

Botrytis squamosa – the causal agent of onion leaf blight in Bosnia and Herzegovina

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SUMMARY

Over the past several decades, necrotic spots, lesions and blight symptoms have been observed on onion leaves in several locations in Bosnia and Herzegovina, where the crop is grown intensively. The type of symptoms indicated a possible infection with *Botrytis squamosa*, a widespread pathogen of onion. As symptoms of leaf spots and necrotic lesions can also be caused by some other biotic and abiotic factors, our research focused on identifying the causal agent of the observed symptoms. The pathogen was isolated from diseased tissue using standard phytopathological procedure and identified based on pathogenic and morphological features. Identification was confirmed by amplification and sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA). The influence of temperature and growth medium on mycelial growth rate of the isolates was also studied.

Keywords: *Botrytis squamosa*; onion leaf blight; identification; Bosnia and Herzegovina

INTRODUCTION

Onion (*Allium cepa* L.), also known as bulb onion or common onion, is a worldwide culinary and therapeutic spice species of the family Liliaceae. Total worldwide area cultivated with onion crops is about 4.9 million ha, while output amounts to 93 Mt (FAOSTAT, 2016). After tomato, onion is the second most important vegetable

crop, and over 140 countries cultivate it worldwide (Anonymous, 2018). Due to its distinctive pungent flavor, it is an essential ingredient of the cuisine in many regions of the world (Sharma et al., 2005). Onion is also a source of various biologically active compounds, such as phenolic acids, thiosulfinates and flavonoids, qualifying it as treatment of various human ailments (Kuetze, 2017).

Fungal diseases are a major factor limiting the production of *Allium* crops. Lorbeer et al. (2004) reported several *Botrytis* species as pathogens on plants belonging to the genus *Allium*, and underlined *Botrytis squamosa*, the causal agent of leaf blight, and *Botrytis allii*, the causal agent of botrytis neck rot, as the most important species. *B. squamosa* had earlier been described by Walker as the causal agent of small sclerotial neck rot of onion (Walker, 1925). However, subsequent studies showed that this species causes onion leaf spots and leaf blight which had been previously described as “blast” (Lorbeer, 1992; Lorbeer et al., 2004). *B. squamosa* epidemics can be divided into two phases: a leaf spotting phase when the number of lesions increases slowly, and a leaf blighting phase when both airborne inoculum and lesion number increase rapidly (Carisse et al., 2008). Initial visible symptoms caused by *B. squamosa* are small distinct necrotic spots, approximately 2 mm in diameter, surrounded by silvery halo, which could be confused with thrips injury, downy mildew, drought, excessive soil moisture, or symptoms caused by *Botrytis cinerea*. The second phase of the disease, leaf blight, results in premature foliage death, and immature and undersized bulbs. The bulb neck also dries improperly, providing an entering point for *Botrytis* neck rot and other fungi (Anonymous, 1990). *B. squamosa* sporulates only on senescent leaf tissues; hence, disease progress is generally slow early in the season when the majority of leaves are green. However, as leaves senesce, the sporulation potential increases rapidly, influencing the rate of disease progress (Carisse et al., 2008).

Necrotic spots, lesions and blight symptoms were observed on onion leaves in several locations of major onion producing areas in Bosnia and Herzegovina. The type of symptoms indicated a possible infection with *B. squamosa*. As symptoms of leaf spots and necrosis can also be caused by some other biotic and abiotic factors,

our research focused on identifying the causal agent of the observed symptoms.

MATERIAL AND METHODS

Sampling and pathogen isolation

Diseased onion plants, showing symptoms of leaf blight, were collected in commercial onion fields at four locations in Bosnia and Herzegovina (Table 1). The samples were taken from fields in which all standard agrotechnical measures, including fungicide application, were performed.

The pathogen was isolated from diseased tissue using standard phytopathological procedure (Dhingra & Sinclair, 1995). Small fragments of diseased tissue, taken from margins of leaf spots, were surface disinfected in 1% sodium hypochlorite solution (NaOCl) for two minutes, placed on sterile potato dextrose agar medium (PDA) and incubated at room temperature for three to seven days. To obtain a pure culture, the developed mycelium was transferred to sterile PDA medium and cleaned using the single hyphal tip method. The derived isolates were kept on PDA slants at 4°C in the Fungal Culture Collection of the Institute of Pesticides and Environmental Protection, Belgrade, Serbia, and transferred to fresh medium once a year.

Pathogenicity testing

Pathogenicity of all obtained isolates was studied by artificial inoculation of fully developed leaves of onion plants at the nine-leaf stage of development (BBCH19) (Meier, 1997). In addition, pathogenicity was checked on onion bulbs and detached leaves of tomato. Mycelial plugs of 4x4 mm, cut from the edge of 4-day-old colonies on PDA, were used for inoculation. To insure a direct

Table 1. *Botrytis squamosa* isolates, their origin, crop size and the number of derived isolates

Location	Coordinates	Field size	Number of isolates	Codes of isolates	Studied isolates
Laktaši (Kosjerovo)	44°59'34" N 17°22'59" E	2.5 ha	5	L101-105	L103, L105
Gradiška (Karajzovci)	45°00'57" N 17°22'01" E	2 ha	5	L106-110	L109, L110
Gradiška (Vakuf)	44°59'30" N 17°22'44" E	1.2 ha	5	L111-117	L116
Srbac (Kukulje)	44°59'54" N 17°23'07" E	5 ha	6	L118-123	L122, L123

contact of the mycelium with plant tissue, the plugs were positioned upside-down on intact surface of onion and tomato leaves, as well as on longitudinal sections of peeled onion bulbs. Inoculated plant parts were incubated in a humid chamber at 20°C for seven days and observed for symptoms daily. Control plant parts were inoculated with sterile PDA plugs. After disease symptoms appeared, the pathogen was re-isolated on PDA, using the same procedure as for isolation. Then, morphological features of the isolated fungi were compared and matched with the original ones used for inoculation.

Identification

The isolates were identified to the species level on the basis of their pathogenicity, morphological characteristics, growth rate and cultural characteristics (daily growth rate, impact of temperature and nutrition medium on growth rate) (Dhingra & Sinclair, 1995). Identification was confirmed by amplification and sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) using ITS1 and ITS4 primers (White et al., 1990).

Morphological characteristics of the resulting colonies were investigated after 15-day incubation on PDA medium at 20°C. The following parameters were observed: colony appearance (color and shape), colony margin appearance, sclerotia formation and sporulation of the isolates (Muntanjola-Cvetković, 1987). For morphological observations of conidiophores and conidia, fully developed onion plants at the nine-leaf stage were inoculated *in situ* using mycelial PDA plugs and incubated in a transparent humid chamber (RH>95%) at room temperature until sporulation of the isolates was observed. Conidiophores and conidia were observed under light microscope (Olympus CX41, Japan) and compared to descriptions given by Hickman and Ashworth (1943).

The growth rate of each isolate at 20°C was estimated on PDA inoculated with 3 mm mycelial plugs from the edge of 4-day-old colonies. After 3 dpi (days post-inoculation) the growth of the isolates was determined by measuring two diameters of each colony at right angles. The experiment was conducted in three replicates and repeated twice.

The influence of four culture media: PDA, Czapek's agar (CzA: NaNO₃, 3 g; K₂HPO₄, 1 g; MgSO₄ × 7H₂O, 0.50 g; KCl, 0.50 g; FeSO₄ × 7H₂O, 0.01 g; sucrose, 30 g; agar, 15 g; H₂O, 1 l), V8 juice agar (fresh tomato juice, 200 ml; CaCO₃, 3 g; agar, 17 g; H₂O, 800 ml) and water agar (WA: agar 17 g, water 1 l), and four PDA temperatures (20, 23, 25, and 27°C) on mycelial growth rate and colony

appearance was assessed 3 dpi. The growth rate of the isolates was determined by measuring two diameters of each colony at right angles. All experiments were conducted in three replicates and repeated twice.

The obtained data were checked for homogeneity of variances and subjected to ANOVA at the 5% probability level with individual pairwise comparisons made using Tukey's test (Sokal & Rohlf, 1995).

DNA extraction

DNA was isolated directly from the colony of the 4-day-old culture on PDA by scraping mycelia with a pipette tip. The mycelia was transferred into 50 µl of PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, CA, USA), vortexed briefly, incubated for 30 min at 56°C and then for 10 min at 100°C, as instructed by the manufacturer, and stored at -20°C until use (Harrington & Wingfield, 1995).

Molecular identification

The identity of isolates was further confirmed by amplification and sequencing of the ITS region of rDNA using primers ITS1 and ITS4 (White et al., 1990). The PCR mix contained 12.5 µl of 2 × Master mix with 2 mM MgCl₂ (Fermentas Life Sciences GmbH, Lithuania), 1 µl of 0.2 mM of each primer, 1 µl of template DNA, and molecular grade water up to a final volume of 25 µl. PCR amplifications were performed with an initial denaturation for 90 s at 94°C, followed by 29 cycles consisting of a denaturation step for 30 s at 94°C, primer annealing for 30 s at 55°C, and extension for 30 s at 72°C. The final extension step was performed at 72°C for 9 min 30 s. Negative controls were included by replacing template DNA with molecular grade water. The PCR products were separated by electrophoresis in 2% agarose gels run in 1 × Tris-borate EDTA buffer at 100 V constant voltage. The gels were stained with ethidium bromide and the products were visualized and photographed under UV light.

Sequencing of rDNA-ITS region

Amplified products were purified using the mi-PCR Purification Kit (Metabion International, Germany), according to the manufacturer's instructions and sequenced directly on automated sequencer (Macrogen Inc., Korea) in both directions using the same primers as for amplification. For each isolate, the consensus sequence, covering the partial rDNA-ITS region, was reconstructed using the ClustalW program. Sequences

of the products were compared with respective sequences available in the GenBank database using the ClustalW program and MEGA6 software (Table 2).

RESULTS

Symptoms

Oval, white or yellowish spots, 1-5 mm in length, surrounded by greenish-white or silvery halos, were observed on onion leaves. The central parts of lesions became sunken, with lengthwise-oriented slots. From the initial infection site, lesions spread along leaves and into lacunas, causing the foliage to turn light tan and then brown, collapsed, and to die within 5 to 12 days after lesions appeared (Figure 1). Under moist conditions, secondary conidial production was observed on necrotic tissue.

Isolates

A total of 23 isolates resembling *Botrytis* spp. were derived from diseased tissue. All isolates formed white uniform colonies with dense aerial mycelium and entire margins.

Pathogenicity

After inoculation with all tested isolates, distinctive whitish lesions typical of *B. squamosa* appeared on fully

developed onion leaves 4-5 dpi. Control leaves remained healthy. The pathogen was successfully re-isolated from the developed lesions, and the same colonies as those of the original isolates were obtained. Pathogenicity of the isolates was thus confirmed. In additional pathogenicity tests on inoculated onion bulbs, all isolates caused localized tissue decay around inoculation point, while no symptoms were recorded 7 dpi on inoculated detached leaves of tomato or plant parts inoculated with sterile PDA plugs (Figure 2). Seven isolates, representing each of four locations, were selected for further studies (Table 1).

Morphological characteristics of isolates

Initially, all isolates formed uniform white aerial mycelia with entire margin on PDA (Figure 3). Morphological differences among the isolates became noticeable 6 dpi. The micelia of non-sclerotial isolates still had equally distributed colonies from both sides. At the same time, whitish spots of immature sclerotia were visible in the colonies of sclerotia-producing isolates (Figure 3). Numerous, fully developed sclerotia were visible 10 dpi. Sclerotia were black, round-shaped and hitched to the medium. Therefore, all isolates could be differentiated into two morphologically distinct groups – mycelial-type isolates that do not produce sclerotia, and sclerotial-type isolates forming numerous sclerotia (Table 3).

None of the isolates sporulated on PDA after incubation of 5 weeks in darkness or under day/night

Table 2. Isolates of *Botrytis squamosa* identified in this study and retrieved from GenBank, the affected host plants, and GenBank accession numbers

Isolate	Gen Bank Accession number	Host	Origin
L103	MK681204 ^a	onion	Bosnia and Herzegovina
L105	MK681205 ^a	onion	Bosnia and Herzegovina
L109	MK681206 ^a	onion	Bosnia and Herzegovina
L110	MK681207 ^a	onion	Bosnia and Herzegovina
L116	MK681208 ^a	onion	Bosnia and Herzegovina
L122	MK681209 ^a	onion	Bosnia and Herzegovina
L123	MK681210 ^a	onion	Bosnia and Herzegovina
Garlic BC-2	EU519205	garlic	China
Onion BC-19	EU519208	onion	China
unknown	AY818331	unknown	Canada
PRI026	AJ716299	unknown	Netherlands
ICMP9334	JX399178	onion	New Zealand
EXGL-19	KC335151	garlic	China

^athis study



Figure 1. *Botrytis squamosa*. Leaf spots and lesions on onion (left and central) and leaf blight (right)



Figure 2. *Botrytis squamosa*. Pathogenicity test: control plant parts inoculated with sterile PDA plugs (left), artificially inoculated fully developed leaves (central), and artificially inoculated onion bulbs and detached leaves of tomato (right)



Figure 3. *Botrytis squamosa*. White uniform aerial mycelium with entire margin after incubation at 20°C for 3 days (left); Mycelial type of isolate after 10 days of incubation (central); Sclerotial type of isolate after 10 days of incubation (right)

Table 3. Morphological and cultural features of *Botrytis squamosa* isolates

Isolate	Morphological type	Number of sclerotia/petri plate	Sporulation	Daily growth rate at 20°C (mm/day)
L103	mycelial	0	no	18.33±0.27 b
L105	sclerotial	81	no	16.42±0.50 a
L109	mycelial	0	no	18.75±1.29 bc
L110	mycelial	0	no	21.33±0.27 cd
L116	sclerotial	126	no	20.17±1.04 d
L122	sclerotial	124.5	no	22.17±0.19 de
L123	sclerotial	183	no	20.42±0.50 e

light regime, while all isolates sporulated intensively on the necrotic tissue of inoculated onion leaves. The conidia were hyaline, 1-cell and ovoid, formed on sporulating branches of conidiophores. Young conidiophores were white, relatively short and compacted with accordion-like degeneration of sporulating branches after conidia have fallen. These branches later fell away, leaving slight projections at contact points with conidiophores, while conidiophores continued to grow, forming another set of sporulating branches and conidia. Older conidiophores became grayish and less compacted with aging.

Cultural characteristics

The isolates kept at 20°C exhibited statistically significant differences in daily growth rates ($F=31.4$, $p<0.001$). The isolate L122 from the location Kukulje had the highest growth rate, 22.17 mm/day, while the isolate L105 from Kosjerovo had the lowest growth rate (16.42 mm/day).

Growth rates of the isolates were significantly affected by temperature ($F=582.72$; $p<0.01$), as shown in Figure 4. The highest growth rate (70.75 mm/3 days) was recorded in the isolate L122 at 25°C, while the lowest was exhibited by the isolate L105 at 27°C (40.75 mm/3 days). Tukey's test showed that differences in average growth rates of the isolates at 20°C, 23°C, and 25°C were not significant. All isolates had the lowest growth rate at 27°C (40.75-49.5 mm/3 days). The shape and appearance of colonies were not affected by the temperatures used.

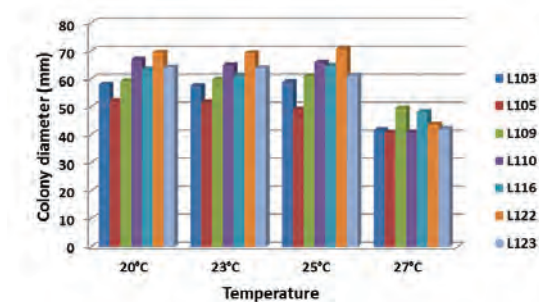


Figure 4. Growth rates of *Botrytis squamosa* isolates on PDA medium at 20, 23, 25 and 27°C

As shown in Figure 5, the growth rate of the isolates was significantly affected by the type of culture medium ($F=529.19$; $p<0.01$). The best medium for mycelial growth of all tested isolates was PDA, on which the highest average growth rate of 61.96 mm/3 days was recorded. The isolates L122 and L110 exhibited the

highest growth rates on this medium, 69.5 and 67 mm/3 days, respectively, while the growth rates of the remaining five isolates were between 52.2 and 64.2 mm/3 days. The lowest growth rate of all isolates was found on V8 juice agar, ranging from 27.2-30.5 mm/3 days (Table 3). The shape of colony was not affected by medium type.

Based on morphological, cultural and pathogenic characteristics all studied isolates were identified as *B. squamosa*, the causal agent of onion leaf blight.

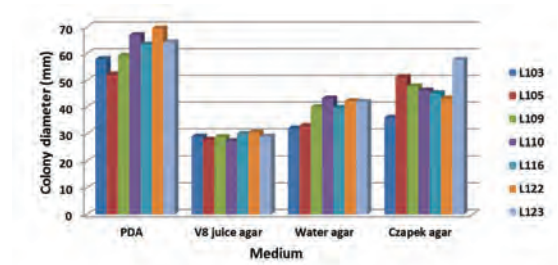


Figure 5. Growth rates of *Botrytis squamosa* isolates on different types of culture medium

Molecular identification of isolates

In order to confirm identification based on morphological features, the ITS region of all seven *B. squamosa* isolates was successfully sequenced. A BLAST analysis showed that the ITS sequence of the studied isolates had 100% nucleotide identity with two *B. squamosa* isolates (Onion BC-19, accession number EU19208 and Garlic BC-2, EU19205) originating from China that are deposited in GenBank. In addition, the isolate L110 exhibited 100% identity with another two isolates originating from Canada (AY818331) and the Netherlands (AJ716299). Therefore, molecular analysis confirmed that the studied isolates belonged to *B. squamosa* species.

DISCUSSION

Diseased onion leaves, collected in several onion fields in Bosnia and Herzegovina, exhibited typical symptoms of leaf blight caused by *B. squamosa*: leaf spots, necrotic lesions, and leaf dieback. However, besides *B. squamosa* as the causal agent of leaf blight disease, symptoms similar to leaf blight can also be caused by several abiotic or biotic factors. For instance, ozone injury, leaf tip dieback caused by hot/dry weather, water stress or other factors, as well as rain and hail injury or

herbicide injury, can all be easily misdiagnosed as leaf blight Anonymous (1990). Therefore, in order to make a precise disease diagnosis, isolation and identification of the causal agent is indispensable. In the present study, 23 isolates, identified later as *B. squamosa*, were derived from diseased tissue exhibiting leaf blight symptoms. All of them were pathogenic to onion leaf fragments and bulbs, but not to tomato leaves. All isolates formed white uniform colonies with entire margin, typical of *B. squamosa*, the causal agent of leaf blight disease (Hickman & Ashworth, 1943; Chilvers & du Toit, 2006). Differentiation of *Botrytis* species that attack onion based on morphological characteristics is possible for most species, except *B. aclada* from *B. allii* (Presly, 1985). Larger conidia and accordion-like degeneration of sporulating branches of conidiophores, as well as the presence of projections on conidiophores, are important features specific for *B. squamosa* (Hickman & Ashworth, 1943). However, precise identification of *B. squamosa* is highly dependent on sporulation, which does not occur on media usually used for fungal culturing. None of the isolates studied in this research sporulated on PDA after incubation for four weeks in darkness or under day/night light regime, confirming previous observations (Chilvers & du Toit, 2006). Although the use of inoculated onion plants to produce conidiophores and conidia appeared to be an adequate method to obtain reproductive structures of *B. squamosa*, combining conventional and molecular methods for species identification would be a more reliable option. Hence, we used the sequence of the ITS region to confirm morphological identification. Our isolates were 100% identical with two *B. squamosa* isolates originating from China.

Pathogenicity tests showed that the studied isolates were highly aggressive to onion leaves, causing fast-spreading lesions, while their aggressiveness to bulbs was weak, causing only localized maceration of tissue around inoculation point. Localized tissue decay caused by *B. squamosa* was also observed by Presly (1985). On the other hand, we found no symptoms on inoculated tomato leaves 7 dpi. As whitish spots and small necrotic lesions on onion leaves, which are typical for *B. squamosa*, are sometimes caused by *B. cinerea* (Anonymous, 1990), the test on tomato leaves can be used for fast differentiation between *B. squamosa* and *B. cinerea*, which is a polyphagous pathogen affecting more than 230 species (Williamson et al., 2007).

Leaf blight is considered to be the most important fungal disease of onion, occurring in many onion growing areas around the world, including Europe, South and North America, Asia and Australia (Ellerbrock & Lorbeer, 1977; De Visser, 1996; Carisse et al., 2011). It

reduces yield, quality and storability of bulbs (Sutton et al., 1986; Tremblay et al., 2003). In some countries, the pathogen and its distribution in onion crops have been well described, and disease epidemiology and management options have been thoroughly studied (Ellerbrock & Lorbeer, 1977; Sutton et al., 1986; Carisse et al., 2005; 2008; 2012; Van der Heyden et al., 2012). However, literature data on leaf blight in Bosnia and Herzegovina and neighboring countries, are either scarce or almost nonexistent. The present study documents the presence of the pathogen in all studied fields, as well as its potential to considerably decrease yield. Over the last 10-15 years, leaf blight incidence has caused significant losses in Ljevče Polje, the most important onion growing area in Bosnia and Herzegovina (M. Koščica, personal communication, 2016). The spreading of the pathogen is enhanced by inadequate growing technology, i.e. frequent monoculture production, lack of spatial isolation between winter and summer onion crops and poor hygienic measures. Hygienic measures are particularly important because nearby onion cull piles and volunteer plants in or near production fields are important inoculum sources (Lorbeer et al., 2004).

Botrytis leaf blight is a polycyclic disease that involves several sequences of events: production and dispersal of primary inoculum, primary infection, repeated cycles of production and dispersal of secondary inoculum and secondary infection and, at the end of the growing season, production of the survival structures of the pathogen (Lorbeer et al., 2004; Carisse et al., 2011). *B. squamosa* overwinters as sclerotia on infected onion leaves and bulb necks remaining in fields as plant debris or cull piles (Lorbeer et al., 2004). Ascospores formed in apothecia that could develop on overwintered sclerotia are not considered an important source of primary inoculum, although they can infect onion leaves. More often, primary infections are established by conidia that are produced on overwintered sclerotia in or around a production field. Sclerotia are able to produce conidia repeatedly, once the produced conidia are removed from sclerotial surface (Holz et al., 2004), resulting in primary inoculum presence in the field for a prolonged period of time in spring and early summer. Since the amount of primary inoculum is a function of the amount of sclerotia overwintered in the field, there is obviously a connection between the final intensity of foliar infection in one year and the amount of primary inoculum available at the start of the next growing season (Carisse et al., 2011). However, in efforts to reduce the amount of primary inoculum, high conidia mobility from surrounding fields (Holz et al., 2004) should also be taken into consideration.

Besides the inoculum sources, some other factors also influence leaf blight disease epidemics. Environmental factors (temperature, rainfall, and leaf wetness duration), crop nutrition and plant phenology, as well as other possible factors that are not well understood, play an important role in epidemic development (Lorbeer et al., 2004). Early infection establishment, followed by plant defoliation at the time of bulb initiation, occurring approximately five weeks before harvest, result in the most severe losses (Carisse et al., 2011). However, there is little information in literature on the relationship between *Botrytis* leaf blight development and decrease in yield. In fungicide trials conducted near Florida, New York, yield losses in untreated plots were between 7 and 30% (Shoemaker & Lorbeer, 1977), while losses found in the Netherlands in the period between 1976 and 1987 varied between 0 and 26% (De Visser, 1996). Thus, the timing and frequency of fungicide application is very important in years with high disease pressure, while it is of minor importance when environmental conditions are not suitable for disease development (Carisse et al., 2008). In some years, up to 16 fungicide spray treatments may be required, although 7-10 treatments, starting from the 3-4 leaves stage until shortly after onion lodging, are sufficient in most cases. In order to avoid unnecessary fungicide applications, several disease prediction systems have been developed (Lacy & Pontius, 1983; Sutton et al., 1986; Vincelli & Lorbeer, 1989; Carisse et al., 2011). However, their use by growers in Bosnia and Herzegovina is not currently feasible. Current disease management in onion growing areas in Bosnia and Herzegovina could be significantly improved by removing and destroying plant debris and volunteer plants in fields and by efficiently destroying cull piles that are important sources of inoculum. In addition, at least a 2-3 year crop rotation scheme, and spatial disconnection between winter and summer onion crops is recommended.

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Botrytis squamosa – prouzročivač lisne pegavosti luka u Bosni i Hercegovini

REZIME

U poslednjih nekoliko godina, na nekoliko lokaliteta u području intenzivne proizvodnje crnog luka u Bosni i Hercegovini uočena je pojava nekrotičnih pega i sušenja lišća. Simptomi oboljenja ukazali su na moguće prisustvo široko rasprostranjenog patogena luka, vrste *Botrytis squamosa*. S obzirom da pegavost i nekrotične lezije na listu luka mogu biti prouzrokovane različitim biotičkim i abiotičkim faktorima, cilj ovog istraživanja bio je da se precizno identifikuje prouzročivač oboljenja. Patogen je izolovan primenom standardnih fitopatoloških metoda i identifikovan na osnovu proučenih patogenih i morfoloških karakteristika dobijenih izolata. Identifikacija je potvrđena amplifikacijom i sekvenciranjem ITS rDNA genomnog regiona. Takođe, proučen je uticaj temperature i hranljive podloge na porast izolata.

Ključne reči: *Botrytis squamosa*; lisna pegavost luka; identifikacija; Bosna i Hercegovina