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Original scientific paper

Morphological, cultural and ecological characterization of *Monilinia* spp., pathogens of stone fruit in Serbia

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SUMMARY

Different brown rot pathogens cause similar symptoms on fruit, which makes it difficult to differentiate them based only on developed symptoms. Therefore, several methods have been described for accurate identification of *Monilinia* species. In spite of the fact that those methods can be reliable, there are several limitations for their use, and the aim of this study was to find out if there are any specific characteristics that could be used as additional features for precise identification and in-depth characterization of *Monilinia* species. The results showed that morphological characteristics on PDA, and mycelium growth rate on PDA medium can be useful characters for differentiation of *Monilinia* species since *M. fructicola* was found to grow faster than the other two test species, while *M. fructigena* grew the slowest. MALT was the optimal medium for *M. laxa* and *M. fructigena* isolates, while PDA medium was optimal for *M. fructicola*. Regarding an extremely acidic medium (pH 2), only *M. laxa* and *M. fructicola* isolates were able to grow in it, while *M. fructigena* isolate was the only that grew in a moderately alkaline medium (pH 9). Also, the results revealed that the optimal temperature for *M. fructigena* and *M. fructicola* growth was 23ºC, while 28ºC was optimal for *M. laxa* isolate. On the other hand, some differences in the sensitivity of *Monilinia* isolates were revealed at extreme temperatures: *M. fructigena* isolate was the most sensitive (grew from 4°C to up to 31°C), while *M. fructicola* isolate was the most resistant (grew at 2°C and 34°C). The obtained results inferred that there are no specific features that can be used for reliable and precise identification of *Monilinia* species, but we observed some differences regarding the effects of extreme temperatures and pH values of culture medium on different species. Further research, involving more isolates, is needed for a final conclusion.

Keywords: stone fruit, brown rot, *Monilinia*, Serbia

INTRODUCTION

Brown rot caused by *Monilinia* species is one of the most threatening diseases that cause important economic losses in pome and stone fruit production worldwide (Batra, 1991; Adaskaveg et al. 2008). Regarding stone fruit, three species of the *Monilinia* genus cause symptoms of brown rot: *Monilinia laxa* (Aderhold and Ruhland) Honey, *Monilinia fructigena* (Aderhold and Ruhland) Honey, and *Monilinia fructicola* (G. Winter) Honey (Byrde & Willetts, 1977; Batra, 1991). *M. laxa* is commonly found in all production areas worldwide, as well as in Serbia, while *M. fructigena,* regularly found in Europe, is a quarantine

pathogen in the United States and Australia. On the other hand, *M. fructicola* is classified as a quarantine pathogen by the European and Mediterranean Plant Protection Organization (EPPO), and by the Serbian and Russian plant protection authorities, but it is widespread in stone fruit in the Americas and some parts of Africa and Asia. However, after its first introduction in France, *M. fructicola* became widespread in the vast majority of European countries, including Serbia. There is also the fourth species that might cause brown rot on stone fruit, *Monilia polystroma*, but this species is mainly detected on apple fruit (EFSA, 2011).

These brown rot pathogens cause similar symptoms on fruit, so that it is difficult to differentiate them based on symptoms alone. On the contrary, colony differences observed under laboratory conditions *in vitro* lead in most cases to accurate identification. Morphological identification, based on the key described by Lane (2002), is the simplest and the most convenient of recommended methods, confirmed in many studies (Fischer et al., 2017; Hrustić et al., 2012, 2015; Lichtemberg et al., 2014; Papavasileiou et al., 2015; Poniatowska et al., 2013). However, morphological characteristics are easily affected by environmental conditions and cannot always be reliable for accurate species discrimination. Considering difficulties in performing accurate identification of *Monilinia* species on the basis of morphological characteristics, other methods have been developed. By far the most reliable results are provided by using molecular methods. The polymerase chain reaction (PCR) was used to develop many protocols for successful identification of *Monilinia* species. Some of these protocols, including the Multiplex protocol that allows differentiation of several *Monilinia* species in one PCR reaction (Côté et al., 2004), have been developed for distinguishing *M. fructicola* as the most destructive *Monilinia* species from other species of that genus (Hughes et al., 2000; Ioos & Frey, 2000). In spite of the fact that molecular methods are more reliable and accurate than morphological identification, there are several limitations to their use, such as their price per sample, and the availability of laboratory equipment and well-trained staff. Taking into account these obstacles, the aims of this study were: a) to find out if there are any specific cultural and/or ecological characteristics of *Monilinia* spp. that could be used as additional features for precise low-cost and reliable identification, and b) to provide more in-depth cultural and ecological characterization of three *Monilinia* species.

MATERIAL AND METHODS

Pathogen

From our previous four-year investigation (Hrustić et al., 2015) of 373 samples of stone fruit with brown rot symptoms that were collected and characterized, we selected three representative isolates of all three economically significant species, *M. laxa*, *M. fructigena* and *M. fructicola*, for further morphological, cultural and ecological characterization (Table 1). The selected isolates were cultured on potato dextrose agar (PDA) medium at 20°C and stored at -80°C in 20% glycerol for long-term storage, and at 4°C on PDA slants for short-term storage (Dhingra & Sinclair, 1995).

Table 1. *Monilinia laxa*, *Monilinia fructigena* and *Monilinia fructicola* isolates used in the study

Species	Location	Origin
Monilinia laxa	Leskovac	Plum
Monilinia fructigena	Belgrade	Sour cherry
Monilinia fructicola	Topola	Nectarine

Morphological characteristics of isolates

Morphological characteristics of the isolates were investigated after 10-day incubation on PDA medium at 22°C, as described by Lane (2002). The following parameters were observed: colony color, margin appearance, rosetting pattern, sporulation and presence of concentric rings of spores, medium pigmentation, and qualitative growth rate. Additionally, conidia produced on MALT medium after ten days incubation were observed and measured under light microscope at 100 × magnification (Olympus CX41, Japan). Also, the suspension of conidia was spread onto water agar (WA; 17 g agar, 1 l H_2O) with sterile cotton swabs and incubated at 24°C in the dark for 18 h. After incubation, the percentage of germinated conidia of each isolate was calculated. A minimum of 50 conidia were examined for each isolate.

Mycelial growth rate assay

Three-millimeter diameter mycelial plugs were cut from the margin of seven-day old colonies, placed on PDA medium and incubated at the temperature of 24°C to asses mycelia growth rate of each tested isolate. Growth rate was calculated after seven days of incubation, and expressed as mm per day. The mean value of three replicates was used to represent each isolate.

Cultural characteristics of isolates

Mycelial growth was tested on five different media: PDA, V8 agar (200 ml V8 juice, 20 g agar, l l H_2O), MALT (500 ml industrial malt, 17 g agar, 500 ml H_2O), WA and CzA (3 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4 \times 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \times 7H_2O$, 1 l H_2O) as described above. Growth rate on each medium was calculated after seven days of incubation at 24°C in darkness, and expressed as mm per seven days. The mean value of three replicates was used to represent each isolate.

For evaluation of the effects of different pH values of the medium on colony growth rates of three *Monilinia* species, the pH of PDA medium was adjusted to range from 2 to 12 by using 0.1 N HCl or 0.1 N NaOH (Dhingra & Sinclair, 1995). Growth rate was calculated after seven days of incubation at 24°C in darkness, and expressed as mm per seven days. The mean value of three replicates was used to represent each isolate.

Ecological characteristics of isolates

The effect of various incubation temperatures on mycelial growth was evaluated on PDA medium. Three-millimeter diameter mycelial plugs were cut from the margin of seven days old colonies, placed on medium and incubated at 18, 20, 23, 25, 28 and 30°C. Additionally, effects of extreme temperatures were studied to determine the lowest and highest temperatures for mycelial growth per species. Temperatures lower than 4°C (0°C - 4°C) and higher than 30°C (30°C - 35°C) were tested. In all tests, colony diameter was measured in two perpendicular directions, after incubation of seven days. The trials were conducted independently in three replicates.

Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) at 5% probability level with individual pairwise comparisons made by Tukey's test using the software *Statistica 10* (2010) (Sokal & Rohlf, 1995).

Molecular identification of isolates

After morphological, cultural and ecological characterization, the identification of selected isolates was confirmed by polymerase chain reaction (PCR) using Multiplex PCR (Côté et al., 2004). Total amount of deoxyribonucleic acids (DNA) of the isolates was extracted from seven days old mycelia of the isolates grown on PDA medium with the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. The PCR reactions were performed in an Eppendorf Master Cycler (Eppendorf, Germany).

RESULTS

Morphological characteristics of isolates

The isolate of *M. laxa* had light- to dark-gray colony with lobed margins without sporulation, *M. fructigena* isolate had cream to yellow colony with rare sporulation and lobate margins, while the isolate of *M. fructicola* developed even margin hazel-colored zonate colonies with abundant sporulation and concentric rings of spores on the surface of PDA. According to

described morphological characteristics determined by Lane (2002), the isolates were identified as *M. laxa*, *M. fructigena* and *M. fructicola*.

In general, the *M. fructigena* isolate produced the biggest conidia, 23.50 μm long and 13.50 μm wide, while *M. laxa* conidia were 15.42 μm long and 11.07 μm wide, and conidia of *M. fructicola* were 16.00 μm long and 10.75 μm wide. There was no statistical difference in the percentage of germinated conidia between the isolates of selected species (Table 2).

Table 2. Comparison of conidia dimensions and percentage of germinated conidia of *Monilinia laxa*, *Monilinia fructigena* and *Monilinia fructicola* isolates

Species	Lenght (μm)	Width (μm)	Germinated conidia (%)
Monilinia laxa	15.42 ± 2.37	11.07 ± 1.54	88.00
Monilinia fructigena	23.50 ± 3.07	13.50 ± 2.40	83.34
Monilinia fructicola	16.00 ± 2.22	10.75 ± 1.26	88.66

Mycelial growth rate

At 24°C temperature, all three tested isolates showed statistically significant differences in colony growth rate ($P<0.01$). In general, the highest growth rate was shown by the isolate of *M. fructicola* (10.55 mm/day), followed by *M. laxa* (9.33 mm/day). On the other hand, the *M. fructigena* isolate showed a significantly slower growth rate of 2.38 mm/day.

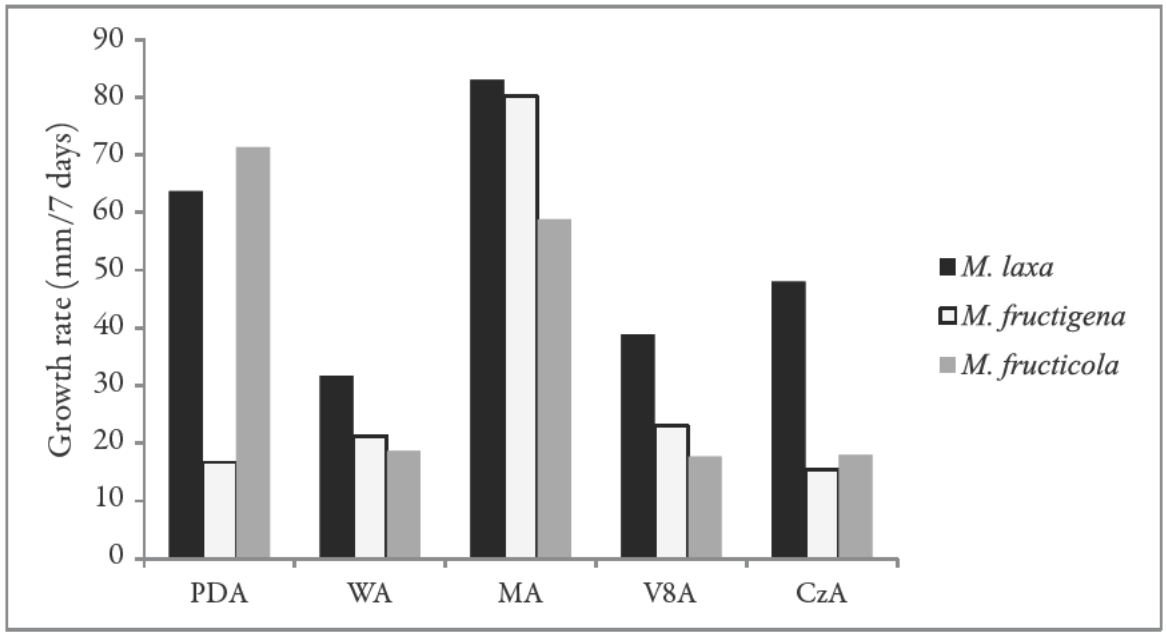

Figure 1. Effects of cultivation media on growth rates of *Monilinia laxa*, *Monilinia fructigena* and *Monilinia fructicola* isolates seven days after inoculation at 24°C

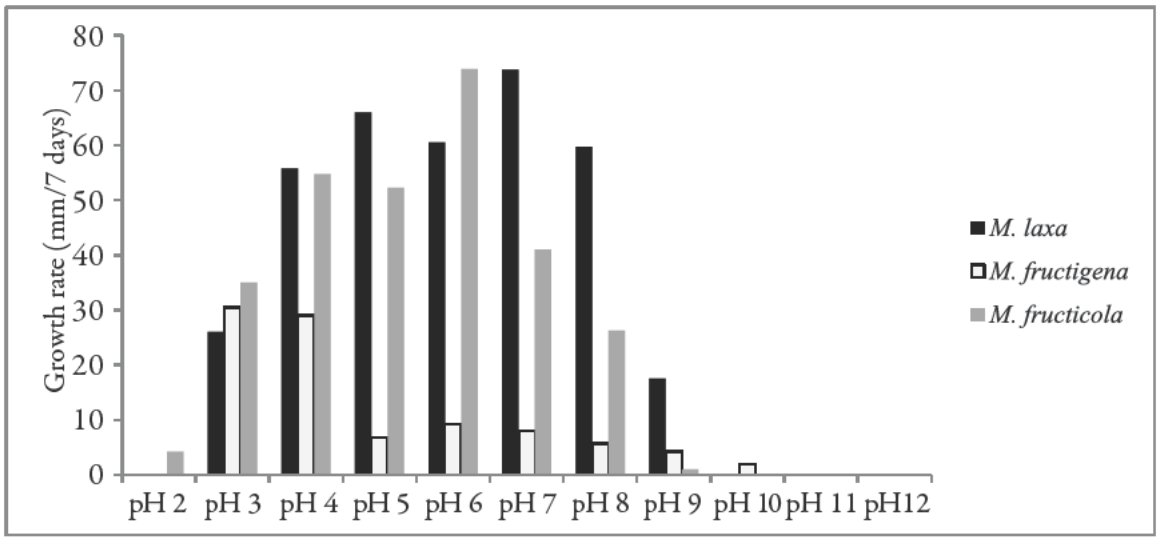

Figure 2. The effect of PDA medium of different pH values on growth rates of *Monilinia laxa, Monilinia fructigena and Monilinia fructicola* isolates seven days after inoculation at 24°C

Cultural characteristics of isolates

Growth rates on five studied media showed that there were significant differences between the isolates of different species ($P<0.05$). All isolates were able to grow on all tested media, but generally the highest growth rate was recorded on MA and PDA media (Figure 1). Conversely, the lowest growth rate was recorded on the WA medium. Besides differences in growth rate, it was notice that all tested isolates had compact mycelium on PDA, MA and V8A media, while mycelium was transparent and sparse on WA and CzA media.

The isolate of *M. laxa* had the highest growth rate on MA medium (83.00 ± 1.79 mm/7 days) and the lowest on WA medium (31.67 ± 1.37 mm/7 days). Also, *M. laxa* produced conidia on MA medium after seven days of inoculation, while it occurred after 10 days of inoculation on PDA medium under daylight. Additionally, the isolate made characteristic rosette pattern colonies on PDA and MA media, while this characteristic pattern was not noticed on the other tested media.

M. fructigena had the highest growth rate (80.17±1.72 mm/7 days) on MA medium, and the lowest (15.33±7.15 mm/7 days) on CzA medium. Similar to *M. laxa*, the *M. fructigena* isolate produced conidia on PDA and MA media, while no conidia were produced on the other test media.

M. fructicola had the highest growth rate on PDA medium (71.33 ± 3.39 mm/7 days), and the lowest (17.67 ± 2.80 mm/7 days) on V8A medium. The isolate produced conidia abundantly on all tested media except WA medium, and concentric rings of conidia were especially evident on PDA medium.

In general, the optimal medium for *M. laxa* and *M. fructigena* isolates was MALT, whereas PDA was the optimal medium for *M. fructicola*.

The results of testing the effects of medium pH showed that there are statistically significant differences in growth rates on media from pH 3 to pH 9 ($P<0.01$). Only *M. laxa* and *M. fructicola* isolates were able to grow on extremely acidic medium (pH 2), and only *M. fructigena* isolate grew on moderately alkaline medium (pH 9) (Figure 2).

M. laxa had the highest growth rate on PDA medium pH 7 (73.75 ± 1.89 mm/7 days), and the isolate did not grow at pH 9 or higher. Additionally, the *M. laxa* isolate made a characteristic colony pattern on PDA medium from pH 5 to pH 8, which was not noticed on the other test media.

On the other hand, the *M. fructigena* isolate showed the highest growth rate on a medium with relatively low pH (pH 3, 30.50 ± 2.83 mm/7 days), and that was the only tested isolate that was able to grow at pH 10. Similar to *M. laxa*, the tested isolate of *M. fructigena* formed typical creamy to yellow colony

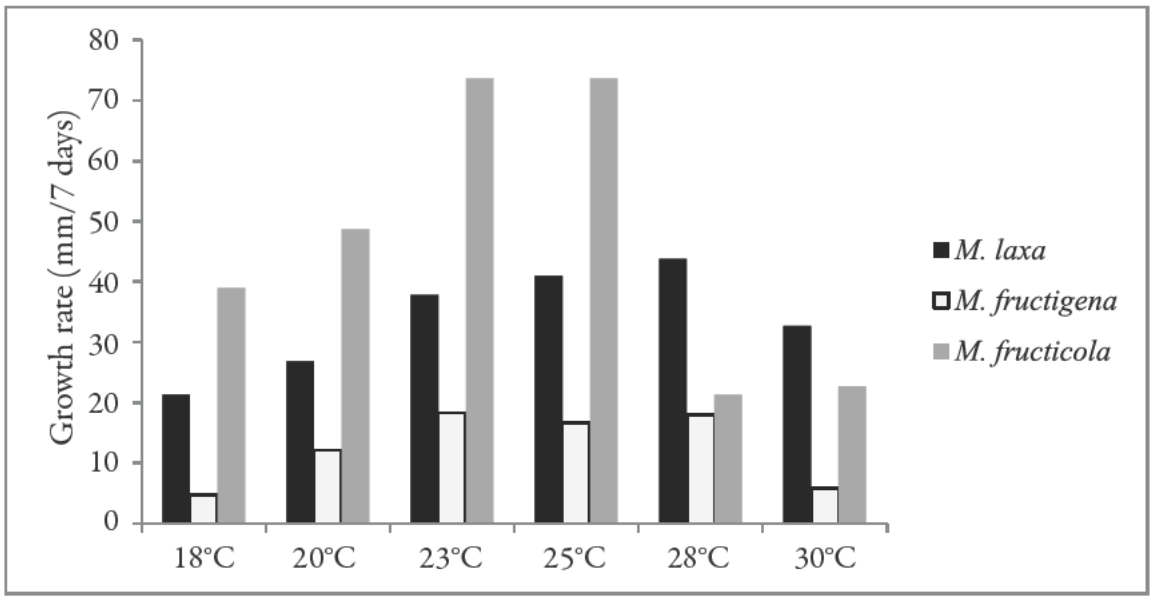

Figure 3. Effects of different temperatures on growth rates of *Monilinia laxa*, *Monilinia fructigena* and *Monilinia fructicola* isolates seven days after inoculation

with concentric rings of conidia, while its colonies were grey with rosette pattern at pH 3 and pH 4.

The *M. fructicola* isolate had the highest growth rate on PDA medium of pH 6, and the lowest at pH 9, and there was no growth on pH exceeding 9. On all test media on which *M. fructicola* colonies were able to grow, they formed a typical colony pattern with concentric rings of conidia.

Ecological characteristics of isolates

The difference in growth rates of the isolates at different temperatures was statistically significant ($P<0.01$). The isolates of *M. fructigena* and *M. fructicola* had the highest growth rate at 23°C, while the most favorable temperature for *M. laxa* was 28°C (Figure 3). On the other hand, the *M. laxa* and *M. fructigena* isolates were found to have the lowest growth rates at 18°C, and the *M. fructicola* isolate at 28°C. Besides differences in growth rates, the isolate of *M. laxa* also showed differences in morphological characteristics of colony shape as its colony had a typical rosette shape characteristic of *M. laxa* isolates at 23 and 25°C, but it had a rounded colony edge at other temperatures. On the other hand, temperature did not affect the colony shape of *M. fructigena* and *M. fructicola* isolates.

Aiming to assess the lowest and highest temperatures in terms of mycelial growth of each tested species, temperatures lower than 4°C and higher than 30°C were tested. The results showed that the *M. fructigena* isolate was the most sensitive to extreme temperatures: the lowest temperature at which *M. fructigena* colony grows was 4°C, and the highest was 31°C. On the other hand, the *M. fructicola* isolate was the most resistant: it grew at 2°C and 34°C. *M. laxa* showed moderate resistance to extreme temperatures: the lowest temperature at which its colony grew was 3°C, while it grew at temperatures lower than 33°C (Figure 4).

Molecular identification of isolates

Using the primers MO368-5, MO368-8R, MO368-10R and Laxa-R2, PCR products of predicted size were amplified: 352 bp for the *M. laxa* isolate, 402 bp for the *M. fructigena* isolate and 535 bp for the *M. fructicola* isolate, providing conformation of identification based on morphological, cultural and ecological characteristics. In the PCR test, no amplicon occurred in a negative control.

DISCUSSION

The results of our previous investigation showed that *Monilinia* spp. were dominant casual agents of brown rot of stone fruit in Serbia, which causes significant losses and damage (Hrustić et al., 2015; 2018). In stone fruit production, more than one *Monilinia* species has been present in complex pathosystems, which makes control of these pathogens more challenging. Over the past eight years, three *Monilinia* species have become widespread on stone fruit in Serbia: *M. laxa*, *M. fructigena* and *M. fructicola*. Our previous paper gave

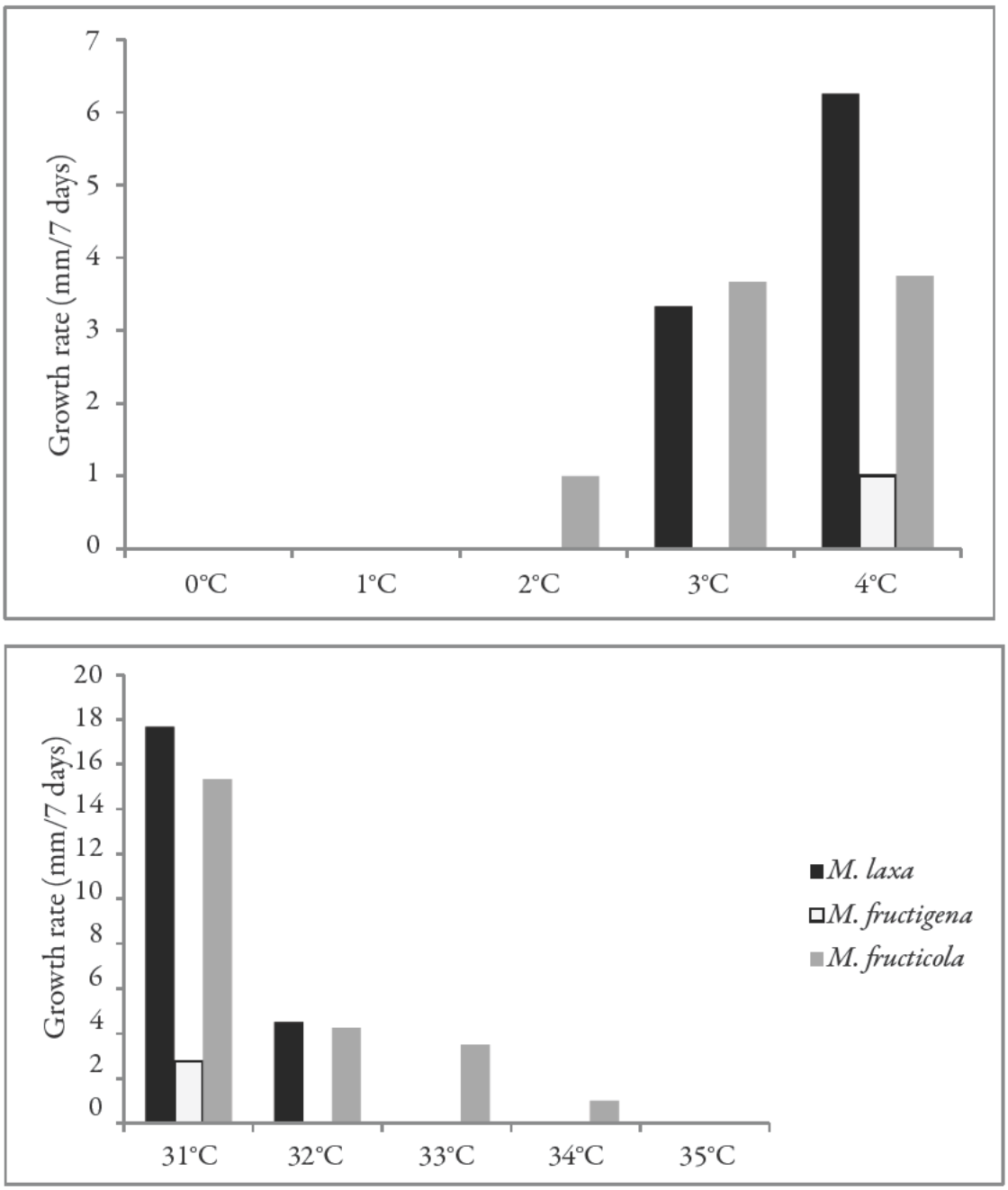

Figure 4. Effects of extreme temperatures on growth rates of *Monilinia laxa*, *Monilinia fructigena* and *Monilinia fructicola* isolates seven days after inoculation

detailed information about their distribution, virulence, some morphological and molecular characteristics (Hrustić et al., 2015), as well as some information on the sensitivity of *Monilinia* spp. to most frequently used fungicides for brown rot control (Hrustić et al., 2018). In the present study, we examined some specific cultural and/or ecological characteristics of *Monilinia* spp. that could be used as additional features for their precise, low-cost and reliable identification.

Morphological characteristics are widely used as identification criteria (Lane, 2002; Vasić et al., 2018; Fischer et al., 2017; De Cal & Melgarejo, 1999; van Leeuwen & van Kesteren, 1998). According to morphological characteristics described by Lane (2002), all isolates tested in this research had colonies with distinctive appearance: the *M. laxa* isolate had gray-colored colony with lobed margins typical for *M. laxa*, the *M. fructigena* isolate had yellow colony color with rare sporulation typical for *M. fructigena*, and the *M. fructicola* isolate had hazel-colored zonate colonies with abundant sporulation typical for *M. fructicola*. According to described characteristics and conformation of identification by using Multiplex PCR, the three chosen isolates were confirmed as *M. laxa*, *M. fructigena* and *M. fructicola*.

Besides morphological characteristics on PDA, mycelium growth rate on PDA medium can also be a useful character for differentiation of species of the *Monilinia* genus (Batra, 1979; van Leeuwen & van Kesteren, 1998; Lichtemberg et al., 2014; Poniatowska et al., 2013). Our results are consistent with some previous studies and showed that *M. fructicola* grew faster than the other two species and that *M. fructigena* grew the slowest.

Cultural and ecological features of different *Monilinia* species have not been widely described, and one of the main goals of this research was to provide an in-depth characterization of three economically important species of this genus. Our results revealed that the optimal medium for the tested *M. laxa* and *M. fructigena* isolates was MALT, whereas PDA was the optimal medium for *M. fructicola*. Similarly, Vasić et al. (2018) and Hu et al. (2011) showed that V8 and PDA media were the most favorable for the growth of *Monilinia* spp. Our results are also in agreement with findings reported by Vasić et al. (2018) that the optimal pH for *M. laxa* was 5-8, for *M. fructigena* 3-4, and for *M. fructicola* 6-8. Holb and Chauhan (2004) also reported that *M. fructigena* growth was faster at lower pH, between 2.5 and 6.5. Obi et al. (2018) reported the highest mycelial growth of a *M. laxa* isolate at pH 6.4, and the lowest at pH 2.4. Also, they proved that *M. laxa* is able to sporulate at a wide range of pH, between 3.5 and 9.5, while the optimum was found to be between pH 4.5 and 5.5.

The results of the present study revealed that the optimal temperature for *M. fructigena* and *M. fructicola* growth was 23°C, and 28°C for the *M. laxa* isolate. These results are partially in agreement with some previous studies (Vasić et al., 2018; Byrde & Willetts, 1977; Pereira et al., 2019; Papavasileiou et al., 2015). Fischer et al. (2017) reported that optimal growth rates from 20°C to 25°C, while Papavasileiou et al. (2015) found it to be at 25°C, and Lichtemberg et al. (2014) at 23.7°C. On the other hand, the ability to grow at extreme temperatures revealed some differences in the sensitivity of *Monilinia* isolates: *M. fructigena* was the most sensitive, while *M. fructicola* was the most resistant to extreme temperatures. Pereira et al. (2019) reported that minimum temperature averages for development of *M. fructicola* and *M. laxa* isolates were 4.5°C and 4.0°C, and the maximum temperature average for those isolates was 36°C, while Papavasileiou et al. (2015) showed that 35°C completely inhibited the growth of tested *M. laxa* and *M. fructicola* isolates from Greece.

Based on the results obtained in this investigation, we concluded that there are no specific cultural and/or ecological features that can be utilized for reliable and precise identification of *Monilinia* species, and that only a comprehensive study including morphological, cultural, ecological and molecular characterization could lead to accurate determination of the causal agent of brown rot in stone fruit. Even though some differences in the effect of extreme temperatures and pH values of culture medium on different species was observed, further research involving more isolates is needed for a final conclusion. Also, our findings are consistent with a previous study (Villarino et al , 2016) in showing that the *M. fructicola* isolate had the greatest growth differences compared to the other two *Monilinia* species.

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Karakterizacija *Monilinia* spp., patogena koštičavih voćaka u Srbiji na osnovu morfoloških, odgajivačkih i ekoloških karakteristika

REZIME

Simptomi koje prouzrokuju vrste roda *Monilinia* lako su prepoznatljivi i karakteristični, međutim samo na osnovu simptoma nije moguće razlikovati vrste ovog roda. U literaturi je opisano nekoliko metoda koje omogućavaju preciznu identifikaciju *Monilinia* vrsta. Uprkos dostupnim metodama, postoji nekoliko ograničavajućih faktora za njihovu upotrebu, pa je cilj ovog istraživanja bio da se utvrdi da li postoji još neka specifičana osobina koja može da se koristi za preciznu identifikaciju, kao i to da se još detaljnije opišu vrste ovog roda. Rezultati su pokazali da morfološke karakteristike na PDA podlozi, kao i brzina porasta micelije mogu biti koristan karakter za razlikovanje vrsta roda *Monilinia* s obzirom da vrsta *M. fructicola* raste značajno brže, kao i da vrsta *M. fructigena* raste najsporije. Optimalna podloga za porast izolata *M. laxa i M. fructigena* bila je MALT podloga, dok je za porast izolata *M. fructicola* bila PDA podloga. Na kiselim podlogama (pH 2) porast su ispoljili izolati *M. laxa i M. fructicola,* dok je na umereno baznoj podlozi (pH 9) porast ispoljio jedino izolat *M. fructigena.* Takođe, rezultati su pokazali da je optimalna temperatura za porast izolata *M. fructigena i M. fructicola* bila 23ºC, dok je za porast izolata *M. laxa* bila 28ºC. Sa druge strane, pri ekstremnim temperaturama vrste roda *Monilinia* ispoljile su određene razlike u porastu: izolat *M. fructigena* bio je najosetljiviji (porast je ispoljio pri temperaturama od 4°C i do 31°C), dok je izolat *M. fructicola* bio najotpornij (porast je ispoljio i pri 2°C, kao i pri 34°C). Rezultati ovih ispitivanja pokazali su da ne postoje specifične osobine izolata koje mogu omogućiti preciznu i pouzdanu identifikaciju vrsta roda *Monilinia,* ali su primećene određene razlike u porastu pri ekstremnim temperaturama i pri različitim pH vrednostima podloge između različitih vrsta ovog roda. Buduća sveobuhvatnija istraživanja sa većim brojem izolata su neophodna za donošenje daljih zaključaka.

Ključne reči: koštičavo voće, mrka trulež, *Monilinia,* Srbija