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Molecular Characterization and Identification of Fungi Causing Stem Canker of Oilseed Rape in Serbia

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Summary: During 2008-2010 the infected *Brassica napus* plant material was collected from nine localities: Karavukovo, Crvenka, Prigrevica, Subotica, Rimski Šančevi, Srbobran, Beška, Banatsko Karađorđevo, and Srpski Miletić. Infected tissue samples were taken from the root of both upper and basal stems, as well as from the leaf, flower and pods. Two reference isolates from Great Britain were used in these studies for the analysis of 119 isolates from Serbia, using polymerase chain reaction (PCR) and PCR-RFLP. Digestion of PCR products was performed with 5 selected endonucleases: *BamH*I, *Hae*III, *Rsa*I, *Eco*RII, and *Alu*I. On the basis of PCR analysis, all isolates originating from Serbia belong to *L.* complex: 111 belong to species *Leptosphaeria maculans*, and 8 to species *Leptosphaeria biglobosa* NA1 (*Leptosphaeria biglobosa brassicae*), respectively.
Keywords: *Brassica napus*, endonucleases, isolates, *Leptosphaeria maculans*, *Leptosphaeria biglobosa*, localities, PCR and PCR-RFLP.

Introduction

Stem canker is an important oilseed rape disease in Europe, Australia, and North America (Fitt et al. 2006); and has been reported worldwide (Howlett et al. 2001; Gosende et al. 2003. The intensity of the disease depends on the climatic factors, agro-technical measures and the resistance of the varieties (Howlett, 2004; Sosnowski et al., 2004; Aubertot et al., 2006;). The disease is caused by two species of phytopathogenic fungi of genus *Leptosphaeria*: *Leptosphaeria maculans* (Desm.) ces. and de Not anamorph *Phoma lingam* (Tode. Fr.) Desmas, causing basal stem canker (Gabrielson 1983; Paul & Rowlinson 1992) and Leptospaeria biglobosa Shoemaker and Brun, which causes symptoms of cancer in the upper stem generally causing less damage, while serious damage occurs in countries with higher summer temperatures (Huang et al. 2005; Fitt et al. 2006). Both species can be found in Africa, Central and South America, and Asia (Anon. 2004 cit loc Fitt et al. 2006). Based on the pathogenicity of isolates, the population of *L.maculans* has previously been divided into two groups – virulent and slightly virulent (McGee & Petrie 1978; Koch et al. 1989; Badawy & Hoppe 1989; Brun et al.1997; Williams & Fitt 1999; Mitrović et al 2012); then into group A and B, based on RFLP analysis (Johnson & Lewis 1990); and finally into Tox$^+$ isolates creating toxic substances – phytotoxins, and Tox^0 which do not reproduce toxic substances (Balesdent et al. 1992). It was determined, based on RFLP analysis and isoenzymatic analysis, that aggressive isolates form a compact group different from the slightly virulent isolates. The same methods showed that slightly virulent isolates can be divided into three subgroups: NA1, NA2, and NA3 (Koch et al. 1991, Gall et al. 1995). Gall et al. (1995) state that NA1 subgroup is predominant in Europe, whereas NA2 is more common in Canada. Balesdent et. al (1998) suggested identification of species and subspecies within the *Leptosphaeria* species complex, based on the ITS restrictive profile using 5 enzymes - AluI, BamHI, HaeIII, EcoRII and RsaI. Based on the obtained results, Balesdent et. al (1998) divided the *Leptosphaeria* species complex into 7 groups: (1.) *Brassica* Tox$^+$, (2.) *Lepidium* Tox$^+$, (3.) *Brassica* Tox^0 NA1 subgroup, (4.) *Brassica* Tox^0 NA2 subgroup, (5.) *Brassica* Tox^0 NA3 subgroup (6.) *Erysimum* Tox^0 and (7.) *Thlaspi* Tox^0. On the basis of molecular characteristics, hosts and geographical origin, Mendes-Pereira et al (2003). divided the *Leptosphaeria* species complex into 7 groups: *Leptosphaeria biglobosa brassicae, Leptosphaeria biglobosa canadensis, Leptosphaeria biglobosa erysimi, Leptosphaeria biglobosa thlaspii, Leptosphaeria biglobosa australensis, Leptosphaeria maculans brassicae,* and *Leptosphaeria maculans lepidii.* The aim of this study was to perform a molecular analysis, identification of pathogenic fungus *Leptosphaeria maculans* and *Leptosphaeria biglobosa* in Serbia.

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Material and Methods

Fungal cultures were isolated from infected plant parts of oilseed rape, which were collected from 9 localities (Karavukovo, Crvenka, Prigrevica, Subotica, Rimski šančevi, Srbobran, Beška, Banatsko Karađorđevo, and Srpski Miletić) during 2008-2010. Tiny fragments were taken from the infected plant organs and immersed into a 3% sodium hypochlorite solution for 3-5 minutes, and then washed with distilled water, and naturally dried under controlled conditions. After drying, fragments of infected tissue were poured in Petri dishes in a box, and placed on a nutrient medium e.g. Potato Dextrose agar (PDA) (Difco Detroit USA). In total, 119 isolates were identified. Isolates from Vojvodina were labelled K, C,L,S,St,GS,and Lj. Two more reference isolates were used in addition, which were received from the Agricultural research centre in Rothamsted UK, and labelled as L.m (*Leptosphaeria maculans*) and L.b (*Leptosphaeria biglobosa*).

DNA extraction

Mycelium fragments and picnides were scraped from the agar and placed into plastic baths. After grinding, 800 µl CTAB buffer (2% (w/v) CTAB 100 µl Tris-HCl pH 8,0, 20 mMEDTA pH 8,0, 1,4 MNaCl and 1% (w/v) polyvinyl-pyrrolidone) was added to the mixture, and transfered into 2 ml test tubes. Tubes were placed in the incubator for 1h at 65 ºC, and each 15 minutes tube contents were vortexed for 5 mins. 600 µl chloroform was added to the tubes after incubation, and vortexed in the incubator for 10s at 25ºC. Tubes were then centrifuged for 10 mins at 1300g. Liquid phase (about 500 µl) was transferred into new tubes, 300 µl isopropanol was added, then it was incubated at room temperature and centrifuged for 10 mins at 1300g. The liquid was poured out of the tubes after centrifuge, and 600 µl of 70% ethanol was added. Tubes were vortexed in the incubator for 10 s, centrifuged for 10 mins at 1300g, and then liquid was removed from the tubes. Open tubes were placed in the drier for 10 mins at 50 ºC. The obtained residue was dissolved in a 25 µl TE buffer, pH 8, and frozen at -20 ºC. DNA was extracted from all isolates using the same method (Day & Shattock 1997).

PCR analysis

Two primers were used for DNA amplification - PN3 (5´CCGTTGGTGAACCAGCGGAGGGATC) and PN-10(TCCGCTTATTGATATGCTTAAG), Balesdent et al. (1998). PCR reaction was performed at the total volume of 25 µl, containing: 1 µl fungal (L. maculans and L. biglobosa) DNA, 11 µl PCR water, 12,5 µl master mix REDTaq (Sigma aldrich) with MgCl2 (Taq plimerase 0,06 U/µl 3 mM MgCl2 0,002% gelatine 0,4 mM dNTP), and 1,25µl of each primer. The mixture was briefly centrifuged, and the tubes were placed in a PCR apparatus (Eppendorf master cycler gradient). Each cycle was carried out for 30 s at 94 ºC, 30 s at 58 ºC, and 1 min at 72 ºC. In total, 37 cycles were performed. After the completed PCR reaction, fragments were visually observed on a 1,5% agarose gel coloured with ethidium bromide.

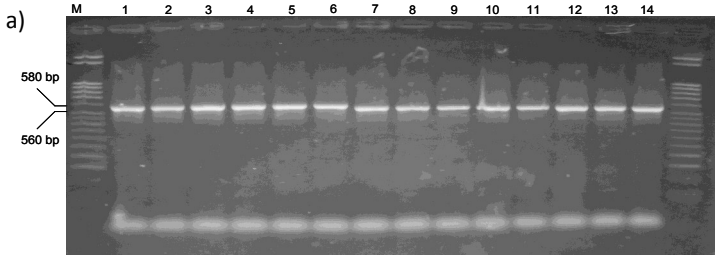

Figure 1a. Amplification with PN3 and PN10 primers. Band M 3000 bp marker, 1 L.b, 2 K-111, 3 K-112,4 K-113, 5 K-115, 6 K-116, 7 K-2, 8 St-16, 9 GS-25, 10 L-5, 11 C-3, 12 Lj-2, 13 S-11, 14 L.m. 560 bp band was used for isolates L.m (reference isolate), K-2, St-16, GS-25, L-5, C-3, Lj-2, S-11, and 580 bp band for isolates L. b. (reference isolate) K-111, K-112, K-113, K-115, K-116.

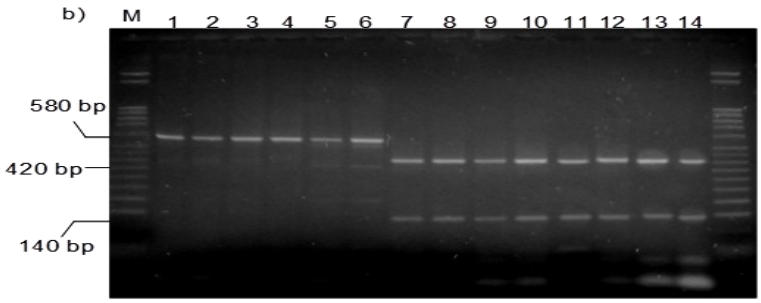

Figure 1b. Digestion of the amplified region with ***BamH*I**. Band M 3000 bp marker, 1 L.b, 2 K-111, 3 K-112,4 K-113, 5 K-115, 6 K-116, 7 K-2, 8 St-16, 9 GS-25, 10 L-5, 11 C-3, 12 Lj-2, 13 S-11, 14 L.m. Restriction enzyme ***BamH*I** in L.b, K-111, K-112, K-113, K-115, and K-116 isolates cuts DNA at 580 bp, while in K-2, St-16, GS-25, L-5, C-3, Lj-2, S-11 and L.m. isolates DNA is cut at 420 and 140 bp.

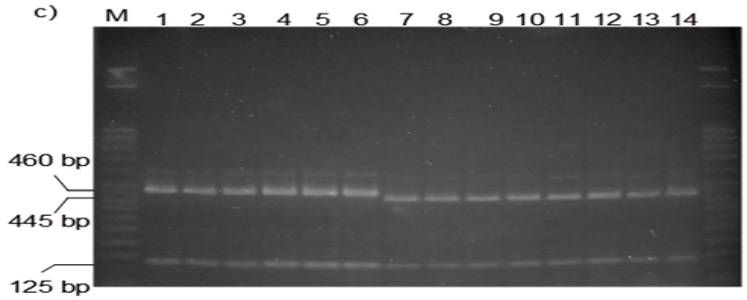

Figure 1c. Digestion of the amplified region with ***Hae*III**. Band M 3000 bp marker, 1 L.b, 2 K-111, 3 K-112,4 K-113, 5 K-115, 6 K-116, 7 K-2, 8 St-16, 9 GS-25, 10 L-5, 11 C-3, 12 Lj-2, 13 S-11, 14 L.m. Restriction enzyme ***Hae*III** in L.b, K-111, K-112, K-113, K-115, and K-116 isolates it cuts DNA at 460 and 125 bp, while in K-2, St-16, GS-25, L-5, C-3, Lj-2, S-11 and L.m. isolates DNA is cut at 445 and 125 bp

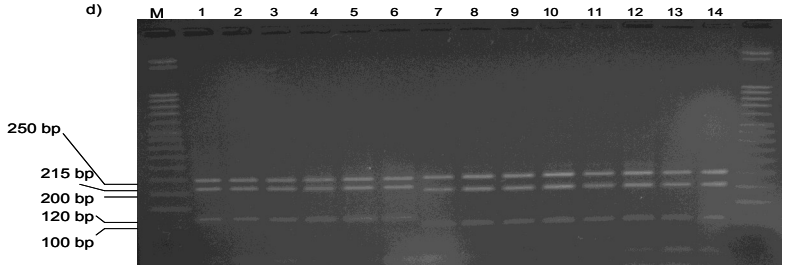

Figure 1d. Digestion of the amplified region with ***Rsa*I**. Band M 3000 bp marker, 1 L.b, 2 K-111, 3 K-112,4 K-113, 5 K-115, 6 K -116, 7 K-2, 8 St-16, 9 GS-25, 10 L-5, 11 C-3, 12 Lj-2, 13 S-11, 14 L.m. Restriction enzyme ***Rsa*I** in L.b, K-111, K-112, K-113, K -115 and K-116 isolates it cuts DNA at 120, 215 i 250 bp, while in K-2, St-16, GS-25, L-5, C-3, Lj-2, S-11, and L.m. isolates it cuts DNA at 100, 200 and 250 bp

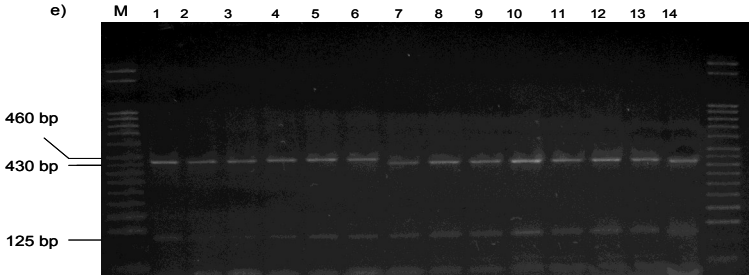

Figure 1e. Digestion of the amplified region with ***EcoR*II**. Band M 3000 bp marker, 1 L.b, 2 K-111, 3 K-112,4 K-113, 5 K-115, 6 K-116, 7 K-2, 8 St-16, 9 GS-25, 10 L-5, 11 C-3, 12 Lj-2, 13 S-11, 14 L.m. Restriction enzyme ***EcoR*II** in L.b, K-111, K-112, K-113, K-115, and K-116 it cuts DNA at 125 and 460 bp,while in K-2, St-16, GS-25, L-5, C-3, Lj-2, S-11 and L.m. isolates it cuts DNA at 125 and 430 bp

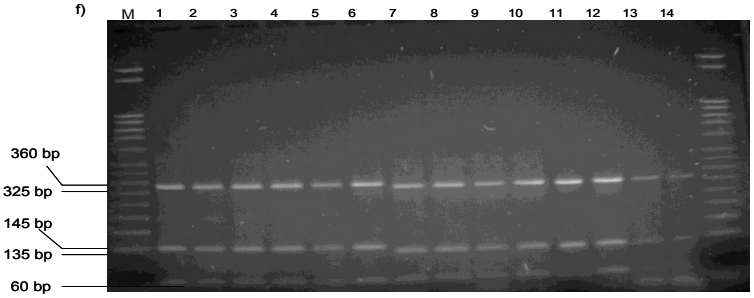

Figure 1f. Digestion of the amplified region with ***Alu*I**. Band M 3000 bp marker, 1 L.b, 2 K-111, 3 K-112,4 K-113, 5 K-115, 6 K-116, 7 K-2, 8 St-16, 9 GS-25, 10 L-5, 11 C-3, 12 Lj-2, 13 S-11, 14 L.m. Restriction enzyme ***Alu*I** in L.b, K-111, K-112, K-113, K-115, and K-116 it cuts DNA at 60, 145 and 360 bp, while in K-2, St-16, GS-25, L-5, C-3, Lj-2, S-11 and L.m. isolates it cuts DNA at 60, 135 and 325 bp

PCR–RFLP analysis

The following isolates were used for the PCR-RFLP analysis: L.b (reference isolate *L. biglobosa*) K-111, K-112, K-113, K-115, K-116, K-2, St-16, GS-25, L-5, C-3, Lj-2, S-11, and L.m. (reference isolate *L. maculans*).

PCR-RFLP was conducted following the producer's recommendations (Fermentas life science) according to Balesdenet et al. 1998. Ten microlitres of PCR product, obtained in the use of PN3 and PN10 primers, were digested in 5 h at 37ºC of 20 U with one of the following enzymes: *BamH*I, *Hae*III, *Rsa*I, *Eco*RII and *Alu*I. After incubation restriction fragments were observed in a 1,5% agarose gel coloured with 20 µl ethidium bromide. Isolates were randomly selected for this analysis. Isolates K-2, St-16, GS-25, L-5, C-3, Lj-2, and S-11 represented a group in these testings, i.e. a plant organ which they were isolated from. Five out of 8 isolates were used for RFLP analysis, labelled K-111, K-112, etc. Given that these 8 isolates showed great morphological similarities with the reference isolate L.b, 5 isolates were used in the PCR-RFLP analysis, in order to identify which subgroup NA-1, NA-2 or NA-3 *L. biglobosa* isolates from Serbia belong to.

Results and Discussion

Based on PCR-RFLP analysis, all tested isolates from Serbia (K-111, K-112, K-113, K-115, and K-116) as well as the reference isolate L.b. belong to *Leptosphaeria biglobosa* species - NA 1 subgroup, while K-2, St-16, GS-25, L-5, C-3, Lj-2, S-11, also from Serbia, and the reference isolate L.m. belong to *Leptosphaeria maculans* species. Tape lengths are expressed in bp (base pairs). PN3 and PN10 primers of tested isolates amplify DNA at 560 bp for *Leptosphaeria maculans* species, and 580 bp for Leptosphaeria biglobosa, which is in accordance with Balesdent et al. (1998). Mendes–Pereira et al. (2003) reported 468 bp for *Leptosphaeria maculans* species (Tox + representative isolates) and 496 bp for *Leptosphaeria biglobosa* species NA1, NA2, and NA3 subgroup. Liu et al. (2006), and Fernando et al. (2016) reported 331-bp *L.maculans* '*brassicae*' isolates and a 444-bp product from *L. biglobosa*'brassicae' isolates. Based on the RFLP profile, these reported species can also be identified. Restriction enzyme *Eco*RI cuts DNA at 430 bp in agressive isolates (*Leptosphaeria maculans*), while in non-agressive isolates (*Leptosphaeria biglobosa*) this fragment was not observed (Koch et al. 1991). The obtained polymorphism enabled identification of tested isolates from Serbia by the use of restriction enzymes. Thus isolates K-111, K-112, K-113, K-115, and K-116 belong to *Leptosphaeria biglobosa* species, while K-2, St-16, GS-25, L-5, C-3, S-11, Lj-2 belong to *Leptosphaeria maculans* species. Besides separating the isolates according to species, polymorphism obtained by restriction enzymes HaeIII, EcoRII and AluI all tested isolates (K-111, K-112, K-113, K-115 and K-116) belong to *Leptosphaeria biglobosa* species, subgroup NA1 dominant in Europe (Gall et al. 1995, Jedryczka et al. 1999, Fitt et al. 2006), which is in accordance with the results (Balesdent et al. 1998).

Conclusions

Based on DNA amplification with PN3 and PN10 primers, K-111, K-112, K-113, K-114, K-115, K-116, K -117 and K-118 are amplified at 580 bp band, while other isolates (K-1 do K-25, St-1 do St-28, GS-1 do GS-27, C-1 do C-6, L-1 do L-10, S-1 do S-11, Lj-1 to Lj-6) are amplified at 560 bp band.In RFLP analysis K-111, K-112, K-113, K-115 and K-116 are different in one or two restriction sites of K-2 St-16 GS-25 L-5 C-3 Lj-2 and S-11 isolates. Based on the conducted research, we can conclude that isolates K-111, K-112, K-113, K-114, K-115, K-116, K-117, and K-118 belong to *Leptosphaeria biglobosa* subgroup NA1 according to the new classification of *Leptosphaeria biglobosa brassicae*, while K-1 to K-25, St-1 to St-28, GS-1 to GS-27, C-1 to C-6, L-1 to L-10, S-1 to S-11, and Lj-1 to Lj-6 belong to *Leptosphaeria maculans* according to the new classification of *Leptosphaeria maculans brassicae*. After the completion of this research, we can conclude that both species present in Serbia (*Leptosphaeria maculans* and *Leptosphaeria biglobosa*) are causal agents of stem canker of oilseed rape (*Brassica napus* var. *oleifera* L).

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Molekularna karakterizacija i identifikacija gljiva uzročnika raka stabla uljane repice u Srbiji

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Sažetak: Kulture gljiva izolovane su iz obolelih biljnih delova uljane repice koji su prikupljeni iz devet lokaliteta (Karavukovo, Crvenka, Prigrevica, Subotica, Rimski šančevi, Srbobran, Beška, Banatsko Karađorđevo, Srpski Miletić) u periodu od 2008 do 2010. godine. Uzorci obolelog tkiva su uzeti sa korena, prizemnog stabla, gornjeg dela stabla i grana, kao i sa lista, cveta, ljuski i semena. Molekularna karakterizacija je sprovedena korišćenjem lančane reakcije polimeraze PCR i PCR-RFLP, a ukupno je ispitivano 119 izolata poreklom iz Srbije i dva referentna izolata iz Velike Britanije. Digestija PCR produkata je sprovedena sa 5 odabranih endonukleaza: *Bam*HI, *Hae*III, *Rsa*I, *Eco*RII i *Alu*I. Na osnovu PCR analize, svi izolati poreklom iz Srbije pripadaju L. kompleksu: 111 pripada vrsti *Leptosphaeria maculans*, a 8 vrsti *Leptosphaeria biglobosa* NA1 (*Leptosphaeria biglobosa brassicae*)

Ključne reči: *Brassica napus*, endonukleaze, izolati, *Leptosphaeria maculans*, *Leptosphaeria biglobosa*, lokalitet, PCR and PCR -RFLP.

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