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Scientific paper

Fusarium oxysporum as Causal Agent of Tomato Wilt and Fruit Rot

Maja Ignjatov, Dragana Milošević, Zorica Nikolić, Jelica Gvozdanović-Varga, Dušica Jovičić and Gordana Zdjelar

Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, Serbia (maja.ignjatov@ifvcns.ns.ac.rs)

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SUMMARY

Tomatoes are parasitized by a number of pathogens, including *Fusarium oxysporum* f. sp. *lycopersici*, causal agent of fusarium wilt. Fresh vegetable fruits can be contaminated with various fungi that produce mycotoxins, which is an important issue for human health. The objective of this paper was to isolate, determine, and identify causal organisms of tomato wilt and fruit rot, based on the pathogens morphological and molecular characteristics. Samples of diseased plants showing symptoms of tomato wilt were collected from different localities in the production region of Vojvodina. Fruits with symptoms of fusarium rot were collected from storage and warehouses. The isolation and morphological determination of the fungus were performed on PDA and Czapek's nutrient media. Isolates from diseased plants growing in field, designated as TFW1-TFW12 and seven isolates from diseased tomato fruits (TFM1-TFM7) were chosen for further investigation. For identification of the fungal isolates, the polymerase chain reaction (PCR) was also used. The EF1/EF2 primer pair was used for molecular identification of *Fusarium* sp. Nine analyzed samples were found to contain DNA fragments 700 bp in size.

Keywords: Wilts; Tomatoes; Nutrients; Isolation; *Fusarium*; Fruits

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is one of economically the most important vegetable crops in Serbia where it is grown both, indoors and outdoors on an area of about 20,000 ha in total. A number of economically important tomato diseases caused by fungi are transmitted by seed or transplants (Ivanović and Ivanović, 2001). Tomatoes are parasitized by a number of pathogens, including *Fusarium oxysporum* Schlecht. f. sp. *lycopersici* (Sacc.) W.C. Snyder et H.N. Hansen, the causal agent of fusarium wilt of tomato (Aleksić et al., 1990; Ivanović and Mijatović, 2003), which is one of the most important species as tomato pathogen (Jones et al., 1982; Agrios, 1988; Smith et al., 1988). In an indoor environment due to high temperature and humidity, *F. oxysporum* f. sp. *lycopersici* can cause significant damage.

The causal agent of fusarium wilt is soil borne pathogen which can persist many years in the soil without a host. Most infections originate from the population associated with infected tomato debris. Healthy plants can become infected by *F. oxysporum* if the soil in which they are growing is infested with the pathogen (Farr et al., 1989; Mijatović et al., 2007). However, pathogenic fungi

of the genus *Fusarium* that are the causal agents of tomato wilt cause root and basal stem deterioration and result in the wilting of vegetable plants. Browning of the vascular tissue is strong evidence of fusarium wilt (Snyder and Hans, 2003). Some isolates of this fungus are pathogenic only to specific plant species (forma specialis) and there is also a large number of physiological races within each of these specialized forms, all of which make the selection for resistance to this pathogen more difficult (Armstrong and Armstrong, 1981). Evaluating tomato material for the resistance, Djordjević et al. (2011) showed that race 1 of fusarium wilt is not a limiting factor for successful tomato production, but race 3 of *Fusarium oxysporum* f. sp. *lycopersici* occurs in Serbia and can seriously endanger tomato production (Djordjević et al., 2011b).

On the other hand, fresh vegetable fruits are quite perishable because their high moisture content makes them vulnerable to microbial decay as well as physiological deterioration (Eckert and Ogawa, 1988). Fresh vegetable fruits can be contaminated with various fungi, including *Alternaria*, *Aspergillus*, *Fusarium*, *Rhizopus*, *Penicillium* and *Trichoderma* species (Brackett, 1988). These species can produce mycotoxins, contaminators of food, which is an important issue for human health. Fusarium rot is one of the most common diseases of fresh tomato fruits in storage and warehouses. The major role in the intensity of rots in tomato fruits in the warehouse is the harvest season. Fruits should be harvested when 70% of the fruits become red. Rot contamination rate increases if harvest can not be carried out for any reason such as inappropriate weather (Yoltas, 1985). The objective of this paper was to perform the isolation and determination of the isolates of causal agent of tomato wilt from an open field and tomato fruit decay in warehouses, based on the pathogens morphological and molecular characteristics.

MATERIAL AND METHODS

Isolation of the pathogen

The samples of diseased tomato plants showing the symptoms of tomato wilt were collected from different tomato varieties at several localities in the production region of Vojvodina from 2009-2011 growing season (Srbobran, Tovariševo, Obrovac, Gložani, Bačka Topola, Senta, Subotica, Begeč). The diseased plants and fruits were analyzed in the laboratory, and pathogen isolation was subsequently performed there as well. Also, fruits with symptoms of fusarium rot were collected from the warehouse during summer and autumn of 2011 and isolation of pathogen was carried out by cutting the fragments, at the border of diseased and healthy tissue (Machado et al., 2002; Mathur and Kongsdal, 2003). The fragments were surface sterilized with 3% NaOCl for 3 minutes, transferred on potato dextrose agar (PDA) and incubated at 25°C for seven days.

Growth characteristics of cultures were studied on PDA and Czapek agar. The cultures were incubated in 90 mm Petri dishes at 25°C in dark, for 7 days. Petri box isolates were then transferred to a thermostat with artificial UV light, at a temperature of 25°C for 10 days, to induce sporulation and pigmentation in culture (Burgess et al., 1994). The light source consisted of three neon tubes measuring 40 W and a black tube „black light" (Philips TLD 36W/08).

Patogenicity test

The pathogenicity of the isolates of fungi was tested by sowing tomato seeds in artificially infected soil (mixture made of the substrate (Klansman 2) and sterile sand at a ratio of 3:1) (Jasnić et al., 2005). Two replicates of each fungal strain were grown on Czapek's medium at 24°C. The suspension for inoculation was prepared by pouring 50 ml of sterile water into each of Petri dishes containing 10-day-old *Fusarium* isolates, stirring the mixture with a sterile glass stick, and pouring it into a glass. The concentration of conidia in the suspension was determined using Türk-Bürger's plate for spore count. It was set to $1x10^6$ conidia/ml. Control plants were sown in soil with sterile distilled water. Incubation was performed at 22-25°C for 14 days.

Molecular identification using PCR

Whether or not the isolates belonged to the species *Fusarium oxysporum* was determined by PCR (*Polymerase Chain Reaction*). The selected isolates were transferred into Petri dishes on PDA medium and allowed to grow for 72 hours at 25°C. After incubation the mycelium was scraped off and transferred to Eppendorf tubes for DNA extraction. The isolation of DNA was performed according to Cenis (1992). The mycelial mat was pelleted by centrifugation for 5 minutes at 13000 rpm, then washed with 500 µl of TE buffer and pelleted again. The TE was decanted and 300 µl of extraction buffer (200 mM tris HCl pH 8.5; 250 mM NaCl; 25 mM EDTA; 0.5 SDS) were added. The mycelium was crushed with conical grinder following by several steps of washing and precipitation. After a wash with 70% ethanol, the pellet was dried and resuspended in 50 µl of TE. The amount of 2 µl was used for PCR reaction. To identify representative strains, polymerase chain reaction (PCR) was preformed with the primer pair: EF1 (5′ATGGGTAAGGA(A/G)GACAAGAC-3′) and EF2 (5′GGA(G/A)GTACCAGT(G/C)ATCATGTT -3′)

(Geiser et al., 2004). The PCR mixture with a total volume of 25 µl consisted of 2x Eppendorf Master Mix (Taq DNA polymerase 1.25 U, 30 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2; 0.1% Igepal-CA630; 0.2 mM dNTP); 0.6 µM of each primer, and 2 µl of fungal DNA. Referent strains of *Alternaria alternata* (DSMZ-62006) were used as negative control and *F. oxysporum* Ft23 as positive control. Cycling conditions were: initial denaturation step at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 2 min. Final DNA extension step was at 72°C for 5 min.

Amplification products were separated by electrophoresis in 2% (w/v) agarose gel containing ethidium bromide (0.5 g/ml) using 1×TBE buffer. Visualization was performed in UV transilluminator and the images were captured with DOC PRINT system (Vilber Lourmat, USA). The appearance of fragments with sized 700 bp indicated that detected isolate belong to *Fusarium oxysporum*. Next step was sequencing and comparing in order to perform final identification.

RESULTS

Symptoms

Most typical symptoms of the disease appeared on tomato plants in field production (Figure 1). The symptoms appeared on older plants during mid-growing season under warm weather conditions. One of the typical signs of the disease was leaf chlorosis. The diseased leaves wilted and dried up. In many cases one side of the plant was affected first. Infection usually occurred on plants in the form of chlorosis, leaf wilting and browning of the vascular system (Figure 2). A cross-section of the stem revealed necrosis of the vessels.

Symptoms of fusarium rot on fruits in the storage and warehouses appeared as white mycelium, usually around the stem, typical for *Fusarium* sp. At the intersection of the fruit mycelium on surrounding tissue was observed (Figure 3). Presence of *Fusarium* sp. was confirmed by microscopic examination of the formed macroconidia. After incubation on PDA, 12 isolates from diseased plants grown in field, designated as TFW1-TFW12 and seven isolates from diseased tomato fruits (TFM1-TFM7) were chosen for further estimation (Table 1).

Table 1. List of isolates from tomato plants and fruits

Isolates	Isolation	Locality
TFW1	tomato xylem	Srbobran
TFW2	tomato xylem	Tovariševo
TFW3	tomato xylem	Obrovac
TFW4	tomato xylem	Gložani
TFW5	tomato xylem	Bačka Topola
TFW6-TFW9	tomato xylem	Senta
TFW10-TFW12	tomato xylem	Subotica
TFM1-TFM3	tomato fruit	Begeč
TFM4-6	tomato fruit	Begeč
TFM7	tomato fruit	Begeč

Figure 1. Symptoms of fusarium wilt on tomato plants in open field

Figure 2. Browning of the vascular system on stem

Figure 3. Tomato fusarium rot symptoms

Growth characteristics

On potato-dextrose medium fungus isolated from stem of diseased tomato plants formed a hyaline, branching mycelium that was white to gray. On Czapek agar isolates formed colonies and mycelium appeared as aerial, grey to light purple in color depending on the isolate (Figure 4 and 5). All observed isolates formed macroconidia as eliptical, gradually pointed or curved edges (pointed end). Macroconidia varied in size from 30-60 μm to 3-5 μm. Most often they were short and had three septa. They formed a large number of unicellular, elyptical, oval-shaped or kidney-shaped microconidia clustered into so-called false heads. All these characteristics pointed to the isolates being of the species *F. oxysporum*.

On PDA medium isolates from diseased fruits formed light pink aerial mycelium and red pigment in the agar. Macroconidia were formed, while microconidia or chlamidispores were not. Macroconidia were hyaline, falcate, 3-7 septate (mostly 5), 30-60 x 2-3 μm in size.

Figure 4. Colonies of *Fusarium* sp. on PDA

Figure 5. Colonies of *Fusarium* sp. on Czapek agar

Patogenicity test

Our results confirmed pathogenicity of isolates TFW1-TFW12 after 14 days. Artificially infected soil had no significant influence on seed germination, but it caused plant wilting after emergence. However, TFM1-TFM7 isolates did not have significant influence on emergence or growing. No significant differences were found related to the uninfected control. Only isolate TFM7 caused root rot similar to "dumping-off" symptoms.

Molecular identification method using PCR

The presence of *F. oxysporum* in the nine isolates, isolated from diseased plant (TFW1-TFW9) was confirmed by comparing the amplified DNA fragments

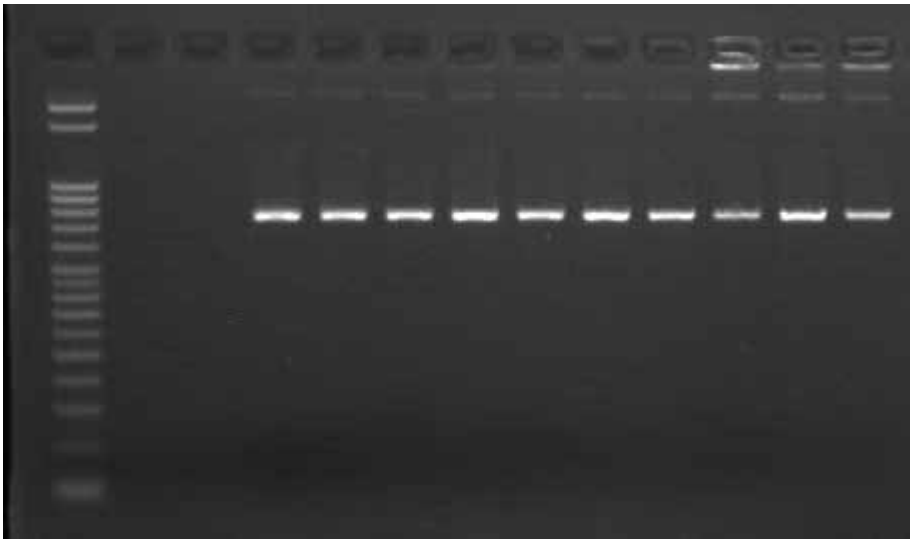

Figure 6. Products of amplification of specific DNA fragment of 700 bp: (M) PCR marker (Step Ladder, 50-3000 bp); (B) blank; (–) negative control DSMZ-62006 (*A. alternata*); (+) positive control strain Ft23 (*F. oxysporum*); Investigated samples: TFW1-TFW9

with the marker and positive control (Figure 6). The analyzed samples were about 700 bp in size. The procedure confirmed that we were dealing with the fungus *F. oxysporum*. Isolates originating from tomato fruit (TFM1-TFM7) were not detected by this method.

DISCUSSION

Fusarium wilt of tomato was observed in a number of tomato-growing areas in Serbia. Previous research on fusarium wilt of tomato in Serbia indicates that this disease occurs in the country frequently but on a smaller scale and that it does not cause any major damage (Balaž et al., 2009; Djordjević et al., 2011a). One of the major causes of poor quality and fruit loss (during storage and transport) are diseases caused by phytopathogenic fungi (Grahovac et al., 2011). Since that pathogen is soil-borne, it can live in the soil for long periods of time, and therefore rotational cropping is not a useful control method. It can also spread through infected dead plant material making cleaning up at the end of the season important. Therefore, control method for *F. oxysporum* could be planting of resistant varieties.

The isolated fungus can be identified on the basis of its morphological characteristics, which is the most difficult step in the process of identification (Rahjoo et al., 2008). In the present study, following the growth characteristics on PDA and Czapek, the fungus formed hyaline, branching mycelium that was white, gray to light pink in color. All tomato fusarium isolates rendered colonies with conidia and mycelia with morphological characteristics typical for *F. oxysporum* (Burgess et al., 1994). The aerial mycelium appears white and may subsequently change in color ranging from gray to violet and dark purple depending on the strain (or special form) of *F. oxysporum* (Smith et al., 1988).

Presence of fusarium species on tomato fruits can cause great damages and economic losses. Also, it can cause human health problems because of production of mycotoxins. Saccardo first described *Fusarium* spp. on tomato. Fungus that was isolated from tomato fruits, originated from northern Italy and was named *Fusarium oxysporum* (Schl.) f. sp. *lycopersici* Sacc. (Walker, 1971).

The concept of integrated pest management (IPM) i.e. sustainable approach in control of causal agents of postharvest fruit rot involves implementation of cultural, physical, biological and chemical measures, to minimize economic, health and risks to consumers and environment (Grahovac et al., 2011). Tanović et al. (2004) investigated essential oils (mint, basil, rosemary, thyme, tea tree) which could be used against soil borne pathogens including *Fusarium oxysporum* f. sp. *lycopersici*. Decay

occurrence in warehouse on tomato fruit could be prevented by earlier harvest. According to Djordjević et al. (2011a), essential oils of *C. carvi* and *G. robertiani* showed high inhibition of mycelial growth of FW *in vitro*, and therefore high potential for implementation *in vivo*.

The development of biomolecular method (Polymerase Chain Reaction, or PCR) made precise and reliable identification of causal organisms possible. The PCR assay used here is suitable for detection of *Fusarium oxysporum*. Using the primers, a specific band at 700 bp was obtained by PCR for nine isolates. Edel et al. (1995) used a number of molecular techniques (RFLP, ERIC-PCR, REP-PCR) in the identification of *F. oxysporum*. They determined that the ERIC and REP PCR techniques were rapid and reliable in identifying *F. oxysporum* isolates, while RFLP analysis served to obtain data on genetic variability within the species itself. Today, there is abundance of data in the literature concerning the use of chain amplification of nucleic acid fragments in the detection of phytopathogenic microorganisms, and a large number of publications that can be found on this subject are the evidence of the topicality of these techniques. In addition to improved pathogen detection, advancements have also been made in taxonomic studies, where relationships among taxonomic categories in particular microorganisms have been better defined (Wallace and Covert, 2000). Since that heterogenicity of the population of pathogens of tomato as well as their presence and harm, especially in the production of tomato in the Serbia, determination of fungal populations has multiple significances from the point of care, breeding and creation of resistant lines and varieties.

CONCLUSIONS

Based on morphological characteristics of fungal isolates, we confirmed presence of *Fusarium* sp. on wilting tomato plants and stored tomato fruits. Based on the results, we conclude that the causal agent of fusarium wilt is *Fusarium oxysporum* f. sp. *lycopersici*. This conclusion was confirmed by polymerase chain reaction technique and because the isolates came from tomatoes, we concluded that they belonged to the specialized form f. sp. *lycopersici*.

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Identifikacija prouzrokovača uvenuća i truleži plodova paradajza

REZIME

Patogene gljive roda *Fusarium*, prouzrokovači uvenuća biljaka, pojavljuju se i kao paraziti povrća uzrokujući propadanje korena, prizemnog dela stabla, kao i uvenuće biljaka. Veći broj patogena parazitira paradajz, među kojima je i *Fusarium oxysporum* f. sp. *Lycopersici*, prouzrokovač fuzarioznog uvenuća. Fuzariozna trulež plodova paradajza redovno se javlja u skladištima i može naneti velike ekonomske gubitke. Cilj ovog rada bio je izolacija, utvrđivanje i identifikacija prouzrokovača uvenuća paradajza i truleži plodova, na osnovu morfoloških i molekularnih karakteristika patogena. Prikupljeni su uzorci obolelih biljaka sa simptomima uvenuća paradajza iz proizvodnih regiona Vojvodine. Sveži plodovi sa simptomima fuzariozne truleži prikupljeni su iz različitih magacina i skladišta. Za izolaciju i morfološku determinaciju gljive korišćene su PDA i Czapek hranljiva podloga. Za dalja proučavanja odabrano je 12 izolata (TFW1-TFW12) poreklom sa biljaka gajenih u polju i sedam izolata (TFM1-TFM7) dobijenih sa zaraženih plodova iz skladišta. Determinacija izolata gljiva obavljena je i pomoću metode, lančane reakcije polimeraze (PCR – Polymerase Chain Reaction). Za molekularnu identifikaciju izolata *F. oxysporum* korišćen je par prajmera EF1/EF2. U devet ispitivanih uzoraka potvrđeno je prisustvo DNK fragmenta veličine 700 bp.

Ključne reči: Uvenuće; paradajz; hraniva; izolacija; Fusarium; voće