MOLECULAR AND MORPHOLOGICAL DETERMINATION OF COLLETOTRICHUM TRIFOLII ISOLATES ORIGINATING FROM ALFALFA

ABSTRACT: Colletotrichum trifolii is a fungal pathogen responsible for anthracnose disease in alfalfa. The isolates of C. trifolii were obtained from diseased alfalfa stems collected from field in Serbia. It was determined by pathogenicity examination that four isolates (Luc-7, Luc-17, Luc-27, Luc-33) can cause stem lesions on inoculated alfalfa plant. Our isolates were compared using reference isolates of C. trifolii (CBS 158.83). Isolates on MA and CDA developed olive green to grey colonies with white margin, while the substrate got dark olive green color. Conidiophores were hyaline, varied in length and produced a succession of conidia apically. Conidia were hyaline, straight, rounded at the ends, and non-septated with average size 7.85x3.87 μm. Average sizes of appressoria were 7.5-16.5 x 5.5 x 8.9 μm. The polymerase chain reaction (PCR) with one set of specific primers was used for the detection of examined isolates of Colletotrichum trifolii. Amplification of desired DNA fragment (590 bp) was determined using specific primer pair TB3-F/TB3-R. Achieved results of amplification indicated that the isolates Luc-7, Luc-17, Luc-27, as well as CBS 158.83, had traits of C. trifolii. Amplification and band on about 430 bp appeared in isolate Luc-33, which also belonged to C. trifolii species.

KEY WORDS: Colletotrichum trifolii, alfalfa, isolates, specific primers

INTRODUCTION

Alfalfa (Medicago sativa L.) is the most important and widely grown forage legume worldwide. One of the most desirable traits of alfalfa is its high nutritive quality as animal feed. Alfalfa is rich in proteins, vitamins and minerals, making it highly favorable for hay production and pasture for livestock, especially dairy cows. Colletotrichum trifolii is a fungal pathogen which causes anthracnose disease in alfalfa. Anthracnose causes significant losses in alfalfa.
crops in Serbia (Vasić, 2007). Infected plants manifest symptoms on stems: straw-colored, brown-bordered, and diamond-shaped lesions in which black acervuli develop (Stuteville and Erwin, 1990). Under favorable conditions, these lesions become enlarged, coalesce, girdle, and kill one or more stems. The fungi then spread internally from lesions on stem bases into crown tissues. A bluish-black discoloration of invaded tissue characterizes the crown rot phase of the disease. Symptoms also include blackening and killing of petioles and formation of a shepherd’s crook when the stem wilts and dies suddenly (Figure 1).

![Fig. 1 – “Shepherd's crook” symptoms](image)

As alfalfa is a perennial, the fungi can persist in stems and crowns of alfalfa grown in warmer areas and re-infect the surrounding plants when conditions are favorable. Anthracnose limits alfalfa production by affecting plant growth, plant vigor, forage yield and quality. Severe infection in susceptible alfalfa varieties can cause 25-30% losses in forage yield (Barnes et al., 1969).

**MATERIALS AND METHODS**

Isolates of *C. trifolii* originated from lesions found on stems of alfalfa sampled Serbia. Several isolates of fungi were obtained out of which four were selected for further observation. The fifth determined isolate, CBS 158.83, originated from the Netherlands. Five selected isolates of *Colletotrichum* spp. (Luc-7, Luc-17, Luc-27, Luc-33 and CBS 158.83) were submitted to intensive observation on nutritive media by methods of Baxter et al. (1983). Culture traits were compared on Malts Agar (MA) and Czapek-Dox Agar (CDA). Five replicates of each isolate per medium were incubated at 25 °C and described
on the tenth day of incubation. Linear increase in colony diameter was recorded as the mean of measurements along two diametrical axes, measured from the third to the tenth day. Texture of aerial mycelium, color of colony edges, zonation and formation of appressoria were described by Hawkins and Graham (1974). Presence or absence of seta in culture was determined according to the methods by Smith and Black (1990). Possibilities of teleomorphic stage forming in the examined isolates were determined according to the method by Baxter et al. (1983).

Final determination of the tested Colletotrichum trifolii isolates was done by the molecular methods (PCR). DNA from mycelia of tested isolates was extracted by the method of Day and Shattock (1997).

Necessary components were added to DNA extract. Detection of Colletotrichum trifolii species was carried out with the primers designed by Chen and Dickman (2002). Used primers were: TB3-F (5'-CGGAATTTCAGTGCAGCGGGTTG) and TB3-R (5'-CGGGATCCACGGATTGTGTTGTCG).

Standard linear DNA (Fermentas, Lithuania) of the fragment size (in bp) (from the bottom: 100; 200; 300; 400; 500; 600; 700; 800; 900; 1031; 1200; 1500; 2000 and 3000) was used. Visualization of amplified products was performed by UV transillumination in the chamber. The appearance of bands in the gel at the appropriate position was considered as a positive reaction.

RESULTS

Morphological characteristics

All colonies on MA medium grew relatively fast, attaining a diameter of 45-77 mm in ten days. Margins were mildly sinuated, marginal zones were closely appressed with pale olive green color, becoming subfelty and alternating with concentric farinaceous bands towards the center, dark olive becoming mouse gray to blackish mouse gray (Figure 2). On the reverse side of the colonies, marginal zones were pale olive-buff becoming iron gray and dark olive-gray in alternating concentric zones in the center.

On CDA, colonies attained diameter of 35-71 mm in ten days and frequently radially folded. Margins were irregular, farinaceous, hair brown towards the central zone which was cinnamon – buff alternating with light cinnamon-drab segments, and benzo brown in the center (Figure 2). Reverse side of the colonies marginal zones were pale olive-buff becoming iron gray and dark olive-gray in alternating concentric zones in the center.

Conidiomata on MA and CDA were small, irregular in size, usually pulvinate, brown to black, setose, formed in concentric bands, and 100-250 μm in diameter. Scattered sclerotia-like aggregations of submerged mycelium also occurred.
Conidiogenous cells were robust, cylindrical, hyaline, becoming pale brown (17-21.5) – 25 x (4-4.1) – 4.5 μm. Conidia were short, cylindrical, relatively broad, both ends obtused mostly 7.85 x 3.87 μm (Figure 3). These traits were similar on both media (MA and CDA).

Initial hyphae often germinate on the apex, or their arms form apresoria or conidium. Apresories are ovoid to obovoid, at first luminous brown or hyaline; oil globes are formed in time, and outer walls are calloused and brown colored. Average dimension of apresoria is 7.5 -16.5 x 5.5 – 8.9 μm.

It was determined that the isolate Luc-17 and reference isolate CBS-158.83 formed setae in cultures, which were septated with 1-3 septs with dimensions 45.5 – 65.45 x 3.2 – 5 μm.

All the observed isolates of Colletotrichum spp. originated from Serbia, and the control isolate CBS 158.83 originated from the Netherlands and it did not form perithecia.
Molecular detection of the examined isolates of Colletotrichum trifolii

PCR method was performed for the final determination of the examined isolates of Colletotrichum trifolii. After the extraction of total DNA of tested isolates, PCR was performed with a pair of specific primers (TB3-F/TB3-R) designed for Colletotrichum trifolii. Visualization of the obtained products was carried out using electrophoretic separation in 1.5% agarose gel, staining with ethidium bromide, and the observation under UV light was performed on transilluminator. The results are shown in Figure 4.

Legend: M – length standard DNA (in bp from the bottom: 100, 200, 300, 400, 500, 600, 700, 800, 900, 1031, 1200, 1500, 2000 and 3000), CBS 158.83 – reference isolate C. trifolii; Luc-7, Luc-17, Luc 27 and Luc-33 – examined isolates.

Fig. 4 – Visualization of amplicons of size 590 bp and 430 bp, obtained using TB3-F and TB3 R primers on 1.5% agarose gel.

Amplification of the desired nucleic acid fragment of about 590 bp PCR with the primer pair TB3-F/TB3-R was done in four examined isolates Luc-7, Luc 17, Luc-27 and CBS 158.83. Pair of designed sequence-specific primers was applied for the amplification of C. trifolii in the ITS region Chen and Dickman (2002). Obtained results of amplification indicated that the isolates Luc-7, Luc 17, Luc-27 and the control isolate CBS 158.83 showed traits corresponding to the descriptions of C. trifolii in literature, thus proving to belong to this species.

Amplification and the appearance of bands at about 430 bp were observed in the isolates of Luc-33, which, according to their morphological and other
properties belong to type *C. trifolii*. This reaction could be caused by insufficient specificity or other lack of applied primers. By using other molecular techniques and genome sequencing, this reaction could be explained. In addition, determined molecular traits of Luc-33 isolates, which differ from the other isolates, may indicate the variability of isolates within individual species of the genus *Colletotrichum*, and are described in the literature (Baxter et al., 1983; Freeman et al., 1998).

Elgin and Ostaszski (1982) reported the existence of two pathogenic races of *Colletotrichum trifolii*. Ariss and Rhodes (2007) described four races in the United States. The existence of differences between the members of the same species may have caused so many doubts over the historical development of taxonomy of the genus *Colletotrichum*.

**DISCUSSION**

Based on morphological traits of the tested isolates grown on MA and CDA media, it can be concluded that these isolates belong to *Colletotrichum trifolii*, which corresponds to the studies of Baxter et al. (1983), Stutville and Erwin (1990), Bailey and Jeger (1992).

Results obtained by amplification indicate that the isolates Luc-7, Luc 17, Luc-27, as well as the control isolate CBS 158.83, exhibit the characteristics that are in accordance with the descriptions of *C. trifolii* in literature, and that they, as such, really belong to this species.

Amplification and the appearance of bands at about 430 bp were present in isolates of Luc-33, which by all morphological and other properties belongs to type *C. trifolii*.

This reaction could be caused by insufficient specificity or other lack of applied primers. By using other molecular techniques and sequencing of the genome, this reaction could be explained. In addition, the determined molecular traits of isolate Luc-33, which to some extent differ from the other examined isolates, may indicate the variability of isolates within individual species of genus *Colletotrichum*. This has already been described in the literature (Baxter et al., 1983; Freeman et al., 2000).

Three races of *C. trifolii* were determined in America and Australia: races 1, 2 and 4 (Ariss and Rhodes, 2007).

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REFERENCES

Ariss, J. J. and Rhodes, L. H. (2007): A New Race of Colletotrichum trifolii Identified on Alfalfa in Ohio. Plant Diseases, Volume 91, Number 10, 1.36.2-1.35.2.


МОЛЕКУЛарна и морфолошка детерминација изолата Colletotrichum trifolii пореклом са луцерке

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Резиме

Colletotrichum trifolii је фитопатогена гљива која проузрокује антракнозу на луцерки. Изолати C. trifolii коришћени у овом раду добијени су изолацијама са стабљика оболелих биљака луцерке пореклом из Србије. У тесту патогености изолати C. trifolii (Luc-7, Luc-17, Luc-27, Luc-33) су проузроковали типичне симптоме стабљичних лезија на тестираним биљкама. На основу морфолошких и молекуларних особина изолати из Србије поређени су са референтним изолатом (CBS 158.83) из Холандије који је детерминисан као врста Colletotrichum trifolii.

На MA и CDA подлогама испитивани изолати формирају ваздушну мицелију зелене боје са рубом крем беле боје, док је у супстратном делу мицелија тамно маслинасто зелене боје. Конидиофоре су безбојне, различите дужине и формирају апикално конидије. Конидије су безбојне, праве са заобљеним крајевима, несептиране, димензија 7.85 x 3.87 µm. Димензије апсорија су 7.5-16.5 x 5.5 x 8.9 µm.

За молекуларну детерминацију испитиваних изолата C. trifolii коришћена је метода ланчане реакције полимеразе (PCR) са једним паром специфичних прајмера.

Амплификација жељеног фрагмента нуклеинских киселина величине око 590 bp вршена је применом PCR методе са TB3-F/TB3-R паром прајмера. Добијени резултати амплификације показују да испитивани изолати Luc-7, Luc-17, Luc-27 поређене са изолатом CBS 158.83 припадају врсти Colletotrichum trifolii. Амплификација фрагмента нуклеинске киселине око 430 bp забележена је код изолата Luc-33, који такође припада врсти C. trifolii.