doi: 10.17221/66/2016-PPS

First Confirmed Report on *Fusarium sporotrichioides* on *Pinus ponderosa* var. *jeffreyi* in Slovakia

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Abstract

Ivanová H., Hrehová Ľ., Pristaš P. (2016): **First confirmed report on** *Fusarium sporotrichioides* **on** *Pinus ponderosa* var. *jeffreyi* **in Slovakia**. Plant Protect. Sci., 52: 250–253.

During 2014–2015, samples from ten 20–25 years old pine (*Pinus ponderosa* var. *jeffreyi*) trees showing wilt symptoms were collected from the Arborétum Mlyňany park. A disease was observed on 20% of the trees. The first symptoms are wilting, stunting, chlorosis, and discolouration of needles, which turned yellow on affected twigs, then red and finally they fell off. Isolations of the pathogen were done from the discoloured tissues of needles (twenty samples from each tree) on Potato Dextrose Agar. Colonies of the fungus (3–4 Petri dishes from each tree) were initially aerial, white or slightly violet, but with age they became red and red pigments were produced in agar. The observed micromorphological characteristics of the fungus, such as presence of simple and proliferating conidiophores with polyphialides, microconidia, macroconidial shape, and chlamydospore presence matched the description of *Fusarium sporotrichioides*. The identity of the fungus was confirmed by phylogenetic analysis of internal transcribed spacer (ITS) sequences. Sequence comparisons placed the fungus to the species *F. sporotrichioides* with similarity of 99.6% at the ITS sequence level.

Keywords: old pine; morphological characteristics; phylogenetic analysis; fungal plant pathogen

Fusarium sporotrichioides Sherb. is a cosmopolitan, ecologically widespread fungal plant pathogen, being found across tropical and temperate regions (Domsch *et al*. 1993). The pathogen is responsible for damage to seedlings causing root rot or cankers (Anderson 1986), and needle dieback on mature trees (Karadžić & Milijašević 2008). This fungus is of notable agricultural and economic importance – it is a significant producer of mycotoxins, particularly trichothecenes, which are known to inhibit protein synthesis in eukaryotes (Leslie & Summerell 2006; Cormick *et al*. 2011).

The aim of this study was to isolate and identify the fungal species associated with the diseased pine species *Pinus ponderosa* Douglas ex C. Lawson, var. *jeffreyi* Balf. ex Vasey, during 2014–2015 and to evaluate the relative tolerance of this species grown in many parks in Slovakia. This study represents the first confirmed report on *F. sporotrichioides* in Slovakia, which is considered as one of the most serious pathogens of *P. ponderosa* var. *jeffreyi* needles.

MATERIAL AND METHODS

The *Fusarium* isolates used in this study were obtained from ten 20–25-years old *P. ponderosa* var. *jeffreyi* trees in the geographic location Arborétum Mlyňany SAS (altitude 200 m a.s.l., cool temperate climate, average daily temperature of 10.6°C, average annual atmospheric precipitation of 541 mm). For the isolation of fungi, diseased tissues of needles (twenty samples from each tree) were surface-sterilised (15 min in a commercial

Supported by by the Scientific Grant Agency of the Ministry of Education of the Slovak Republic and the Slovak Academy of Sciences – VEGA, Grants No. 2/0071/14 and No. 2/0069/14.

doi: 10.17221/66/2016-PPS

solution containing 1.5% sodium hypochlorite) and then rinsed well with sterile distilled water. Small pieces of tissue, cut from the edges of lesions, were placed directly onto PDA medium (Merck, Darmstadt, Germany), and incubated at 23°C for 7 days in a versatile environmental test chamber MLR-351H–Sanyo (Sanyo, Osaka, Japan). Fungi resembling those in the genus *Fusarium* were transferred to Petri dishes (PD) containing PDA (or Malt extract agar – MA, Agar-agar, Kobe I – AA; Carl Roth GmbH + Co KG, Karlsruhe, Germany). The study of fungal structures was performed with a BX41 clinical light microscope (Olympus, Tokyo, Japan) under 400× and 1000× magnifications. Measurements were done through the medium of QuickPhotomicro 2.2 programme and the morphometric values were compared with previously published data for the taxa (Leslie & Summerell 2006). Fungi were identified to the level of the genus using the taxonomic guide of Barnett and Hunter (1972). Selected isolates of *Fusarium* were identified to the species level using monographs of Samson *et al*. (1981) and Gerlach and Nirenberg (1982).

The studied fungus was identified using the phylogenetic analysis of internal transcribed spacer (ITS) sequences. Total DNA was isolated from a fresh mycelium (2 PD) using microwave treatment and subsequent Triton X-100 lysis (Goodwin & Lee 1993). The isolated DNA (10 ng) was used as a substrate in PCR reaction with fungal universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') for amplification of the ITS1-5.8S-ITS2 ribosomal DNA regions (White *et al*. 1990). PCR amplifications were performed in a Bio-Rad MJ Mini Personal Thermal Cycler (Bio-Rad, Santa Clara, USA) under conditions specified elsewhere (Ivanová *et al*. 2016). Amplified products were analysed by electrophoresis on 1% agarose gels in TAE buffer (Sambrook *et al*. 1989). The amplified product was purified from agarose gel using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA) and sequenced by the dideoxytermination method with each primer used in the PCR reaction at GATC-Biotech (Konstanz, Germany). DNA sequences were assembled using DNA Baser software (Heracle BioSoft SRL, Arges, Mioveni, Romania) and submitted to the GenBank database under accession No. KU948933. DNA sequences were compared against the GenBank database using the BLASTn algorithm (Altschul *et al.* 1990). For the analysis of phylogenetic relatedness ITS sequences of related *Fusarium* spp. were downloaded from the GenBank database, aligned using the MUSCLE algorithm (Edgar 2004) and phylogenetic relationships were constructed using the Neighbour-Joining method available in the MEGA software Version 6 (Tamura *et al*. 2013). Phylogenetic robustness of observed trees was tested by bootstrap analysis after 1000 replications.

Dried samples of infected needles of *P. ponderosa* var. *jeffreyi* are deposited at the herbarium, specimen R 5377, a pure culture of the fungus *F. sporotrichioides* (leg. K. Pastirčáková, det. H. Ivanová, P. Pristaš, Arborétum Mlyňany, 2015) can be obtained from the culture collection of the Institute of Forest Ecology of the SAS, Branch for Woody Plant Biology in Nitra.

RESULTS AND DISCUSSION

In our study we confirm the first report on *F. sporotrichioides* in Slovakia, as one of the most serious pathogens of *P. ponderosa* var. *jeffreyi* needles. It is a fast-growing fungus, usually able to grow up to 8–9 cm in diameter within four days. Optimal growth temperature ranges from 22.5°C to 27.5°C and the minimum humidity level required for vegetative growth is 88% (Domsch & Gams 1970; Gagkaeva 2008). Colonies of the fungus (3–4 PD from each tree) were initially aerial, white or slightly violet, later pinkish to brownish red. Growth of the mycelium, which is usually able to grow up to 6–7 cm in diameter within 5 days after inoculation, depends on the used medium (Figure 1). This fast-growing fungal mycelium obtained from *Pinus* needles was identified as *F. sporotrichioides* based on phylogeny, cultural and morphological features. The presence of typical abundant macroconidia with 1–3 septa with dimensions of 23(29)37 × 4–5(6) µm and with 4 septa of *Fusarium* with dimensions of 40-43 × 4–5 µm

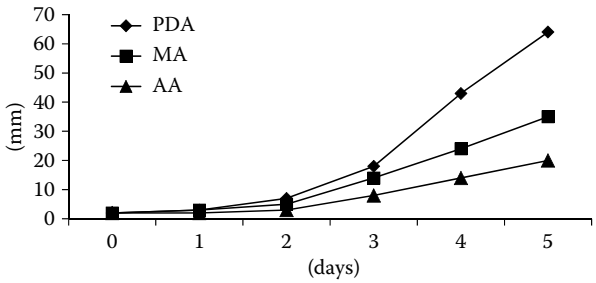

Figure 1. Comparison of growth rates of mycelium *F. sporotrichioides* on different medium

PDA – Potato dextrose agar, MA – Malt extract agar, AA – Agar-agar, Kobe I

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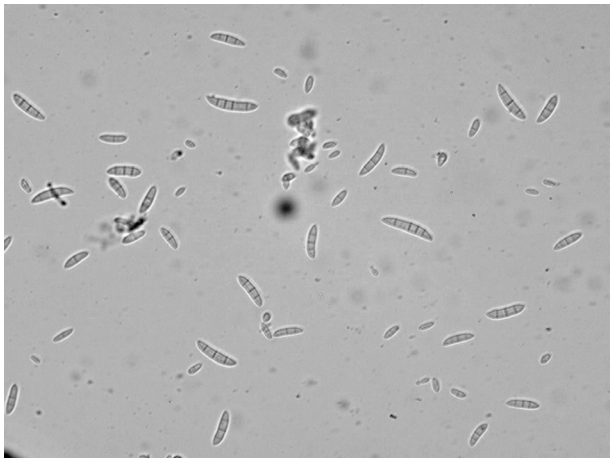

Figure 2. Presence of typical abundant macroconidia with 1–3 and 4 septa and 1-celled ovoid, oval to pyriform, spindle-shaped or pip-shaped microconidia of *Fusarium sporotrichioides* on damaged needles of *Pinus ponderosa* var. *jeffreyi*

was observed on damaged needles. Macroconidia formed on hyphae and on sporodochia were falcate with apical curved, pointed cell and basal notched or foot-shaped cell. One-celled ovoid, oval to pyriform, spindle-shaped or pip-shaped microconidia with dimensions of 5–8(15) × 2–3(6) µm were also observed among macroconidia (Figure 2). They were abundantly produced from polyphialides on tree-like conidiophores in the aerial mycelium. The third spores – chlamydospores were thick-walled with 7–15 µm in diameter, globose, brown, often produced in chains or clumps. Our results are comparable with data acquired by Wollenweber (1931) in research on *Fusarium* through a systematic life-long study of a wide range of cultures from different climatic regions. The author observed the fungus *F. sporotrichioides*, in which the dimensions of 1-septum macroconidia were 12 × 3.6 (9–20 × 2.5–5.0) µm, 3-septum conidia 30 × 5 (18–29 × 3–5.5 µm), and 4–5-septum conidia 36 × 5.4 (30–40 × 4–6) µm. According to Refai *et al.* (2015) colonies of *F. sporotrichioides* produce the profuse white to pale red mycelium. Macroconidia are abundant in orange sporodochia, falcate to lunate, 3–5 septate, apical cell curved and tapering, basal cell poorly developed. Microconidia produced from mono- or polyphialides are pyriform and 0–1 septate. Chlamydospores are abundant. According to Ellis and Ellis (1997) macroconidia in this culture are slightly curved, falcate with 3–5 septa. Apical cell is curved and pointed, basal cell is notched or foot-shaped. The fungus is compared with taxonomically related species, which show the presence of polyphialides that produce pyriform as well as fusiform microconidia and blastospores (Chelkowski *et al*. 1989). An important feature for their distinction from other fusaria is numerous brown and globose chlamydospores, which are thick-walled, filled with lipid-like material serving to carry the fungus over winter in soil when no suitable host is available. The chlamydospores may be borne singly, in pairs, in clumps, or in chains, and the outer wall may be smooth or rough (Samson *et al*. 1981).

Amplification and the sequence comparisons of the ITS region have become a common approach to the molecular identification of fungi (Nilsson *et al*. 2008). ITS sequence of the R5377 isolate was obtained and compared against GenBank database. The comparisons placed the isolate clearly to the *F. sporotrichioides* group. ITS sequence comparisons showed similarities of 546/548 nucleotides to *F. sporotrichioides*, 545/548 nucleotides to *F. armeniacum*, and 502/548 nucleotides to *F. circinatum* ITS sequences. Similar intraspecies ITS sequence similarities (99.3–99.8%) were observed e.g. for the isolates of *F. culmorum*

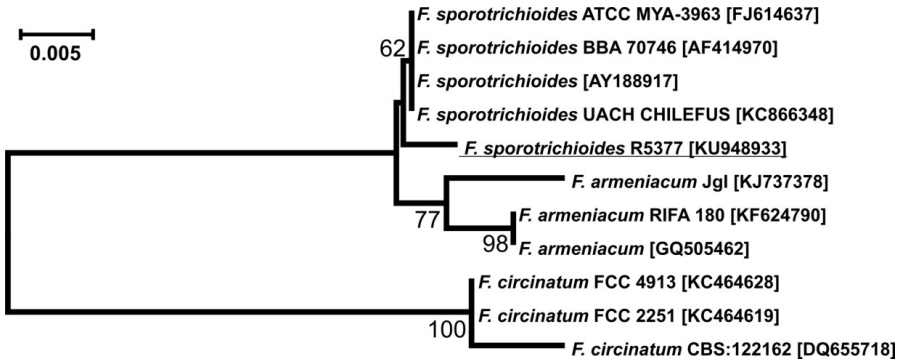

Figure 3. Phylogenetic relationship of selected *Fusarium* spp. internal transcribed spacer sequences. The tree was constructed using Neighbor Joining algorithm. The numbers at nodes are bootstrap values after 1000 repetitions. The sequence of R5377 isolate described in this study is underlined

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(Mishra *et al*. 2000). Multiple sequence alignment (Figure 3) confirmed that the R5377 isolate belongs to the species *F. sporotrichioides*.

During an investigation on the mycoflora of pine trees in park greenery we isolated on *Pinus ponderosa* var. *jeffreyi* the fungus with distinctive characteristics which was identified – on the basis of light-microscope morphological studies and by phylogenetic analysis – as a new recorded fungus *F. sporotrichioides* occurring in Slovakia.

References

Altschul S.F., Gish W., Miller W., Myers E.W., Lipman D.J. (1990): Basic local alignment search tool. Journal of Molecular Biology, 215: 403–410.

Anderson R.L. (1986): New method for assessing contamination of slash and loblolly pine seeds by *Fusarium moniliforme* var. *subglutinans*. Plant Disease, 70: 452–453.

Barnett H.L., Hunter B.B. (1972): Illustrated Genera of Imperfect Fungi. Minneapolis, Burgess Publ. Co.

Cormick S.P., Stanley A.M., Stover N.A., Alexander N.J. (2011): Trichothecenes: from simple to complex mycotoxins. Toxins, 3: 802–814.

Chelkowski J., Malgorzata M., Kwasna H., Viscont A., Golinski P. (1989): *Fusarium sporotrichioides* Sherb., *Fusarium tricinctum* (Corda) Sacco and *Fusarium poae* (Peck) Wollenw. – cultural characteristics, toxinogenicity and pathogenicity towards cereals. Journal of Phytopathology, 124: 155–161.

Domsch K.H., Gams W. (1970): Pilze aus Agraböden. Stuttgart, Gustav Fischer Verlag.

Domsch K.H., Gams W., Anderson T.H. (1993): Compendium of soil fungi. Vol. 1. Eching, IHW-Verlag.

Edgar R.C. (2004): MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research, 32: 1792–1797.

Ellis M.B., Ellis P.J. (1997): Microfungi on Land Plants: an Identification Handbook. Slough, Richmond Publishing.

Gagkaeva T. (2008): Introduction to *Fusarium* taxonomy. *Fusarium,* Genes and Toxin. Available at http://fou02.bioforsk.no/fusarium/Presentasjoner/Introduction%20to%20Fusarium%20taxonomy.pdf (accessed Jan 5, 2016).

Gerlach W., Nirenberg H.I. (1982): The Genus *Fusarium* – A Pictorial Atlas. Berlin-Dahlem, Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft.

Goodwin D.C., Lee S.B. (1993): Microwave miniprep of total genomic DNA from fungi, plants, protists and animals for PCR. BioTechniques, 15: 438, 441–442, 444.

Ivanová H., Pristaš P., Ondrušková E. (2016): Comparison of two *Coniochaeta* species (*C. ligniaria* and *C. malacotricha*) with a new pathogen of black pine needles – *Sordaria macrospora*. Plant Protection Science, 52: 18–25.

Karadžić D., Milijašević T. (2008): The most important parasitic and saprophytic fungi in Austrian pine and scots pine plantations in Serbia. Bulletin of the Faculty of Forestry, 97: 147–170.

Leslie J.F., Summerell B.A. (2006): The *Fusarium* Laboratory Manual. Ames, Blackwell Publishing Professional.

Mishra P.K., Fox R.T.V., Culham A. (2000): Application of nrDNA ITS sequence for identification of *Fusarium culmorum* isolates. EPPO Bulletin, 30: 493–498.

Nilsson R.H., Kristiansson E., Ryberg M., Hallenberg N., Larsson K.H. (2008): Intraspecific *ITS* variability in the Kingdom fungi as expressed in the international sequence databases and its implications for molecular species identification. Evolutionary Bioinformatics Online, 4: 193–201.

Refai M., Hassan A., Hamed M. (2015): Monograph on the Genus *Fusarium*. Available at https://scholar.cu.edu.eg/?q=hanem/files/monograph_on_the_genus_fusarium.pdf

Sambrook J., Fritsch E.F., Maniatis T. (1989): Molecular Cloning: A Laboratory Manual. 2nd Ed. New York, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Samson R.A., Hoekstra E.S., van Oorschot C.A.N. (1981): Introduction to Food-borne Fungi. Utrecht, Centraalbureau voor Schimmelcultures. Institute of the Royal Netherlands, Academy of Arts and Sciences.

Tamura K., Stecher G., Peterson D., Filipski A., Kumar S. (2013): MEGA 6: Molecular evolutionary analysis version 6.0. Molecular Biology and Evolution, 30: 2725–2729.

White T.J., Bruns T., Lee S., Taylor J. (1990): Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M., Gelfand D., Sninsky J., White T. (eds): PCR Protocols: a Guide to Methods and Applications. Orlando, Academic Press: 315–322..

Wollenweber H.W. (1931): *Fusarium* monograph. Parasitic and saprobic fungi. Zeitschrift für Parasitenkunde, 3: 269–516.

Received: 2016–04–25
Accepted after corrections: 2016–07–07
Published online: 2016–08–12

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