Diversity and pathogenic characteristics of the Fusarium species isolated from minor legumes in Korea

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Abstract

Several legume plants exhibiting wilt symptoms were obtained from legume plantations in Korea from 2020 to 2021. Different fungal genera, including *Fusarium* spp.; *Colletotrichum* spp., *Macrophomina* spp., *Rhizoctonia* spp., *Pythium* sp., *Phytophthora* sp., and *Lasiodiplodia* sp., were isolated from the internal tissues of the obtained plant roots and stems. *Fusarium* spp. were the most dominant, accounting for 71% (29) of the isolates. The *Fusarium* isolates were identified via morphological characteristics and molecular identification using the DNA sequence of translation elongation factor 1 alpha and RNA polymerase II second largest subunit regions. In the pathogenicity test, *Fusarium oxysporum* and *Fusarium fujikuroi* generally exhibited high virulence, and *Fusarium falciforme*, *Fusarium metavorans*, and *Fusarium ipomoeae* species exhibited low or no virulence. The NC20-738, NC20-739, and NC21-950 isolates demonstrated the widest host range as they infected all the nine crop plants. Conversely, the NC20-772 isolate induced disease only in adzuki beans and exhibited the narrowest host range. The diverse information regarding the *Fusarium* spp. obtained in this study will provide the basis for implementing management strategies for *Fusarium*-induced plant diseases to minimize economic loss associated with minor legume crops in Korea.

Introduction

Legumes are plants belonging to the family Fabaceae whose seeds are known as pulses when used as dry grains [1]. Approximately 751 genera and 19,000 legumes species are known [2, 3]. They are widely distributed and comprise the third largest land plant family following Orchidaceae and Asteraceae, accounting for ~ 7% flowering plant species [4, 5]. Well-known legumes include soybean (*Glycine max* (L.) Merr), chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* Medik.), mung bean (*Vigna radiate* (L.) Wilczek), adzuki bean (*V. angularis* L.), and clover (*Trifolium repens* L.). Legumes are primarily cultivated for livestock forage and silage, soil-improving green manure, and human consumption, as they are a nutritious food source containing high minerals, carbohydrates, fibers, and protein [2, 6]. Accordingly, legumes are grown in numerous countries, such as India, Canada, Myanmar, China, Russia, Türkiye, and Korea, as important protein sources and means of reducing dependency on synthetic pesticides and nitrogen fertilizers [7, 8]. According to the crop production statistics published by the National Statistical Office of the Republic of Korea, although domestic legume production has continuously decreased from 172,000 tons in 2013, there has been an increase since 2017, with domestic legume production of 129,925 tons and cultivation area of 63,956 ha in 2021 [9].

In India, the world's largest producer of legumes, 10–15% food legume production is lost due to diseases [8]. Various factors cause diseases in legumes, such as parasitic weeds, nematodes, pests, viruses, bacteria, and fungi. Among them, fungi constitute the largest and most important pathogens that affect all plant parts at all growth stages [10]. The chief fungal legume diseases include rust (*Uromyces* spp., *Phakopsora* spp., and *Puccinia* spp.), powdery mildews (*Erysiphe* spp. and *Podosphaera* spp.), downy mildews (*Peronospora* spp.), ascochyta blight (*Ascochyta* spp. and *Phoma* spp.), botrytis gray mold (*Botrytis* spp.), anthracnoses (*Colletotrichum* spp.), damping-off (*Pythium* spp., *Rhizoctonia* spp., and *Fusarium* spp.), root rot (*Aphanomyces euteiches*, *Rhizoctonia solani*, *Fusarium* spp., etc.), collar rot (*Sclerotium rolfsii*), vascular wilt (*Fusarium* spp.), and white mold (*Sclerotinia* spp.) [11]. There have already been studies regarding Fusarium wilt of soybean in South Korea [12]. However, to the best of our knowledge, this is the first study regarding the diversity and pathogenic characteristics of *Fusarium* spp. isolated from minor legumes in Korea.

Link introduced the *Fusarium* genus in 1809 [13]; several species in this genus reportedly induce diseases in plants, humans, and livestocks [14]. As plant pathogens, *Fusarium* spp. have caused several significant social impacts in the past, such as the Fusarium wilt caused by *Fusarium oxysporum* f. sp. *Cubense* [10] in the 1960s, which nearly devastated the commercial banana industry. In 2018, the American Phytopathological Society's List of Plant Diseases reported that > 81 of the 101 economically important plant species had had at least one associated *Fusarium* disease [15]. At least 19 *Fusarium* spp. have been isolated from leguminous crops, and these are mainly involved in symptoms such as wilt, root rot, sudden death syndrome, and damping-off [16]. Likewise, various *Fusarium* spp. are involved in legume diseases; among them, *F. oxysporum* is considered the major pathogen that causes Fusarium wilt [17].

The most suitable strategy to control Fusarium wilt is cultivating resistant varieties [18]. However, the precise identification and pathogenic characteristics of *Fusarium* spp. distributed in minor legume cultivation fields in Korea have not been investigated extensively. Therefore, the objectives of this study were to report the isolation rate of fungal genera, diversity of *Fusarium* spp., and pathogenic characteristics of the isolates obtained from domestic minor legumes with wilt symptoms.

Results

Symptoms and fungal isolation

Wilt symptoms were observed in 14 major legume cultivation fields in Chungnam, Chungbuk, Gyeongnam, and Jeonnam during 2020–2021 in Korea. However, there was a difference in the severity of the wilt depending on the cultivation fields. The results revealed that the incidence of wilt symptoms in all the legume fields was 1–5%, mostly occurring during the high-temperature period of June–September, following the middle growth period of the legume plants. The observed wilt symptoms of the legume plants included yellowing of leaves, browning of stems and roots, root rot, stunting, wilting, and plant death. The main fungal genera recovered from the symptomatic legumes were *Fusarium* spp. (71%); *Colletotrichum* spp. (10%), *Macrophomina* spp. (7%), *Rhizoctonia* spp. (5%), *Pythium* sp. (2%), *Phytophthora* sp. (3%), and *Lasiodiplodia* sp. (2%). Of the 41 obtained isolates, 29

(71%) were *Fusarium* spp. (Fig. 1). These *Fusarium* isolates were detected in kidney beans, mung beans, adzuki beans, and sword beans from seven regions in Korea.

Morphological and cultural characteristics

Following the examination of the morphological and cultural characteristics of macroconidia, microconidia, sporodochia, and aerial mycelia, 29 isolates of *Fusarium* were classified into 4 species complexes: *F. oxysporum* species complex (FOSC), *F. solani* species complex (FSSC), *F. fujikuroi* species complex (FFSC), and *F. incarnatum-equiseti* species complex (FIESC).

Among the 29 *Fusarium* isolates, 13 (45%) were identified as FSSC. The macroconidia of these isolates were plump without any significant curvature with three to five septa, they had oval or obovoid microconidia. The sporodochia of FSSC formed on carnation leaf agar (CLA) were white to beige and usually had longer conidiophores than *F. oxysporum* (Supplementary Fig. S1). The detailed cultural and morphological characteristics of isolates belonging to FSSC are described in Supplementary Table S1. However, the sporodochia of the NC20-729 and NC20-745 isolates rarely formed and their microconidia were not observed. Among them, the macroconidia of the NC20-729 isolate were considerably larger and thinner than those of other isolates of FSSC. In addition, the color of sporodochia in the NC20-743 isolate was pale orange rather than white to beige, which differed from the general characteristics of FSSC. Seven isolates (24%) were identified as FOSC, exhibiting macroconidia curved with parallel walls with three to four septa, while the microconidia were oval or clavate. In this species complex, sporodochia were generally absent, and when present, they were orange. However, the NC20-772 isolate specifically formed white to beige–colored sporodochia (Supplementary Fig. S2). The detailed cultural and morphological characteristics of the FOSC isolates are described in Supplementary Table S2. In addition, seven isolates (24%) were identified as FFSC, which formed slender macroconidia with no significant curvature. The microconidia were formed in chains and did not form chlamydospore (Supplementary Fig. S3). The detailed cultural and morphological characteristics of the FFSC isolates are described in Supplementary Table S3. Only the NC21-948 isolate formed short chains of microconidia, whereas all the other FFSC isolates formed long chains of microconidia (data not shown). Finally, two isolates (7%) were identified as FIESC, which formed elongated and whip-like macroconidia but did not form microconidia. The sporodochia observed on CLA were orange to beige in color (Supplementary Fig. S4). The detailed cultural and morphological characteristics of the FIESC isolates are described in Supplementary Table S4.

The cultural characteristics of *Fusarium* isolate tended to be similar within the same species. However, some species exhibited different growth rates, phenotypes, and pigmentation owing to their intraspecies diversity. For example, unlike most *Fusarium* spp., NC20-729 of *F. azukicola* and NC20-745 of *F. phaseoli* exhibited a slow growth rate. The previously described morphological and cultural characteristics of 14 *Fusarium* spp. comprising 29 isolates are described in Supplementary Table S5.

Molecular identification

For accurately identifying the 29 *Fusarium* isolates to the species level, the nucleotide sequences of the translation elongation factor 1 alpha (TEF) and RNA polymerase II second largest subunit (RPB2) regions were analyzed and their amplification sizes were 600–800 bp and 1,800–2,000 bp, respectively (Supplementary Fig. S5). The phylogenetic tree for all the 29 isolates was divided into 4 identical groups according to morphological characteristics (Supplementary Fig. S6). Then, a phylogenetic tree was additionally created for each species complex for detailed species identification (Fig. 2). The FSSC isolates included *F. vanettenii*, *F. azukicola*, *F. falciforme*, *F. solani*, *F. phaseoli*, *F. oblongum*, *F. ferrugineum*, *F. liriodendri*, and *F. metavorans*. The FFSC isolates included *F. fujikuroi*, *F. concentricum*, and *F. proliferatum*. However, all seven FOSC isolates included *F. oxysporum*, and two FIESC isolates included *F. ipomoeae*. The full list of these isolates with their hosts and accession numbers are provided in Supplementary Table S6. Our results reveal that 14 *Fusarium* spp. were recovered from the minor legumes exhibiting typical wilt symptoms, among which *F. oxysporum* was the most common species (seven isolates) followed by *F. fujikuroi* (four isolates).

Pathogenicity test

The pathogenicity test on the 29 obtained isolates revealed that each isolate exhibited different pathogenicity even when they belonged to the same species (Table 1). The isolates evaluated as highly virulent had an average disease index of ≥ 3, including five isolates of *F. oxysporum*, four isolates of *F. fujikuroi*, and a single isolate for each of *F. solani*, *F. azukicola*, *F. vanettenii*, and *F. concentricum*. These *Fusarium* isolates completely rotted the roots of their corresponding host plants and interfered with water absorption, thereby inhibiting the growth of the host plants (Supplementary Fig. S7). Moreover, only the first leaf was grown in the aboveground parts of the plants. Conversely, the *F. falciforme*, *F. metavorans*, and *F. ipomoeae* isolates were less virulent or nonpathogenic.

Table 1
Pathogenicity of the 29 Fusarium isolates obtained from wilted minor legumes against their original hosts

Original host	Isolate[a]	Species[b]	Mean of disease index[c]			
			Kidney bean	Mung bean	Adzuki bean	Sword bean
Kidney bean	NC20-728	*F. vanettenii**	2.8			
	NC20-732	*F. fujikuroi**	4			
	NC20-733	*F. oxysporum**	4			
	NC20-734	*F. vanettenii**	3			
	NC20-745	*F. phaseoli*	2.7			
	NC20-746	*F. oxysporum**	3.8			
Mung bean	NC20-729	*F. azukicola**		4		
	NC20-730	*F. oxysporum*		3.2		
	NC20-731	*F. falciforme*		0.3		
	NC20-742	*F. oxysporum*		2.2		
	NC20-743	*F. solani*		4		
	NC20-776	*F. oblongum**		1.8		
	NC20-777	*F. ipomoeae*		1.3		
	NC20-779	*F. oxysporum*		3.8		
	FD00137	*F. falciforme*		0.7		
Adzuki bean	NC20-737	*F. fujikuroi**			4	
	NC20-738	*F. fujikuroi**			4	
	NC20-739	*F. fujikuroi**			3	
	NC20-772	*F. oxysporum**			1.5	
	NC20-773	*F. oxysporum**			3.2	
	NC20-774	*F. falciforme*			0.8	
	NC21-982	*F. metavorans*			1.2	
	NC21-983	*F. metavorans*			0.8	
Sword bean	NC21-948	*F. concentricum**				4
	NC21-949	*F. proliferatum*				0
	NC21-950	*F. proliferatum**				3
	NC21-951	*F. solani*				1
	NC21-952	*F. ferrugineum*				1
	NC21-953	*F. liriodendri*				2.7
	CONTROL		0	0	0	0

[a] Disease indices of NC20-737, NC20-738, and NC20-739 were cited from Ha, et al.[41]

[b] *, New pathogens that have not been reported in Korea so far, in each host.

[c] Disease index 0 = no symptoms, 1 = root necrosis and root loss < 30%, 2 = root necrosis and root loss 31–60%, 3 = root necrosis, root loss > 61%, and poor growth, and 4 = complete necrosis of root tissue and no roots or plants death.

Host range investigation

19 *Fusarium* isolates were selected to investigate the host range based on the results of the pathogenic characteristics of all the *Fusarium* spp. (Table 2). Investigating the host range of nine plants belonging to the leguminous and gramineous plants using these selected isolates revealed a very diverse host range for each isolate (Table 3). The FSSC isolates did not cause wilt disease in rice and maize as gramineous hosts even if these isolates were highly virulent. However, in FOSC, the NC20-730 and NC20-773 isolates caused wilt disease in rice as well as legumes (Supplementary Fig. S8).

Unlike other species complexes, all the FFSC isolates caused wilt disease in rice and generally had a wide host range. Specifically, the NC20-738 and NC20-739 isolates of *F. fujikuroi* and the NC21-950 isolate of *F. proliferatum* demonstrated significant pathogenicity in all the nine plants. Conversely, the NC20-772 isolate, which exhibited low virulence in the pathogenicity assay, did not cause wilt disease in all the tested plants except for adzuki beans. Duncan's Multiple Range Test (DMRT) using R program (Lucent Technologies, USA) revealed the difference in the incidence of the 19 isolates at a 5% significance level (Table 3).

Table 2
List of the pathogenic *Fusarium* isolates that were selected for host range investigation

Species complex[a]	Species	Isolate	Region	Pathogenicity[b]
FSSC	*F. vanettenii*	NC20-728	Boryeong, Chungnam	++
	F. azukicola	NC20-729	Yeosu, Jeonnam	+++
	F. vanettenii	NC20-734	Boryeong, Chungnam	++
	F. solani	NC20-743	Hongseong, Chungnam	+++
	F. phaseoli	NC20-745	Hongseong, Chungnam	++
	F. oblongum	NC20-776	Miryang, Gyeongnam	+
	F. liriodendri	NC21-953	Hwasun, Jeonnam	++
FOSC	*F. oxysporum*	NC20-730	Yeosu, Jeonnam	+++
	F. oxysporum	NC20-733	Boryeong, Chungnam	+++
	F. oxysporum	NC20-746	Hongseong, Chungnam	+++
	F. oxysporum	NC20-772	Miryang, Gyeongnam	+
	F. oxysporum	NC20-773	Miryang, Gyeongnam	+++
	F. oxysporum	NC20-779	Miryang, Gyeongnam	+++
FFSC	*F. fujikuroi*	NC20-732	Seocheon, Chungnam	+++
	F. fujikuroi	NC20-737	Yeosu, Jeonnam	+++
	F. fujikuroi	NC20-738	Yeosu, Jeonnam	+++
	F. fujikuroi	NC20-739	Yeosu, Jeonnam	++
	F. concentricum	NC21-948	Hwasun, Jeonnam	+++
	F. proliferatum	NC21-950	Hwasun, Jeonnam	++

[a] FSSC, *F. solani* species complex; FOSC, *F. oxysporum* species complex; FFSC, *F. fujikuroi* species complex; FIESC, *F. incarnatum-equiseti* species complex.

[b] +: disease index 1–2, ++: disease index 2.1-3, +++: disease index 3.1-4

Table 3
Host range of 19 selected *Fusarium* spp. isolates that were collected from the wilted minor legumes

Species complex[a]	Isolate	Origin	Mean of disease index[b] or occurrence of disease[c] on hosts															
			Kidney bean		Sword bean		Mung bean		Adzuki bean		Cowpea		Soybean		Lentil bean		Rice	Corn
FSSC	NC20-728	Kidney	2	de[d]	1	d	1	f	2	de	1.5	e	0.9	gh	1	g	-	-
	NC20-729	Mung	4	a	2	c	4	a	4	a	4	a	3.7	ab	2.5	de	-	-
	NC20-734	Kidney	2	de	2	c	1	f	0.6	fg	2.5	d	1.8	ef	1	g	-	-
	NC20-743	Mung	1.7	e	4	a	2	e	0.4	fg	1	ef	3	bcd	0.8	g	-	-
	NC20-745	Kidney	4	a	1	d	3.2	bc	3	b	4	a	0.6	h	4	a	-	-
	NC20-776	Mung	3	b	2	c	1.8	e	1	f	2.5	d	3	bcd	1.7	f	-	-
	NC21-953	Sword	1.6	e	3.6	a	0.8	fg	4	a	1.4	e	2.7	cd	2	ef	-	-
FOSC	NC20-730	Mung	3	b	2.4	bc	4	a	2.8	bc	2.8	cd	4	a	3	cd	+	-
	NC20-733	Kidney	3	b	2.3	bc	3	cd	4	a	3.6	ab	3.3	abc	3.8	ab	-	-
	NC20-746	Kidney	2.7	bc	1.2	d	2.4	de	2	de	2.6	d	2.4	de	2.5	de	-	-
	NC20-772	Adzuki	0	g	0.2	e	0.2	gh	1	f	0	g	1	gh	0.1	h	-	-
	NC20-773	Adzuki	4	a	3.5	a	3.8	ab	3	b	2.7	cd	3.4	abc	2.1	ef	+	-
	NC20-779	Mung	1	f	1	d	2.1	e	0.8	fg	0.4	fg	1.4	fg	3	cd	-	-
FFSC	NC20-732	Kidney	2.4	cd	3.4	a	0.2	gh	1.8	e	0.6	fg	2.3	de	2.7	d	+	+
	NC20-737	Adzuki	3.2	b	2.8	b	3	cd	2.2	cde	3.4	abc	3	bcd	2.7	d	+	-
	NC20-738	Adzuki	4	a	4	a	4	a	4	a	4	a	4	a	4	a	+	+
	NC20-739	Adzuki	4	a	3.8	a	4	a	2.6	bcd	3	bcd	4	a	3	cd	+	+
	NC21-948	Sword	4	a	4	a	4	a	4	a	4	a	4	a	4	a	+	-
	NC21-950	Sword	4	a	4	a	4	a	4	a	3.9	a	2.8	cd	3.4	bc	+	+
Control			0	g	0	e	0	h	0	g	0	g	0	h	0	h	-	-

[a] FSSC, *F. solani* species complex; FOSC, *F. oxysporum* species complex; FFSC, *F. fujikuroi* species complex; FIESC, *F. incarnatum-equiseti* species complex.

[b] Disease index 0 = no symptoms, 1 = root necrosis and root loss < 30%, 2 = root necrosis and root loss 31–60%, 3 = root necrosis, root loss > 61%, and poor growth, 4 = complete necrosis of root tissue and no roots or plants death.

[c] +, Occurrence of disease; -, nonoccurrence of disease.

[d] Duncan's Multiple Range Test (DMRT) for mean comparison of disease index within each host ($P < 0.05$).

Discussion

We conducted various assays to elucidate the diversity and pathogenic characteristics of *Fusarium* spp., which cause Fusarium wilt in minor legumes in Korea. The results confirmed that not only the well-known *Fusarium* spp. but also species that had not been reported in Korea so far currently caused Fusarium wilt in legumes in Korea.

By sampling wilted minor legumes and isolating the fungi, several fungal genera were obtained, including *Fusarium* spp., *Colletotrichum* spp., *Macrophomina* spp., *Rhizoctonia* spp., *Pythium* sp., *Phytophthora* sp., and *Lasiodiplodia* sp. Among these fungal genera, *Fusarium* spp. accounted for 71% of the recovered isolates, indicating that this genus was the dominant and major causal pathogen of wilt symptoms. A previous study involving Fusarium wilt of soybean in Korea from 2014 to 2016 reported that *Fusarium* spp., *Colletotrichum* sp., *Rhizoctonia* sp., *Macrophomina* sp., *Phytophthora* spp., and *Calonectria ilicicola* have been isolated from the soybean; in addition, the *Fusarium* genus isolation rate was 79% [12]. These results are consistent with this study, probably attributable to the fact that the cultivation area and environment were almost the same in both studies. On the other hand, in a Chinese study of soybean-related *Fusarium* spp., the isolation of *Fusarium* spp., *Alternaria* sp., *Aspergillus* sp., *Botryosphaeria* sp., *Colletotrichum* sp., *Corynespora* sp., and *Diaporthe* sp. have been reported [19]. And only *Fusarium* spp. and *Colletotrichum* sp. were similar to the species isolated in a Korean study [12]. This is thought to be because of differences in the distribution and dominant species of fungal pathogens depending on region, environment, and cultivation method.

Among the various fungal genera mentioned above, *Fusarium* is one of the most important plant pathogens owing to its wide host range and it being the main causal pathogen of wilt disease [19]. However, as some *Fusarium* spp. are similar, identifying these isolates at the species level remains confusing and complicated [20]. Therefore, herein, the *Fusarium* isolates were identified at the species complex level by observing their morphological features, and genetic analysis was additionally performed to identify them at the species level. The 29 *Fusarium* isolates were classified into 4 species complexes: FOSC, FSSC, FFSC, and FIESC. In a similar previous study conducted in Korea [12], the *Fusarium* strains isolated from soybeans differed from the current study only in being identified as *F. graminearum* species complex and not as FIESC. Species identification through the molecular sequencing of the fungal DNA revealed 14 *Fusarium* spp. in this study. A much greater variety of *Fusarium* spp. were isolated than the five species from soybeans in a previous study [12]. Among the 14 species, *F. oxysporum* was the most common with 7 isolates. These results indicate that *F. oxysporum* remains the major causal pathogen of Fusarium wilt in legumes.

The NC20-745 isolate was not clearly distinguished from *F. phaseoli* and *F. crassistipitatum* owing to the lack of difference in the nucleotide sequence for molecular identification. However, according to Aoki, et al. [21], *F. crassistipitatum* is distinguished from *F. phaseoli* by its yellowish colonies on potato dextrose agar (PDA). Therefore, the NC20-745 isolate has been identified as *F. phaseoli* based on these morphological features. The major pathogen of Fusarium wilt is reportedly, *F. oxysporum*; however, various other *Fusarium* spp. have also been frequently reported as wilt and root rot pathogens [12, 16, 22, 23]. In the UK, *F. solani*, *F. coeruleum*, *F. oxysporum*, *F. redolens*, *F. avenaceum*, *F. graminearum*, *F. sambucinum*, and *F. equiseti* have been isolated from legumes exhibiting root rot symptoms [24]. Furthermore, in China, *F. fujikuroi*, *F. proliferatum*, *F. luffae*, and *F. sulawense* have been isolated from the rotten pods of soybean [25]. As such, the *Fusarium* spp. involved in legume wilt and root rot are very diverse. As this distribution is primarily affected by the cultivation environment, it is necessary to investigate the diversity of *Fusarium* spp. and understand the characteristics of such fungal pathogens to prevent future disease outbreaks.

However, because not all of these isolated *Fusarium* spp. are pathogenic, a pathogenicity test was performed to distinguish between the pathogenic and nonpathogenic isolates. The results revealed that four isolates of *F. fujikuroi* (100%), five of *F. oxysorum* (71%), one of *F. solani*, *F. proliferatum*, and *F. ipomoeae* (50%), two of *F. vanettenii* (100%), and one of *F. phaseoli*, *F. concentricum*, *F. oblongum*, and *F. azukicola* (100%) had a disease severity of > 2.5. Whereas *F. falciforme* (three isolates), *F. metavorans* (two isolates), *F. proliferatum* (one isolate), and *F. ferrugineum* (one isolate) were nonpathogenic. Thus, not all the 14 isolated *Fusarium* spp. were pathogenic, and even the same species showed different pathogenicity depending on the isolate. Arias et al.[16] reported that only one isolate of the 14 *F. oxysporum* isolates from infected soybeans caused the root rot disease; and that other *Fusarium* spp. also showed considerable differences in pathogenicity by isolate. These results are consistent with the results of this study. Moreover, according to the *Fusarium* spp., the degree of disease severity also differed in different studies. In the US, *F. graminearum* is the most pathogenic followed by *F. virguliforme*, *F. proliferatum*, *F. sporotrichioides*, and *F. solani* [16]. However, in China, *F. proliferatum* is reportedly the most pathogenic followed by *F. fujikuroi*, *F. sulawense*, and *F. luffae*. [25] These results indicate complex differences among different countries, including those in the dominant *Fusarium* spp., types of cultivated legumes, cultivation environment, and cropping system. Therefore, it is advisable that an in-depth investigation of the diversity and pathogenic traits of *Fusarium* spp. according to their isolation regions, crops, soil, and cropping system should be conducted in Korea.

Based on the results of the pathogenicity assay, 19 *Fusarium* isolates were selected whose host range was investigated on 7 leguminous and 2 gramineous plants. Most *Fusarium* isolates were evaluated as having a broad host range. Most *Fusarium* isolates did not cause wilt disease in corn, but two isolates of *F. fujikuroi* and one isolate of *F. proliferatum* caused wilt disease in all the nine crops, including corn, and were thus evaluated as having the widest host range. Similarly, a previous study by Amatulli et al.[26] reported that *F. fujikuroi* and *F. proliferatum* had a wide host range, including corn, asparagus, fig, onion, palm, pine, and rice. Therefore, according to the results of this and the current study, it can be concluded that there are at least 14 host species of *F. fujikuroi* and *F. proliferatum*. Furthermore, all *Fusarium* spp. belonging to FFSC caused wilt disease in rice. However, *F. fujikuroi* reportedly causes bakanae disease in rice. Therefore, we additionally performed immersion inoculation [27] to confirm whether *F. fujikuroi* isolated from legumes can cause bakanae disease, which showed that all the four *F. fujikuroi* isolates increased the height of rice and caused bakanae disease (data not shown). This is consistent with the results of the previous study conducted by Choi, et al.[12], which confirmed that *F. fujikuroi* isolated from soybeans had caused bakanae disease in rice. Three isolates of *F. oxysporum*, known as the representative pathogen of Fusarium wilt,

and one isolate of *F. azukicola*, reported for the first time in Korea, also caused wilt disease in all seven legumes. With respect to *F. azukicola*, Aoki, et al. [23] reported that eight strains of *F. azukicola* isolated from Japan also caused root rot in adzuki beans, kidney beans, mung beans, and soybeans. Thus, it is likely to become a problematic fungal pathogen in the near future. As such new pathogens may exist in the future, continuous pathogen identification and host range monitoring are highly recommended.

Currently, research regarding the Fusarium wilt of legumes in Korea is insufficient; hence, information regarding the existing *Fusarium* pathogens is lacking. Therefore, this study revealed some previously unreported Fusarium wilt pathogens and their pathogenic characteristics and host range. The results of this study can be used for future research in effective Fusarium wilt management strategies, including breeding of wilt-resistant varieties and cultivation control methods such as crop rotation.

Methods

Sample collection and isolation of the fungi

Experimental research and field studies on plants (either cultivated or wild), including the collection of plant material, complied with relevant institutional, national, and international guidelines and legislation. And we have permission to collect legumes. From 2020 to 2021, ~ 50 samples exhibiting wilt symptoms from minor legumes such as kidney beans, adzuki beans, mung beans, sword beans were collected from 14 domestic legume plantations, in Hongseong, Boryeong, Seocheon, Yeosu, etc. (Fig. 3). To isolate the fungi from the samples, the discolored internal tissues of the root and stem were cut into small pieces (5 × 5 mm) using a sterilized scalpel, surface-sterilized with 1% sodium hypochlorite (NaOCl), and then washed twice with sterile distilled water. The surface-sterilized sample pieces were placed on water agar (WA) and incubated at 25℃ in the dark. After 3–5 days of incubation, the growing fungal colonies from the sample pieces were observed under a stereomicroscope. Single spore isolation from these sample pieces was conducted by partially modifying the single spore isolation method [28]. First, the spores of hyphomycetes were picked up directly from the sample pieces using a platinum hook. The spores were mixed with sterile water on a sterilized slide glass to prepare a spore suspension. The spore suspension was dropped on one end of the WA, an English capital letter “E” was drawn with a flame-sterilized platinum loop (sterilized for each stroke), and the entire medium was streaked. The unsealed plate was incubated at 25℃ for 21–24 h. Once the spores were germinated, the agar block containing the germinated single spore was lifted using a sterilized platinum hook and placed on the WA. When the colony grew 1–2 cm, a small piece of mycelium with agar was cut and transferred to another PDA plate to assess contamination. Then, only pure fungal cultures were transferred to PDA slants and stored at 10℃ till further use in the following assays.

Morphological identification and characterization of fungal isolates

To investigate the morphological characteristics, the isolates were cultured on CLA media [29, 30] at 25℃ for 14 days under near ultraviolet (NUV)/dark (12 h/12 h) incubation conditions to investigate the morphological characteristics of the fungal isolates. Following incubation, the morphological characteristics, such as the shape and size of microconidia, macroconidia, presence or absence and color of sporodochia, were investigated [31]. To investigate the cultural characteristics, the isolates were inoculated on PDA and cultured at 25℃ in the dark for 7 days. Following incubation, the cultural characteristics, including colony growth rate, aerial mycelial color and texture, and colony pigmentation, were investigated [31].

DNA extraction

Genomic DNA was extracted from the mycelial powder using Maxwell® RSC PureFood GMO and Authentication Kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Each fungal isolate was individually inoculated by placing three to five pieces of PDA with mycelia into 20 ml potato dextrose broth (Difco, Bergen, USA) and then incubated at 25℃ for 5–7 days. Following incubation, the growing fungal mycelia were filtrated using a sterilized piece of miracloth. The harvested mycelia were completely dried via freeze-drying overnight, and then ground using sterilized beads and a homogenizer to prepare mycelial powder. The mycelial powder was vortexed with 20 µL RNase A and 30 µL proteinase K and then incubated in a heating block at 65℃ for 30 min. After incubation, they were centrifuged at 14,000 rpm for 5 min, and 400 µL supernatant was recovered. The fungal genomic DNA was extracted from this supernatant using Maxwell® RSC Kit, and stored at − 20℃ till further use in the subsequent assays.

Polymerase chain reaction amplification

For the molecular identification of the *Fusarium* isolates, the TEF and RPB2 coding regions were selected [20]. TEF was amplified using EF1 and EF2 primers, and RPB2 was amplified using 5f2 and 11aR primers [32] (Supplementary Table S7). Polymerase chain reaction (PCR) amplification was performed using 50 µL mixture, containing 3 µL DNA templates (33ng/µL), 3 µL each F/R primer (5 pmoles/µL), 5 µL 10X *n*Taq-Tenuto buffer (Mg^{2+} plus), 5 µL 2 mM dNTP mixture, 30 µL sterile water, and 0.5 µL *n*Taq-Tenuto polymerase. The PCR conditions for DNA amplification of the TEF region included initial denaturation at 95℃ for 4 min; 34 cycles of denaturation at 95℃ for 30 s, annealing at 58℃ for 45 s, extension at 72℃ for 50 s; and a final extension at 72℃ for 7 min [33]. For the RPB2 region, the PCR conditions were 95℃ for 2 min; 34 cycles of denaturation at 95℃ for 30 s, annealing at 56℃ for 30 s, extension at 72℃ for 2 min; and a final extension at 72℃ for 5 min [34].

DNA purification and sequence analysis

The final PCR products were observed via electrophoresis on a 1.4% agarose gel at 100V for 30 min. When the target and nontarget bands were formed and appeared as multiple bands, gel purification was performed, and PCR purification was conducted only when the target band appeared. PCR and gel purification were conducted via Wizard® SV gel and PCR Clean-up System Kit (Promega, San Luis Obispo, CA, USA) according to the manufacturer’s

instructions. The purified PCR product was sequenced in both directions via Bionics Co., Ltd. (Seoul, Korea) using EF1 and EF2 primer for TEF and 5f2, 7cr, 7cF, and 11aR primers for RPB2 (Supplementary Table S7). The consensus sequences were assembled and revised using the Seqman program (DNASTAR, Madison, USA) [35]. The novel sequences generated in this study were deposited in National Centre for Biotechnology Information (NCBI) GenBank.

Phylogenetic analysis

To identify the species of the isolates, the sequence alignments of the TEF and RPB2 regions were conducted using the MUSCLE algorithm of MEGA-X software [36, 37] with other reference sequences of *Fusarium* spp. obtained from the NCBI GenBank. The phylogenetic trees were constructed based on the Maximum likelihood and Kimura 2-parameter model [38, 39] and verified by 1,000 bootstrap replicates [29]. The *F. staphyleae* strain NRRL 22316 was used as an out group. Information regarding the reference and outgroup strains is summarized in Supplementary Table S8.

Pathogenicity test

The pathogenicity test of the 29 isolates was conducted via the soil inoculation method using cornmeal sand inoculum to the original host (host from which each isolate was collected) [40]. The cornmeal sand inoculum was prepared by mixing 240 g dry sand, 26 g cornmeal, and 65 ml distilled water in 500-mL Erlenmeyer flasks, autoclaving twice at 121℃ for 30 min, and adding 15 PDA disks (5-mm diameter) with pathogen mycelium. In the control treatment, pure PDA disks were added instead of inoculated disks. The inoculum was incubated at 25℃ for 4 weeks without shaking. Following inoculation, the cornmeal sand inoculum and autoclaved soil were mixed at a volume ratio of 3:7 and then divided into 200 mL for each pot (72 × 72 × 100 mm). Two germinated seeds were planted in each pot, and three replicates pots were used for each treatment. All plants were grown in the controlled plant growth room at 25℃–27℃, with a photoperiod of 12 h/day. Three weeks after sowing, the disease index was scored on a 0–4 scale for each host according to the degree of root damage (Fig. 4).

Investigation of host range

The host range investigation was conducted with selected isolates based on their pathogenicity; hence, certain isolates with low virulence were also included. In total, 19 isolates were investigated, including 6 isolates collected from kidney beans, 5 from mung beans, 5 from adzuki beans, and 3 from sword beans. The host range investigation assay was also conducted using the soil inoculation method with cornmeal sand inoculum, similar to the pathogenicity test. However, there were some differences in the experimental procedures. In this assay, on preparing the cornmeal sand inoculum, 450 mL dry sand, 26 g finely ground cornmeal (for food), and 70 mL distilled water were mixed in a 1-L Erlenmeyer flask, and 30 5-mm diameter PDA disks inoculated with pathogens were added. After incubation for 4 weeks at 25℃, the mixture of cornmeal sand inoculum and sterilized soil in a 2:8 volume ratio was placed in 100 × 40 mm plant culture dishes with holes in their bottom, and eight seeds were planted for each crop. The plants used for this host range assay included nine crop plants, involving seven leguminous plants and two gramineous plants. The leguminous crops included kidney bean, mung bean, lentil, sword bean, soybean, adzuki bean, and cowpea. Furthermore, the gramineous plants included rice and corn. The disease index was evaluated 3 weeks after inoculation according to the degree of root damage, similar to the pathogenicity test. Then, Duncan's DMRT was performed at a 5% significance level using the R program to statistically confirm whether there was a significant difference in the incidence of strains for the host.

Declarations

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Author contributions

M.S.H did the study design, data collection and analysis, manuscript writing, and preparation of the draft of manuscript and revised manuscript. H.R did the study design, data collection, and statistical analysis. H.J.J did the study design and English proofreading of the manuscript. H.-W.C did the study design, data collection and analysis and preparation of revised manuscript. All authors approved the final version.

Data availability

All sequences produced in this study are publicly available in NCBI GenBank Database (https://www.ncbi.nlm.nih.gov/genbank/) and accession numbers are available in Supplementary Table S6. The datasets used and/or analyzed during the current study were available from the corresponding author on reasonable request.

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Figures

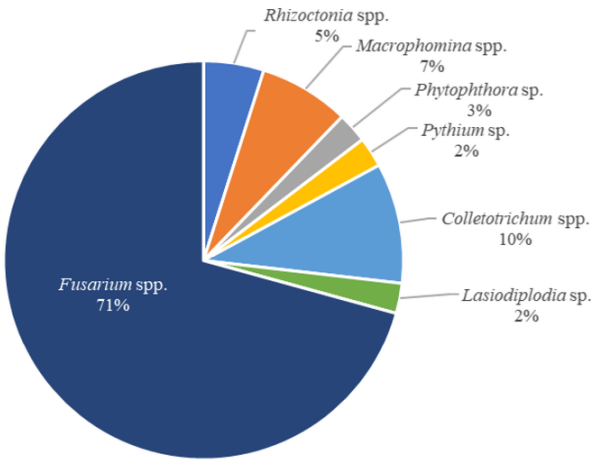

Figure 1

Isolation rate of fungi obtained from legume plant exhibiting wilt symptoms. Among 41 isolates, *Fusarium* was the dominant genus, accounting for 71% (29) of the isolates.

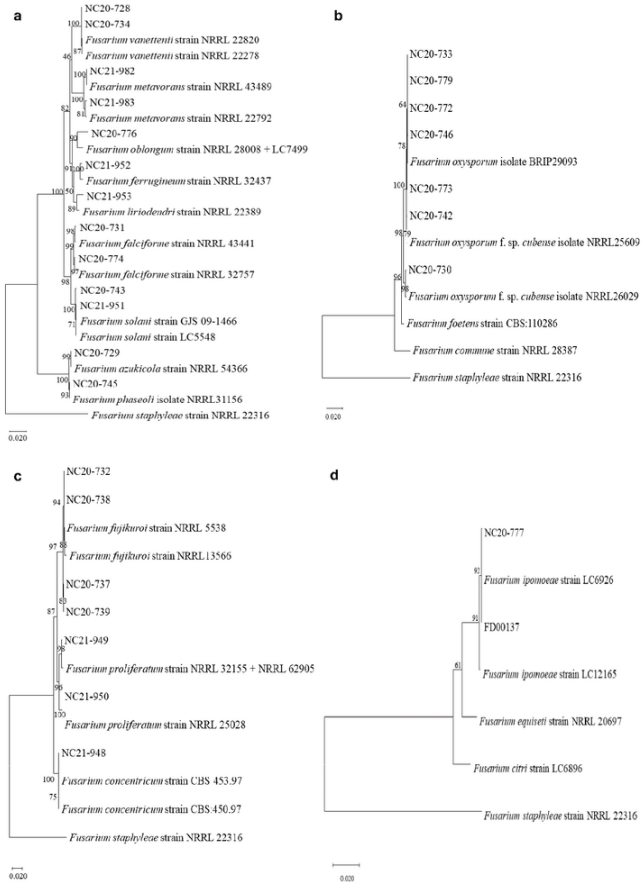

Figure 2

Phylogenetic trees for each species complex of the 29 *Fusarium* isolates. The phylogenetic tree of a) *Fusarium solani* species complex (FSSC), b) *F. oxysporum* species complex (FOSC), c) *F. fujikuroi* species complex (FFSC), and d) *F. incarnatum-equiseti* species complex (FIESC). The trees were generated using Maximum likelihood analysis of translation elongation factor 1α (TEF) and RNA polymerase second largest subunit (RPB2) gene nucleotide sequences. The number in each branch indicates bootstrap values obtained following a bootstrap test with 1,000 replications. The scale bar represents 0.02 nucleotide substitution per site.

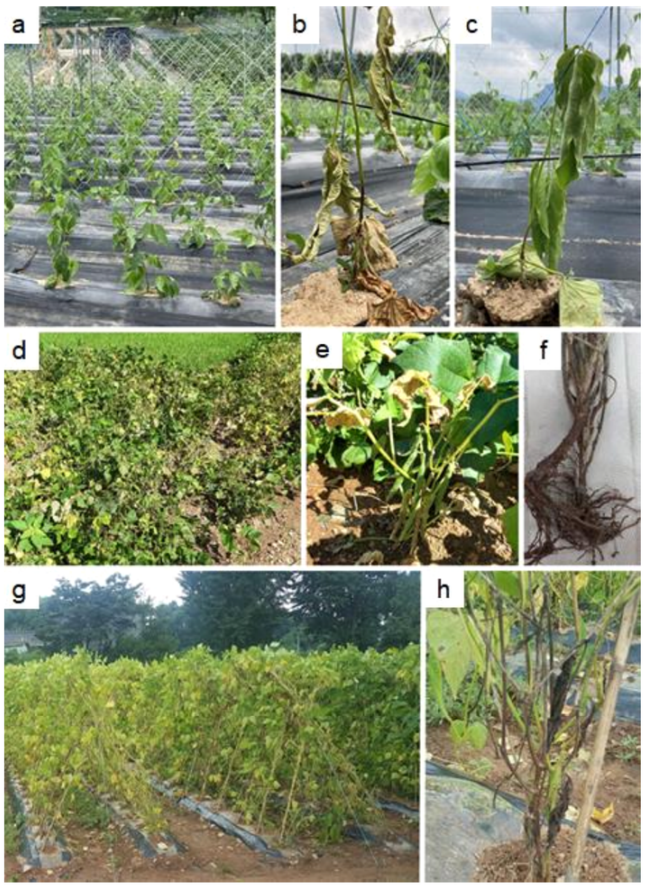

Figure 3

Legumes showing typical wilt symptoms observed in 14 domestic legume plantations in Korea. a–c) Kidney beans in Hongseong, d–f) Adzuki beans in Yeosu, and g–h) Sword beans in Hwasun.

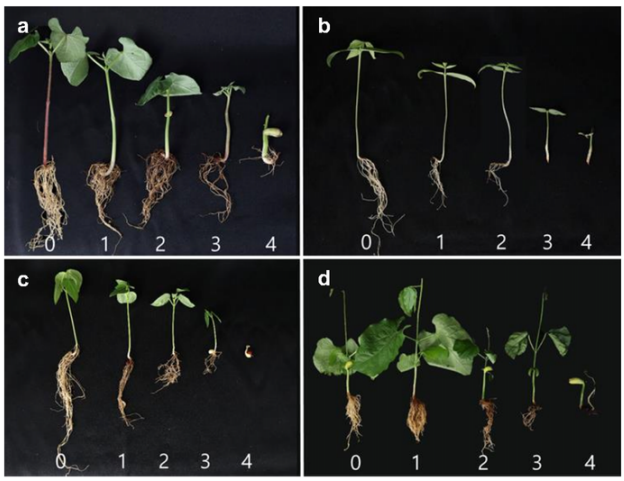

Figure 4

Disease index of four leguminous crops, which are the original hosts of the isolates. The disease index of a) kidney beans, b) mung beans, c) Adzuki beans, and d) sword beans. Disease index 0 = no symptoms, 1 = root necrosis and root loss <30%, 2 = root necrosis and root loss 31%–60%, 3 = root necrosis, root loss >61%, and poor growth, and 4 = complete necrosis of root tissue and no roots or plants death.

Supplementary Files

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