UDC 575.630
DOI: 10.2298/GENSR1601187V
Original scientific paper

VEGETATIVE COMPATIBILITY AND RFLP ANALYSIS OF *Colletotrichum destructivum* ISOLATES FROM ALFALFA AND RED CLOVER

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Vasić, T., Krnjaja, V., Jevremović, D., Stanković, S., Terzić, D., Milenković, J., Marković, J. (2016): *Vegetative Compatibility and RFLP analysis of Colletotrichum destructivum isolates from alfalfa and red clover*.- Genetika, Vol 48, No. 1, 187-198.

A total of 17 isolates of *Colletotrichum* from alfalfa (*Medicago sativa* L.) and red clover (*Trifolium pratense* L.) plants with anthracnose symptoms were collected from 11 districts in Serbia during 2005-2010 and tested for variability in vegetative compatibility groups (VCGs) and restriction fragment length polymorphisms (RFLP). Nitrate non-utilising (nit) mutants were isolated from each of investigated *C. destructivum* isolates by selecting chlorate-resistant sectors on medium with chlorate. The isolates were grouped in five VCGs while one isolate was self-incompatible. No relationship was found between VCGs and geographical origin of the isolates. Restriction Fragment Length Polymorphism (RFLP) analysis of a 900 bp intron of the glutamine synthetase (GS) gene revealed a unique polymorphic profile of *C. destructivum* isolates, distinct from the profiles of other *Colletotrichum* species. An identical profile was produced for all *C. destructivum* isolates, regardless of their host and geographical origin. PCR-RFLP failed to detect some the Serbian *C. destructivum* isolates.

Key words: RFLP; VCGs; *M. sativa; T. pratense*; *C. destructivum*

INTRODUCTION

Colletotrichum destructivum O'Gara is a causal agent of anthracnose disease of alfalfa (*Medicago sativa* L.), a serious constraint for alfalfa cultivation in North America, Europe and Africa) (BOLAND and BROCHU, 1989). Conidia form in the acervuli on stem lesions. Rainsplash or wind carries the conidia to the growing petioles and stems. The fungus grows down from infected stems into the crown and taproot, tissue dieback, predisposition to winter injury, wilting and plant

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death. Soil infections of alfalfa by *C. destructivum* have caused considerable yield losses in North America (BOLAND and BROCHU, 1989), Europe (VASIĆ, 2013; VASIĆ *et al*., 2015), North and South Africa (KOCH *et al.,* 1989). The host range of *C. destructivum* is wide and includes a number of legumes (*Phaseolus lathyroides*, *Glycine max, Trifolium* spp., and others), tobacco and dodder (LATUNDE-DADA *et al.,* 1996).

The vegetative compatibility is a biological characteristic of the life cycle of many filamentous fungi that manifests with formation heterokaryons between two different fungal isolates. These isolates belong to the same vegetative compatibility group – VCG (LESLIE, 1993). If the heterokaryosis is not realised between different isolates it is called vegetative incompatibility which is controled by heterokaryon incompatibility or vegetative incompatibility genes (*het* or *vic* genes) (GLASS *et al.,* 2000). The knowledge of vegetative compatibility groups (VCGs) is of particular interest in fungi such as *Colletotrichum* spp. because VCGs divide the population into groups that can change genetic material by heterokaryosis and the parasexual cycle. It has not been definitively demonstrated that vegetative compatibility is operative in nature. The usefulness of VCGs is that they can be used as markers for the genetic analysis and the study of biology of fungal populations (BROOKER *et al.,* 1991).

Vegetative compatibility can be tested in different ways, but complementary auxotrophic mutants, capable of forming a prototrophic heterokaryon are predominantly used. The *nit* mutants are considered excellent genetic markers for determination of vegetative compatibility and grouping of isolates or clones of a fungus into VCGs (KRNJAJA *et al.,* 2013).

Different molecular methods are used for identifying *Colletotrichum* spp. Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) is a very useful method for the analysis of nucleotide sequences of the internal transcribed spacer (ITS) regions (VALERIO *et al.,* 2005). Applying the DNA for determing the ribosomal RNA complex (rDNA) is used as important technique for identification of species of fungi; ITS sequences serve for separating the 16S, 5.8S and 28S fungal rRNA subunits (ABBAS, 2013). For describing the genetic variability of phytopathogenic fungi, an important and fast method as the amplification of ITS region and its restriction is used (FIGUEIREDO *et al.,* 2012). A comprehensive taxonomic revision of *Colletotrichum* based on molecular and phylogenetic data has been provided in the last years (CANNON, 2012; DAMM *et al.,* 2012 a and b). These studies have resulted in a much better understanding of phylogenetic relationships and diversity within the genus and led to the identification of a new species (PHOULIVONG *et al.,* 2010; FAEDDA *et al.,* 2011; DAMM *et al.,* 2012a, VASIĆ *et al.,* 2014). All taxa have been defined genetically on the basis of multi-gene phylogeny as many of the species can not be reliably distinguished using ITS regions alone (SCHENA *et al.,* 2013). The aim of this research was to determine the genetic structures of *C. destructivum* isolates population, originating from alfalfa and red clover, using vegetative compatibility and restriction fragment length polymorphisms.

MATERIALS AND METHODS

Fungal isolates

A total of 17 isolates of *C. destructivum* have been collected during 2005-2010 from alfalfa and red clover crops in 11 districts in Serbia (Table 1). Small fragments of diseased plant tissue (stem, crown and root) were surface-disinfected, put on Petri plate (9 cm in diameter) containing potato dextrose agar (PDA) and kept in the incubator at 25ºC. All isolates were purified using the single-spore isolation procedure.

VCG analysis

Vegetative analysis vegetative compatibility groups (VCGs) were determined in all single-spore isolates of *C. destructivum* (Coll-3, Coll-8, Coll-9, Coll-10, Coll-11, Coll-18, Coll-29, Coll 31, Coll-32, Coll-35, Coll-37, Coll-38, Coll-48, Coll-68, Coll-75, Coll-AŠ, Coll-BK and CC657) based generation of *nit* mutants on chlorate medium, identification *nit* mutant phenotypes and complementation tests.

The following media were used in VCG analysis: basal medium (BM), minimal or nitrate medium - basal medium with 2 g $NaNO_3$ 1000 ml^{-1} (MM), minimal medium with chlorate – minimal medium with 1.6 g L-asparagine and depending on the isolate 15-60 g $KClO_3$ 1000 ml^{-1} (MMC), nitrite medium - basal medium with 0.5 g of $NaNO_2$ 1000 ml^{-1} and hypoxanthine medium - basal medium with 0.2 g of hypoxanthine 1000 ml^{-1}.

The basal medium (BM) was prepared as follows (per litre of distilled water): 30 g of sucrose, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 10 mg of $FeSO_4 \cdot 7H_2O$, 20 g of agar and 0.2 ml of the trace element solution. The trace element solution contained (per 95 ml of distilled water): 5 g of citric acid, 5 g of $ZnSO_4 \cdot 7H_2O$, 1 g of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.25 g of $CuSO_4 \cdot 5H_2O$, 50 mg of $MnSO_4 \cdot H_2O$, 50 mg of H_3BO_3 and 50 mg of $NaMoO_4 \cdot 2H_2O$ (PUHALLA, 1985).

Generation of nit mutants

Small mycelial fragments of 7 day old culture of each isolate cultivated on PDA were placed in the centre of a Petri plate with MMC. Ten MMC plates with one mycelial fragment were used per isolate. The plates were incubated during 7-14 days at 25˚C in the dark. After that period the chlorate-resistant sectors were transferred to MM. Colonies with thin expansive growth and no aerial mycelium on MM were considered as *nit* mutants (auxotrophic mutants) (CORREL *et al.*, 1987).

Nit mutant phenotypes

The *nit* mutants were identified in different phenotypic types on the basis of their growth on media with one of three different nitrogen sources. Fragments of chlorate-resistant sectors were put in the centre of Petri plates with media with different sources of nitrogen, nitrate (MM), nitrite and hypoxanthine medium and incubated as described above. Based on the growth and appearance of mycelium on substrates with different source of nitrogen, different types of *nit* mutants (*nit1*, *nit*3 and NitM) were identified. Then, the identified *nit* mutants were maintained and propagated for further analyses on MM.

Complementation tests

The complementation test of *nit* was mutants is performed on MM that contains nitrate as a nitrogen source. Pairing of two isolates occurs on this medium - one isolate is of the *nit1* type and the other is of the NitM type or *nit3*, if NitM was not isolated from the same isolate of one species, *nit1* x NitM or *nit1* x *nit*3 respectively. In these tests, for each investigated isolate one *nit1* and NitM mutant were used. Mycelial fragment of NitM of investigated single-spore isolate was put in the centre of Petri plate with MM. Mycelial fragments of *nit1* mutants were placed around it at a distance of 2 to 4 cm maximum, but one of *nit1* mutants was of the same single-spore isolate as mutant NitM (test of autocompatibility or test of self-compatibility), and other *nit1* mutants of

different single-spore isolates (test of compatibility). Test of mutants matching was performed in two replications for each isolate studied. Seeded Petri plates were incubated at room temperature, and the score of complementarity between isolates was assessed after 7-14 days.

DNA extraction

All *C. destructivum* isolates (Table 1) and two reference isolates of *C. gloeosporioides* (AVO-37-4B) and *C. dematium* (CC560) were further examined by PCR-RFLP analysis.

Fungal DNA was extracted with a method described by DAY and SHATTOCK (1997). Cubes of mycelia-covered agar (0.5 cm^2) were frozen in liquid nitrogen and then incubated at 65°C for 60 minutes in 800 µl CTAB buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA and 0.2% β-mercaptoethanol) with agitation on every 15 min. After incubation, samples were mixed with the same volume of chloroform and centrifuged at 10.000g for 10 min. Supernatant was pipetted to a new Eppendorf tubes with 0.6 volume of ice-cold isopropanol, mixed and centrifuged at 10.000g for 15 min. DNA pellet was rinsed with 1 ml of ice-cold ethanol and dried at room temperature and resuspended in 100 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8).

Table 1. Isolates of C. destructivum from Serbia examined in this study.

Isolates	*District*	*Host*	Year
Coll-3	Rasina	Alfalfa	2005
Coll-8	Raška	Alfalfa	2005
Coll-9	Rasina	Alfalfa	2005
Coll-10	Rasina	Alfalfa	2006
Coll-11	Central Banat	Alfalfa	2006
Coll-18	Nišava	Alfalfa	2007
Coll-29	Podunavlje	Alfalfa	2008
Coll-31	Central Banat	Alfalfa	2008
Coll-32	Central Banat	Alfalfa	2008
Coll-35	South Banat	Alfalfa	2009
Coll-37	Šumadija	Alfalfa	2009
Coll-38	Podunavlje	Alfalfa	2009
Coll-48	Pomoravlje	Alfalfa	2010
Coll-68	Pčinj	Alfalfa	2010
Coll-75	South Backa	Alfalfa	2010
Coll-Aš	South Backa	Red clover	2010
Coll-Bk	Srem	Red clover	2010

PCR-RFLP analysis

A primer pair GSF1-GSR1 was used to amplify a 900 bp intron region of the GS gene (LIU *et al.*, 2007). Each reaction mixture contained 2 µl DNA, 2.5 µl 10x PCR buffer, 2.5 µl of 2.5 mM dNTPs, 0.25 µl of each 10 µM primer, 1.5 µl of 25 mM $MgCl_2$, 0.25 µl of rTaq DNA polymerase (5U/ µl, GE Healthcare, UK), and 15.75 µl RT-PCR grade water to a total volume of 25µl. PCR reaction was performed in Tpersonal thermal cycler (Biometra, Germany) according to

the following conditions: initial denaturation step at 94°C for 4 min; 35 cycles (94°C for 60 s, 60°C for 60 s, 72°C for 90 s); and final extension for 5 min on 72°C.

Amplified PCR products were digested with two enzyme combinations: (a) *Hind*III + *Hinf*I + *Hae*III and (b) *Hind*III + *Hinf*I + *Msp*I. PCR products were mixed with 2 µl of 10x fast digest buffer, 1 µl of each of the three enzymes per combination (Fermentas, Lithuania) and RT-PCR grade water to a total volume of 20 µl. Reaction mixture was incubated on 37°C for 60 min. Digested fragments were electrophoretically separated in a 5% polyacrylamide gel in 0.5x Tris-acetate-EDTA buffer on 150 V for 2.5 h and stained with silver nitrate. DNA fragments ranging 40-500 bp were marked for their presence/absence on the obtained profiles.

RESULTS

Nit mutant isolation and phenotype identification

Chlorate-resistant sectors were recovered from all 17 isolates after 7-14 days when cultured on MMC. The number of chlorate-resistant sectors per isolate varied from 5 (Coll-11) to 28 (Coll-48). Two types of *nit* mutants (*nit1* and NitM) from all investigated isolates were isolated from chlorate-resistant sectors. Mutant *nit1* was characterized with sparse colony without dense aerial mycelium on MM and wild-type colony with dense mycelium on nitrite and hypoxanthine medium, whereas mutant NitM was characterized by thin colony on MM and hypoxanthine medium, a dense mycelium colony on nitrite medium. In our study, *nit3* mutants were not isolated. *Crn* mutants were isolated from chlorate-resistant sectors. However, due to wild-type colony on MM, they were discarded in complementation tests.

In all investigated isolates average frequency of *nit1* mutants was higher (8.57%) compared to NitM mutants (4.43%). The number of *nit1* mutants ranged from two (Coll-3) to 17 (Coll-32), and in NitM from one (Coll-38 and Coll-48) to 12 (Coll-29). *Crn* mutants were identified with frequency from 0 (Coll-10, Coll-11 and Coll-35) to 20 (Coll-3) (data not presented).

Complementation tests

Complementation between *nit* mutants was identified by the development of the prototrophic heterokaryon which is characterized with dense mycelium in place of contact and anastomoses of mycelia. Formation of heterokaryons between *nit1* and NitM mutants of the same isolate was assessed as self-compatibility (autocompatibility) (Figure 1). No complementation between *nit* mutants recovered from the same isolate was assessed as heterokaryon self-incompatibility (HSI). Forming of heterokaryon between *nit1* and NitM mutants of various isolates was evaluated as compatibility of the investigated isolates and these isolates were classified in the same VCG.

One of the 17 *C. destructivum* isolates turned out to be self-incompatible (Coll-48), therefore it was discarded in complementation test. Other 16 self-compatible isolates were grouped in five VCGs. Determined VCGs had different number of isolates, two VCGs had one isolate each (Coll-68 and Coll-75, respectively) and the remaining three VCGs comprised three isolates each (Coll-9, Coll-32, Coll-38), five (Coll-18, Coll-29, Coll-35, Coll-37, Coll-BK) and six isolates (Coll-3, Coll-8, Coll-10, Coll-11, Coll-AŠ, Coll-31), respectively. No correlation was found between VCGs and the geographic origin of the isolates. . Determined VCGs were found in more that one district.

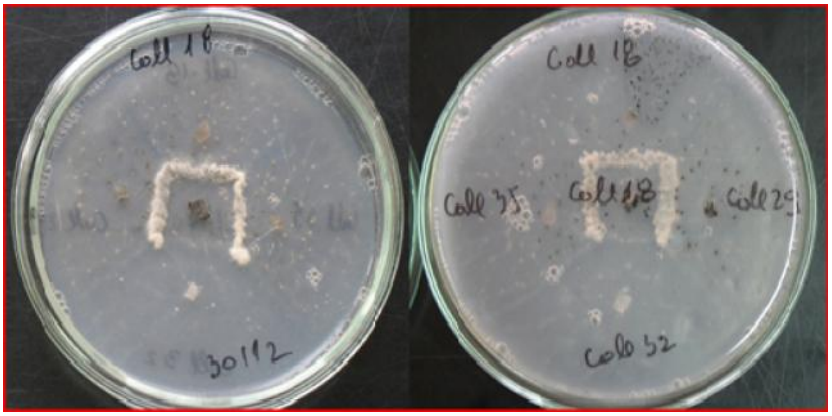

Figure 1. Complementation tests on MM between auxotrophic mutants of *C. destructivum*: prototrophic heterokaryons between NitM and *nit1* mutants of isolate Coll-18 (autocompatibility) and between a NitM mutant of isolate Coll-18 and *nit1* mutants of isolate Coll-29 and Coll-35, respectively (compatibility); NitM mutant of isolate Coll-18 and *nit1* mutant of isolate Coll-32 did not form heterokariotic prototrophic mycelium (incompatibility)

RFLP-PCR analysis

PCR products were successfully amplified from 11 tested isolates (Coll-Aš, Coll-3, Coll-9, Coll-10, Coll-11, Coll-18, Coll-29, Coll-35, Coll-37, AVO-37-4B and CC560). Both enzyme combinations (a) *Hind*III + *Hinf*I + *Hae*III and (b) *Hind*III + *Hinf*I + *Msp*I produced highly polymorfic profiles. Comparison of GS intron RFLP profiles indicated three clear distinct profiles corresponding to the three analysed *Colletotrichum* species.

The first enzyme combination produced polymorphic profile of eight polymorphic bands for *C. destructivum* isolates (Figure 2). All *C. destructivum* isolates (8 from alfalfa and one isolate from red clover) gave the same polymorphic profile regardless their geographic origin.

The second enzyme combination also produced polymorphic profile for tested isolates (Figure 3). Clear qualitatively difference was observed between profiles of isolates belonging to *C. destructivum*, *C. gloeosporioides* and *C. dematium*. A profile with seven polymorphic bands was observed for *C. destructivum* isolates. As for the first enzyme combination, all *C. destructivum* isolates gave the same polymorphic profile regardless their geographic origin.

The DNA fragments obtained from eight isolates (Coll-8, Coll-31, Coll-32, Coll-38, Coll-48, Coll-68, Coll-75 and Coll-Bk) were hardly visible on the gel and were not of sufficient quality for RFLP analysis.

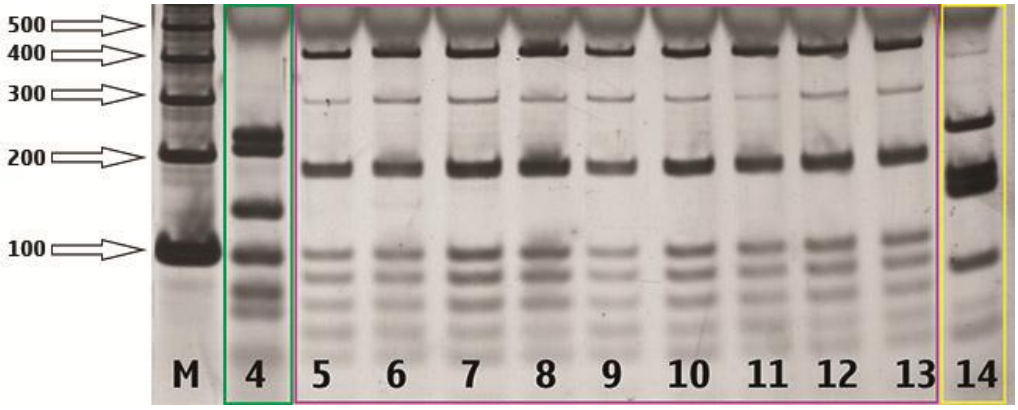

Figure 2. RFLP analysis of 900 bp intron GS genes of *C. destructivum* isolates. PCR product (≈900 bp) was treated with a combination of three enzymes *Hind*III + *Hinf*I + *Hae*III, and analysed in polyacrylamide gel. (4- reference isolate of *C. gleosporioides* AVO-37-4B, 5- isolate Coll-Aš, 6- isolate Coll-3, 7- isolate Coll-9, 8- isolate Coll-10, 9- isolate Coll-11, 10- isolate Coll-18, 11- isolate Coll-29, 12- isolate Coll-35, 13- isolate Coll-37, 14- reference isolate of *C. dematium* (CC560)) M- 100 bp DNA Ladder.

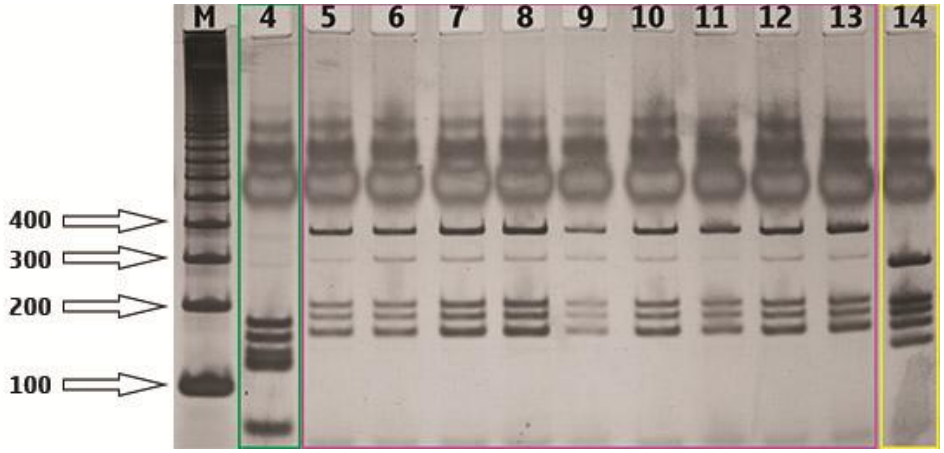

Figure 3**.** RFLP analysis of 900 bp intron GS genes of selected isolates. Multiplied PCR product (≈900 bp) treated with combination of three enzymes (b): *Hind*III + *Hinf*I + *Msp*I (4- reference isolate *C. gleosporioides* (AVO-37-4B), 5- isolate Coll-Aš, 6- isolate Coll-3, 7- isolate Coll-9, 8-isolate Coll-10, 9- isolate Coll-11, 10-isolate Coll-18, 11- isolate Coll-29, 12- isolate Coll-35, 13-isolate Coll-37, 14- reference isolate *C. dematium* (CC560)) and analysed in polyacrylamide gel. M- marker-100 bp DNA Ladder

DISCUSSION

VCG analysis

Applying the VCG method, from chlorate-resistant sectors, in all investigated isolates of *C. destructivum*, two phenotypic types of *nit* mutants, *nit1* and NitM were identified. In the present study, the frequency of *nit1* was higher than of NitM which is in concordance with the results of CORRELL *et al.* (2000); KATAN (2000); VARZEA *et al.* (2002); PIECZUL and RATAJ-GURANIWSKA (2004); KRNJAJA *et al.* (2008); LEVIĆ *et al.* (2008). Contrary to these results, BROOKER *et al.* (1991); BARCELOS *et al.* (2011) have isolated in the highest number *nit3* mutants in investigated *Colletotrichum* isolates.

Among 17 investigated isolates of *C. destructivum* originating from two hosts, alfalfa and red clover, five VCGs and one self-incompatible isolate were identified. Two VCGs included isolates originating from the same host (alfalfa) but from different districts, Pčinja district (Coll-68) and South Bačka district (Coll-75), respectively. Two VCGs included isolates from different and distant locations (districts) of Serbia as well as isolates originating from different hosts. One VCG included six isolates: four isolates originated from alfalfa from two districts Rasina (Coll-3 and Coll-10) and Central Banat (Coll-11 and Col-31) and two isolates from different hosts and different districts. The isolate Coll48 from alfalfa from district Pomoravlje was self-incompatible. The isolates originating from same VCG had similar or identical multi locus haplotypes and belonged to the same clonal line and were more genetically similar than isolates from different VCGs (KATAN, 2000; LESLIE and SUMMERELL, 2006). In this research, it was determined that there was genetic variability in investigated isolates of *C. destructivum* originated from alfalfa and red clover from Serbia. This is similar to results of BARCELOS *et al.* (2011) which have confirmed high diversity among 47 isolates of *C. lindemuthianum* classified in 45 VCGs and originating from the same geographic origin. Also, BEEVER *et al.* (1995) identified 11 VCGs in a population of 32 isolates of *C. gloesporioides* from fruits. Contrary to SANEI and RAZAVI (2011), only two VCGs were established, i.e. homogenicity of the olive *C. gloesporioides* population. Considering that species of the genus *Colletotrichum* cause anthracnose on a wide host range and show high variability in morphological characters, relative high variability of the investigated population of *C. destructivum* in regard to the genetic structure is justified in this study.

PCR-RFLP analysis

We analysed the restriction digestion of the GS intron of 9 isolates belonging to *C. destructivum* and one isolate of *C. gloeosporioides* and *C. dematium*. Restriction profiles obtained with two enzyme combinations (*Hind*III + *Hinf*I + *Hae*III and *Hind*III + *Hinf*I + *Msp*I, respectively) revealed clear differences in profiles of tested isolates belonging to the three species. Polymorfic profiles of the isolates AVO-37-4B (*C. gloeosporioides*) and CC560 (*C. dematium*) are identical to the profiles of the isolates belonging to these species as described in the study of LIU *et al.* (2007). Polymorphic profiles of all tested *C. destructivum* isolates were identical. These unique profiles did not correspond to the profiles of many *Colletotrichum* species (*C. orbiculare, C. lindemuthianum, C. trifolii, C. orbiculare* from Xanthium, *C. malvarum, C. dematium, C. acutatum* and *C. magna*) examined by LIU *et al.* (2007). RFLP diversity between the *Colletotricum* species from different hosts was previously described in several studies (GUERBER *et al.*, 2003; LIU *et al.*, 2007; MAHARAJ and RAMPERSAD, 2012). *Colletotrichum orbiculare, C. trifolli, C. lindemuthianum* and *C. malvarum* are closely phylogenetically related based on the GS intron sequences, but produce different polymorphic profiles of the GS intron.

The identification of the species from genus *Colletotrichum* is not an easy task because of the great morphological variations. RFLP analysis of studied species with two enzyme combinations confirmed polymorphism. In the paper of FIGUEIREDO *et al.* (2012) it was demonstrated that RFLP analysis may show a correlation between the genetic groups and geographical origin of the *C. gloesporioides* isolates. In our study, polymorphic profiles of all *C. destructivum* isolates were identical, both from alfalfa and red clover from different geographic locations in Serbia.

PCR reactions yield no or very faint products with eight analysed *C. destructivum* isolates (Coll-8, Coll-31, Coll-32, Coll-38, Coll-48, Coll-68, Coll-75 and Coll-Bk). The reason for this is probably caused by nucleotide mismatches at the primer binding sites in these isolates. Extracted genomic DNA was of high quality that was confirmed in the PCR and sequence analysis using other primer pairs (VASIĆ, 2013; VASIĆ *et al.*, 2015).

The polymorphic profiles of all tested *C. destructivum* isolates were identical, and clearly distinct from profiles of other studied *Colletotrichum* species. These results confirm that RFLP analysis may be used as useful diagnostic method for differentiation of *Colletotrichum* species infecting alfalfa and red clover in Serbia. Due to the genetic diversity of some Serbian *C. destructivum* isolates RFLP method may not be fully reliable for this purpose. Both of these methods, however, are not very reliable for the taxonomy of *Colletotrichum*, due to significant variability within the *C. destructivum* species.

ACKNOWLEDGEMENTS

Ministry of Education, Science and Technological Development Republic of Serbia, project TR 31057.

Received February06th, 2015
Accepted December 25th, 2015

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VEGETATIVNA KOMPATIBILNOST I RFLP ANALIZA *Colletotrichum destructivum* IZOLATA SA LUCERKE I CRVENE DETELINE

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Izvod

Dobijeno je ukupno 17 izolata, iz obolelih biljaka lucerke i crvene deteline, sa simptomima antraknoze. Kolekcionisani su u periodu 2005-2010. godine, u 11 okruga u Srbiji. Primenom metoda vegetativne kompatibilnosti i polimorfizma dužine restrikcionih fragmenata (RFLP) ispitivana je genetička varijabilnost izolata.

Iz hlorat-rezistentnih sektora kod svih proučavanih izolata izolovani su mutanti koji su se odlikovali retkom, tankom supstratnom micelijom na MM podlozi. Ispitivani izolati su grupisani u pet vegetativno kompatibilnih grupa (VCGs) dok je jedan izolat nekomopatibilan (samo-inkomopatibilan). Nije utvrđena korelacija između VCGs i geografskih lokaliteta ispitivanih izolata. Na osnovu RFLP analize fragmenata od 900 bp nastalog umnožavanjem introna gena za (GS) gena, otkriven je jedinstven restrikcioni profil za vrstu *C. destructivum*, koji se razlikuje od restrikcionih profila drugih *Colletotrichum* vrsta, koje su poslužile u ovim istraživanjima kao referenti. Identičan profil napravljen je za sve proučavane izolate *C. destructivum*, bez obzira na domaćina i geografsko poreklo. Pomoću RFLP metode nisu mogli da se detektuju svi ispitivani izolati *C. destructivum*.

Primljeno 06.II. 2015.
Odobreno 25. XII. 2015.