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S A D R Ž A J

N a u č n i r a d o v i

CONTENTS

Scientific papers

Plant Protection
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Scientific paper

IN VITRO EFFECTIVENES OF DIFFERENT ESSENTIAL OILS IN CONTROL OF *ALTERNARIA ALTERNATA*

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The effectiveness of volatile phase of essential oils of *menthe*, *eucalyptus* and *rosmarinus* in control of *A. alternata*, a postharvest pathogen on fruits and vegetables, expressed through inhibition of mycelium growth, in vitro, has been tested. The inhibitory effect of tested oils has been determined four days after setting the trial by calculating the percentage of inhibition of radial growth of pathogen mycelium (PIRG), while the minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) have been determined seven i.e. fourteen days later. The highest value of PIRG (100%) was found in essential oil of menthe when applied in concentration of 0,15µl/ml of air and essential oil of rosmarinus applied in 0,6µl/ml of air.

Essential oil of eucalyptus had the highest PIRG value (89,74%) when applied in concentration of 0,6µl/ml of air. MIC of essential oil of menthe was 0,3µl/ml of air while other two essential oils did not show total inhibitory effect in tested concentrations (MIC >0,6µl/ml of air). Essential oil of menthe did not have a fungicide effect on pathogen, not even in the highest concentration (MFC > 0,6µl/ml of air). In other two oils MFC has not been determined since they did not express the inhibitory effect in the first. Therefore, menthe essential oils could be an alternative to chemicals to control *A. alternata*, a postharvest pathogen on fruits and vegetables, and can control this pathogen in vitro. These results will help in further testing of effectiveness of essential oils in vivo.

Key words: mentha, *eucalyptus*, *rosmarinus*, inhibition, biological control.

INTRODUCTION

Alternaria alternata is one the most common pathogens in the vegetable crop production pre and post harvest. This pathogen jeopardizes production of different vegetable crops in all phases of production, starting from seed to plants in vegetation to fruits in storage (Agrios, 2005; Abd-Alla et al., 2009; Bulajić et al., 2009). As a postharvest pathogen, *A. alternata* causes Alternaria rot that causes great losses. These losses go up to 30% per year and even up to 43% of the total production of tomatoes (Abd-Alla et al., 2009). It is important to note that storage conditions necessary to preserve the quality of fruits for a long time are also favorable for the development of this pathogen. Particularly, the increased humidity which is necessary in order to prevent the occurrence of fruit shrivel is also very good for the development of the pathogen, and the fact that *A. alternata* develops even at low temperatures supports the fact that this is a very important pathogen (Barkai-Golan and Pasteur, 2008a; El-Sheshtawi et al., 2010).

Apart from direct economic considerations, diseased fruits pose a potential health risk. *A. alternata* is known to produce mycotoxins under certain conditions. The occurrence of Alternaria mycotoxins has been recorded in tomatoes, peppers, melons as well as in several processed fruits including tomato products (Barkai-Golan, 2008b). The major mycotoxins that can appear in the fruits of vegetables when stored are: alternariol ($C_{14}H_{10}O_5$), alternariol methyl ether ($C_{15}H_{12}O_5$) and altenuene ($C_{15}H_{16}O_6$), which are benzopirone derivates; also tenuazonic acid ($C_{10}H_{15}NO_3$), which is a tetramic acid derivate; and altertoxin-I ($C_{20}H_{16}O_6$), a perylene derivate (Andersen and Frisvad, 2004).

Several kinds of synthetic fungicides have been used to control the postharvest decay caused by Alternaria rot. Using fungicides for a long time results in the development of resistant strains (Rosslenbroich and Stuebler, 2000). At the same time, many of these fungicides gradually become ineffective (Spotts and Cervantes, 1986). As a result, it is easy to conclude that this pathogen is difficult to control. With the continued loss of currently used postharvest decay control measures (fungicides), there is a perpetual need to search for alternatives (Abd-Alla et al., 2009). Given the trend of modern plant protection to limit the use of fungicides, in order to protect consumers and the environment, it is very important to find new methods and environmentally friendly ways to control pathogens that will be equally effective (Djordjevic et al ., 2010a, 2010b). One possible solution is to apply the essential oils of some plants. The impact of these oils on certain pathogenic fungi of plants and fungi important in the food industry was investigated by several authors (Abd-Alla et al., 2009; Aslam *et al*., 2010; Parven *et al*., 2010; Simić *et al.*, 2008; Soković *et al*., 2008, 2009a, 2009b; Veljić *et al*., 2009; Zhang *et al.*, 2009). Given that plants are rich sources of antifungal

compounds, they could be an appropriate alternative to conventional fungicides if these compounds are formulated correctly (Tanovic et al., 2005, Wilson et al., 1997). Before any pesticide's application *in vivo*, biological or conventional, it is first necessary to determine its toxicity, i.e. its efficiency *in vitro*.

The aim of this study is to determine the antimicrobial activity, i.e. the *in vitro* efficiency of some essential oils in controlling the postharvest pathogen of vegetable crops, *Alternaria alternata*, to determine their toxicity and determine the approximate concentration as the starting point for *in vivo* studies.

MATERIALS AND METHODS

The pathogen identification required isolation of pure fungi culture by repetitive single-spore transfers followed by microscopic examination.

The antifungal activity of essential oils of *Mentha piperita*, *Eucaliptus citriodora*, *Rosmarinus officinalis* was investigated to *Alternaria alternata*, by exposing mycelium of pathogen to volatile phase of these oils (Soylu *et al.,* 2006; Tanović *et al.*, 2009). Mycelial plug (5x5mm) was transferred to the center of the Petri plate (R=9cm), after which the plates were turned upside down. The oils were applied as a drop onto the inner side of the plate covers on the sterile filter paper (R=0,5cm), which was placed in the center on the cover glass, at the concentrations of 0.04, 0.06, 0.1, 0.15, 0.3 i 0.6 µl/ml of air inside the Petri plates using micropipette. In order to enable the contact of volatile phase of oils and pathogen, the Petri plates were kept upside down. The plates were sealed with self-adhesive foil in order to prevent release of oil vapors out of the plates. The Petri plates were also kept at 23 °C. As control, a Petri plate with a drop of sterile distilled water instead of oil was used.

After four, seven and fourteen days, the radial growth of pathogen mycelium in the treated plates and control were measured. After four days of exposure to oil vapor, the percent of inhibition of radial growth of pathogen mycelium (PIRG) was calculated. After seven days from the exposure, the plates were observed for initial growth of mycelium without measuring. The concentration of oil which completely inhibited mycelium growth after seven-day exposure was considered fungistatic and the lowest of these concentrations were determined as the minimum inhibitory concentration (MIC). After that, the plates were opened and ventilated in the sterile laminar flow for 30 minutes in order to remove volatiles of oils in order to determine fungicidal effect of oils. The fungicidal concentrations were those which suppress mycelial growth even after seven days from ventilation. The lowest concentrations were considered as minimal fungicidal concentration (MFC).

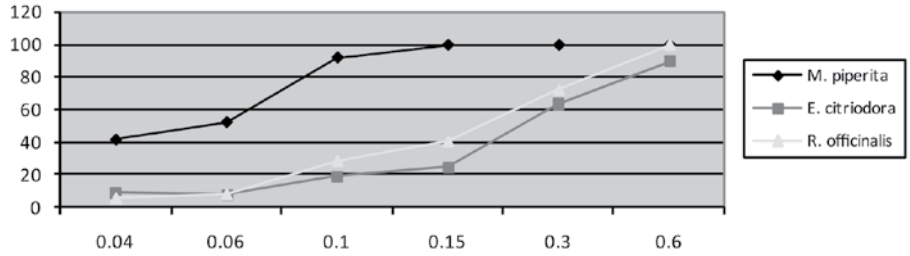
All experiments were performed twice with five replications of each oil concentration. The percentage of inhibition of radial growth of pathogens mycelia were calculated using the following formula:

$$PIRG(\%) = \{g_c - g_t / g_c\} \times 100 ,$$

where ***gc*** is the growth of mycelium in control plates, ***gt*** the growth of mycelium in treated plates. The analysis of variance and significance of differences using Duncan's Multiple Range Test (P=0.05) was done by using mathematical program MATLAB Ver. 7.0.

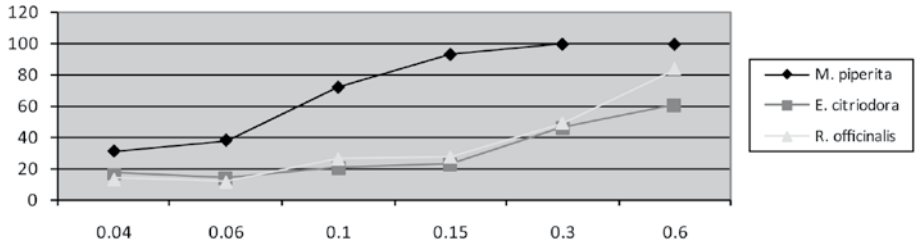
RESULTS

The antifungal activity of essential oils of *Mentha piperita*, *Eucaliptus citriodora* and *Rosemarinus officinalis* against postharvest pathogen *Alternaria alternate* were investigated. The oils were treated to each bioassay plate which allows only volatiles to be causative agents for any microbial inhibition. Four days after exposure of pathogen to oils, the highest percentage of inhibition was demonstrated in the essential oil of *M. piperita*, which showed total inhibition at a concentration of 0.15 µl/ml of air. The essential oil of *R. officinalis* expressed the same level of inhibition of *A. alternate*, applied at a concentration of 0.6 µl/ml of air. Meanwhile, the essential oil of *E. citriodora* demonstrated the highest percentage of inhibition in the concentration of 0.6 µl/ml of air and the value of inhibition was 89.74% (Graph. 1).



Graf. 1 - Inhibitorno dejstvo isparljive faze eteričnih ulja *Mentha piperita*, *Eucaliptus citriodora* and *Rosemarinus officinalis* prema skladišnom patogenu Alternaria alternata nakon 4 dana.

Graph. 1 - Inhibitory activity of volatile phase of essential oils of *Mentha piperita*, *Eucaliptus citriodora* and *Rosemarinus officinalis* toward postharvest pathogen Alternaria alternata after 4 days of exposure.



Graf. 2 - Inhibitorno dejstvo isparljive faze eteričnih ulja *Mentha piperita, Eucaliptus citriodora* and *Rosemarinus officinalis* prema skladišnom patogenu Alternaria alternata nakon 7 dana.

Graph. 2 - Inhibitory activity of volatile phase of essential oils of *Mentha piperita*, *Eucaliptus citriodora* and *Rosemarinus officinalis* toward postharvest pathogen Alternaria alternata after 7 days of exposure.

Seven days after the exposure of pathogen to the effects of volatiles of essential oils, minimum inhibitory concentration (MIC) based on the percentage of inhibition was determined (Graph. 2). The lowest value of MIC was detected with the *M. piperita* oil and 0.3 µl/ml of air, while the other two oils did not have this value in the observed range of concentrations and the MIC was higher than 0.6 µl / ml of air (MIC> 0.6 µl/ml of air). Since the oil of *M. piperita* showed total inhibition of growth after seven days, MFC is determined only for this oil seven days after removing the oil phase, i.e. seven days after the determination of MIC and the Petri plates ventilation. After this period MFC was not determined because of the occurrence of growth of mycelium of *A. alternata* (MFC > 0,6 µL/ml of air) (Table 1.).

Table 1 - Values of MIC and MFC of *Mentha piperita*, *Eucaliptus citriodora* and *Rosemarinus officinalis*

Tabela 1 - MIC i MFC eteričnih ulja *Mentha piperita*, *Eucaliptus citriodora* and *Rosemarinus officinalis*

Essential oils (Eterična ulja)	**MIC***	**MFC****
M. piperita	0,3µl/ml of air	> 0,6µl/ml of air
E. citriodora	> 0,6µl/ml of air	> 0,6µl/ml of air
R. officinalis	> 0,6µl/ml of air	> 0,6µl/ml of air

*MIC minimal inhibitory concentration,
**MFC minimal fungicidal concentration
*MIC minimalna inhibitorna koncentracija
**MFC minimalna fungicidna koncentracija

DISCUSSION

Biological control has been consider as one of the most promising alternatives to fungicides (Abd-Alla et al., 2009; Feng and Zheng, 2007; Đorđević et al., 2010). Natural pesticides based on plant-essential oils may represent alternative crop protectans whose time has come, but according to some this claim has yet to be substantiated through controlled experiments and scientific investigation (Isman, 2000). Several studies have explored the potential of essential oils as antifungal agents (Abdolahi et al., 2010; Tanović et al., 2005; Lee et al., 2007; Fawzi et al., 2009). In this study, we investigated the antifungal activities of essential oils of *Mentha piperita*, *Eucaliptus citriodora* and *Rosemarinus officinalis* against postharvest pathogen *Alternaria alternata* by exposure to volatile phases of the oils, *in vitro*. All of the oils had the inhibition effect on mycelial growth of *A. alternate*, more or less. As observed, the antifungal activities of essential oils were dependent on the type of essential oil and oil concentration. *In vitro* tests showed that the essential oil of *Mentha piperita* had the highest inhibition effect. However, the antifungal properties of this essential oil at used concentrations were fungistatic, not fungicidal. The oil of *R. officinalis* showed strong inhibition but not strong enough to have fungistatic or fungicidal effects on this pathogen. The weakest inhibition effect was seen in the essential oil of *E. citriodora*. In the research of Abd-Alla et al. (2009), the essential oil of *M. piperita* showed the inhibition of mycelial growth of *A. alternata* at maximal rate of application with growth reduction of 46%. The method in that research considers incorporation of oils in medium and, therefore, the differences ensue from that fact. Because of that, the essential oil of *E. citriodora* did not show any inhibitory effect on *A. alternate* in the research of Feng and Zheng (2007) while in this research it had growth reduction of 89,74% when applied in 0,6µl/ml of air. In other studies conducted to test antifungal effects of *E. citriodora* oil, evaluating the effect of volatile phase, Lee et al. (2007) proved that this phase has a strong inhibitory effect on *Botritis cinerea* and *Rhizoctonia solani*. In the same study, the essential oil of *R. officinalis* did not have any inhibitory effect on investigated pathogens while in our study it had 100% inhibition rate applied at 0,6µl/ml of air. Also, the essential oil of *M. piperita* in the study of Lee et al. (2007) did not show any fungistatic effect whatsoever, but in our study it had a very strong fungistatic effect. In the research of Tanović et al. (2005), which investigated antifungal effect of volatile phase of these oils on *Botritis cinerea*, it had a strong inhibition effect. These differences are showing that comparing results of different studies is difficult because of the differences in plant extract composition and in methodologies of assessments of microbial activity (Arslan and Dervis, 2010).

Although some studies have reported on the antifungal activity of essential oils, the mechanism(s) of action of such oils is (are) poorly understood. However, some researchers reported that there is a relationship between the chemical structure of the most abundant compounds in the essential oils and the antimicrobial activity. According to Faid et al. (1996), the antimicrobial activity of major oil compounds happens in the following order: phenols (highest activity) > alcohols > aldehydes > ketones > ethers > hydrocarbons. The chemical composition of these essential oils was the subject of several studies (Soylu et al., 2006; Lee et al., 2007). Based on the research of Pitarokili et al. (2002), the composition of oil may vary on different localities. Because of that, the analysis of composition of these oils will be the subject of further research.

Alternaria alternata is a postharvest pathogen that causes severe losses of vegetables in storage. Due to the fact that chemical compounds are becoming ineffective and that they are harmful for consumers, control strategy for this pathogen in the future is applying biological means of control. Using essential oils of different plant species is one of these potential control strategies. Based on the results of this study, we can confirm this statement and encourage future research of implementation of these and other potential essential oils *in vitro* as well as *in vivo*.

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IN VITRO EFIKASNOST POJEDINIH ETERIČNIH ULJA U SUZBIJANJU *ALTERNARIA ALTERNATE*

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REZIME

Ispitivana je efikasnost isparljive faze etarskih ulja *mente*, *eukaliptusa* i *ruzmarina* u suzbijanju *A. alternata*, patogena uskladištenih plodova povrtarskih i voćarskih kultura, izraženo kroz inhibiciju porasta micelije, *in vitro*. Inhibitorni efekat posmatranih ulja izračunavan je četiri dana nakon postavljanja ogleda i izražen je procentom inhibicije radijalnog porasta micelije patogena (PIRG) dok su minimalna inhibitorna koncentracija (MIC) i minimalna fungicidna koncentracija (MFC) izračunavane nakon sedam odnosno četrnaest dana. Etarsko ulje *mente* imalo je najveću vrednost PIRG (100%) pri koncentraciji ulja od 0,15µl/ml vazduha. Stoprocentni inhibitorni efekat (PIRG) ulje *ruzmarina* ispoljilo je pri koncentraciji 0,6µl/ml vazduha, dok je ulje *eukalipusa* imalo najvišu vrednost PIRG (89,74%) pri koncentraciji 0,6µl/ml vazduha. Najnižu vrednost MIC imalo je ulje *mente* (0,3µl/ml vazduha) dok ulja *eukaliptusa* i *ruzmarina* nisu pokazala totalni inhibitorni efekat u ispitivanim koncentracijama (MIC > 0,6µl/ml vazduha). Etarsko ulje *mente* nije pokazalo fungicidni efekat prema posmatranom patogenu ni u najvišoj koncentraciji (MFC > 0,6µl/ml vazduha). Kod druga dva ulja MFC nije ni određivana jer nisu imala ni inhibitorni efekat prema miceliji patogena. Na osnovu ovih rezultata možemo zaključiti da je primena etarskog ulja *mente* u cilju kontrole *A. alternata*, patogena uskladištenih plodova, opravdana i da se na taj način može kontrolisati ovaj patogen, *in vitro*. Ovi rezultati će poslužiti kao polazna tačka za dalja ispitivanja u cilju primene etarskih ulja i *in vivo*.

Key words: menta, eukaliptus, ruzmarin, inhibicija, biološka kontrola.

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UTICAJ JEDINJENJA UGLJENIKA I AZOTA NA PORAST IZOLATA *COLLETOTRICHUM* SPP.

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U Srbiji je tokom poslednjih godina zabeležena pojava antraknoze ploda kruške, jabuke, višnje i paradajza. Iz obolelih plodova su izolovane gljive roda *Colletotrichum*. Za komparativna istraživanja je odabrano 20 monosporijalnih izolata, kao i referentni sojevi *C. acutatum* (CBS 294.67) i *C. gloeosporioides* (CBS 516.97) koji su predstavljali kontrolu. Fiziološka proučavanja su obuhvatila i određivanja uticaja različitih izvora ugljenika i azota na porast kultura *C. acutatum* i *C. gloeosporioides*. Rezultati su pokazali da je karboksimetilceluloza najadekvatniji izvor ugljenika za rast izolata *Colletottrichum* spp. Od tri proučavana izvora azota, najveći porast svih testiranih gljiva zabeležen je na podlogama sa kazeinhidrolizatom.

Ključne reči: *Colletotrichum* spp., ugljenik, azot, porast.

UVOD

Gljive roda *Colletotrichum* (teleomorf *Glomerella*), su kosmopolitske vrste umerenog i tropskog klimatskog područja, prisutne na velikom broju domaćina: voću, povrću, leguminozama, žitaricama, šumskom i ukrasnom bilju (Bailey et al., 1992). Na biljkama mogu prouzrokovati nekrozu korena i stabla, pegavost i uvijenost lista, defolijaciju, palež i nekrozu cvasti, kao i antraknozu i trulež plodova (Wharton and Uribeondo, 2004). Simptomi se manifestuju tokom vegetacije, ali je češći slučaj ostvarivanja latentnih infekcija koje nakon berbe plodova

i tokom naedekvatnih uslova skladištenja kulminiraju pojavom nekroze i truleži (Freeman et al., 1998). Gubici nastali usled truljenja antraknoznih plodova voća i povrtarskih kultura mogu iznositi od 10 do 80%, što u nekim zemljama u razvoju ozbiljno ugrožava tržišnu ekonomiju (Than et al., 2008).

U Srbiji su poslednjih godina sa atraknoznih plodova voća i povrća izolovana, a potom morfološki i molekularno identifikovana dva, ekonomski najznačajna patogena roda *Colletotrichum*: *C. acutatum* i *C. gloeosporioides* (Ivanović et al., 2007; Živković i sar., 2008, 2009, 2010; Živković, 2011).

Ugljenik i azot predstavljaju esencijalne komponente neophodne za sintezu proteina, rast ćelija i razmnožavanje gljiva (Oritsejafor, 1986). Međutim, poznato je da svi izvori ugljenika i azota ne manifestuju podjednako stimulativan efekat na razvoj i sporulaciju, kao i da neke gljive poseduju veću afinitet prema određenim jedinjenjima ovih elemenata. S obzirom da su vrstama *C. acutatum* i *C. gloeosporioides*, kao i ostalim fitopatogenim gljivama za odvijanje normalnih metaboličkih i reproduktivnih funkcija neophodni ugljenik i azot, od velikog je značaja utvrditi u kom obliku se ovi elementi mogu najefikasnije iskoristiti.

Osnovni cilj rada je ispitivanje uticaja različitih izvora ugljenika i azota na rast izolata *Colletotrichum* spp. Dobijene informacije o nutritivnim zahtevima gljiva roda *Colletotrichum* su od značaja za sagledavanje odnosa patogen – biljka, jer su infekcioni potencijal patogena i osetljivost biljke domaćina u direktnoj zavisnosti od raspoloživih izvora ugljenika i azota.

MATERIJAL I METODE

Ispitivanje usvajanja jedinjenja ugljenika i azota. Za ispitivanje nutritivnog efekta ugljenika i azota, odabrano je 20 izolata *Colletotrichum* spp. poreklom sa različitih domaćina (Tabela 1). U komparativna proučavanja su uključena i dva referentna soja: *C. acutatum* (CBS 294.67) i *C. gloeosporioides* (CBS 516.97) iz kolekcije Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, Holandija. Sve kulture su gajene su na osnovnoj podlozi obogaćenoj sa 10 g/l ugljenika, odnosno 2 g/l azota (Paterson and Bridge, 1994). Kao izvori ugljenika korišćeni su: karboksimetilceluloza (KMC), skrob i pektin, a od izvora azota: kazeinhidrolizat (KH), natrijumnitrat ($NaNO_3$) i amonijumdihrogenfosfat ($NH_4H_2PO_4$). Zasejavanje podloga je izvršeno nanošenjem fragmenata ispitivanih izolata iz kultura starih 7 dana, odgajenih na krompir-dekstroznom agaru (KDA). Diskovi micelija su postavljani su u centar Petri kutija, a zasejane podloge su potom inkubirane u termostatu na temperaturi od 25°C. Ogled je izveden u 3 ponavljanja. Porast izolata *Colletotrichum* spp. ocenjen je sedmog dana merenjem prečnika kolonije, a dobijene vrednosti su preračunate u procente poređenjem sa vrednostima porasta izolata na osnovnoj podlozi (bez dodatih jedinjenja ugljenika i azota).

Statistička obrada rezultata. Statistička obrada rezultata obavljena je upotrebom paketa STATISTICA v. 6. Analiza uticaja dva faktora je izvršena primenom parametarskog modela analize varijanse (ANOVA/MANOVA). Provera adekvatnosti ovog modela za konkretnu analizu sprovedena je na osnovu vrednosti koeficijenata varijacije (Cv) i Leveneovog testa za homogenost varijansi. Pojedinačna poređenja tretmana sprovedena su na bazi Duncan multiple range testa. Nakon obrade rezultata svi ispitivani izolati *Colletotrichum* spp. grupisani su modelom klaster analize, baziranoj na Euklidskoj distanci i kompletnom povezivanju.

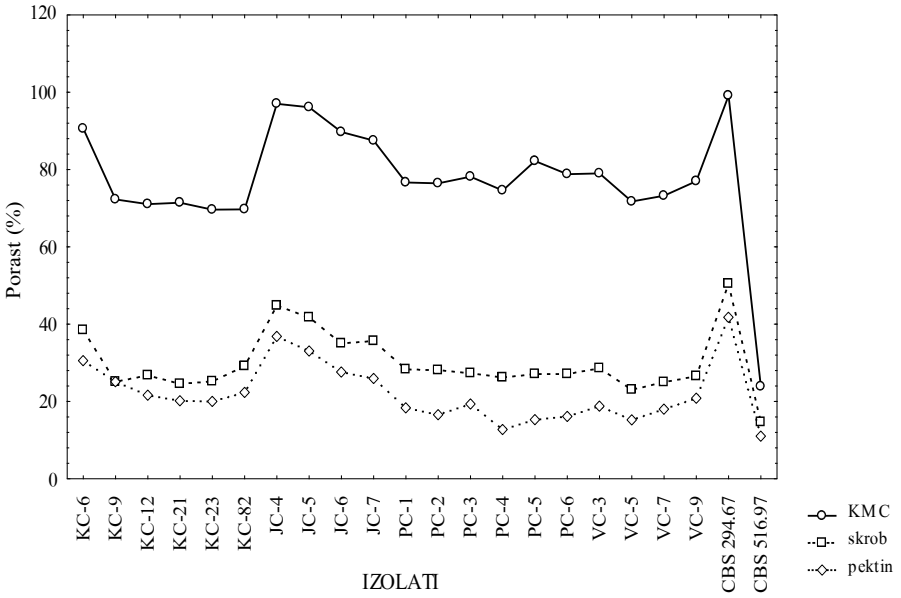
Tabela 1 - Izolati *Colletotrichum* spp.

Table 1 - Isolates of *Colletotrichum* spp.

Izolat Isolate	Vrsta Species	Domaćin Host	Godina izolacije Year of isolation
KC-6	*C. gloeosporioides*	kruška/pear	2005
KC-9	*C. gloeosporioides*	kruška/pear	2005
KC-12	*C. gloeosporioides*	kruška/pear	2005
KC-21	*C. acutatum*	kruška/pear	2006
KC-23	*C. acutatum*	kruška/pear	2006
KC-82	*C. acutatum*	kruška/pear	2007
JC-4	*C. acutatum*	jabuka/apple	2007
JC-5	*C. gloeosporioides*	jabuka/apple	2007
JC-6	*C. gloeosporioides*	jabuka/apple	2008
JC-7	*C. gloeosporioides*	jabuka/apple	2008
VC-3	*C. gloeosporioides*	višnja sour cherry	2008
VC-5	*C. gloeosporioides*	višnja sour cherry	2008
VC-7	*C. gloeosporioides*	višnja sour cherry	2008
VC-9	*C. gloeosporioides*	višnja sour cherry	2008
PC-1	*C. acutatum*	paradajz/tomato	2008
PC-2	*C. acutatum*	paradajz/tomato	2008
PC-3	*C. acutatum*	paradajz/tomato	2007
PC-4	*C. acutatum*	paradajz/tomato	2007
PC-5	*C. acutatum*	paradajz/tomato	2008
PC-6	*C. acutatum*	paradajz/tomato	2008

REZULTATI

Ispitivanje usvajanja jedinjenja ugljenika. Od tri testirana izvora ugljenika, izolati *Colletotrichum* spp. najveći porast ostvaruju na podlozi sa KMC (69,70-97,01%), značajno manji na supstratu obogaćenom skrobom (23,04-38,50%), dok je najmanji porast zabeležen u prisustvu pektina (12,70-36,82%), (Graf. 1). Referentni sojevi *C. acutatum* i *C. gloeosporioides* veoma specifično reaguju na prisustvo jedinjenja ugljenika u podlozi. Za *C. acutatum* (CBS 294.67) je karakteristično da u najvećem procentu usvaja sva tri izvora ugljenika, za razliku od referentnog soja *C. gloeosporioides* (CBS 516.97) koji je gotovo indiferentan na prisustvo ovih jedinjenja i izdvaja se od ostalih po veoma slabom porastu na podlozi sa KMC-om (23,85%), skrobom (14,68%) i pektinom (11,01%).



Graf. 1 - Porast izolata *Colletotrichum* spp. na različitim izvorima ugljenika.

Chart 1 - Growth of isolates of *Colletotrichum* spp. on different sources of carbon.

Leveneovim testom utvrđena je nehomogenost varijansi izolata *Colletotrichum* spp. (F=1,752; p=0,003). Rezultati obrađeni analizom varijanse (MANOVA) kao dvofaktorijalni ogled ukazuju da uzmeđu izolata, izvora ugljenika i njihovih interakcija postoje statistički veoma značajne razlike (Tabela 2).

Tabela 2 - Analiza varijanse porasta *Colletotrichum* spp. na različitim izvorima ugljenika.

Table 2 - Analysis of variance of mycelial growth of *Colletotrichum* spp. on different sources of carbon.

Izvori varijacije Source of variation	Stepeni slobode	Sredine kvadrata	Količnik	Nivo značajnosti
	df	MS	F	p-level
izolati/isolates	21	0,08	466,12	0,000**
izvori C/sources of C	2	5,93	32605,76	0,000**
izolati x izvori C isolates x sources of C	42	0,01	59,12	0,000**
greška/error	132	0,00	-	-

** Statistički veoma značajna razlika ($p<0,01$).
** Statistically very significant diference ($p<0,01$).

Na osnovu Duncanovog testa određeni su nivoi značajnosti porasta izolata *Colletotrichum* spp. na svakom ispitivanom izvoru ugljenika (Tabela 3).

Na podlozi sa KMC-om izolati sa ploda kruške (osim KC-6) u međusobnim poređenjima ne manifestuju statistički značajne razlike. Takođe, razlike u porastu ne postoje ni između ovih kultura i izolata VC-5 i VC-7. Parnim poređenjima patogena JC-4 i JC-5, kao i JC-6 i JC-7 nisu konstatovane statistički značajne razlike u pogledu porasta kultura na supstratu sa KMC-om. Duncanovim testom nisu utvrđene značajne razlike ni između izolata sa paradajza i većine izolata poreklom sa višnje. Referentni soj *C. acutatum* (CBS 294.67) na podlozi sa KMC-om nije statistički značajno različit jedino od patogena JC-4 i JC-5, a *C. gloeosporioides* (CBS 516.97) je po izrazito slabom porastu na ovom izvoru ugljenika, statistički značajno različit od ostalih ispitivanih izolata *Colletotrichum* spp.

Na podlozi obogaćenoj skrobom nisu konstatovane statistički značajne razlike u porastu izolata KC-9, KC-12 i KC-21. Ove kulture se istovremeno ne razlikuju od većine izolata sa paradajza i višnje. Hromogeni izolati KC-23 i KC-82, na osnovu porasta na podlozi sa skrobom, takođe nisu statistički značajno različiti. Patogeni *Colletotrichum* spp. sa paradajza u međusobnim poređenjima ne manifestuju značajne razlike, a takođe ni u poređenjima sa izolatima VC-7 i VC-9. Referentni sojevi CBS 294.67 i CBS 516.97, su po svom porastu na podlozi sa skrobom statistički značajno različiti od ostalih izolata *Colletotrichum* spp.

Tabela 3 - Uticaj različitih izvora ugljenika na porast izolata *Colletotrichum* spp.

Table 3 - Effect of different carbon sources on growth of *Colletotrichum* spp.

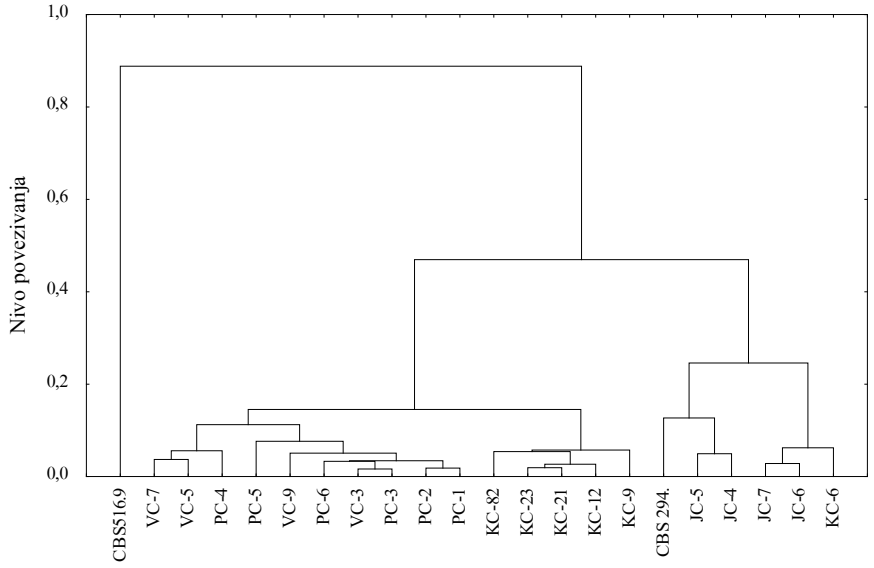
Izolat Isolate	Izvori ugljenika Sources of carbon					
	KMC KMC		Skrob Starch		Pektin Pectin	
KC-6	90.61	a*	38.50	p	30.52	m
KC-9	72.28	b	25.09	a	25.09	a
KC-12	71.06	bc	26.74	ab	21.61	b
KC-21	71.43	bcd	24.54	abc	20.15	bc
KC-23	69.63	bcde	25.19	abcd	20.00	bd
KC-82	69.70	bcdef	29.17	be	22.35	b
JC-4	97.01	g	44.78	r	36.82	n
JC-5	96.12	g	41.75	s	33.01	o
JC-6	89.72	ah	35.05	f	27.57	e
JC-7	87.50	h	35.65	f	25.93	ae
PC-1	76.67	i	28.33	beg	18.33	cdf
PC-2	76.45	ij	28.10	begh	16.53	fg
PC-3	78.15	ijk	27.31	abdeghi	19.33	cdfh
PC-4	74.59	bijl	26.23	abcdghij	12.70	i
PC-5	82.20	p	27.12	abcdeghijk	15.25	gj
PC-6	78.81	ijkm	27.12	abcdeghijkl	16.10	gjk
VC-3	79.02	ijkmn	28.57	beghijklm	18.75	cdfhl
VC-5	71.74	bcdefo	23.04	acdn	15.22	gjk
VC-7	73.25	bcdlno	25.00	abcdijklno	17.98	cdf-ghkl
VC-9	76.99	ijklm	26.55	abcdeghij-klmo	20.80	bcdhl
CBS 294.67	99.13	g	50.43	t	41.74	p
CBS 516.97	23.85	r	14.68	u	11.01	i

* Vrednosti u koloni označene istim slovom nisu statistički značajno različite na osnovu Duncan multiple range testa ($p<0.05$).

* Means in column followed by the same letter are not statistically significantly different by Duncan's multiple range test ($p<0.05$).

Na podlozi sa pektinom većina ispitivanih izolata su statistički značajno različiti. Izolati sa ploda kruške, KC-12, KC-21 i KC-23 u prisustvu pektina manifestuju porast koji nije statistički značajno različit. Ovi patogeni nisu značajno različiti ni od izolata PC-1, PC-3, VC-3, VC-7 i VC-9. Izolati JC-6 i JC-7, po porastu na podlozi obogaćenoj pektinom, takođe nisu statistički značajno različiti. Većina patogena poreklom sa ploda paradajza i višnje formira kolonije koje po veličini nisu međusobno statistički značajno različite. Referentni soj *C. acutatum* (CBS 294.67) se na ovom ispitivanom izvoru ugljenika statistički značajno razlikuje od svih ostalih kultura *Colletotrichum* spp., a porast referentnog soja *C. gloeosporioides* (CBS 516.97) nije značajno različit jedino od izolata PC-4.

Na osnovu ispoljenih sličnosti/razlika u pogledu porasta izolata *Colletotrichum* spp. na tri različita izvora ugljenika, urađen je dendrogram baziran na Euklidskoj distanci i kompletnom povezivanju (Graf. 2). Svi ispitivani izolati su svrstani u tri klastera. S obzirom na izrazito slabo usvajanje ispitivanih



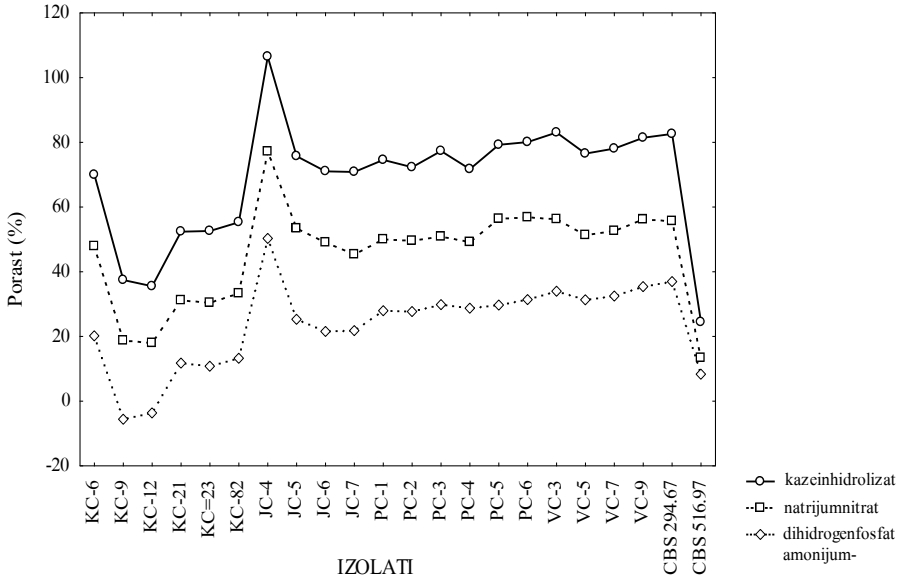
Graf. 2 - Dendrogram izolata *Colletotrichum* spp. na osnovu usvajanja različitih izvora ugljenika.

Chart 2 - Dendrogram of isolates of *Colletotrichum* spp. based on utilization of different sources of carbon.

jedinjenja ugljenika, referentni soj *C. gloeosporioides* (CBS 516.97) je izdvojen i formira prvi zasebni klaster. Drugi klaster je sačinjen od dva subklastera, sa 15 izolata poreklom sa različitih domaćina. Prvi subklaster obrazuju izolati porek-

lom sa višnje i paradajza, a drugom subklasteru osim hromogenih izolata pripada i KC-12, takođe sa ploda kruške. Treći klaster, takođe grade dva subklastera. Prvom suklasteru pripada referentni soj *C. acutatum* (CBS 294.67) i izolati JC-4 i JC-5. Drugi subklaster obrazuju preostala dva patogena poreklom sa jabuke (JC-6 i JC-7) i izolat KC-6 sa kruške. Osim izrazite specifičnosti referentnog soja *C. gloeosporioides*, ostale kulture *Colletotrichum* spp. su na formiranom dendogramu u većini slučajeva grupisane prema poreklu, odnosno biljci domaćinu.

Ispitivanje usvajanja jedinjenja azota. Od tri testirana jedinjenja azota, izolati *Colletotrichum* spp. u najvećem procentu usvajaju kazeinhidrolizat (24,42-100%), potom $NaNO_3$ (13,36-77,11%), a znatno manje $NH_4H_2PO_4$ (8,29-50,25%), (Graf. 3). Referentni soj *C. gloeosporioides* (CBS 516.97) na podlogama sa ispitivanim izvorima azota, kao i na podlogama sa ugljenikom, reaguje veoma specifično i u veoma niskim procentima usvaja navedena jedinjenja. Izolat JC-4 sa ploda jabuke, od svih ispitivanih kultura manifestuje najveći porast na podlogama sa kazeinhidrolizatom, $NaNO_3$ i $NH_4H_2PO_4$. Porast kultura KC-9 i KC-12 na osnovnoj podlozi sa $NH_4H_2PO_4$ je za 5,62, odnosno 3,66% manji nego na kontrolnoj podlozi bez prisustva jedinjenja azota.



Graf. 3 - Porast izolata *Colletotrichum* spp. na različitim izvorima azota.

Chart 3 - Growth of isolates of *Colletotrichum* spp. on different sources of nitrogen.

Leveneovim testom utvrđena je homogenost varijansi izolata *Colletotrichum* spp. (F=1,406; p=0,051). Rezultati obrađeni analizom varijanse (MANOVA) kao dvofaktorijalni ogled ukazuju da uzmeđu izolata, izvora azota i njihovih interakcija postoje statistički veoma značajne razlike (Tabela 4).

Tabela 4 - Analiza varijanse porasta *Colletotrichum* spp. na različitim izvorima azota.

Table 4 - Analysis of variance of mycelial growth of *Colletotrichum* spp. on different sources of nitrogen.

Izvori varijacije Source of variation	Stepeni slobode	Sredine kvadrata	Količnik	Nivo značajnosti
	df	MS	F	p-level
izolati/isolates	21	0,21	1055,926	0,000**
izvori N/sources of N	2	3,34	16139,052	0,000**
izolati x izvori N isolates x sources of N	42	0,01	22,872	0,000**
greška/error	132	0,00	-	-

** Statistički veoma značajna razlika (p<0,01).
**Statistically very significant diference (p<0,01).

Na osnovu Duncanovog testa određeni su nivoi značajnosti porasta izolata *Colletotrichum* spp. na svim ispitivanim izvorima azota (Tabela 5).

Na podlozi obogaćenoj kazeinhidrolizatom, nisu utvrđene statistički značajne razlike u međusobnim poređenjima izolata KC-6 i patogena JC-6 i JC-7, poreklom sa jabuke i PC-2 i PC-4 sa ploda paradajza. Takođe, parnim poređenjima porasta kultura KC-9 i KC-12, kao i hromogenih izolata KC-21 i KC-23, nisu utvrđene značajne razlike. Izolati sa jabuke (JC-5, JC-6 i JC-7) u pojedinim kombinacijama sa izolatima sa paradajza, ali i izolati sa ovog domaćina u poređenjima sa kulturama poreklom sa ploda višnje, ne manifestuju razlike koje su statistički značajne. Referentni soj *C. gloeosporioides* (CBS 516.97) je značajno različit u odnosu na ostale ispitivane izolate, a *C. acutatum* (CBS 294.67) se po porastu na podlozi sa kazeinhidrolizatom statistički ne razlikuje od PC-6, VC-3 i VC-9.

Tabela 5 - Uticaj različitih izvora azota na porast isolata *Colletotrichum* spp.

Table 5 - Effect of different nitrogen sources on growth of *Colletotrichum* spp.

Izolat Isolate	Izvori azota Sources of nitrogen					
	KH		$NaNO_3$		$NH_4H_2PO_4$	
KC-6	69.95	a*	47.89	a	20.19	a
KC-9	37.45	b	18.73	b	-5.62	b
KC-12	35.53	b	17.95	b	-3.66	b
KC-21	52.38	c	31.14	c	11.72	c
KC-23	52.59	c	30.37	c	10.74	cd
KC-82	55.30	p	33.33	p	13.26	cd
JC-4	106.47	r	77.11	r	50.25	r
JC-5	75.73	d	53.40	d	25.24	e
JC-6	71.03	ae	49.07	a,e	21.50	af
JC-7	70.83	aef	45.37	s	21.76	af
PC-1	74.58	dg	50.00	aef	27.92	g
PC-2	72.31	aefgh	49.59	aefg	27.69	egh
PC-3	77.31	di	50.84	efgh	29.83	ghi
PC-4	71.72	aefh	49.18	aefghi	28.69	ghij
PC-5	79.24	ij	56.36	j	29.66	ghij
PC-6	80.08	jl	56.78	jl	31.36	ijlm
VC-3	83.04	m	56.25	jlm	33.93	mn
VC-5	76.52	dgijn	51.30	defghin	31.30	ijlmno
VC-7	78.07	dijln	52.63	dhn	32.46	imno
VC-9	81.42	jlmo	56.19	jlmo	35.40	np
CBS 294.67	82.61	lmo	55.65	jlmo	36.96	p
CBS 516.97	24.42	s	13.36	t	8.29	d

* Vrednosti u koloni označene istim slovom nisu statistički značajno različite na osnovu Duncan multiple range testa ($p<0.05$).

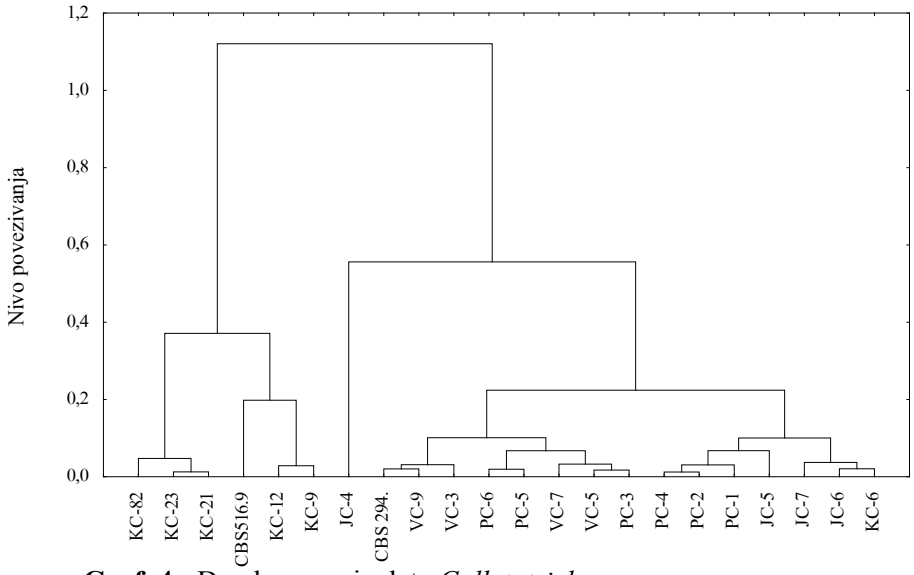
* Means in column followed by the same letter are not statistically significantly different by Duncan's multiple range test ($p<0.05$).

Na podlozi sa $NaNO_3$ ne postoje statistički značajne razlike u porastu izolata KC-6, JC-6 i tri izolata sa paradajza, PC-1, PC-2 i PC-4. Statistički se značajno ne razlikuju ni hromogeni izolati KC-21 i KC-23. Patogen JC-5, sa ploda jabuke

se na podlozi obogaćenoj ovim azotnim jedinjenjem značajno ne razlikuje od kultura sa višnje (VC-5 i VC-7), a izolat JC-6 od većine kultura *Colletotrichum* spp. sa paradajza (PC-1, PC-2, PC-3 i PC-4). Ova četiri izolata sa paradajza u međusobnim poređenjima ne manifestuju statistički značajne razlike, a razlike nisu konstatovane ni između ovih kultura i izolata VC-5. Takođe, porast kultura PC-5 i PC-6 nije statistički značajno različit od porasta izolata VC-7 i VC-9. Referentni soj *C. acutatum* (CBS 294.67) se na osnovu porasta na podlozi sa $NaNO_3$ značajno ne razlikuje od izolata PC-5, PC-6, VC-3 i VC-9, za razliku od *C. gloeosporioides* (CBS 516.97) koji je na ovoj podlozi statistički značajno različit od izolata *Colletotrichum* spp.

Na osnovu porasta na podlozi obogaćenoj sa $NH_4H_2PO_4$, izolati KC-6, JC-6 i JC-7, zatim KC-9 i KC-12, kao i svi hromogeni izolati sa ploda kruške, nisu statistički značajno različiti. Takođe, značajnih razlika nema ni između izolata JC-6 i JC-7 i kultura *Colletotrichum* spp. sa paradajza. Izolati VC-3, VC-5 i VC-7 poreklom sa višnje, u međusobnim poređenjima ne manifestuju statistički značajne razlike, a izolat VC-9 po porastu na podlozi sa $NH_4H_2PO_4$ nije značajno različit od referentnog soja *C. acutatum* (CBS 294.67). Porast referentnog *C. gloeosporioides* (CBS 516.97) u prisustvu ovog jedinjenja azota, nije statistički značajno različit jedino od izolata KC-23, sa ploda kruške.

Na osnovu ispoljenih sličnosti/razlika u pogledu porasta izolata *Colletotrichum* spp. na tri različita izvora azota, urađen je dendrogram baziran na Euklidskoj distanci i kompletnom povezivanju (Graf. 4). Svi ispitivani izolati su svrstani u četiri klastera. Prvi klaster grade dva subklastera sa ukupno 6 izolata *Colletotrichum* spp. Hromogeni izolati, KC-21, KC-23 i KC-82 su u okviru prvog subklastera, a referentni soj *C. gloeosporioides* (CBS 516.97) i izolati KC-9 i KC-12 za koje je karakterističan veoma slab porast na podlogama obogaćenim jedinjenjima azota, pripadaju drugom subklasteru. S obzirom na izrazito visok procenat usvajanja sva tri izvora azota treći, zasebni klaster gradi izolat JC-4. Preostalih 15 izolata, uključujući i referentni soj *C. acutatum* (CBS 294.67) pripada četvrtom klasteru. U okviru ovog klastera formirana su dva subklastera sa izolatima koji se u većini slučajeva po porastu na podlogama sa azotom, statisitčki značajno ne razlikuju. Prvi subklaster obrazuju CBS 294.67, svi izolati sa ploda višnje i tri izolata sa paradajza (PC-3, PC-5 i PC-6). Drugom subklasteru pripadaju preostali izolati sa paradajza, jabuke i kruške. Kao i u prethodnom eksperimentu, klaster analiza je potvrdila heterogenost ispitivanih populacija *Colletotrichum* spp.



Graf. 4 - Dendrogram izolata *Colletotrichum* spp. na osnovu usvajanja različitih izvora azota.

Chart 4 - Dendrogram of isolates of *Colletotrichum* spp. based on utilization of different sources of nitrogen.

DISKUSIJA

Najznačajniji energetski izvori neophodni za rast i razmnožavanje gljiva su soli neorganskog porekla: kalcijumnitrat, natrijumnitrat i amonijumnhlorid; različita organska jedinjenja: kazein, kvaščev ekstrakt, malc ekstrakt i urea; ugljeni hidrati u obliku monosaharida (glukoza, fruktoza, galaktoza), disaharida (maltoza i saharoza), polisaharida (skrob i rafinoza), kao i alkoholi manitol i glicerol (Adejoye et al., 2006; Omanor et al., 2008). Navedena jedinjenja su primarni snabdevači ćelija ugljenikom i azotom, a od stepena njihove iskoristivosti zavisi vitalnost i infekcioni potencijal patogena.

U našim istraživanjima kulture *Colletotrichum* spp. na podlogama obogaćenim sa tri različita jedinjenja ugljenika, u najvećem procentu usvajaju KMC, potom skrob, a znatno manje pektin. Referentni sojevi *C. acutatum* i *C. gloeosporioides* se veoma specifično ponašaju u prisustvu testiranih izvora ugljenika. U poređenju sa ostalim izolatima, referentni soj *C. acutatum* (CBS 294.67) na supstratima sa KMC-om, skrobom i pektinom formira kolonije najvećeg prečnika. S druge strane, *C. gloeosporioides* (CBS 516.97) je gotovo indiferentan

i izdvaja se od ostalih kultura po veoma niskim procentima usvajanja navedenih jedinjenja ugljenika.

Od tri jedinjenja azota, ispitivani izolati *Colletotrichum* spp. u najvećem procentu usvajaju kazeinhidrolizat, potom $NaNO_3$, a znatno manje $NH_4H_2PO_4$. Prečnik kolonija izolata KC-9 i KC-12 (*C. gloeosporioides*) na osnovnoj podlozi obogaćenoj sa $NH_4H_2PO_4$ je manji, nego na kontrolnoj podlozi bez prisustva ovog jedinjenja, pa se može govoriti o genetskoj varijabilnosti i netolerantnosti ovih kultura prema $NH_4H_2PO_4$.

Dobijeni rezultati su u saglasnosti sa istraživanjima autora koji su ispitivali uticaj različitih izvora ugljenika i azota na porast izolata *Colletotrichum* spp. Swart (1999) ističe da od testiranih izvora ugljenika izolati *C. gloeosporioides* procentualno najviše usvajaju KMC, potom skrob, pektin, manitol i sorbitol; a od jedinjenja azota kazeinhidrolizat i $NaNO_3$, zatim $NaNO_2$, a znatno manje $NH_4H_2PO_4$ i ureu. Sangeetha and Rawal (2008), konstatuju da izolati *C. gloeosporioides* od više testiranih jedinjenja ugljenika u najvećem obimu usvajaju manitol, fruktozu i saharozu, a azot najčešće u formi nitratnih soli.

Iako su gljive roda *Colletotrichum*, a naročito *C. acutatum* i *C. gloeosporioides*, genetski veoma varijabilni i heterogeni organizmi, u ovim istraživanjima nisu konstatovane značajne razlike između ove dve vrste kada je u pitanju njihov razvoj na supstratima sa različitim izvorima ugljenika i azota.

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EFFECT OF CARBON AND NUTRIGEN SOURCES ON GROWTH OF *COLLETOTRICHUM* SPP.

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SUMMARY

In Serbia, the occurrence of anthracnose on pear, apple, sour cherry, and tomato fruits has been recorded during the last several years. *Colletotrichum* spp. were isolated from all diseased fruits. Twenty monoconidial isolates were selected for comparative studies, and the reference strains of *C. acutatum* (CBS 294.67) and *C. gloeosporioides* (CBS 516.97) were used as a control. The physiological studies have included the investigation of the effect of different sources of carbon and nitrogen on growth of *C. acutatum* and *C. gloeosporioides*. The results indicated that carboxymethyl cellulose was the most appropriate source of carbon for the growth of isolates of *Colletottrichum* spp. Of the three nitrogen sources studied, the best growth of all tested fungi was recorded on the media with casein hydrolysate.

Key words: *Colletotrichum* spp., carbon, nitrogen, growth.

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DISTRIBUTION OF ALDER YELLOWS PHYTOPLASMA ON COMMON AND GRAY ALDER (*ALNUS GLUTINOSA* AND *ALNUS INCANA*) IN SERBIA

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Alder yellows (AldY) phytoplasma associated with common alder (*Alnus glutinosa*) and grey alder (*A. incana*) belongs to the ribosomal RNA group16SrV. This phytoplasma is closely related to the *Flavescence dorée* (FD) phytoplasma, a quarantine pathogen of economic importance that affects vineyards of southern Europe including Serbia. To date, alder yellows phytoplasma has been reported in many European countries including France, Germany, Switzerland, Austria, Italy and the Baltic region. Infected alders are exhibiting symptoms such as leaf yellowing, small leaves, reduced foliage, or sometimes they remain symptomless. To investigate occurrence and distribution of this phytoplasma, a survey was conducted on a wide territory of Serbia. Results confirmed wide distribution of alder yellows phytoplasma in Serbia in both symptomatic and asymptomatic trees. From the 72 plants sampled, 54 were positive for the presence of phytoplasmas. RFLP profiles of the 16S rRNA gene indicated presence of 16SrV-C phytoplasma subgroup. Further characterization by PCR-RFLP analysis of the ribosomal protein gene operon of all phytoplasma positive isolates tested confirmed presence of the 16SrV-C phytoplasma subgroup. Implication of the wide distribution of AldY phytoplasma to the epidemiology of FD phytoplasma as well as disease management are discussed.

Key words: 16S rRNA, disease epidemiology, *Flavescence dorée*, PCR-RFLP, *rpl22-rps3*, symptoms.

INTRODUCTION

Phytoplasmas are wall-less, phloem-limited, non-culturable prokaryotes of the class Mollicutes that are associated with diseases in several hundred plants species. Because they cannot be cultured on an artificial medium and lack measurable phenotypic characters, classification of phytoplasmas has been based primarily on molecular analyses of highly conserved 16S rRNA gene sequences (Lee et al., 1998).

Alder yellows is a disease caused by phytoplasma genetically closely related to the *Flavescence dorée* phytoplasma, that affects vineyards in many south European countries. To date, severe outbreaks caused by *Flavescence dorée* (FD) have been reported from France, Italy, Spain (Daire et al., 1997; Martini et al., 1999; Angelini et al., 2001; Arnaud et al., 2007), including Serbia (Krnjajić et al., 2007). Recently this phytoplasma has been reported from Portugal, Switzerland, Austria and Croatia (De Sousa et al., 2009; Schaerer et al., 2007; Reisenzein and Steffek, 2011; Škorić et al., 2011) showing trend of continuous spreading throughout vineyard regions across southern Europe. In general, the outbreaks of the *Flavescence dorée* in Europe are caused by two genetic entities, where both belong, in a wider sense, to elm yellows phytoplasma or 16SrV group (Lederer and Seemüller 1991; Maurer et al., 1993). In east Europe, including northern parts of Italy, the vineyards are affected with phytoplasma belonging to the 16SrV-C subgroup (FD-C), while in France and western part of Italy with phytoplasma belonging to the 16SrV-D subgroup (FD-D). Both phytoplasmas are quarantine diseases of the grapevine, epidemically transmitted by the leafhopper *Scaphoideus titanus* Ball (Angelini et al., 2003; Arnaud et al., 2007). However, occasionally grapevine could be inoculated with alder yellows phytoplasma by leafhopper *Oncopsis alni* (Schrank), which causes Palatinate grapevine yellows (PGY) disease, known from Palatinate region in South West Germany (Maixner and Reinert, 1999; Maixner et al., 2000). In addition, finer molecular and phylogenetic studies strongly support hypothesis that phytoplasmas from ribosomal 16SrV-D subgroup affecting vineyards in west Europe are originating from alder trees (Malembic-Maher et al., 2007; Arnaud et al., 2007).

The importance of alder yellows phytoplasma in epidemiology of grapevine yellows disease becomes obvious after reported great diversity within field infected alders. Cvrković et al. (2008) reported the presence of alder yellows phytoplasma from two sites in Serbia. Even though 16SrV-C phytoplasmas affecting vineyards in Serbia were found in *Clematis vitalba* (Filippin et al., 2009), different genotypes associated with *Alnus glutinosa* and *Alnus incana* may represent potential threat in epidemiology of grapevine yellows diseases caused by phytoplasmas from 16SrV group. Thus, in this paper, we performed a study to determine incidence of alder yellows phytoplasma associated with alder trees in Serbia.

MATERIALS AND METHODS

The material for this study was collected between 2006 and 2010. Leaves with petioles from *Alnus glutionsa* and *Alnus incana* trees were collected on the forests margins and riverbanks in several districts of Serbia. A total of 12 sites were surveyed (Picture 1). Leaves with petioles from six randomly selected trees per site, expressing symptoms of yellowing or being symptomless were sampled for phytoplasmas detection by PCR-RFLP analysis. Fresh midribs and petioles were dissected, distributed in 1 gram aliquots and stored at -20ºC prior to analyses. DNA was extracted according to previously reported protocols (Angelini et al., 2001).

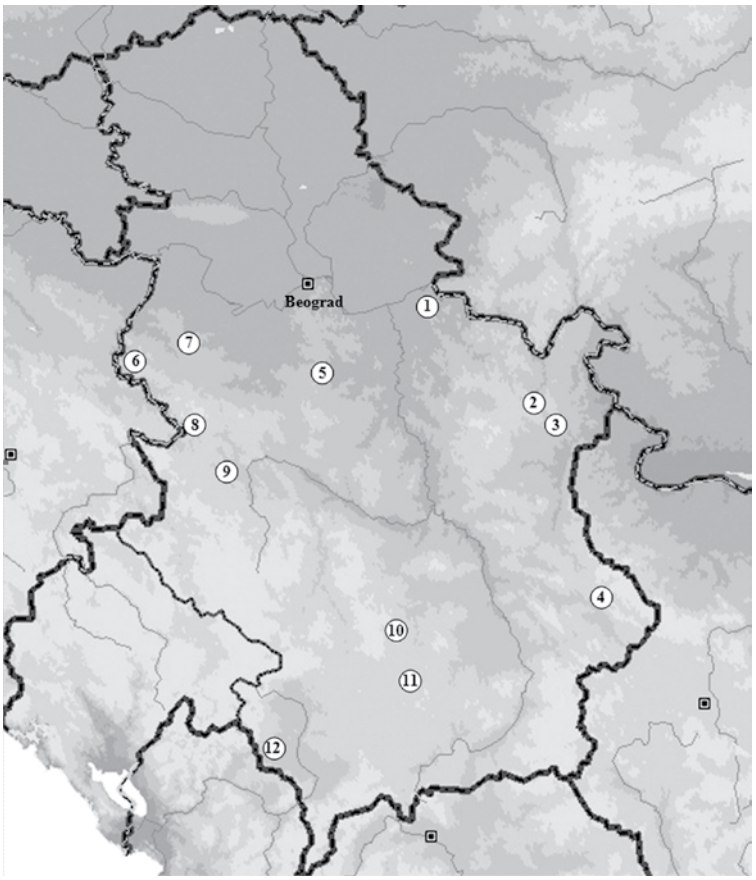
Initial phytoplasma identification was conducted using a nested PCR assay on the 16S ribosomal gene with P1/P7 (Deng and Hiruki, 1991; Smart et al., 1996) and 16r758f/M23Sr (Gibb et al., 1995; Padovan et al., 1995) primers according to Angelini et al. (2001). PCR products were separated on 1% agarose gels, stained with ethidium bromide and visualized under UV transilluminator. Samples producing an amplicon in nested PCR were subjected to restriction digestion with *Taq*I endonuclease (Fermentas) according to the manufacturer's instructions. The restriction digestion products were subsequently separated electrophoretically on 13% polyacrylamide gels in TBE buffer (Tris-Borate 90mM, EDTA 1mM), stained with ethidium bromide and visualized under UV light.

Diversity of phytoplasmas associated with alders in Serbia was estimated by characterization of ribosomal protein gene operon comprising *rpl22* and *rps3* genes, using the primer pair rp(V)F1/rpR1 in direct PCR followed by nested PCR with rp(V)F1A/rp(V)R1A primers (Lee et al., 2004). The reaction mixture (3mM $MgCl_2$, 0.3mM each dNTPs, 0.6µM each primer, 0.75 U of Ampli*Taq* Gold polymerase (Applied Byosistem)) was subjected to 34 cycles with the following steps: 10 min at 95ºC for enzyme activation, 1 min (90 s for the first cycle) at 94ºC for denaturation, 2 min at 50ºC for annealing and 3 min (10 min for the last cycle) at 72ºC extension. All parameters were identical in direct and nested PCR. PCR products were separated in 1% agarose gel, stained with ethidium bromide and visualised under a UV transilluminator, then subjected to RFLP analyzes with *Mse*I restriction enzyme (Fermentas). The restriction products were separated in 13% polyacrylamide gel, stained and visualized as described above.

RESULTS

Survey and collection. Common alder (*Alnus glutinosa*) is a wide distributed and common tree in Serbia, often found at river and stream banks and mesic foothills sites. To estimate incidence of alder yellows phytoplasma in Serbia we have sampled alder trees from twelve sites: Šuvajić (near Veliko Gradište) (1),

Debeli Lug (near Majdanpek) (2), Jabukovac (near Negotin) (3), waterfall Bigar (Monastery St. Onufrije near Temska) (4), vicinity of Topola (5), Mt. Radalj (near Loznica) (6), Zavlaka (near Osečina) (7), Okletac (near Bajina Basta) (8), Potpeći (near Užice) (9), Aleksandrovac (10), Kraljevo (11) and Prijepolje (12) (Picture 1).



Picture 1 – Map of Serbia showing localities surveyed for alders with Alder yellows phytoplasmas between 2006-2010: (1) Šuvajić (near Veliko Gradište); (2) Debeli Lug (near Majdanpek); (3) Jabukovac (near Negotin); (4) Waterfall Bigar (Monastery St. Onufrije near village Temska); (5) vicinity of Topola; (6) Mt. Radalj (near Loznica); (7) Zavlaka (near Osečina); (8) Okletac (near Bajina Bašta); (9) Potpeći (near Užice); (10) Aleksandrovac; (11) Kraljevo; (12) Prijepolje.

Slika 1 – Mapa Srbije sa lokalitetima sakupljanja jova i Alder yellows fitoplazme između 2006. i 2010. godine: (1) Šuvajić (u blizini Velikog Gradišta); (2) Debeli Lug (u blizini Majdanpeka); (3) Jabukovac (u blizini Negotina); (4) Vodopad Bigar (Manastir Sveti Onufrije u blizini sela Temska); (5) okolina Topole; (6) Planina Radalj (u blizini Loznice); (7) Zavlaka (u blizini Osečine); (8) Okletac (u blizini Bajine Bašte); (9) Potpeći (u blizini Užica); (10) Aleksandrovac; (11) Kraljevo; (12) Prijepolje.

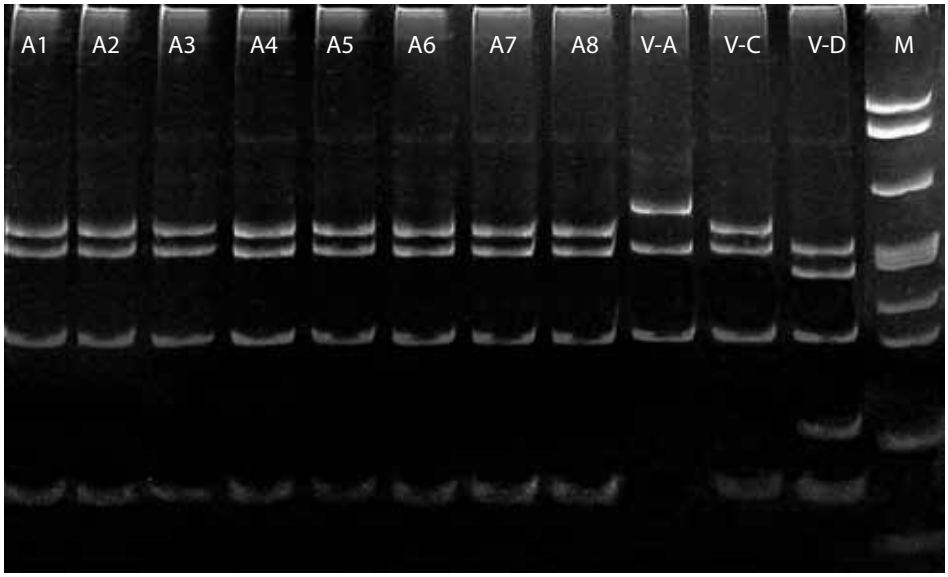
Table 1 – Occurrence of AldY phytoplasmas in symptomatic and asymptomatic alders collected on 12 localities in Serbia between 2006 and 2010.

Tabela 1 – Prisustvo AldY fitoplazme u simptomatskim i asimptomatskim jovama sakupljenim na 12 lokaliteta u Srbiji između 2006. i 2010. godine.

Locality Lokalitet	Alder species Alder vrsta	Symptoms observed Registrovani simptomi	PCR positive/ analyzed samples[a] PCR pozitivni/ analizirani uzorci[a]
Šuvajić	Alnus glutinosa	leaf yellowing	5/6
Debeli Lug	*Alnus glutinosa*	leaf yellowing, asymptomatic	6/6
Jabukovac	*Alnus incana*	asymptomatic	4/6
Monastery Sveti Onufrije	*Alnus glutinosa*	leaf yellowing, asymptomatic	5/6
Topola	*Alnus glutinosa*	leaf yellowing, asymptomatic	6/6
Mt. Radalj	Alnus glutinosa	leaf yellowing,	4/6
Zavlaka	*Alnus glutinosa*	leaf yellowing, asymptomatic	4/6
Okletac	*Alnus glutinosa*	leaf yellowing, asymptomatic	5/6
Potpeći	*Alnus glutinosa*	asymptomatic	4/6
Aleksandrovac	*Alnus glutinosa*	asymptomatic,	4/6
Kraljevo	*Alnus glutinosa*	asymptomatic	3/6
Prijepolje	*Alnus glutinosa*	leaf yellowing, asymptomatic	4/6
In total Ukupno			54/72

(a) identification of AldY phytoplasmas was performed using nested PCR with primer pairs P1/P7 and 16r758f/M23Sr followed by restriction analysis with *Taq*I endonuclease.
(a) identifikacija AldY fitoplazme je izvršena pomoću nested PCR analize sa P1/P7 i 16r758f/M23Sr i restrikcionom analizom sa *Taq*I endonukleazom.

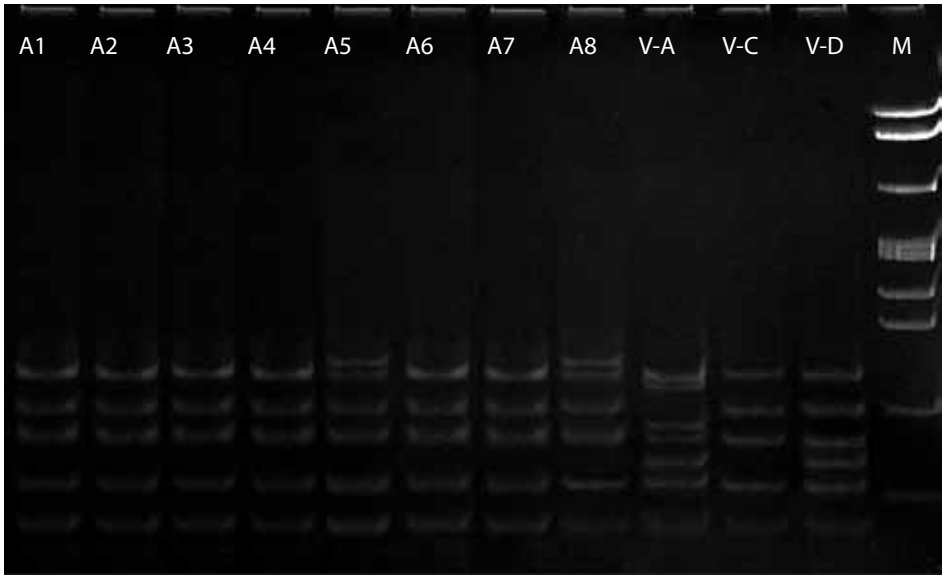
Molecular analyses. A total of 72 alder samples were subjected to nested PCR analyses with P1/P7 and 16r758f/M23Sr primers, of which 54 tested positive for the presence of alder yellows phytoplasma (Table 1). RFLP profiles of 16S rRNA gene showed the presence of phytoplasmas of the 16SrV-C subgroup (Picture 2) in all positive samples. Further characterization of the ribosomal protein gene operon showed two different *Mse*I RFLP profiles, one similar to FD-C



Picture 2 – RFLP analyses of the 1050 bp 16SrRNA gene amplified by nested PCR with primer pair P1/P7 followed with 16r758f/M23Sr primers, digested with *Taq*I and separated by electrophoresis through 13% polyacrylamide gels. A1-4: alder samples from Central Serbia; A5-8: alder samples from East Serbia; V-A: elm yellows (EY) phytoplasma of the 16SrV-A ribosomal subgroup maintained in periwinkle (provided by W.A. Sinclair, New York); V-C: *Flavescence dorée* phytoplasma of the 16SrV-C subgroup (FD-C) isolated from naturally infected grapevine from Central Serbia; V-D: *Flavescence dorée* phytoplasma of the 16SrV-D subgroup (FD-D) isolated from naturally infected grapevine from Veneto region, Italy (provided by E. Angelini, Conegliano); M: marker, ΦX174 DNA/HaeIII digested, Fermentas.

Slika 2 – RFLP analiza 1050 bp nested PCR produkata 16S rRNK gena umnoženih pomoću P1/P7 i 16r758f/M23Sr para prajmera digestovanih sa *Taq*I endonukleazom i elektroforetski razdvojenih kroz 13% poliakrilamidni gel. A1-4: uzorci jova iz centralne Srbije; A5-8: uzorci jova iz istočne Srbije; V-A: elm yellows (EY) fitoplazma ribozomalne podgrupe 16SrV-A održavana u perivinki (W.A. Sinclair, New York); V-C: *Flavescence dorée* (FD-C) fitoplazma ribozomalne podgrupe 16SrV-C izolovana iz prirodno inficirane vinove loze iz centralne Srbije; V-D: *Flavescence dorée* (FD-D) fitoplazma ribozomalne podgrupe 16SrV-D izolovana iz prirodno inficirane vinove loze iz Veneto regiona u Italiji (E. Angelini, Conegliano); M: marker, ΦX174 DNA/HaeIII digested, Fermentas.

and one (only in samples from eastern Serbia) similar to the AldY strain previously described by Lee *et al.*, 2004 (Picture 3). RFLP profiles showed the presence of phytoplasmas of the 16SrV-C subgroup in all samples collected from alder trees with characteristic symptoms of yellowing, but also in certain number of trees which were symptomless.



Picture 3 – RFLP analyses of the 1200 bp ribosomal protein operon sequence comprising *rpl22-rps3* genesamplified by nested PCR with primer pair rp(V)F1/rpR1 followed by rp(V)F1A/rp(V)R1A primers, digested with *Mse*I, and separated by electrophoresis through 13% polyacrylamide gels. Abbreviations of the isolates and reference strains are the same as described on Picture 2.; M: marker, ΦX174 DNA/HaeIII digested, Fermentas.

Slika 3 – RFLP analiza 1200 bp nested PCR produkata operona ribozomalnih proteina *rpl22-rps3* umnoženih pomoću rp(V)F1/rpR1 i rp(V)F1A/rp(V)R1A para prajmera digestovanih sa *Mse*I endonukleazom i elektroforetski razdvojenih kroz 13% poliakrilamidni gel. Oznake izolata i referentnih sojeva su iste kao na Slici 2.; M: marker, ΦX174 DNA/HaeIII digested, Fermentas.

According to recently published data, there is some extent of confirmation about phytoplasma exchange between wild alders and grapevine in west Europe, particularly in France and West Germany, as it is shown by Maixner et al. (2000) and Arnaud et al. (2007). These findings objectively launched hypothesis that first FD outbrakes observed in France in the mid 50's, after accidental introduction of the leafhopper *S. titanus,* could originate from alder trees driven by intermediary transmission of alder yellows phytoplasma by the leafhopper *Oncopsis alni* to grapevine (Arnaud et al*.,* 2007). It is worth noting that alder trees and vineyards are often in close contacts in some regions of France or in Palatinate region in West Germany.

DISCUSSION

Phytoplasma exchange from the wild flora and agricultural crops is not a rare phenomenon, and is usually correlated with changing in behavior or ecological demands of particular hemipteran vectors. Host shift of *Reptalus panzeri* from shrubs to maize, for example, lead to severe outbreaks of Stolbur phytoplasma on maize (Jović et al., 2009). Similar situation has been reported for *Hyalesthes obsoletus* involvement in rapid and severe outbreaks of stolbur phytoplasma on lavender (*Lavandula angustifolia*) (Sforza et al., 1999; Gaudin et al., 2011) in France or on potato crop in Serbia (Jović et al., 2011). In last decade, increasing number of reports about accidental or sometimes permanent, but low rate presence of phytoplasmas in different crop systems became relatively common event. This is obviously in correlation with substantial changes in assemblages of Hemipteran vectors inside or around particular agro-ecosystems. One of the possible reasons should be an intensive fertilization practices used in agriculture in past 50 years which lead to an increase of concentrations of total nitrogen, amino acid and organic compounds in both the crops and weeds (Jović et al., 2009), resulting in a nutritional balance that facilitates leafhopper fecundity and better survival of the offspring (Brodbeck et al., 1999; Olmstead et al., 1997).

Following the experience of the researchers involved in the study of epidemiology of grapevine yellows disease in west Europe, there is a strong indication that alder yellows phytoplasma should be considered as a potential threat. Our study clearly indicates that alder yellows phytoplasmas are widely distributed in Serbia with high incidence in alders. On the other hand, there are substantial differences in ecology of alder trees in Serbia and west Europe, i.e. alders are obviously related with mesic and cold habitats which are obviously not suitable habitats attractive for Serbian vine growers. Thus, there is a big spatial gap in topology which strictly separates alder's compartments from vineyards, giving less possibilities of accidental transmission of alder yellows phytoplasma by its natural vector *Oncopsis alni*.

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RASPROSTRANJENJE ALDER YELLOWS FITOPLAZME NA CRNOJ I BELOJ JOVI (*ALNUS GLUTINOSA* I *ALNUS INCANA*) U SRBIJI

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IZVOD

Alder yellows (AldY) fitoplazma koja je u asocijaciji sa crnom jovom (*Alnus glutinosa*) i belom jovom (*A. incana*) pripada 16SrV ribozomalnoj grupi fitoplazmi. Ova fitoplazma je srodna fitoplazmi zlatastog žutila vinove loze *Flavescence dorée* (FD), koja je karantinski patogen od ekonomskog značaja u vinogradima južne Evrope uključujući i Srbiju. Do sada je prisustvo alder yellows fitoplazme utvrđeno u mnogim evropskim zemljama uključujući Francusku, Nemačku, Švajcarsku, Austriju, Italiju i Baltički region. Inficirane jove ispoljavaju simptome žutila listova, malih listova, redukcije lisne mase, ili ponekad ne ispoljavaju simptome inficiranosti. U cilju utvrđivanja prisustva i rasprostranjenja ove fitoplazme na široj teritoriji Srbije, sprovedeno je uzorkovanje simptomatskih i asimptomatskih jova. Rezultati istraživanja su potvrdili široku distribuciju alder yellows fitoplazme u Srbiji i prisustvo fitoplazme kako u simptomatskim tako i u asimptomatskim stablima. Od ukupno 72 uzorkovane biljke, 54 su bile inficirane fitoplazmom. Analizom RFLP profila 16S rRNK gena utvrđeno je prisustvo 16SrV-C podgrupe fitoplazmi. Dalja karakterizacija PCR-RFLP analizom operona ribozomalnih proteina svih pozitivnih izolata potvrdila je prisustvo 16SrV-C podgrupe fitoplazmi. U diskusiji je istaknut značaj širokog rasprostranjenja AldY fitoplazme i uticaja na epidemiologiju FD fitoplazme kao i na kontrolu bolesti.

Ključne reči: 16S rRNA, epidemiologija bolesti, *Flavescence dorée*, PCR-RFLP, *rpl22-rps3*, simptomi.

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UDK: 595.78(549.1)
Scientific paper

BEMBECIA BUMBURETA SP.N. - A NEW SPECIES OF CLEARWING MOTHS FROM NORTH-WESTERN PAKISTAN (LEPIDOPTERA, SESIIDAE)

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A new species, *Bembecia bumbureta* sp. n., is described. It is similar to the West Caucasian species *Bembecia syzcjovi* Gorbunov, 1989, and *Bembecia pagesi* Toševski, 1993 described from North India. The new species is collected in North West Pakistan, Chitral province using pheromone trap. Bionomics and host plant are unknown.

Key words: *Bembecia bumbureta* sp. n., Sesiidae, Lepidoptera, Chitral, Pakistan.

INTRODUCTION

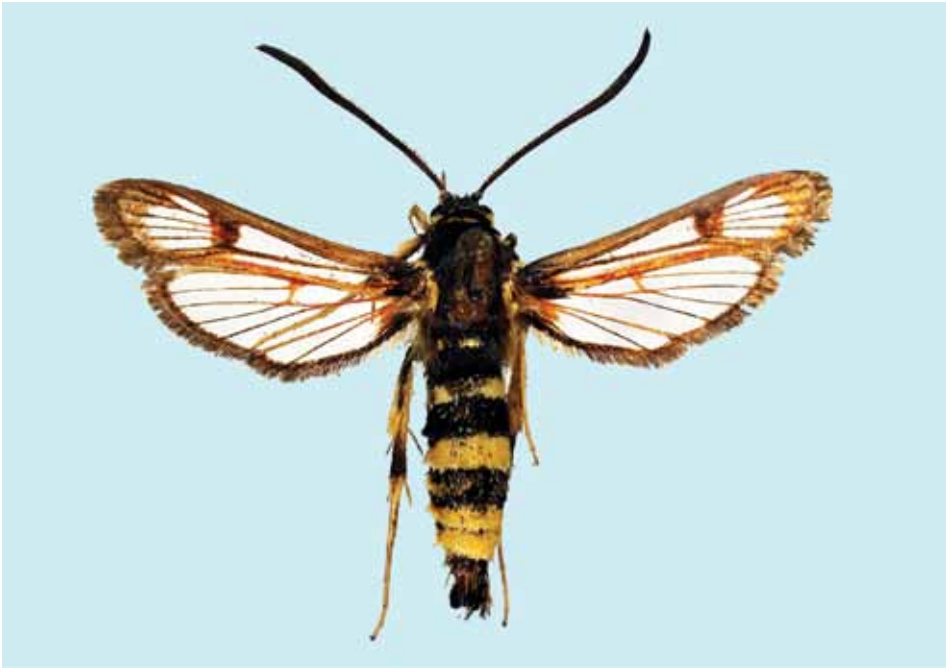
The genus *Bembecia* Hübner 1819 is consisted of over 70 species distributed mainly in central and west Palearctic (Špatenka et al., 1999). The larvae are univoltine or biennial. The larvae develop in the roots, rarely in the stems of different plants of the family Fabaceae. Sexual dimorphism is relatively common event in *Bembecia* species, together with absence of transparency of the wings in females, which is a homoplasius character (Laštůvka 1992). The distribution center of the genus *Bembecia* probably lies in the southern parts of the central Palearctic (Špatenka et al., 1999). For this reason it is expected that the list of *Bembecia* species from southern part of central Palearctic will increase in future. The genus *Bembecia* should be divided in several species groups of which the most numerous is *B. ichneumoniformis*-group with over 40 described

species. In addition, several species from this region were described during late 90's (Špatenka, 1997). In this paper, a new species from above mentioned group-species is described. The specimen has been collected using commercial pheromone traps (Wageningen, Netherlands) (in Chitral province (North West Pakistan).

Bembecia bumbureta sp. n.

M a t e r i a l **.** Holotypus, male, North West Pakistan, Bumburet, Chitral, 3200 m, 28-31.06.1997, lgt. Jerome Pages, in collection of MNHN (Muséum National d'Histoire Naturelle, Paris, France)

Description of Holotype (Fig. 1 a). Alar expanse 31 mm, body length 18 mm, forewing length 14 mm, antenna 6 mm.



Picture 1 - *Bembecia bumbureta* sp. n. Holotypus, male, North West Pakistan, Bumburet, Chitral, 3200 m, 28-31.06.1997, lgt. Jéróme Pagés,

Slika 1 - *Bembecia bumbureta* sp. n. Holotip, mužjak, Severozapadni Pakistan, Bumburet, Čitral, 3200 m, 28-31.06.1997, lgt. Jéróme Pagés,

Antenna black. Head black with retrocephalic pale yellow hairs like scales; frons pale brown with whitish gray scales above; vertex black, mixed with pale yellow scales posteriorly; labial palpus white, dorsally with black bristle scales among second segment ventrally and laterally.

Thorax black with blue sheen, tegula with broad yellow spot along outer margin; patagia shining black; prothorax and mesothorax black, metathorax black with golden-yellow hair-like scales dorsally and medially. Fore coxa brown black with yellowish scales exteriorly, femur black; tibia black proximally, yellow distally; tarsi yellow with some black scales. Hind coxa black; femur black with whitish scales exteriorly, tibia yellow, black proximally and with distinct black ring distally; spurs yellowish. Tarsi yellow with some black scales.

Abdomen: brown black with extensive blue sheen; tergites 2, 4, 6 and 7 with broad yellow posterior margins, while tergite 5 with yellow scales medially; all sternites with yellow posterior margin; anal tuft black, suffused with yellow scales.

Ground color of forewing pale brown with all three transparent areas well developed; anterior transparent area (ATA) and posterior transparent area (PTA) well developed and transparent along their length; costal margin pale brown, anal margin brown covered with yellow and orange-yellow scales; discal spot dark brown with semilunar orange-red design along outer margin; external transparent area (ETA) large, divided into 5 cells; apical area narrow cowered with orange red and brown scales; outer margin black brown, fringes brown. Hind wing transparent, discal spot orange brown, triangularly shaped, reaching conjunction of M_3- Cu_1; outer margin narrow, brown black; fringes brown.

Male genitalia (Fig. 2). Scopula androconialis well developed; gnathos distinct with all crista well developed; medialis crista rounded; external margin of lateral crista gnathi wave-shaped medially. Valva nearly rectangular, crista sacculi straight, obliquely situated, moderately raised above internal valva surface, reaching little more than 1/2 of the valva length. Aedeagus bulbous basally, significantly longer than valva length, somewhat curved proximally.

Picture 2 - *Bembecia bumbureta* sp. n., male genitalia: uncus-tegumen with aedeagus (left), valva (right).

Slika 2 - *Bembecia bumbureta* sp. n., genitalije mužjaka: uncus-tegumen aedeagusom (levo), valva (desno).

Differential diagnosis. Habitually, the new species is very similar to *Bembecia pagesi* Toševski, 1993 described from Northern India and *Bembecia syzcjovi* Gorbunov, 1989, described from Georgia. Both species possess characteristic large ETA area of fore wing and extremely narrow apical area. In *B. bumbureta* sp. n. ETA area is smaller and apical area is distinctly broad. From both species, newly described species clearly differs in genital morphology. *B. pagesi* and *B. syzcjovi* belong to the species from the *Bembecia dispar*-group (Špatenka et al., 1999) while *B. bumbureta* to the *Bembecia ichneumoniformis*-group. From the *B. diamerica* Toševski, 2011 (in press), *B. bumbureta* sp.n. differs by dark brown

fore wings and brown black discal spot and different morphology of crista sacculi which is straight in the former and distally slightly bent in the latter.

Etymology. The new species is named after type locality Bumburet (Chitral district) in the Khyber-Pakhtunkhwa province of NW Pakistan.

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BEMBECIA BUMBURETA SP. N.– NOVA VRSTA STAKLOKRILCA (LEPIDOPTERA, SESIIDAE) IZ SEVEROZAPADNOG PAKISTANA

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REZIME

U ovom radu prikazan je opis vrste *Bembecia bumbureta* sp. n. Novoopisana vrsta je slična vrsti *Bembecia syzcjovi* Gorbunov, 1989 iz zapadnog Kavkaza i vrsti *Bembecia pagesi* Toševski, 1993, koja je opisana iz severne Indije. Nova vrsta je ulovljena na feromonske klopke u Čitral provinciji (severno-zapadni Pakistan). Biologija i biljka domaćin je nepoznata

Ključne reči: *Bembecia bumbureta* sp. n, Sesiidae, Lepidoptera, Chitral, Pakistan.

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A manuscript, double-spaced printed, contains: chapter, title, the name of an author and addresses, abstract, key words, text (including the chapters: introduction, material and methods, results, discussion, references and summary followed by key words), tables and graphs, photographs and drawings.

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