Soybean sudden death syndrome (SDS), caused by members of the Fusarium solani species complex, is an important disease leading to substantial yield reductions. The threat of soybean SDS is becoming serious in Korea, probably due to changes in the climate favoring the prevalence of this disease. In this study, after isolating Fusarium spp. from root rot-symptomatic soybean, we determined that F. solani was a causal agent of soybean SDS based on morphological and molecular characterization as well as Koch’s postulate. From 2009 to 2011, a total of nine isolates of Fusarium solani were collected from major soybean fields, Korea. Morphological and TEF-1α sequence analyses confirmed a variety of nine F. solani isolates. All F. solani isolates were close with F. solani species from Asia and mostly belonged to F. solani f. sp. pisi (SSLP14, 15, 16,19, and 20). Based on homology analysis of both ITS and TEF-1α, some isolates (SSLP2, 18, and 22) were similar to F. solani causing human eye keratitis, indicating a shared pathogenicity both on humans and plants. In a pathogenicity test, we observed hyphae in both xylem and phloem tissues from discolored roots and basal stems of plants exhibiting foliar SDS symptoms, indicating its contribution to foliar symptoms. Based on the differential reactions of soybean plant genotypes to F. solani isolates, one indigenous F. solani isolates, SSLP15 was identified having the highest levels of virulence. In addition, Danhaekkong and Jinpumkong 2 soybean were found to be resistant to SDS as demonstrated by slight symptom with less than 20% foliage affected. The evaluation of SDS resistance could be beneficial to support varietal improvement through extensive soybean breeding program in Korea.

Keywords: Fusarium solani, soybean, sudden death syndrome
preliminary report suggests the involvement of various races of F. solani in the severity of this disease (Mueller et al., 2002).

As current climate conditions change in Korea becoming more favorable for the development of SDS, isolating pathogenic F. solani and screening for soybean genotypes resistant to SDS are the first steps in a soybean breeding program aimed at controlling this disease. A number of indigenous F. solani strains from Korea was explored (Gopal et al., 2012) but not for SDS evaluation purpose. Therefore, the present work was undertaken to isolate and identify Fusarium species based on morphological and molecular approaches using internal transcribed spacer (ITS) and transcription elongation factor (TEF)-1α, notably F. solani, from diseased soybean plants with SDS-like symptoms or root rot. In addition, we assessed the virulence of F. solani isolates against different soybean genotypes under artificially controlled conditions.

MATERIAL AND METHODS

Collection and isolation of Fusarium species

Soybean plants with SDS-like symptoms which were characterized by discolored stems and root rot, were collected at the R5 to R6 growth stages from two different fields, in Suwon (latitude:37°17’27”N, longitude:127°00’32”E, elevation above sea level:58 m) and Daegu (latitude:35°52’13”N, longitude:128°35’22”E; elevation above sea level:45m), Korea, over a 3-year period, from 2009 to 2011. Both areas have similar long winter and high soil moisture. Suwon has the average rainfall of 1311 mm, average annual temperature of 11.6 °C and maximum and minimum temperature of -5 to -10 °C and around 30 °C, respectively. While in Daegu, the average rainfall is 1055 mm and the average temperature is 13.6 °C, the average minimum temperature is -2 to -4 °C while maximum temperature is around 30 °C. These fields experienced continuous soybean cultivation. Small pieces (1-2 × 1-2 mm) of basal stems and roots were surface-sterilized in 1% sodium hypochlorite and rinsed in sterile water. The tissue sections were then air-dried on filter paper and plated onto potato dextrose agar (PDA) medium supplemented with 500 mg/L streptomycin to suppress bacterial growth. The culture plates were incubated at 25 °C under a 12 h light/dark regime. The obtained isolates were purified by sub-culturing single spores, and the pure fungal isolates were stored in 30% glycerol at -80 °C until use.

Molecular and morphological characterization of F. solani

For genomic DNA isolation, each fungal strain was grown in liquid complete medium (CM) at 25 °C on a rotary shaker (150 rpm) for 3 days, and the mycelial mass was harvested and lyophilized. DNA was extracted with a cetyltrimethylammonium bromide protocol (Leslie and Summerell, 2006). All fungal isolates were first observed their morphoscopic morphology. These isolates were then identified based on their sequences of internal transcribed spacer (ITS) region of ribosomal DNA amplified and sequenced with an ITS primer pair, ITS4 (5’-TCC TCC GCT TAT TAT GAT GC-3’) and ITS5 (5’-GGA AGT AAA AGT ATC ATG ATG TAT CAC G-3’)(White et al., 1990). F. solani species were further characterized by using DNA sequences of partial translation elongation factor (TEF)-1α amplified with specific primers e1 (forward primer: 5’-ATG GGT AGT CTA GGG CTT CAC ACC TAT CC-3’) and e2 (reverse primer: 5’-GGA (G/A) GT ACC AGT GC (G/C) AT CAT GTT-3’) (O’Donnell et al., 1998). Oligonucleotides were synthesized by the Bioneer oligonucleotide synthesis facility (Daejon, South Korea). The amplified PCR product showing a single band on agarose gel electrophoresis was purified and used as a template for sequencing using a BigDye Terminator Cycle Sequencing Kit V.3.1 (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed with an automatic sequencer, Model ABI 3730 (Applied Biosystems) and the resulting sequences were analyzed using SeqScape software v. 2.0 (Applied Biosystems). For homology analysis, the sequences were compared against the non-redundant sequence data using BLAST. Sequences of several Fusarium species in the public database were included for phylogenetic tree construction. A neighbor-joining (NJ) tree was constructed with MEGA 4.0 using the bootstrap method with 1,000 replications (Tamura et al., 2007).

The morphological differences among the fungal isolates of F. solani were characterized with examining several parameters (colony, conidia and conidiophores, perithecia, and chlamydospores). Fungal isolates were grown on PDA for 8 days to observe fungal colony. Morphology of conidia, conidiophores, chlamydospore, and perithelia was observed from 6-14-day-old carnation leaf-piece agar (CLA) cultures (Leslie and Summerell, 2006). CLA cultures were incubated at 25 °C under near UV light (wavelength: 365 nm; HIkv Import & Export Co., Ltd.) for about 10 days to induce sexual reproduction. Differential interference contrast (DIC) images were obtained with a DE/Axio Imager A1 microscope (Carl Zeiss, Oberkochen, Germany).

Evaluation of SDS susceptibility

Five soybean genotypes that have been primarily used as parents for genetic mapping populations were selected for pathogenicity testing using nine F. solani isolates. In addition to the five genotypes, four genotypes of a USDA germplasm collection including SDS-resistant genotype (PI 536636: Ripley), SDS-susceptible genotype (PI 652935: BARC-19), as well as Haiiro (PI 243530) and Spencer (PI 525454) were also included against three selected F. solani isolates (SSLP2, 20, and 22). F. solani isolates were grown at 25 °C for 7 days and used to infest red sorghum seeds that had been soaked overnight and autoclaved twice. Five plugs (4 mm in diameter) of mycelia were used for inoculation. The seeds were incubated with the mycelia at 24 °C for 2 weeks. A mixture of sterilized fertilizer:soil (1:1) was mixed and placed into 10×10×10 cm3 tubes. The drain hole of each tube was plugged with non-absorbent cotton, the tube was prefilled with sterile soil mixture, and 7 g of infected sorghum seeds were evenly distributed onto the soil mixture. Additional soil mixture was added to each tube, directly covering the infected seeds, at a depth of 2 cm. Soybean seeds were planted in the soil mixture, and the seeds were covered with additional soil mixture. Non-inoculated sorghum seeds were used as a control. A pathogenicity test was conducted in a growth chamber programmed for 12 h day/night, a temperature of 25 °C, and a relative humidity of 80%, with three replications per treatment. After seedling emergence, the soil moisture was maintained near water holding capacity. Four weeks after planting, each plant was evaluated for SDS leaf symptoms using disease rating according to standard criteria (Hartman et al., 1997). The criteria were determined as follows: 1 = no symptoms (high resistant), 2 = slight symptom showing 1 to 20% foliage affected (resistant), 3 = 21 to 50% foliage affected (moderate), 4 = heavy symptom with 51 to 80% foliage affected (susceptible), and 5 = severe symptom showing 81 to 100% foliage affected (high susceptible).

Colonization of F. solani in soybean

The stems and roots of F. solani-inoculated plants showing SDS symptoms were collected 4 weeks after planting for observation via microscopy. The plants were rinsed in running tap water and prepared for microscope sectioning. Both F. solani-inoculated and control samples were fixed with modified Karnovsky’s fixative solution (2% paraformaldehyde supplemented with 2% glutaraldehyde in 0.05 mol/L sodium cacodylate buffer) and washed in 0.05 mol/L sodium cacodylate buffer. The samples were then post-fixed in 1% osmium tetroxide in 0.05 mol/L sodium cacodylate buffer and briefly washed twice in distilled water. The tissues were infiltrated with stain and embedded overnight by en bloc staining with 0.5% uranyl acetate at 4 °C. After the samples were dehydrated in a graded ethanol series and propylene oxide and embedded in Spur’s resin, they were polymerized at 70 °C for 24 h. Thick sections were generated with an ultra microtome and observed by light microscopy.

Statistical analysis

All data were subject to analysis of variance (ANOVA) using SAS software (SAS, Cary, NC, USA). The statistical difference in infection rate was tested by the least significant differences (LSD) at P≤0.05 (SAS, 2002).

RESULTS

Isolation of F. solani

All fungal colonies grew rapidly (± 9 mm in diameter) at room temperature, and macroconidia appeared one week after inoculation. Most isolates produced Fusarium-shaped conidia and possessed cottony aerial mycelia with various color from whitish, yellowish-white or pale to yellow, brownish, pinky to bluish, sometimes with purple were observed. A comparison of the genomic sequences of the ITS regions revealed that all isolates shared high sequence homology with known Fusarium species at a level ranging from 99 to 100% (Table 1). Of the 54 isolates examined, nine isolates were identified as Fusarium solani, and the rest were F. equiseti (31 isolates), Gibberella moniliformis (anamorph: F. verticillioides; 13 isolates), and Gibberella zeae (anamorph: F. graminearum Schwabe; one isolate). All F. solani were further characterized their molecular and morphological properties.

Molecular and morphological characterization of F. solani

TEF sequences of nine F. solani isolates (SSLP2, 14, 15, 16, 17, 18, 19, 20, and 22) with other Nectria haematococca-F. solani species isolated from various hosts were used for the phylogenetic analysis (O’Donnell, 2000). All of the strains identified in this study were included in the clade 3 which contained Asian F. solani species (Fig. 1). Five isolates (SSLP14, 15, 16, 19, and 20) were grouped with F. solani i. sp. psi strains. SSLP2/22 and SSLP18 strains were similar in TEF sequences with F. solani strains causing human eye keratitis at USA and India, respectively (O’Donnell et al., 2007). SSLP17 was out grouped with Neocosmospora africana (another sexual stage name of F. solani) which was isolated from South African soil (O’Donnell, 2000). Based on the virulence level, these 54 isolates were divided into three groups. SSLP15 and SSLP20 tend to be the same subclade indicating their close genetic relationship to support their same environment in Daegu where this site is more favorable for SDS than.
Suwon. While isolates having lower virulence seemed to distribute in several branches.

Pathogenicity of *F. solani*

All *F. solani* isolates were examined for their virulence on various soybean genotypes. The isolates produced various levels of typical SDS symptoms, such as intercellular chiasm and necrosis on young leaves, when inoculated on soybean plants grown in a growth chamber. Typical SDS symptoms were observed on soybean leaflets and roots. Notably, the *F. solani* pathogen was re-isolated from surrounding area of the infected soybean plants and identified as the same species, demonstrating Koch’s postulated.

After inoculation with the isolates SSLP2, SSLP20, and SSLP22, SDS-resistant ‘Ripley’ showed an average SDS severity value of 0.03%, while a value of 21.9% was observed in SDS-susceptible ‘BARC-19’ which was comparable with ‘Spencer’ (21.4%). None of the tested soybean genotypes exhibited lower values of SDS severity than Ripley or higher values than BARC-19, which was especially high when the later genotype was infected with SSLP22 (48.23%). Even though a bit less, this severity rating using SSLP22 indigenous from Korea is in good agreement with the USDA examination that BARC has been categorized as susceptible genotype. Hairo critically exhibited resistance to SDS, with mean severity value of 12.2% against the three isolates, respectively (Table 2). This result suggests that different geographical and environmental conditions contribute the distinct virulence of *F. solani* strains.

The average SDS severity of Korean soybean genotypes was examined in plants grown in a growth chamber 4 weeks after inoculation with *F. solani* (Table 3). The main effect that showed significant differences was the percent of leaflets with SDS symptoms (P < 0.05), which depended on the fungal isolate used for inoculation. SSLP15 showed the greatest pathogenicity, with levels of 24.0%, whereas SSLP17 had the lowest pathogenicity at only 1.3%. In addition to Pureunkong, SSLP15 had the highest SDS severity (98.9%), while Pureunkong had the highest SDS severity (7.9%), which was significantly lower than the other genotypes.

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Table 3 SDS severity of host soybean plant genotypes against *F. solani* isolates

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<th>Isolates</th>
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<td>SSLP2</td>
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<td>SSLP22</td>
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*Within fungal inoculum and host soybean genotype combinations, means (column or row) not followed by the same letter are significantly different at *P*<0.05 based on LSD. **The categories of SDS severity rating are the same as those on Table 2.

Figure 1 Phylogenetic tree of *F. solani* species complex produced by examining the sequence homology of the partial translation elongation factor (TEF)-1α using the neighbor-joining method with bootstrap values from 1000 replications.

![Phylogenetic tree of *F. solani* species complex](image1.png)

Figure 2 Mycelial growth of *F. solani* strains on potato dextrose agar (PDA) incubated at 25°C. Pictures were taken 8 days after inoculation on PDA.

![Mycelial growth of *F. solani* strains](image2.png)

Figure 3 Morphological characters of *F. solani* strains. A. sporodochia produced on carnation leaf pieces. Macroconidia (B), phialide (C), and microconidia (D) produced by SSLP14, 15, 16, 17, 18, 19, or 20 strains. Macroconidia (E), phialide (F), and microconidia (G) produced by SSLP2 or 22 strains. H. chlamydospores produced by *F. solani* strains. I. red/orange perithecia produced by SSLP2, 17, or 22 strains. J. rossete asc dissected from perithecia. Pictures were taken 6–14 days after inoculation on carnation leaf agar (CLA) cultures.

![Morphological characters of *F. solani* strains](image3.png)

Figure 4 Colonization of *F. solani* hyphae in cross sections of tissue from a soybean plant with foliar, basal stem, and root symptoms of SDS. A. *F. solani* hyphae in phloem tissue. B. *F. solani* hyphae in xylem tissue. C. Colonization of *F. solani* in effective and non-effective zones in the xylem and phloem tissues of soybean, respectively.

![Colonization of *F. solani* hyphae](image4.png)
DISCUSSION

Sudden death syndrome (SDS) is thought to be optimal in favored high soil moisture and low temperature during the early growing season of soybean followed by relatively high temperature. Longer watered and higher than the hot climate during summer seem to attribute the occurrence of SDS and root rot symptom soybean crop in Korea recently. As demonstrated in this current study, during 2009–2011, SDS-like symptom with root rot was observed in some fields of cultivated soybean in Korea, leading to isolate the fungal pathogen. We identified predominant species of Fusarium solani F. equiseti, G. moniliformis, F. solani, and G. Zeae based on molecular and morphological approaches. Morphological observation is helpful to identify this Fusarium species because literally, the morphological characteristics assisted to initially differentiate fungal species even though easy misidentification usually occurred. Morphological identification of Fusarium species (Marasas et al. 2001; Hsuan 2011) is able to sort important species especially F. solani group before employing another method (Leslie and Summerrell, 2006) or molecular approach. In particular, the identified F. solani from SDS-diseased soybean plants agreed with previous studies that this fungus causes soybean SDS. These suggest that cool soil temperatures and higher humidity levels during the 3-year sampling period, have a favorable environment for SDS pathogen development. Moreover, these fields contain historical hot spots for soybean diseases, with continuous soybean planting. This condition can be more severe since some plant-parasitic nematodes are interacted with SDS development (Xing and Westphal, 2013), however, F. solani was also reported to be pathogenic to the nematode eggs of Heterodera glycines (McLean and Lawrence, 1995). In addition, F. equiseti and G. moniliformis species complex are the most common diseases reported in agricultural crops worldwide, including soybean (Jasnic et al., 2005; Hsuan et al., 2011). Recently, F. graminearum was also reported to be pathogenic and a causal agent of SDS in cereal crops as well as soybean (Marinetti et al., 2013; Brar et al., 2011).

Based on the criterion of the morphology of microconidia produced on the conidiophore and macroconidia formed on sporodochia, nine F. solani in our study showed typical characters (Fig. 3). These morphological characteristics could be a basic information to classification into formae specialis of fungal isolation. Morphological characters of some isolates were matched with F. solani f. sp. pisi described previously (Jung et al., 1999). These initial morphological characteristics are very potential for genetic studies on the molecular basis of pathogenicity. Moreover, part of ascospore could be seen clearly among isolates, might be responsible for their survival as shown by their morphological structure. Phylogenetic tree analysis revealed that a variety of soybean SDS-causing F. solani strains were found in Korea. All isolates seem genetically closer to the F. solani from Asia than other continents. This SDS agent, F. solani isolates from Korea could be specific because they had far distance with F. solani species complex, such as F. tucumanciae and F. virguliforme from South America which are well known as SDS agent as well. A number of isolates (SSLP14, 15, 16, 19 and 20) which belong to F. solani f. sp. pisi suggests that the habitat of this species varies, not only pea, ginseng, mulberry, chickpea (Matuo and Suyodo, 1973) but also soybean. As supported by a prior study that F. solani f. sp. pisi was also found on soybean and showed remarkable virulence in pea seeding (Jung et al., 1999). Another finding showing one of a sexual stage of F. solani, Nectria haematococca was also observed. Morphological characters of some isolates were matched with F. solani f. sp. pisi described previously (Yamada et al., 2003). Moreover, early infection at the seedling stage enables the effective development of SDS fungus in xylem tissues, leading to foliar SDS symptoms (Gao et al., 2006). The current study demonstrates that the presence of SDS fungus in root tissues is associated with foliar symptoms, which supports a previous study showing a strong association between SDS disease and foliar infection (Yamada et al., 2003). Moreover, early infection at the seedling stage enables the effective development of SDS fungus in xylem tissues, leading to foliar SDS symptoms (Gao et al., 2006). The current study demonstrates that the presence of SDS fungus in root tissues is associated with foliar symptoms, which supports a previous study showing a strong association between SDS disease and foliar infection (Yamada et al., 2003).

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