MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF A NEW ALFALFA PARASITE - COLLETOTRICHUM LINICOLA IN SERBIA

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Abstract
Alfalfa (Medicago sativa L.) is economically the most important forage crop in Serbia. During the period of 2005-2010, intense occurrence of alfalfa anthracnose was observed in Serbia (Srpska Crnja, South Banat District). Alfalfa plants showed characteristic symptoms of anthracnose disease (“shepherd’s crook”) including wilting and dying of the upper parts of the stems. Collected isolates formed light green to dark olive-green colonies on potato dextrose agar (PDA) and developed black acervuli around the center of the colony. In cultures on PDA medium acervuli were formed. Conidia were hyaline, aseptate, straight with one end pointed and the other slightly rounded, with dimensions 12.5 to 25.0 × 2.5 to 7.5 μm (mean 19.83 × 4.42 μm). In cultures on PDA medium after 5 days, numerous setae were formed. The setae were slightly darker at the bottom and lighter at the top, septate with 3 septa. Setae dimensions were with dimensions 100 to 185.5 × 2.5 to 5 μm (average 160.9 × 3.12 μm). PCR amplification using ITS1-ITS4, GSF1-GSR1, GDF1-GDR1 and T1-Bt2b primer pairs yielded fragments of approximately 495 base-pairs (bp), 900 bp, 200 bp and 750 bp, respectively. Based on the morphological characteristics and molecular characterization, the analyzed isolate Coll-44 from alfalfa was determined as Colletotrichum linicola Pethybr. & Laff.. According information we have on disposal this is the first report of C. linicola causing alfalfa anthracnose in Serbia.

Keywords: Anthracnose; Alfalfa; Colletotrichum linicola; PCR analysis

Introduction
Many diseases cause the dying of alfalfa plants, reduction of yield, and negative impact on quality of animal feed. Anthracnose of alfalfa is one of the most important diseases that reduce alfalfa yield (O'Neill et al., 1997; Vasić, 2013) and is caused by fungi of the genus Colletotrichum. Damage induced by presence and development of the pathogen causes the reduction of quality and quantity of fresh mass from 10-30% depending on the alfalfa cultivar, species of pathogen, climatic and edaphic factors (Latunde-Dada et al., 2007; Vasić, 2013). C. trifolii and C. destructivum spread rapidly during warm, wet weather. Conidia forms in the acervuli on stem lesions. Wind and rain splash carry conidia to the growing petioles and stems. Infected hyphae proliferate within susceptible host tissue and form oval-shaped lesions. In hot, dry weather, infected stems may wilt and die. The fungus grows down in infected stems into the crown and taproot, causing killing of tissue, predisposition to winter injury, wilting or plant dieback (Vasić, 2013). Anthracnose of alfalfa, caused by C. destructivum is prevalent on wide area in Serbia and the induced damages are of great economic importance (Vasić, 2013).
**Material and methods**

**Morphological traits**

The isolates studied in this survey were obtained from infected alfalfa plants collected from 2005-2010 in Serbia (Srpska Crnja, South Banat District). All samples were placed in paper bags and delivered to the phytopathological laboratory of the Institute for Forage Crops, Kruševac. The pathogen was isolated from the stem, the top of the root, and root of alfalfa. Potato dextrose agar (PDA) medium was used for the isolation of the pathogen (Dhingra and Sinclair, 1995). Petri dishes were incubated in thermostat at 24 ± 2°C in the dark.

Morphological traits of selected isolates of *Colletotrichum* sp. were studied on PDA and carnation leaf agar (CLA) (Waller et al. 1998) according to the method by Baxter et al. (1983). Hyphal appearance was prepared according to the method by Baxter et al. (1983) on PDA medium and in hanging drops over the glass according to the method described by Hawksworth (1974). Size of conidiomata was determined by measuring the diameter (10 fully formed acervuli on PDA) and calculating the mean values. The presence or absence of setae in the cultures was determined by the method of Smith and Black (1990) by observation of 10-day-old cultures under the light microscope. The form and dimensions of conidia in the selected isolates of *Colletotrichum* sp. were examined according to the method of Smith and Black (1990). Dimensions of conidia were determined by measuring the length and width of 30 randomly selected conidia in the selected isolates grown on PDA, using a light microscope (Olympus CX41). Morphological traits of appresoria of the studied isolates were determined using a modified method by Hawksworth (1974). The shape, color and dimensions of appresoria were studied in selected isolates. Twenty-five appresoria per isolate were observed and measured. To observe the formation of teleomorph stage, studied isolates were grown on PDA. Cultures were incubated at 25°C in the day and night cycle, and the formation of perithecia was observed on three occasions, after 30 days, 6 months and 12 months. Petri dishes were kept in a thermostat at 25°C. The trial was set to 10 repetitions per isolate.

**Molecular detection and identification**

Isolates were grown on PDA in the dark, at 25°C for 7 days. DNA extraction was done according to the method described by Day and Shattock (1997). Polymerase chain reaction (PCR) was done using four sets of primers. The 5.8S rDNA, ITS1 and ITS2 region (ITS) was amplified with the primer pair ITS1-ITS4; an intron region of the glutamine synthetase gene (GS) with GSF1-GSR1 primer pair; an intron region of the glyceraldehyde-3-phosphate dehydrogenase gene (GPDH) with the primer pair GDF1-GDR1; and 5’ end of the β-tubulin gene (TUB2) with T1-Bt2b primer pair (Table 1).

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences 5’-3’</th>
<th>Fragment length</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS1</td>
<td>TCCGTAGGTTAACCTGC</td>
<td>~495 bp</td>
<td>Freeman et al. (2000)</td>
</tr>
<tr>
<td>ITS4</td>
<td>TCCCTCCGTTATGATTG</td>
<td>~900 bp</td>
<td>Liu et al. (2007)</td>
</tr>
<tr>
<td>GSF1</td>
<td>ATGGCCGATACATCTGG</td>
<td>~200 bp</td>
<td>Glass et al. (1995)</td>
</tr>
<tr>
<td>GSR1</td>
<td>GAACCGTGAAATTCAC</td>
<td>~200 bp</td>
<td>Glass et al. (1995)</td>
</tr>
<tr>
<td>GDF1</td>
<td>GCCGTCACCACTTCATTA</td>
<td>~700 bp</td>
<td>O’Donnell and Cigelnik (1997)</td>
</tr>
<tr>
<td>GDR1</td>
<td>GGGTGGAGTGACATTCG</td>
<td>~200 bp</td>
<td>Glass et al. (1995)</td>
</tr>
<tr>
<td>T1</td>
<td>AACATGCAGGTGAGTTG</td>
<td>~750 bp</td>
<td>O’Donnell and Cigelnik (1997)</td>
</tr>
<tr>
<td>Bt2b</td>
<td>ACCCTCAGTGAGTTGACCCTT</td>
<td>~750 bp</td>
<td>Glass et al. (1995)</td>
</tr>
</tbody>
</table>
PCR tests were performed in a TPersonal thermocycler (Biometra, Germany). PCR products were analyzed by 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light on a transilluminator (Biometra, USA). The appearance of the fragment of the expected size was considered as a positive reaction. Amplified products of the ITS and TUB2 regions of Coll-44 isolate were custom sequenced (Macrogen, the Netherlands). Obtained sequences were compared with the available sequences of Colletotrichum sp. from the NCBI GenBank database using BioEdit software (Hall, 1999).

Results and discussion

Morphological traits
On PDA medium, the isolate Coll-44 was characterized as C. linicola. It formed cottony colonies, velvety gray to light olive green in color. Analyzed isolate of C. linicola formed fruiting bodies - acervuli. Dimensions of acervuli were ranged from 100-280 μm. Similar results were earlier reported by Tunali et al. (2008), Vasić et al. (2014) and Damm et al. (2014). The Coll-44 isolate formed setae within conidiomata, slightly darker at the bottom and lighter at the top, having from 1 to 3 septae. Dimensions of septae were 100-185.5 x 2.5-5 μm (average 160.9 x 3.12 μm). The isolates identified as C. linicola formed cylindrical conidia, tapered at one end and rounded at the other, dimensions 10-25 x 2.5-7.5 μm. Our result is in accordance with Tunali et al. (2008) and Vasić et al. (2014). During this study it was found that C. linicola isolates forms septa in the equatorial part of the conidia during germination, which is a characteristic trait of this species. Based on this characteristic traits, C. linicola differ from close related species which do not form septae. Latunde-Dada and Lucas (2007) and Tunali et al. (2008) noted that the species C. linicola forms septae during germination of conidia. The Coll-44 isolate formed numerous appresoria, dimensions 5-17.5 x 2.5-7.5 μm (average 11.8 x 5.9 μm). The results obtained from our study coincide with the results of Latunde-Dada and Lucas (2007), Tunali et al. (2008), Vasić et al. (2014) and Damm et al. (2014). During this experiment was performed the isolate Coll-44 from Serbia (Srpska Crnja, South Banat District) did not form perithecia. This result is in accordance with Baxter et al. (1983), Latunde-Dada and Lucas (2007). Pure culture of the tested isolate was deposited in the public collection of CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands (specimen no. CBS 138125).

Molecular detection and identification
The polymerase chain reaction (PCR) has been successfully applied for the identification of the isolates from alfalfa, including C. linicola isolate Coll-44. Universal primer pair ITS1-ITS4 yielded a product of approximately 495 bp in all tested isolates (Figure 1).

![Figure 1](image-url)
The presence of the fragment of approximately 900 bp was determined for most of the tested isolates using the primer pair GSF1-GSR1, an intron region of the GS gene had poor efficiency for Coll-44 isolate (Figure 2).

Figure 2. Electrophoretic analysis of PCR products with GSF1-GSR1 primer pair. Lines: 18 - isolate Coll-44, M- 100-base pair ladder (Amersham Biosciences).

PCR analysis with GDF1-GDR1 resulted in a positive reaction for tested isolates. Using these primer set, an intron region of the GPDH gene was successfully amplified for Coll-44 isolate (Figure 3).

Figure 3. Electrophoretic analysis of PCR products with GDF1-GDR1 primer pair. Lines: 13 - isolate Coll-44, M - 100-base pair ladder (Amersham Biosciences).

In fourth PCR reaction with the primer pair T1-Bt2b the expected fragment of 750 bp was obtained for Coll-44 isolate (Figure 4).

Figure 4. Electrophoretic analysis of PCR products with T1-Bt2b primer pair. Lines: 13 - isolate Coll-44, (-) - negative control, M- 100-base pair ladder (Amersham Biosciences).

The extracted fungal genomic DNA was of high quality and suitable for PCR amplification, allowing successful detection of isolates selected for this study. By using different primer pairs for PCR detection C. linicola isolate was successfully detected. Amplified PCR products of ITS and TUB2 regions of the isolate Coll-44 were sequenced and obtained sequences were deposited
in the NCBI Genbank under accessions numbers JX908364 and KJ556347, respectively. The sequences of Coll-44 isolate were compared with the available sequences from the GenBank database. Blast analysis confirmed 100% nucleotide identity of the Coll-44 ITS sequence with the GenBank accessions AB046609 and JQ005765 of C. *linicola*. Additionally, Coll-44 TUB2 sequence showed 99.6% nucleotide identity with the GenBank accession JQ005849 of C. *linicola* isolate CBS 172.51.

Freeman et al. (2000) have successfully distinguished 230 different isolates of *Colletotrichum* species using universal primers ITS1-ITS4 and sequencing PCR products of 450-490 bp of the tested isolates. Johnston and Jones (1997) used an analysis of LSU rDNA sequences of isolates derived from different crops in New Zealand. Moriwaki et al. (2002) successfully distinguished 236 isolates by studying ITS-2/LUS rDNA of *Colletotrichum* species in Japan using rDNA ITS1 region for sequencing products of size of approximately 157-190 bp. Liu et al. (2007) stated that the use of RFLPs of a intron regions in the glutamine synthetase (GS) gene has proved successful for the identification and characterization of closely related species within the genus *Colletotrichum* which were previously difficult to distinguish based on morphological traits. PCR reaction with GSF1-GSR1 primer pair had poor efficiency for Coll-44 isolate and gave product of not sufficient quality for further RFLP analysis. On the other hand, the intron region of the gene for GS is suitable for the detection and further characterization of Serbian *C. destructivum* isolates (Vasić, 2013). Detection of the isolates using the GDF1/GDR1 primers proved to be reliable and allowed the amplification of a portion of the second intron of the GPHD gene. These primers produced fragments of about 200 bp in all tested isolates. Talhinhas et al. (2002) performed the first multilocus phylogenetic analysis of the genus *Colletotrichum* by studying *C. acutatum* community on lupins (*Lupinus* sp.) using ITS, TUB2 and HIS4 sequences, while Guerber et al. (2003) used the intron region of a gene for GS and GPHD nucleotide sequences for the analysis of *C. acutatum*.

**Conclusions**

Based on morphological characteristics and molecular detection and characterization, the analyzed isolate from alfalfa was determined as *C. linicola*. Molecular detection with the primer sets targeting different genomic regions is useful tool for the detection of *C. linicola* in alfalfa, but sequence analysis is needed to distinguish this from other species. According information we have on disposal this is the first report of *C. linicola* causing alfalfa anthracnose in Serbia.

**Acknowledgment**

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**References**


