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Časopis izlazi u elektroničkom izdanju dva puta godišnje, krajem lipnja i prosinca, a predviđena su i dva specijalna izdanja tijekom godine iz biotehničkog područja.
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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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SADRŽAJ:

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

Katarina Martinko[1]*, Ivona Novaković[2]

prethodno priopćenje (preliminary communication)

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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).

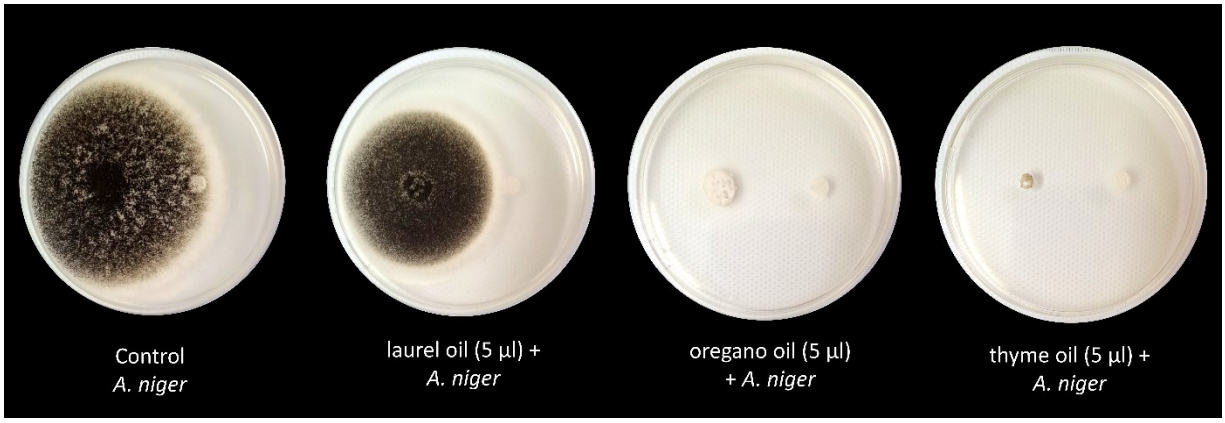

Figure 1. *Antifungal effect of laurel, oregano and thyme oil against Aspergillus niger after 7 days.*

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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