



Original Scientific Paper

Susceptibility of Serbian plum cultivars to indigenous bacterial and *Monilinia laxa* isolates

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ABSTRACT:

The susceptibility of Serbian plum cultivars to pathogens originating from their phyllosphere was evaluated by inoculating detached young leaves and mature fruits. The virulence of indigenous isolates of the bacteria *Pseudomonas syringae*, *Pseudomonas congelans*, *Erwinia persicina*, *Clavibacter michiganensis* and *Rhizobium nepotum* was tested on detached leaves of four Serbian plum cultivars (Ranka, Požegača, Čačanska leptotica and Čačanska rodna). The *Pseudomonas syringae* isolates formed intense symptoms within 48 hours on all tested cultivars with severity index values in the range of 41 – 47%. The other isolates had significantly lower severity values or no symptoms were developed. This study demonstrates for the first time pathogenicity of *Pseudomonas congelans* on plum, with symptom intensity not significantly different from *P. syringae* after 96 h of incubation. Virulence of *Monilinia laxa* isolates was tested on mature fruit of the Čačanska rodna and Požegača cultivars and was detected in both of them. Higher susceptibility to *M. laxa* was recorded for the Požegača cultivar, with a fruit infection rate between 43 and 66%. In the case of the Rodna cultivar, no statistically significant difference in the fruit infection rate was detected between the four tested *M. laxa* isolates. These data indicate significant susceptibility of Serbian plum cultivars to indigenous *P. syringae* and *M. laxa* isolates.

Keywords:

plum, leaf, fruit, bacterial
phytopathogens, *Monilinia laxa*

UDC: 582.711.713:581.2

Received: 13 May 2020

Revision accepted: 01 July 2020

INTRODUCTION

Plum (*Prunus domestica* L.) has a long tradition of cultivation and holds first place in the fruit production of Serbia (MATKOVIĆ 2015). One part of the annual yield is used in fresh and dried form, but the bulk of it is processed into slivovitz, the traditional plum brandy (MILATOVIĆ *et al.* 2015). Among various Serbian plum cultivars, autochthonous Požegača and Ranka are the best raw materials for producing high-quality slivovitz. In order to retain suitable characteristics, Požegača was crossed with the “Wangenheim” and “Stanley” plum cultivars in breeding programs that resulted in the hy-

brids Čačanska leptotica (hereafter referred to as Leptotica) and Čačanska rodna (hereafter referred to as Rodna) (MILOŠEVIĆ & MILOŠEVIĆ 2012). Serbian plum trees, mainly those of autochthonous cultivars, are affected by different diseases that have a strong impact on their annual yield (MATKOVIĆ 2015; GLIŠIĆ *et al.* 2016).

The plum phyllosphere is inhabited by a plethora of microorganisms, including pathogenic bacteria and fungi (JANISIEWICZ *et al.* 2013; JANAKIEV *et al.* 2019, 2020). Under climatic conditions favourable for the development of diseases, these epiphytic populations can initiate disease outbreaks and strongly affect the yield during the season (WENNEKER *et al.* 2012; PONIATOWS-

KA *et al.* 2013). Among the bacterial diseases of plum, the most devastating is bacterial canker, caused by *Pseudomonas syringae* (WENNEKER *et al.* 2012). Necroses formed by this pathogen impede nutrient flow and consequently cause the drying of branches and, eventually, of the whole tree (GAVRILOVIĆ 2009). Also, earlier studies reported the presence of *Xanthomonas arboricola*, causing bacterial spot, and *Erwinia amylovora*, causing fire blight, on plum plants as an emerging risk to plum seedlings (PALACIO-BIELSA *et al.* 2011; VEGH *et al.* 2012). Besides bacteriosis, the economically most important plum disease is brown rot caused by *Monilinia* species. Plum plants are extremely vulnerable to brown rot, especially the mature fruits as the most sensitive stage in the pre-harvest period, but also in storage conditions (LINO *et al.* 2016). *Monilinia laxa* is recognised as the causative agent of brown rot in Serbian plum orchards, with 89% incidence among detected *Monilinia* spp. (HRUSTIĆ *et al.* 2015).

In our previous studies on bacterial and fungal diversity (JANAKIEV *et al.* 2019, 2020), potentially pathogenic bacteria and *M. laxa* were isolated from the phyllosphere of the Lepotica, Rodna, Požegača and Ranka cultivars. Because the epiphytic population can initiate disease outbreaks during favourable climatic conditions and affect the welfare of plum trees in orchards, the pathogenicity of indigenous isolates should be investigated. In the studies of IVANOVIĆ *et al.* (2012) and HRUSTIĆ *et al.* (2015), the pathogenicity of bacterial and fungal pathogens isolated from plum orchards in Serbia was evaluated on different hosts. However, susceptibility of the Lepotica, Rodna, Požegača and Ranka cultivars to economically significant phytopathogens from their phyllosphere was not investigated. Therefore, the aims of the present study were: 1) to examine the susceptibility of those four Serbian plum cultivars to bacterial pathogens and *Monilinia laxa* isolated from their phyllosphere; and 2) to assess the virulence of these indigenous pathogens.

MATERIAL AND METHODS

Experimental design. To assess the virulence of indigenous bacterial strains, detached young leaves of the four studied plum cultivars were inoculated. Susceptibility of the cultivars to bacterial pathogens was evaluated on the basis of symptoms that developed on the treated detached leaves. From each cultivar, five leaves were treated in triplicate. For experiments testing the virulence of *Monilinia laxa*, detached mature fruit of the Požegača and Ranka cultivars was inoculated. Susceptibility of the cultivars to *M. laxa* in the experiment was judged according to the brown rot symptoms that developed. From each cultivar, five mature fruits were inoculated in triplicate. The experiments were repeated twice independently.

Plant material. Plant material was collected from four Serbian plum cultivars, the autochthonous varieties Požegača and Ranka and the hybrid varieties Lepotica and Rodna, grown in an orchard not treated with pesticides in the last 10 years, located in Saraorci (municipality of Smederevo, Serbia; N 44° 29' 11" and E 21° 04' 34"). Samples of young leaves were collected from the Požegača, Ranka, Lepotica and Rodna cultivars. Fruits were collected in the mature phenological stage from the Rodna and Požegača cultivars. Collected leaves and fruits were stored for a short time at 4°C until they were inoculated.

Bacterial and fungal strains. The 22 isolates of *Pseudomonas* spp., *Erwinia persicina* ČL3/16_1 and *Clavibacter michiganensis* R3/1 belong to the collection isolated from the phyllosphere of four Serbian plum cultivars as described by JANAKIEV *et al.* (2020). Briefly, leaves and fruits collected from the Lepotica, Rodna, Požegača and Ranka cultivars were washed with 1×PBS and the proper dilution was plated on Luria–Bertani (LB) agar plates. Isolates were identified by sequencing of the partially amplified 16S rRNA gene sequence. One strain of *Rhizobium nepotum* (V1/16_2), which originated from pear (*Pyrus communis* ‘Williams’), was also included in the investigation. All strains were grown on LB agar plates and incubated for 48 h at 25°C.

The three isolates of *M. laxa* were also part of the collection isolated from the phyllosphere of Serbian plum cultivars (JANAKIEV *et al.* 2019). Briefly, *M. laxa* was isolated from 1×PBS washings of plant material and the proper dilution was plated on potato dextrose agar (PDA). Molecular identification was performed by sequencing amplicons of the ITS1 and ITS3 regions. One virulent *M. laxa* isolate originating from cherry (*Prunus avium* L.) was included as a control. All isolates were cultured on PDA for 7 days at 25°C. Their pure cultures were maintained on PDA slants at 4°C until further use.

Hypersensitive Reaction on Tobacco. The ability of *Pseudomonas* spp. isolates to induce a hypersensitive reaction (HR) on tobacco leaves was tested on the “White Burley” cultivar of *Nicotiana* tobacco. Bacterial suspensions were adjusted in sterile distilled water to approximately 10⁷ CFU/ml. The suspensions were infiltrated between two lateral nerves of mature leaves using a medical syringe. Sterile distilled water was used as a control. The tobacco plants were kept in a growth chamber at 25°C under conditions of a cycle consisting of 12 h of light and 12 h of darkness. Leaves were observed for necrotic lesions 2 days after treatment. The experiments were repeated twice independently, with three replications for each bacterial isolate.

Test of bacterial pathogenicity on plum leaves and determination of infection severity. The ability of the

potential pathogens *P. syringae* ČL2/2 and P7/16_2, *P. congelans* ČR1/16_2, *E. persicina* ČL3/16_1, *C. michiganensis* R3/1 and *R. nepotum* V1/16_2 to induce necrosis was tested according to MORAGREGA *et al.* (2003) by inoculating detached leaves sampled from the four studied plum cultivars. Five leaves of each plum cultivar were inoculated for each isolate. The leaves were sterilised for 2 minutes in 70% ethanol and washed twice in sterile water. On the back of the leaf, between the lateral nerves, a wound was inflicted with a sterile scalpel. The wounds were inoculated with 20 µl of bacterial suspension adjusted to 10⁸ CFU/ml. For a negative control, leaves were inoculated with 20 µl of sterile water. The inoculated leaves were placed on a piece of filter paper soaked in water and incubated in closed transparent boxes to maintain a high percentage of moisture in the humid chamber's atmosphere. The leaves were incubated at 25°C for 48 and 96 hours under daylight conditions. The lengths of necroses were measured and the isolate virulence (severity index, SI) was determined according to the formula:

$$SI = \frac{\sum_{n=1}^N In}{N \times I_{max}} \times 100,$$

where *In* is the corresponding severity index, *N* is the number of inoculated leaves and *I_{max}* is the highest severity index (corresponding to number 4). The severity index, in a range of 0 to 4, was determined based on the presence of symptoms according to the following scale: 0 - no infection; 1 - lesion on leaf up to 5 mm; 2 - lesion between 5 and 10 mm; 3 - lesion between 10 and 20 mm; and 4 - more than 20 mm of leaf length affected by infection. The experiments were repeated twice independently, with three replications for each bacterial isolate.

Test of *Monilinia laxa* pathogenicity on plum fruit and virulence assessment. The pathogenicity of three isolates of *M. laxa* (M1, M8 and M13) was tested on mature fruits of the Rodna and Požegača cultivars. The fruits were disinfected for 2 minutes in 70% ethanol and washed twice in sterile water. Wounds 6 mm in diameter were punctured on the fruits using a cork borer and inoculated with mycelial plugs (5 mm in diameter) sampled from the periphery of 7-day-old cultures. Fruits with fragments of sterile PDA medium were placed as negative controls. A *Monilinia laxa* isolate originating from cherry was used as a positive control. Five fruits were inoculated for each isolate. The fruits were placed on a piece of moist cotton wool in boxes that were sealed to maintain high humidity levels and incubated at 25°C under daylight conditions. After 72 hours of incubation, the percentage of fruit surface covered with the *M. laxa* mycelium was estimated in relation to total surface of the plum fruit. The percentage of fruit infected with mycelium was evaluated in a range of from 0%, representing the complete absence of any

mycelium, up to 100%, where the total fruit surface was covered with mycelium of *M. laxa*. The experiments were repeated twice independently, with three replications for each *M. laxa* isolate.

Data analysis. The obtained data were subjected to analysis of variance (one-way ANOVA), and separation of mean percentages of necroses on plum leaves and fruits was analysed using Tukey's HSD (honest significant difference) test with a significance level of *p* < 0.05 in IBM SPSS Statistics, v. 23 (SPSS, Inc.).

RESULTS

Pathogenicity of bacterial isolates and host susceptibility. Out of 22 *Pseudomonas* spp. isolates tested for hypersensitivity of tobacco, *P. syringae* ČL2/2, *P. syringae* P7/16_2 and *P. congelans* ČR1/16_2 induced necrotic lesions on tobacco leaves, the treated tissue becoming dry and white in the injected area. They were selected for pathogenicity testing on detached plum leaves, together with the isolates *E. persicina* ČL3/16_1 and *C. michiganensis* R3/1 from plum and *R. nepotum* V1/16_2 from pear. Symptoms on detached leaves were observed 48 h and 96 h after inoculation (Fig. 1). The highest severity values were detected with *Pseudomonas* spp. isolates on all of the tested plum cultivars. *Pseudomonas syringae* isolates (ČL2/2 and P7/16_2) caused progressive necrosis within 48 hours, with severity values in the range of 41–47%. The other isolates had SI values significantly lower, which was evident on all cultivars. After 96 h of incubation, no significant changes in SI values for *P. syringae* were detected (Fig. 2). The lesions induced by *P. congelans* ČR1/16_2 expanded and reached SI values of 44% on leaves of the Lepotica, Ranka and Požegača cultivars. The leaf damage caused by *P. congelans* on the Rodna cultivar was significantly lower than that induced by *P. syringae*.

Compared to *Pseudomonas* spp. isolates, other strains were less virulent on plum leaves, or no symptoms were observed. Detached leaves of the Lepotica and Ranka cultivars were susceptible to *E. persicina* ČL3/16_1, while no necrotic tissues were observed on Rodna and Požegača leaves. On leaves of the Lepotica cultivar, from whose phyllosphere the *E. persicina* ČL3/16_1 isolate originated, the severity value after 96 hours of incubation was 27%. On the Ranka cultivar, severity values were significantly lower (Fig. 2). Isolate *R. nepotum* V1/16_2 showed virulence on leaves of all cultivars except Lepotica. The highest severity value (27%) was detected on leaves of the Rodna cultivar. The isolate *C. michiganensis* R3/1 caused no visible symptoms on the tested cultivars.

Pathogenicity of *M. laxa* isolates and host susceptibility. After three days of incubation, fruits of both



Fig. 1. Pathogenicity of *Pseudomonas syringae* ČL2/2 isolate on leaves of the following cultivars: Ranka (A); Čačanska leptica (B); Požegača (C); Čačanska rodna (D). Necrotic changes after 96 hours of incubation are shown.

plum cultivars showed susceptibility to the tested *M. laxa* isolates, with development of typical brown rot symptoms regardless of the *M. laxa* isolate or the plum cultivar (Fig. 3). The Rodna cultivar showed similar susceptibility to all *M. laxa* isolates, with a percentage of plum infection varying between 43 and 49% after 72 h of incubation (Fig. 4). There was no statistically significant difference in the percentage of infection between the four tested isolates on the Rodna cultivar. The Požegača cultivar showed a higher susceptibility to *M. laxa*, with a fruit infection rate between 43 and 66%. The lesions induced by the *M. laxa* M13 isolate were statistically significantly larger than those induced by the *M. laxa* M8 isolate and in the positive control. No symptoms of brown rot were observed on the fruits inoculated with sterile PDA plates.

DISCUSSION

Plum cultivars used in Serbia for processing of high-quality slivovitz are significantly affected by various diseases. Considering that fact, we carried out *ex situ* testing of the susceptibility of two autochthonous and two hybrid cultivars to potentially pathogenic bacterial and *M. laxa* isolates that originated from their phyllosphere.

Among the tested isolates, the most intense necroses on detached leaves were caused by those of *P. syringae*. These experimental data are in accordance with the results of previous studies dealing with the pathogenicity of *P. syringae* on detached leaves of different hosts, including plum (GILBERT *et al.* 2010; HULIN *et al.* 2018). *Pseudomonas syringae* has been detected in orchard surveys as a serious pathogen of fruits in Serbia.

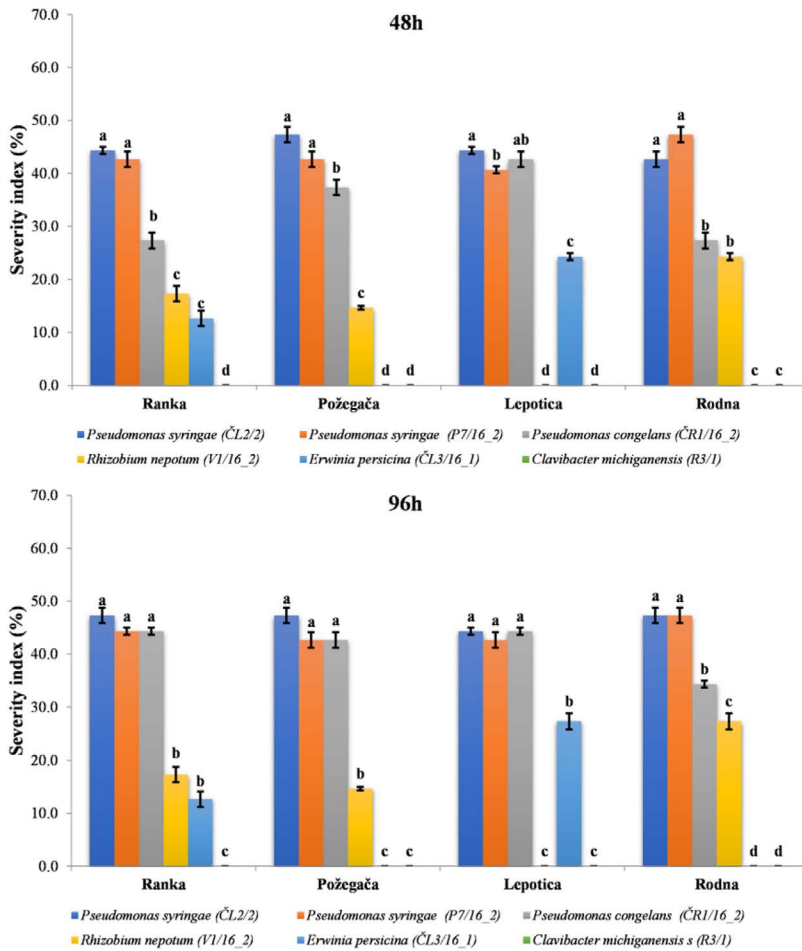


Fig. 2. Severity index obtained for indigenous bacterial isolates on the Lepotica, Rodna, Ranka and Požegača plum cultivars after 48 and 96 hours. Values indicated by the same letter are not statistically significant ($P < 0.05$) according to Tukey's HSD test.

GAVRILOVIĆ *et al.* (2008) reported drying of branches of the Lepotica and Rodna cultivars caused by *P. syringae* in orchards located in Western Serbia. Another study (IVANOVIĆ *et al.* 2012) also reported the presence of *P. syringae* in samples collected from different areas of plum cultivation in Serbia. Pathogenicity of *P. syringae* isolates originating from infected plum in this study was confirmed by artificial inoculation of immature fruit of pear, cherry, tomato, lemon, and bean. The present study was focused on investigating the susceptibility of plum by inoculating detached plum leaves with phytopathogens originating from their phyllosphere, including *P. syringae* isolates. Besides inducing the most intense necroses on detached leaves, the *P. syringae* isolates also showed the absence of cultivar specificity. The wide range of cultivar susceptibility to indigenous strains of *P. syringae* could be used as epidemiological data for future disease management in the analysed orchard.

Another *Pseudomonas* species from the plum phyllosphere, *P. congelans*, had significant virulence intensity that after 96 hours of incubation reached the same level as that of the effect caused by *P. syringae* isolates. There are no available data in the literature reporting

pathogenicity of this species. *Pseudomonas congelans* is classified in the *P. syringae* phylogenetic group, currently comprised of several closely related plant-pathogenic species, including *P. syringae* (DILLON *et al.* 2019). The potential to cause necrosis on plum leaves indicates that *P. congelans* is probably part of the plum pathobiome that is emerging as a new species on the list of *Pseudomonas* spp. pathogens.

The other isolates tested had significantly lower or no virulence manifested, as in the case of the *C. michiganensis* isolate. *Clavibacter michiganensis* is the causal agent of bacterial wilt and canker of tomato (DE LEÓN *et al.* 2011). On the other hand, *Erwinia persicina* isolate ČL3/16_1 induced necrosis on leaves of the Lepotica and Ranka cultivars. The primary hosts of *E. persicina* are different legumes, so these experimental data showed its pathogenic potential to be broader than previously reported (ZHANG & NAN 2014). The obtained data indicate that known phytopathogens should be tested for pathogenicity on plants from whose microbiome they were isolated, regardless of the phytopathogen's primary host. *Rhizobium nepotum*, a species known to cause crown gall on leaves of plum and other species of the

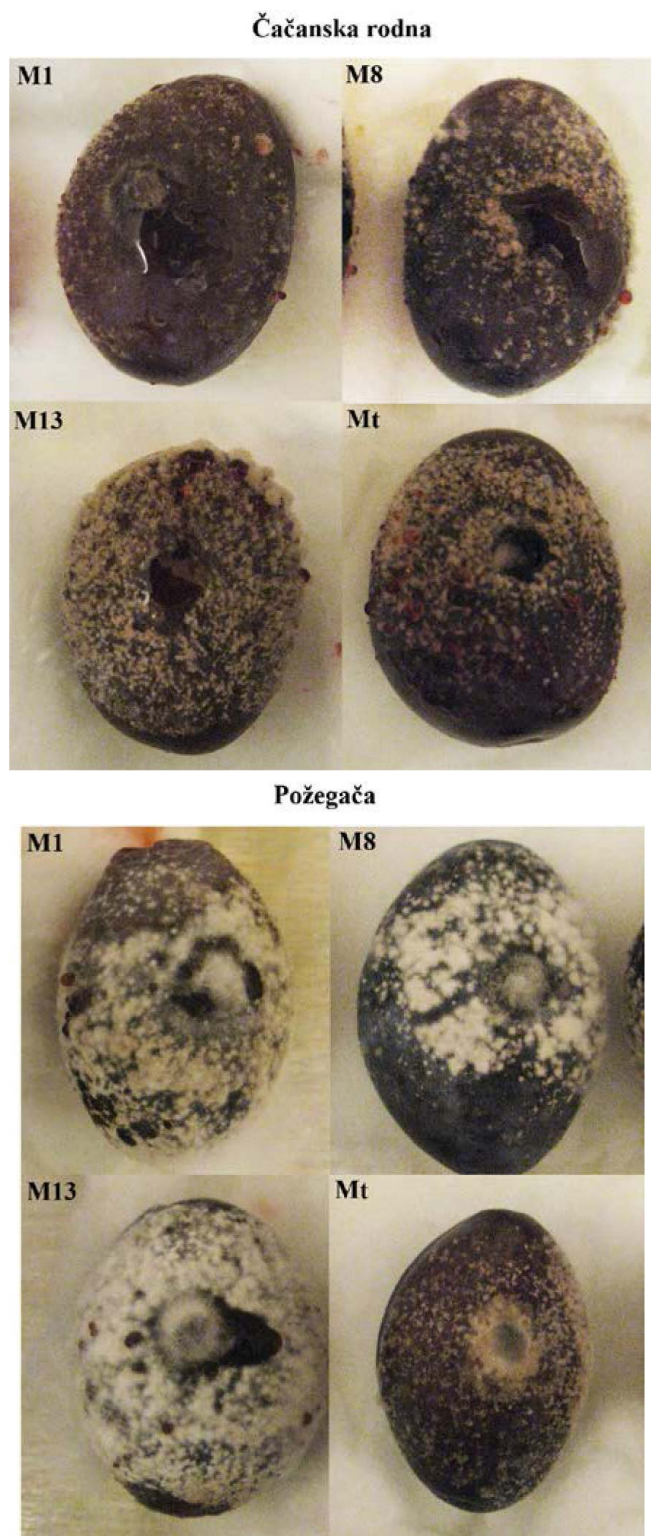


Fig. 4. Percentage of infection of fruit of the Čačanska rodna and Požegača cultivars caused by three *Monilinia laxa* isolates originating from plum. The *Monilinia laxa* Mt isolate was used as a control. Values indicated by the same letter are not statistically significant ($P < 0.05$) according to Tukey's HSD test.

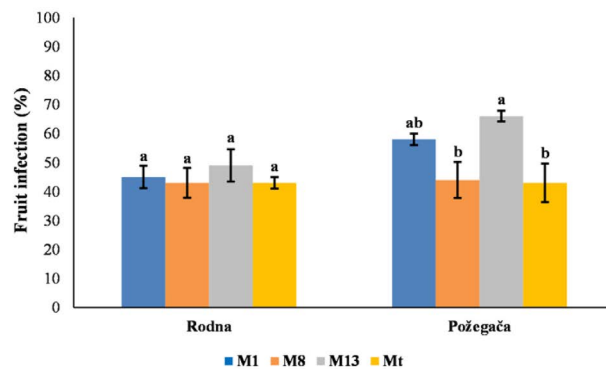


Fig. 3. Symptoms of brown rot caused by *Monilinia laxa* on fruits of the Čačanska rodna and Požegača cultivars. Isolates M1, M8 M13 originating from plum were tested. Fruits inoculated with isolate Mt originating from cherry was used as a control.

genus *Prunus* (PUŁAWSKA *et al.* 2016), was also tested. The Požegača, Rodna and Ranka cultivars showed moderate susceptibility to *R. nepotum*. An exception was the Lepotica cultivar, which exhibited resistance. One of the reasons for different susceptibility of the cultivars could lie in differences in their genotypes, which might include genes regulating plant disease resistance (LORANG *et al.* 2007).

In addition to being an effective way of evaluating susceptibility to bacterial pathogens, the *ex situ* method on detached plant material proved to be an efficient system of screening cultivars for susceptibility of their fruit to *M. laxa*. Moreover, this experimental system showed itself to be unambiguous, since no latent infection occurred on fruit inoculated with a sterile PDA plug as a negative control. The ability of indigenous *M. laxa* isolates to cause typical symptoms on artificially inoculated mature fruits of the Rodna and Požegača cultivars agrees with field observations of plum susceptibility to *M. laxa* in Serbian plum orchards (HRUŠTIĆ *et al.* 2015; GLIŠIĆ *et al.* 2016). The obtained data are also in accordance with the results of studies from other plum-producing areas across the Europe, where *M. laxa* was commonly detected in plum orchards affected by brown rot (GELL *et al.* 2007; SZÓDI *et al.* 2012; PONIATOWSKA *et al.* 2013). The observed differences in the virulence of *M. laxa* isolates on fruits of the Rodna and Požegača cultivars is possibly attributable to the existence of different plum genotypes, causing higher resistance of the Rodna cultivar. The genotype of the host plant has been reported to influence the degree of fungal pathogenicity (SACRISTAN & GARCIA-ARENAL 2008). All three *M. laxa* isolates showed significant pathogenicity, despite differences in the plum cultivars tested, suggesting that natural isolates can spread throughout the orchard and cause significant damage.

CONCLUSION

The present study demonstrates significant susceptibility of four Serbian plum cultivars to selected indigenous *P. syringae* and *M. laxa* isolates. Future investigation should therefore be focused on management of the diseases caused by these potential threats to organic orchards and prevention of their potential spread to other orchards. Appropriate and ecologically safe disease management would include development of biological control agents based on the use of indigenous bacterial or fungal strains of antagonistic to these important pathogens and thereby capable of suppressing them.

Acknowledgement – This work was supported by the Ministry of Education, Science and Technological Development of Serbia (Grants No. 451-03-68/2020-14/200178).

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REZIME



Botanica
SERBICA

Osetljivost srpskih sorti šljive na autohtone izolate bakterija i *Monilinia laxa*

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Osetljivost srpskih sorti šljive na patogene koji potiču iz njihove filofsere procenjena je inokulacijom ubranih mladih listova i zrelih plodova. Ispitivana je virulencija autohtonih bakterijskih izolata *Pseudomonas syringae*, *Pseudomonas congelans*, *Erwinia persicina*, *Clavibacter michiganensis* i *Rhizobium nepotum* na ubranim listovima četiri srpske sorte šljive (Ranka, Požeगाča, Čačanska leptotica i Čačanska rodna). Izolati *P. syringae* su u okviru 48 časova formirali intenzivne simptome na svim testiranim sortama sa indeksima intenziteta virulencije u opsegu od 41 – 47%. Ostali izolati su imali značajno niže indekse virulencije ili su simptomi izostali. Ova studija je prvi put pokazala patogenost *Pseudomonas congelans* na šljivi, sa intenzitetom simptoma koja se nije značajno razlikovala od *P. syringae* posle 96 časova inkubacije. Virulencija izolata *Monilinia laxa* testirana na zrelih plodovima sorti Čačanska rodna i Požeगाča je uvrđena na obe sorte šljive. Zabeležena je veća osetljivost na *M. laxa* kod sorte Požeगाča sa procentom inficiranosti ploda između 43 i 66%. Na sorti Rodna nije postojala statistički značajna razlika u procentu inficiranosti ploda između četiri testirana izolata *M. laxa*. Ovi podaci ukazuju na značajnu osetljivost sorti srpske šljive na autohtone izolate *P. syringae* i *M. laxa*.

KLJUČNE REČI: šljiva, list, plod, bakterijski fitopatogeni, *Monilinia laxa*