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Original scientific article

THE MORFOLOGICAL AND MOLECULAR IDENTIFICATION OF *Fusarium verticillioides* CAUSING FUSARIOSIS ON WHEAT GRAIN

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During the 2014-2017 period, wheat samples were collected from discoloured spikes affected by Fusarium head blight (FHB) from 20 locations in Serbia. After isolation, fungi were cultivated on potato dextrose agar (PDA) at 25ºC for 7 days. Based on the in situ identification on PDA, 36 isolates of the section Liseola were selected for further analyses. The pathogenicity of all isolates was confirmed on wheat leaves. The virulence of isolates was determined by measuring the lengths of spots formed at the inoculation leaf site. In order to prove the presence of the species *Fusarium verticillioides*, a pair of primers FV-F2/FV-R was used. This pair of primers amplifies the sequence of the *gaoB* gene, and it proved to be specific for the stated species. Moreover, for the same purpose, a pair of primers VER1-VER2 based on the calmodulin partial gene was used. The reference isolate RBG 1603 Q27 was used as a positive control. The pair of primers VER1-VER2 produced a band of the expected size - 578 bp in 18 isolates, while using FV-F2/FV-R, a 370bp long band confirmed the presence of *F. verticillioides* in 16 samples. Sixteen out of 18 isolates verified with VER1-VER2 were also identified as *F. verticillioides* with FV-FS/FV-R. No amplification was observed in a negative control.
Keyword: fungi, *Fusarium verticillioides*, identification, pathogenicity, primers, wheat

INTRODUCTION

Wheat is one of the most important cultivated plant species and can be easily grown in different climatic regions. Nowadays, wheat is grown on 218 million hectares worldwide, which is the greater land area than any other commercial crop is grown on, and continues to be the most

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important source of food grain for humans. Global wheat production currently amounts to 774 million tons (FAO, 2021). Europe, China, India, Russia and the USA are the main centres of wheat production (STATISTA, 2021). Wheat has a predominant role in the grain trade and is utilised as food (67%), feed (20%), seed (7%), and industrial products (6%) (GRUNDAS, 2003). In Serbia, wheat is grown on about 580,000 hectares with the average yield of 4.389 t/ha, and the total annual production of approximately 2.5 million tons (FAO, 2019).

Fusarium, *Aspergillus*, *Penicillium* and *Alternaria* species are the most common fungal causal agents of decay of wheat grains in the field (ALKADRI *et al*., 2014). *Fusarium* is one of the most economically important genera of phytopathogenic fungi. Several *Fusarium* species can infect small grain cereals such as wheat, barley and oat causing Fusarium head blight (FHB) (LOGRIECO *et al*., 2002; VAN DER LEE *et al*., 2015). Secondary metabolites and mycotoxins synthesised by the species of the genus *Fusarium* lead to reduced grain quality, and thus to significant economic losses (PARRY *et al*., 1995; CHAMPEIL *et al*., 2004; WEGULO *et al*., 2015).

The predominant species can vary over crop species involved, geographic regions and environmental conditions. In Europe, the species mostly found associated with head blight of wheat are *F. graminearum* Schwabe, *F. culmorum* (W.G. Smith) Sacc., and *F. avenaceum* (Fr.) Sacc. Furthermore, *F. poae* (Peck) Wollen., *F. equiseti* (Corda) Sacc. and *F. sporotrichioides* Sherb. are less frequently isolated species. Species of the section Liseola such as *F. verticillioides* (Sacc.) Nirenberg, and *F. proliferatum* (Mats.) are sporadically isolated. (BOTTALICO and PERRONE, 2002; WAGACHA and MUTHOMI, 2007; VOGELGSANG *et al*., 2008).

F. verticillioides is distributed worldwide and has a broad host range. The primary host of this species is maize, on which it causes seedling decay, stalk rot and ear rot (LESLIE and SUMMERELL, 2006). Besides maize, it is also found on wheat, on which it causes FHB (STANKOVIĆ *et al*., 2007; TANČIĆ, 2009), then on rice, on which it causes bakanae disease and on sorghum (BURGESS *et al*., 1994; BLACUTT *et al*., 2018). In Serbia, the presence of this species has been recorded on maize (LEVIĆ and STOJKOV, 2002), hop (STANKOVIĆ *et al*., 2008) and wheat (STANKOVIĆ *et al*., 2007).

This species is particularly important due to the synthesis of secondary metabolites of fumonisins, which cause severe animal diseases and are implicated in human diseases (LOGRIECO *et al*., 2002; BLACUTT *at al*., 2018). In Serbia, as well as in the world, fumonisins were isolated for the first time in wheat spikes in 2009 (STANKOVIĆ *et al*., 2011).

Since symptoms of discoloured spikes, which are a sign of fusariosis, were recorded on grains of winter wheat in Serbia, the aim of this study was to identify a causal agent of wheat decay based on morphological and molecular characteristics in order to confirm its presence.

MATERIALS AND METHODS

The research within this study encompassed a survey of wheat crops during the four-year period (2014-2017). Plants with symptoms of FHB were used as a source material for isolation of pathogens. The samples were collected in the following 20 locations in Serbia: Apatin, Batajnica, Bela Crkva, Dužine, Jaša Tomić, Kikinda, Kraljevo, Martinci, Mionica, Omoljica, Padinska Skela, Panonija, Sombor, Srbobran, Stara Pazova, Svilajnac, Zemun, Zemun Polje, Žabare and Vrbovsko.

The collected samples of kernels were sterilised with a bleach and water solution (1:3) and then fungi were incubated on PDA, at 25 ºC in thermostat for 7 days. Based on the locations analysis, 36 isolates identified as species of the section Liseola were purified to the monospore culture and used in further analyses.

Test of pathogenicity and aggressiveness of isolates in vitro

In situ detached wheat leaf assay was used to investigate the potential pathogenicity and agressiveness of selected Liseola section isolates (IMATHIU *et al*., 2009). Kernels of wheat variety Aurelia were surface sterilised, sown in pots (27cm x 15cm) and grown in the germinator at 20°C and photoperiod of 12h. The first wheat leaf was excised, sterilised with 70% ethanol and placed into a Petri dish on a filter paper, previously moistened with 2mL of sterile distilled water. Leaves were placed in Petri dishes (8 leaves per a dish) in four replicates. For inoculation purposes, isolates were cultivated on PDA at 25°C in the thermostat for 7 days. The suspension was made by scraping off mycelia and adding sterile distilled water up to the concentration of $1x10^6$ spores/mL. An injury was made with the tip of the pipette in the centre of the leaf, on which 10µl of inoculum was later applied.

Leaves inoculated with the fungal spore suspension were used as a positive control, while leaves inoculated with sterile distilled water were used as a negative control. Incubation at 25°C and photoperiod of 12h lasted 7 days, after which the spot length was measured. The pathogen was reisolated in order to confirm Koch's postulates.

Morphological identification

Isolates were subcultured on three nutritive media - potato dextrose agar (PDA), synthetic nutrition agar (SNA) and carnation leaf agar (CLA). Macroscopic characteristics, such as a colour, a mycelium appearance and an average colony diameter (after 7 days), were observed on PDA. Isolates were cultured at 25°C in the thermostat for 7 days. Microscopic characteristics, especially the width and the length of macroconidia, and presence of monophyalides, were examined on SNA and CLA. Isolates were cultured at 25°C and a 12h light/dark regime. PDA and CLA were prepared according to BURGESS *et al.* (1994), while SNA was prepared according to NIRENBERG (1976).

Molecular identification and characterisation

Total genomic DNA was extracted from the 7-day-old mycelia using DNeasy Plant Mini Kit (250), QIAGEN, Hilden, Germany). The DNA concentration was determined using Eppendorf Bio Photometer D30. The molecular identification of *Fusarium verticillioides* was done using primers already verified as species specific in previous studies (MULE *et al*., 2004; FARIA *et al*., 2012). MULE *et al*. (2004) designed primers VER1 (5′-CTTCCTGCGATGTTTCTCC-3’) and VER2 (5′-AATTGGCCATTGGTATTATATATCTA-3’) for several *Fusarium* species including *F. verticillioides* based on the sequence of *calmodulin* gene. Moreover, FARIA *et al*., (2012) showed that the primer pairs FV-F2 (5′-CACTGGTGGTAACGATGCG-3’) and FV-R (5′-CACCCTGAGTGCCCTTGGTG-3’) designed targeting the *gaoB* gene sequence amplified a DNA fragment only from the *F. verticillioides* genomic DNA. The RBG 1603 Q27 was used as a reference isolates. The PCR reaction for the primer pair VER1-VER2 was conducted in 50µl

volume mixture containing: 1xbuffer, 15pmol primers, 2.5M each dNTP, 1.25U DreamTaq Green DNA Polymerase, ThermoFisher Scientific, USA and 2 µl (~10ng) of template DNA. PCR conditions were as follows: denaturation at 95°C for 5 min; 35 cycles of denaturation at 94°C for 50 s, annealing at 56°C for 50 s, extension at 72°C for 1 min; final extension at 72°C for 7 min.

The reaction mixture for PCR with the primer pair FV-F2/FV-R was prepared in a final volume of 50µl and consisted of: 1xbuffer, 25 pmol each primer, 2.5M each dNTP, 1.25U DreamTaq Green DNA Polymerase, ThermoFisher Scientific, USA and 2 µl (~10ng) of template DNA. The thermal cycling program was performed with 25 cycles of 1.5 min at 94°C, 1.5 min at the recommended annealing temperature 64°C and 2 min at 72°C. For initial denaturation the samples were heated at 94°C for 5 min, and after the cycles, the samples were incubated at 72°C for 10 min for final extension. As a negative control, a reaction mixture was used in which RNase- free water was added instead of the DNA sample.

All PCR reactions were performed in Biometra Thermal Cycler, Germany and products were verified on a 2% agarose gel stained in ethidium bromide solution (0.25 µg/mL). The size of the bands was estimated according to GeneRuler 100bp DNA Ladder, ThermoFisher Scientific, USA. Gels were photographed under the UV light using a BioDocAnalyze Live gel documentation system, Biometra, Germany.

RESULTS AND DISCUSSION

On PDA, the tested isolates formed colonies with cotton aerial mycelium, powdery appearance due to the presence of a mass of microconidia, and white to grayish-purple color. The pigment in the agar was dark yellow to purple-grey (Figure 1). The average colony diameter on PDA measured after the 7-day growth at 25ºC in thermostat was 71 mm. Macroconidia were observed on CLA and SNA. They were three septate with the average width and length of 4.85µm and 15.44 µm, respectively. Sixteen out of 36 isolates formed long chains and monophialides, which is a distinctive feature of *Fusarium verticillioides.* These results are in accordance with results gained by LESLIE and SUMMERELL (2006).

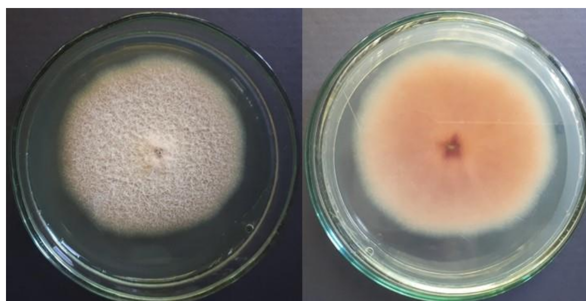

Figure 1. Colony appearance of the isolate MRI 10 (a-colony surface; b-colony reverse) after 7-day incubation at 25°C in the dark on PDA

All tested isolates expressed pathogenicy on wheat leaves. The symptoms are manifested such as necrotic or chlorotic areas on the affected plant part. The virulence of isolates was determined by measuring the length of formed spot at the inoculation site. The average spot length was 2.91 mm. The shortest (2.17 mm), i.e. the longest (4.33 mm) spot length was measured in isolates MRI 1, i.e. MRI 7, respectively (Figure 2).

Figure 2. Isolate MRI 7: occurrence of brown spots on leaves of wheat variety Aurelia, 7 days after artificial inoculation

Correspondingly to our results, WANG *et al.* (2014) determined pathogenicity of *F. temperatum* and *F. subglutinans* on wheat. The rates of spread on wheat spikes differed (1.41-2.38). IMATHIU *et al.* (2009) confirmed successful infection and disease development by all examined *F. langsethiae* isolates. According to PFORDT *et al.* (2020) all observed isolates of *F. temperatum* and *F. subglutinans* affected winter wheat and caused moderate to severe FHB symptoms. OPOKU *et al.* (2011) were assessing the aggressiveness of 20 different *F. langsethiae* isolates on wheat leaves by measuring the lesion length formerd at the inoculation site. All 20 isolates used for the experiment caused visible lesions. The length of lesion ranged from 3.00 to 7.33 mm.

A total of 36 isolates of *Fusarium* species were tested using two species-specific primers for the presence of *Fusarium verticillioides*. The primers were constructed to detect this fungal species in maize kernels and in pure cultures of fungal isolates. This study validated their applicability in the detection of *F. verticillioides* in other crops (wheat, barley). Results of amplification are shown in Figures 3 and 4. The primer pair VER1-VER2 (MULÉ *et al.*, 2004) produced a band of expected size − 578 bp in 18 isolates, while using FV-F2/FV-R (FARIA *et al.*, 2012), 370 bp long band confirmed the presence of *F. verticillioides* in 16 samples. Sixteen out of 18 isolates verified with VER1-VER2 were also identified as *F. verticillioides* with FV-FS/FV-R.

Figure 3. PCR reaction products using VER1 / VER2 pair of primers (MULE *et al*., 2004). Expected fragment size: 578bp. 1-36 number of samples, M - DNA size marker (100bp), samples: 1, 4, 5, 7, 10, 13, 17, 18, 23, 25, 26, 27, 28, 29, 30, 31, 34, 35 – *Fusarium verticillioides*, K – blank control, R – reference isolate

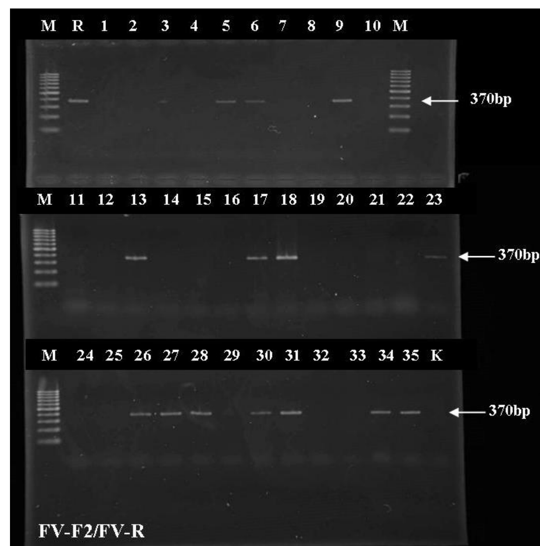

Figure 4. PCR reaction products using FV-F2 / FV-R pair of primers (FARIA *et al*., 2012). Expected fragment size: 370bp. 1-36 number of samples, M - DNA size marker (100bp), samples 1, 4, 5, 7, 10, 13, 17, 18, 23, 26, 27, 28, 30, 31, 34, 35 – *Fusarium verticillioides*, K – blank control, R – reference isolate

Based on morphological, molecular and pathogenic properties, 16 isolates were identified as *F. verticillioides*. The species *F. verticillioides* isolated from wheat grain in Serbia, for the first time was proven by molecular methods. PCR is a frequently used method for precise and prompt identification of *Fusarium* species. PCR may be a substitute or a complement to the identification based on morphological traits (DEMEKE *et al*., 2005; SPANIC *et al*., 2010; FARIA *et al*., 2012; SUGA *et al*., 2013).

CONCLUSION

The swift, accurate and precise identification of *Fusarium* spp. is important for forecasting the mycotoxigenic risk of this pathogen. *F. verticillioides* is a significant agent that causes decay, not only maize, in wheat but in other cereals too. Since early identification of this pathogen may prevent the synthesis of mycotoxins, the risk of intoxication in humans and animals can be reduced. A further apprehending of the distribution and mechanisms of invasion may lead to the establishment of efficient strategies to control this important pathogen.

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MORFOLOŠKA I MOLEKUALRNA IDENTIFIKACIJA *Fusarium verticillioides* PROUZROKOVAČA FUZARIOZE NA ZRNU PŠENICE

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Izvod

Tokom perioda od 2014. do 2017. godine sakupljani su uzorci pšenice sa fuzarioznih klasova sa 20 lokacija u Srbiji. Nakon izolacije, gljive su gajene na KDA podlozi 7 dana u termostatu na temperaturi od 25°C. Na osnovu identifikacije *in situ* na KDA (krompir dekstrozni agar), 36 izolata sekcije Liseola odabrano je za dalje analize. Potvrđena je patogenost svih izolata na listovima pšenice. Virulentnost izolata utvrđena je merenjem dužina formirane pege na mestu inokulacije. Za dokazivanje prisustva vrste *Fusarium verticillioides* korišćen je par prajmera FV-F2/FV-R koji umnožava sekvence *gaoB* gena i koji se pokazao kao specifičan za navedenu vrstu. Takođe, sa istim ciljem korišćen je par prajmera VER1-VER2 dizajniran na osnovu dela genske sekvence za kalmodulin. Kao pozitivna kontrola korišćen je referentni izolat RBG 1603 Q27. Par prajmera VER1-VER2 obrazovao je traku očekivane veličine (578bp) kod 18 izolata, dok je pomoću para prajmera FV-F2/FV-R traka dužine 370bp potvrdila prisustvo *F. verticillioides* kod 16 izolata. Šesnaest od 18 izolata koji su identifikovani VER1-VER2 parom, takođe su identifikovani i FV-FS/FV-R prajmerima. U negativnoj kontroli nije bilo amplifikacije.

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