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PHYTOPATOGENIC FUNGI OF ALFALFA IN SERBIA

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Abstract

Alfalfa (*Medicago sativa* L.) occurs in nature as a cultivated and wild species. As one of the oldest and most important perennial forage crops, alfalfa has the potential to produce large amounts of high-quality forage. This paper presents the preliminary results of the population study of phytopathogenic fungi on alfalfa. A total of 625 samples of alfalfa plants were collected between 2002 and 2019 in Serbia from 17 locations. Standard phytopathological isolation was performed from the transition of healthy to diseased tissue on potato dextrose agar (PDA) with streptomycin. After incubation at 22°C and a light regime of 12 hours day/12 hours night, the obtained cultures were examined microscopically and the morphological identification of fungi to the genus level was carried out using standard keys. Checking the pathogenicity of the obtained isolates was done through artificial inoculation with damage to plant parts. Inoculation was performed with 7-day-old fungi isolates, and the inoculated plants were stored at a temperature of 20 to 25°C. Based on the morphological and pathogenic characteristics of the obtained isolates, it was determined that alfalfa pathogens were dominant in Serbia *Colletotrichum, Fusarium, Verticillium, Rhizoctonia, Sclerotinia* and *Phoma*. As the causative agents of alfalfa diseases are not sufficiently studied in Serbia, the conducted research represents a significant contribution to defining the role of phytopathogenic fungi in the symptoms they cause on alfalfa, all with the aim of contributing to finding adequate measures for their successful suppression.

Key words: *alfalfa, phytopathogenic fungi.*

Introduction

The genus *Medicago* has more than 60 species, of which about 70% are annuals. Among the field plants for feeding cattle, alfalfa has the most important place, so many call it the "queen of fodder plants". After destroying the areas under alfalfa, large amounts of nitrogen and organic matter remain in the soil, the decomposition and mineralization of which improve the physical, chemical, and microbiological properties of the soil (Đukić *et al*., 2009).

At least 62 parasitic fungi that cause alfalfa diseases have been described in the world, of which only 19 types of disease-causing fungi have been described in our country. According to the part of the plant that primarily endangers, parasites on alfalfa are classified as those that inhabit the above-ground parts of alfalfa with the manifestation of symptoms on the leaves and stems, and parasites that inhabit the ground part of the tree, the crown, and the root (Balaž and Popović, 2005; Vasić, 2013). The most widespread and harmful parasitic fungi that threaten alfalfa are: *Colletotrichum trifolii, C. destructivum, C. truncatum* and *C. dematium* – causes of anthracnose; *Pseudopeziza medicaginis* and *Stemphylium botryosum* – causes of alfalfa leaf spotting; *Peronospora trifoliorum* – the causative agent of alfalfa blight, *Uromyces striatus* – the causative agent of alfalfa rust, *Cercospora medicaginis* – the cause of leaf

spotting; *Leptotrochila medicaginis* – the cause of yellowing of alfalfa leaves; *Fusarium oxysporum* f. sp. *medicaginis*, *Verticillium albo-atrum* and *V. dahliae* – causes of wilting of alfalfa; *Rhizoctonia solani*, *Sclerotinia trifoliorum* and *S. rolfsii* – causative agents of root and ground rot (Mackie *et al*., 2003; Ivanović, 2005; Krnjaja *et al*., 2005a; Vasić, 2007; Vasić *et al*., 2010; Vasić *et al.,* 2011a, 2011b; Vasić, 2013). In addition to the aforementioned pathogenic fungi, alfalfa root and crown rot is caused by *Phytophthora megasperma*, *Phoma medicaginis* – the cause of the blackness of the tree and leaf spotting of alfalfa, as well as species from the genus *Pythium* – the cause of seedling blight and rotting of alfalfa seeds. The most commonly isolated *Fusarium* species that cause root rot and root neck are *F. oxysporum*, *F. solani* – the causes of fusarium wilt of alfalfa (Stuteville and Erwin, 1990; Krnjaja *et al*., 2005a; Krnjaja, 2005b). In addition to fungi, bacteria also cause significant damage to alfalfa production, including *Pseudomonas syringae* pv. *syringae* – the cause of bacterial stem spotting; *Pseudomonas marginalis* pv. *alfalfae* – the cause of bacterial root rot; *Clavibacter michiganensis* susp. *insidiosum* – the cause of bacterial wilt; *Xanthomonas campestris* pv. *alfalfae* – the cause of bacterial leaf spot and others (Stuteville and Erwi1990; Arsenijević, 1997; Balaž and Popović, 2005). Numerous phytopathogenic viruses that cause diseases have been described on alfalfa, such as Alfalfa mosaic virus, AMV; Cucumber mosaic virus, CMV; Clover yellow mosaic virus, CYMV and others (Stuteville and Erwin, 1990; Šutić, 1995; Jasnić, 2005). Damages resulting from the presence and development of pathogenic agents are expressed through a reduction in the quantity and quality of green mass from 10 to even 70% depending on the alfalfa variety, pathogen type, climatic and edaphic conditions. Reduction of the assimilation surface, falling of leaves, immaturity of seeds, the presence of harmful pathogen metabolites are also consequences of the presence of pathogens on alfalfa leaves. Diseases of the crown and roots cause a weakening of the plant's vitality, an increase in sensitivity to frost, premature decay, an increase in weediness, and thus a decrease in hay quality (Vasić, 2013). During a longer period of monitoring (2002-2019), it was determined that in some locations in Serbia there were large losses in alfalfa fields, with typical symptoms of root rot, necrosis, chlorosis and wilting of alfalfa plants. The phytopathogenic fungi affecting alfalfa have not been extensively studied in Serbia. Therefore, a research initiative was undertaken to identify and characterize the fungal species responsible for alfalfa diseases. The objective of this study is to contribute to the development of effective management strategies for the successful control of these fungal pathogens.

Materials and Methods

The isolates analyzed in this study were obtained from diseased alfalfa plants collected over the period from 2002 to 2019. The collection of samples was carried out in the main production areas of alfalfa in the territory of the Republic of Serbia, including a total of 17 localities: Čurug, Ašanja (Southern Bačka District), Srpska Crnja, Farkaždin (Southern Banat District), Trnavci (Nišava District), Vraneši (Raška District), Banatsko Karađorđevo, Aleksandrovo (Middle Banat District), Banovci (Srem District), Markovac (Danube District), Selo Varvarin, Kobilje, Globoder, Bela Voda (Rasina District), Kloka (Šumadija District), Davidovac (Pčinj District) and Dobrichevo (Pomeranian District). During field sample collection, plants exhibiting symptoms of chlorosis, necrosis, rot, and withered tops were selected. In total, 625 samples were collected for analysis. The pathogen was isolated from the stem and roots of alfalfa. After bringing the samples to the laboratory, the samples were first washed with running water, and after washing, parts of the stem and roots were cut into pieces measuring 0.5-1 cm. The samples thus prepared were disinfected with 96% ethanol for 10 seconds, 1% sodium hypochlorite (NaOCl) for 1 min. and washed three times in sterile distilled water. They were then dried on sterile filter paper and placed on potato dextrose agar

(PDA) with streptomycin. In each Petri dish, five plant parts were placed, in two repetitions. They were kept in a thermostat at 22°C and a light regime of 12 hours day/12 hours night. The developed mycelia were transplanted onto a new PDA medium (Dhingra and Sinclair, 1995). A microscopic examination was performed using an Olympus CX31 microscope. Morphological identification of fungi to genus was performed using standard keys.
In order to determine the pathogenic properties of selected isolates obtained from infected alfalfa plants, a pathogenicity test was performed by inoculating injured alfalfa plants (Vasić, 2007). Before inoculation, all plants were wounded at the base of the stem with a sterile spear needle. In this way, the injured plants were inoculated by placing small fragments of the colony of studied isolates in the wounds. Inoculation was performed with isolates of the obtained mushrooms, 7 days old, grown on PDA medium. Plants inoculated in this way were stored at a temperature of 20-25°C. The experiment was set up in two replicates with five plants per isolate. Alfalfa plants that were injured in the same way served as a control, after which they were inoculated with small fragments of the substrate without mycelia.

Results and discussion

After checking the alfalfa crop, several alfalfa fields showed signs of wilting. Plants with symptoms were collected from 17 locations in Serbia. All the plants from which the fungi was isolated clearly showed symptoms in the form of necrotic spots and lesions on the stem. Fungi of the genus *Fusarium* were isolated from these plants. Similarly, necrosis with white aerial mycelium in the lower third of the stem was observed in some plants, and fungi of the genus *Sclerotinia* were isolated from these plants. On some plants, black fruiting bodies-pycnidia belonging to the genus *Phoma* were observed on the stem. When fungi of the genus *Colletotrichum* are isolated from alfalfa, the tips of the stems are usually bent down. Fungi are also distinguished based on morphological and pathogenic characteristics, in addition to the symptoms of plants present in the field, the causative agents of alfalfa diseases were determined (Table 1).

Table 1. Morphological and pathogenic characteristics of the obtained isolates on alfalfa plants

Types of fungi	**Morphological characteristics**	**Pathogenic characteristics**
Fusarium spp.	52.09–84.35 x 0.86–9.25μm	On the roots, the alfalfa plant test causes typical symptoms of root system rot
Rhizoctonia spp.	12.87–85.8 x 3.6–8.56 μm	Causes root neck rot on alfalfa test plants
Sclerotinia spp.	6–9 x 10–20 μm	On the test plants, it causes rot from the roots towards the root neck and stem
Phoma spp.	5.45–7.14 x 2.98–4.18 μm	Black fruiting bodies develop on the test plants
Verticillum spp.	6–12 x 2.5–3 μm	The pathogen on the test plants causes symptoms in the form of discoloration of the vascular tissue of the roots
Colletotrichum destructivum *C. trifolii* *C. linicola*	10–25 x 2.5–7.4 μm 12.5–17.5x 5.75 μm 12.5–25x 2.5–7.5 μm	On the test plants, lesions were observed in the ground part of the tree, most often on the lower third, light to dark brown in color, irregular in shape, with dark brown edges

The roots of the plants showed symptoms in the form of light brown necrosis, and fungi of the genera *Fusarium* and *Rhizoctonia* were isolated from these plants. Discoloration of the conducting tissue has also been observed in some root system plants and fungi belonging to the genus *Verticillium* have been isolated from these plants.

Based on the morphological and pathogenic characteristics of the obtained isolates, it was determined that fungi of the genus *Colletotrichum* are prevalent on alfalfa in Serbia, followed by fungi belonging to the genera *Fusarium*, *Verticillium*, *Rhizoctonia*, *Sclerotinia* and *Phoma*. The obtained results (Table 1) of morphological and pathogenic characteristics of our isolates are in agreement with the results obtained by Harvey, 1965; Graham *et al*., 1979; Latunde-Dada *et al*., 1997; Boland and Hall, 1994; Krnjaja *et al*., 2005a; Krnjaja, 2005; Rhodes and Sulch, 2005; Cedeño *et al*., 2006; Castell-Miller *et al*., 2007; Vasić, 2013; Reich *et al*., 2017; Xu *et al*., 2019; Ling *et al*., 2019. Species of the genus *Fusarium* are soil fungi and are common wherever alfalfa, red clover and other perennial forage legumes are grown. In the presence of other stress factors, *Fusarium* spp. caused by root rot, often followed by root neck rot or a combination thereof, causing the death of diseased plants. Symptoms on the upper parts of the plant are similar to other types of root rot. *F. oxysporum*, *F. solani* and *F. roseum* are most often isolated from diseased alfalfa roots (Graham *et al*., 1979; Krnjaja *et al*., 2005a; Ling *et al*., 2019). In Serbia, 11 different species of the genus *Fusarium* were isolated from diseased alfalfa plants (Krnjaja, 2005b). Root of the root, tree and root neck caused by *Rhizoctonia solani* is a soil fungus, the most destructive phase of the disease occurs when *R. solani* infects the root neck and as a result of this development the plants die and the yield decreases. Although it can cause root neck rot alone, it is most often associated with other fungi (*Fusarium* spp., *Phoma* spp. and *Colletotrichum* spp.), which also cause root neck tissue death (Mijušković, 1993; Rodiso and Sulc, 2005; Krnjaja *et al*., 2005a).

Root neck and stem rot is a disease of alfalfa caused by *Sclerotinia trifoliorum* (syn. *Sclerotinia sclerotiorum*) - the damage caused by this fungus on alfalfa is significantly less than with *Trifolium* species, especially red clover (Graham *et al*., 1979). Disease symptoms appear most often in early spring, and hard winter frosts promote the spread of rot from the roots to the root neck and trunk (Graham *et al*., 1979; Reich *et al*., 2017).

Alfalfa root disease caused by the *Phoma sclerotioides* was discovered in the United States in 1996. Symptoms of the disease most often appear in three-year-old alfalfa. In addition to alfalfa, this fungus also attacks *Trifolium* species. The fungus in the form of pycnosclerotia maintains vitality in plant residues in the soil. Crop rotation with non-host plant species is balanced, with fertilization, early mowing and winter cultivation of cultivars less sensitive to low temperatures being the most common pathogen control methods (Gray *et al*., 2004; Cedeño *et al*., 2006). Verticillium wilt caused by *Verticillum albo-atrum* is the most economically important alfalfa disease in the world. If this disease is detected in alfalfa, it means that 50% of the plants are already infected. The pathogen causes symptoms in the form of discoloration of the vascular tissue of the roots, burning and wilting of individual leaves, and chlorosis of the leaf tip in the shape of the letter "V". The fungus maintains vitality in soil, plant debris and seeds (Graham *et al*., 1979; Krnjaja *et al*., 2005a; Xu *et al*., 2019). Symptoms of anthracnose caused by fungi of the genus *Colletotrichum* (*C. destructivum*, *C. trifolii* and *C. linicola*) occurred most often after the second cutting, regardless of weather conditions. The tops of the stems are usually bent down, the leaves dry, turn yellow and then turn pink, which gives the whole field a pink appearance in case of strong attack (Latunde-Dada *et al.*, 1997; Lantunde-Dada *et al*., 1999; Johnston, 2000; Latunde-Dada and Lucas, 2007; Vasić, 2013). In temperate regions *C. trifolii*, *C. destructivum* and *C. linicola* persist in alfalfa stems and crowns (Vasić, 2013).

Conclusion

The obtained results indicate that alfalfa is susceptible to attack by a large number of phytopathogenic fungi that can significantly affect the reduction of its yields and quality.
Since the pathogenic species of fungi that cause diseases on alfalfa have not been sufficiently studied in Serbia, research was carried out with the aim of determining the species that cause diseases on alfalfa, as well as their more detailed morphological and pathogenic determination, all with the aim of contributing to finding adequate measures for their successful suppression.

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