Characterization of *Fusarium oxysporum* Isolated from Paprika in Korea

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In the present study we first report in Korea the identification and characterization of *Fusarium oxysporum* isolated from rotten stems and roots of paprika (*Capsicum annuum* var. *grossum*) at Masan, Kyungsangnamdo in 2006. The fungal species produced white aerial mycelia accompanying with dark violet pigment on PDA. The optimal temperature and pH for the growth of the species was 25°C and pH 7, respectively. Microscopic observation of one of isolates of the species shows that its conidiophores are unbranched and monophialides, its microconidia have oval-ellipsoidal shape with no septate and are of 3.0–11 × 1.5–3.5 µm sizes, its macroconidia are of 15–20 × 2.0–3.5 µm sizes and have slightly curved or slender shape with 2–3 septate. The results of molecular analysis show that the ITS rDNA of *F. oxysporum* from paprika shares 100% sequence identity with that of known *F. oxysporum* isolates. The identified species proved its pathogenicity by causing rotting symptom when it was inoculated on paprika fruits. The growth of *F. oxysporum* from paprika was suppressed on PDA by agrochemicals such as benomyl, tebuconazole and azoxystrobin. The identified species has the ability of producing extracellular enzymes that degrade cellobiose and pectin.

KEYWORDS: Extracellular enzymes, *Fusarium oxysporum*, Fungicides, Paprika

Paprika (*Capsicum annuum* var. *grossum*) referred to as bell pepper or sweet pepper is a valuable vegetable crop which is principally used as an ingredient in a broad variety of dishes. Since 1994 this wellbeing-style vegetable crop has been cultivated in Korea. Although this vegetable is not highly popular in Korean food market, its cultivation and production have continuously been increased to export to Japanese market as a highly profitable crop. With the increase of its cultivation, reports on the occurrence of diseases in paprika have also been increased in Korea. The diseases of paprika reported in Korea include Fusarium rot by *Fusarium solani* (telemorph *Nectria haematococca*) (Jee *et al.*, 2005) and sclerotial disease by *Sclerotinia sclerotiorum* (Jeon *et al.*, 2006) and virus diseases such as Cucumber mosaic virus (Kim *et al.*, 2002), Potato virus Y (Choi *et al.*, 2005), and Tomato spotted wilt virus (Kim *et al.*, 2004).

Since its introduction by Link (1809), *Fusarium* has been one of genera containing many recalcitrant plant-pathogenic species that are not easy to differentiate and control. In Solanaceae, especially in *Capsicum* L., wilt by *F. annuum* (Anonymous, 1960; Leyendecker and Nakayama, 1956), fruit and stem rot and wilt by *F. oxysporum* (Alfieri *et al.*, 1984), and damping-off by *Fusarium* sp. (Raabe *et al.*, 1981; Grand, 1985) were known be problems in the United States. In Korea, fruit rot of red pepper by *Fusarium* listed in Korean Plant Disease List (2004), but spe-

cies name of the pathogenic fungi has not been known yet. Thus, among *Fusaria* that are pathogenic to *Capsicum* plants, *F. solani* from paprika is the only species that its species name is known in Korea. In a disease survey of paprika at the region of Masan, Kyungsangnam-Do in July of 2006, we isolated fungi from rotten stems and roots of wilted paprika in commercial greenhouses. We here first report the occurrence of *F. oxysporum* on paprika in Korea. With the identification of the species, its morphological and physiological characteristics were described.

Materials and Methods

Fungi isolation. Diseased stems and roots of paprika were collected in July of 2006 from two greenhouses located in Masan, Kyungsangnam-Do. In the two greenhouses paprika was cultivated in soil. For fungal isolation, pieces of paprika tissues around diseased lesions were dissected, subjected to surface-disinfection with 5% sodium hypochlorite for 1 minute, rinsed with sterile distilled water and then air-dried on a clean bench. The dried samples were placed on PDA (Difco, USA) and incubated at 25°C for few days. Mycelial tips of the fungal isolates grown on the medium were transferred on new PDA plates. Single spore isolation was performed from the colony by picking conidia using dilution method on WA (water agar) and the obtained spores were cultured on PDA. A single spore isolate (coded as DUCFO-1) was

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used for identification and characterization works.

Observation of cultural and morphological characteristics. Colony characteristics of the fungal isolate were observed after the cultures were grown on PDA for 5 days. Morphological features of the isolate were examined under a phase-contrast microscope (Karl Zeiss, Axioskop 40) after growing the culture on carnation agar and PDA at 25°C for 1–4 weeks. Growth rate was determined using PDA, MEA (malt extract agar, Difco, USA), and OA (oatmeal agar, Difco, USA). Mycelial growth was recorded periodically by measuring diameters of colony. Optimal temperature and pH ranges for mycelial growth of the fungal isolate were examined on PDA by incubating the cultures at 20, 25, 30 and 37°C, respectively and at pH5, 7, and 10 for a week.

Genomic DNA extraction, PCR amplification and nucleotide sequencing. Genomic DNA of the fungal isolate was extracted by using a drilling method (Kim *et al.*, 1999). The ITS ribosomal DNA regions were amplified by PCR using universal primer pairs, ITS1-ITS4 (White *et al.*, 1990). PCR reaction mixture (a total volume of 50 μ l) contained 200 ng fungal genomic DNA, 20 pmol of each primer, 10 mM (each) of the four deoxynucleotide triphosphates (dNTPs), 1 $\times$ PCR buffer (10 mM Tris-Cl [pH 8.0], 1.5 mM MgCl₂, 50 mM KCl), 1 unit Thermostable polymerase (Solgent Corp.). Amplification was done in a Gene Amp-950 thermal cycler (ABI, USA). PCR conditions were programmed as follows: one cycle of denaturation at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and final one cycle of extension at 72°C for 10 min. The amplified DNA product was sequenced on Applied Biosystems ABI 373 DNA sequencer. Both strands of the PCR-amplified DNA fragments were sequenced using the PRISM Ready Reaction DyeDeoxy termination cycle sequencing kit and DNA sequences were determined in an Applied Biosystems ABI 373 DNA sequencer. The obtained nucleotide sequences were searched through BLASTN at GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Pathogenicity test. Pathogenic ability test was done against mature fruits of two paprika cultivars, Special (red colored) and Derby (yellow colored). Fungal conidia suspension prepared from the fungal cultures grown on PDA was applied to paprika fruits using a sterile brush. Both wound-treated fruits of paprika were inoculated with conidial suspension (1×10^6 conidia/ml). Control treatment was done with sterile water. The inoculated fruits were incubated in a humid chamber at 25°C for 14 days and rotting of fruit tissues were evaluated by observing the changes of color and softness in the fruit tissues. The inoculated

fungus was re-isolated from the rotted fruits tested, and its identity was confirmed by microscopic observation.

Fungicide test. The fungal isolate was precultured on PDA at 25°C for 5 days, and agar cores (5 mm diameter) of the grown cultures were placed on PDA plates containing each of different fungicides and grown for 7 days. As fungicides, benomyl, tebuconazole, dimethomorph, azoxystrobin, and triflumizole were used at the concentration of 1, 10, 100, and 1000 ppm, respectively. Sensitivity to these fungicides was evaluated by periodically measuring colony diameters on a light box.

Extracellular enzyme activity test. The fungal isolate was precultured on PDA at 25°C for 5 days. For the observation of fungal extracellularase activity, the preculture was transferred onto the media containing each of 0.5% D-cellobiose (Sigma, USA), pectin (MP Biomedicals, USA), starch (Sigma, USA), xylan (Sigma, USA) as enzymatic carbon source, 0.1% yeast nitrogen base (Difco, USA) as fundamental nitrogen source, 0.5% dyes (Congo Red, Sigma, USA) for chromogenic reaction, and 1.5% agar powder. After 5–7 days of culturing at 25°C, evaluation of enzyme activity was performed by observing clear zone (plaque) formed around the fungal colony by reaction between the enzymes secreted by the fungus and chromogenic substrates. Clear zone was observed by naked eyes or documented by taking a photo. Photos were

Fig. 1. Symptoms of *Fusarium* disease of paprika by *Fusarium oxysporum*. Darkly discolored and browned stems (A and B). Dried and rotten tissues are shown after dissecting basal parts of wilted paprika roots (C and D).

taken after mounting the plate onto a light box.

Results and Discussion

Symptom. At the greenhouses diseased plants were somewhat wilted in their roots and stems in comparison with healthy plants. Severely infected plants were wilted or blighted under the humid and hot greenhouse's environmental condition that was favorable to the disease development. Discoloring or dark browning of infected tissues in stems was shown later according to disease development (Fig. 1A~B). Wilt of basal parts of roots also is shown as dark-brown with the disease development (Fig. 1C~D). These rotting symptoms are similar to that caused by *F. solani*.

Identification and characterization of *F. oxysporum*.

Fungal isolates from the rotten stem and roots of paprika in this study were identified as *Fusarium oxysporum* by analyzing mycological characters and rDNA sequences. The colony formed with white aerial mycelia that later produce dark violet pigment on PDA which is one of well known properties of *F. oxysporum* (Fig. 2A~B). Microconidia produced on microconidiophores have elliptical shape and no septate (Fig. 2C~D). When the fungus was cultured on carrot agar at 25°C for 10 days, its macroconidia were abundantly formed in sporodochia on the surface of agar. Macroconidia were straight to slightly curved in shape with 3 septa (Fig. 2F). Chlamydospores are formed singly from the fungus grown on carrot agar for 5 days (Fig. 2E). The observed morphological characteristics of the identified species are summarized at Table 1 and compared with the reports of Booth (1970) and descriptions in a *Fusarium* manual book (John and Brett, 2006). As we can see at Table 1, most of the mycological features of the *Fusarium* isolate from this study agree with the known features of *F. oxysporum*.

To further support the identification results we amplified by PCR the ITS rDNA of the identified isolate and sequenced. Sequence comparison in the GenBank DNA database showed that the determined sequence shares

Fig. 2. Morphological features of *Fusarium oxysporum* isolated from rotten stems of paprika. The fungal isolate was grown on PDA or carrot agar for microscopic observation. Colony shape: front (A) and back (B) side of a PDA plate. Microconidia (C), Microconidiophores (D), and a single chlamydospore (E) formed on PDA ($\times 1000$). Macroconidia and microconidia formed on carrot agar (F) ($\times 400$). Scale bars represent 10 μm .

100% sequence identity with that of *F. oxysporum* and 86~98% with other *Fusarium* species (Table 2). Together with molecular and morphological data, the isolated species was identified as *F. oxysporum*.

F. oxysporum includes many representatives that are pathogenic to plants often causing vascular wilt diseases, damping off problems, and crown and root rots. Many *F. oxysporum* isolates appear to be host specific, which has

Table 1. Comparison of morphological characters of the present isolate with *Fusarium oxysporum* previously described

Species	Shape of conidiophores	Shape and size (μm) of		Shape and size (μm) of chlamydospores
		Microconidia	Macroconidia	
Present isolate	unbranched monophialides	no septate, oval-ellipsoidal 3.0~11 $\times$ 1.5~3.5	2~3 septate, Slightly curved, slender 15~20 $\times$ 2.0~3.5	globose-ellipsoidal, singly 5.0~15 $\times$ 5.0~13.0
<i>F. oxysporum</i> ^a	unbranched and branched monophialides	no septate, oval-ellipsoidal, straight to curved 5.0~12 $\times$ 2.2~3.5	usually 3~5 septate, fusoid-subulate and pointed at both ends 27~46 $\times$ 3.0~4.5	globose-ellipsoidal, singly or in pairs 4.0~16 $\times$ 4.0~12

^aBooth (1970).

Table 2. Nucleotide sequence identity of the ITS rDNA among *Fusarium* spp.

	Isolate	<i>F. oxysporum</i>	<i>F. redolens</i>	<i>F. acutatum</i>	<i>F. ambrosium</i>	<i>F. solani</i>
Isolate*	100	100	98	98	87	86
<i>F. oxysporum</i>		100	98	98	87	86
<i>F. redolens</i>			100	98	86	83
<i>F. acutatum</i>				100	87	86
<i>F. ambrosium</i>					100	96
<i>F. solani</i>						100

*The isolate is DUCCF0-1. GenBank accession numbers of the ITS rDNA sequences of reference *Fusarium* species are AY928419 (*F. oxysporum*), EF495234 (*F. redolens*), AY213653 (*F. acutatum*), AF178398 (*F. ambrosium*), and AF178408 (*F. solani*).

Table 3. Comparison of ITS rDNA sizes in *Fusarium oxysporum*

<i>F. oxysporum</i> isolate	Host, Source	size of ITS region (bp) ^a
DUCCF0-1	Paprika, This study	447
AY928419 ^b	Watermelon, China	447
AY667487 ^b	Pimento plant, China	446
AY669121 ^b	Cucumber, USA	460
DQ016211 ^b	Conifer nursery, USA	467
AF440543 ^b	Tomato, Canada	470
AF132800 ^b	Soybean, USA	470

^aITS region includes ITS1, 5.8S, and ITS2. DUCCF0-1 is from this study.

^bAccession number in GenBank.

resulted in the subdivision of the species into *formae speciales* and races that reflect the apparent plant pathogenic specialization (Leslie and Summerell, 2006). Over 100 *formae speciales* and races of *F. oxysporum* have been described. When we compared the size of ITS rDNA, *F. oxysporum* from paprika and watermelon has the same ITS size of 447 bp. *F. oxysporum* from tomato and soybean has the longest ITS sequences (Table 3). Table3 shows that size of ITS rDNA varies among *F. oxysporum* isolates from different host plants.

Characterization of *F. oxysporum*.

Pathogenicity: To evaluate the pathogenic ability of the identified *F. oxysporum*, the fungus was inoculated on fruits of paprika. The fungus successfully colonized on wound-treated fruits of the two tested cultivars and produced mycelium mass (Fig. 3C–D). But no colonization was observed on control inoculation (Fig. 3A–B). The infected parts of the fruits became soft and little soaked. The presence of the inoculated fungus was found inside of fruit tissues (Fig. 3E–F) and from the tissues the fungus could be re-isolated fulfilling Koch’s postulation. Although the fungus was isolated from stems and roots, it shows its ability to infect fruits too. Thus, *F. oxysporum* we isolated are likely to infect to the stems, roots, and fruits of paprika plants.

Growth properties: When we grew *F. oxysporum* on PDA, MEA, OMA, the fungus grew best on OMA (Fig.

Fig. 3. Pathogenicity test on two different paprika varieties, Special and Derby. Control: no fungal colonization is observed from wound-treated fruits of Special (A) and Derby (B) that are inoculated with sterile water. Fungal colony development by *F. oxysporum* from this study on wound-treated fruits of Special (C) and Derby (D) is shown 7 days after inoculation. Fungal colonization is also shown in inside parts of wound-treated fruits of Special (E) and Derby (F) 14 days after inoculation.

4A). The slowest growth was found on MEA. The optimum temperature for mycelial growth of *F. oxysporum* on OMA was 25°C (Fig. 4B). The fungus has hardly grown at 37°C. In preliminary study the fungal isolate showed its ability of growth at broad ranges of pH. Thus narrow pH ranges of 5, 7, 10, were compared to define more precise optimum pH. The highest mycelial growth was shown at pH 7 (Fig. 4C). Overall, the growth properties of the paprika isolate generally agreed with those of known *F. oxysporum*.

Sensitivity to fungicides: To understand its agrochemical sensitivity we grew the *F. oxysporum* with different

Fig. 4. Growth properties of *F. oxysporum* from this study. Mycelial growth of the fungus on different medium (A), at different temperature (B), and at different pH (C).

fungicides at diverse parts per million (Table 4). The 5 fungicides were chosen from commercially available fungicides for red pepper (*Capsicum annuum*) disease control in Korea. The fungus was sensitive to benomyl, tebuconazole and azoxystrobin than triflumizole and dimethomorph. The least sensitive was shown to dimethomorph. Thus, for the control of *F. oxysporum* occurring in paprika, benomyl, tebuconazole and azoxystrobin can be used as a primary choice.

Enzymatic activity: Considering the *F. oxysporum* can infect and causes rot symptom on not only stem and root

Table 4. Sensitivities of *F. oxysporum* isolates from this study to 5 different fungicides known to be used for disease control of pepper

Fungicide tested	Mycelial growth (cm)			
	0 ppm	10 ppm	100 ppm	1000 ppm
Benomyl	3.8	N	N	N
Tebuconazole	3.7	N	N	N
Dimethomorph	3.8	2.2	1.8	1.4
Azoxystrobin	3.7	N	N	N
Triflumizole	3.7	0.8	N	N

N : No growth. All values are means of five replicates.

Fig. 5. Observation of the ability of producing extracellular enzymes by *F. oxysporum* from this study. The fungus was grown on chromogenic media that contain carbon substrates D-cellobiose (A), pectin (B), starch (C) and xylan (D). Clear zones formed on media indicate the presence of extracellular enzyme activities. Bars point out clear zones.

but also fruits of paprika, we postulate that this fungus may use extracellular enzymes to penetrate the host plant. Thus, we examined its ability to grow on chromagenic media containing different polymeric carbon sources. Clear zone that indicates the presence of extracellular enzyme activity was observed from all the tested chromagenic substrate media containing D-cellobiose, pectin, starch and xylan (Fig. 5). The color of congo red-contained media was changed from red to dark violet, red to white or formation of clear zone. The size of clear zone formed was higher in the media containing D-cellobiose and pectin than starch and xylan. This result shows that the fungus can produce high amount of extracellular β -glucosidase and pectinase. Since pectinase is one of well known enzymes used by many plant pathogenic fungi as

a pathogenic factor, this enzyme might be important in the infection of *F. oxysporum* to paprika.

Conclusion

We isolated and identified *F. oxysporum* that caused wilt and rot diseases in paprika. This is first report of its occurrence in *Capsicum* plant in Korea. *F. oxysporum* is the most widely dispersed species among *Fusarium* genus and can be recovered from most soils-Arctic (Kommedahl *et al.*, 1988), tropical (Joffe and Palti, 1977) or dessert (Mandeel *et al.*, 1995), and cultivated (McMullen and Stack, 1983) or not (McMullen and Stack, 1984). The presence of the species in Korea also reported from many other crops (The Korean Society of Plant Pathology, 2004). However, interestingly, in spite that several red pepper cultivars have been cultivated across Korea for so many years, disease caused by *F. oxysporum* has been not reported from red pepper in Korea. We don't know yet that whether there is disease in field but no report has been done or the species really cannot cause disease to red pepper. At this time, it is not known that whether *F. oxysporum* from paprika could infect red pepper or not. In the near future its host range should be examined.

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