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Molecular Detection of *Monilinia fructigena* as Causal Agent of Brown Rot on Quince

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SUMMARY

Species of the genus *Monilinia* are important causal agents of fruit rot on pome and stone fruits in Serbia. The pathogen is very harmful, especially on small properties and cottage plantations where intensive control measures are not applied. Quince is important host for the pathogens of this genus. During spring 2010, intensive occurrence of mummified fruits overwintering on branches of the quince was observed. The pathogen was isolated using standard phytopathological methods. Pathogenicity of eight obtained isolates was tested by artificial inoculation of injured apple fruits. Identification was performed according to pathogenic, morphological and ecological properties, and was confirmed by Multiplex polimeraze chain reaction, PCR. All the isolates studied caused brown rot on inoculated apple fruits. The isolates form light yellow colonies with lobate margins, with single-celled, transparent, elliptical or oval conidia in chains, regardless temperature or light presence. Sclerotia are observed in 14 days old cultures. The highest growth rate of most of the isolates is at 27°C and in dark. Based on studied pathogenic, morphological and ecological characteristics, it was found that the *Monilinia fructigena* is causal agent of brown rot of quince. Using specific primers (MO368-5, MO368-8R, MO368-10R, Laxa-R2) for detection of *Monilinia* species in Multiplex PCR reaction, the expected fragment 402 bp in size was amplified, which confirmed that the studied isolates belonged to the species *M. fructigena*.

Keywords: Species; Rots; Quinces; Properties; *Monilinia*; Light; Isolation; Fruit; Browning

INTRODUCTION

Quince (*Cydonia oblonga* Mill.) is an old pome fruit species which has been cultivated for over 4000 years, most often as a side culture. According to international Food and Agriculture Organisation (FAO), quince is relatively poorly present in world production of temperate fruits. The total world production is several tens of times smaller compared to apple or pear (Mratinić, 2010).

In most European countries extensive production in small area is dominant. Nominally, the largest producers of quince in Europe are: Spain, Serbia, Russia, Romania, Ukraine and Greece. Serbia is in the first place in Europe according to quince growing areas and in second according to production amount. Its production is present with 19.8% in European, which is 2.8% in world fruit production. Quince production is relatively small in Serbia, and in total fruit production its share is 1.3% (Mratinić, 2010).

Quince is very valuable fruit for household as well as for industrial processing. Its fruits are an excellent raw material for a variety of processing, particularly for making preserves, compotes, jellies, juices, liqueurs, and lately increasingly demanded quince brandy (Mratinić et al., 2009; Nikolić, 2009). Contrary to small consumption as table fruit, processing industry appreciates quince for high biological value of the fruit, i.e. very favorable chemical content for different types of processing (Mratinić, 2010).

In spite its many advantages over other fruit species (it is one of the last fruit trees to blossom avoiding late spring frosts; it bears every year which is not the case with many tree fruit species; it has very large fruits enabling easy harvest up to two weeks long) and high utility value of fruits, quince is grown to a limited extent in Serbia, and in the last few years the production is slightly decreasing (Nikolić, 2009). The total production of quince in 2011 was 13.955 t (Anonymous, 2011). Although agroecological conditions in Serbia favor quince growing, the producers are reluctant to raise new plantations given that quince is highly susceptible to fire blight caused by *Erwinia amylovora* (Postman, 2008; Papachatzis et al., 2011). Besides *E. amylovora*, species of the genus *Monilinia* are also among the most important quince pathogens, causing twig blight and fruit rot.

Species of the genus *Monilinia* are well known plant pathogens that affect fruit production worldwide, causing significant damage and losses, particularly on species from *Rosaceae* family (Byrde and Willetts, 1977). Three species of this genus are economically important and can cause damages on all pome fruit species: *M. fructigena* (Aderhold and Ruhland) Honey, *M. laxa* (Aderhold and Ruhland) Honey and *M. fructicola* (Winter) Honey (Byrde and Willetts, 1977). Besides mentioned species, *M. linhartiana* (Prill. and Delacr.) Buchw. was described as a pathogen of quince (Balaž, 2000). Morphology and biology of these species are very similar which can often lead to wrong identification of the disease causal agents (Byrde and Willetts, 1977; Corazza et al., 1998). *M. fructigena* primarily causes rot of pome fruits before and during storage, while flower, twig and branch infections are rarely observed (Anonymous, 2004). On the other hand, *M. laxa* is economically important on stone fruits: peach, apricot, sour and sweet cherry, and plum on which it causes blossom and twig blight, as well as fruit brown rot (Byrde and Willetts, 1977). However, *M. laxa* was also detected on pome fruits (Muñoz et al., 2008). *M. fructicola* is a widespread pathogen in Australia, New Zeland, South Africa, North and South America, Japan (Anonymous, 1997). In Europe, this pathogen is on the A2 list of quarantine organisms (Anonymous, 2009), while in Serbia it has not been documented on any fruit species.

Species of the genus *Monilinia* as pathogens of quince are poorly documented. At present, only the presence of *M. fructigena* and *M. linhartiana* has been reported in Serbia (Balaž, 2000), while neither *M. fructicola*, nor *M. laxa* has been detected. However, detail investigations of the species of this genus isolated from quince have not been conducted. Given the damage caused by these species in quince production on one, and poor information on their distribution and pathogens characteristics on the other hand, the main objectives of this paper were to identify and characterize isolates obtained from mummified quince fruits from different localities, and to establish and introduce a protocol for species-specific identification by conventional and molecular methods.

MATERIAL AND METHODS

Pathogen isolation

The pathogen was isolated from mummified quince fruits collected at three different localities (Radmilovac, Bijeljina and Dvorovi) on territory of Serbia and

Republic of Srpska, by using standard phytopathological isolation techniques. Fragments of mummified tissues were surface sterilized by immersion in 4% solution of sodium-hypochlorite (NaOCl) for two minutes and placed on sterile potato dextrose agar (PDA) in Petri dishes and incubated for seven days. The obtained monosporic isolates were subcultured on potato-dextrose-agar medium (PDA) at 20°C and stored on slants at 4°C (Dhingra and Sinclair, 1995).

Pathogenicity test and reisolation

The obtained isolates were tested for pathogenicity by artificial inoculation of injured apple fruits. Mycelial fragments, cut from the edge of seven-day old PDA cultures, were placed on previously surface-sterilised and injured healthy apple fruits as it was described by Vignutelli et al., (2002). Fruits inoculated with sterile fragments of PDA medium were used as a control. Inoculated fruits in two replicates were incubated in a humid chamber at room temperature, and the symptom occurrence was observed daily during 10 succesive days. After rot symptoms occurred, the pathogen was reisolated from inoculated fruits, using the same methods and conditions as for the isolation.

Morphological characteristics of isolates

Morphological characteristics of the isolates were investigated after 10-day incubation on PDA medium at 22°C, as it was described by Lane (2002). The following parameters were observed: colony appearance, color, margin and colony shape, sporulation, presence of concentric ring, medium pigmentation, qualitative growth rate. Conidiophores and conidia were observed under light microscope (Olympus CX41, Japan) at 100 x magnification.

Ecological characteristics of isolates

Three-millimeter-diameter mycelial plugs were cut from the margin of seven-day-old colony, grown on PDA at 24°C in dark, placed on PDA medium and incubated at temperatures of 20°C, 23°C, 25°C and 27°C to test the influence of temperature on the isolates growth rate. The effect of light was investigated by incubation of the isolates under day light regime and in dark at 24°C. Colony diameter was measured in two perpendicular directions, after incubation of seven days. The trial was conducted in two independent experiments with four replicates, and the data were processed by analysis of variance using software packet Statistica (StatSoft Inc, 2001). Duncan's multiple range test was used to test significance of difference among means of colony diameters.

DNA extraction

DNA was obtained directly by scraping mycelia with a pipette tip from seven-day-old culture on PDA. The mycelia was transferred into 50 µl of PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, CA USA), vortexed briefly, incubated 30 min at 56°C followed by 10 min incubation at 100°C and stored at -20°C until use (Harrington and Wingfiled, 1995). The DNA quality from each isolate was confirmed to be suitable for polymerase chain reaction (PCR) by generation of a single band with universal primers ITS1 and ITS4 (Whiteet al., 1990).

Molecular identification of isolates

The pathogen was identified on the bases of its pathogenic, morphological and ecological characteristics which was confirmed by PCR. The following specific primers were used for detection: MO368-5, MO368-8R, MO368-10R and Laxa-R2**,** according to protocol Côté et al. (2004). The primers used in the experiment are shown in Table 1. Reaction mix of 25 µl final volume consisted of: 1 X Master mix (Fermentas, Lithuania) (0.625 U Taq polymerase, 2 mM $MgCl_2$, 0.2 mM of each dNTP-a), 1 µl of each primer (20 µM) and 1 µl of extracted DNA. Reaction PCR mix without added DNA served as a negative control. PCR reactions were performed in Eppendorf Master Cycler and the reaction conditions were as follows: initial denaturation 2 min at 95°C, followed by 35 cycles of 15 s at 95°C, 15 s at 60°C, 1 min at 72°C. Final elongation was three minutes at 72°C. The amplification products were analyzed by 1% agarose gel electrophoresis (0.6 g agarose in 40 ml TBE buffer) in TBE buffer (86 mM Tris, 89 mM H_3BO_3, 2 mM EDTA) at 100V constant voltage, stained with 0.1% ethidium bromide solution and visualised under UV light. Molecular weight of the obtained PCR product was determined according to its position in relation to 1kb DNA marker (Fermentas, Lithuania). The presence of amplicons estimated at 402 bp in size was considered as positive reaction for the presence of *M. fructigena*.

Table 1. Primers used for molecular detection of *Monilinia* species using polymerase chain reaction

Species	Primer Name	Sequence (5'-3')
common reverse primer	MO368-5	GCA AGG TGT CAA AAC TTC CA
M. fructigena and *Monilia polystroma*	MO368-8R	AGA TCA AAC ATC GTC CAT CT
M. fructicola	MO368-10R	AAG ATT GTC ACC ATG GTT GA
M. laxa	Laxa-R2	TGC ACA TCA TAT CCC TCG AC

RESULTS

Pathogen isolation

Eight light yellow colonies with lobate margins were obtained from collected mummified fruits using previously described method. On PDA medium, the isolates formed compact, light yellow mycelium with lobate margins, similar to the mycelium of *Monilinia* spp. (Figures 1 and 2).

Pathogenicity test

All tested isolates caused brown, two-cemtimeter wide spreading lesions on inoculated apple fruits after incubation of three days. Rot spread concentrically from inoculation site, and after ten days affected entire fruits on which gray sporodochia were formed (Figure 3). The difference among the isolates in terms of virulence was not observed. The pathological changes in the control were noticed.

Figure 1. *Monilinia fructigena*: mummified quince fruits

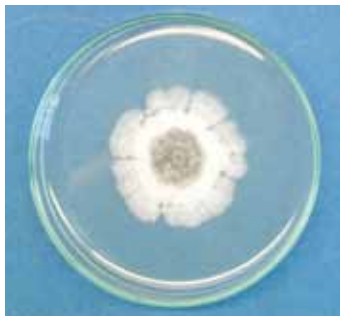

Figure 2. *Monilinia fructigena*: compact light yellow mycelium with lobate margins on PDA medium seven days after inoculation

Figure 3. *Monilinia fructigena*: artificially inoculated apple fruits four (left) and 10 days (in the middle) after inoculation and negative control (right)

Morphological characteristics of isolates

The results of studied morphological macroscopic and microscopic characteristics of the isolates are shown in Table 2. All the isolates formed slowly growing, light yellow colonies with lobate margins on PDA medium. Even after 14 days of culturing at 22°C the diameter of colonies did not reach 90 mm Petri dishes. All tested isolates exhibited abundant sporulation, and concentric rings of aerial mycelium with conidia were visible on the colony surface, after three days of incubation. In 14-day-old cultures rare black sclerotia, mainly distributed along the colony margins, were observed. Neither pigment formation which stain the mediumn or presence of a black line at the margin of the colony were observed.

The conidia (12.5-22.5 x 7.5-15 μm) were hyaline, one-celled, elliptical to oval arranged in chain in which the youngest conidium is at the chain base (Figure 4).

Table 2. Macroscopic and microscopic characteristics of studied isolates

Isolates	Macroscopic characteristics							Microscopic characteristics
	Colony color	Growth rate[1]	Colony shape[2]	Sporulation[3]	Concentric ring of spores[4]	Colony margin[5]	Black margin[6]	Dimension of conidia (μm)
1DM	yellow	S	-	+	+	L	-	17.5-22.5 × 7.5-15
2DM	yellow	M	-	+	+	L	-	12.5-17.5 × 7.5-10
3DM	yellow	F	-	+	-	L	-	12.5-17.5 × 7.5-10
5DM	yellow	S	-	+	+	L	-	15-20 × 7.5-10
6DM	yellow	S	-	+	+	L	-	12.5-15 × 7.5-10
7DM	yellow	S	-	+	+	L	-	15-20 × 7.5 × 12.5
8DM	yellow	S	-	+	+	L	-	12.5-17.5 × 7.5-10
9DM	yellow	S	-	+	+	L	-	17.5-20 × 10-12.5

[1] Colony growth rate: S (slow) < 70 mm, M (medium) 70-80 mm, F (fast) > 80 mm after 10 days at 22°C;
[2] Colony shape: R-rosette, - not rosette;
[3] Sporulation: + abundant, - scarce;
[4] Concentric ring of spores: + present, - absent;
[5] Colony margin: L-lobate, E- entire;
[6] Black marginal line: +present, - absent.

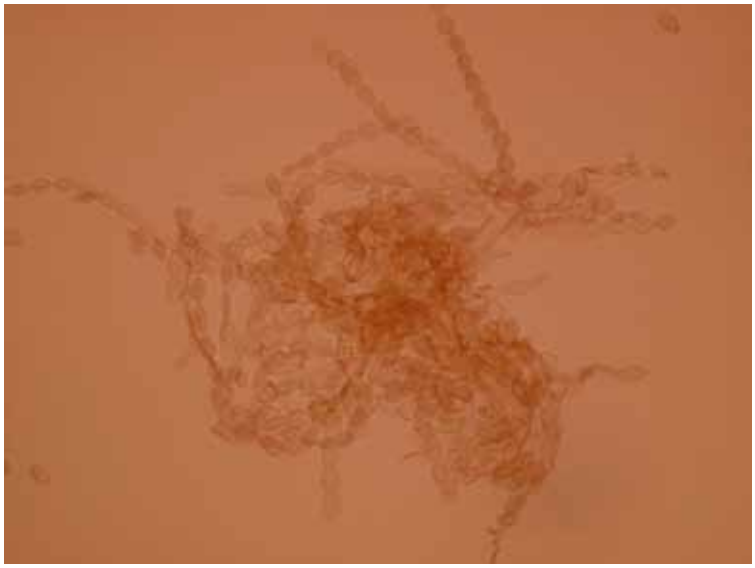

Figure 4. *Monilinia fructigena*: the conidia in branching chains

Ecological characteristics of isolates

The difference in growth rate of the isolates at different temperatures was statistically significant.

From eight tested isolates, six had the highest growth rate at 27°C. The isolate 7DM showed the highest growth rate at 20°C, while for the isolate 9DM the most favorable temperature was 23°C. Seven days after incubation, the lowest growth rate (10.17 mm) was recorded for the isolate 5DM at 20°C, while the isolate 9DM showed the highest growth rate (59.83 mm) at 23°C (Table 3, Figure 5).

Table 3. Influence of different temperatures on the growth rates of the *Monilinia fructigena* isolates on PDA medium after seven days of incubation

Isolates	Colony diameter (mm) MS ± SD*			
	20°C	23°C	25°C	27°C
1DM	11.33±2.87 Be	29.67±8.53 Ad	28.67±12.72 Acd	30.50±11.87 Ac
2DM	39.92±12.19 Bab	45.75±6.28 Bb	44.83±6.13 Ba	54.00±3.36 Aa
3DM	37.58±18.23 Abc	37.67±8.66 Ac	37.33±9.48 Ab	43.25±5.10 Ab
5DM	**10.17±0.98 De**	18.50±1.64 Ce	21.83±1.17 Bd	24.83±5.85 Ad
6DM	26.67±10.07 Ad	22.58±8.66 Ae	31.58±17.23 Abc	35.25±21.74 Abc
7DM	47.75±12.17 Aa	39.92±10.2 Bbc	33.17±6.99 Bbc	32.08±11.19 Bbc
8DM	33.83±3.46 Abcd	36.08±6.05 Ac	31.08±4.85 Abc	36.33±11.70 Abc
9DM	28.92±12.48 Dcd	**59.83±8.17 Aa**	49.54±2.71 Ba	42.25±4.83 Cbc

* Results are means value ± standard deviation. For each attribute the mean values with the same lowercase letters among isolates in the same column are not significantly different at 5% level of probability (Duncan's multiple range test) and with the same uppercase letters among temperatures in the same row are not significantly different.

Figure 5. *Monilinia fructigena*: effect of temperature on growth of 1DM isolate seven days after inoculation (left to right: 20°C, 23°C, 25°C and 27°C)

Significant difference in growth of the isolates under conditions of day light and dark was recorded (Table 4).

Table 4. Influence of light and darknes on the growth rates of the *Monilinia fructigena* isolates on PDA medium after seven days of incubation

Isolates	Colony diameter (mm) MS ± SD*	
	Light	Dark
1DM	10.00±4.62 Bbc	23.50±1.29 Ab
2DM	10.00±1.15 Bbc	16.25±0.96 Ac
3DM	12.25±1.50 Bab	24.25±0.50 Aab
5DM	14.00±0.00 Aa	10.25±2.06 Bd
6DM	13.50±0.58 Ba	**25.75**±1.50 Aa
7DM	9.00±1.82 Bcd	15.50±0.58 Ac
8DM	7.00±0.00 Bde	9.50±1.73 Ad
9DM	**4.75**±0.50 Be	9.00±0.82 Ad

* Results are means value ± standard deviation. For each attribute the mean values with the same lowercase letters among isolates in the same column are not significantly different at 5% level of probability (Duncan's multiple range test) and with the same uppercase letters among light regime in the same row are not significantly different.

Seven isolates exibited higher average growth rates in dark, while only one isolate (5DM) grew faster in the presence of daylight. The highest recorded growth rate is 25.75 mm (isolate 6DM) measured in dark, while the lowest growth of 4.75 mm was recorded in the presence of light (isolate 9DM). The formation of concentric rings of aerial mycelium with conidia was specific for the colonies of all isolates which grew in the presence of light, while in the colonies isolates which grew in the dark the rings were not formed (Figure 6).

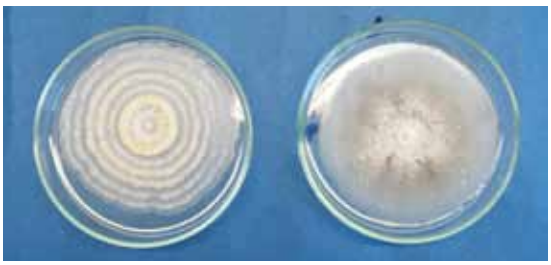

Figure 6. *Monilinia fructigena*: effect of light and darkness on growth of 6DM isolate (left-light, right-dark)

Molecular identification of isolates

Using primers MO368-5, MO368-8R, MO368-10R and Laxa-R2, PCR products of the the size of 402bp were detected in isolates from mummified quince fruits. In PCR test no amplicons occurred in negative control. PCR results for tested isolates are shown in Figure 7.

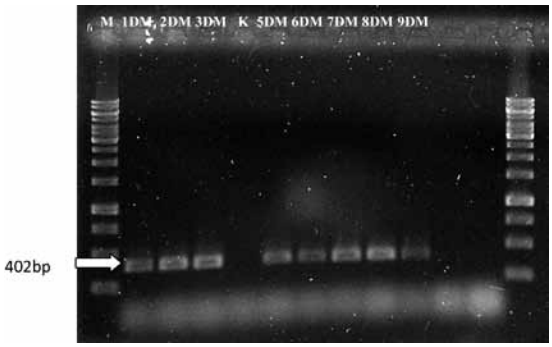

Figure 7. Visualisation of the amplicons obtained by multiple PCR with primers MO368-5, MO368-8R, MO368-10R and Laxa-R2 in 1% agarose gel. Columns 1-3 and 5-9 isolates from mumified quince fruits (1DM, 2DM, 3DM, 5DM, 6DM, 7DM, 8DM, 9DM); Column 4- negative control

Based on morphological and ecological characteristics, as well as, on the result of the PCR reaction, the isolats were identified as *Monilinia fructigena*.

DISCUSSION

Monilinia spp. are widely distributed plant pathogens. Causing significant damages and losses in fruit production, they are classified among economically the most important pathogens (Amiri et al., 2009). They are the object of extensive investigations for over 150 years, and in North America for about a century (Balaž, 2000). Besides *M. fructigena,* which is the most common parasite of pome fruits, *M. linhartiana* is also encountered on quince and is specific only for this fruit species. Symptoms caused by *M. linhartiana* are observed very early in spring mainly on leaves that are next to the flower. Infected leaves and fruits start to wilt and die, and the infection readily spreads on young fruits. Dried fruits turn into mummies that remain to hang on the branches (Balaž, 2000). Identical symptoms on quince fruits were observed in early spring 2010 on several localities in Serbia and Republic of Srpska. However, the investigation showed that all the isolates obtained from collected samples belong to the species *M. fructigena*.

All isolates tested in this investigation formed colonies with distinctive appearance, typical for *M. fructigena*, light yellow, with lobate margins and abundant sporulation which was easily noticed in form of concentric rings. Also, formation of sclerotia was observed in 14-day-old cultures. Batra and Harada (1986) detected formation of black, irregularly shaped sclerotia

in cultures old one month which confirmed previous investigations (Harada, 1977; Batra, 1979) in which sclerotia formation was considered a specific characteristic of *M. fructigena* isolates. Specific features of *M. laxa* colonies such as rosette shape, grey color, black marginal line and spore absence, were not related to any of the isolates originating from quince. All tested isolates formed abundant large, one-celled, elliptical conidia in chains characteristic for *M. fructigena* (van Leeuwen and van Kesteren, 1998; Lane, 2002; Anonymous, 2009). Presence of disjunctor between conidia in chains characteristic for *M. linhartiana* (Balaž, 2000) was not observed in cultures of the tested isolates.

Mycelium growth rate on PDA medium can be a useful character for differentiation of species of *Monilinia* genus. Batra (1979) found that *M. laxa* can be distinguished from *M. fructigena* and *M. fructicola* by slower growth rate: *M. fructigena* grows 80 mm after seven days of incubation, while *M. laxa* grows 40 mm after six days.

Growth of *Monilinia* species on PDA medium is significantly affected by conditions of light and dark (De Cal and Melgarejo, 1999). As stated in their study, high UV light wavelengths significantly inhibit isolate growth compared to the growth in the absence of light. Growth inhibition of *M. fructigena* isolates ranges from 80% to 93%. On the other hand, conditions of day light do not inhibit mycelial growth of *M. fructigena* isolates (Harada, 1977). In this investigation, the average maximum growth of the isolates was recorded in the absence of light. Significant difference in growth and morphological appearance between investigated isolates was observed, depending on the light presence.

However, morphological and ecological characteristics are not reliable parameters for species identification. In the Muñoz et al. (2008) study, only 72% of the isolates primarily identified according to morphological characteristics were confirmed by molecular technique. Methods of molecular biology, particularly PCR technique, are necessary for rapid and reliable detection and identification of different pathogens. Detection of species of the genus *Monilina* using primers and protocols described by Còté et al. (2004), is a useful, fast and reliable technique for specific detection of the isolates in single reaction. Specific primers MO368-5, MO368-8R, MO368-10R and Laxa-R2 enable visualisation of amplicons with different sized: 402bp specific for *M. fructigena*, 535bp for *M. fructicola*, 351bp for *M. laxa* and 425bp for *M. polystroma*. The size of the amplicons obtained in Multiplex PCR reaction, in which isolates collected from mummified quince fruits were used, is in agreement with the results obtained by Còté et al. (2004).

Based on pathogenic, morphological and ecological characteristics, it was found that the isolates belong to the species *M. fructigena*. Using specific primers for detection of species of *Monilinia* genus in Multiplex PCR reaction, the expected fragment 402 bp in size was amplified, suggesting that studied isolates do belong to the species *M. fructigena*.

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Molekularna detekcija *Monilinia fructigena* prouzrokovača truleži ploda dunje

REZIME

Vrste roda *Monilinia* su značajni prouzrokovači truleži ploda jabučastog i koštičavog voća u našoj zemlji. Parazit nanosi velike štete, posebno na malim posedima i u vikend zasadima u kojima se ne primenjuje redovna zaštita. Dunja je značajan domaćin patogena ovog roda. Tokom proleća 2010. godine ustanovljena je intenzivnija pojava mumificiranih plodova prezimelih na granama. Cilj ovog rada bio je da se identifikuje prouzrokovač mumifikacije plodova dunje, prikupljenih sa različitih lokaliteta. Patogen je izolovan primenom standardnih fitopatoloških metoda. Patogenost osam dobijenih izolata proverena je veštačkom inokulacijom povređenih plodova jabuke. Identifikacija je obavljena na osnovu patogenih, morfoloških i ekoloških osobina, a potvrđena je primenom Multiplex PCR. Svi proučavani izolati su na inokulisanim plodovima jabuke prouzrokovali trulež smeđe boje. Proučavani izolati na KDA podlozi formiraju svetlo-žute kolonije režnjevitog oboda. U kulturi gljiva, nezavisno od uticaja temperature i prisustva ili odsustva svetlosti, formira jednoćelijske, providne, eliptične ili ovalne konidije u nizovima. Sklerocije se uočavaju u kulturama starosti 14 dana. Većina izolata ostvaruje najbolji porast na temperaturi od 27°C i u odsustvu svetlosti. Na osnovu proučenih patogenih, morfoloških i odgajivačkih odlika, utvrđeno je da izolati pripadaju vrsti *Monilinia fructigena*. Korišćenjem specifičnih prajmera (MO368-5, MO368-8R, MO368-10R, Laxa-R2) za detekciju vrsta roda *Monilinia* u Multiplex PCR reakciji, amplifikovan je očekivani fragment veličine oko 402 bp čime je potvrđeno da proučavani izolati pripadaju vrsti *M. fructigena*.

Ključne reči: Vrste; trulež; dunja; osobine; *Monilinia*; svetlost; izolacija; plod