ABSTRACT: During summer and autumn, easily recognizable diseased plants can be detected in alfalfa fields. Diseased plants have characteristically curved apical part, commonly known as “shepard’s hook”. Using the standard mycological methods, several fungi isolates were obtained. Four of these isolates were used for further study. Two standard mycological methods were used for determination of pathogenicity of Colletotrichum spp. isolates: with and without damaging of the stems. During these studies, it was determined that all of the isolates were causing the symptoms of alfalfa anthracnose. All of the selected isolates form germinative bodies — acervulae — on mycelium. Acervulae can be grouped or scattered across the colony area. Their size in the culture was 100—250 mm. Conidia were short, cylindrical, relatively broad, with both end obtuse 7,85 x 3,85 mm. Appressoria were ovate to obovoid, pale to medium brown 7,5—16,5 x 5,5—8,9 µm.

KEY WORDS: Colletotrichum trifolii Bain et Essary, alfalfa, morphology, pathogenicity

INTRODUCTION

Colletotrichum trifolii Bain et Essary, inducer of alfalfa anthracnose, is widespread in many areas in Central Serbia and Vojvodina. Considering that and, also, the damage it can cause, anthracnose is economically very important disease (Robottić i sar., 1983; Robottić i Klokocar-Šmit, 1983). The damage caused by this pathogen agent can be seen trough decreased quantity and quality of green mass from 10 to 70%, depending on the alfalfa cultivar, pathogen species and edaphic factors (Stuteville and Erwin, 1990).

The effect of this disease is a decrease of the alfalfa field duration. Anthracnose decreases vigor of the individual plants and thins out the plant popu-
lation. During summer and autumn, the diseased plants start to appear in the alfalfa fields. These diseased plants have characteristic appearance. Several stems of the plant have yellow to silverfish color and start to show the signs of wilting. The apical part of the plants is curved down, forming the so-called “shepard’s hook”. These symptoms correspond with symptoms described in the other countries by various authors (Ostazeski et al., 1969; Devine et al., 1971). In our country, authors that have studied this fungus are Robotic sar. (1983a); Milijić i sar. (1986); Vasić i sar. (2005).

Considering the damage that anthracnose induces in the alfalfa fields in our country and considering the fact that this disease was not studied in detail, this study was performed. The aim of this study was to collect the isolates of Colletotrichum spp. in Serbia and to study its pathogenicity and morphological characteristics. A detailed study of alfalfa anthracnose inducer enables us to understand pathologic processes and possibilities for eradication of this pathogen agent.

MATERIAL AND METHODS

Using standard mycological methods, several fungi isolates were obtained. For the further study, four of these isolates were selected, while the fifth isolate CBS 158.83 was imported from the Netherlands. These five selected Colletotrichum spp. isolates (Luc-7, Luc-17, Luc-27, Luc-33 and CBS 158.83) were further tested by using two different methods. Alfalfa cultivar K-28 was used for this experiment. The plants were sampled from fields and transplanted into vessels. Two methods of inoculation were used. The first method (A) is based on inoculation of the damaged alfalfa plants by applying fragments of Colletotrichum spp. colonies of the studied isolates. The second method (B) is based on inoculation of the undamaged alfalfa plants by applying colonies fragments, too. The marks used for the evaluation of the plants range from 1 to 5 (Ostazeski et al., 1969).

The macroscopic and microscopic morphological characteristics of the selected isolates were studied. The basic characteristics of the colonies were considered and described for macroscopic study. The morphological traits of the five selected Colletotrichum spp. isolates (Luc-7, Luc-17, Luc-27, Luc-33 and CBS158.83) were studied on the nutrient medium using the method of Baxter et al. (1983). The morphological traits of appressoria of the studied isolates were determined using a modified method after Hawkins (1974). The presence or absence of sets in the culture was determined using the method by Smith and Black (1990). Also, the possibility of forming teleomorphic state in the isolates was studied according to Baxter et al. (1983).
RESULTS

Symptoms

During a three year period, in summer and autumn, it has been determined that diseased plants appear in the alfalfa fields. These plants have characteristic appearance. Several stems of one plant or shrub have yellow or silvery color and start to wilt. The diseased plants have characteristic curved apical parts, which form the so-called “shepard’s hook”.

At the lower parts of the diseased plant, the most often at the lower third, light to dark brown spots with black borders can be seen. Mycelium of the parasite grows right down through the stem, and when the stem withers, the so-called “crown anthracnose” starts to show. The infection of the plant and root crown is characterized by dry rotting that stains the infected tissue in blue-black color (Fig. 1.).

Pathogenicity assessment

All of the studied isolates have caused the symptoms of alfalfa anthracnose on the plants that were treated using both quoted methods (A and B). The control group of plants did not develop these symptoms, since they were inoculated with the substrate that did not contain mycelium. However, the intensity of the symptoms was different between the used methods after 15 days. The intensity of the symptoms in the first method (A) was somewhat stronger than the intensity of the symptoms in the second method (B). This can be explained by the fact that conidia have needed some time to start germinating, to form the appressoria, to penetrate cuticle and to infect the plant. Thirty days after the infection, there weren’t any significant differences in the expression of the symptoms between the quoted inoculation methods (tab. 1).
Tab. 1 — The pathogenicity of the five *Colletotrichum* spp. isolates on the alfalfa, assessed by using two different inoculation methods.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Method A</th>
<th>Method B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After 15 days</td>
<td>After 30 days</td>
</tr>
<tr>
<td>Luc-7</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Luc-17</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Luc-27</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Luc-33</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>CBS158.83</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

LEGEND: — no lesions (class 1), + lesions are present on the stem, but there are no withered stems (class 2), ++ numerous lesions on the stem, stem starts to wilt (class 3), +++ stems are withered and dry (class 4).

*Morphological characteristics*

On the very first day, on the KDA medium, studied isolates Luc-7, Luc-17, Luc-27 and Luc-33 form white mycelia 3—4 mm in diameter. When the colonies reach 40—55 mm in diameter, the middle part of the colony starts to turn darker and gains green or olive green coloration, while the colony border still keeps white coloration.

![Fig. 2 — The appearance of the colonies on the KDA nutritive medium](image)

Also, as it ages, the colony develops uniformly. It gains olive green to gray coloration (Fig. 2), on the entire surface. Also, it creates acervulae and pink spore mass. The studied *Colletotrichum* spp. isolates formed germinative bodies — acervulae. Acervulae were small in size, misshapen or round, pale to dark brown, almost black in color and stromatic. Acervulae that were formed in these cultures were 100—250 mm in diameter. It was also determined that
the isolates Luc-17 and CBS 158.83 formed sets in the conidiomates. Sets were light to dark brown in color, usually straight, with smooth or lightly wrinkled surface, often wider in the basis and more pointed and somewhat darker at the top. Sets were septated with 1—3 septs, and 45.5—65.45 x 3.2—5 mm in size (Fig. 3).

The average colony size was 7.85 x 3.87 µm. Insignificant differences between isolates originating from different part of Serbia were determined, while CBS 158.83 isolate has formed significantly larger conidia. Based on the quoted morphological criteria, all of the studied isolates were positioned into isolates with dominant cylindrical conidia, smooth at both ends (Fig. 4).

During germination, conidia undergo certain morphological changes. First, the conidia swell up, after which they often lose...
their granular content and become more transparent, while in their equatorial part there is no development of the septum (Fig. 5).

On the apical parts of the germinated initial hyphae or their branches, appressoria or secondary conidia can be often formed. Formed appressoria were light brown or hyalic at first, but, in time, oil globules are formed, and their outer walls become thicker, after which they become dark brown in color. Average dimensions of the appressoria were 7.5—16.5 x 5.5—8.9 µm (Fig. 6).

All of the studied Colletotrichum spp. isolates obtained from Central Serbia and Vojvodina, as well as the control isolate CBS 158.83 obtained from the Netherlands did not form perithecia.

**DISCUSSION**

After the study of pathogenicity of the *C. trifolii* isolates obtained from Serbia it was determined that there had been expression of the symptoms of anthracnose in the inoculated plants. Baxter et al. (1983) point that *Colletotrichum trifolii*, when cultivated on KDA medium, forms acervulae. The same is with our results. According to Baxter et al. (1983), Stutveille and Erwin (1990), conidia are cylindrical, rounded at both ends, 11—15 x 3—5 µm in size, which corresponds to our results. Graham et al. (1976) point that phytopatogen fungus *C. trifolii* rarely forms sets in the culture. The similar results were achieved in our study. Studies of Bailey et al. (1992) and Baxter et al. (1983) point that phytopatogen fungus of *Colletotrichum* spp. genus has the ability to form appressoria when infectious hyphae touches solid surface. This fact also corresponds to our results. Baxter et al. (1983) point that teleomorphic state was never found in association with anamorphic state in the culture.

On the basis of the symptoms found in fields, and pathogenic and morphologic traits of the five selected isolates of the studied fungus, it has been determined that they are similar to *Colletotrichum trifolii* Bain et Essary spe-
cies. According to Sutton (1992), the morphology is the base for differentiation of the species of Colletotrichum genus. However, according to this author, it is necessary to use molecular methods to create a reliable system for identification of species of this genus. Identification of the obtained Colletotrichum spp. isolates using somewhat modified Cano et al. (2004) method has shown that the isolates Luc-7, Luc. 17, Luc. 27 and Luc-33 are indeed C. trifolii.

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REFERENCES


МОРФОЛОШКЕ И ПАТОГЕНЕ ОДЛИКЕ
COLLETOTRICHUM TRIFOLII BAIN ET ESSARY,
ПРОУЗРОКОВАЧА АНТРАКНОЗЕ ЛУЦЕРКЕ

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Резиме

У току лета и јесени у луцершту су запажене оболеле биљке лако препознатљиве по боји и изгледу. По неколико стабала на једној биљци добијају сламасто жуту до сedefасту боју и вену. Оболеле биљке имају карактеристичан врх повијен надоле, у виду тзв. “пастирске куке”. Из заражених биљака луцерке, стандардним миколошким поступцима, добијено је више изолата гљиве, од којих су за даља проучавања одабрана четири. За проверу патогености испитиваних изолата Colletotrichum spp. коришћен су две стандардне миколошке методе: са повређивањем стабљика луцерке и без повређивања стабљика. Током ових проучавања утврђено је да су сви проучавани излати проузроковали симптоме карактеристичне за анtrakнозу луцерке. Сви одабрани изолати на мицелији обраzuju плодонасна тела — ацеруле, које у оквиру колонија могу бити груписане у једном делу или разбачане по целој површини колоније. Њихова величина у култури је износила 100—250 μm. У ацерулама се формирају многобројне једнолијске конидије које су излуженог цилиндричног облика, заобљене на крајевима, величине 7,85 x 3,85 μm. После кљињања конидија сва четири проучавају апсорије, чије су димензије у просеку 7,5—16,5 x 5,5—8,9 μm, што је једна од главних карактеристика фитопатогеног гљива из рода Colletotrichum.