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RAPESEED FLOWERS WILT CAUSED BY PATHOGENIC FUNGI LEPTOSPHAERIA MACULANS IN SERBIA

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Abstract

Stem canker (blackleg) is economically the most important disease of oilseed rape worldwide. In addition to the cotyledons, stem, leaf and pods in favorable climates conditions (humid and rainy weather) parasite can cause wilt flowers of rapeseed. In 2010, with the flowers of rapeseed more isolated strains of fungi. In these studies investigated the morphology and pathogenic properties that is associated with the molecular identification. On PDA medium at 25 ± 1^0C were studied morphological characteristics of the isolates: growth, appearance, color and appearance of the edge of the colony, the forms of pigments as well as structure and appearance of mycelium, the size, shape and color pycnidiospores and pycnidia 9 isolates (isolated in Serbia C -1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9). All study strains on nutrient medium formed irregular colonies, they have slow growth and a strong sporulation. Pycnidiospore are unicellular, hyaline, mostly straight, with or without a drop of oil. All tested isolates causing disease symptoms on cotyledons cultivar Banaćanka Molecular identification was performed application the PCR technique using primers PN3/PN10. In addition to these isolates were used and two reference strains obtained from the Centre for agricultural studies, Rothamsted, UK, and which are marked with L.m (*Leptosphaeria maculans*) and L.b (*Leptosphaeria biglobosa*). These studies showed that all the strains isolated with wilt flowers of rapeseed belonging to the species *Leptosphaeria maculans*.

Key words: *Brassica napus*, *Leptosphaeria maculans*, morphological and pathogenic properties, molecular identification.

Introduction

Blackleg and stem canker is economically the most important disease of oilseed rape in Europe, Australia and North America (Fitt et al., 2006). This disease is caused by two species of plant pathogenic fungi of the genus *Leptosphaeria*: *Leptosphaeria maculans* (Desm.) Ces. de Not anamorphic stadium: Phoma lingam (Tode ex Fr.) Desm and *Leptosphaeria biglobosa* Shoemate and Brun. Both types are present on all the continents (Anon 2004 loc cit. Fitt et al., 2006). The intensity of the disease depends on climatic factors, agro-technical measures and resistance varieties (Howlett, 2004; Aubertot et al., 2006; Sosnowski et al., 2004). The parasite causes symptoms in the emergence and maturation of rapeseed. On cotyledons, leaves, and pods symptoms manifest themselves in the form of leaf spot, while the stem and root fungus causes cancer, or dry rot (Gabrielson, 1983; Paul & Rowlinson, 1992). During the autumn, the fungus causes leaf infection by ascospores that are released from mature pseudothecia (Huang et al., 2003a; Marcroft et al., 1999; Hammond et al., 1985). In addition to ascospores in Australia pycnidiospores pathogens cause the symptoms of the cotyledon and hypocotyl (Barbetti & Khangura, 2000 loc cit. West et al., 2001; Gosende et al., 2003). During the spring the mycelium from the leaf continue to grow and over the petiole infects a stem, causing the symptoms of cancer (Hammond et al., 1985; Thürwächter et al., 1999; Paul

and Rawlinson, 1992). On nutrient medium isolates of group A (*L. maculans*) are slow and irregular increase compared to group B (McGee and Petrie, 1978; Humpherson-Jones, 1983; 1986; Koch et al., 1989). However, there are also reports that some isolates of group A have a faster growth of group B, it is clearly not possible to distinguish reliable on the basis of colony growth rate in vitro (Delwiche, 1980 cited by Williams in 1992; Kharbanda and Stevens, 1993; Salisbury et al., 1995). In addition to the symptoms of the disease seems to plants,cultural characteristics or analyses of secondary metabolite(sirodesmin PL),the value of recent advances in nucleic acid technology in particular the polymerase chain reaction (PCR) in the detection and characterization of plant pathogen (Janse, 1995). Molecular characterization of *L. maculans* has received considerable attention and results from several methods: RFLP profiling, DNA sequence analysis and several PCR-based methods, provide compelling evidence that *L. maculans* consists of more than one species (Johnson and Lewis, 1990; Koch et al., 1991; Taylor et al., 1991; Jedryczka et al., 1997).

Material and Methods

Isolation of fungi and obtaining single-spores isolates

Fungal strains were isolated from diseased canola flowers that were collected from three sites (Crvenka, Rimski šančevi, Srbobran) from Serbia during 2010 year. Fragments of the diseased plant flowers were dipped into a solution of 3% sodium hypochlorite for 3 to 5 minutes, and then were washed with distilled water, and naturally dried in controlled conditions. After drying, the parts of the diseased tissue were placed on the PDA (potato dextrose agar) medium (Difco Detroit USA. The nutrient medium was added to 50 mg of streptomycin sulphate (Galenika Belgrade, Serbia) per liter. Petri dishes were kept at a temperature of $25^oC \pm 1^oC$. Obtaining single-spore isolates was performed on the follows: Pycnidiospores, which are released from the pycnidia in form droplets, the tip of the needle were transferred to plastic tubes containing previously added 2 ml of sterile water. The suspension pycnidiospores was poured on water agar. After 48^h, germinated pycnidiospore, together with a part of the substrate was transferred on the PDA medium, and placed in controlled conditions at $25\ ^oC \pm 1^oC$ in order to develop single-spores isolates. In this study, was investigated 9 isolates (C -1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9). In addition to these isolates were used and two reference strains obtained from the Centre for agricultural studies, Rothamsted, UK, and which are marked with L.m (*Leptosphaeria maculans*) and L.b (*Leptosphaeria biglobosa*) and two isolates originating from Serbia, which are marked with K-117, K-118.

Morphological characteristics

On PDA medium at a temperature $25^oC \pm 1^0C$ were studied morphological characteristics of the isolates: growth, appearance, color and appearance of the edge of the colony, formation of pigment as well as the structure and appearance of mycelium,then the size, shape and color pycnidiospora and pycnidia (Muntanjola-Cvetkovic 1987). Mycelium growth of the tested isolates were expressed in cm after 5, 10 and 15 days.

Pathogenicity tests

In addition to the morphological characteristics were investigated and pathogenic properties of isolates.In jiffy pots, which are were previously filled with compost,seedlings were planted at 5 germinated rapeseed cultivar Banaćanka. After germination (6-7 days) cotyledons were punctured with a sterile needle. On each wound a drop 5 µl of pycnidiospore suspension 10^6/ml was placed (Koch et al. 1991). The plants inoculated were transferred in controlled conditions at a temperature of 20^0C and 95% RH for 12^h photoperiod. After 48^h the plants were placed in the greenhouse. The first review of the plants was performed after 5 days and

the onset of symptoms and changes in plants have been observed in the next 20 days. Pathogenicity of isolates was assessed with + (causing the symptoms of the cotyledons) and - (no symptoms). Pycnidiospores suspension was prepared as follows: each isolate was placed on PDA medium in three replications. After 10-15 days based on binocular reviews in Petri dishes were added to 10 ml of sterile distilled water (Bonman et al. 1981. Sterile glass rod was gently withdrawn over the surface of pycnidia and mycelium to obtain his release pycnidiospores. Pycnidiospore released from Petri dishes were filtered through 10 MESHA sieve into a sterile plastic tube.The suspension pycnidiospores was kept 56h under controlled conditions at a temperature of 20^{o} C ± 1^{o} C and 12^{h} photoperiod.

Fungal DNA extraction

Total DNA isolates was extracted using the method described by O'Gorman et al. (1994) with the following modification by Kuusk et al. (2002). Mycelium and pycnidia were harvested from two-to-three week old PDA plates and frozen in liquid nitrogen. By Day and Shattock (1997) material was subsequently ground to powder and suspended in 800 µl extraction buffer (2% (w/v) CTAB 100 µl Tris-HCl pH 8,0, 20 mMEDTA pH 8,0, 1,4 MNaCl i 1% (w/v) polyvinyl pyrrolidone). After incubation at 65^{0}C for 1h, the mixture was added 600 µl of chloroform and vortexed for 10 seconds in the incubator at 25°C. The tubes were then centrifuged at 1300 g for 10 min. The liquid phase (approximately 500 ul) was transferred into a new tube and added to 300 µl isopropanol. Re-incubated for 10 min at room temperature and then centrifuged for 10 min at 1300 g. Following centrifugation the liquid was decanted from the tube and then added 600 µl of 70% ethanol. The tubes were vortexed for 10 seconds in an incubator and then centrifuged for 10 min at 1300 g. Open tube placed in a dryer at 50°C for 10 min. The resulting precipitate was dissolved in 100 µl TE buffer pH 8.0 and kept for several minutes at room temperature and then frozen at-20°C.

PCR analysis

Primers used for ITS amplification were deduced from flanking sequences of *Saccharomyces cerivisiae,* the 18S rDNA for primer PN3 (forward) (5´CCGTTGGTGAACCAGCGGAGGGATC) and the 28S rDNA PN10 (reverse) (TCCGCTTATTGATATGCTTAAG). Amplifications were performed in a total volumeof 25 µl containing: 1 µl fungal DNA, 11 µl H_2O, 12.5 µl master mix REDTaq (Sigma aldrich) with $MgCl_2$ (Taq polymerase 0.06 U/µl 3 mM $MgCl_2$ 0.002% gelatin 0.4 mM dNTP), 1.25µl each primer.(Balesdent et al.1998). The mixture is centrifuged briefly and then the tubes were placed in PCR (Eppendorf master cycler gradient).The reaction was performed for 37 cycles.Each cycle consisted of 30 s at 94ºC, 30 s at 58ºC and 1 min at 72ºC. PCR amplified fragments were visually observed on the 1.5% agarose gels stained with ethidium bromide .Marker (M)=DNA Length Standard 3000 bp, Eurofines, Gene Scan.

Results and disscusion

Symptoms

Leptosphaeria maculans is causing symptoms to the rapeseed plants from the stages cotyledon until maturity of crops. In addition to (hypocotyls, cotyledon, stem, upper stem, pod) parasite causes wilt and decay of flowers. In the early stages of the parasitic fungus causing wilt flowers (fig.1). Later, the flowers become rudimentary brown colour and remain on floral branches. At this stage, the parts of the flower and floral sprigs can be observed pycnidial fungi.

Fig.1. Rapeseed flowers wilt caused by the fungus *L. maculans* (initial and final symptom)

Morphological characteristics

On PDA medium after 5 days (at a temperature of $25^0C \pm 1^0C$) isolates form a roundish irregular colonies off-white color with an irregular edge. After 10 days, the center of the colony is black color because of the presence of pycnidia. Sustratna colony is also the form of irregular, off-white, with early accumulation of black pigment. After 15 days colonies due to strong sporulation have a brown color, and only certain parts of the edge of the colony are dirty white. With the age of the colony of the edge becomes more irregular. All the tested isolates and reference with LM have slow growth on PDA medium. Pycnidia are roundish, dark brown, singular or in stromatic, diameter 220 x 510 um. Pycnidiospores released from the pycnidia in the form of drops of gelatin, yellow to pink color. Pycnidiospores are unicellular, hyaline, shortly cylindrical, mostly straight, a slightly curved, with or without a drop of oil, the diameter of 1.30 x 2.20 x 2.80 to 5.40 um. Mycelial growth and sporulation of tested isolates is shown in Tab. 1.

Table 1. Diameter (cm) and sporulation of tested isolates of L. maculans on PDA medium and temperature of $25^0C \pm 1^0C$.

Isolate	**After 5 days**	**Sporulation**	**After 10 days**	**Sporulation**	**After 15 days**	**Sporulation**
L.m	1.51	-	3.30	++	4.70	+++
L.b	1.55	-	4.30	-	9	+

C-1	1.39	-	2.40	+	3.35	+++
C-2	1.45	-	2.95	++	4.60	+++
C-3	1.40	-	2.70	++	4.35	+++
C-4	1.53	-	3.00	+	4.10	+++
C-5	1.57	-	2.50	++	4.05	+++
C-6	1.80	-	3.40	++	4.80	+++
C-7	1.6O	-	2.90	++	4.50	+ + +
C-8	1,48	-	2,95	+ +	3,90	+ + +
C-9	1,50	-	2,40	+	3,05	+ + +
K-117	1,60	-	4,40	+	9	++
K-118	1,57	-	4,45	-	9	+

- No sporulation; + low sporulation; + + middle sporulation; + + + strong sporulation

Pathogenicity isolates

The first appearance of symptoms (after 5 days) could be observed on the inoculated part of the cotyledon in the form of chlorosis tissue. After 7 days, chlorosis covered the entire cotyledon. On the infected part of the tissue appeared grayish spots edged with dark margin (fig.2). After 15 days the center of the spot it had a pronounced greyish white color. During this period was observed the beginning of the formation of pycnidia in the center of the spots. In this study, all tested isolates showed high aggressiveness.

Fig. 3. Control (on the left) and symptoms of the disease (on the right)

Molecular characteristics

Size polymorphism of the ITS region was evident following amplification of fungal DNA using the PN3 and PN10 primers.The tested isolates (C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9), as well as reference strains L.m (*L. maculans*), PN3 and PN10 primers givig a 560 bp band (fig.3). Based on obtained fragments size tested isolates (C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9) belonging to the species L. maculans. With reference strains Lb (*L. biglobosa*) and K-117, K-118 size of the amplified fragment were 580bp

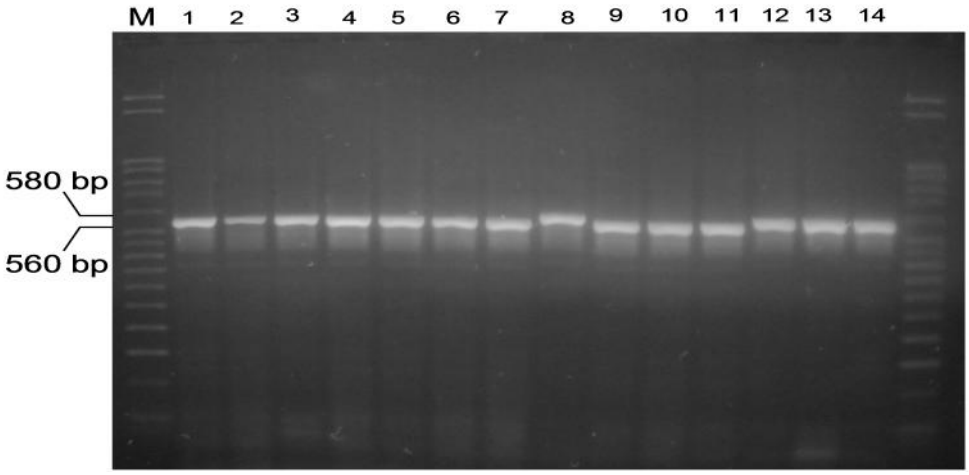

Fig.3. PCR amplified fragments with PN3 and PN10 primers. M 3000 bp marker, 1 C-1; 2 C-2; 3 C-3;4 C-4; 5 C-5; 6 C-6; 7 L.m; 8 L.b; 9 C-7; 10 C-8; 11 C-9; 12 L.b; 13 K-117; 14 K-118.

Among the many diseases, stem cancer is an economically important disease of oilseed rape in the world (Fitt et al., 2006). The parasite causes the symptoms of disease of the stages cotyledons until maturity rapeseed (Petrie, 1979; Paul and Rawlinson, 1992). Fungi in nature are maintained by pycnidia, mycelia and pseudothecia (Williams, 1992). The favorable climatic conditions for fungi (wet and rainy weather) pathogen can cause collapse flowers. In the initial stages of the disease occurs in the form of wilting flowers. Later, the flowers become rudimentary brown color and remain glued to twigs. At this stage floral sprigs and flowers become grayish white color on which were observed pycnidial fungi (Mitrovic and Trkulja, 2010).

On PDA medium, in the initial stages of development, and later, all of the isolates tested (C - 1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9) forming mycelium gray-white color which is in accordance with Williams & Fitt (1999) and Fitt et al. (2006) Aging colony forming black pigment in the medium (Pound, 1947; Humpherson-Jones, 1986; Williams and Fitt 1999; Mitrović and Marinković, 2007). Mycelia of all tested strains have slow and erratic increase in the PDA medium with a rim, which is often with irregular edges, which is in accordance with Pound (1947), Koch et al. (1989), Mitrović and Marinković (2007), Williams and Fitt (1999). However, there are report that some isolates (*L. maculans*) have a rapid increase in the nutrient medium (Delwiche 1980 cit. Loc. Williams 1992; Kharbanda and Stevens 1993, Salisbury et al. 1995), which is not the case with tested isolates. All the tested isolates on PDA medium, forming roundish pycnidia dark brown, singular or in stromatic Pycnidia diameter of 220 to 510 μm which is in accordance with Punithalingam and Holliday (1972). Pycnidiospores released from the pycnidia in the form of drops of gelatin, yellow to pink color. Pycnidiospores are unicellular, hyaline, shortly cylindrical, mostly straight, a slightly curved, with or without a drop of oil, the diameter of 1.30 x 2.20 x 2.80 to 5.40 um. Similar results were presented by other authors (Punithalingam and Holliday, 1972; Williams, 1992; Mitrović and Marinković, 2007; Mitrović, 2013). The appearance of spots on the infected cotyledons is very similar to the other authors (Hall, 1992; West et al. 1999; Brun et al. 1997; Mitrović, 2013). PN3 and PN10 primers in the tested isolates amplified DNA fragments of about 560 bp for species Leptosphaeria maculans and 580 bp for *Leptosphaeria biglobosa* type which is consistent with the results (Balesdent et al. 1998) while Mendes–Pereira et al. (2003) reported size of the amplified fragment were 468 bp for *Leptosphaeria maculans* (Tox^+ isolates representative) and 496 bp of *Leptosphaeria biglobosa*.

Conclusion

The tested isolates, from infected plants, are causing symptoms in the form of light gray to gray spots bordered by a dark rim within which are pycnidia. On PDA medium, colonies grow slowly and irregularly. Pycnidia are roundish black with a diameter of 220 to 510 um. Pycnidiospores are unicellular, hyaline, shortly cylindrical, mostly straight and slightly curved, with or without a drop of oil, the diameter of 1:30 x 2:20 x 2.80 to 5:40 m. PN3 and PN10 primers in the tested isolates amplified DNA fragments of about 560 bp. Based on the conducted research it can be concluded that all isolates belong to the species Leptosphaeria maculans.

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