ISSN 2623-6575

UDK 63

GLASILO FUTURE

PUBLIKACIJA FUTURE - STRUČNO-ZNANSTVENA UDRUGA ZA PROMICANJE ODRŽIVOG RAZVOJA, KULTURE I MEĐUNARODNE SURADNJE, ŠIBENIK

VOLUMEN 7 BROJ 4

LISTOPAD 2024.

Glasilo Future

Stručno-znanstveni časopis

Nakladnik:
FUTURA
Sjedište udruge: Šibenik

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Grafička priprema: Ančica Sečan, mag. act. soc.
Objavljeno: 09. listopada 2024. godine.
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Riječ gostujućeg urednika

Poštovani čitatelji Glasila Future,

pred Vama je specijalno izdanje časopisa posvećeno profesoru emeritusu Bogdanu Cvjetkoviću. Svojim znanstvenim i stručnim radom, koji traje više od pola stoljeća, prof. Cvjetković ostavio je značajan i neizbrisiv trag ne samo u Hrvatskoj nego i svjetskoj fitopatologiji i fitofarmaciji. Ostavljajući iza sebe brojne generacije diplomiranih inženjera agronomije svojim entuzijazmom i predanošću fitopatologiji uspio je „zaraziti“ te biti predani mentor 18 magistara znanosti te 8 doktora znanosti. Širokog znanja i znanstvenih interesa magistrirao je na Prirodoslovno-matematičkom fakultetu u Zagrebu iz području biljne virologije, a doktorirao na svojem *alma mater* Agronomskom fakultetu u Zagrebu kod profesora Josipa Kišpatića na području biljne mikologije. U želji da ovim brojem djelomično oslikamo široko područje interesa prof. Cvjetkovića, ovo specijalno izdanje obuhvaća tri izvorna znanstvena rada te dva prethodna priopćenja iz područja biljne mikologije, bakteriologije te virologije. Kraj ovog specijalnog izdanja posvećen je crticama iz života dr. Željka Jurjevića, jednog od doktora znanosti koji je doktorirao pod mentorstvom prof. Cvjetkovića, a trenutno s uspješnom karijerom u Sjedinjenim Američkim državama (EMSL Analytical, Inc.). Izrazito mi je drago da su se sudjelovanju u ovom broju odazvali znanstvenici koje se bave fitopatologijom na području Hrvatske, ali i kolege iz inozemstva, dajući svoj značajan doprinos kvaliteti ovog specijalnog izdanja, ali i izražavajući pijetet prof. Cvjetkoviću.

Prvi rad kolegica Dušice Kovačević, Katarine Zečević te Ivane Stanković s Poljoprivrednog fakulteta Univerziteta u Beogradu govori o djelomičnoj molekularnoj karakterizaciji izrazito polifagnog virusa mozaika krastavca izoliranoga iz dvije biljke božura sa simptomima mozaika i klorotičnih prstenova. Nakon potvrde virusa serološkim i molekularnim metodama sekvenciranjem dijela genoma proteinskog omotača utvrđeno je da izolati iz božura pripadaju u podgrupu IA. Autorice skreću pozornost da bi božur kao trajnica mogao imati značajnu epidemiološku ulogu u kontekstu značajnog izvora ovog virusa.

Rad kolega Kirila Bahcevandzieva te Antónia A. Monteira (Research Centre for Natural Resources, Environment and Society - CERNAS, Portugal) vodi nas u područje fenotipskih i genotipskih interakcija između različitih kupusnjača te ekonomski značajnog uzročnika plamenjača kupusnjača (*Hyaloperonospora brassicae*). Kroz istraživanje je utvrđeno da izolati navedenog patogena iz različitih područja Europe pokazuju različite stupnjeve patogenosti. Analizirani model gen-za-gen otvara nove mogućnosti istraživanja rezistentnosti kod različitih kupusnjača te gena za patogenost uzročnika plamenjače.

Da su na gljivične patogene osjetljive i invazivne biljne vrste govori rad autora Darija Ivića i Adrijane Novak (Hrvatska agencija za poljoprivredu i hranu). Analizom stabala pajasena sa simptomima

sušenja i propadanja na području Nacionalnog parka Krka utvrđena je prisutnost 15 različitih vrsta polifagnih gljiva iz rodova *Diaporthe*, *Diplodia*, *Dothiorella*, *Fomitiporia*, *Fusarium*, *Paraconiothyrium*, *Peroneutypa*, *Rosellinia*, *Schizophyllum* te *Verticillium*. Autori ističu da je ulogu utvrđenih gljiva u sušenju i propadanju ove invazivne vrste potrebno utvrditi testovima patogenosti.

Prethodno priopćenje doktorice znanosti Katarine Martinko i studentice Ivone Novaković sa Sveučilišta u Zagrebu Agronomskog fakulteta donosi preliminarne rezultate *in vitro* istraživanja protugljivičnog djelovanja esencijalnih ulja timijana, divljeg mažurana i lovora na uzročnika crne truleži plodova različitih poljoprivrednih kultura (*Aspergillus niger* Tiegh.). Autorice zaključuju da prvenstveno eterična ulja timijana i divljeg mažurana imaju veliki potencijal kao fumiganti u kontroli crne truleži uskladištenih poljoprivrednih proizvoda, te kao takvi predstavljaju svojevrsnu alternativu trenutno često korištenim fungicidima.

Prethodno priopćenje doktorice znanosti Jelene Plavec (Hrvatska agencija za poljoprivredu i hranu) opisuje uzročnika bakterioznog paleža lijeske (*Xanthomonas arboricola* pv. *corylina*) utvrđenog metodom lančane reakcije polimerazom iz rasadnika i komercijalnih nasada lijeske na području Hrvatske. Imajući u vidu sve veću popularnost ove kulture u našoj zemlji autorica skreće pozornost da će u budućnosti biti potrebno povesti više pažnje u praćenju ovog ekonomski značajnog patogena svrstanog na listu reguliranih nekarantenskih štetnika ne samo lijeske, već i drugih vrsta iz roda *Corylus*.

Crtice doktora znanosti Željka Jurjevića sažimlju različite dijelove profesionalnog razvoja prof. Cvjetkovića, ali ujedno predstavljaju i jednu toplu životnu priču protkanu zajedničkim trenucima provedenima s profesorom.

Vežući se na životopisne crtice dr. Jurjevića, i osobno kao jedan od doktoranada, mogu reći da bi se o liku i djelu prof. Cvjetkovića mogla napisati ne jedna, nego više knjiga. Na kraju mogu reći da mi je bila iznimna čast i zadovoljstvo intenzivno surađivati s profesorom sve do njegovog odlaska u mirovinu. I danas, sa životopisnim pričama i neograničenim praktičnim iskustvom, dragi mi je sugovornik na Zavodu za fitopatologiju u čiji razvoj je utkao značajno razdoblje svojega života i kojem je dao svoj neprocjenjivi obol!

Prof. dr. sc. Darko Vončina

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Stručno-znanstveni časopis

FUTURA – stručno-znanstvena udruga za promicanje održivog razvoja, kulture i međunarodne suradnje, Bana Josipa Jelačića 13 a, 22000 Šibenik, Hrvatska

(2024) 7(4) 01–68

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Partial molecular characterization of cucumber mosaic virus isolate infecting garden peony (*Paeonia officinalis*) in Serbia

Dušica Kovačević[1]*, Katarina Zečević[1], Ivana Stanković[1]

izvorni znanstveni rad (original scientific paper)

doi: 10.32779/gf.7.4.1

Citiranje/Citation[2]

Abstract

Paeonia officinalis (family Paeoniaceae), known as the garden peony, is a very popular flowering plant with large, showy flowers grown in many gardens in Serbia. In May 2021, peony plants showing chlorotic ringspot and severe mosaic of leaves were observed in a private garden in Zemun (District of Belgrade, Serbia). Symptomatic leaves from two plants were collected and analysed for the presence of cucumber mosaic virus (CMV), tobacco rattle virus (TRV), alfalfa mosaic virus (AMV) and tomato spotted wilt virus (TSWV) using commercial ELISA kits. CMV was detected serologically in both peony samples and no other plant virus was identified. The causal agents from both ELISA-positive samples were successfully mechanically transmissible to *Chenopodium quinoa* and *Nicotiana glutinosa* plants. CMV infection in symptomatic garden peony plants was also confirmed by RT-PCR with CMV-specific primers amplifying the complete coat protein (CP) gene and parts of the 3′- and 5′-UTRs. Selected ELISA-positive sample (318-21) was Sanger sequenced using the same primer as in RT-PCR and phylogenetic tree based on complete CP sequences showed that Serbian CMV isolate from garden peony belongs to the CMV subgroup IA.

Key words: peony, viruses, *Cucumovirus*, ELISA, bioassay, RT-PCR, phylogenetic analysis.

Introduction

Species of the genus *Paeonia* are amongst the most popular garden plants in regions with a temperate climate. They have been cultivated for several thousand years in China and their cultivation spread to many countries, including Serbia. This plant genus is divided into woody and herbaceous species based on their morphological characteristics and life forms (Kamenetsky and Dole, 2012). There are numerous peony hybrids and varieties, each with their own unique characteristics. One of them is *Paeonia officinalis*, the garden peony, which attracts attention with its large flowers. It is known that

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[2] Kovačević, D., Zečević, K., Stanković, I. (2024). Partial molecular characterization of cucumber mosaic virus isolate infecting garden peony (*Paeonia officinalis*) in Serbia. *Glasilo Future, 7*(4), 01–13.

these plants can be infected by various pathogenic microorganisms. Among the phytopathogenic fungi, *Botrytis paeoniae* and *B. cinerea*, which cause bud blast, stand out (Rogers, 1995). In addition to these fungi, phytopathogenic viruses also play an important role and represent serious constraint to garden peony production worldwide. The most common reported virus in peony species is tobacco rattle virus (TRV), which causes peony ring spot disease. Its presence has been confirmed worldwide (Europe, USA, Japan, and New Zealand) (Chang et al., 1976; Robertson et al., 2009; Samuitien et al., 2009). Additionally, alfalfa mosaic virus (AMV) was detected in plants grown in the botanical garden of the University of Parma (Bellardi et al., 2003). In France, cucumber mosaic virus (CMV) was isolated and identified on *P. lactiflora* (Cardin et al., 2010), while tomato spotted wilt virus (TSWV), the species *Orthotospovirus tomatomaculae,* can also lead to infection in plants of this genus. However, the range of viruses that peonies can harbor have been expanded considerably with the development of high-throughput sequencing technology, including grapevine leafroll-associated virus-3 (GLRaV-3), the most recently discovered virus infecting peonies (Mischenko et al., 2023).

CMV (genus *Cucumovirus,* family *Bromoviridae*) is widespread throughout the world and is particularly prevalent in regions with a temperate climate. It is one of the most economically important viruses affecting numerous cultivated plant species worldwide. CMV has a broad host range and infects more than 1200 species from at least 100 plant families. It persists in many perennial cultivated and weed plants, and its spread is facilitated by insect vectors. The transmission primarily occurs in a non-persistent manner by aphids, with over 80 aphid species involved, of which *Myzus persicae* and *Aphis gossypii* are the most efficient. Additionally, transmission via seeds of certain cultivated and weed plants and mechanical sap transmission have also been documented (Palukaitis et al., 1992; García-Arenal and Palukaitis, 2008).

The genome of CMV consists of three linear, positive sense RNAs, designated RNA 1 to RNA 3. RNA 1 and RNA 2 code for viral replicase proteins 1a and 1b, respectively. RNA 2 codes for protein 2b, which is involved in the suppression of gene silencing, expression of symptoms and the spread of pathogen. Bicistronic RNA 3 encodes protein 3a (MP - movement protein), facilitating virus movement within the plant and aphid-mediated transmission. Additionally, it encodes protein 3b, also known as coat protein (CP), which encapsulates RNAs but also enables the cell-to-cell and systemic movement, and aphid transmission (Palukaitis et al., 1992; Jacquemond, 2012).

CMV strains are divided into two subgroups: I and II. Subgroup I is further subdivided into subgroups IA and IB. Isolates of subgroups IA and II are distributed worldwide, while isolates of subgroup IB originate mainly from East Asia, although some isolates have also been found in other parts of the world (Jacquemond, 2012; Giakountis et al., 2018). The virus may also encapsidate a small linear single-stranded satellite RNAs (satRNAs), that may enhance or attenuate symptoms induced by CMV, as has been recorded in lethal necrosis syndrome in tomato plants (García-Arenal and Roossinck,

2019). So far, more than 180 sequence variants of CMV satRNAs are associated with CMV I and II subgroup isolates. They are classified in three main phylogenetic clusters: necrogenic satRNAs, non-necrogenic satRNAs, and larger satRNAs which can be either necrogenic or non-necrogenic (Palukaitis and García-Arenal, 2019).

In Serbia, CMV is one of the most frequently detected and economically most important virus of numerous vegetable and field crops (Stanković et al., 2011; Vučurović et al., 2011, 2012; Milojević et al., 2013b; Milošević et al., 2017; Nikolić et al., 2018; Milošević et al., 2020; Stanković et al., 2021), as well as in various ornamental plant species (Milojević et al., 2013a; Milojević et al., 2014; Milošević et al., 2015; Milojević et al., 2016, ; Zečević et al., 2024). In addition, CMV was recently recorded for the first time in garden peony (Zečević et al., 2023b).

The aim of this study was to determine the genetic relationship of the new Serbian CMV isolate from garden peony with isolates available from GenBank database, including previously identified Serbian isolates. This information is the first step towards a better understanding of the epidemiology of the virus and the development and implementation of appropriate control measures.

Material and methods

Samples collection and serological detection

Two peony plants with virus-like symptoms were noticed in a private garden in Zemun (District of Belgrade, Serbia) in May 2021. Two samples of symptomatic leaves (one sample per plant) were collected and transported to the Laboratory for virology and mycology at Faculty of Agriculture, University of Belgrade.

Collected samples were tested with double-antibody sandwich (DAS)-ELISA using commercially available diagnostic kits (Loewe Biochemica, Sauerlach, Germany) against the common peony viruses: CMV, TRV, AMV and TSWV. Briefly, samples were homogenized with extraction buffer at a ratio of 1:10 (weight/volume) using a cold mortar and pestle. Absorbance at 405 nm was determined using an ELISA microplate reader (DAS srl, Italy). Positive samples had absorbance value that was two times higher than the absorbance of the negative control. Commercial positive and negative controls were included in each ELISA test.

Mechanical transmission

Five plants of each of the two test species Chenopodium quinoa and Nicotiana glutinosa were mechanically inoculated with the crude sap extracted from two ELISA-positive samples (isolates 318-21 and 319-21) using 0.01 M phosphate buffer (pH 7.0) and silicon carbide abrasive. The test plants were inoculated at the 2 to 3 true leaf stage and kept in a greenhouse at 22-25°C for up to four weeks

post-inoculation. The presence of CMV in test plants was verified serologically four weeks post-inoculation.

Molecular detection

Total RNAs Serbian CMV isolates 318-21 and 319-21 were extracted using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and subjected to reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was caried out using the One-Step RT-PCR kit (Qiagen, Hilden, Germany) and primers, CMVCPfwd and CMVCPrev (Milojević et al., 2012), which amplifies an 871-bp fragment of the entire CP gene and parts of the 3′- and 5′-UTRs. Serbian CMV isolate from Cucurbita pepo 'Olinka' (GenBank Accession Number HM065510) was used as a positive control, while the PCR mix with RNase-free water served as a negative control.

RT-PCR was done in a total volume of 25 µl, containing 1x Qiagen OneStep RT-PCR buffer, 400 µM dNTP mix, 0.6 µM of each primer, 1 µl Qiagen OneStep RT-PCR enzyme mix, and 50-100 ng of RNA template. Amplification was performed in the Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific, USA) with the following conditions: reverse transcription at 50°C for 30 min, initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 1 min, with final extension step at 72°C for 10 min. The size of the amplified products was determined by 1% agarose gel electrophoresis in TBE buffer after staining in ethidium bromide (EB) solutions and visualization on ETX-F20.M UV transilluminator (Vilber Lourmat, France).

Detection of CMV satRNAs

Possible presence of satRNAs in two garden peony samples was tested using RT-PCR and primers CMVsat-fwd/CMVsat-rev (Škorić et al., 1996). Serbian CMV satRNAs isolate (KM358138) was used as a positive control, while RNase-free water served as a negative control. RT-PCR reactions were performed in a volume of 25 µl as described previously. PCR amplification of CMV satRNA was performed under the following conditions: 2 cycles at 94°C for 1 min, 42°C for 1 min, 72°C for 1 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The size of the amplified products was determined as described above.

Sanger sequencing and phylogenetic analysis

RT-PCR product obtained from a CMV-positive sample 318-21 was directly sequenced in both directions using the same primers as in RT-PCR assay. The obtained sequence was deposited in GenBank and compared with CMV isolates available using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

Further characterization was performed by reconstruction of phylogenetic tree using 29 complete CP sequences of CMV isolates retrieved from the GenBank (table 1) and the CMV CP sequence generated in this study. Phylogenetic tree was constructed using the maximum parsimony method implemented in MEGA X software (Kumar et al., 2018) with the default parameter applying 1000 rounds of bootstrapping and bootstrap values <60% were omitted. An isolate of peanut stunt virus (Acc. No. U15730) was used as the outgroup sequence. Based on MODELTEST implemented in MEGA X, Kimura 2-parameter model with Gamma distribution (K2+G) was selected for calculation of the diversity within and between subgroups.

Table 1. *Coat protein (CP) gene sequences of cucumber mosaic virus (CMV) used in the phylogenetic analysis.*

Isolates	**Country**	**Host plant**	**GenBank Accession Number**
253-15	Serbia	*Solanum lycopersicum*	KC847071
207-09		*S. lycopersicum*	MN656189
471-09		*Capsicum annuum*	KC847073
581-11		*C. annuum*	KC414926
1-12		*Peperonia tuisana*	KC505441
473-12		*Citrullus lanatus*	KC878465
79-13		*Tulipa* sp.	KJ854451
Trk7	Hungary	*Trifolium repens*	L15336
NS		*Nicotiana glutinosa*	AJ511990
I17F	France	*S. lycopersicum*	Y18137
R		*S. lycopersicum*	Y18138
Ri-8	Spain	*S. lycopersicum*	AM183119
Tfn	Italy	*S. lycopersicum*	Y16926
TN	Japan	*S. lycopersicum*	AB176847
PF		/*	AB368501
Y		*N. tabacum*	D12499
Ly2	Korea	*Lilium longiforum*	AJ296154
NT9	Taiwan	*S. lycopersicum*	D28780
PoCMV7-7	Syria	*S. tuberosum*	AB448695
RZ	China	*N. tabacum*	EF159146
CTL		*Brassica chinensis*	EF213025
Cb7		*S. lycopersicum*	EF216867
Tsh		*S. lycopersicum*	EF202597
Q	Australia	*C. annuum*	M21464
LY		*Lupinus angustifolius*	AF198103
S	South Africa	*Cucurbita pepo*	AF063610
LS	USA	*Lactuca sativa*	AF127976
FNY		*Cucumis melo*	D10538
N1-03		*Vinca minor*	JF918966

* Unknown host plant.

Results and discussion

Symptoms and virus detection using DAS-ELISA

In May 2021, chlorotic ringspot and severe mosaic on leaves (figure 1) were observed on two peony plants grown in a private garden in Zemun (District of Belgrade, Serbia). Serological assays s revealed that CMV was the only virus detected in both collected samples, the new Serbian isolates were identified as 318-21 and 319-21. No other tested viruses were detected.

CMV has a wide host range, including numerous ornamental plants (Palukaitis et al., 1992; García-Arenal and Palukaitis, 2008). The symptoms caused by CMV as well as the severity of the disease vary depending on CMV molecular characteristics, including the presence of a satellite RNA, the host genotype, the growth stage, the time of infection, and environmental factors (Mochizuki, 2012; Zhao et al., 2016). In some cases, CMV infection may occur asymptomatic. Conversely, it can lead to systemic necrosis (Jacquemond, 2012). Generally, in ornamental plants, CMV most commonly induces mosaic. Additionally, ring spots, mottling of flowers, bud necrosis, and stunted growth of plants may occur (Ashfaq et al., 2017). On *Peonia lactifera* species, CMV causes very pronounced mosaic and the appearance of chlorotic rings (Cardin et al., 2010), which were also recorded in this study.

Figure 1. *Symptoms of cucumber mosaic virus (CMV) infection recorded in garden peony from Zemun (Serbia) with severe mosaic and chlorotic rings. (PHOTO: I. Stanković, 2021).*

Bioassay

In order to biologically characterize the CMV ELISA-positive isolates 318-21 and 319-21, crude sap extract from symptomatic peony plants were used to mechanically inoculate five plants each of two species: *C. quinoa* and *N. glutinosa*. All inoculated plants reacted uniformly and showed characteristic symptoms of CMV infection, which is in correlation with previous reports (Milojević et al., 2013b, 2014; Choi et al., 2015). Local chlorotic spots in mechanically inoculated *C. quinoa* and severe mosaic and leaf malformations in *N. glutinosa* plants were noticed seven- and 14-days post-inoculation, respectively. CMV infection in all mechanically inoculated plants was confirmed using DAS-ELISA.

RT-PCR assay

The result of RT-PCR testing revealed that both symptomatic garden peony samples yielded an amplicon of 871 bp confirming the presence of CMV. The primer pair CMVsat-fwd/rev was unable to amplify amplicons from both garden peony CMV isolates, indicating that no satRNA sequences are associated with selected CMV isolates.

Various pathogenic microorganism can cause serious economic losses in ornamentals industry, but viruses are a major constraint for most ornamental plants, especially for species which are exclusively vegetatively propagated due to the accumulation of viruses in propagative material (Valverde et al., 2012; Mitrofanova et al., 2018). CMV is the plant virus with the broadest host range, infecting several agriculturally important crops such as tomato, tobacco, cucurbits, and legumes, as well as a variety of ornamental plants (Palukaitis et al., 1992). So far, CMV has only been detected in *P. lactiflora* in France (Cardin et al., 2010) and this study is the second detection of the virus in peony plants.

Sequence analysis and phylogeny

The RT-PCR product of the selected isolate 318-21 was purified and bi-directional sequenced as described above (PP818664). BLAST results showed that CP sequence of the new Serbian CMV isolate has the highest nt identity of 99.54% with Serbian isolate 514-11 (KT270567) from *Cucumis sativus*.

Phylogenetic tree (Figure 2) showed clustering of the selected isolates into tree subgroups IA, IB and II supported by bootstrap values of 100% and an overall level of nucleotide diversity of 0.256±0.019. Subgroup I was further subdivided into two subgroups, IA and IB. The genetic diversity among subgroups ranged from 0.0553±0.0068 to 0.3470±0.0370, whereas diversity within each group was lower (IA-0.022±0.003; IB- 0.054±0.007; II-0.012±0.002). The Serbian CMV isolate originating from garden peony belonged to subgroups IA. According to the previous study on the genetic population in Serbia (Milošević et al., 2017; Stanković et al., 2021; Vučurović et al., 2012; Zečević et al., 2023a), the isolates of CMV subgroup IA are widespread. Therefore, the majority of isolates worldwide belong

to subgroup IA (Roossinck, 2002; Bonnet et al., 2005; García-Arenal et al., 2008; Jacquemond, 2012). Subgroup II isolates have also been found in ornamental plants in our country (Milošević et al., 2015) and tomato (Stanković et al., 2021), but their frequency is much lower. The characterization of CMV isolates is most often based on the CP gene (Roossnick, 2002), but recent studies have shown the importance of genetic characterization based on each of the three RNAs, because genetic recombination and reassortment events are significant for evolution of viruses with multiple genomes as CMV (Lin et al., 2003; García-Arenal and Palukaitis, 2008). This is only a partial characterization of CMV isolate from garden peony. Further research should be carried out to determine the variability within the CMV population in Serbia, based on the analysis of all five genes of CMV. This will provide more precise information on its population structure in our country.

Considering the fact that garden peonies are widespread and popular garden plants in Serbia, the presence of CMV could be a limiting factor for the cultivation of these plants. In addition, this finding has significant implications for the successful production of various ornamental plants, since peonies, as perennial plants, serve as a virus reservoir and additional source of inoculum and represent an important link in the epidemiology of CMV.

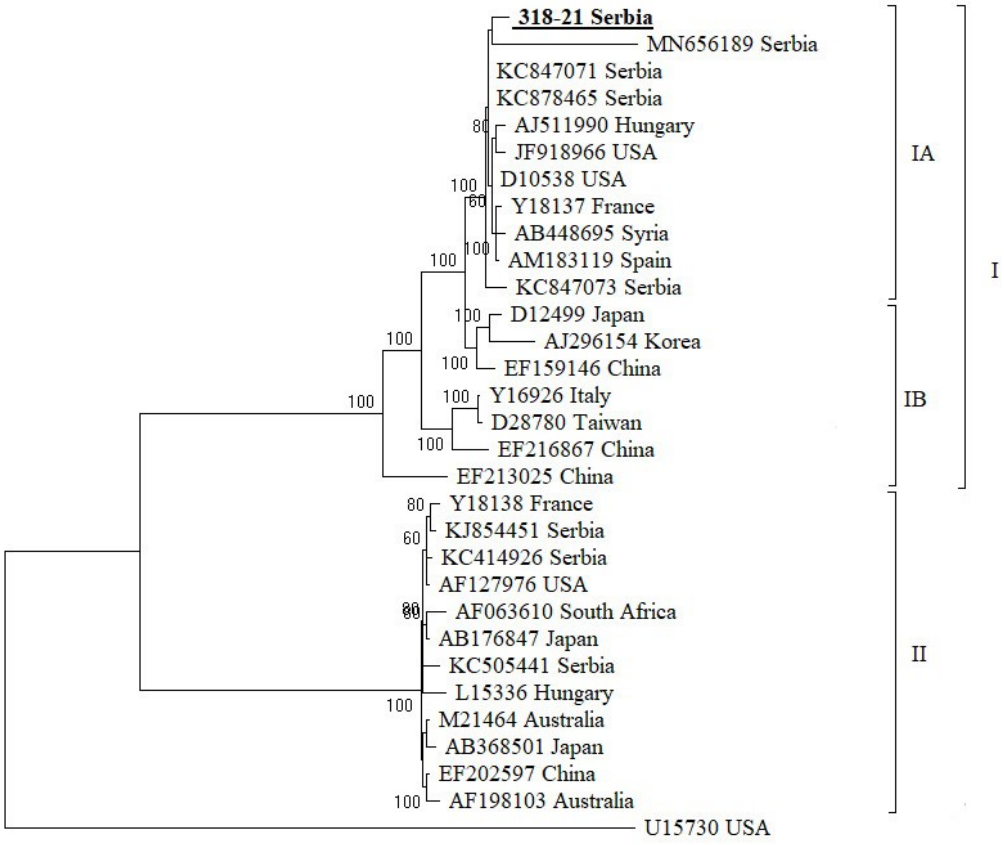

Figure 2. *Phylogenetic tree comparing on the complete CP sequences of 30 cucumber mosaic virus (CMV) isolates using MEGA X and 1,000 interations. Bootstrap values greater than 60% are indicated on the corresponding branches. The CMV isolate from this study is underlined and in bold.*

Conclusion

Having previously reported the occurrence of CMV in garden peony (Zečević et al., 2023b), we have now partially characterized the virus for the first time by Sanger sequencing and phylogenetic analysis of the complete CP gene. The new Serbian isolate, which originates from the garden peony, belongs to the subgroup IA. This is epidemiologically very important because garden peony, as a new perennial host of CMV, could represent a significant reservoir of the virus and an additional source of inoculum in our country. Due to the great damage CMV causes to various hosts worldwide, including many vegetable and ornamental plants, constant control and monitoring of the virus is necessary.

Acknowledgements

This research was supported by grant of Ministry of Science, Technological Development, and Innovation, Republic of Serbia (number 451-03-65/2024-03/200116).

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Primljeno: 18. svibnja 2024. godine. ***Received:*** *May 18, 2024.*

Prihvaćeno: 24. rujna 2024. godine. ***Accepted****: September 24, 2024.*

Identification of *Brassica oleracea* accessions with specific reaction to *Hyaloperonospora brassicae* isolates

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izvorni znanstveni rad (original scientific paper)

doi: 10.32779/gf.7.4.2

Citiranje/Citation[3]

Abstract

The variability or polymorphism of the response between the host and the parasite is controlled by the gene-to-gene system, which is responsible for the "genetic wars" between the host and parasite populations, the mechanism by which the host recognizes a locus or some loci particulars in the parasite. A standard set of *B. oleracea* plants at the cotyledon stage, which could aloww a reproducible classification of *H. brassicae* pathotypes, is poorly characterised. The aim of this study was to identify differentiating plants selected from a collection of *B. oleracea* accessions characterised by possible specific reactions with *H. brassicae* isolates to verify the existence of races among the isolates and to serve as a basis for differentiation among brassica cultivars. The experiment was divided into two tests to evaluate the host-isolate interaction. In the first test, the interactions between thirteen brassica accessions and three *H. brassicae* isolates were evaluated. In the second test, sixteen accessions were inoculated with eight different isolates and analysed. Seven-day-old seedlings were inoculated by applying two 10 µl drops of the spore suspension of the different isolates to each cotyledon. The resistance found in some accessions showed different frequencies with respect to the isolates, ranging from 10% (HRI4302 with H501 and CGN18451 with Hb006) to 93% (ISA 207 with Hb006). In the second test in group D2, accession KB01 showed specific resistance to Hb005, in D3 accession KB091 was resistant to isolate Hb-FP06 and in group D4, accessions KB14/00, KB566 and KB092 showed resistance to isolate Hb-FP06. Two gene-to-gene models presented in this work were designed to explain the relationships between differentiated accessions and *H. brassicae* isolates. These resistant accessions were considered as potential sources of downy mildew resistance and for that purpose need to be genetically characterised and further exploited in breeding programmes.

Key words: host resistance, *Brassica oleracea*, downy mildew, cotyledon inoculation, gene-to-gene.

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Introduction

The phenotypic expression of the interaction between the host and the pathogen depends on the interaction between the genotypes of the two in an environment that favours their development. Variations in this phenotypic expression may result from individual characteristics of the host and/or pathogen. This interaction is so close that it is sometimes difficult to recognize whether the parasite influences the host or the host influences the performance of the parasite (Lebeda and Schwinn, 1994).

Hyaloperonospora brassicae (syn. *Hyaloperonospora parasitica* subs. *brassica*) causing a downy mildew in brassica crops, is an economically destructive disease and can be found in many regions of the world where these crops are grown. Comparison of the pathogenic variability of *H. brassicae* isolates with the interaction phenotype of brassica plants is a likely indicator for finding accessions with specific reactions to the isolates, and provides a basis for defining whether potential parasite races exist.

Brassica oleracea plants from different regions of the world show different interaction phenotypes with *H. brassicae* isolates (Moss et al., 1991; Dias et al., 1993; Nashatt and Awasthi, 1995; Nashatt and Rawlinson, 1994; Branca et al., 2005; Coelho et al., 2012), which has been a challenge to better define the behaviour of this parasite.

Some variability in the pathogenicity of *H. brassicae* isolates to *Brassica oleracea* plants has been reported (Natti et al., 1967; Monteiro and Williams, 1989; Ohguchi et al., 1990; Dias et al., 1993a; Silué et al., 1995; Leckie et al., 1996; Masheva, 1996a; Masheva, 1996b; Silué et al., 1996; Sousa, 1996 and Jensen et al., 1999; Branca et al., 2005; Coelho et al., 2012), recognizing the existence of potential races of this parasite in Europe and the USA (Gaumann, 1918; Gaumann, 1926; Felton and Walker, 1946; Wang, 1949; Natti et al., 1967; McMeekin, 1969, Thomas and Jourdain, 1990; Thomas and Jourdain, 1992). This specific interaction between *H. brassicae*, and different Brassica hosts was also observed in *Arabidopsis* ecotypes (Koch and Slusarenko, 1990).

The evaluation of resistance of nuclear collection accessions (Bahcevandziev, 2003) showed the possibility of heterozygote interaction phenotypes with *H. brassicae* isolates. These accessions showed that among *B. oleracea* plants there is a specific reaction material capable of producing good sources of differentiating plants. The study of such variable material is only possible if there is a host-specific parasite reaction by comparing different isolates in relation to a defined host (Monteiro at al., 2005; Coelho et al., 2018).

In brassicas it is possible to apply two isolates to the same seedling, each isolate being inoculated on a different cotyledon. This technique allows an analysis of pathogenicity differences between the isolates and the potential hosts with differentiating characteristics (Bahcevandziev, 2003).

The evaluation methods used to define the presence of potential races in *H. brassicae* were based on numerical scales (Thomas and Jourdain, 1990; Thomas and Jourdain, 1992; Silué et al., 1995; Silué et al., 1996), observing the phenotypic host response (Wang, 1949; Natti et al., 1967) or simply in the form of *H. brassicae* spores (Gauman, 1918; Gauman, 1926; McMeekin, 1969). To date, no author has conducted a systematic study to detect potential *H. brassicae* races on different *B. oleracea* accessions.

The interaction between brassica hosts and *H. brassicae* isolates is considered specific because it is qualitative in nature. To evaluate this type of interaction, a scale with discrete classes based on the description of the interaction phenotype (IP) should be used. This evaluation mode enables the detection of genes leading to specific IP. This type of evaluation facilitates more detailed distinction between different isolates and better determines hosts as potential differentiators.

The aim of this study was to identify differentiating plants, selected from a collection of *B. oleracea* accessions characterized by possible specific reactions with *H. brassicae* isolates, to verify the existence of putative strains among the isolates and to serve as a basis for differentiation between brassica cultivars.

Material and methods

The work consisted of two evaluation tests of the host-isolate interaction. In the first test, interactions between thirteen accessions and three *H. brassicae* isolates (Tables 1 & 2) were evaluated. In the second test, sixteen accessions were inoculated with eight different isolates and analysed (Tables 4 & 5).

Two tests were performed to confirm the existence of plants with a differential reaction and to compare Portuguese isolates with those from Europe, which already showed different molecular characteristics (Casimiro, 2001).

Seedling inoculation

The *H. brassicae* inoculum was prepared by placing sporulated cotyledons of the susceptible Portuguese variety ‘Coração de Boi’ (*Brassica oleracea* var. *capitata*), separated from the plantlets and used as a maintenance stock, in 40 ml of distilled water with agitation for 2 min with a vortex to dislodge the conidia. The spore suspension was filtered through cheesecloth and centrifuged at 2,000 rpm for 3 min. The supernatant was discarded, and the pellet re-suspended in distilled water and centrifuged again. This was repeated two times. Finally, the spore concentration was adjusted to 5×10^4 spores/ml with a haemocytometer (Neubauer Improved). Seeds from three brassica host plants were sown in multicell trays (3×5×5 cm cells) filled with peat-based substrate Levington M2 (Fysons, UK) and maintained in a growth room at 20±1°C, 70% RH, 20h photoperiod under cool white light (Osram) at 250 μmol m^{-2} s^{-1}. The emerged seedlings were randomly thinned to one seedling per cell. Cotyledons of seven-day-old seedlings were inoculated with a micropipette by applying two 10 μl

drops of the spore suspension. One of the two cotyledons was marked with a pinch, to separate the isolates. Normally, the pinched cotyledon was inoculated with a Portuguese isolate when possible. After inoculation each tray was enclosed in a black polyethylene bag and placed in an incubation chamber at 16± 1°C and 100% RH to induce infection of the cotyledons with the isolate. In the case of Hb005, CrGC 3.4 (*Brassica oleracea* var. *acephala*) seedlings were used for inoculum preparation, as Portuguese variety 'Coração de Boi' showed resistance to this isolate.

1st experiment

The three isolates used in this experiment were Hb501, Hb005 and Hb006 (Table 1). For this test six styrofoam trays were used as repetition. In each tray seeds from thirteen accessions were sown with 10 seedlings each, randomly distributed, thus occupying 130 cells per tray, totalling 60 seedlings/accession. Inoculations were performed by applying two different isolates on the cotyledons of the same seedling, placing one drop of the conidia suspension from each isolate on a respective cotyledon. Thus, each plant was inoculated simultaneously with two isolates (Hb501 x Hb005; Hb501 x Hb006; Hb005 x Hb006). This facilitated the analysis of the differences that might occur between the isolates with respect to their pathogenicity in the same accession.

Table 1. *Isolates of Hyaloperonospora brassicae (Hb) used in the 1st experiment.*

Isolate	Origin
Hb501	ISA (Portugal)
Hb005	HRI Wellesbourne (United Kingdom)
Hb006	HRI Wellesbourne (United Kingdom)

The 'Coração de Boi' (*Brassica oleracea* var. *capitata*) is a commercial variety (Soares and Rebelo, Portugal) while CrGC 3.4 (*Brassica oleracea* var. *acephala*) was used as a control plant.

Table 2. *Brassica oleracea accessions used in the 1st experiment.*

Nr	Code	Variety	Comon name	Origin
1	ISA 207	*Tronchuda*	Couve Algarvia	Portugal
2	HRI 4302	*Acephala*	Covo	Zimbabwe
3	HRI 5389	*Gongylodes*	Cavolo	Italy
4	HRI 5443	*Gongylodes*	Cavolo forte	Italy
5	HRI 5555	*Acephala*	Arsis Ra F1	Netherland
6	HRI 5652	*Capitata*	Shetland cabbage	United Kingdom
7	HRI 6226	*Acephala*	Giant Gersey kale	United Kingdom
8	HRI 6254	*Botrytis*	Tasman	Australia
9	CGN 18450	*Capitata*	Raketa	Chech Republic
10	CGN 18451	*Capitata*	Predzvest	Chech Republic
11	Commercial	*Capitata*	Coração de Boi	Portugal
12	ISA 62	*Tronchuda*	Murciana	Portugal
13	CrGC 3.4	*Acephala*	Rapid Cycling Brassica	USA

Resistant phenotype was defined by the appearance of necroses (mild and/or dense) on the adaxial side of the cotyledons, with no sporulation on the abaxial side. On the other hand, conidiophores formation on the abaxial side was considered as a susceptible phenotype (Table 3). The same scale was used in both tests.

Table 3. *Scale used to evaluate host x parasite interaction.*

Reaction to the parasite	DI	IP	Characteristics
Resistant	**0**	**NN**	Lack of host response and sporulation
	1	**HN**	Small necrosis; absence of sporulation
	3	**FN**	Necrosis confined to the inoculation site; absence of sporulation
Susceptible	**5**	**SS**	Necrosis confined to the inoculation site; weak sporulation (up to 5 conidiophores), confined to the inoculation site
	6	**CS-C**	Necrosis confined to the inoculation site; sporulation confined to the site of inoculation
	7	**CS-D**	Absence of localized necrosis; sporulation dispersed throughout the cotyledon
	9	**HS**	Absence of localized necrosis; abundant and dispersed sporulation throughout the cotyledon

DI – disease index; IP – interaction phenotype.

2nd experiment

In this assay, eight *H. brassicae* isolates (Table 4) originated from various countries were used to inoculate sixteen brassica accessions (Table 5).

The accessions used in this second test were sown on six styrofoam trays (repetitions). In each tray sixteen accessions were randomly distributed with 10 seedlings per tray. Two different isolates were inoculated onto the same seedling, thus adding 60 seedlings/accession/isolate. The inoculations were performed as described in the 1st experiment.

Table 4. *H. brassicae (Hb) isolates used in the 2nd experiment.*

	Isolate	Origin
1	Hb501	ISA (Portugal)
2	Hb005	HRI Wellesbourne (UK)
3	Hb006	HRI Wellesbourne (UK)
4	Hb-FP06	University of Bretagne (France)
5	Hb-Italian	Universidade of Catánia (Italy)
6	Hb-Murcia	Nunhems (Germany)
7	Hb502	ISA (Portugal)
8	Hb517	ISA (Portugal)

Some of the accessions used in this assay resulted from resistant plants selected from the nuclear collection assessments (Bahcevandziev, 2003), which were then self-pollinated (S2 and S3) or crossed (F2 and F3). Therefore, these accessions carry the same codes as their parents, (ISA 62, ISA 207, HRI 5443, and HRI 4302). The KB 91 and KB 92 accessions were provided by prof. Ales Lebeda from the University

of Prague (Czech Republic). The KB 4/00 accession was a traditional variety. The accessions 'Coração de Boi' (*Brassica oleracea* var. *capitata*), (Soares and Rebelo, Portugal),'Caramba' (*Brassica oleracea* var. *capitata*) (Germiplanta, Portugal), 'Beira' (*Brassica oleracea* var. *tronchuda*) (Germiplanta, Portugal) and 'Hunter Hybrid' (*Brassica oleracea* var. *botrytis*) (Yates, Australia) were the only commercial varieties used in the trial. The CrGC 3.4 accession was used as a control plant. (Table 5).

Table 5. *Brassica oleracea accessions used to identify the differentiator sources.*

Nº	Code	Variety	Common name	Origin/cross
1	**KB 207/00**	*tronchuda*	Couve Algarvia (S_3)	Portugal / S_3 ISA207 (FN)
2	**KB 62/00**	*tronchuda*	Murciana (S_3)	Portugal / S_3 ISA 62 (HS)
3	**KB 01**	*capitata*	Coração de Boi	Portugal / Soares e Rebelo
4	**KB 85**	*capitata*	Caramba	Portugal / Germiplanta
5	**KB 87**	*tronchuda*	Beira	Portugal / Germiplanta
6	**KB 4/00**	*tronchuda*	Tronchuda (S_2)	Portugal / S_2 Horto (FN)
7	**KB 5/00**	*gongylodes*	Cavolo forte (F_2)	F_2 (CrGC x HRI 5443) (FN)
8	**KB 14/00**	*tronchuda*	Couve Algarvia (F_2)	F_2 (CrGC x ISA 207) (FN)
9	**KB 91**	*capitata*	Predzvest	Univ. Praga / Chech Rep.
10	**KB 92**	*capitata*	Raketa	Univ. Praga / Chech Rep.
11	**KB 10/00**	*gongylodes*	Cavolo forte (F_3)	F_3 (CrGC x HRI 5443) (FN)
12	**KB 16/00**	*acephala*	Covo (F_2)	F_2 (CrGC x HRI 4302) (FN)
13	**KB 11/00**	*gongylodes*	Cavolo forte (F_3)	F_3 (CrGC x HRI 5443) (FN)
14	**KB 13/00**	*gongylodes*	Cavolo forte (F_3)	F_3 (CrGC x HRI 5443) (SS)
15	**KB 566**	*botrytis*	Hunter Hybrid	Austrália / Yates
16	**CrGC 3.4**	*acephala*	Rapid Cycling Brassica	USA

Interaction phenotype assessments were performed by applying the same scale defined in the 1st experiment (Table 3).

Trial design and statistical analysis of the obtained results

Sampling and distribution of accessions in both trials was performed in randomized blocks with 6 replicates, testing a total of 60 plants per accession. To compare the pathogenicity of the isolates, the percentage of susceptible plants was calculated in relation to the total number of plants infected with the same isolate. The aggressiveness of the isolates was analyzed by calculating the disease index (DI) using the analysis of variance (ANOVA) from STATISTICA, version 12.0. The Scheffé test was applied to compare the means of accession DI.

Results

1st experiment

The IP evaluation showed a clear interaction between host and isolate genotypes. 5 reaction groups were identified, which were designated as G1, G2, G3, G4 and G5 to facilitate analysis of the results (Tables 6 & 7).

A resistant accession was considered when more than 10% of the seedling/accession had a resistant phenotype. With more than 10% of resistant plants in an accession, the systematic behaviour of the accession was considered, thus showing the presence of genes responsible for the determination of resistance in the host. Susceptible accessions were classified in group G1 and accessions that were resistant to the three isolates were classified in group G5 (Table 6).

Table 6. *B. Oleracea accession groups with differentiator reaction to the isolates.*

Groups	**Characteristics**
G1	Accessions susceptible to all isolates
G2	Accessions susceptible to isolates H501 and H006
G3	Accessions susceptible to isolates H501 and H005
G4	Accessions susceptible to isolate H501
G5	Accessions resistant to all isolates

Four genotypes resistant to one of the isolates (groups G2 and G3) were observed. The commercial 'Coração de Boi' variety showed resistance to Hb005 (Group G2), while accessions CGN 18451, HRI5652 and CGN18450 were only resistant to Hb006 (Table 7).

Table 7. *Accessions distributed in groups with differentiating reactions, the percentages of seedlings resistant (-) to the three H. brassicae isolates and the DI.*

Group	**Accessions**	**Percentage of seedlings (%) resistant to respective isolate ***		
		Hb501	**Hb005**	**Hb006**
		(+)	(+)	(+)
G1	CrGC 3.4	0	0	0
	ISA 62	0	0	0
		(+)	(-)	(+)
G2	Coração de Boi	3	70	0
		(+)	(+)	(-)
G3	CGN 18451	3	0	10
	HRI 5652	0	0	12
	CGN 18450	0	4	58
		(+)	(-)	(-)
G4	HRI 5555	0	20	13
	HRI 6254	8	27	27
		(-)	(-)	(-)
	HRI 4302	10	47	59
	HRI 5389	15	34	37
G5	HRI 6226	20	12	34
	HRI 5443	39	67	43
	ISA 207	66	82	93
	DI**	**6,9 a**	**6,1 b**	**6,3 b**

* (-) resistant; (+) susceptible.

** In each sub-column the mean values followed by the same letter did not differ significantly (P≤0.05).

Among the 21 accessions analysed in this test, no plants resistant only to the Portuguese isolate and susceptible to the two UK isolates were found. Host-isolate interactions in resistant plants showed the existence of resistance genes (Table 8). These genes were expressed through phenotypes with necrosis and without any sporulation.

Table 8. *Presence of possible resistance genes to the isolates of H. brassicae in B. oleracea accessions analysed in the 1st experiment.*

Group	**Accessions**	**H. brassicae isolates ***		
		Hb501	**Hb005**	**Hb006**
G1	CrGC 3.4 ISA 62	0	0	0
G2	Coração de Boi	0	1	0
G3	CGN 18451 HRI 5652 CGN 18450	0	0	1
G4	HRI 5555 HRI 6254	0	1	1
G5	HRI 4302 HRI 5389 HRI 6226 HRI 5443 ISA 207	1	1	1

***** (1) Presence of resistance gene (s); (0) lack of resistance gene (s)

To elucidate the relationship between differentiating accessions and H. brassicae isolates, Table 9 was constructed. This model posits the involvement of three resistance (R) genes and three corresponding avirulence (Avr) genes.

Table 9. *Gene-to-gene relationship between the accessions and the three H. brassicae isolates.*

					Isolates with avirulence genes (A)		
					Hb501	**Hb005**	**Hb006**
Group	**Accessions**	**Resistance Genes (R)**			**A1** **.** **.**	**.** **A2** **.**	**.** **.** **A3**
G1	CrGC 3.4 ISA 62	**.**	**.**	**.**	+	+	+
G2	Coração de Boi	**.**	**R2**	**.**	+	**-**	+
G3	CGN 18451 HRI 5652 CGN 18450	**.**	**.**	**R3**	+	+	**-**

G4	HRI 5555 HRI 6254	**.**	**R2**	**R3**	+	**-**	**-**
G5	HRI 4302 HRI 5389 HRI 6226 HRI 5443 ISA 207	**R1**	**R2?**	**R3?**	**-**	**-**	**-**

+ = compatible reaction (susceptibility); - = incompatible reaction (resistance).

The r1 gene present in hri 4302, hri 5389, hri 6226, hri 5443 and isa 207 confers resistance to isolate hb501 which has the avirulence gene a1.The 'Coração de boi' gene r2 confers resistance to isolate hb005, the latter with avirulence gene a2 and the same gene confers resistance in accessions hri5555 and hri6254 to isolate hb005, which has the same avirulence gene a2. The r2 gene is probably found in the g5 group accessions, where it expresses incompatible interactions with the same isolate hb005, this with the a2 avirulence gene. The r3 gene characterizes the hri5652, cgn18450 and cgn18451 accessions and confers resistance to isolate hb006, which has the avirulence gene a3. The r3 gene is present in the g4 group and probably in the g5 group accessions, where it expresses interactions incompatible with the hb006 isolate (Table 9).

2nd experiment

The accessions that had more than 10% of the seedling/accession with the hn and fn interaction phenotype were considered resistant and therefore carrying possible resistance genes.

Based on the host-isolate interaction, the accessions were divided into 8 groups (Table 10). The accessions belonging to each of these groups were characterized by differential and specific reactions with the isolates.

Table 10. *Groups of B. oleracea accessions with differential reaction to the H. brassicae isolates.*

Groups	Characteristics
D1	Accessions susceptible to all isolates
D2	Accessions resistant to isolate 2
D3	Accessions resistant to isolate 4
D4	Accessions resistant to isolate 5
D5	Accessions resistant to isolates 2, 4 and 5
D6	Accessions susceptible to isolates 1, 3 and 6
D7	Accessions susceptible to isolates 7 and 8
D8	Accessions resistant to all isolates

In D1 group were placed susceptible accessions on the eight isolates. Five accessions from D2, D3 and D4 groups showed resistance to a different isolate. The segregation of resistance found in KB 4/00 (Group D5) showed that its genotype recognized three of the eight isolates used. The accessions from group D6 and those from D7 showed greater differentiation from the eight isolates (Table 11).

The resistance to the eight H. brassicae isolates characterized the accessions from group D8, where 'Cavolo forte' (HRI 5443) offspring predominated (Table 11).

Table 11. *Sixteen accessions distributed in groups with differential reactions and the percentages of seedlings resistant (-) to the eight H. brassicae isolates, characterized by their respective ID.*

		Percentage (%) of seedlings resistance to each isolate*							
Gr.	**Accessions**	**Hb501**	**Hb005**	**Hb006**	**Hb-FP06**	**Hb-Italian**	**Hb-Murcia**	**Hb502**	**Hb517**
D1		(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
	CrGC 3.4	0	0	0	0	0	0	0	0
	KB 62/00	0	0	0	0	0	0	0	0
D2		(+)	(-)	(+)	(+)	(+)	(+)	(+)	(+)
	KB 01	0	45	0	0	0	0	0	0
D3		(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)
	KB 091	0	0	0	15	0	0	0	0
D4		(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)
	KB 092	0	0	0	0	16	0	0	0
	KB 566	0	0	0	0	21	0	0	0
	KB 14/00	0	0	0	0	42	0	0	0
D5		(+)	(-)	(+)	(-)	(-)	(+)	(+)	(+)
	KB 4/00	0	30	0	20	33	0	0	0
D6		(+)	(-)	(+)	(-)	(-)	(+)	(-)	(-)
	KB 11/00	0	31	0	35	67	0	21	19
D7		(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)
	KB 16/00	60	26	15	25	50	43	0	0
	KB207/00	53	44	41	38	63	27	0	0
D8		(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
	KB 5/00	15	60	35	35	85	28	37	38
	KB 10/00	28	74	85	74	95	47	67	67
	KB 13/00	19	29	22	55	58	29	29	19
	KB 85	78	80	95	22	70	53	52	53
	KB 87	78	18	90	18	84	59	82	85
DI**		**6,4ab**	**5,5ab**	**5,9ab**	**5,8ab**	**5,2a**	**6,1ab**	**6,7b**	**6,6b**

* (-) resistant; (+) susceptible; ** In each sub-column the mean values followed by the same letter did not differ significantly (P≤0.05).

The pathogenicity, recognized by DI, matched with the degree of virulence of the isolates. Isolates Hb502 and Hb517 showed the highest pathogenicity and were significantly different from Hb-Italian. The pathogenicity of the remaining five isolates was similar, showing no significant differences with the three isolates reported (Table 11).

For resistance, accessions with differentiating reaction were observed. In group D2 there was specific resistance in accession KB01 to Hb005, in group D3 accession KB091 was resistant to isolate Hb-FP06 and in group D4 accessions KB14/00, KB566 and KB092 expressed resistance to isolate Hb-Italian (Table 11). The accessions of these three groups showed different degrees of resistance (% resistant plants) in relation to their isolates.

Group D5 was characterized by specific resistance expressed in KB4/00 to isolates Hb005, Hb-FP06 and Hb-Italian, while in group D6 specific resistance was observed in the accession KB11/00 in relation to Hb502 and Hb517 (Table 11).On the other hand, accessions from group D7 showed specific resistance to isolates. Therefore, accessions KB207/00 and KB16/00 showed resistance type with Hb501, Hb005, Hb006, Hb-FP06, Hb-Italian and Hb-Murcia. In group D8 accessions, non-specific resistance was observed. The Hb-Italian isolate showed a high incompatibility with all accessions in this group (Table 11).

The virulence of the isolates was defined by the distinguishing characteristics of the accessions in relation to the isolates. When evaluating the degree of virulence (number of accessions resistant to the respective isolate), the three Portuguese isolates showed a higher virulence compared to the other isolates. To this group we can add Hb-Murcia and Hb006, which had almost the same degree of virulence with the accessions. The least virulent of all was the Hb-Italian.Within the groups, accessions KB5/00 and KB10/00 were identified that presented more stable resistance in relation to the eight isolates (Table 11).

The differentiating reactions in the accessions related to 8 isolates were expressed by the incompatible interaction phenotype which was characterized by necrosis on the adaxial side and no sporulation on the abaxial side of the cotyledons. These interactions in resistant accessions showed the existence of resistance genes (Table 12).

Table 12. *Possible presence of resistance genes to eight H. brassicae isolates in the sixteen B. oleracea accessions analysed in the 2nd experiment, characterized with their degree of virulence.*

		H. brassicae isolates*				
Group	**Accessions**	**Hb502 Hb517**	**Hb501 Hb006 Hb-Murcia**	**Hb005**	**Hb-FP06**	**Hb-Italian**
D1	**CrGC 3.4 KB 62/00**	0	0	0	0	0
D2	**KB 01**	0	0	1	0	0
D3	**KB 091**	0	0	0	1	0
D4	**KB 092 KB 566 KB 14/00**	0	0	0	0	1
D5	**KB 4/00**	0	0	1	1	1
D6	**KB 11/00**	1	0	1	1	1

D7	**KB 16/00 KB 207/00**	0	1	1	1	1
D8	**KB 5/00 KB 10/00 KB 13/00 KB 85 KB 87**	1	1	1	1	1
	Degree of virulence	**6**	**7**	**10**	**10**	**12**

***** (1) presence of resistance gene(s); (0) lack of resistance gene(s).

To try to explain the differentiating relationships between the 16 accessions and the eight H. brassicae isolates, a model presented in Table 13 was created. This model shows the expression of 5 resistance genes in the accessions and five avirulence genes in the isolates.

Table 13. *Gene-to-gene relationships between sixteen B. oleracea accessions and eight H. brassicae isolates.*

							Isolates with avirulence gene (A)			
							Hb502 Hb517	**Hb501 Hb006 Hb-Murcia**	**Hb005**	**Hb-FP06**	**Hb-Italian**
							A1	**. A2 . . .**	**A1 A2 A3 . .**	**A1 A2 . A4 .**	**A1 A2 . . A5**
Gr.	**Accession**	**Resistance genes (R)**									
D1	**CrGC 3.4 KB 62/00**	**.**	**.**	**.**	**.**	**.**	+	+	+	+	+
D2	**KB 01**	**.**	**.**	**R3**	**.**	**.**	+	+	-	+	+
D3	**KB 091**	**.**	**.**	**.**	**R4**	**.**	+	+	+	-	+
D4	**KB 092 KB 566 KB 14/00**	**.**	**.**	**.**	**.**	**R5**	+	+	+	+	-
D5	**KB 4/00**	**.**	**.**	**R3**	**R4**	**R5**	+	+	-	-	-
D6	**KB 11/00**	**R1**	**R2 ?**	**.**	**.**	**.**	-	+	-	-	-
D7	**KB 16/00 KB 207/00**	**R1 ?**	**R2**	**.**	**.**	**.**	+	-	-	-	-
D8	**KB 5/00 KB 10/00 KB 13/00 KB 85 KB 87**	**R1**	**R2**	**.**	**.**	**.**	-	-	-	-	-

+ = compatible reaction (susceptibility); - = incompatible reaction (resistance).

Discussion

The aim of combining of *H. brassicae* isolates from different European countries and analyzing them in the same laboratory under uniform conditions was to compare the virulence of the isolates and to verify whether these parasitic *H. brassicae* races are putative.

The *B. oleracea* accessions showed great variability in the interaction between the host and *H. brassicae* isolates. According to Milgroom and Fry (1997), host-parasitic interaction variability characterizes hosts as genetically heterogeneous populations. Regarding the variability in the reaction with the respective isolates, the accessions in the two tests were divided into 5 (first test) and 8 groups (second test) after evaluation.

The accessions belonging to groups 2, 3 and 4 (first test), and those from groups 2, 3, 4, 5, 6 and 7 (second test) showed a different reaction with the isolates due to host-parasite interactions. These accessions recognized different isolates. This recognition is in agreement with Beynon (1997), who considers that certain plants have the particular resistance gene that is able to recognize some isolates (or races), while others do not. According to this author, the parasite has genes with which it interacts with host genes and this interaction can lead to resistance or susceptibility.

The commercial variety 'Coração de Boi' was resistant to isolate H005 in both tests. The gene that confers resistance in 'Coração de Boi' to Hb005 is probably the only resistance gene that this variety has. According to Kuhn (1987) differentiating accessions usually have a single resistance gene. Thus, there is a possibility that 'Coração de Boi' could be used as a differentiating plant for the Hb005 isolate. In this sense, group G3 accessions from the first test can be used as differentiating plants for Hb006. On the other hand, in the second test, accessions KB092, KB566 and KB14 / 00 can probably be considered differentiating plants for Hb-Italian, such as KB 091 for Hb-FP06.

The accessions HRI 4302, HRI 5389, HRI 6226, HRI 5443, and ISA 207 used in the first test showed different levels of incompatibility with the three isolates, which was also confirmed in the second test with the accessions KB 05/00, KB 10/00, KB 13/00, KB 85 and KB 87 in relation to the eight isolates. According to Nielsen and Tikhomirov (1993), incompatibility does not define races; these accessions were recognized as resistant. The mentioned accessions probably do not have specific genes that can specify the isolates, but according to Moseman et al. (1984) and Nielsen and Tikhomirov (1993) must have other genes or the combination of two or more genes that condition their resistance response. On the other hand, the incompatibility between the hosts and the isolates (groups G5 and D8) was highly variable in their expression, showing low (10 or 15%) and high (93 or 95%) levels. This shows that the resistance observed in these accessions can be controlled by different genes. According to Roelfs (1984), variable incompatibility characterizes the heterozygous genotype that defines resistance.

Following this author, we can say that the *H. brassicae* isolates used in the tests do not have the same genotype for pathogenicity with the different accessions.

Isolates from different geographical regions showed a high degree of host specificity. According to Sherriff and Lucas (1987), *H. brassicae* shows specificity for its hosts. According to Nielsen and Tikhomirov (1993), the specificity of the host-parasite interaction, represented by different IFs, characterizes genetically different races. According to Cardwell and Wehrly (1997), parasite populations are composed of several races with different frequencies of host affinity. According to Slusarenko and Mauch-Mani (1991), *H. brassicae* is a complex race with at least two avirulent loci.

If we analyze the specificity of the host-isolate interaction in the two tests, we can say that the isolates Hb005, Hb-Italian, Hb502, Hb517 and Hb-FP06 can be considered as races due to their specific reaction with the accessions that are not fully recognized by the hosts.

The resistance observed in the accessions was characterized by necrosis at the inoculation site and without sporulation of the parasite. Necrosis, according to Rethage et al. (2000), depends directly on the host-parasite interaction. According to Bittner-Edy et al. (1999), this phenotype determines the presence of race-specific resistance in the host and is controlled by a gene-to-gene interaction.

According to Person et al. (1976) and Crute et al. (1985) parasite variability awakens the function of gene-to-gene interaction. Lebeda and Schwin (1994) consider that pathogenic variability in *H. brassicae* is expressed through gene-to-gene interaction, which is essential for the recognition of physiological races (Parlevliet, 1985; Kema et al., 1996). According to De Wit (1992), the two types of resistance, specific and non-race specific, are closely related to gene-to-gene interaction.

The two gene-to-gene models presented in Tables 9 and 13 should explain the relationships between the differentiated accessions and the *H. brassicae* isolates.

In the first model, three pairs of resistance and avirulence genes were determined. The R1 gene was found in the G5 group accessions. This gene is the only one that conferring resistance to Hb501 in the accessions and could originate from *B. oleracea* var. gongylodes, *B. oleracea* var. *acephala* and *B. oleracea* var. *tronchuda*. The R2 gene was found in 'Coração de Boi' (group G2) and in G4 group accessions where tohether with R3 it confers resistance to the two UK isolates. The R3 gene confers resistance to the isolate Hb006 in accessions from group G3. The three genes R1, R2 and R3 probably confer partial quantitative resistance to the three *H. brassicae* isolates in the G5 group accessions, which is also defended by Bahcevandziev et al. (2015), and may represent an interesting type of durable resistance.

The model presented in Table 13 is based on five pairs of resistance and avirulence genes. Several hypotheses were made to explain some of the accession-isolate interactions. The most important genes

were defined as R1 and R2. The R1 gene confers resistance to the isolates Hb502, Hb517, Hb005, Hb-FP06 and Hb-Italian in the accessions of groups D6 and D8 and probably to the isolates Hb501, Hb006 and Hb-Murcia in the accessions of group D7. This gene could originate from the genomes of *B. oleracea* var. *gongylodes* and *B. oleracea* var. *tronchuda*. The R2 gene confers resistance to the isolates Hb501, Hb006, Hb-Murcia, Hb005, Hb-FP06 and Hb-Italian in the accessions of groups D7 and D8 and probably to Hb502 and Hb517 in accession KB11 / 00. Its origin coincides with that of the R1 gene. In the D8 group accessions these two genes together conferred partial quantitative resistance to the eight *H. brassicae* isolates, which could be part of a durable resistance (Bahcevandziev et al., 2015).

The R3 gene is the only one that confers resistance to the isolate Hb005 in 'Coração de Boi'. The R4 gene confers resistance to the Hb-FP06 isolate in ‘Predzvest’, originating from this accession. The R5 gene confers resistance to the Hb-Italian isolate in the D3 group accessions. These three genes together (R3, R4 and R5) are also present in KB4 / 00 where they confer resistance to the isolates Hb005, Hb-FP06 and Hb-Italian.

Previously published studies show the existence of two *H. brassicae* races in relation to *Brassica oleracea* plants (Felton and Walker 1946; Wang 1949; McMeekin 1969; Natti et al. 1967; Thomas and Jourdain 1990; Thomas and Jourdain, 1992), but neither has been found in Europe. The only paper referring to the presence of three *H. brassicae* races in Europe, particularly in Switzerland, was published by Gaümann in 1926. Since then, nothing more has been published on *H. brassicae* races in Europe. This work should be considered as a preliminary basis for future studies on the potential of *H. brassicae* races in Europe.

Conclusions

The results obtained in this study regarding the phenotypic/genotypic interactions between the different *Brassica oleracea* accessions and *H. brassicae* isolates from different regions in Europe, showed the existence of the downy mildew putative races. These isolates were distinguished by different levels of virulence and by the expression of genomes in the accessions, which demonstrated their specificity.

The gene-to-gene model analysed in this work can lead to a deeper investigation of the resistance genes present in brassicas and their avirulence genes that can be found in *H. brassicae* isolates.

This model, which identifies a potential source of downy mildew resistance in *Brassica oleracea* plants, needs to be genetically characterised and further exploited in breeding programmes.

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Primljeno: 09. svibnja 2024. godine. ***Received:*** *May 09, 2024.*

Prihvaćeno: 24. rujna 2024. godine. ***Accepted****: September 24, 2024.*

Fungi associated with declining tree of heaven (*Ailanthus altissima*) in Krka National Park

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izvorni znanstveni rad (original scientific paper)

doi: 10.32779/gf.7.4.3

Citiranje/Citation[2]

Abstract

Tree of heaven (*Ailanthus altissima*) is an oriental tree listed as an invasive alien species of Union concern. Its establishment and spread may be especially harmful in protected and ecologically sensitive environments. In 2021, a survey was carried out in Krka National Park to determine the presence of fungi on tree of heaven plants showing a decline, dieback or wilt symptoms. Eighteen symptomatic *A. altissima* plants were found in five locations within Krka National Park. These symptomatic trees were sampled and analysed for the presence of fungi. Fungi isolated were identified by morphology and ITS1/ITS4 sequencing. Fifteen fungal species were identified: *Diaporthe chamaeropis*, *D. eres*, *D. foeniculina*, *D. ravennica*, *Diplodia mutila*, *D. seriata*, *Dothiorella viticola*, *Fomitiporia mediterranea*, *Fusarium oxysporum*, *F. solani*, *Paraconiothyrium brasiliense*, *Peroneutypa scoparia*, *Rosellinia corticium*, *Schizophyllum commune* and *Verticillium dahliae*. The isolated fungi range in their ecological niches from secondary colonisers of the declining tree of heaven wood, to possible endophytes, and plant pathogens.

Key words: invasive species, tree of heaven, mycobiota, plant pathogens, endophytes, saprophytes.

Introduction

Invasive alien species are a major threat to biodiversity (McGeoch et al., 2010; Gentili et al., 2021). Tree of heaven (*Ailanthus altissima* (Mill.) Swingle) is a representative example of an invasive alien plant and is widely recognized as biologically harmful to sensitive ecosystems in Europe and other areas it has invaded (Simberloff et al., 2013; Sladonja et al., 2015). *Ailanthus altissima* is included in the list of invasive alien species of Union concern, as defined by Regulation (EU) 1143/2014 of the European Parliament and of the Council. One of the criteria to be included in this respective list is that the invasive species is likely to cause a 'significant adverse impact on biodiversity or the related

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ecosystem services'. Tree of heaven is a highly adaptable and opportunistic species able to successfully establish itself in new environments (Kowarik and Säumel, 2007; Novak and Kravarščan, 2011). It is native to China and was introduced into Europe during the 18th century as an ornamental plant (Kowarik and Säumel, 2007).

Tree of heaven is present in all Croatian counites (Novak and Novak, 2017). It has been described as especially invasive in the coastal part of the country (Novak and Novak, 2017). Fast spread, competition with indigenous plants and its effects on natural ecosystems make tree of heaven especially harmful to protected areas such as nature parks or national parks. Novak and Novak (2017) noted that tree of heaven has colonized relatively large areas of Krka National Park. The authors state that *A. altissima* within Krka National Park evidently "endangers native species, disturbs the stability of the ecosystem, alters the environment, and defaces the appearance of a landscape".

While the invasion of an alien species may be initially fast, various biotic and abiotic factors may alter such spread over time. In their natural range, plants are exposed to herbivorous vertebrates, insects and pathogens that are coevolutionary adapted to their hosts. Alien plants in new environments often initially lack natural enemies. Kowarik and Säumel (2007) state that "outside of its native range, *Ailanthus altissima* is usually subject to a low herbivore pressure...". While insects damaging to alien plants are usually easier to note and investigate, microorganisms like plant pathogenic fungi, bacteria or phytoplasmas may remain less evident. For *A. altissima*, much attention has been attributed to a wilt disease caused by a highly specialized fungus *Verticillium nonalfalfae* Interbitzin et al. (Kasson et al., 2014; Maschek and Halmschlager, 2018). However, many other fungi have been reported on this plant outside its natural range. Ding et al. (2006) reported 65 fungi found on tree of heaven. Some of them are presumed to be pathogenic, possibly affecting the invasive potential of this plant. This study aimed to check the eventual presence of *V. nonalfalfae* on *A. altissima* in Krka National Park, Croatia. Further, it aimed to identify fungi associated with the dead or declining tree of heaven plants, as the first step to identify the potential pathogens which may impact the spread of this invasive species. Finally, this was the first study on fungi naturally occurring on tree of heaven in Croatia.

Materials and methods

Visual examinations and collection of *A. altissima* samples were carried out in early October 2021. Five localities (Oćestovo, Ivoševci, Ključ, Lozovac and Skradin) within the Krka National Park were surveyed (figure 1). Tree of heaven plants on each locality were searched. Two types of plants were selected for sampling (table 1): 1 – dead plants (plants completely dead, no leaves and/or adventive shoots); 2 – declined plants (part of a crown or individual branches dead, crown partially alive, with or without individual shoots drying). Internal symptoms on selected plants were assessed by cutting branches and checking cross-cuts for the presence of discoloration, necrosis or wood rot. On trunks,

the bark was cut with a knife and internal symptoms inside the cambium and deeper in the wood were inspected. Eighteen plants, dead or showing decline symptoms, were sampled. Samples of wood cuts from trunks and 5-10 cm long fragments of thicker branches were taken from each plant. Plants were marked, designated as PjK and a number (table 1), and their GPS position was recorded.

Samples were analysed in the Laboratory for Mycology, Centre for Plant Protection – CAAF, in the city of Zagreb, Croatia. Each sample was analysed by two parallel procedures. Branch fragments were debarked, rinsed with distilled water, surface sterilized in 70 % ethanol for one min and incubated in moist chambers at 22 °C for 7-10 days. The presence of mycelium, fruiting bodies or other sporulating structures was examined by use of a dissecting microscope. If present, spores were morphologically analysed by light microscopy utilizing a compound microscope at 100x magnification. If possible, preliminary identification to the genus level was carried out following descriptions by Ellis and Ellis (1997) and Kirk et al. (2008). Fungi determined were compared to isolates obtained by the second procedure, described as follows.

Trunk fragments were cut to rectangular 2-4 mm chips, which were rinsed with water and surface sterilized as described above. Five to 15 chips were prepared from each sample. After drying in laminar flow, chips were placed on carrot-piece agar (CPA) and incubated for seven days at 20 °C. Plates were inspected for the development of fungal colonies. To obtain pure isolates, the edge of each colony was transferred to plates with potato-dextrose agar (PDA) and incubated for 10-14 days at 22 °C in darkness.

Fungal colonies developed on PDA were analysed for colony appearance, growth rate, hyphal morphology and the eventual presence of sporulating structures. If present, spores were microscopically examined. After a comparison of isolates with fungi developed on branches incubated in moist chamber, 25 isolates were selected for identification at the species level by molecular methods.

Mycelium was collected from cultures on PDA, frozen at -80 °C for a minute and ground to a fine powder utilizing a manual mortar and pestle. Total DNA extraction was performed by DNeasy® Plant Mini Kit (Qiagen) according to manufacturer instructions. Extracted DNA was quantified by spectrophotometry (Jenway® 7415 Nano). Five µl of extract was used for polymerase chain reaction (PCR) in the mixture of 12,5 µl EmeraldAmp Max® mix (Takara), 5,5 µl of water, and 1 µl of ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') primers (White et al., 1990). Reaction parameters were modified from White et al. (1990): 95 °C/2 min, 95 °C 20 sec, 55 °C 25 sec, 72 °C 50 sec (35 cycles) and final elongation at 72 °C for 10 min. Reactions were performed in Applied Biosystems® 2720 Thermal Cycler. Products were visualised in 1 % agarose gel, purified by GenElute® PCR-Clean-up Kit (Sigma-Aldrich) and prepared for sequencing. Sequencing was performed in Macrogen Europe®. Species were identified by comparing the similarity of sequences with those retrieved from GenBank® using the BLAST option.

Results

In total 15 fungal species were isolated from dead or declining tree of heaven trees in Krka National Park (Table 1). *Diaporthe ravennica* Thambug., Camporesi & K.D. Hyde was determined in four samples from three locations (Oćestovo, Ivoševci and Skradin). *Peroneutypa scoparia* (Schwein.) Carmarán & A.I. Romero (former name *Eutypella scoparia* (Schwein.) Ellis & Everh.) was identified in three samples from two locations (Oćestovo and Skradin). Species *Rosellinia corticium* (Schwein.) Sacc., *Fomitiporia mediterranea* M. Fisch., *Schizophyllum commune* Fr., *Diaporthe eres* Nitschke and *Fusarium solani* (Mart.) Sacc. were found in two samples. *Diplodia mutila* (Fr.) Fr., *Diplodia seriata* De Not., *Paraconiothyrium brasiliense* Verkley, *Fusarium oxysporum* Schltdl., *Dothiorella viticola* A.J.L. Phillips & J. Luque, *Verticillium dahliae* Kleb., *Diaporthe foeniculina* (Sacc.) Udayanga & Castl. and *Diaporthe chamaeropis* (Cooke) R.R. Gomes, Glienke & Crous were determined in one sample each.

Figure 1. *Locations surveyed (yellow dots) for dead or declined tree of heaven (*Ailanthus altissima*) in Krka National Park.*

Table 1. *Tree of heaven (*Ailanthus altissima*) sample identification label, locations and fungal species identified on dead or symptomatic plants.*

Sample (plant)	Location	Category	Fungal species found
PjK-1	Oćestovo	dead plant	*Fusarium solani*
PjK-3	Oćestovo	declined plant	*Diplodia mutila* (= *Botryosphaeria stevensii*)
PjK-5	Oćestovo	dead plant	*Rosellinia corticium* *Diaporthe eres*
PjK-7	Oćestovo	dead plant	*Eutypella scoparia* (= *Peroneutypa scoparia*)
PjK-9	Oćestovo	dead plant	*Eutypella scoparia* (= *Peroneutypa scoparia*)
PjK-10	Oćestovo	dead plant	*Rosellinia corticium* *Paraconiothyrium brasiliense*
PjK-11	Oćestovo	declined plant	*Fusarium oxysporum*
PjK-12	Oćestovo	declined plant	*Diaporthe ravennica*
PjK-14	Ivoševci	declined plant	*Dothiorella viticola* *Diaporthe eres*
PjK-15	Ivoševci	dead plant	*Diaporthe foeniculina* *Schizophyllum commune*
PjK-16	Ivoševci	declined plant	*Diaporthe ravennica* *Fusarium solani*
PjK-17	Ivoševci	declined plant	*Diaporthe ravennica*
PjK-21	Ključ	declined plant	*Diplodia seriata*
PjK-22	Ključ	dead plant	*Schizophyllum commune*
PjK-24	Lozovac	declined plant	*Fomitiporia mediterranea* *Diaporthe chamaeropis*
PjK-26	Skradin	declined plant	*Diaporthe ravennica*
PjK-27	Skradin	dead plant	*Eutypella scoparia* (= *Peroneutypa scoparia*)
PjK-28	Skradin	dead plant	*Verticillium dahliae* *Fomitiporia mediterranea*

Discussion

All fungal species isolated from the dead or declining tree of heaven plants have been described as plant pathogens in some plant host systems such as *Fomitiporia mediterranea* on grape (*Vitis* spp.) (Fischer, 2002). However, many of them are also widely considered as endophytes, secondary parasites or saprophytic species developing on dead plant tissues (Leslie and Summerell, 2006; Gomes et al., 2013; Petrini et al., 2013; De Errasti et al., 2014). Since pathogenicity tests were not performed, it cannot be presumed to which extent particular fungal species contributed to the decline or dieback of *A. altissima* plants. The role of the fungi in a decline of trees is often complex. Generally, only a relatively small number of fungal species are severe pathogens able to kill an adult tree. Abiotic and biotic factors more often show cumulative and interconnected effect, contributing jointly to tree death or decline. Symptomatic tree of heaven plants may have been stressed or damaged by shocks like freezing or drought (Kowarik and Säumel (2007), making them vulnerable to fungal infections. Fungi from genera like *Diplodia* and *Diaporthe*, as well as wood-rotting species like *F. mediterranea* or *S. commune* are sometimes described as pathogenic to plants damaged by stress (Slippers and Wingfield, 2007; Gomes et al., 2013; Moretti et al., 2021).

All fungal species found on tree of heaven are polyphagous, described on numerous woody plants. Among them, *Fusarium solani* and *F. oxysporum* are probably the most ubiquitous (Leslie and Summerell, 2006). These *Fusarium* species are frequently isolated from numerous annual and perennial plants (Leslie and Summerell, 2006). Depending on the strain and the host they are infecting, they may be destructive pathogens, weak secondary invaders or only saprophytes (Leslie and Summerell, 2006). Similarly to *Fusarium* species, the genus *Diaporthe* also comprises severe plant pathogens, weakly pathogenic species, endophytes and saprobes (Gomes et al., 2013; Udayanga et al., 2014). It may be noted that pycnidia of all *Diaporthe* species found in the present study developed abundantly on debarked and discoloured wood of *A. altissima* samples in the laboratory. *Diaporthe* species are found on many woody plants, but taxonomic uncertainties arising from their morphological similarity have brought confusion on a host range of particular species (Udayanga et al., 2014). Certain *Diaporthe* species have been described both as endophytes and pathogens. For example, *D. eres*, found in one sample, has been described as a common endophyte on a number of plant hosts (Udayanga et al., 2014). However, it has also been found as a pathogen, e.g., on blackberry (Vrandečić et al., 2011) or hazelnut (Arciuolo et al., 2021). Three other *Diaporthe* species were found in the present study, *Diaporthe ravennica*, *D. foeniculina* and *D. chamaropis*. They may form a complex of closely related *Diaporthe* species on *A. altissima*, as it is known in the case of other woody species (Dissanayake et al., 2017; Chen et al., 2014; Udayanga et al., 2014; Gomes et al., 2013). *Diaporthe ambigua* has been recorded as a pathogen on kiwi (Auger et al., 2013) and grapevine (van Niekerk et al., 2005). *Diaporthe foeniculina* has been described as a pathogen of chestnut (Annesi et al., 2016), avocado (Guarnaccia et al., 2016) or eucalyptus (Deidda et al., 2016). This species has been found on *A. altissima* in Italy (Dissanayake et al., 2017), but without an assessment of its potential pathogenicity.

Two widespread wood-rotting basidiomycetes were found in this study, *S. commune* and *F. mediterranea*. *Schizophyllum commune* is one of the most common secondary colonizer of declining or dead woody plants (Ohm et al., 2010), and it could be presumed that it has a similar role on tree of heaven. *Fomitiporia mediterranea* is known as a much more potent pathogen (Moretti et al., 2021), reported as the most important causal agent of grapevine esca disease (Fischer, 2002; Moretti et al., 2021). Basidiocarps of *F. mediterranea* were noted on the trunks of both *A. altissima* plants from which the fungus was isolated.

Species *Rosellinia corticium* and *Peroneutypa scoparia* have been found in samples of *A. altissima* with distinctive internal symptoms. Plants were dead, and black discoloration was visible under the bark. Both fungal species have been described as endophytes, secondary colonizers and saprophytes on numerous woody plants (Acero et al., 2004; Petrini, 2013; de Errasti et al., 2014; Ćelepirović et al., 2020). Relatively recently, *P. scoparia* has been confirmed as pathogenic to kiwifruit (Castilla-Cayuman et al., 2018).

Fungi which are most likely to be pathogenic on tree of heaven in this research are Botryosphaeriaceae species (*Diplodia* and *Dothiorella* genera) and *Verticillium dahliae*. *Diplodia mutila* is known as a frequent causal agent of cankers and dieback of woody plants (Sutton, 1980; Slippers and Wingfield, 2007). It has been associated with the decline of oak (Ragazzi and Mesturino, 1978), ash (Kraj et al., 2013) and pines (Mohali and Encinas, 2001). In agriculture, *D. mutila* was reported as one of the causal agents of grapevine Botryosphaeria dieback (Kaliterna et al., 2011), as well as a pathogen of stone fruits, pome fruits and walnuts (Slippers et al., 2006; Chen et al., 2014; Diaz et al., 2018). *Diplodia seriata* has been reported on more than 200 woody hosts (Farr and Rossman, 2013). It is well-known as a grapevine pathogen (Úrbez-Torres, 2011), but its pathogenicity has also been confirmed on many other plant species (Phillips et al., 2007). *Dothiorella viticola* has been described in 2005 (Luque et al., 2005). It has been reported as a causal agent of canker disease on some crops, such as citrus (Adesemoye et al., 2014) or grapevine (Luque et al., 2005).

Verticillium dahliae is a cosmopolitan pathogen causing wilt disease in many plant hosts (Inderbitzin et al., 2011; Inderbitzin and Subbarao, 2014). The fungus has been reported to cause progressive wilt of tree of heaven in Toscana, Italy (Pisuttu et al., 2020). The pathogenicity of *V. dahliae* on *A. altissima* has been proven (Pisuttu et al., 2020). The authors are stating that *V. dahliae* has been probably found as a causal agent of *A. altissima* wilt in Italy long ago, as they found in description of Goidanich (1935). Another *Verticillium* species, *V. nonalfalfae*, is causing wilt of tree of heaven and has a perspective as a biocontrol agent against this invasive plant. The efficacy and host specificity of *V. alfalfae* was proven in studies conducted in the USA (Kasson et al., 2014) and Austria (Maschek and Halmschlager, 2018; Lechner et al., 2023).

All fungi found in this study are reported on various woody hosts, many of them present in the natural habitats of Krka National Park. Other plants probably serve as an inoculum for tree of heaven infection or colonization, whether as pathogens, weak pathogens, endophytes or saprophytes. Plant pathogenic fungi may naturally influence the spread of *A. altissima* and its ability to invade new areas. Although it is not realistic to expect that the fungi described could significantly contain the spread of *A. altissima*, the results show how invasive alien plants are exposed to various microorganisms in newly invaded areas.

Conclusion

In newly invaded areas, tree of heaven (*Ailanthus altissima*) is exposed to various naturally present fungi. Many fungal species living as pathogens, endophytes or saprophytes on other wood plants can attack or colonize tree of heaven. Fifteen fungi were found on dead tree of heaven plants, or plants showing decline symptoms in Krka National Park. Their role cannot be known without pathogenicity tests.

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Primljeno: 12. svibnja 2024. godine. ***Received:*** *May 12, 2024.*

Prihvaćeno: 24. rujna 2024. godine. ***Accepted****: September 24, 2024.*

Antifungal activity of thyme, oregano and laurel essential oils against *Aspergillus niger* Tiegh.

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prethodno priopćenje (preliminary communication)

doi: 10.32779/gf.7.4.4

Citiranje/Citation[3]

Abstract

A range of pathogenic fungi are associated with postharvest fruit spoilage in storage that cause significant economic losses every year. To control postharvest mycoses, fungicide application is the usual practice. However, the use of essential oils as an alternative strategy to chemical fungicides has been considered for management of the postharvest fruit decay in order to ensure nonnegative impact on human health. Mycotoxigenic fungi *Aspergillus niger* Tiegh. is economically important pathogen which causes postharvest black rot on various fruits. The present study evaluated the vapor phase antifungal activity of three essential oils - thyme (*Thymus vulgaris* L.), oregano (*Origanum vulgare* L.) and laurel (*Laurus nobilis* L.) against postharvest pathogen *A. niger.* The antifungal activity of selected essential oils were evaluated by disc volatilization method on PDA medium. The vapor phase of all tested oils showed a significant ($p < 0.05$) fungistatic growth inhibition of pathogen *A. niger*, where thyme oil achieved the highest pathogen inhibition (99.8%), while laurel oil was less effective (36.3%). The obtained results suggest that the tested vapor phase (especially of thyme and oregano oils) could potentially be extremely useful fumigants to prevent and control the fruit rot in storage.

Key words: antifungal activity, *Aspergillus niger*, essential oils, postharvest, fungistatic.

Introduction

The problems of a modern food industry are manifested in the postharvest deterioration of fruit caused by phytopathogenic fungi, which are responsible for almost 85% of fruit diseases (Santos et. al, 2020). Fungi have a significant impact on fruit during storage, making it unsuitable for human consumption by reducing its nutritional value and sometimes producing mycotoxins. Fruits with a high-water content are susceptible to attack by postharvest fungi, the best known of which is *A. niger*, which

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causes black rot on stored fruits (Dos Santos et al., 2012; De Sousa et al., 2013; Jahani et al., 2020). The production of mycotoxins like ochratoxin A, fumonisin, sterigmatocystin, cyclopiazonic acid and patulin, is refer to the specie *A. niger* (Plascencia-Jatomea et al., 2014). On the other hand, *A. niger* quickly develops resistance, which limits the spectrum of available and effective fungicides. The use of chemical fungicides is increasingly limited and therefore alternative methods need to be developed to reduce the negative impact on the environment (Phillips et al., 2012; Císarová et al., 2016). In addition, consumers' negative impressions of chemical fungicides are directing their attention to biological control methods (Sharma and Tripathi, 2008). According to the Croatian Phytosanitary Information System (FIS, https://fis.mps.hr/fis/javna-trazilica-szb/), only one fungicide is currently registered for the control of *Aspergillus* sp., causative agent of black rot in grapevine. It is therefore important to find effective and ecologically safe methods for postharvest fungal control (Shao et. al, 2013). In order to protect the fruits from decay, much attention is being paid to essential oil research (Farzaneh et al., 2015). An alternative solution is the use of essential oils extracted from medicinal plants as a source of antifungal compounds, which are characterized by volatility, ecological acceptability and consequently consumer acceptance (Tzortzakis and Economakis, 2007). The use of essential oils for postharvest control of pathogenic fungi is a cost-effective and environmentally friendly method that can be used by farmers to extend the shelf life of their products (Prakash et al., 2015; Kaddes et al., 2019).

Essential oils are aromatic, volatile liquids that are extracted from certain plants. Due to their chemical composition, essential oils have been shown to act as antioxidants and antimicrobial compounds (Reyes-Jurado et al., 2019; Jahani et al., 2020). The antifungal effect of essential oils in the volatile phase depends on the volatility, composition and effect of their secondary metabolites (Hyldgaard et al., 2012; Císarová et al., 2016; Reyes -Jurado et al., 2019). This is confirmed by numerous studies that have shown that some essential oils such as oregano (*Origanum vulgare* L.), laurel (*Laurus nobilis* L.) and thyme (*Thymus vulgaris* L.), exhibit antifungal activity against postharvest pathogens *in vitro*. It is interesting that note some oil formulations are already used in the food industry (for preservation) as plant-based fumigants for stored food, which is why they belong to the "GRAS" category, which is recognized by the Food and Drug Administration (FDA) as safe under the prescribed conditions of use (Císarová et al., 2016; Oliveira et al., 2020; Jahani et al., 2020). Today, due to their volatile effect, the essential oils of many plants from the genus *Thymus* are used in the food and pharmaceutical industry, where the most famous is thyme oil (Chrpova et al., 2010). The goal of this research was to evaluate the antifungal activity of vapor phase of three essential oils using the disc volatilization method against postharvest fungus *A. niger*.

Materials and methods

Isolation and determination of the pathogen

The pathogen A. niger was isolated from onion bulb with symptoms of black mould. The strain was purified, maintained on PDA (Potato Dextrose Agar, Sigma – Aldrich, USA) and incubated in the dark at 23 °C in a climate chamber. The isolate was molecularly analysed to species level using the DNA extraction method according to Elias et al. (2004), and conventional PCR conditions according to Henry et al. (2000), and subsequently sequenced by Macrogen Europe (The Netherlands).

Oils

Concentrated thyme, oregano and laurel oils without syntetic chemicals were obtained from Aromara d.o.o. (Šenkovec, Croatia) and stored at 4 °C, in dark, until the first use.

Laboratory volatile bioassays

The effect of three oils (thyme, laurel and oregano) was tested against the pathogen A. niger in vitro using the disk volatilization method according to Tzortzakis and Economakis (2007). For the vapor phase method, Petri plates (85 mm ⌀) with PDA medium (Sigma – Aldrich, Darmstadt, Germany) (10 ml) were inoculated with mycelial disc (5 mm ⌀) of the test pathogen cut from the margin of 7-day-old culture. A sterile filter paper disc (Whatman, no. 1,5 mm ⌀, Sigma-Aldrich, USA) was placed on PDA medium of inoculated Petri plate, 4 cm from the mycelial disc. Test plates were containing filter disc with applied oil (5 µL/plate), and plates with distilled water instead of oil, served as a control (according to Arrebola et al., 2010). Afterwards, the Petri plates were double wrapped with parafilm to avoid vapour evaporation and incubated at 23 °C, in dark, 7 days (Wu et al., 2011). The assay was performed in ten replicates for each essential oil. In order to test the difference between the cidal and static effects of oil volatiles against A. niger, non-growing mycelial discs were transferred to a new PDA plate (incubation: 7 days/ 23 °C/dark) (according to Sellamuth et al., 2013). In any Petri plate in which no pathogen growth was observed , the oil was considered fungicidal, while any pathogen growth was considered fungistatic.

Measurement of mycelial growth area

On the seventh day, Petri plates photographs were processed with Image J computer program (Schneider et al., 2012) according to Martinko et al. (2022). Mean values (cm^2) of pathogen growth area were obtained, and inhibition index (I= %) was calculated to quantify antifungal effect of tested essential oils.

Statistical analysis

Pathogen growth dana in test and control Petri plates were presented with their mean values and standard deviations (SD). Data that conformed to a normal distribution were statistically analyzed using One Way ANOVA, and differences between treatments were evaluated using Tukey's test ($p \leq 0.05$) in the statistical program SPSS (IBM, version 15.0, Chicago, IL, USA).

Results and discussion

Mycelial growth of *A. niger* at a concentration of 5 µL/plate was noticed in all Petri plates containing tested oils. The variant with thyme and oregano oil shows pathogen growth suppression by 99.8% and 97.7%, respectively, while the variant with laurel oil inhibited growth by 36.3% compared to the control (table 1., fig. 1.). The mycelial growth area was significantly inhibited in all variants with oils compared to the control group (Tukey test, $p \leq 0.05$).

Table 1. *Vapour phase effect of laurel, oregano and thyme oil on micellar area of Aspergillus niger after 7 days.*

	Control	Test (5 µl)		
	A. niger	laurel oil + *A. niger*	oregano oil + *A. niger*	thyme oil + *A. niger*
$\bar{x}$ (cm^2) ± SD	47.4 ± 0.5 [a]	30.2 ± 3 [b]	1.1 ± 1.4 [c]	0.09 ± 0.1 [c]
I (%)	0	36.3	97.7	99.8

* Mean values marked with the same letter within columns are not significantly different at $p \leq 0.05$ (Tukey's test).

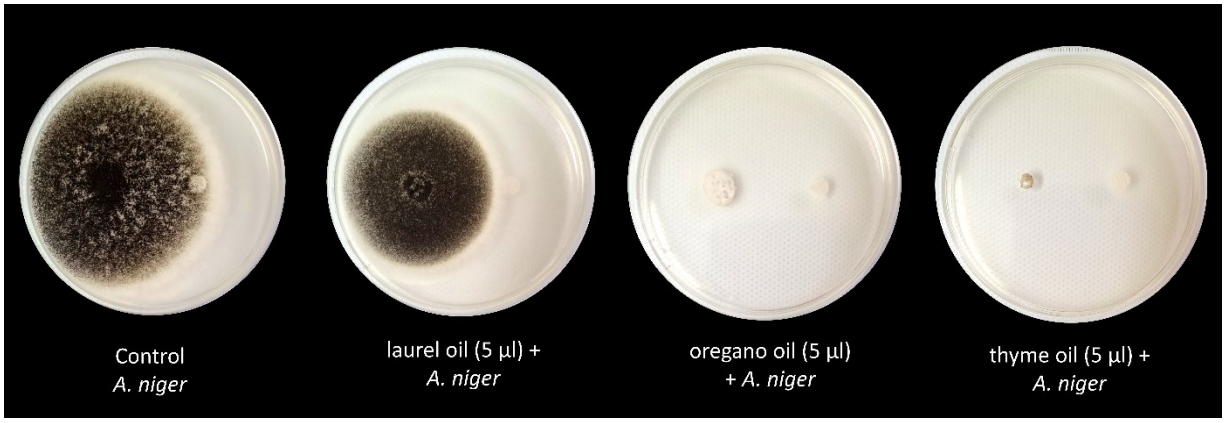

Figure 1. *Antifungal effect of laurel, oregano and thyme oil against Aspergillus niger after 7 days.*

All tested oils (thyme, oregano and laurel) showed significant antifungal activity on *A. niger* growth in their vapor phase, after exposure to 5 µL/ plate after 7 day-incubation (fig. 1., table 1). The mycelial discs transfer from test Petri plates on freshly poured PDA medium, where the pathogen began to

grow 4 days after, confirms that all tested oils have a fungistatic effect. Also, the *A. niger* sporadicity was observed in the presence of thyme and oregano oil. Also, thyme oil inhibits mycelial growth of various postharvest pathogens such as *Alternaria alternata* (Wu et al., 2011), *Fusarium oxysporum, Colletotrichum gloeosporioides, Rhizoctonia solani* (Lee et al., 2007; Sellamuthu et Al., 2013), *Rhizopus stolonifer, Botrytis cinerea*, and *Monillinia fructicola* (Svircev et al., 2007; Sellamuthu et al., 2013). Rasooli et al. (2006) state that the effect of thyme oil is probably due to thymol biological action as the most abundant component in that oil. The study by Behbahani et al. (2013) confirmed the significant antifungal effect of thyme oil vapor phase using the same method (disc volatilization method) and concentration (5 μL/Petri) used in this study. There are several studies (Angelini et al., 2006; Koc and Kara, 2014) that confirm thyme oil significant antifungal effect against *Aspergillus* species, while laurel oil has the lowest reported activity, which is in accordance with the results of this research. In study of Çetin et al. (2011), the essential oil of oregano showed significant inhibition of 23 out of 26 isolates of human and phytopathogenic bacteria, 13 out of 14 tested fungi and yeast species, including *A. niger*, while all tested microorganisms were inhibited by thyme oil. Several authors (Lambert et al., 2001; Caputo et al., 2017) confirm that the antifungal activity of oils depends on the content of antimicrobial compounds. Oregano and thyme have been extensively studied for their antimicrobial activity due to their higher content of carvacrol and thymol. In addition, the activity of the oil depends on the phase in which the pathogen is exposed to the oil. As confirmed by Inouye (2003), the vapor pressure of the volatile compounds interferes with spore respiration. When the vegetative hyphae are exposed to the volatile phase of the essential oil, they are likely to segment in order to survive. Similarly, some oils, such as oregano oil, have a long-lasting effect on fungi, in contrast to oils that have a temporary effect (spearmint oil), although both oils have shown a fungistatic effect on treated fungal pathogens. Inouye (2003) also notes that the actual concentration of oil volatiles is much lower than the nominal concentration and that only a portion of the evaporated oil may be effective against microorganisms. The mechanism of antifungal action of essential oils has been widely investigated, although it is still poorly understood. Recent studies have demonstrated that hydrophobic essential oils increase the permeability of cell membranes, leading to leakage of cell contents and cell death (Khaneghah et al., 2018; Guo et al., 2020), along with DNA damage (Salehi et al., 2020). The pathway of action of the antifungal mechanism of essential oils is not clear, but there are two or more directions at the same time (Ju et al., 2019). The results of the *in vitro* antifungal effect of the tested essential oils depend on the concentration, phase and composition of the oils used and the target pathogen, and the results presented in this research can serve as a reference for further research. In any case, it has been proven that the tested essential oils have potential activity against *A. niger*, but further research is needed to ensure their safe use in food preservation.

Conclusion

In summary, the present study proves that the vapor phase of the tested essential oils effectively controlled postharvest pathogen *A. niger in vitro*. Therefore, thyme, oregano and laurel oils in the volatile phase could be used as an alternative to chemical post-harvest fungicides for the control of black rot in stored fruit. However, it is necessary to additionally test selected oils *in vivo* and evaluate the potential use, especially of oregano and thyme oil, as additives to extend food safety and shelf life.

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Primljeno: 26. travnja 2024. godine. ***Received:*** *April 26, 2024.*

Prihvaćeno: 24. rujna 2024. godine. ***Accepted****: September 24, 2024.*

Xanthomonas arboricola pv. corylina, uzročnik bakterioznog paleža lijeske (*Corylus avellana*) u rasadnicima i komercijalnim nasadima lijeske u Hrvatskoj

Xanthomonas arboricola pv. corylina, the causative agent of bacterial blight of hazelnut (*Corylus avellana*) in nurseries and commercial plantations in Croatia

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prethodno priopćenje (preliminary communication)

doi: 10.32779/gf.7.4.5

Citiranje/Citation[2]

Sažetak

Xanthomonas arboricola pv. *corylina* (*Xac*) je uzročnik bakterioznog paleža lijeske (*Corylus avellana L.*). Iako je bolest najznačajnija na običnoj lijeski, mogu biti zaražene i druge vrste roda *Corylus* spp., kao što su *C. maxima, C. pontica* i *C. colurna*. Budući da prisutnost *Xac* na ovim biljkama za sadnju može izazvati "neprihvatljiv ekonomski učinak", bakterija je svrstana od 2019. g. na listu reguliranih nekarantenskih štetnika (eng. *Regulated Non-Quarantine Pest*, RNQP). Bakteriozni palež lijeske rasprostranjen je u svim glavnim područjima uzgoja lijeske diljem svijeta. Bolest je posebno štetna u rasadnicima i mladim nasadima, jer su biljke osjetljivije dok su mlade. Međutim, jake zaraze mogu se vidjeti i u starijim proizvodnim nasadima. Kako se površine pod lijeskom posljednjih godina povećavaju, u drugim zemljama tako i u Hrvatskoj, povećala se i učestalost i značaj ove bolesti.

Ključne riječi: bakteriozni palež, identifikacija, kontrola, RNQP, fitosanitarne mjere.

Abstract

Xanthomonas arboricola pv. *corylina* (*Xac*) is the causal agent of bacterial blight of hazelnut (*Corylus avellana* L.). Although the disease is most significant on European hazelnut, other *Corylus* spp., such as *C. maxima, C. pontica*, and *C. colurna* can also be infected. As its presence on plants intended for planting can cause an “unacceptable economic impact”, the bacterium has been listed as a regulated non-quarantine pest (RNQP) since 2019. Bacterial blight of hazelnut is widespread in all major hazelnut-growing regions worldwide. The disease is especially detrimental in plant nurseries and

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young orchards, as the plants are more susceptible in their early stages. However, severe infections can also occur in orchards later during exploitation. As the area of hazelnut orchards has increased in many countries, including Croatia, in recent years, the incidence and importance of this re-emerging disease has also increased.

Key words: bacterial blight, identification, control, RNQP, phytosanitary measures.

Uvod

Xanthomonas arboricola pv. *corylina* (*Xac*) je uzročnik bakterijske paleži lijeske (*Corylus avellana* L.). Iako je bolest najznačajnija na običnoj lijesci, *C. maxima*, *C. pontica* i *C. colurna* (EPPO, 2004) također mogu biti zaraženi. Uslijed porasta intenzivnog uzgoja lijeske posljednjih godina u svijetu (FAO, 2020), porasla je i učestalost bolesti uzrokovane *Xac*. U Republici Hrvatskoj lijeska se također sve više uzgaja te je od 2012. godine s 2400 hektara zabilježeno povećanje na 8230 hektara u 2022. godini (https://ec.europa.eu/eurostat).

Simptomi bakteriozne paleži javljaju se na listovima, pupovima, plodovima i drvenastim dijelovima biljke. Prvi simptomi uočljivi su u proljeće na pupovima koji se ne otvaraju, ili se ubrzo nakon početnog razvoja suše (Miller et al., 1949; Lamichhane i Varvaro, 2014). Karakteristični simptomi bolesti vidljivi su na listovima kao sitne, nepravilne, nekrotične pjege okružene klorotičnim prstenom. U povoljnim uvjetima temperature i vlažnosti, pjege se šire i spajaju, te se stvaraju veće nekrotične zone nepravilnog oblika. Listovi se suše i rubovi se oštećuju te biljke izgledaju kao da su spaljene plamenom, po čemu je bolest i dobila naziv (Miller et al., 1949; Arsenijević, 1997). Blago uleknute, tamne, vodenaste pjege mogu se pojaviti i na ovojnici (egzokarpu) ploda, no rijetko zahvaćaju jezgru (Miller i et al., 1949). Mlade grane se suše, a njihova zaraza ima i najveći gospodarski značaj jer je to dio biljke koji daje plod (Miller i sur., 1949). Na deblu i većim granama dolazi do pojave rana ispod kojih je nakon uklanjanja kore vidljiva tamna nekroza koja difuzno prelazi u zdravo tkivo. Na tim se mjestima tijekom razdoblja visoke vlažnosti mogu vidjeti i kapi ljepljivog žućkastog bakterijskog eksudata (iscjetka) koji se tijekom vegetacije prenosi kapima kiše te tako omogućava sekundarne zaraze (Miller et al., 1949; Arsenijević, 1997). Intenzitet zaraze, uz okolišne uvijete, posebno ovisi o starosti biljke. Najveće su štete u rasadnicima i mladim nasadima (1-4 godine), gdje zaraza često završava slabljenjem i odumiranjem biljaka iako su značajne štete zabilježene i u starijim nasadima (Slika 1). Gubitci prinosa prosječno iznose oko 10%, ali zabilježeni su i drastični gubitci od 90 do 100% (Miller et al., 1949; Prunier et al., 1976; Lamichhane i Varvaro, 2014).

Općenito, bakterijski biljni patogeni prenose se vodom, odnosno kišnim kapima, vjetrom, različitim vektorima (uključujući i čovjeka) no zaraženi sadni materijal glavni je put unošenja i širenja ovog uzročnika (Lamichhane i Varvaro, 2014). Kontrola bolesti se postiže fitosanitarnim certificiranjem sadnog materijala. Bakterija *Xac* je od 2019. klasificirana kao regulirani nekarantenski štetni

organizam (RNQP). RNQP je definiran kao „nekarantenski štetnik čija prisutnost u biljkama za sadnju utječe na namjeravanu upotrebu tih biljaka s ekonomski neprihvatljivim učinkom i koji je stoga reguliran na teritoriju ugovorne stranke uvoznice“ (Picard et al., 2017), a razvijeni su i kategorizirani brojni prikladni dijagnostički alati i laboratorijske procedure kojima se dijagnosticira pojedine bakterijske vrste iz ove skupine ili im se pomoću njih analizira genetička raznolikosti i struktura populacija te mehanizmi patogenosti (Catara et al., 2021). Budući da je prag RNQP na sadnom materijalu lijeske 0%, na sadnom materijalu lijeske *Xac* ne smije biti prisutan. Novim sustavom u području biljnoga zdravstva i službenih kontrola, na snazi od kraja 2019. godine, kontrola RNQP na bilju namijenjenom sadnji u nadležnosti je ovlaštenih specijaliziranih subjekata. Kako bi se provjerila usklađenost sadnog materijala proizvedenog u Hrvatskoj sa zahtjevima u području biljnoga zdravstva, u 2023. godini po prvi se puta provodio program posebnog nadzora usmjeren na rasadnike. U okviru navedenog programa uzeti su uzorci lijeske za analizu na *Xac* iz dva rasadnika, 3 uzorka iz jednog te 7 uzoraka iz drugog rasadnika, ukupno 10 uzoraka.

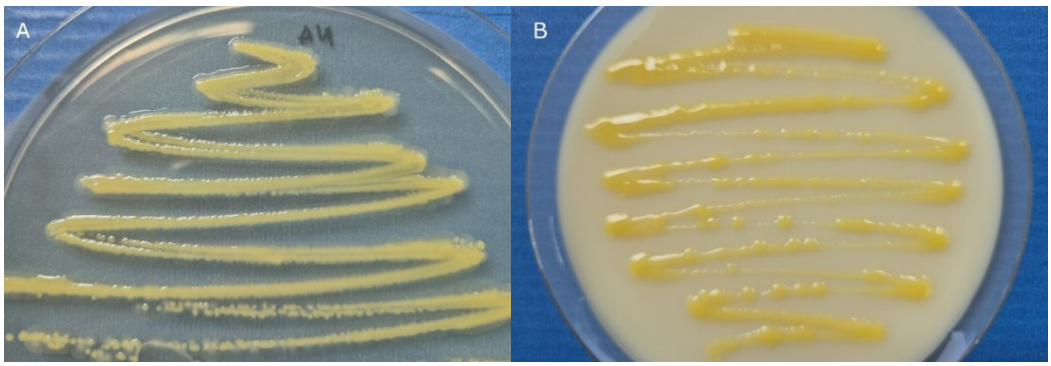
Slika 1. Sušenje dijela stabla lijeske uzrokovano bakterijom *Xanthomonas arboricola* pv. *corylina* (Foto: Dario Ivić).
Figure 1. *Drying of part of the hazelnut caused by the bacterium Xanthomonas arboricola* pv. *corylina (Photo: Dario Ivić).*

Materijali i metode

Dijelovi oboljelog tkiva lista, pupoljka i kore grančice macerirani su u sterilnoj destiliranoj vodi. Macerat je naslojen na ploče s hranjivim agarom (NA) i inkubiran na 27 °C 48 h. Karakteristične pojedinačne žute kolonije su zatim precijepljene na hranjivu podlogu ekstrakta kvasca-dekstroze-CaCO3 (YDC) (Schaad et al., 2001) i inkubirane pod istim uvjetima. Izolirane pojedinačne žute, konveksne kolonije upotrijebljene su za molekularnu potvrdu. Izdvajanje DNA iz bakterijskih kolonija izvedeno je pomoću komercijalno dostupnog kompleta DNeasy Plant Mini Kit (Qiagen, Hilden, Njemačka) prema uputama proizvođača. Radi potvrde izolata konvencionalnom lančanom reakcijom polimerazom (eng. *Polymerase Chain Reaction*, PCR) izvedena su dva testa, jedan koji je specifičan za predstavnike vrste *X. arboricola* i cilja gen *qumA* korištenjem para početnica XarbQ-F/XarbQ-R (Pothier i sur., 2011) te drugi, koji ima visoku specifičnost prema *corylina* sojevima a cilja dio *ftsX* gena, korištenjem para početnica XapY17-F/XapY17-R (Pagani, 2004; Pothier et al., 2011). Umnoženi produkti razdvojeni su elektroforezom u 1% -tnom agaroznom gelu, obojeni otopinom GelRed (Olerup SSP, West Chester, PA, SAD) i vizualizirani pod UV transiluminatorom (UViTech, Cambridge, Engleska, Ujedinjeno Kraljevstvo). U sve PCR testove uključena je DNA izolirana iz referentnog soja *X. a.* pv. *corylina* NCPPB 3037 (*Corylus avellana* - Ujedinjeno Kraljevstvo, 1978) kao pozitivna kontrola (K^+), a reakcijska smjesa s dodatkom vode umjesto uzorka (W) predstavljala je negativnu kontrolu. Od produkata umnožavanja dobivenih PCR-om odabrana su po 2 izolata s obje lokacije i poslana na sekvenciranje (Genewiz, Njemačka).

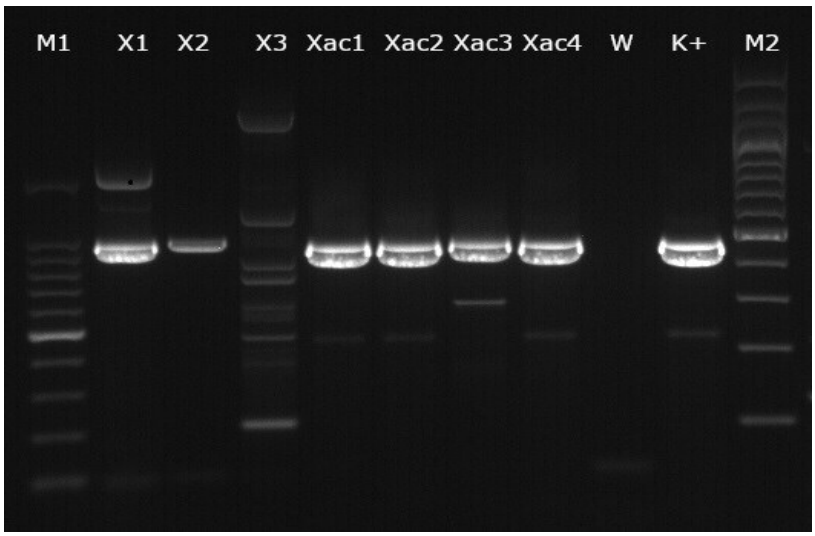
Rezultati

Iz prikupljenih uzoraka izolirani su različiti bakterijski sojevi na pločicama s hranjivim agarom. U 9 od 10 prikupljenih uzoraka utvrđeno je prisustvo bakterija koje formiraju male, okrugle, žute i sjajne kolonije, karakterističnih za rod *Xanthomonas* (slika 2, A i B).



Slika 2. Izgled kolonija *X. arboricola* pv. *corylina* na (A) hranjivom agaru (NA) i (B) na hranjivoj podlozi ekstrakta kvasca-dekstroze-CaCO3 (YDC) nakon 72 h (Foto: Jelena Plavec).
Figure 2. *Appearance of X. arboricola* pv *corylina colonies on NA (A) and YDC (B) medium after 72 h (Photo: Jelena Plavec)*.

U 9 od 10 analiziranih bakterijskih sojeva umnoženi su PCR produkti očekivane veličine. Produkt od 402 bp dobiven korištenjem para početnica XarbQ-F/XarbQ-R potvrdio je pripadnost vrsti *X. arboricola*, dok je drugim parom početnica (XapY17-F/XapY17-R) umnožen fragment *ftsX* gena očekivane veličine od 943 bp koji je karakterističan za vrstu *Xac* (slika 3). Analiza odabranih sekvenci dobivenih iz produkata umnoženih početnicama XapY17-F/XapY17-R potvrdila je prisutnost *Xac*. Hrvatski izolati pokazali su od 99,88% do 100% nukleotidnu identičnost s *Xac* sojem IVIA 3978 (CP076534) izoliranim iz lijeske u Španjolskoj. Na temelju izgleda kolonija na hranjivim podlogama NA i YDC, te na temelju molekularne detekcije, analizirani izolati prikupljenih u dva rasadnika u Hrvatskoj najvjerojatnije pripadaju bakteriji *X. arboricola* pv. *corylina*. Radi konačne identifikacije, uz ove preliminarne metode, trebalo bi provesti i ostale biokemijsko-fiziološke analize i test patogenosti izolata. Navedene uzorke bi ubuduće trebalo testirati novim protokolom za specifičnu detekciju *Xac*-a koja uključuje konvencionalni PCR te PCR u realnom vremenu (Kałużna et al., 2023).



Slika 3. PCR produkt veličine 943 bp umnožen početnicama XapY17-F/XapY17-R; X1, X2, X3 - uzorci prikupljeni u rasadniku 1; Xac1, Xac2, Xac3, Xac4 -uzorci prikupljeni u rasadniku 2; W - negativna kontrola; K+ pozitivna kontrola; M1 –marker 100 bp, M2 –marker 200 bp (oba markera Dye Plus, Takara, Japan).
Figure 3. *PCR product size 943 bp amplified with primers XapY17-F/XapY17-R; X1, X2, X3 - samples collected in nursery 1; Xac1, Xac2, Xac3, Xac4 - samples collected in nursery 2; W - negative control; K+ positive control; M1 – marker 100 bp, M2 – marker 200 bp (both markers Dye Plus, Takara, Japan).*

Rasprava i zaključci

Raniji rezultati su pokazali da je *Xac* prisutan u komercijalnim nasadima lijeske u kontinentalnom dijelu Hrvatske (neobjavljeni rezultati), te su uslijed povoljnih okolišnih uvjeta za razvoj bolesti, zabilježene i značajnije štete. Slična iskustva prijavljena su i u zemljama regije (Prokić, 2014; Popović et al., 2023) posljednjih godina. Međutim, preliminarni rezultati izloženi u ovom radu potvrđuju da je

Xac prisutna i u rasadnicima u Hrvatskoj, što je u skladu s ranije objavljenim tvrdnjama da je zaraženi sadni materijali uobičajeni izvor ove bolesti u cijelom svijetu (Lamichhane i Varvaro, 2014).

Kad je bolest već prisutna u nasadima, preporučuje se rezidba i uklanjanje zaraženih dijelova biljaka i zatvaranje rana. Bakrovi spojevi, koji se i inače koriste za kontrolu biljnih bakterioza (Agrios, 2005) koriste i za zaštitu od *Xac*, uglavnom u kombinaciji s ditiokarbamatima. Premda primjena tih pripravaka daje većinom zadovoljavajuće rezultate, uglavnom ako se radi o površinskim populacijama *Xac*, pojava na bakar otpornih sojeva *Xac* trenutno predstavlja glavnu prijetnju učinkovitom upravljanju bakterioznom paleži lijeske u ugroženim nasadima (Gardan, 1983; Lamichhane i Varvaro, 2014).

Ipak, najučinkovitija kontrola bakterioza i dalje je prevencija. Uz sadnju otpornih kultivara, kojih je uslijed povećanja globalne proizvodnje lješnjaka i nasada lijeske također sve više (Webber et al., 2021), najvažnija preventivna mjera je korištenje zdravog sadnog materijala (Janse i Wenneker, 2002; Lamichhane i Varvaro, 2014). Zemlje EPPO regije (eng. *European and Mediterranean Plant Protection Organization*) usvojile su propise kojima se može značajno poboljšati zdravstveno stanje sadnog materijala, a uključuju provođenje fitosanitarnih mjera, nadzor rasadnika i provedbu certifikacijskih shema (Janse i Wenneker, 2002). U kontekstu pregleda rasadničkog materijala na latentne infekcije, koje bi inače ostale neotkrivene i postale izvor primarne infekcije u novim nasadima (Catara et al., 2021; Kałużna et al., 2023) posebno je važan razvoj brzih, osjetljivih i vrlo specifičnih metoda, odnosno dijagnostičkih alata za preciznu dijagnostiku *Xac*.

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Primljeno: 17. svibnja 2024. godine. ***Received:*** *May 17, 2024.*

Prihvaćeno: 24. rujna 2024. godine. ***Accepted****: September 24, 2024.*

Sjećanja na razdoblje provedeno u suradnji s profesorom Bogdanom Cvjetkovićem na Sveučilištu u Zagrebu Agronomskom fakultetu

Memories of the period spent in collaboration with Professor Bogdan Cvjetković at the University of Zagreb, Faculty of Agriculture

Željko Jurjević[1]*

crtice (dashes)

Dr. sc. Bogdan Cvjetković, prof. emeritus, diplomirao je na Poljoprivrednom fakultetu u Zagrebu, 1966. godine. Bio je pripravnik, asistent, profesor, direktor OOUR-a Instituta za zaštitu bilja Fakulteta poljoprivrednih znanosti te predstojnik Zavoda za fitopatologiju. Prethodno je završio poznatu i cijenjenu Šibensku gimnaziju te je govorio dva strana jezika što mu je olakšavalo put k svjetskoj fitopatološkoj znanosti. U mladosti, već od osnovne škole je preferirao prirodoslovlje, a kao većina tadašnjih „starih" Šibenčana volio je i ptice. Za šibenski i kninski rodni kraj, ljude, starinu i običaje ostao je doživotno vezan.

Specijalizirao se u brojnim svjetski poznatim fitopatološkim centrima: Istituto di Patologia vegetale, u više navrata 1976, 1978 i 1979. godine, Bologna-IT; International Mycological Institute London-GB 1979. godine; Centraalbureau voor Schimmelcultures, Baarn 1985. godine-NL) i na studijskim boravcima u Lenjingradu 1986. godine, Francuskoj 1988. godine, SAD-u te Kanadi 1997. godine.

Osim na matičnom fakultetu sudjelovao je i u nastavi na sveučilišnom preddiplomskom studiju „Mediteranska poljoprivreda" u Splitu. Bio je prvi nastavnik „fitopatologije" na Agronomskom fakultetu Sveučilišta u Mostaru i na Veleučilištu "Marko Marulić" u Kninu.

Studenti i kolege su ga percipirali kao vrhunskog stručnjaka i znalca koji je neprestano spajao struku i znanost. Od studenata je tražio znanje, ali im je prikladnim pedagoškim metodama i primjerima s terena olakšavao polaganje opsežnog i složenog gradiva. Ostao im je u dobrom sjećanju, kao profesor i nadasve kao čovjek. Znao je prepoznati, zainteresirati i nagraditi najbolje studente, a neki od njih su danas njegovi nasljednici, profesori i stručnjaci diljem svijeta.

U Hrvatskoj znanstvenoj bibliografiji CROSBI je navedeno oko 460 njegovih bibliografskih jedinica, gdje su navedeni brojni stručni i znanstveni radovi, knjige i drugo. Uz to je napisao oko 170 popularnih članaka. U člancima i knjigama opisao je nekoliko patogenih organizama (više vrsta gljiva, 2 virusa, 2 fitoplazme, 1 bakteriju) na biljkama, do tada ne opisanih u Hrvatskoj i time obogatio saznanja o *tim patogenima.*

[1] EMSL Analytical, Inc., 200 US-130 North Cinnaminson, New Jersey, Sjedinjene Američke države.
* E-mail: zjurjevic@emsl.com (dopisni autor).

Bio je voditelj brojnih domaćih i stranih projekata, član brojnih domaćih i stranih udruženja. U nekoliko domaćih časopisa bio je član uredništva i glavi urednik Glasila biljne zaštite 2007 - 2017. god. Bio je delegat, kasnije član predsjedništva u „*Mediterranean Phytopathological Union"* (MPU) i jedan mandat član uredništva „Phytopathologia Mediterranea", glasila MPU. Profesor Bogdan Cvjetković je i redoviti član ugledne Akademije poljoprivrednih znanosti koja je utemeljena 2017. godine u Zagrebu.

Za njegov doprinos znanosti, struci i nastavi uručeno mu je više nagrada i priznanja, a navodim samo najznačajnije: **Hrvatski sabor:** Državna nagrada za znanost - za životno djelo. **Grad Knin:** Nagrada za zapažene rezultate u znanstvenom, nastavnom i stručnom radu na promicanju lika i djela dr. Lovre Montija. **Sveučilište u Zagrebu Agronomski fakultet:** Povelja Agronomskog fakulteta za posebne zasluge za doprinos na unapređenju znanstvenog, nastavnog i stručnog rada širenju ugleda Fakulteta i agronomske struke u nas i u svijetu. **Hrvatsko društvo biljne zaštite:** Povelja uz zlatnu plaketu - Prvom predsjedniku - Hrvatskog društva biljne zaštite za utemeljenje, vođenje i promicanje društva (2006). Priznanje USDA za uspješno vođenje američko-hrvatskog projekta i mnoge druge.

Doprinos prof. Cvjetkovića u razvoju fitopatološke znanosti i fitofarmacije u Hrvatskoj je nemjerljiva. Profesor je značajno doprinio razvoju suvremene fitopatologije kroz rad s novim generacijama znanstvenika koji su ili će nastaviti njegovim putem. Moj prvi „pravi" posao na Institutu za zaštitu bilja Fakulteta poljoprivrednih znanosti u Zagrebu je započeo 16. rujna 1991. godine gdje je profesor Cvjetković bio predstojnik Zavoda za fitopatologiju. Nakon odvajanja Instituta za zaštitu bilja 30. travnja 1992. godine nastavio sam raditi kao asistent, a kasnije kao docent u Zavodu za fitopatologiju Agronomskog fakulteta Sveučilišta u Zagrebu do 19. veljače 2002. godine. Osobno sam, tijekom 11 godina suradnje s profesorom Cvjetkovićem mogao iz prve ruke posvjedočiti njegovoj velikoj predanosti razvoju Zavoda za fitopatologiju bilo kroz implementaciju novih tehnologija, ili podršci njegovim asistentima na daljnjoj izobrazbi u prestižnim svjetskim institucijama. Njegovi suradnici su se usavršavali na sljedećim institucijama: Željko Tomić (Centraalbureau voor Schimmelcultures, Baarn-NL); Ljubo Isaković (Centraalbureau voor Schimmelcultures, Baarn-NL); Snježana Topolovec-Pintarić (Wageningen University-NL); Edyta Đermić (Max-Planck-Institut für Zellbiolog ie, Rosenhof, Ladenburg-DE). Tihomir Miličević dva puta (Università degli Studi di Bari Aldo Moro Bari-IT); Dario Ivić (Consiglio nazionale delle ricerche Rim-IT) i (*Istituto tossine e micotossine da parassiti vegetali di Bari-IT)*; Darko Vončina (Centro di ricerca e sperimentazione in agricultura "Basile Caramia" Locorotondo-IT; Consiglio nazionale delle ricerche Bari-IT, te Universita degli Studi di Bari "Aldo Moro" Bari-IT); Joško Kaliterna (Università degli Studi di Bari Aldo Moro Bari-IT). Željko Jurjević (Institute Foreign Disease-Weed Science Research Fort Detrich, Maryland-SAD; *Istituto tossine e micotossine da parassiti vegetali di Bari-IT*; International Atomic Energy Agency, Beč-Austrija, te Fulbrigtova zaklada University of Georgia-SAD).

Usavršavanja mladih suradnika ne samo da su doprinijeli napretku Zavoda za fitopatologiju kroz uvođenje novih suvremenih tehnika i tehnologija u znanosti fitopatologije (molekularna dijagnostika, istraživanju mikotoksina itd.), već su utjecala i na njihova životna usmjeravanja u znanosti. Jedan od primjera je i moj osobni. Zavod je imao brojne suradnje s praksom i pojedinim svjetskim institucijama, a jedan od takvih je bila i suradnja s ARS-USDA koja je bila organizirana kroz dvotjednu razmjenu hrvatskih i znanstvenika iz SAD-a. Jednom takvom prilikom bio je red na Zavod za fitopatologiju da delegira osobu koja će posjetiti USDA Foreign Disease, Fort Detrich, Maryland, SAD 1994. godine. Profesor Cvjetković je tada došao u moj ured i kazao mi da ja idem u SAD. Ja sam se kao 26-godisnji mladi asistent smrznuo od straha i odgovorio da ja ne mogu ići, jer dobro ne vladam s engleskim jezikom, budući ga ranije nisam učio u školi. Profesor mi je odgovorio: „*Naravno da nećeš imati problema, pa i prije tebe su drugi išli znajući manje engleskog od tebe, primi se knjige i vjeruj u sebe, ti to možeš*!“. Pri isteku moje dvotjedne posjete bio sam upitan od domaćina da li bih želio produžiti moj boravak. Moj odgovor je bio: „*Rado, ali trebam odobrenje od moje institucije i profesora Cvjetkovića.*“. Već idućeg jutra, dan prije mog zakazanog povratka, moj domaćin kada me je ugledao pri dolasku na posao na drugoj strani hodnika je imao ogroman osmjeh i mahao s listom papira kojeg je upravo primio na faks uz riječi: „*Bogdan je odobrio tvoj ostanak još tri mjeseca.*“. Ta odluka profesora Bogdana Cvjetkovića je usmjerila moju daljnju karijeru kao znanstvenika. Jedna od veliki profesorovih ljubavi u mladim danima je bila i ljubav prema motociklima i mototrkama. Tu njegovu ljubav prema brzoj vožnji sam iskusio i osobno. Na jednom od naših brojnih putovanja za postavljanje poljskih pokusa, on je malo brže ušao u zavoj pri čemu je došlo do malo snažnijeg kočenja. Nakon par trenutaka on me pita: „*Pa nisi se valjda prepao*?“. O dragom profesoru Bogdanu Cvjetkoviću se može napisati još mnogo redaka, ali i sažeti, profesor Cvjetković se može opisati u tri riječi - Mentor, Kolega i Prijatelj! Čovjeka koji je uvijek spreman pomoći. Naposljetku, svome profesoru koji je još uvijek aktivan u svojoj struci želim dobro zdravlje i da još dugo uživa u zasluženoj mirovini.

Upute autorima

Stručno znanstveni časopis Futura objavljuje znanstvene i stručne radove iz biotehničkih znanosti (poljoprivrede, šumarstva, drvne tehnologije, prehrambene tehnologije, nutricionizma, biotehnologije i interdisciplinarne biotehničke znanosti) kao i društvene vijesti, bibliografije, zatim prikaze knjiga i radova, popularne znanstvene radove, polemike i dr. Objavljuju se samo radovi koji nisu drugdje predani za objavljivanje, niti objavljeni. Znanstveni radovi se kategoriziraju: – izvorni znanstveni rad (original scientific paper) – pregledni znanstveni rad (scientific review) – prethodno priopćenje (preliminary communication) – konferencijsko priopćenje (conference paper) – rad prethodno prezentiran na konferenciji. Radove recenziraju dva ili više znanstvenika iz odgovarajućeg područja.

Rad ne smije imati više od 17 tipkanih stranica, veličina slova 11, font Times New Roman, prored 1,5, margine 2,5. Izuzetno, uz odobrenje uredništva, neki interdisciplinarni ili uredništvu interesantni radovi mogu sadržavati do 25 ili više tipkanih stranica. Rukopisi se predaju u elektroničkom obliku na hrvatskom ili engleskom jeziku (e-mail: urednistvo@gazette-future.eu).

Izvorni znanstveni rad treba sadržavati: puna imena i prezimena autora s nazivima institucija, adresom i e-poštom; naslov, sažetak, abstract, uvod, materijale i metode, rezultate istraživanja, diskusiju, zaključak i literaturu podebljano za naslove. Radovi napisani na engleskom jeziku se predaju bez naslova na hrvatskom jeziku i hrvatskog sažetka.

Naslov rada treba biti što kraći, na hrvatskom i engleskom jeziku. Kategoriju rada predlažu autori, a potvrđuju recenzenti i glavni urednik.

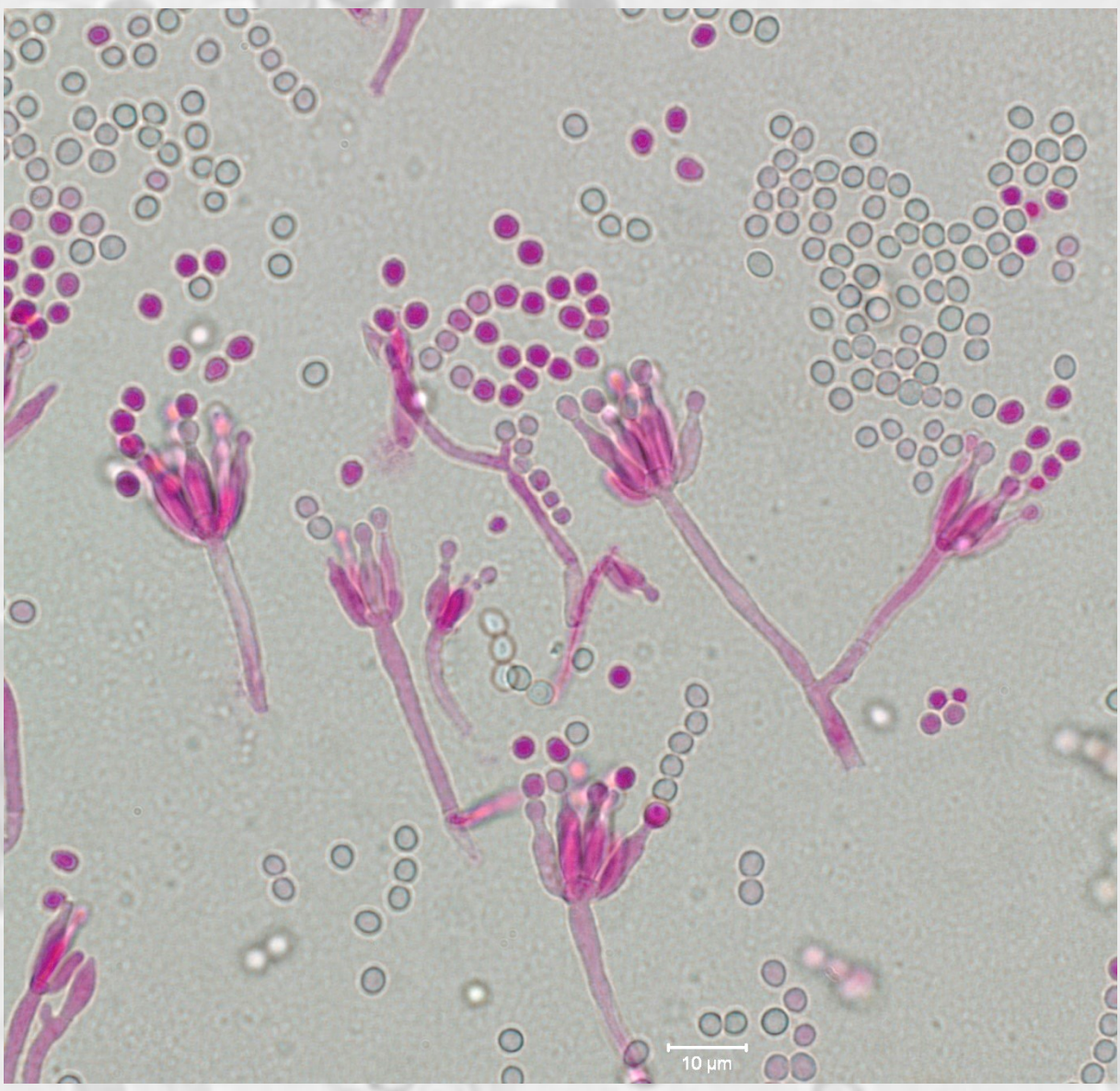
Sažetak treba sadržati opći prikaz, metodologiju, rezultate istraživanja i zaključak. Rad je potrebno pisati u trećem licu s min. 3 do 5 ključnih riječi. Obim sažetka ne bi smio biti veći od 250 riječi. Abstract je prijevod sažetka s ključnim riječima.

Uvod treba sadržavati što je do sada istraživano i što se željelo postići danim istraživanjem. Materijale i metode istraživanja treba ukratko izložiti. U rezultatima i diskusiji (raspravi) potrebno je voditi računa da se ne ponavlja iznijeto. U zaključcima je potrebno izložiti samo ono što pruža kratku i jasnu predstavu istraživanja. Literaturu treba poredati prema abecednom redu autora i to: prezime i početno slovo imena autora ili Anonymous (nepoznat autor), godina izdanja u zagradama, naslov knjige ili članka, naziv časopisa te broj ili godište, kao i mjesto izdavanja i oznaku stranica od–do. Više od deset autora se u literaturi navodi kao npr. Prezime et al. (2018). Fusnote u radu treba izbjegavati ili eventualno koristiti za neka pojašnjenja. Autori se u tekstu citiraju sukladno APA standardu npr. (Prezime, 2018); (Prezime1 i Prezime2, 2016); (Prezime et al., 2018) (više od dva autora). Citate prate navodnici ("n") i stranica preuzimanja citiranog teksta (Prezime, 2018, str. 44).

Tablice se numeriraju i navode iznad na hrvatskom i u kurzivu na engleskom jeziku.

Slike se numeriraju i navode ispod na hrvatskom i u kurzivu na engleskom jeziku.

Rezolucija slika (grafikon, fotografija, crtež, ilustracija, karta) treba iznositi najmanje 300 dpi.



Fotografija: *Penicillium cvjetkovicii.*

Izvor: Željko Jurjević.