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Diaporthe eres Nitschke IS THE ONLY *Diaporthe* SPECIES FOUND ON BLUEBERRY IN CROATIA

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SUMMARY

Increase in blueberry (*Vaccinium corymbosum*) cultivation in Croatia during the last several years raised an interest in pests and pathogens occurring on this fruit crop. Among them, fungus *Diaporthe vaccinii* (*Phomopsis vaccinii*) is currently regulated as a quarantine harmful organism. *Diaporthe vaccinii* is the causal agent of Phomopsis shoot blight and canker on blueberry, and the status of this regulated pathogen in Croatia has been unknown. From 2016 to 2018, an official survey was carried out in Croatian blueberry orchards to determine the eventual presence of *D. vaccinii*. Thirty-three visual inspections were performed in 24 blueberry orchards in 11 counties and the City of Zagreb, and 66 samples of twigs showing symptoms resembling Phomopsis twig blight and canker were collected. *Diaporthe* spp. were detected in 20 samples, and 20 isolates were collected. In the majority of samples (49) fungi from Botryosphaeriaceae family were found, alone or co-occurring with *Diaporthe*. Phylogenetic analysis of ITS1-5.8S-ITS2 ribosomal DNA sequences showed that all *Diaporthe* isolates collected can be identified as *Diaporthe eres*. The survey confirmed the status of *D. vaccinii* as the pathogen not known to occur in Croatia.
Key words: Vaccinium corymbosum, Diaporthe (Phomopsis), survey, Diaporthe eres, Diaporthe vaccinii

Diaporthe eres Nitschke JEDINA JE *Diaporthe* VRSTA NAĐENA NA BOROVNICI U HRVATSKOJ

SAŽETAK

Povećanje površina pod američkom borovnicom (*Vaccinium corymbosum*) u Hrvatskoj tijekom posljednjih nekoliko godina dovelo je do zanimanja za štetnike i uzročnike bolesti na toj voćarskoj kulturi. Među njima, gljiva *Diaporthe vaccinii* (*Phomopsis vaccinii*) trenutno je regulirana kao karantenski

štetni organizam. *D. vaccinii* je uzročnik sušenja izdanaka i raka borovnice, a status tog reguliranog patogena u Hrvatskoj bio je nepoznat. Od 2016. do 2018., u domaćim nasadima borovnice proveden je program posebnog nadzora s ciljem utvrđivanja moguće prisutnosti gljive *D. vaccinii*. U 24 nasada američke borovnice u 11 županija i u Gradu Zagrebu provedena su trideset i tri vizualna pregleda te je sakupljeno 66 uzoraka biljaka sa simptomima sušenja izdanaka. *Diaporthe* spp. je utvrđen u 20 uzoraka, iz kojih je sakupljeno 20 izolata. U većini uzoraka (49) utvrđene su gljive iz porodice Botryosphaeriaceae, same ili zajedno s gljivama iz roda *Diaporthe*. Filogenetske analize sekvenci ITS1-5.8S-ITS2 ribosomske DNA pokazale su da se svi sakupljeni *Diaporthe* izolati mogu identificirati kao *Diaporthe eres*. Poseban nadzor potvrdio je status *D. vaccinii* kao štetnog organizma čija prisutnost u Hrvatskoj nije poznata.

Ključne riječi: Vaccinium corymbosum, Diaporthe (Phomopsis), poseban nadzor, Diaporthe eres, Diaporthe vaccinii

INTRODUCTION

Fungi from the genus *Diaporthe* are widespread saphrophytes, endophytes and pathogens associated with numerous plant hosts (Gomes et al., 2013; Udayanga et al., 2011). Taxonomy of the genus and species description have undergone major changes during the last decade, mostly as a result of applying molecular phylogeny tools like multilocus sequence analysis (Udayanga et al., 2014; Gomes et al., 2013; Udayanga et al., 2011). Many new species and new species-host associations are constantly being described and reported, with multiple species increasingly reported on the same host (Lombard et al., 2014; Elfar et al., 2013; Gomes et al., 2013).

Despite the growing number of species described, still only a limited number of *Diaporthe* spp. are considered to be plant pathogens of economic importance on cultivated woody hosts. Among them, *Diaporthe vaccinii* Shear (anamorph *Phomopsis vaccinii* Shear) is currently the pathogen regulated as a quarantine harmful organism in the European Union (EU, 2000). *Diaporthe vaccinii* is the causal agent of twig blight, canker and fruit rot of blueberry and cranberry (*Vaccinium* spp.), and it is listed in the Annex IIAI of Directive 2000729/EC (EU, 2000). Up to about two decades ago, *D. vaccinii* was considered to be the species of restricted worldwide distribution. It was regarded to be widespread only in North America, where it has been reported as a severe pathogen of blueberry crops (EFSA PLH Panel, 2014; Farr et al., 2002). However, during the last several years, studies have shown that *D. vaccinii* is present also in Europe (Cardinaals et al., 2018; Michalecka et al., 2017; Lombard et al., 2014). Beside *D. vaccinii*, other species of *Diaporthe* (*Phomopsis*) have been recently reported on blueberry, and some were shown to be pathogenic (Lombard et al., 2014; Elfar et al., 2013).

The eventual presence of quarantine harmful organism *D. vaccinii* in Croatia was unknown. Šubić (2011) is referring to *D. vaccinii* as the causal agent of blueberry shoot dieback in Međimurje, but without laboratory identification of the fungus. To confirm its presence or absence, phytosanitary survey was carried out in Croatian blueberry orchards. The main objective was to determine the status of *D. vaccinii* in Croatia, but also to get an insight into fungal species potentially associated with twig dieback and canker of this increasingly popular berry fruit crop.

MATERIALS AND METHODS

Multi-annual survey on *Diaporthe vaccinii* presence in Croatia was carried out from 2016 to 2018. In a three-year period, visual inspections and sampling were performed in blueberry (*Vaccinium corymbosum* L.) orchards in Croatia. Number of orchards inspected in different counties and the number of samples collected is presented in Table 1. Visual inspections were focused on detecting symptoms resembling those described for Phomopsis shoot dieback and canker. If symptoms were observed, symptomatic shoots were collected and analysed in the laboratory.

Totally 66 samples were collected. Shoots were examined under the stereomicroscope for the eventual presence of fungal fruiting bodies (pycnidia). After the examination, shoots were washed in tap water and cut in chips 5-10 mm in diameter. Remaining parts of the shoots were incubated in moisture chamber at room temperature to induce the eventual formation of pycnidia. Woody chips were surface-sterilised in 1 % NaOCl, rinsed, dried, and inoculated on potato-dextrose agar (PDA). PDA plates were incubated at 21 °C for 7-10 days in darkness. After incubation, plates were checked for *Phomopsis*-like colonies (Gomes et al., 2013; Farr et al., 2002). Each *Phomopsis*-like colony was transferred to pure half-strength PDA culture by hyphal tip isolation from the colony edge and was considered an isolate. Twenty potentially *Diaporthe* (*Phomopsis*) sp. isolates were collected.

Isolates were examined for their morphology in PDA culture (growth, appearance, colour and sporulation). All 20 isolates were identified as *Phomopsis* (*Diaporthe*) sp. according to the morphology. As morphological features of many *Diaporthe* species are overlapping (Udayanga et al., 2014; Udayanga et al., 2011; EPPO, 2009), morphological analysis of pycnida and condidia was not performed and PCR-sequencing of ITS1-5.8S-ITS2 ribosomal DNA region was used for species identification. Fungal DNA from pure cultures was extracted using DNeasy® Plant Mini Kit (Quiagen Inc., USA) according to manufacturer's instructions. PCR amplification was performed as described by EPPO (2009) using primer pair ITS1 and ITS4 (White et al., 1990). PCR products were purified and sent for sequencing to Macrogen Europe (Amsterdam, Netherlands). Sequences were checked and corrected in Sequencher®

software (Gene Codes Corporation, USA), and were subjected for phylogenetic analysis using MEGA 5.05 software (Tamura et al., 2011). Sequences of reference *D. vaccinii* isolates CBS 118571 (Acc. No. KC343223) and CPC 23812 (Acc. No. KJ160587), *D. eres* isolate CBS 524.82 (Acc. No. JQ807448.1) and *D. baccae* CBS 136972 (Acc. No. KJ160565) retrieved from GenBank® were used for comparison. *Godronia cassandrae* strain OVB 13-001 (Acc. No. KC595271) was used as an outgroup in construction of UPGMA phylogenetic tree. Grouping of isolates and sequence similarities were used for identification of isolates.

RESULTS AND DISCUSSION

From 2016 to 2018, 24 blueberry orchards in 11 counties and the City of Zagreb were visually inspected. Some orchards were inspected several times in three years, while other were inspected only once. Symptoms resembling Phomopsis shoot blight and canker were observed in 17 orchards and totally 66 samples of dried twigs were collected (Table 1.).

Table 1 Counties, locations, number of visual inspections and number of samples collected in *Diaporthe vaccinii* surveys in blueberry orchards in Croatia from 2016 to 2018.
Tablica 1. Županije, lokacije, broj vizualnih pregleda i broj uzoraka sakupljenih u okviru posebnog nadzora *Diaporthe vaccinii* u nasadima borovnice u Hrvatskoj od 2016. do 2018.

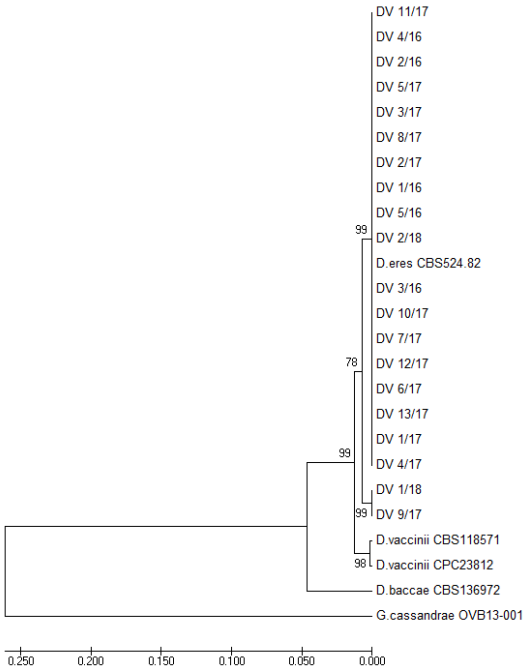
County *Županija*	**Locations** *Lokacije*	**No. of visual inspections** *Broj vizualnih pregleda*	**No. of samples** *Broj uzetih uzoraka*
Bjelovar bilogora	Čazma	3	7
Sisak Moslavina	Topusko, Kutina, Veliki Gradac	7	18
Zagreb	Donja Bistra, Križ, Donja Zelina, Ivanić Grad	1	17
Zadar	Gračac	1	-
Virovitica Podravina	Pitomača	2	3

Međimurje	Mursko Središće, Štrigova	3	1
Varaždin	Petrijanec, Šijanec, Domitrovec, Sigetec Ludbreški	4	7
City of Zagreb	Botinec, Kupinečki Kraljevec	3	9
Koprivnica Križevci	Đurđevac, Glogovec	2	-
Karlovac	Krnjak, Jaškovo	2	2
Istria	Vrvari	1	2
Požega Slavonia	Dereza	1	-
11 counties + City of Zagreb	**24 locations**	**30 visual inspections**	**66 samples**

Diaporthe sp. was isolated from 20 out of 66 samples (30 %) and 20 isolates were collected (five in 2016, 13 in 2017 and two isolates in 2018). In eight samples, *Diaporthe* sp. and fungi from the Botryosphaeriaceae family were found to co-occur. Only Botryosphaeriaceae were found in 41 samples, while in five samples other unidentified fungi were recorded, not belonging to *Diaporthe* or Botryosphaeriaceae.

Isolates of *Diaporthe* sp. grown on half-strength PDA were typical of *Diaporthe* (*Phomopsis*) according to the descriptions (Udayanga et al., 2014; Udayanga et al., 2011; EPPO, 2009; Farr et al., 2002), but some variability in culture appearance was noted. The majority of isolated were fast growing, developed white fluffy aerial mycelium and abundant conidiomata containing both α- and β-conidia, and were whitish to brown or whitish with brown zones in reverse. Several isolates were sterile and developed brownish aerial mycelium.

Phylogenetic analysis of ITS1-5.8S-ITS2 ribosomal DNA sequences showed grouping of all 20 *Diaporthe* isolates collected into the clade distinct from two *D. vaccinii* reference isolates (Picture 1). The majority of isolates grouped together with the reference *D. eres* isolate CBS 524.82. The results of phylogenetic analysis confirmed that all *Diaporthe* isolates collected from blueberry in Croatia can be identified as *D. eres*. Closely related, but regulated *D. vaccinii* was not found.



Picture 1 UPGMA phylogenetic tree derived from sequences of ITS1-5.8S-ITS2 ribosomal DNA of reference *Diaporthe* isolates deposited in GenBank© and isolates from blueberry analysed in the study. Bootstrap interior-branch values are based on 500 replicates.

Slika 1. UPGMA filogenetsko stablo na temelju sekvenci ITS1-5.8S-ITS2 ribosomske DNA referentnih *Diaporthe* izolata iz GenBank® baze podataka i izolata s borovnice iz istraživanja. Vrijednosti na ograncima temelje se na 500 ponavljanja.

D. *eres* is cosmopolitan *Diaporthe* species which has often been regarded as a species complex rather than a single, well-defined species (Udayanga et al., 2014; Gomes et al., 2013). Udayanga et al. (2014) divided *D*. *eres* complex into nine different phylogenetic species, including both *D*. *eres* and *D*. *vaccinii*. Besides demonstrating close relationship between *D*. *eres* and *D*. *vaccinii*, the mentioned study showed that phylogenetic analysis of several different genes might be needed to resolve *D*. *eres* complex and to identify the species *sensu stricto*. In study on *Diaporthe* species occurring on blueberry in Europe, Lombard et al. (2014) also used sequences of several different genomic regions to identify the species. These studies have shown the complexity of precise identification of *Diaporthe* spp. on different hosts, which are aggravating circumstances in any kind of phytosanitary activity related to plant quarantine.

D. *eres* is reported to be relatively frequently found on *Vaccinium* plant hosts in Europe (Lombard et al., 2014; Cardinaals et al., 2018; Michalecka et al., 2017), and these results are in line with the present study. Pathogenicity of *D*. *eres* on blueberry was shown to be very weak (Cardinaals et al., 2018), and it is doubtful

whether this species can be regarded as a blueberry pathogen. Isolation of *D*. *eres* from dried twigs and shoots during the survey in Croatia might be a consequence of endophytic nature of the fungus, or *D*. *eres* may be secondary, opportunistic invader of weakened or stressed plant tissue. On the other side, some studies are indicating that *D*. *eres* may be pathogenic, at least as a part of fungal complex involved in blueberry canker. In Croatia, *D*. *eres* was reported as a pathogen of blackberry (Vrandečić et al., 2011), while Kaliterna et al. (2012) reported *D*. *eres* as a moderate pathogen of grapevine

Quarantine status of *D*. *vaccinii* is implying phytosanitary control on import, movement and production of *Vaccinium* host plants in the European Union. Beside this, the status of *D*. *vaccinii* is implying phytosanitary measures of eradication or containment in case of finding. Surveys and control measures are usually expensive and demanding for national plant health system. Recent studies on *Diaporthe* species on blueberry and some expert opinions are addressing the need for a re-assessment of quarantine status of *D*. *vaccinii* in Europe (Cardinaals et al., 2018; EFSA PLH Panel, 2014; Lombard et al., 2014). Lombard et al. (2014) showed that the complex of *Diaporthe* species is occurring on blueberry in Europe, and that several different species might be involved in development of twig dieback and canker. Similarly, several *Diaporthe* species were reported as blueberry pathogens in Chile (Elfar et al., 2013). These studies showed that *D*. *vaccinii* is not the only *Diaporthe* species causing twig blight and canker on blueberry. In pathogenicity tests, Cardinaals et al. (2018) showed that *D*. *vaccinii* is causing relatively mild symptoms when inoculated on shoots of blueberry cultivars Duke and Liberty. The authors are stating that "*D. vaccinii* may not be a major threat to blueberry production in Europe", and that the quarantine status of the fungus might be revised (Cardinaals et al., 2018). Finally, EFSA PLH Panel (2014) concluded that "*D. vaccinii* does not have the potential to be a quarantine pest as it does not fulfil one of the pest categorisation criteria defined in International Standard for Phytosanitary Measures No 11, that of having a severe impact".

In survey carried out in Croatia, twig blight and canker symptoms were shown to be only sporadically present in Croatian blueberry plantations, and this obviously does not represent a serious problem. Moreover, it was shown that fungi from Botryosphaeriaceae family are more frequently isolated from dried twigs than *Diaporthe* (*Phomopsis*) species. It can be argued whether the eventual presence of *D*. *vaccinii* in Croatia should have a severe impact and whether any eventual phytosanitary measures should be justified in such case.

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