Short communication
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Fungi associated with canker diseases on olive in Istria (Croatia)

Gljive povezane s bolestima gljivičnog raka na maslini u Istri (Hrvatska)

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ABSTRACT

A survey in olive orchards in Istria (Croatia) was carried out from 2016 to 2018 to investigate pathogens potentially associated with olive decline and dieback. In nine orchards, canker-like symptoms were recorded on olive trunks and branches. Six fungal species were isolated from cankers: *Geosmithia* sp., *Diaporthe* sp., *Diatrype stigma*, *Diplodia seriata*, *Neofusicoccum parvum*, and *Pseudophaeomoniella oleae*. Pathogenicity tests were conducted in a greenhouse, on branches of 4-year-old local cultivar 'Buza'. Eight months after branch inoculation, *D. seriata* showed to be the most aggressive, causing an average of 67 mm long lesion. *N. parvum* caused 31 mm long lesion, while *Diaporthe* sp. showed only weak aggressiveness, causing 3 mm long lesion. *Diatrype stigma*, *P. oleae* and *Geosmithia* sp. did not cause any symptoms on inoculated plants. This is the first report of *Neofusicoccum parvum* and *Diaporthe* sp. as pathogens on olive in Istria (Croatia).

Keywords: *Botryosphaeriaceae*, inoculation, isolates, olive decline, pathogenicity test

SAŽETAK

Terensko istraživanje maslinika u Istri (Hrvatska), provedeno je od 2016. do 2018. godine s ciljem utvrđivanja potencijalnih patogena povezanih s odumiranjem i sušenjem maslina. Šest vrsta gljiva izolirano je s drvenastih dijelova masline sa simptomima gljivičnog raka: *Geosmithia* sp., *Diaporthe* sp., *Diatrype stigma*, *Diplodia seriata*, *Neofusicoccum parvum* i *Pseudophaeomoniella oleae*. Test patogenosti proveden je u plasteniku, na granama 4-godišnjih sadnica maslina autohtone sorte Buža. Osam mjeseci nakon inokulacije, *D. seriata* pokazala se kao najagresivnija vrsta uzrokujući lezije duljine 67 mm, *N. parvum* je uzrokovao lezije duljine 31 mm, a *Diaporthe* sp. pokazao se kao najslabiji patogen, uzrokujući lezije duljine 3 mm. Vrste *D. stigma*, *P. oleae* i *Geosmithia* sp. nisu uzrokovale simptome na inokuliranim maslinama. Prema našim spoznajama, ovo je prvi nalaz vrsta *Neofusicoccum parvum* i *Diaporthe* sp. kao patogena masline u Istri (Hrvatska).

Ključne riječi: *Botryosphaeriaceae*, inokulacija, izolati, odumiranje masline, test patogenosti

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INTRODUCTION

Olive is the most important crop in coastal regions of Croatia, representing an agricultural landmark and cultural symbol of Istria and Dalmatia since the ancient Roman times. The emergence of olive quick decline caused by *Xylella fastidiosa* Wells et al. 1987 in southern Italy (Carlucci et al., 2013a) imposed mandatory plant health surveys on olive. These surveys raised an interest in olive decline and dieback research, in the scientific community and among olive producers. The other important reason for olive dieback and decline studies in Croatia was an increasing number of similar studies in Italy (Carlucci et al., 2013b), California (Úrbez-Torres et al., 2013), and Spain (Moral et al., 2007), identifying new olive pathogens and providing new insights into the etiology of olive diseases.

Intensified research on olive diseases in Croatia resulted in detection and description of several olive pathogens new to Croatia, like *Verticillium dahliae* Kleb. (Kaliterna et al., 2016), *Armillaria mellea* (Vahl) P. Kumm. (Ivić and Godena, 2017) or *Pleurostomophora richardsiae* (=*Pleurostoma richardsiae* (Nannf.) Reblova & Jaklitsch) (Ivić et al., 2018). The aim of this study was to identify and to assess pathogenicity of fungi found on canker-like symptoms on olive in Croatia, possibly contributing to olive decline or dieback syndrome.

MATERIALS AND METHODS

Surveys, sampling and isolation of fungi

Visual inspections of olive orchards were carried out from 2016 to 2018 in the main olive-growing regions in Istria. Twenty-five orchards have been inspected for the presence of wilt, decline, dieback or canker symptoms on olive trees. Among locations inspected, canker-type symptoms have been recorded in nine orchards (Table 1). They were assessed by observing colour changes and eventual depressions on the outer bark. The outer bark has been cut to detect inner wood necrosis. Cankers were partially cut open with a knife, 0.5 – 1.0 cm deep, in order to encircle the edges of necrotic tissue and adjacent, asymptomatic, visually healthy tissue. Symptomatic bark or branch parts were cut and taken as samples for laboratory analysis.

In one orchard, a heavy infestation of the olive bark beetle *Hylesinus toranio* Danthoine, was noticed. In beetle galleries under the bark, whitish mycelia became visible, suggesting the presence of ambrosia fungi such as *Geosmithia* spp. Symptomatic branches were taken as samples.

In laboratory, cut fragments of bark, branches or cankers, approximately 3 – 6 mm in size, were surface disinfected in 70% ethanol for approximately half a minute, rinsed with sterile water, dried and placed onto carrot-piece agar (CPA). Colonies emerging from inoculated wood pieces within three to five days of incubation at 22 °C were transferred to potato dextrose agar (PDA), and incubated for two weeks in darkness at the same temperature. Totally 34 fungal isolates were collected and identified according to morphological features and molecular data.

Identification of isolates

Out of 34 isolates, 12 were identified as *Geosmithia* sp. according to the colony appearance and morphology of conidiophores and conidia (Kolařík et al., 2007). All 12 isolates were isolated from olive bark beetle galleries. Four isolates were determined as belonging to *Botryosphaeriaceae*, out of which three were preliminarily identified as *Diplodia* sp., and one as *Neofusicoccum* sp. (Phillips et al., 2013). Three isolates were *Diaporthe*-like (Udayanga et al., 2011). Six isolates were *Phaeomoniella*-like but could not be identified to the genus level with confidence. Nine morphologically similar isolates did not sporulate and could not be identified according to morphological characters.

Isolates were grouped by locations of origin and morphology in culture (Table 1). DNA was extracted from the biomass of representative isolates by using DNeasy Plant Mini Kit® (Quiagen Inc., Valencia, USA) according to the manufacturer's instructions. PCR reactions were performed by using Promega PCR Master Mix® (Promega, USA) and amplification primers ITS1/ITS4 (White et al., 1990) with annealing temperature 58 °C, followed by sequencing. Sequencing was carried out by Macrogen Europe BV® (Amsterdam, The Netherlands). Sequences

were edited in Sequencher® (Gene Codes Corporation, USA) and compared with sequences retrieved from GenBank® database.

Pathogenicity tests

Pathogenicity tests were carried out in a greenhouse on 4-year-old olive plants of local cultivar 'Buza'. Sets of ten plants were included either with *Neofusicoccum parvum* MSG-3 (Acc. No. OP090502), *Diplodia seriata* MSG-1 (Acc. No. OP090495), *Diaporthe* sp. MSG-18 (Acc. No. OP093624), *Diatrype stigma* MSG-12 (Acc. No. OP090406), *Pseudophaeomoniella oleae* MSG-26 (Acc. No. OP090503) or *Geosmithia* sp. DI-4 (Acc. No. OP090501). Each isolate was inoculated once on each plant. Inoculum was prepared on PDA incubated at 22 ° C for two weeks. On randomly chosen branches of each plant, 10-mm diameter circular lesions about one mm deep were made with a borer by removing the outer bark. Mycelial plugs of the same diameter were cut from PDA cultures, placed into wounds, and sealed with parafilm. Non-inoculated pure PDA plugs were used as a control.

Inoculated plants were kept in a greenhouse for approximately eight months, from November 2017 to July 2018, and monitored for the presence of symptoms. After eight months, inoculated branches were cut. Superficial lesion lengths were measured, if developed. Subsequently, bark was removed and the length of internal wood necrosis, if present, were measured. Internal lesion length values were used for statistical analysis. Length values were compared using ANOVA and Newman-Keuls multiple comparison test. Re-isolation of fungi from lesions was performed similarly as described for the original isolation of pathogens.

RESULTS

Symptoms observed in the field

Olive canker symptoms observed in the field were relatively similar in all orchards where detected. Colour change from naturally grey to reddish-brown or brownish-purple was visible on the outer bark. Lesions were sometimes slightly depressed. When the bark was removed, brown internal necrosis was visible in cambium, following the line of colour change visible outside (Figure 1). Cankers were typically encountered on two- to three-years old branches about two to six cm in diameter. In one case, canker lesion was visible on the main trunk, developing almost from the basal part of the trunk to the tree top. Bark cracking on cankered side of the trunk was observed. In an orchard where olive bark beetle attack was recorded, inner lesions were visible among numerous bark beetle galleries and exit holes.

Figure 1. Canker on olive trunk. *Neofusicoccum parvum* was isolated from the canker (photo: D. Ivić)

Identification of species

Species identified in the study were *Diplodia seriata* De Not., *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, *Pseudophaeomoniella oleae* Nigro, Antelmi & Crous, *Diatrype stigma* (Hoffm.) Fr., *Diaporthe* sp. and *Geosmithia* sp. On PDA, all isolates belonging to the same species were morphologically more or less indistinguishable. The number of isolates and locations (orchards) where particular species were found are presented in Table 1.

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Table 1. Locations surveyed, fungal species identified and the number of isolates collected from particular locations where olive canker symptoms were detected in Istria, Croatia

Location	Cultivar[a]	Fungal species found	No. of isolates collected
Bale	Buza	*Diatrype stigma*	2
Rovinj I	Buza	*Diplodia seriata*	1
Rovinj II	Buza	*Diplodia seriata*	1
		Pseudophaeomoniella oleae	2
Rovinj III	Buza	*Diplodia seriata*	1
		Pseudophaeomoniella oleae	2
Rovinj IV	Buza	*Pseudophaeomoniella oleae*	2
Poreč	Buza	*Diatrype stigma*	7
Vodnjan I	Karbonaca	*Neofusicoccum parvum*	1
Vodnjan II	Buza	*Diaporthe* sp.	3
Vodnjan III	Buza	*Geosmithia* sp.	12

[a] Cultivar showing canker symptoms

Pathogenicity

Three weeks after the inoculation, development of red-brownish lesion was visible on all *D. seriata* inoculation points. Three months after the inoculation, red-brownish lesion were visible on plants inoculated with *D. seriata* and *N. parvum*, and leaves attached on branches become chlorotic (Figure 2). Five months after the inoculation, dieback of leaves on single *D. seriata*-inoculated branches was recorded on four out of 10 plants. Bark cracking also started to develop on *D. seriata* lesions. Leaves on branches inoculated with *N. parvum* turned yellow-green, but no browning and wilting was observed. On plants inoculated with *Diaporthe* sp., only small depressed lesions were visible around inoculation sites. On inoculation sites of other fungi and control inoculation sites, no lesions and no specific symptoms were recorded.

Mean lesion length was the highest for *D. seriata*, which showed to be the most aggressive species in the study (Table 2).

Mean lesion for *N. parvum* was about two-times smaller than for *D. seriata*. *Diaporthe* sp. caused visible lesion only about 3 mm in length after eight months, confirming its pathogenicity, but indicating weak aggressiveness. No visible lesions formed on plants inoculated with *D. stigma*, *P. oleae*, *Geosmithia* sp. and PDA control.

Figure 2. Necrosis under the bark after an inoculation of olive branches with six species of fungi. From bottom to top: *Diplodia seriata* MSG-1, *Neofusicoccum parvum* MSG-3, *Diaporthe* sp. MSG-18, *Diatrype stigma* MSG-12, *Geosmithia* sp. DI-4 and *Pseudophaeomoniella oleae* MSG-26. In the case of inoculation with *D. seriata* and *N. parvum* the development of necrosis is clearly visible, *D. neotheicola* caused small necrosis from the inoculation site, while *D. stigma*, *Geosmithia* sp. and *P. oleae* did not develop beyond the inoculated site (photo: D. Ivić).

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Table 2. Mean internal lesion length caused by six fungal species isolated from olive trees in Istria, Croatia, on 4-year-old 'Buza' olive trees eight months after inoculation

Species and isolate	Mean lesion length (mm)[a]
Diplodia seriata MSG-1	66.7 ± 4.2 a
Neofusicoccum parvum MSG-3	31.0 ± 2.7 b
Diaporthe sp. MSG-18	2.8 ± 0.1 c
Diatrype stigma MSG-12	0.0 d
Pseudophaeomoniella oleae MSG-26	0.0 d
Geosmithia sp. DI-4	0.0 d
Uninoculated control	0.0 d

[a] Values followed by the same letter do not differ significantly at 0.05 level

DISCUSSION

The results of the study showed that particular pathogens associated with olive canker-type diseases in Croatia are the same as reported in some olive-growing regions in Italy, Spain and USA. *Neofusicoccum parvum* has been reported as olive pathogen and considered as one of the causal agents of olive decline in Southern Italy (Carlucci et al., 2013b). The same species has been found to be the most aggressive canker pathogen on oleaster (*Olea europaea* subsp. *europaea* var. *sylvestris* (Mill.) Lehr) in Sardinia (Manca et al., 2020). *Diplodia seriata* has been described previously in Croatia as a pathogen associated with olive dieback (Kaliterna et al., 2012), and has also been isolated from blighted olive shoots in California (Moral et al., 2010). *Neofusicoccum parvum* and *D. seriata* have also been identified as pathogenic to olive fruits (Lazzizera et al., 2008; Moral et al., 2008), indicating their potential economic importance in olive crops.

Diaprothe sp. isolate in the present study showed pathogenicity, but weak aggressiveness to olive plants. Many *Diaporthe* species are associated with canker diseases of woody plants (Udayanga et al., 2011). *Diaporthe neotheicola* was determined as the causal agent of olive twig canker in Apulia, Italy (Frisullo et al., 2015). Carlucci et al. (2013) reported 1.7% frequency of *Diaporthe* spp. isolations from cankers and sub-cortical brown streaking on olive trees in Italy, while Moral et al. (2017) isolated *Diaporthe* spp. from olive branch dieback symptoms in Spain. *Diaporthe* species were also reported on olive trees showing twig and branch dieback in California, along with *Diatrype stigma*, *Diplodia* spp. and *Neofusicoccum* spp. (Úrbez-Torres et al., 2013).

Pseudophaeomoniella oleae has only been described recently (Nigro et al., 2015), found for the first time on olives in Apulia, Italy. Only recently, *P. oleae* has been shown to be associated with wood streaking and olive decline in Greece, and has been confirmed as an olive pathogen (Markakis et al., 2022).

Geosmithia species have not been identified as olive pathogens so far. During the recent decade, two *Geosmithia* species associated with bark beetles have been shown to be pathogenic to their plant hosts, *Geosmithia morbida* M. Kolařík, Freeland, C. Utley & Tisserat on walnuts (Kolařík et al., 2011) and *G. pallida* (G. Sm.) M. Kolařík, Kubátová & Pažoutová (G. Sm.) on *Quercus agrifolia* Née in Southwestern USA (Lynch et al., 2014). However, most *Geosmithia* species are described as bark beetle symbionts, not as plant pathogens (Kolařík et al., 2007). It is possible that this is the case with *Geosmithia* isolates found in the present study, as no disease symptoms were observed in pathogenicity tests. Olive bark beetle damage might be responsible for the dieback symptoms observed in the orchard where *Geosmithia* isolates were found.

Diatrype stigma and *Pseudophaeomoniella oleae* did not cause any symptoms to inoculated plants, and were not confirmed as olive pathogens in the present study. However, Markasis et al. (2022) reported that *P. oleae* can cause wood discolorations on inoculated olive plants. *Diatrype stigma* was assessed as weakly pathogenic on inoculated olive branches by Úrbez-Torres et al. (2013). On the other hand, the results of pathogenicity tests obtained for *N. parvum*, *D. seriata* and *D. neotheicola* are mostly in accordance with those from other similar studies. *Neofusicoccum* and *Diplodia* species are regularly reported as highly pathogenic to olive wood tissue, while *Phomopsis* species show intermediate to weak pathogenicity (Moral et al., 2010; Úrbez-Torres et al. 2013, Moral et al., 2017).

These species are now confirmed as the causal agents of olive canker in Croatia. To the best of our knowledge, this is the first report of *Neofusicoccum parvum* and *Diaporthe* sp. as pathogens on olive in Istria (Croatia).

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