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Investigation of host-specificity of phytopathogenic fungi isolated from woody plants

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SUMMARY

Host-specificity is an important characteristic of fungal pathogens. Changing climate could create more appropriate environmental conditions for phytopathogens, thus formerly host-specify fungi could be able to colonize new hosts. Noxious plant pathogen fungi, which can infect several plant species are well-known worldwide. These genera may expand their range of hosts because of the appearance in new geographic areas due to climate change. This new exposure can result in serious problems in agriculture because of the lack of immunity. The susceptibility of apple tree was studied through testing pathogenicity in vitro with species isolated from walnut twigs and nuts, and identified by ITS sequences. Three of four tested species, Botryosphaeria dothidea, Diaporthe eres and Diplodia seriata colonized and necrotized the infected apple branches, while Juglanconis juglandina was not able to infect the twigs. Members of Botryosphaeriaceae were the most virulent, causing the largest lesions in the fastest way. This experiment draws attention to the threat of new host-pathogen connections, which can arise because of the favourable weather conditions and can spread between neighbouring cultures.

Keywords: *apple, artificial inoculation, climate change, host-specificity, pathogenicity, woody tissues*

INTRODUCTION

Fungi are present in all groups of plants as saprophytes or parasites, and some of them cannot grow without any host (Alexopoulos et al., 1996). They can spread on a global scale, which depends heavily on environmental factors. Their spore can move by rain, irrigation water, wind and insects, which allows to travel from one plant to another. However, diseases can spread slowly this way, new fungal diseases may appear in an another continent via infected seeds (Bandyopadhyay et al., 1998). Moreover, the role of human activities in fungal spore move is not negligible (Yarwood, 1983).

Host-specificity of phytopathogenic fungi and their ability to adapt a new plant is an interesting concept among plant pathologist. Host-specify fungi can grow only on one particular plant species, which phenomenon was studied also on a molecular biology basis and developed gene for gene thesis (Flor, 1971).

Climate change is threatening the whole world through extreme weather conditions, as increased temperature, monumental rainfall, droughts, hurricane or flooding (Costello et al., 2009). Because of the changed environment plants may contact with new pathogen, which never had been exposed before to, causing the lack of immunity. This means infections can spread into new hosts and new geographical areas (Hoberg and Brooks, 2015). Chestnut blight was introduced in similar way into the USA as well as sudden oak death (Emiko et al., 2007).

Plant diseases cause serious losses on a yearly basis, amounting to 10–16% of global food production (Strange and Scott, 2005; Chakraborty and Newton, 2011). Some changed environmental factors, such as higher humidity can provide favourable condition for fungi boosting disease risk and contribute to the development of diseases (Huber and Gllespie, 1992). Pathogens can overwinter more likely because of the increasing temperature, which predict plant protection problems after the dormant season (Coakley et al., 1999). Already for twenty years ago, researchers found, that some fungi cause more severe symptoms in warmer years (Brasier, 1996; Sutherland et al., 1997). The geographic ranges of pathogens may can alter due to climatic changes, as a result new host – pathogen connection can appear (Davis and Shaw, 2001), just like when plants were introduced in new continents in the past (Valent, 1990).

Members of the genus *Botryosphaeria* have been described in the United States as a pathogenic fungus responsible for apple decay, but were isolated from cankers of apple trees in Argentina, in Australia and in Brazil (Shay and Sitterly, 1954; Sutton, 1991). It has been observed, that the most harmful species of the genus colonize several hosts or are geographically widespread, because these species were often the most pathogenic in the case of artificial inoculation (de Wet et al., 2000; Pavlic et al., 2007; van Niekerk et al., 2004). Researchers have observed as well, that not all species cause infections in all areas, that have a broad host range. For example, *Botryosphaeria dothidea* causes serious infections in fruits and nuts in the United States, but is not at all or rarely found in these hosts in South Africa and other regions (Michailides, 1991; Pavlic et al., 2007; Slippers et al., 2004).

Diaporthe species caused cankerous lesions on apple tree in Japan as well as in South Africa, in Urugay, and on peach tree in Greece (Satoko et al., 1999; Smit et al., 1996; Thomas et al., 2009; Cloete et al., 2011; Sessa et al., 2017).

Diplodia genus are also able to colonize tissues of apple tree. Species were isolated from cankered parts in British Columbia and in Iran (Abdollahzadeh, 2015; Úrbez-Torres et al., 2016).

In 2017, a new genus named *Juglanconis* was described in *Juglandaceae*, separated from *Melanconis* genus, in addition new species were identified,

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however there may be further hidden groups (Voglmayr, 2017). *Juglanconis juglandina* is known for dieback of walnut (*Juglans* spp.) in Europe (Belisario, 1999). Regarding variable weather conditions in Hungary, the purpose of our study was to study host-specificity of some fungal genera and to reveal new possible host-pathogen relationships by (i) identify microbiome of woody tissues of apple tree and (ii) testing susceptibility of the plant against disjunctive pathogens, which can become potential threat of Hungarian agriculture due to changed environmental impacts.

MATERIALS AND METHODS

Collection of fungal isolates

In order to study the fungal microbiome of apple trees, samples were collected in Pallag, in the Horticulture Investigational Station of University of Debrecen. Seven tree were sampled which showed cankerous symptoms on trunks and branches. The bark tissues of collected plant pieces were removed, then they were disinfested in 10% chlorogen-sesquihydrate (Neomagnol)-Tween20 solution for one minute, and then the samples were washed in sterile distilled water twice. The woody tissues were placed on potato-dextrose agar medium (Biolab) containing streptomycin-sulphate to minimize bacterial growth. Fungal colonies were transferred to fresh PDA media, following seven days of incubation at room temperature.

During the pathogenicity test, pathogen isolates were originated from walnut (*Juglans regia*) in similar way to the method mentioned above. The sampled orchard was near Jánkmajtis, where symptomatic twigs fruits were collected.

Morphological and molecular biological identification

Isolates were identified genus-level based on their cultural characteristics. Main morphological markers, colours and texture of colonies were studied on PDA.

Seven days old fungal colonies grown on PDA were used for DNA-extraction. Fungal mycelia were scraped with a sterile inoculation loop into tubes containing bashing beads. Genomic DNA was extracted with NucleoSpin Plant II (Macherey-Nagel) Kit. The ITS1-4 primers (IDT) of internal transcribed spacer (ITS) loci (White et al., 1990) were chosen to amplify variable sections of fungal gene with polymerase chain reaction. The reaction volume was 25 µL: 12,5 µL Green Master Mix (Thermo Fisher), 0,5 µL of each primer (10 pmol/ µL), 10,5 µL nuclease-free water and 1 µL DNA (10 µg/µL). PCR products were purified with NucleoSpin Gel, PCR Clean Up Kit DNA (Macherey-Nagel) sequencing was performed in Microsynth GmBh. Sequences were blast in National Center for Biotechnology Information (NCBI) resulting a list of the most similar strains.

Artificial inoculation of apple twigs

Pathogenicity tests were performed to investigate the susceptibility of plants, the manner and the rate of the spreading of fungal pathogens. To study whether canker-causing species isolated from other host plants could cause symptoms on apple tree, twigs were collected in Pallag. Twigs were washed in tap water and soaked in 70% ethanol for 3 minutes. After rinsing twice with sterile distilled water, the inoculation was performed. A V-shaped incision was formed in the centre of the sample with a scalpel, then a mycelium plug was placed in the wound, which was covered with laboratory film (Parafilm), as well as the cut ends of the twigs. The test was performed in triplicate. The inoculated branches were placed in a plastic box with Petri dish filled with sterile water to provide humidity. (Tang et al., 2011).

Measurements were carried out two, three, and four weeks later to determine the extent of pathogens in the samples. During the first two times, only the condition of the cortex was examined, and the internal tissues was examined at fourth week.

Pathogens were reisolate to determine, whether the inoculated fungi colonized the inner tissues of branches. The process was the same as described above in 'Collection of fungal isolate'.

RESULTS AND DISCUSSIONS

Fungal community of apple twigs

To crawl the present host-pathogen connections, fungal microbiome of apple tree was studied. In the cankerous apple branches, two *Botrystis* spp., four *Diplodia* spp., and *Alternaria* isolates were on the basis of colony morphology and conidial characters.

Isolates were collected for artificial inoculation of apple twigs, from plant parts of walnut, *Botryosphaeria* spp. and *Diaporthe* spp. from rotted walnuts, as well as *Diplodia* and *Juglanconis* genus from symptomatic walnut branches were cultured.

Colonies of *Botrytis* sp. were greyish and powdery, and the conidia were ovate. Members of *Diplodia* genus produced dark green pigments and ellipsoid conidia, as well as smaller aerial mycelia. *Alternaria* species created greenish colonies and this genus is well-known for their conidia septate horizontally and vertically.

Cultures of *Botryosphaeria* were dark grey and rich in thick aerial mycelia, *Diaporthe* species were recognizable about their dirty white mycelia and black sclerotia. *Juglanconis* spp. generated bright yellow cultures on PDA.

For more accurate results, isolates were identified also with a molecular genetic method, which results confirmed our observations during genus-level identification. Based on ITS sequences, *Alternaria alternata*, *Botrytis cinerea* and *Diplodia seriata* were present in the apple branches. As far as walnut tree samples were concerned, *Botryosphaeria dothidea* and *Diaporthe eres*, *Diplodia seriata* and *Juglanconis juglandina* species were identified. These deposited isolates are summarized in *Table 1*.

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Table 1

Species collecting from symptomatic apple branches and pathogens using during artificial inoculation

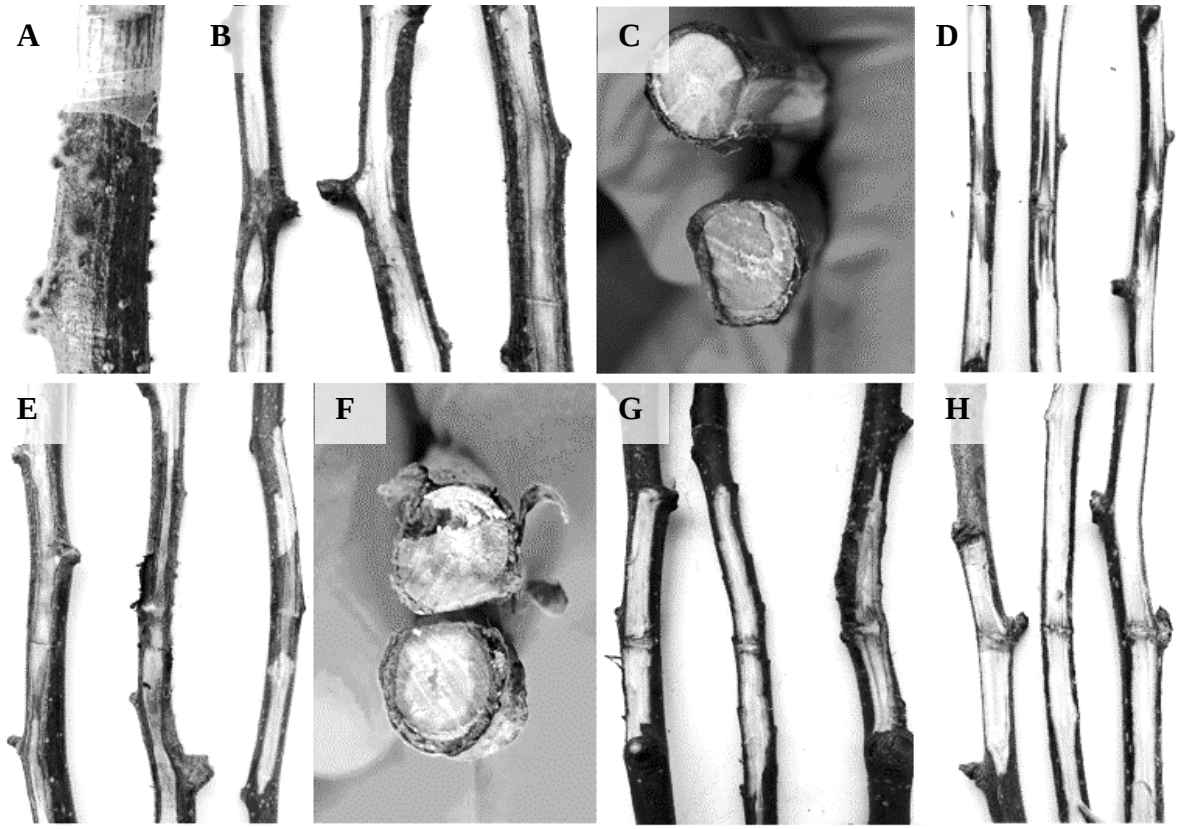
Species	Host	Symptom	Geographical origin	GenBank Accession Number
Alternaria alternata	*Malus domestica*	Cankered branch	Pallag, Hungary	MN706186
Diplodia sp.	*Malus domestica*	Cankered branch	Pallag, Hungary	MN706187
Diplodia sp.	*Malus domestica*	Cankered branch	Pallag, Hungary	MN706188
Diplodia seriata	*Malus domestica*	Cankered branch	Pallag, Hungary	MN706189
Botrytis cinerea	*Malus domestica*	Cankered branch	Pallag, Hungary	MN706190
Botrytis cinerea	*Malus domestica*	Cankered branch	Pallag, Hungary	MN706191
Botryosphaeria dothidea	*Juglans regia*	Rotted kernel	Jánkmajtis, Hungary	MN706192
Diaporthe eres	*Juglans regia*	Rotted kernel	Jánkmajtis, Hungary	MN706193
Diplodia seriata	*Juglans regia*	Symptomatic branch	Jánkmajtis, Hungary	MN706194
Juglanconis juglandina	*Juglans regia*	Symptomatic branch	Jánkmajtis, Hungary	MN706195

Pathogenicity test

Botryosphaeria dothidea, Diaporthe eres, Diplodia seriata and *Juglanconis juglandina* from walnut were selected to setup pathogenicity test (*Picture 1*, *Table 1*). Two, three and four weeks after inoculation, the condition of the infected branches and the spread of the pathogens in tissues were examined.

Every tested pathogen caused symptoms on apple twig samples except *Julganconis juglandina*. (*Picture 1/*G). In every case, the effect of artificial inoculation was manifested on the cortex (*Picture 1/A*) as well as in the vascular tissues (*Picture 1/*B, *1/*C, *1/*D, *1/*E, *1/*F). Control branches treated with empty PDA plug did not show lesions (*Picture 1/*H).

Picture 1: **Effect of artificial inoculation. A: Pycnidia of *Botryosphaeria dothidea* on twig. B: Necrotic lesions caused by *B. dothidea*. C, D: *Diaporthe eres* induced symptoms in the phloem. E, F: Pathogenicity of *Diplodia seriata*. G: Symptomless branches treated with *Juglanconis juglandina*. H: Controls.**



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The spread of the lesions by weeks is illustrated in the *Figure 1*. Isolates belong to the *Botryosphaeriaceae* induced the most length lesions with the fastest spread. *B. dothidea* were more aggressive than *D. seriata* until the third week, however sizes of discolorations were almost the same at the fourth week. The negative effect of *Diaporthe eres* was much slower and the extent of the necrotic area were smaller also. Larger lesions were induced in the vascular tissues, when branches were treated with mycelia of *B. dothidea*, but mainly with *D. eres*. Although *D. seriata* produced serious necrotic symptoms in the phloem as well.

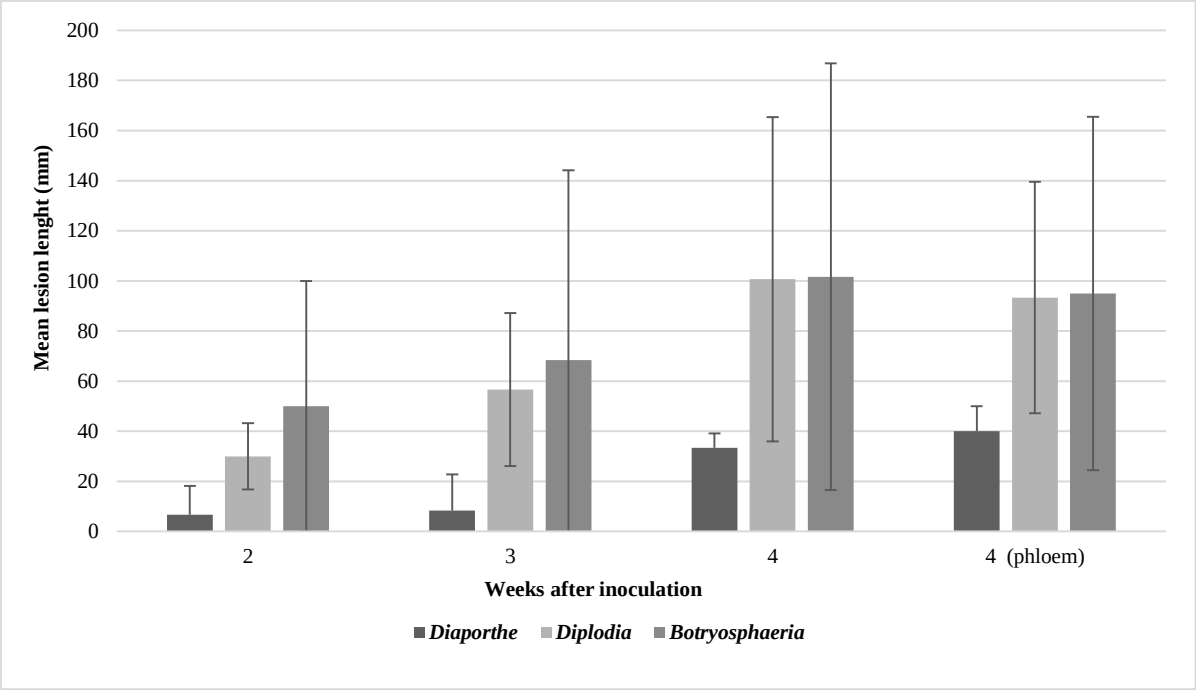
The reisolation of necrotic lesion induced fungi was successful. The fungal population of samples inoculated with *Juglanconis juglandina* was the same as the control, consequently this species was not able to colonize the apple twigs.

Canker-causing fungi, which had wide range of host could colonize and produce symptoms on woody tissues of apple. Despite belonging to *Diaporthales* order, *J. juglandina*, as its name show its pathogenicity may limit to member of *Juglans* spp.

D. seriata was present in symptomatic apple branches, which fact elucidated the high virulence of *Botryopshaeriaceae* during the pathogenicity test and supported the susceptibility of the host.

High standard deviation among triplicates require the repetition of the analysis with more replicates.

Figure 1: **Summary results of the pathogenicity test. Error bar represent standard deviation**



CONCLUSIONS

New host-pathogen relationships could appear because of the result of favourable environmental conditions for phytopathogenic fungi. Virulence of isolates were tested, which were present in apple twigs microbiome, and genera which were reported in apple woody tissues mainly in countries with warmer climate (Shay and Sitterly, 1954; Sutton, 1991; Smit et al., 1996; Thomas et al., 2009; Cloete et al., 2011; Abdollahzadeh, 2015; Sessa et al., 2017) and originated from rotted walnuts and symptomatic twigs.

After artificial inoculation, *Botryosphaeria dothidea*, *Diaporthe eres* and *Diplodia seriata* species not only colonized the woody tissues of apple, but also caused lesions. *Juglanconis juglandina* did not cause any symptoms in inoculated branches and did not colonize them, which is confirmed by unsuccessful reisolation. However, *D. seriata* was isolated from this orchard, *B. dothidea* and *D. eres* are considered a possible new host-pathogen interaction, which can result in devastating diseases later, being aware of their virulence on this tree.

Furthermore, the results of this paper support the importance of the monitoring of symptoms on neighbouring plant cultures due to possible cross-infection.

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