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Characterization, pathogenicity, and extracellular enzymes of *Fusarium solani* f. sp. *phaseoli* associated with common bean in Khorasan Razavi Province of Iran

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Abstract

Members of the genus *Fusarium* are considered highly destructive pathogens of common beans (*Phaseolus vulgaris*). This study aimed to identify the causal agent of the disease, confirm its pathogenicity following Koch's postulates, and investigate the extracellular enzymes of *F. solani*. After conducting a field survey on common bean plants in Khorasan Razavi Province in August and September of 2021 and 2022 and isolating fungi, the isolates were identified as *F. solani* f. sp. *phaseoli* based on morphological traits and molecular analysis of the internal transcribed spacer region. Pathogenicity tests revealed the isolates causing symptoms such as yellowing, rotting, and wilting on common bean seedlings (cv. Derakhshan). Moreover, extracellular destructive enzymes, including pectinase and cellulase, were quantitatively assessed. The isolates exhibited cellulase activity more quickly and at higher levels than pectinase. Our findings indicated that the enzymes are key in inducing disease symptoms in the host plant.

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Introduction

Legumes are considered one of the most important human food sources after cereals. Asia leads in legume cultivation, followed by Africa, North America, and Latin America. Broughton et al. (2003) identify beans as the third key legume crop, after soybeans and peanuts. Various types of beans are rich sources of protein (22%), carbohydrates (50-56%), vitamins, and essential minerals such as iron, zinc, and manganese (Broughton et al., 2003). In Iran, nearly 40% of the country's legume production comes from bean cultivation (Ahmadi et al., 2020). A range of diseases adversely impacts bean cultivation, with fungal rot of crown and root tissues being among the most significant threats to bean yield. Fusarium species including *Fusarium avenaceum* (Fr.) Sacc., *F. acuminatum* Ellis & Everh, *F. proliferatum* (Matsush.) Nirenberg, *F. culmorum* (Wm.G. Sm.) Sacc., *F. crookwellense* L.W. Burgess, P.E. Nelson & Toussoun, *F. equiseti* (Corda) Sacc., *F. redolens* Wollenw, and *F. anthophilum* (A. Braun) Wollenw, have also been reported as the causal agents of bean crown and root rot, worldwide (Montiel-Gonzalez et al., 2005). Other fungal species, including *F. oxysporum* Schltdl., *F. solani* (Mart.) Sacc. and *F. semitectum* Berk. & Ravenel have been identified as the causal agents of crown and root rot in bean production regions of Zanjan Province (Safarloo & Hemmati, 2014).

Members of this genus are commonly found in soil and have considerable activity at a pH of 7; however, only a limited number of species are active in relatively alkaline and slightly acidic soils (Saeedi & Jamali, 2021). The richness of organic compounds in the soil is a crucial factor in promoting the germination of

chlamydospores and their population increase (Dastjerdi et al., 2004). The optimal temperature for the spread of *F. solani* on beans is between 28-32 °C; however, they have also been identified in soils with temperatures ranging from 15-32 °C (Cramer, 2000). Some members of this genus have shown the ability to secrete mycotoxins due to their diversity, wide distribution, and ability to inhabit various environments such as soil, water, plant systems, and human habitats (Coleman, 2016; Urbaniak et al., 2018). These mycotoxins can disrupt protein synthesis and lead to a range of health problems in humans, including kidney disease, as well as symptoms like general weakness, nausea, and even fatalities in animals (Pestka, 2010; Nicolaisen et al., 2009). Additionally, through the secretion of mycotoxins, members of this genus lead to genetic alterations and significant economic losses due to contamination at different stages of food processing (Geiser et al., 2013).

One significant factor contributing to the decline of bean cultivation globally is the infection of plants by *F. solani* f. sp. *phaseoli* (Burkholder) Snyder & Hansen (Knodel et al., 2007). Crown and root rot of beans caused by *F. solani* is prevalent in numerous bean growing areas, such as Mexico, Brazil, Colombia, Peru, Ecuador, Venezuela, and Kenya (Kraft et al., 1981; Godoy et al., 2004) and America (Andres Ares et al., 2006; De Jensen et al., 2002). This disease is the most critical threat to beans, globally (Abawi, 1989). In Iran, this disease was first reported on beans in 1968 in Zanjan (Naseri, 2008) and occurred on various bean types in different regions, including Lorestan (Dadgar, 2009), Khorasan Razavi (Kraft et al., 1981), Markazi (Kocheki & Banayan-aval, 1993), Fars, Zanjan, East Azerbaijan, Isfahan (Naseri, 2008), Qazvin (Dehghani et al., 2018; Saremi et al., 2011), and Chaharmahal and Bakhtiari (Ershad, 2009). According to the reports of Faraji and Okhovvat (2005), the *F. solani* isolates from Tehran and East Azerbaijan Provinces exhibited the highest levels of pathogenicity and prevalence on beans. These findings were confirmed by Naseri (2008) in Zanjan.

Pathogenicity of *Fusarium* spp. is related to diverse mechanisms, such as production of extracellular enzymes and many toxins (Taheri, 2018). Various researchers have demonstrated the importance of extracellular enzymes from *Fusarium* spp. and other fungal phytopathogens during the infection of different plant species in Iran and other regions, worldwide (Samandri Najafabadi et al., 2024; Maghsoudi et al., 2024; Wanyoike et al., 2002).

This study aimed to isolate the pathogen causing crown and root rot of common bean (*Phaseolus vulgaris*) in Khorasan Razavi Province based on morphological and molecular characteristics, investigate complete Koch's postulates for the causal pathogen, and evaluate the activity of destructive extracellular enzymes of the pathogen, such as cellulase and pectinase.

Materials and Methods

Sampling, fungal isolation and morphological identification

In 2021 and 2022, common bean plant samples suspected of *Fusarium* infection were collected from different regions of Khorasan Razavi Province via M-shaped movement in the fields (Mohammadi & Kazemi, 2002). Plants were collected based on disease symptoms including yellowing, stunted growth, and rotting of crown and root tissues. After recording the complete details of the samples (collection location, date of collection, type of plant tissue), they were transferred to the laboratory in paper bags. For fungal isolation, the tissues underwent a thorough washing with running water to remove soil and contaminants. Then, 5 mm small pieces of plant tissues were superficially disinfected with a 0.5% sodium hypochlorite solution for two min, and after three consecutive washes with sterile distilled water for two min, they were dried on sterile filter paper. Then, the samples were cultured in Petri dishes containing general media, including Potato Dextrose Agar (PDA) (Booth, 1977), and specific media such as Nash and Snyder Agar (Nash & Snyder, 1962), and Peptone-Pentachloronitrobenzene Agar (PPA) containing 20 mg/L of streptomycin sulfate. The cultures were maintained in a incubator for seven days at 28 ± 2 °C with 12/12 h of light/darkness (Nirenberg, 1976). The isolates were identified based on macroscopic and microscopic morphological characteristics, including colony color and growth rate on PDA (Leslie & Summerell, 2006), as well shape and dimensions of microconidia and macroconidia, and presence of chlamydospores on Spezieller Nährstoffarmer Agar (SNA) (Nirenberg, 1976; Leslie & Summerell, 2006) and Carnation Leaf-piece Agar (CLA) media (Fisher et al., 1982). For long-term preservation, sterile filter papers colonized by the fungi were stored in sterile microtubes at -20 °C (Valent et al., 1991).

DNA extraction, sequencing and phylogenetic analysis

Initially, the fungus (isolate FS20) was cultured on PDA and incubated at 28 °C for seven days. About 100 mg of mycelium was collected from the colony surface and finely grinded by adding liquid nitrogen. Genomic DNA was extracted according to the method of Zhang et al. (2010) via cetyltrimethyl ammonium bromide (CTAB). The internal transcribed spacer (ITS) region was amplified via primer sets ITS1/ITS4 (TCC GTA GGT GAA CCT GCG G / TCC TCC GCT TAT TGA TAT GC) (White et al., 1990). In a 25 μL amplification reaction, 12.5 μL of master mix (Amplicon), 1 μL of each primer (10 pM), 8.5 μL of deionized water, and 2 μL of template DNA (50 ng) were combined. The PCR was conducted in a Biometra thermal cycler (Göttingen, Germany), starting with a three min initial denaturation at 95 °C, followed by 35 cycles consisting of 30 s of denaturation at 95 °C, 45 s of annealing at 50 °C, and 90 s of extension at 72 °C, and a final extension at 72 °C for 10 min (Sekhar et al., 2018). The PCR products were analyzed on 1% agarose gels and subsequently sequenced by Macrogen Co. (Seoul, Korea) with the same primers. The consensus sequences were analyzed by BLASTN against previously deposited sequences in GenBank (NCBI). The ITS regions were analyzed by MEGA 7 software based on the maximum likelihood method according to the Tamura-Nei model, with displaying 1000 bootstrap values on the branches (Chitrampalam & Nelson, 2016).

Pathogenicity tests

To conduct pathogenicity tests, seeds of common bean cv. Derakhshan were obtained from the Markazi Agricultural and Natural Resources Research and Education Center in Markazi Province of Iran. The seeds were sterilized with 1% sodium hypochlorite solution for one min, followed by three rinses with sterile distilled water. The seeds were then stored at 28°C for 24-48 h on moist sterile filter paper. After germination, five seeds were planted in each pot containing a mix of sterilized soil, sand, and leaf compost in a 1:1:1 ratio (v/v) (Amini et al., 2013). The pots were maintained in a greenhouse for 24 days at of 30 ± 4 °C, and 65% relative humidity, with a 16/8 h of light/darkness.

Two isolates (FS20 and FS35) were cultured on PDA medium and incubated at 28 ± 2 °C for seven days. Forty grams of mung beans in an Erlenmeyer flask containing 1 L of boiling water (mung bean broth medium) for 10 min, the contents of the flask were filtered through sterile muslin tissue. The resulting extracts (Zhang et al., 2013a) were then combined with SNA culture medium (including 1 g KH2PO4, 1 g KNO3, 0.5 g KCl, 0.5 g MgSO4.7H2O, 0.2 g glucose, 0.2 g sucrose, 18 g agar in 1 L of distilled water) (Nirenberg, 1976) according to the methods of Zhang et al. (2013b) and Koch et al. (2013). The isolates were inoculated with a macroconidial suspensions with the final concentration of 1 × 105 conidia/ml. The common bean seedlings at four-leaf stage were removed from the pots, and their roots were immersed in the suspensions containing 0.05% (v/v) Tween 20 (Gargouri-Kammoun et al., 2009). The control seedlings were inoculated in the same manner with sterile distilled water containing 0.05% (v/v) Tween 20. The seedlings were transferred to the pots and kept for 24 days at 25 °C and 65% relative humidity in with a 16/8 h of light/dark. At the end of the experiment, the seedlings were removed from the soil and washed with water. Disease severity was classified into five levels following: 0 = no discoloration; 1 = 1 to 25% discoloration; 2 = 26 to 50%; 3 = 51 to 75%; 4 = more than 75% discoloration on crown and root; and 5 = seedling death (Fernandez and Chen, 2005). Disease index (DI) was also evaluated based on the formula described by Taheri and Tarighi (2010).

Disease severity (%) =

$$(0n0+1n1+2n2+3n3+4n4+5n5)/5N\times100$$

n0: number of seedlings with disease score 0; n1: number of seedlings with disease score 1; n2: number of seedlings with disease score 2; n3: number of seedlings with disease score 3; n4: number of seedlings with disease score 4; n5: total number of seedlings with disease score 5; N: total number of the seedlings in this assay. The experiment was repeated three times, with three replicates. Fungal re-isolation was carried out from all crowns and roots to confirm Koch's postulates and the isolates were identified as described above.

Quantitative cell wall degrading enzymes (CWDEs) assays

The activity of extracellular destructive enzymes was assessed in laboratory conditions 10 days after inoculation (Ortega et al., 2013; Kikot et al., 2009; Xiao et al., 2013).

Extracellular destructive enzymes

Pectinase assay

Pectinase activity was evaluated in liquid culture media containing 4.6 g of pectin, 5 g of yeast extract, 5 g of peptone, and 5 g of KH2PO4 in 1 L of distilled water (Macmillan & Voughin, 1964; Khairy et al., 1964).

After inoculating the media with 10 mm hyphal plugs from each of the isolates (FS20 and FS35), the cultures were placed on a rotary shaker at 150 rpm for 10 days at 28 ± 2 °C. The activity of pectinase was assessed by measuring the decrease in D-galacturonic acid levels and quantifying its concentration through the dinitrosalicylic acid (DNS) colorimetric method. The enzymatic activity of pectinase was then assessed at wavelength of 540 nm. The enzyme activity units for pectinase (Colowich, 1995) were defined as the amount of enzyme that releases one micromole of galacturonic acid per min, based on the standard curve. The standard curve was created based on the absorbance of varying concentrations (µg/mL-1) of D-galacturonic acid.

Cellulase assay

Cellulase activity was investigated via a medium of carboxymethyl cellulose (Abdel-Razik, 1970) containing 4.6 g of carboxymethyl cellulose (CMC) instead of citrus pectin (Ding et al., 2011). The cultures were inoculated with 10 mm hyphal plugs from the isolates and subsequently maintained on a rotary shaker at 150 rpm for a duration of 10 days at a temperature of 28 ± 2 °C. Cellulase activity was investigated according to the technique Wood and Bhat (1988). Absorbance was assessed at a wavelength of 550 nm, and the amount of sugar reduction was measured from the glucose standard curve. One unit of cellulase activity was defined as the amount of enzyme that catalyzed 1.0 µ mol of glucose per minute during the hydrolysis reaction.

Statistical analysis

The disease index data were statistically analyzed with Minitab 17 via the Least Significant Difference (LSD) test to identify significant differences, set at a significance level of P≥0.05. Each experiment was repeated three times, and each treatment was evaluated with three replicates.

Results

Morphological identification of the fungal isolates

A total of twenty fungal isolates were collected, and among them, the FS20 and FS35 isolates were identified as *F. solani* based on their morphological characteristics. The isolates showed rapid growth on PDA medium at 28 ± 2 °C, with a 6.5 ± 0.5 cm colony diameter after seven days. The hyphae exhibited considerable dispersion, typically appearing white to creamy with a brownish color, and occasionally showing green or blue regions in the colony's center (Figs. 1A, 1B). Microconidia were usually single-celled, with dimensions of 5.2 -18.6 × 2.4-4.2 µm, occasionally two-celled, measuring 6.8-26.8 × 3.5 µm, oval, or ovoid (Fig. 1C). Macroconidia, consisting of four to five cells (featuring a rounded apical cell and a foot-shaped basal cell), were broad, elongated, and slightly rod-like, measuring 30.5-53.2 × 4.4-7.2 µm, formed on white to creamy-colored cushions (Fig. 1D). Brown, spherical to oval chlamydospores were often formed singly, in pairs, or sometimes in chains, abundant in number, measuring 6.4-11.4 µm in diameter, located in the middle of the hyphae or at the ends (Fig. 1E). Microconidia were abundantly formed in false heads on long phialides (Fig. 1F).

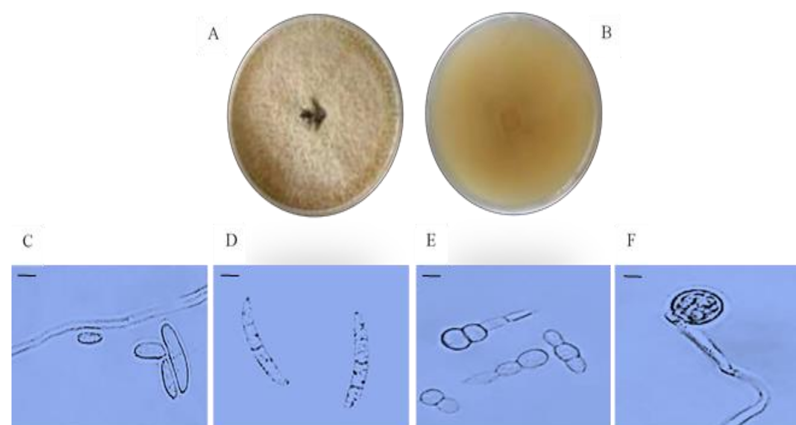

Fig. 1. Cultural and microscopic characteristics of the colony of *Fusarium solani* f. sp. *phaseoli* on PDA medium. (A: Obverse, and B: Reverse). Microconidia (C); Macroconidia (D); Chlamydospores (E); Phialids morphology (F); Scale bars: 20 µm.

Molecular phylogenetic analysis

The identification of FS20 as *F. solani* f. sp. *phaseoli* was confirmed based on molecular analysis of the ITS genomic regions. Both isolates (FS20 and FS35) showed a PCR product of about 600-700 bp, however, only one of the isolates (FS20) was sequenced. The sequences were analyzed with the BLASTn tool from National Center for Biotechnology Information (NCBI), confirming the identity of the pure culture. The BLASTn findings demonstrated a full 100% alignment with *F. solani* sequences from GenBank, and morphological identification was validated via molecular analysis of the ITS genomic regions. The ITS sequence accession number PQ516997 was obtained from NCBI after the sequence was submitted to GenBank. The ITS region underwent phylogenetic analysis in combination with the ITS4 sequence, by MEGA 7 software based on the maximum likelihood method according to the Tamura-Nei model, with 1000 bootstrap values. In this tree, *F. solani* isolates were placed in a separate clade from other members of this genus due to their high genetic similarity (Fig. 2).

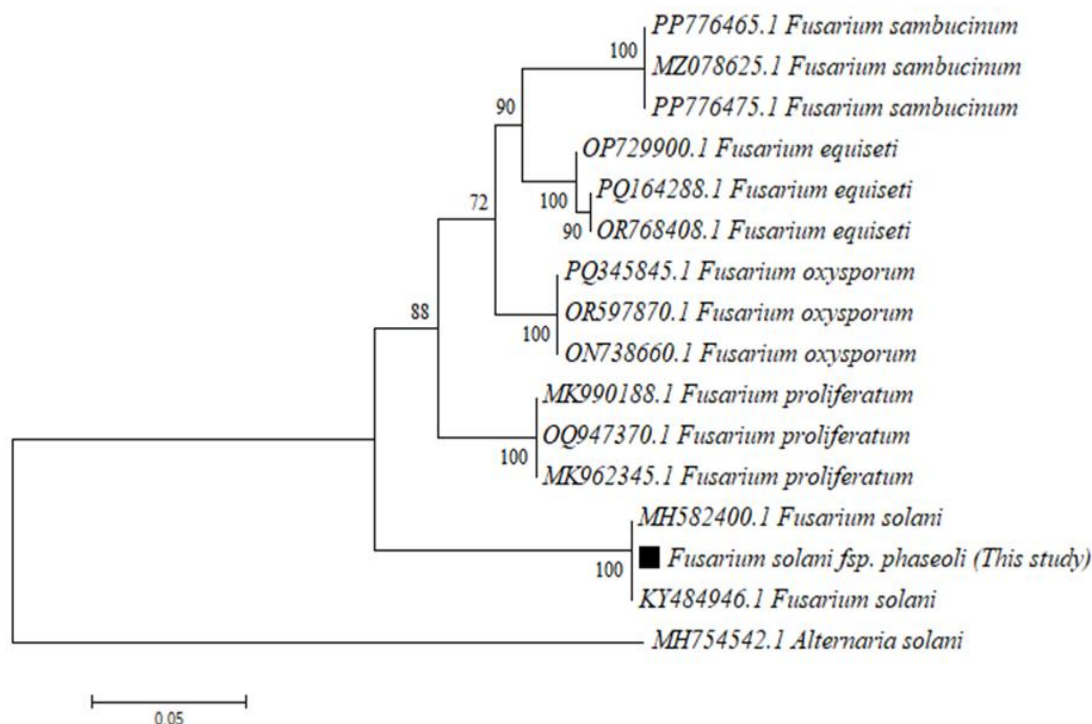

Fig. 2. Phylogenetic analysis of internal transcribed spacer (ITS) sequence of *Fusarium solani* f. sp. *phaseoli* obtained from bean roots via the maximum likelihood method in MEGA 7. Bootstrap values supporting the branches resulted from 1000 replicates.

Pathogenicity tests

Both selected isolates (FS20 and FS35) showed significant virulence on seedlings 24 days after inoculation, with the bean plants exhibiting yellowing, reduced growth, and wilting. The crown and root tissues displayed a discoloration from red to brown, with evidence of cracking and necrosis in the longitudinal sections (Fig. 3A). Ultimately, FS20 and FS35 isolates exhibited disease indices of 88.20 and 82.65, respectively. Subsequently, the agents responsible for the disease were isolated from the plant tissues, and following the application of Koch's postulates, pathogenicity in beans was proved after 24 days of inoculation (Fig. 3A,B).

Analysis of the CWDEs activity

Based on the analysis of the CWDEs activity, the isolates displayed the ability to secrete CWDEs. Pectinase and cellulase showed the highest activity at 144 and 72 h post-cultivation in liquid medium, respectively. Following these peaks, their activity gradually decreased, then increased and decreased sinusoidally, and finally stabilized until the end of

cultivation. During the maximum activity intervals, the CWDE activity levels were recorded between 3855 and 4535 µg/ml for pectinase (Fig. 4A) and from 820 to 935 µg/ml for cellulase (Fig. 4B). Moreover, pectinase activity increased with a more gradual trend, while the isolates achieved maximum cellulase activity more rapidly.

Fig. 3. Symptoms of crown and root rot of common bean seedlings caused by *Fusarium solani* f. sp. *phaseoli*, 24 days after inoculation (A), Control (B).

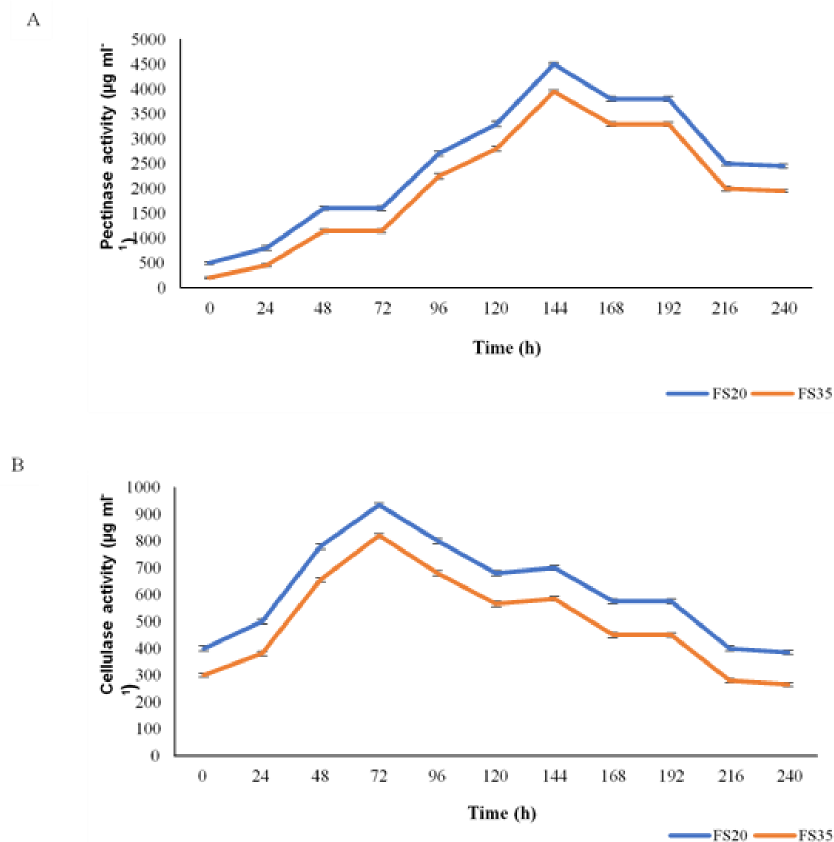

Fig. 4. Assessment of activities of CWDEs produced by *Fusarium solani* f. sp. *phaseoli* isolates over a maximum incubation duration of 240 h. Pectinase activity (A), Cellulase activity (B). Values are means of three replicates.

Discussion

Fusarium species with a wide host range cause hypocotyl rot, seeds and seedlings decay at pre and post-emergences (Mukankusi et al., 2011; Nzungize et al., 2012) and wilting in common bean and are considered one of the main factors contributing to the destruction of crown and root tissues in bean (Aoki et al., 2014). Members of this genus are found as cosmopolitan pathogens in all climatic zones (Backhouse & Burgess, 1995). However, they prefer warm tropical areas (Sangalang et al., 1995). The isolates of this fungus, with the highest prevalence, have been identified as one of the most significant damaging agents to bean fields in Zanjan, Qazvin, East Azerbaijan, and Lorestan Provinces (Naseri, 2008; Saremi et al., 2011; Dehghani et al., 2018). The soil-borne fungus *F. solani* is one of the damaging factors for beans in Khorasan Razavi, which causes up to 85% yield loss in some regions (Ahari Mostafavi et al., 2009).

In this study, *F. solani* isolates were obtained from bean fields in Khorasan Razavi Province, and their morphological and phylogenetic characteristics were investigated, together with determining their pathogenicity levels. The activity of cellulase and pectinase produced by the fungal isolates were also determined, which are involved in pathogenicity. Two fungal isolates (FS20 and FS35) were identified based on morphological techniques, and the morphological identification of one of the isolates (FS20) was confirmed by molecular analysis. Chehri et al. (2015) showed the possibility of separating members of this genus based on the ITS region, with strong bootstrap values. In accordance with results of the present research, Eke et al. (2016) and Toghueo et al. (2016) confirmed the phylogeny of *F. solani* strains through sequencing of the rDNA-ITS region. Based on the pathogenicity tests, the *F. solani* isolates showed a high level of pathogenicity on bean seedlings. In line with our results, Eke et al. (2016) reported the aggressiveness and destructiveness of *F. solani* on bean plants. Additionally, the reports by Montiel-González et al. (2005) and Aoki et al. (2014) confirmed the pathogenicity of *F. solani* isolates on beans. In line with our findings, Rahkhodaei et al. (2023) identified *F. solani* from beans based on morphological characteristics and molecular methods through sequencing of ITS and the translation elongation factor 1α (*tef-1α*) gene regions, and proved its pathogenicity on beans in the Markazi Province.

The activity of CWDEs, including pectinase and cellulase, was evaluated over different time points since these enzymes play considerable roles in pathogenicity of fungi, such as *F. solani* on the host plants (Kikot et al., 2009; Ortega et al., 2013). These enzymes are also important for phytopathogenic fungi lacking specialized penetration structures (Gibson et al., 2011). Throughout the investigation of CWDEs, cellulase activity reached its peak more quickly, while pectinase showed maximum activity after a longer duration. In line with these findings, Ortega et al. (2013) and Khaledi et al. (2017) reported the maximum level of pectinase activity after a longer incubation duration compared to the other enzymes investigated. *Fusarium solani* is a key factor in reducing the quality of beans, thus, it is vital to identify this pathogen in different regions of Iran to implement control measures and prevent its adverse effects on the key agricultural products.

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Conflict of interest

The authors declare that there are no conflicts of interest present

CRediT author statement

B. Bagherieh: Laboratory works & writing original draft. **P. Taheri:** Supervision, methodology, writing, reviewing & editing.

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