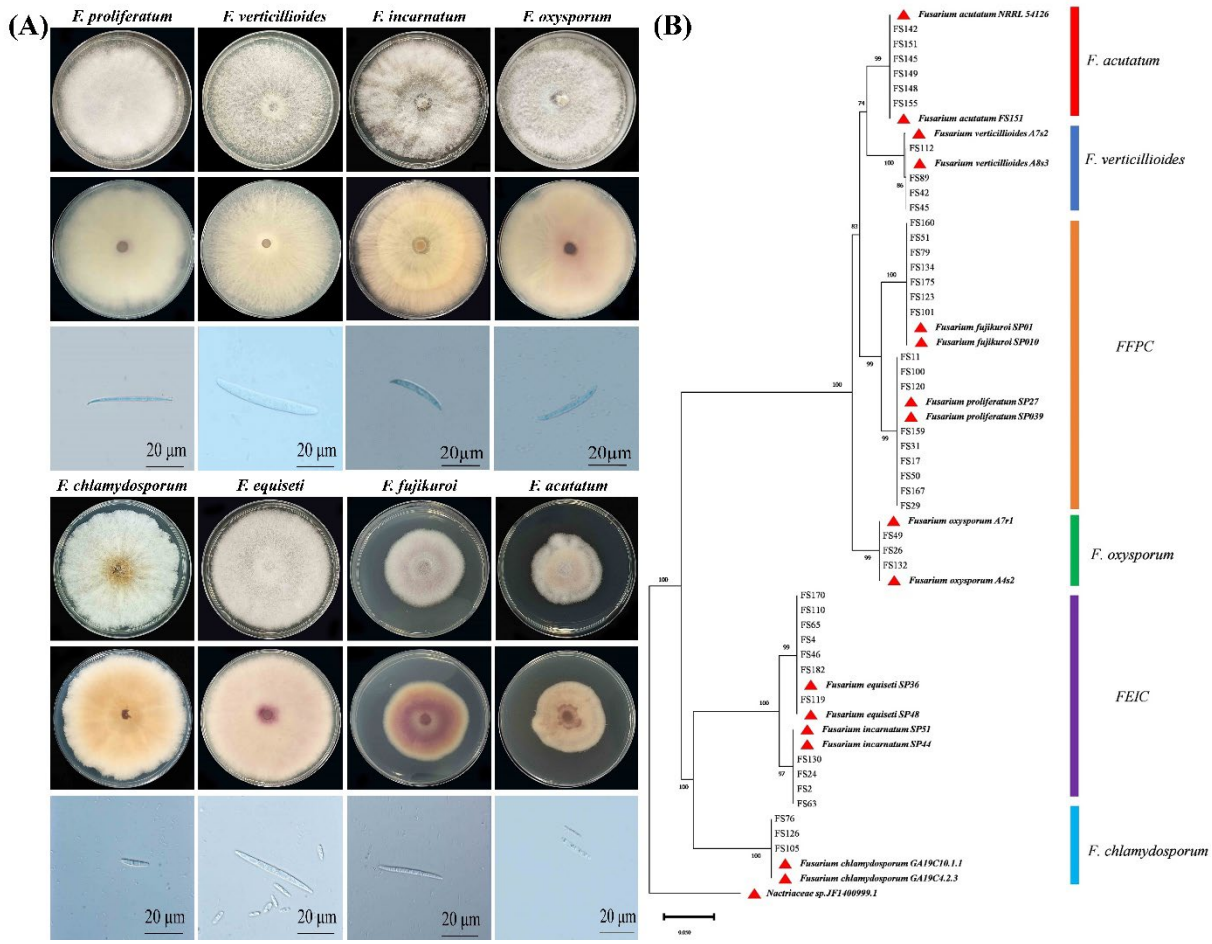

Figure 2. Morphological and molecular identification of *Fusarium* isolates associated with soybean pods. (**A**) Characterization of colonies and conidium of *Fusarium* isolates. Typical colonies of *Fusarium* species were observed after 7 days, and conidium spores (down line) were visualized under microscopy after 10 days of growth on PDA. Scale bars are 20 μm. (**B)** Phylogenetic tree of *Fusarium* isolates based on *rDNA ITS* fragments. A maximum likelihood (ML) tree was constructed by MEGA X (Pennsylvania State University). Bootstrap support values were ≥50% from 1000 replications, which are shown at the nodes**.**

Table 1. Morphological characters of *Fusarium* species cultured on PDA medium.

***Fusarium* species**	**Shape**	**Macroconidia**		**Septum**	**Colony characterization**	**Growth rate (cm/day)**
		Width (μm)	**Length (μm)**			
F. equiseti	**Falcate**	3.10±0.02c, 3.02-3.7	39.25±1.81a, 38.23-45.98	3-5	Pale grey color (front), ginger yellowish (back)	4.88 ± 0.41b
F. incarnatum	Falcate	3.98±0.44a, 5.67-2.72	36.98±3.63a, 45.55-36.62	3-4	Pale grey color(front), yellowish color (back)	5.32 ± 0.39a
F. verticillioides	Fusiform	3.40±0.9b, 3.8-3.33	23.25±0.2b, 20.21-25.90	2-3	pale grey colonies, reverse pale grey	4.9 ± 0.3b
F. proliferatum	Falcate, fusiform	3.60±1.12b, 5.41-2.96	39.12±6.54a, 48.56-32.66	3-4	Pale grey color (front), pale grey (back)	4.50 ± 0.03c
F. fujikuroi	Falcate	2.42±0.46e, 3.12-2.28	39.92±1.98a, 43.82-38.94	3.-5	Pale grey color (front), pale yellowish color (back)	4.76 ± 0.32c
F. oxysporum	Falcate, with a foot spore	3.1±0.16c, 2.31-4.82	26.9±1.6b, 28.62-22.23	3	pale grey (front) pale purple on the back	5.4 ± 0.3a
F. chlamydosporum	Falcate	3.20±0.82c, 3.90-3.22	25.45±0.20b, 28.12-23.95	2-3	Brown, light pink (Front) purple (back)	5.23 ± 0.01a

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F. acutatum	Falcate	2.79±1.62d, 3.20-1.98	23.24±0.8b, 24.56-20.86	3-5	white grey (front) purple (back)	4.39 ± 0.02c

Note: All data are the average value from three independent replicates. Different lower-case letter in the identical column reveals a significant variation at the level of $P > 0.05$.

3.3. Identification of Colletotrichum Species Associated with Soybean Pods

Morphological analysis of 132 fungal isolates identified 37 isolates as *Colletotrichum* species. Colonies exhibited white, cottony mycelia, while conidia varied in shape (fusiform, cylindrical and oval/ellipsoidal) presented in Figure 3A and Table 2. There isolates were classified into six morphological groups. Furthermore, *Colletotrichum* species was confirmed using a six-locus molecular approach including *rDNA ITS*, *CHS*, *GAPDH*, *ACT*, *CAL* and *TUB*2 genes. BLASTn analysis revealed maximum sequence similarity with six distinct *Colletotrichum* species. Phylogenetic analysis based on these loci employed maximum parsimony and ML methods (1000 bootstrap replicates). The tree included 37 isolates from this study, 12 reference isolates and *Monilochaetes infuscans* (CBS:869.96) as an outgroup (Table S5). Our results demonstrated that all isolates clustered within a single major clade but resolved into six well-supported species: *C. truncatum*, *C. karstii*, *C. cliviicola*, *C. plurivorum*, *C. boninense*, and *C. fructicola* and their accession numbers were obtained from GenBank (Figure 3B, Table S6).

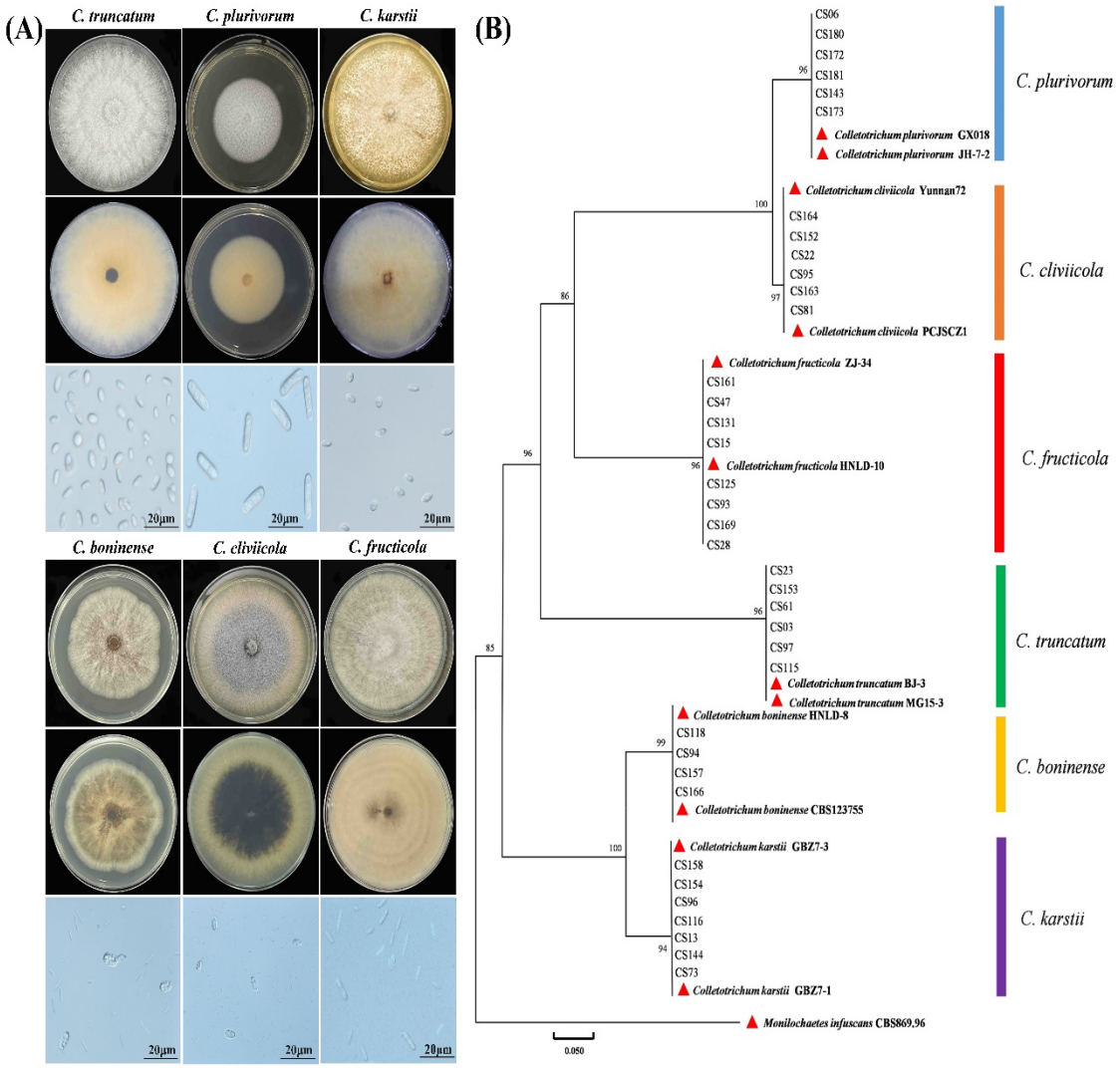

Figure 3. Morphological and molecular identification of *Colletotrichum* isolates associated with soybean pods. (A) The morphological traits of *Colletotrichum* species isolated from soybean. Colonies of *Colletotrichum* species were examined after 7 days and spores after 15 days of incubation. (B) Phylogenetic tree of *Colletotrichum* isolates

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based on *rDNA ITS*, *CHS*, *GAPDH*, *ACT*, *CAL* and *TUB*2 genes. The tree was constructed using the ML method by MEGA X (Pennsylvania State University). Bootstrap support values were ≥50% from 1000 replications, which are shown at the nodes.

Table 2. Morphological characters of *Colletotrichum* species cultured on PDA medium.

Species name	Texture	Conidial shape	Conidia size		Growth rate (cm/day)
			Length (μm)	Width (μm)	
C. truncatum	**Cottony**	**Fusiform**	23.20±0.56a 24.65-16.22	5.56±0.35b 5.90-4.32	6.90 ± 0.12a
C. karstii	Cottony	Cylindrical	15.5±0.20b 18.20-14.90	6.80±0.23a 8.56-5.52	5.63 ± 0.27b
C. cliviicola	Cottony	oval/ellipsoidal	13.35±0.02c 14.22-12.86	3.62±0.06c 4.56-3.22	6.12 ± 0.25a
C. plurivorum	Cottony and white	Fusiform	13.75±0.12c 15.78-12.66	3.4±0.02c 4.45-3.56	6.30 ± 0.09a
C. boninense	Medium brown	Cylindrical	15.10±0.2b 16.20-14.75	5.30±0.45b 6.60-4.25	6.15±0.02a
C. fructicola	Greyish black	Fusiform	12.90±0.32c 14.56-10.86	6.80±0.23a 8.56-5.52	6.4±0.60a

Note: All data are the means of three independent replicates: Different lower-case letter in the identical column reveals a significant variation at the level of $P > 0.05$. n=50.

3.4. Isolation Frequency of Fusarium and Colletotrichum Species

For isolation frequency, *F. proliferatum* (20.93%), *F. fujikuroi* (16.27%) and *F. equiseti* (16.27%) were most prevalent, followed by *F. acutatum* (13.95%), *F. verticillioides* (9.3%) and *F. incarnatum* (9.3%) among *Fusarium* genera. Compared to other species, *F. oxysporum* and *F. chlamydosporum* were the least frequent, with the isolation frequency of 6.97% each (Figure 4A). Among *Colletotrichum* species, *C. fructicola* was predominant (21.62%), followed by *C. truncatum* and *C. karstii* (18.91% each), *C. cliviicola* and *C. plurivorum* (16.21% each). *Colletotrichum boninense* was the least isolated and accounts for 10.81% of total *Colletotrichum* isolates (Figure 4B). Hence, the *F. proliferatum* and *C. fructicola* were dominant species isolated from soybean pods in Southwest China.

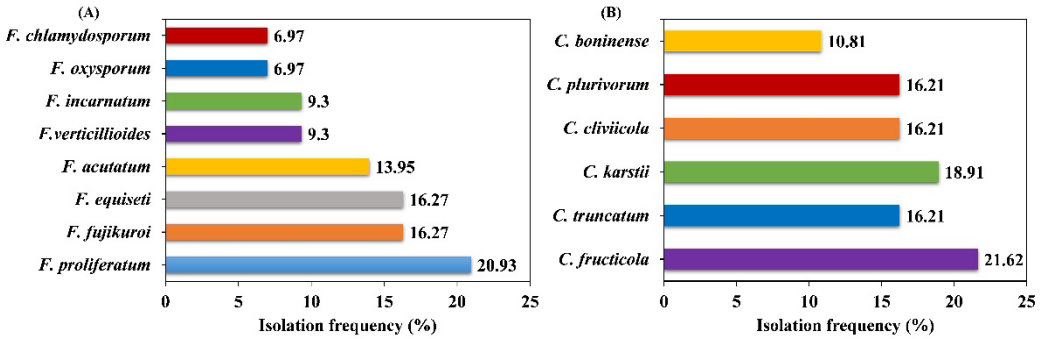

Figure 4. The isolation frequency of *Fusarium* and *Colletotrichum* species from intercropped soybean pods. (A) *Fusarium* sp. and (B) *Colletotrichum* sp.

3.5. Pathogenicity of Fusarium Species on Soybean Pods and Seeds

Pathogenicity assays were conducted to evaluate the effects of *Fusarium* species on soybean pods (Figure 5 and Table 3) and seeds (Figure S1 and Table S7). As shown in Figure 5, all *Fusarium* species successfully penetrated soybean pods, causing varying degrees of internal seed decay. Among them, *F. acutatum* and *F. verticillioides* (100%) resulted in complete PMC (100%), followed by *F. proliferatum*

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(90.66%), *F. equiseti* (88.33%), *F. oxysporum* (55%), *F. fujikuroi* (46.66%), *F. incarnatum* (26.66%), and *F. chlamydosporum* (23.33%). Interestingly, *F. proliferatum*, *F. acutatum*, and *F. verticillioides* exhibited a DSI of 100%, while *F. oxysporum* had a DSI of 83.33%, *F. fujikuroi*, *F. chlamydosporum*, and *F. equiseti* all had a DSI of 75%. *F. incarnatum* showed the lowest DSI (33.33%). Additionally, infected pods exhibited a reduced weight compared to un-inoculated controls, likely due to mycelial overgrowth (Table 1). Internal seed rot was observed with *F. proliferatum* and *F. fujikuroi*, while other species caused external rot with minimal discoloration compared to control pods. Seven days post-inoculation, the seeds showed partial to complete coverage by white mycelium (with noted color variation), correlating with species-specific virulence (Figure S1, Table S7). Our results demonstrate that *F. proliferatum*, *F. acutatum* and *F. verticillioides* were the most virulent species.

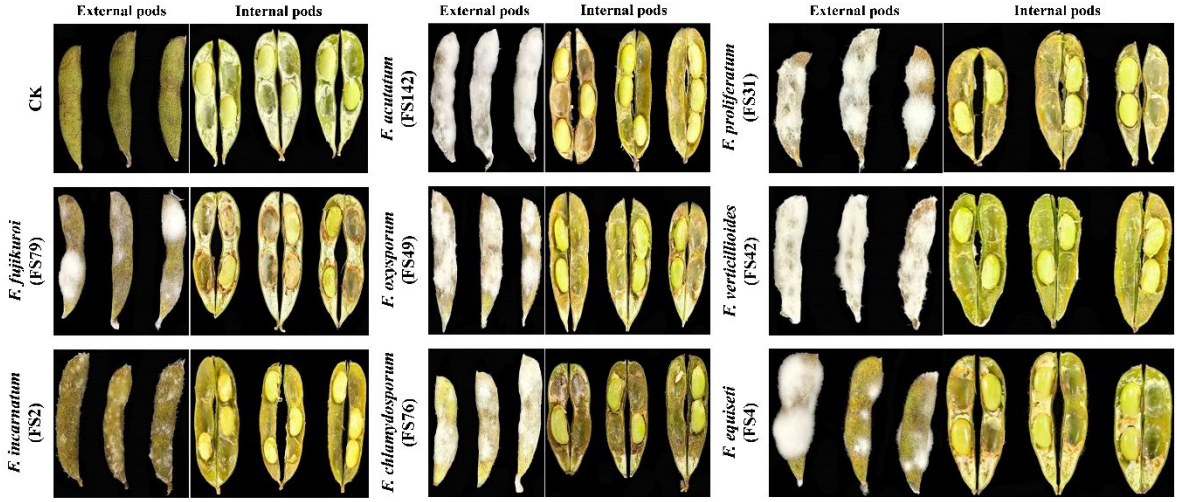

Figure 5. The pathogenicity of different *Fusarium* species on soybean pods. Soybean pods were inoculated with the selected *Fusarium* isolates by a pod-soaking inoculation method at a final concentration of 1×10^5 spores per mL. The disease symptoms were observed after 7 days of post inoculation.

Table 3. Pathogenicity of *Fusarium* species on soybean pods isolated from intercropped soybean pods.

Isolates	Disease incidence (%)	PMC (%)	DSI (%)	Pod Weight (g)
Control (CK)	0	0±0e	0±0d	1.89±0.04d
F. proliferatum (FS31)	100	90.66±0.40a	96.66±4.71a	1.79±0.31c
F. proliferatum (FS120)	100	89.66±0.47a	100±0a	1.71±0.05c
F. proliferatum (FS167)	100	78.33±2.35a	93.33±4.71a	1.74±0.01c
F. fujikuroi (FS79)	100	43.33±2.35b	66.66±11.78b	2.48±0.27b
F. fujikuroi (FS101)	100	46.66±4.71b	75±0ab	2.37±0.24b
F. fujikuroi (FS123)	100	23.33±2.35c	58.33±11.78b	2.71±0.09b
F. equiseti (FS4)	100	88.33±2.35a	75±11.78b	2.21±0.07b
F. equiseti (FS65)	100	41.66±2.35b	50±11.78b	2.19±0.02b
F. equiseti (FS170)	100	21.66±2.35c	50±20.41b	2.13±0.03b
F. acutatum (FS142)	100	100±0a	100±0a	2.64±0.09b
F. acutatum (FS151)	100	95±4.08a	100±0a	2.47±0.31b
F. acutatum (FS155)	100	98.33±2.35a	100±0a	2.57±0.12b
F. verticillioides (FS42)	100	98.33±2.35a	100±0a	2.29±0.12b
F. verticillioides (FS89)	100	100±0a	100±0a	2.25±0.04b
F. verticillioides (FS112)	100	100±0a	100±0a	2.29±0.06c
F. incarnatum (FS2)	100	26.66±2.35c	33.33±11.78c	3.34±0.19a
F. incarnatum (FS24)	100	20±4.08dc	25±0c	3.29±0.05a
F. incarnatum (FS130)	100	18.33±6.23d	33.33±11.78c	3.25±0.06a

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F. oxysporum (FS49)	100	55±4.08b	83.33±11.78a	1.84±0.27c
F. oxysporum (FS26)	100	45±4.08b	66.66±11.78b	1.86±0.04c
F. oxysporum (FS132)	100	50±4.08b	58.33±11.78b	1.89±0.16c
F. chlamydosporum (FS76)	100	18.33±2.35d	75±0ab	1.68±0.16c
F. chlamydosporum (FS105)	100	23.33±6.23c	50±20.41b	1.57±0.05c
F. chlamydosporum (FS126)	100	16.66±2.35d	66.66±11.78b	1.69±0.08c

Note: The data are the average value from three independent replicates. Lowercase in the same column indicate significant difference. Significant difference was analyzed using Duncan's multiple range assay at the level of $P > 0.05$.

3.6. Pathogenicity of Colletotrichum Species on Soybean Pods and Seeds

All *Colletotrichum* species caused diseases on soybean pods, characterized by rotted pods, discolored, and abundant mycelial coverage on pods (Figure 6). All representative isolates of *Colletotrichum* species resulted in 100% disease incidence on soybean pods (Table 4). *Colletotrichum karstii* (91.66%) *C. fructicola* (90%) exhibited the highest PMC, followed by *C. truncatum* and *C. cliviicola* (both 81.66%). In contrast, *C. boninense* (11.66%) and *C. plurivorum* (10%) showed minimal PMC. DSI was highest for *C. fructicola* (100%), followed by *C. truncatum* and *C. karstii* (both 83.33%), *C. cliviicola* (66.66%), and *C. plurivorum* and *C. boninense* (both 33.33%). Similarly, pod weight varied significantly upon species. *C. boninense* recorded the highest weight (2.98 g), followed by *C. karstii* (2.67 g), *C. plurivorum* (2.59 g), *C. cliviicola* (2.21 g), *C. truncatum* (2.16 g), and *C. fructicola* (2.09 g). Furthermore, internal seed rot with discoloration occurred in pods infected by *C. fructicola*, *C. truncatum*, *C. karstii* and *C. cliviicola,* while *C. plurivorum* and *C. boninense* caused only external discoloration externally. Based on disease severity metrics and symptoms, *C. fructicola* emerged as the most virulent pathogen on soybean pods (Figure 6). Similarly, *Colletotrichum* species also caused severe damage to inoculated soybean seed (Figure S2), with all species covering seeds with mycelium. Among these species, representative isolates of *C. fructicola* had the highest PMC (100%), followed by *C. cliviicola* (96.66%) and *C. boninense* (93.33%). However, maximum DSI occurred in *C. fructicola* and *C. truncatum* (both 91.66%), trailed by *C. boninense* (81.66%) and *C. cliviicola* (70%). Comparably, *C. karstii* and *C. plurivorum* exhibited the lowest pathogenicity, with DSI of 58.33% and 38.33%, respectively. In contrast, *C. plurivorum* and *C. boninense* caused only external damage with slight discoloration (Table S8). Thus, *C. fructicola* was the most virulent species on both soybean pods and seeds.

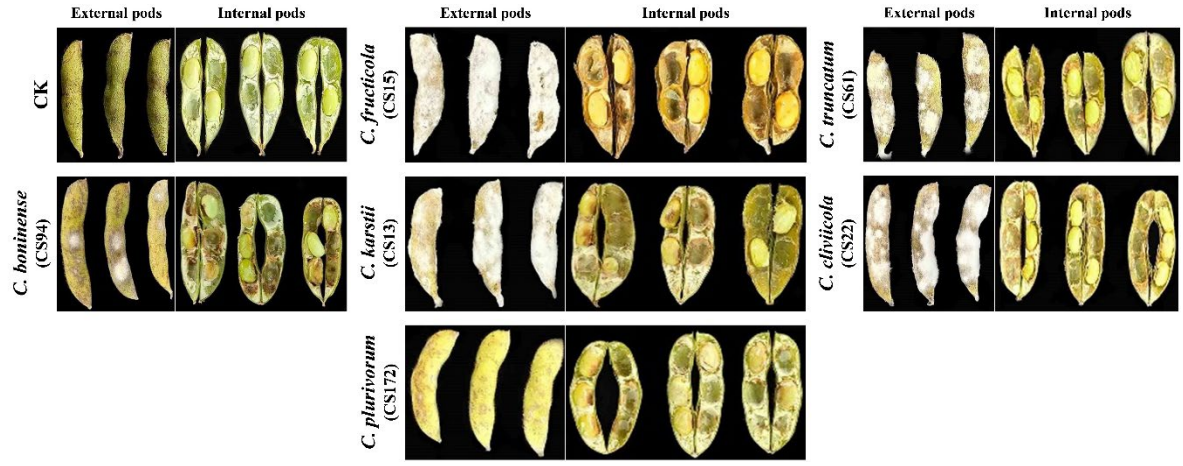

Figure 6. The pathogenicity of different *Colletotrichum* species on soybean pods. Soybean pods were challenged by the chosen *Colletotrichum* isolates by using the pod-soaking inoculation method with the final concentration of 1×10^5 spores per mL. The disease symptoms were noted after 7 days of post inoculation.

Table 4. Pods pathogenicity of *Colletotrichum* species isolated from intercropped soybean pods.

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Isolates	Disease incidence (%)	PMC (%)	DSI (%)	Pod weight (g)
Control (CK)	0	0±0d	0±0e	1.86±0.040d
C. fructicola (CS15)	100	90±4.08a	100±0a	2.51±0.04b
C. fructicola (CS93)	100	86.66±6.23a	91.66±11.78a	2.52±0.02b
C. fructicola (CS169)	100	76.66±2.35ab	100±0a	2.51±0.01b
C. truncatum (CS03)	100	78.33±4.71ab	75±0b	2.09±0.02c
C. truncatum (CS61)	100	80±4.08a	83.33±11.78b	2.11±0.01c
C. truncatum (CS153)	100	81.66±2.35a	66.66±11.7b	2.16±0.08c
C. karstii (CS13)	100	91.66±2.35a	83.33±11.78b	2.67±0.05b
C. karstii (CS 96)	100	86.66±2.35a	75±0b	2.47±0.08b
C. karstii (CS 158)	100	80±7.07a	75±0b	2.67±0.03b
C. cliviicola (CS 22)	100	81.66±2.35a	66.66±11.78c	2.13±0.12c
C. cliviicola (CS95)	100	76.66±2.35ab	58.33±11.78c	2.21±0.08c
C. cliviicola (CS 164)	100	65±4.0b	50±20.41c	2.26±0.09c
C. plurivorum (CS06)	100	10±4.08c	25±0d	2.60±0.02b
C. plurivorum (CS172)	100	8.33±2.35c	33.33±11.78d	2.59±0.01b
C. plurivorum (CS180)	100	6.66±2.35c	25±20.41d	2.61±0.01b
C. boninense (CS94)	100	10±4.08c	33.33±11.78d	2.96±0.01a
C. boninense (CS118)	100	11.66±6.23c	25±0d	2.98±0.02a
C. boninense (CS166)	100	10±4.08c	25±0d	2.95±0.03a

Note: The data are the mean value from three independent replicates. Lowercase in the same column indicate significant difference. Significant difference was calculated by applying Duncan's multiple range assay at the level of $P > 0.05$.

4. Discussion

It is well known that fungal diseases causing soybean seed and pod deterioration significantly reduce global yield and quality [61]. The current study employed morpho-molecular characterization and multi-locus phylogenetic analysis to investigate fungal pathogens in Sichuan province, Southwest China. And we focused on two major genera, *Colletotrichum* and *Fusarium* species. Several previous studies documented the occurrence and pathogenic effects of individual *Fusarium* species [8,11,25,62], our work expands understanding by characterizing the fungal diversity across both genera. We identified eight *Fusarium* species (*F. verticillioides*, *F. incarnatum*, *F. equiseti*, *F. proliferatum*, *F. fujikuroi*, *F. oxysporum*, *F. chlamydosporum*, and *F. acutatum*) and confirmed the presence of the FPFC and FEIC species complexes (Figure 2), aligning with reports from Sichuan province [8]. Additionally, *Colletotrichum,* the 8th most devastating and wide-spectrum plant pathogen [35], causes anthracnose in soybean and related legumes [63]. We identified six species including *C. truncatum*, *C. karstii*, *C. cliviicola*, *C. plurivorum*, *C. boninense*, and *C. fructicola* (Figure 3).

Furthermore, accurate pathogen identification is essential for disease management [40]. Advances in fungal disease identification have significantly improved the ability to identify a range of pathogenic plant fungi employing specific gene sequence analysis and improved molecular techniques [64,65]. It is well-established that both *Fusarium* and *Colletotrichum* species often appear in complexes, sharing similar morphological characteristics (e.g., colony and spore shape) worldwide [66]. To characterize these species, the amplification of two or more genes has emerged as a standard method for accurately identifying specific fungal species within widely spread genera [67]. By witnessing morphological features, molecular procedures, and phylogenetic analysis of *RPB2* and *EF1-α* gene sequences, we recognized 43 different *Fusarium* species. It has been assumed that two sequencing sections, *EF1-α* and RNA polymerase largest subunit *RPB1* and/or *RPB2*, are indispensable for *Fusarium* species characterization [68]. multiple recent studies have used these regions for the precise identification of *Fusarium* isolates complexes [9,23,24,69]. For *Colletotrichum* species identification, we used multilocus analysis of *ACT*, *CHS*, *ITS*, *GAPDH*, *TUB2* and *CAL* genes.

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employing these genes, we recognized six different *Colletotrichum* species, including *C. boninense*, *C. truncatum*, *C. cliviicola*, *C. karstii*, *C. fructicola* and *C.* plurivorum. A parallel method has been used to identify the *Colletotrichum* isolated infecting olive trees and Tea-Oil Camellia (*Camellia oleifera C. Abel*) [25,26].

Pathogenicity evaluation exposed tissue-specific virulence. Among *Fusarium* isolates, *F. acutatum* and *F. verticillioides* exhibited the highest aggressiveness (PMC and DSI) on soybean seeds. Earlier studies have revealed that *F. verticillioides* has the ability to diminish soybean seed quality [52], while *F. acutatum* causes root rot and crown , leading to yellowing and plant death in tomato plants [27]. Among *Colletotrichum* isolates, *C. truncatum* and *C. fructicola* were the furthermost virulent isolates when inoculated on soybean seeds. Interestingly, few past studies have pronounced the *C. fructicola* as a non-host specific pathogen proficient of infecting an extensive range of plants and crops, including blueberry [22], sugarcane [29], and apple [70]. Except for *C. truncatum* and *C. fructicola*, our results presented that *C. karstii* acted as a moderately aggressive pathogen toward soybean pods and seeds. In line with our study, *C. karstii* has been reported to cause anthracnose in soybean in China [71]. Many studies have documented that soybean pod diseases lead to seed deterioration, which negatively affects seed germination and reduces grain yield in soybean fields [72]. The pathogenicity outcomes from current study confirmed that the isolated *Fusarium* and *Colletotrichum* isolates are lethal and capable of decaying soybean pods and seeds, directly impacting PMC, DSI and seed weight. We predict that *F. acutatum*, *F. verticillioides*, *C. fructicola*, and *C. truncatum* are destructive plant pathogens responsible for soybean pod and seed decay. Therefore, understanding their infection mechanisms through molecular analysis provides critical insights for developing targeted management strategies.

5. Conclusions

This study underpins the significance of managing fungal pathogens in soybean pods to protect yields. The infected pods from five different locations in Sichuan Province, Southwest China, yielded *Colletotrichum* and *Fusarium* species. Through morphological indices and molecular markers analysis, we identified 43 *Fusarium* isolates. Additionally, six other molecular markers were amplified to identify 37 *Colletotrichum* isolates. Pathogenicity tests highlights that *F. verticillioides*, *F. acutatum* and *F. proliferatum* were the most destructive, causing noteworthy pod and seed damage, and reduced the seed vigor. Among *Colletotrichum* isolates, *C. fructicola* and *C. truncatum* were the highly aggressive by exhibiting severe symptoms on soybean seeds and pods. These findings underscore the necessity of developing resistant soybean varieties through targeted breeding, particularly against the most aggressive fungal species in high-risk regions like Southwest China, where environmental conditions favor pathogen proliferation, and would greatly reduce seed and pods rot, improving germination rates, and hamper yield losses, thus enhancing sustainability of soybean production.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/doi/s1. Table S1: The primers used for amplification and identification of *Fusarium* and *Colletotrichum* species. Table S2: Reference sequences of *rDNA ITS* gene from NCBI used for the homology analysis of isolated fungal diversity. Table S3: Reference sequences of *RPB2* and of *EF1-α* gene from Fusarium MLST, GenBank and FUSARIUM-ID used for the homology analysis of isolated *Fusarium* species. Table S4: The GenBank accession numbers of *RPB2* and *EF1-α* of *Fusarium* species obtained from soybean pods. Table S5: Reference sequences of *ITS*, *CHS*, *GADPH*, *ACT*, *CAL* and of *TUB2* gene from NCBI GenBank used for the homology analysis of isolated *Colletotrichum* species. Table S6: The gene bank accession numbers of *CHS*, *ITS*, *GADPH*, *ACT*, *CAL* and *TUB2* gene from NCBI. Table S7: Seed pathogenicity of *Fusarium* species isolated from intercropped soybean pods. Table S8: Seeds pathogenicity of *Colletotrichum* species isolated from intercropped soybean pods. Figure S1: Seed symptoms after inoculation with the representative isolates of different *Fusarium* species from intercropped soybean pods. Figure S2: Seed symptoms after inoculation with the representative isolates of different *Colletotrichum* species from intercropped soybean pods.

Author Contributions: Conceptualization, M.M. and X.L.; methodology, M.M.; software, M.M. and F.Y; validation, M.M., X.L. and W.Z.; formal analysis, M.M. and T.Y; investigation, M.M and X.L.; resources, X.L.;

 doi:10.20944/preprints202508.1045.v1

data curation, M.M. and Y.L.; writing—original draft preparation, M.M and M.N.; writing—review and editing, X,L., Z.S. and M.N.; visualization, X.L., S.Y. and X.W.; supervision, X.L.; project administration, X.L.; funding acquisition, X.L. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by the National Key R&D Program (2023YFD1401000), Key Research and Development Plan of Sichuan Province (23ZDYF3037), and Guangxi Key Research and Development Program (AB23026107).

Data Availability Statement: The gene sequence information of *Fusarium* and *Colletotrichum* isolates in this study are available in the database of NCBI.

Acknowledgments: We are thankful to Dr. Gulshan Irshad for his kind guidance during fungal isolation.

Conflicts of Interest: The authors declare no conflict of interest in this work. All forms of financial support are acknowledged in the contribution. This work does not involve any human participants or animals. All authors have offered the consent to submission.

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