DOI: 10.2478/10004-1254-63-2012-2227

Scientific paper

IDENTIFICATION OF *FUSARIUM* SPECIES ISOLATED FROM STORED APPLE FRUIT IN CROATIA*

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Received in February 2012
CrossChecked in August 2012
Accepted in September 2012

Several species of the genus *Fusarium* can cause apple fruit to rot while stored. Since *Fusarium* taxonomy is very complex and has constantly been revised and updated over the last years, the aim of this study was to identify *Fusarium* species from rotten apples, based on combined morphological characteristics and molecular data.

We identified 32 *Fusarium* isolates from rotten apple fruit of cultivars Golden Delicious, Jonagold, Idared, and Pink Lady, stored in Ultra Low Oxygen (ULO) conditions. Fusarium rot was detected in 9.4 % to 33.2 % of naturally infected apples, depending on the cultivar. The symptoms were similar in all four cultivars: a soft circular brown necrosis of different extent, with or without visible sporulation. *Fusarium* species were identified by the morphology of cultures grown on potato-dextrose agar (PDA) and carnation leaf agar (CLA). Twenty one isolates were identified as *Fusarium avenaceum* and confirmed as such with polymerase chain reaction (PCR) using specific primer pair FA-ITSF and FA-ITSR. *F. pseudograminearum, F. semitectum*, *F. crookwellense*, and *F. compactum* were identified by morphological characteristics. *F. avenaceum* can produce several mycotoxins and its dominance in Fusarium rot points to the risk of mycotoxin contamination of apple fruit juices and other products for human consumption.

Pathogenicity tests showed typical symptoms of Fusarium rot in most of the inoculated wounded apple fruits. In this respect *Fusarium avenaceum*, as the dominant cause of Fusarium rot in stored apple fruits is a typical wound parasite.

KEY WORDS: F. avenaceum, *Fusarium rot, morphology, mycotoxins, pathogenicity, PCR*

Fusarium species are primarily known as the causal agents of plant diseases, but fungi of this genus also produce many secondary toxic metabolites that can cause acute or chronic diseases in humans and domestic animals (1, 2). *Fusarium* toxicoses directly affecting humans have occured in history, for instance in Athens in the 5th century BC (3) and in the Soviet Union during World War II (4). The most prominent plant disease outbreaks caused by the *Fusarium* species occurred in commercial banana industry in the 1960s (5) and in wheat and barley in the USA during the 1990s (6).

* The subject of this article has partly been presented at the International Symposium "Power of Fungi and Mycotoxins in Health and Disease" held in Primošten, Croatia, from 19 to 22 October 2011.

Types of plant diseases caused by *Fusarium* spp. vary and may include root, stem, fruit or seed rots, leaf diseases, cancers, and wilts (7). Fusarium rot occurrs on apples and other fruits while they are stored and shelved (8, 9). It is a brown, soft, and watery circular necrosis, that gradually spreads over infected tissue, which becomes slightly sunken, sometimes with dense whitish mycelium (8, 9). According to the literature, the most common causal agents of Fusarium rot on apples and pears are *Fusarium avenaceum* (Fr.) Sacc., *F. culmorum* (W. G. Sm.) Sacc., *F lateritium* Nees, and *F. solani* (Mart.) Sacc. (8, 9). *Fusarium* taxonomy is very complex and has constantly been revised and updated over the last 100 years. Traditional diagnostic methods for detection and identification of *Fusarium* species are based only on morphological characteristics observed on selective media under specific incubation conditions (10). Morphological identification requires considerable expertise, especially in distinguishing closely related *Fusarium* species, as their morphological features may overlap. Recent introduction of molecular analysis has advanced detection and screening of many *Fusarium* species (7). Today, *Fusarium* species are usually identified by combining the morphological, biological, and molecular data.

The objectives of this study were to determine *Fusarium* species isolated from stored rotten apple fruits using morphological and molecular data, test their pathogenicity, an to determine the extent of Fusarium rot on the affected fruits.

MATERIALS AND METHODS

Apple fruit origin, sampling, and isolation of Fusarium *species*

Apples analysed in this study included four cultivars (Golden Delicious, Jonagold, Idared and Pink Lady) from two orchards (Bjelovar and Ivanić Grad), stored in Ultra Low Oxygen (ULO) conditions during the 2009/2010 season. Conditions in the ULO chambers were: temperature 1 °C, relative humidity >95 %, oxygen concentration 1.7 %, and carbon dioxide concentration 2.0 %. After five, six, seven, eight or nine months of storage, apple fruits of each cultivar showing decay symptoms were removed from the selection line during packing operations. In total, 500 rotten fruits were analysed. Fruits with circular, brown, watery necrosis with visible whitish, yellowish or pink mycelia covering the lesions or fruits with only brown watery necrosis were considered infected with *Fusarium* spp. and taken to the laboratory, placed in a moist chamber, and incubated for 4 days to 8 days at room temperature. After incubation, fruits with Fusarium rot symptoms were recorded and their proportion in total decayed fruits calculated. *Fusarium* colonies on symptomatic apple fruits were examined with a stereomicroscope and a microscope. Single-spore *Fusarium* isolates were recovered from sporulating colonies using a procedure described by Leslie and Summerell (7). Isolates were grown on carnation leaf-piece agar (CLA) at room temperature for seven to 20 days. After incubation, spores were suspended in 1.5 mL tubes by adding a small scrape of sporodochia formed on CLA to 100 µL of sterile water. Spore suspensions were spread on 100 mm diameter Petri dishes with 2 % water agar (WA). Plates were incubated overnight, right-side up, at 25 °C. Germinated spores were examined under a dissecting microscope, excised with a sterile needle, and transferred to Petri dishes containing potato dextrose agar (PDA). For each isolate three replicates were done. In total 32 single-spore *Fusarium* isolates were obtained from randomly selected fruits with sporulating colonies.

Identification of Fusarium *species*

Species were identified according to the morphology on PDA and CLA, using the descriptions of Leslie and Summerell (7). Isolates were grown on PDA at 22 °C for 14 days in order to describe colony morphology and pigmentation as secondary criteria for *Fusarium* identification. On CLA, isolates were incubated for 7 days to 20 days, after which macroconidia, microconidia, phialides, and other features were examined under a light microscope. Isolates ID-FUS 1 and ID-FUS 2 did not sporulate on CLA and were therefore grown on carrot agar (CA). After incubation at 22 °C temperature for 14 days, those isolates also produced sporodochia with macroconidia, and were identified based on morphological characters of the macroconidia and formation of perithecia.

Isolates morphologically identified as *F. avenaceum* were confirmed by polymerase chain reaction (PCR) using specific primer pair FA-ITS F (5'-CCA GAG GAC CCA AAC TCT AA-3') and FA-ITS R (5'-ACC GCA GAA GCA GAG CCA AT-3') (11, 12). Fungal DNA was extracted from pure mycelial cultures of 21 isolates using Extract-N-Amp Plant PCR Kit (Sigma-Aldrich Co., USA) following the manufacturer's

instructions. Sterile water was used as negative control. PCR was performed in 20 μL of reaction mixture containing 4 μL of DNA extract, 10 μL Extract-N-Amp PCR Ready Mix (Sigma-Aldrich Co., USA), 1 μL of each primer (in the concentration of 25 pmol μL^{-1}), and 4 μL of pure water. DNA amplification involved the following cycles : initial 2 min denaturation at 94°C, 30 cycles of 1 min at 94 °C, 1 min at 59 °C, and 2 min at 72 °C, followed by a final extension of 5 min at 72 °C. PCR products were separated by electrophoresis in 1.5 % agarose gel and stained in ethidium bromide.

Pathogenicity tests

All *Fusarium* isolates were tested for pathogenicity. Inoculations were done on wounded and unwounded apple fruit Jonagold, in three replicates for each isolate. Conidia of *Fusarium* isolates were transferred to 1.5 mL tubes containing 500 μL of sterile distilled water. Conidial concentration was determined with a haemocytometer and adjusted to $1x10^5$ mL^{-1}. Apple fruits were first rinsed with 96 % ethanol, then with sterile water, and then air-dried. The fruits were then wounded with a sterile needle and inoculated with 2 μL of spore suspension using a pipette. Unwounded apples were inoculated with fragments of CLA (5 mm x 5 mm) containing sporodochia with macroconidia, which were sealed on the surface of fruits with parafilm. Fruits inoculated with sterile water and sterile fragments of CLA were used as control. In total, 194 apples were inoculated and incubated at 22 °C. The experiment was performed two times. The appearance of symptoms was recorded, and lesion diameters were measured two and three weeks after inoculation. Average lesion growth was calculated and expressed in mm per day. Growth was analysed with the analysis of variance (ANOVA) using ARM 7 software (13) and average growth values compared with Duncan's test.

RESULTS

During the season 2009/2010, Fusarium rot was determined on all four apple cultivars (Golden Delicious, Jonagold, Idared and Pink Lady). Symptoms were similar: soft circular brown necrosis of different extent, covered with whitish, yellowish or pink sporulation (Figure 1), or without visible sporulation. The frequency of Fusarium rot among decayed fruits varied between the cultivars from 9.4 % to 33.2 % (Table 1). The highest was recorded on Pink Lady; it was almost twice as high as on Jonagold and three times as high as on Golden Delicious.

Table 1 *Fusarium rot (%) on decayed apple fruit stored in ULO conditions.*

Cultivar	Fusarium rot / %				
	Storage in ULO conditions / months				
	5	6	7	8	9
Idared	-	-	-	9.6	16.8
Jonagold	-	15.0	16.4	-	-
Golden Delicious	-	9.4	12.4	-	-
Pink Lady	33.2	-	29.2	27.4	-

Figure 1 *Symptoms of Fusarium rot on apple fruit Jonagold*

According to morphological features, 21 out of 32 isolates were identified as *F. avenaceum*, two as *F. pseudograminearum* O'Donnell & T. Aoki, three as *F. semitectum* Berk. & Ravenel, four isolates as *F. crookwellense* L.W. Burgess, P.E. Nelson & Toussoun, and two as *F. compactum* (Wollenw) Raillo (Table 2). Isolates ID-FUS1 and ID-FUS2 had morphological characters identical to two species, *F. pseudograminearum* O'Donnell & T. Aoki and *F. graminearum* Schwabe (7). Nevertheless, those species could be distinguished by the formation of perithecia. *F. graminearum* is homothallic and most strains produce perithecia more quickly and in greater number on CA than on CLA, while *F. pseudograminearum* is heterothallic and cannot produce perithecia (7). Since the ID-FUS1 and ID-FUS2 isolates did not produce perithecia on either medium, they were both identified as *F. pseudograminearum. F. avenaceum* was the prevalent *Fusarium* species, and its isolates were therefore

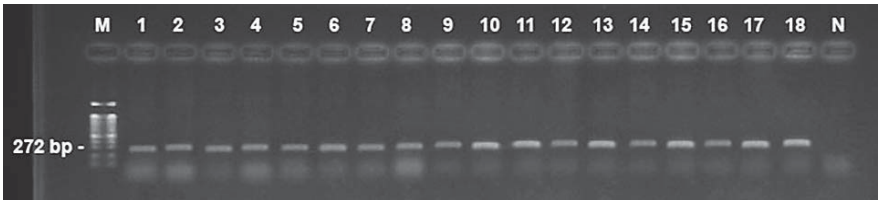

Figure 2 *Agarose gel showing specific detection of 18* Fusarium avenaceum *isolates with primer pair FA-ITSF and FA-ITSR. Amplificons of the expected size (272 bp) were obtained from all tested samples. Lanes 1-18: isolates DA-F3, JON-F1, JON-F2, JON-F3, GD-F1, GD-F2, GD-F3, ID-F2, ID-F3, ID-F4, ID-FUS5, ID-FUS6, C2-F1, C2-F2, C2-F3, C3-F1, C3-F3 and C3-F4. Lane N: negative control. Lane M: 100 bp ladder, (Intron Biotechnology, South Korea)*

Table 2 *Fusarium isolates, species determined, and pathogenicity*

***Fusarium* isolate**	**Source (cultivar)**	**Species**	**Pathogenicity tests Average lesion growth / mm per day**	
			Wounded fruit	**Unwounded fruit**
DA – F1	Idared	*F. avenaceum*	1.53 ^{gh*}	0.00 ^{d}
DA – F2	Idared	*F. semitectum*	0.27 ^{jkl}	0.00 ^{d}
DA – F3	Idared	*F. avenaceum*	0.67 ^{ij}	0.00 ^{d}
DA – F4	Idared	*F. avenaceum*	0.00 ^{l}	0.00 ^{d}
DA – F5	Idared	*F. avenaceum*	0.60 ^{ijk}	0.00 ^{d}
JON – F1	Jonagold	*F. avenaceum*	2.43 ^{cd}	0.00 ^{d}
JON – F2	Jonagold	*F. avenaceum*	1.43 ^{h}	0.00 ^{d}
JON – F3	Jonagold	*F. avenaceum*	1.90 ^{efg}	0.00 ^{d}
GD – F1	Golden Delicious	*F. avenaceum*	2.10 ^{def}	0.80 ^{c}
GD – F2	Golden Delicious	*F. avenaceum*	2.30 ^{cde}	0.00 ^{d}
GD – F3	Golden Delicious	*F. avenaceum*	3.67 ^{a}	2.30 ^{a}
ID – F1	Idared	*F. compactum*	1.67 ^{gh}	0.00 ^{d}
ID – F2	Idared	*F. avenaceum*	2.47 ^{bcd}	1.47 ^{b}
ID – F3	Idared	*F. avenaceum*	1.53 ^{gh}	0.00 ^{d}
ID – F4	Idared	*F. avenaceum*	2.20 ^{cde}	0.00 ^{d}
ID – FUS 1	Idared	*F. pseudograminearum*	0.17 ^{kl}	0.00 ^{d}
ID – FUS 2	Idared	*F. pseudograminearum*	0.17 ^{kl}	0.00 ^{d}
ID – FUS 3	Idared	*F. crookwellense*	0.00 ^{l}	0.00 ^{d}
ID – FUS 4	Idared	*F. crookwellense*	0.20 ^{kl}	0.00 ^{d}
ID – FUS 5	Idared	*F. avenaceum*	0.93 ^{i}	0.00 ^{d}
ID – FUS 6	Idared	*F. avenaceum*	0.97 ^{i}	0.00 ^{d}
C1 – F1	Pink Lady	*F. semitectum*	0.30 ^{jkl}	0.00 ^{d}
C2 – F1	Pink Lady	*F. avenaceum*	2.13 ^{de}	0.00 ^{d}
C2 – F2	Pink Lady	*F. avenaceum*	0.37 ^{jkl}	0.00 ^{d}
C2 – F3	Pink Lady	*F. avenaceum*	0.87 ^{i}	0.00 ^{d}
C2 – F4	Pink Lady	*F. semitectum*	0.33 ^{jkl}	0.00 ^{d}
C3 – F1	Pink Lady	*F. avenaceum*	0.53 ^{ijk}	0.00 ^{d}
C3 – F2	Pink Lady	*F. crookwellense*	0.37 ^{jkl}	0.00 ^{d}
C3 – F3	Pink Lady	*F. avenaceum*	2.87 ^{b}	0.00 ^{d}
C3 – F4	Pink Lady	*F. avenaceum*	2.60 ^{bc}	0.00 ^{d}
C3 – F5	Pink Lady	*F. crookwellense*	0.23 ^{jkl}	0.00 ^{d}
LM -3/2	Pink Lady	*F. compactum*	1.70 ^{fgh}	0.00 ^{d}
Control	-	-	0.00 ^{l}	0.00 ^{d}
LSD (P=0.05)			0.396	0.151

** Values followed by the same letter do not differ significantly (P<0.05, ANOVA)*

subjected to PCR to confirm identification. All isolates yielded the expected 272 bp fragment (Figure 2).

Statistical analysis of pathogenicity tests has shown significant differences between the *Fusarium* isolates inoculated on wounded and unwounded apple fruits (P=0.05). When inoculated on wounded fruit, only one *F. avenaceum* isolate (DA-F4) and one *F. crookwellense* isolate (ID-FUS3) did not cause any symptoms. Two *F. pseudograminearum* isolates (ID-FUS1, ID-FUS2) and one *F. crookwellense* isolate ID-FUS4 were the least aggressive, while isolate *F. avenaceum* GD-F3 was the most aggressive. When inoculated on unwounded apple fruit, only three *F. avenaceum* isolates (GD-F1, GD-F3 and ID-F2) had average lesion growth statistically different from control. In both tests, isolate GD-F3 showed the highest level of pathogenicity and significantly differed from all other isolates and control.

DISCUSSION

The most common causal agents of apple fruit deterioration during storage are fungi (8, 9, 14, 15). Among them, *Fusarium* species are considered of minor importance and have received little attention, especially in ULO storage conditions (8, 9). Similarly in Croatia, causes of stored apple fruit diseases have poorly been investigated (14, 15) or the available information is old (16-19).

Recently, *Fusarium* species determined in Greece in decayed stored and shelved apple fruits were also shown to be of minor importance (20). Tournas and Uppal-Memon (21) reported that only 3 % of apple fruit showed internal spoilage associated with *Fusarium* species. However, our results have shown a relatively high incidence of *Fusarium* spp. on all four apple cultivars. The most susceptible cultivar was Pink Lady with frequency of Fusarium rot from 27.4 % to 33.2 %. Our results suggest that susceptibility of apple cultivars to *Fusarium* spp. should be evaluated, and Fusarium rot may be a more important postharvest disease in ULO storage conditions than expected.

The toxigenic potential of many *Fusarium* species emphasizes the need for accurate identification on the species level. According to Leslie and Summerell (7), *Fusarium* species determined can be identified based on morphology alone. However, identification based on molecular data is considered more reliable and accurate than morphological identification and has become much more important in diagnostics of the fungi from the genus *Fusarium* (7). In our study, *F. avenaceum* species-specific PCR matched morphological identification and showed that *F. avenaceum* was the dominant species causing Fusarium rot of stored apple fruit. Our results are similar to reports from Slovenia (22) and Greece (20). One of the differences is that we report a larger number of *Fusarium* species on stored apple fruit in Croatia. Beside *F. avenaceum*, we identified *F. crookwellense*, *F. semitectum*, *F. compactum*, and *F. pseudograminearum*. The Greek study (20) reports only *F. avenaceum* and *F. proliferatum*, while the Slovene study (22) has isolated *F. avenaceum* as the only species on apples affected with *Fusarium* wet core rot. Snowdon (9) mention several species beside *F. avenaceum* that may cause Fusarium rot such as *F. culmorum*, *F. lateritium* and *F. solani*, but these species were not determined in our study.

Postharvest apple diseases are a result of latent infections in the field or of wounds infected by pathogens during the harvest, handling, and storage (23). *Fusarium* species can latently infect fruit through lenticels (9), but our study has shown that most of the *Fusarium* isolates only caused Fusarium rot when inoculated on wounded apple fruits (Figure 3).

Figure 3 *Pathogenicity test: inoculated fruit Jonagold after two, three, and six weeks of incubation (isolate C2-F2)*

Statistical analysis of average lesion growth has shown significant differences and variability between 30 *Fusarium* isolates and control, while only two isolates (DA-F4 and ID-FUS3) did not cause any symptoms and were identical to control when inoculated on wounded apple fruit (Table 2). On the other hand, only three *F. avenaceum* isolates (GD-F1, GD-F3 and ID-F2) caused necrosis when inoculated on unwounded fruits, while other 29 isolates were equal to control (Table 2). In other words *F. avenaceum*, is generally a wound parasite.

High incidence of *F. avenaceum* found in this study stresses the risk of mycotoxin contamination of apples and apple products. Sorensen et al. (24) detected thirteen *F. avenaceum* metabolites, including moniliformin, antibiotic Y, aurofusarin, and enniatins in artificially inoculated and naturally infected apples with wet apple core rot. After 14 and 21 days of incubation most of the metabolites reached significantly high levels, showing that *F. avenaceum* can produce high amounts of mycotoxins. Moniliformin is the most important mycotoxin produced by *F. avenaceum* (7); it is considered cytotoxic (25) and can cause different disorders and mortality in domestic animals (25).

In conclusion, our results have raised doubt that Fusarium rot may not be as rare as thought earlier. This in turn, points to a higher risk of moniliformin contamination of stored apples. This contamination may be minimised by improving control measures in the field.

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Sažetak

IDENTIFIKACIJA VRSTA RODA *FUSARIUM* IZOLIRANIH S PLODOVA JABUKE NAKON SKLADIŠTENJA

Fuzarijsku trulež ploda jabuke može uzrokovati veći broj vrsta roda *Fusarium*. Budući da je taksonomija roda *Fusarium* vrlo kompleksna te je podložna neprestanim promjenama posljednjih godina, cilj ovog rada bio je identificirati vrste roda *Fusarium*, izolirane sa zaraženih plodova jabuke na temelju morfoloških karakteristika i molekularnih analiza.

Skupljena su ukupno 32 izolata roda *Fusarium* sa zaraženih plodova jabuke kultivara Golden Delicious, Jonagold, Idared i Pink Lady, nakon skladištenja u ULO („Ultra Low Oxygen“) uvjetima. Fuzarijska je trulež zabilježena na 9,4 % do 33,2 % plodova zahvaćenih skladišnim bolestima. Simptomi fuzarijske truleži bili su slični kod sva četiri kultivara, a javljali su se u obliku smeđe, mekane nekroze koja se postepeno širi, uz pojavu ili bez pojave vidljive sporulacije na površini ploda. Vrste su identificirane na temelju morfoloških karakteristika na krumpirsko-dekstroznom agaru (KDA) i agaru s listićem karanfila (LKA). Ukupno je 21 izolat determiniran kao vrsta *Fusarium avenaceum*, što je potvrđeno i lančanom reakcijom polimerazom (PCR) uz uporabu specifičnog para početnica FA-ITSF i FA-ITSR. Vrste *F. pseudograminearum, F. semitectum*, *F. crookwellense* i *F. compactum* identificirane su na temelju morfologije. Istraživanjem je utvrđeno da je *F. avenaceum*, vrsta koja je potencijalni proizvođač nekoliko mikotoksina, dominantan uzročnik fuzarijske truleži ploda jabuke nakon skladištenja. Visok postotak zaraze fuzarijskom truleži može izazvati kontaminaciju sokova jabuke i ostalih proizvoda mikotoksinima, sekundarnim metabolitima vrsta *Fusarium* štetnih za ljudsko zdravlje.

Testovi patogenosti pokazali su da većina izolata uzrokuje pojavu tipičnih simptoma fuzarijske truleži nakon inokulacije na oštećeni plod. Navedeno upućuje na činjenicu da je *F. avenaceum,* kao dominantan uzročnik fuzarijske truleži ploda jabuke tipičan parazit rana.

KLJUČNE RIJEČI: F. avenaceum, *fuzarijska trulež, mikotoksini, morfologija, patogenost, PCR*

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