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THE POSSIBILITY OF SPREADING *LEPTOSPHAERIA MACULANS* AND *LEPTOSPHAERIA BIGLOBOSA* BY RAPESEED SEED

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SUMMARY

Stem canker (blackleg) is economically the most important disease of oilseed rape worldwide. This disease is caused by two species of pathogenic fungi of the genus *Leptosphaeria*: *Leptosphaeria maculans* (Desm.) ces. and de Not anamorf *Phoma lingam* (Tode. Fr.) Desmas, that causes stem canker and blackleg root and *Leptospaeria biglobosa* Shoem and Brun, that causes symptoms such as cancers of the upper part of the stem usually causing less damage, and can cause serious damage in countries with higher summer temperatures. Pathogenic fungi from one area to another are transmitted by ascospores and contaminated (infected) seed. In order to prove the modes of transmission of parasites, the test is done transmitting the parasite by seeds. Disinfected seeds of oilseed rape cultivars Quinta were submerged in the suspension of pycnospores. Submerged seeds were kept at 20 º C ± 1 º C and 12 h photoperiod for 48 h. After planting in plastic containers seeds were kept at 25 º C ± 1 º C and 12h photoperiod. In this experiment the following isolates were used: C-5, L-5, K-7, LJ-3, S-11, St-1, GS-3 and Lm as a reference isolate (*L. maculans*) followed by K-113, K-115 and Lb as a reference isolate (*L. biglobosa*). The symptoms were assessed after 7 and 14 days after emergence. Ratings were made with + which meant visible symptoms on cotyledons or hypocotyl and – what referred to healthy plants. After 7 days, the isolates (Lm, C-5, L-5, K-7, LJ-3, S-11, St-1, GS-3) were caused disease symptoms on plants of oilseed rape. This pathogenicity is reflected in the number of seedlings, leaf spots and stalk lodging cotyledons. At izolate Lb (reference isolate *L. biglobosa*) K-113 and K-115 after 7 and 14 days was not observed pathogenicity on rapeseed plants. The control variety seeds were soaked in distilled water. The trial was set in 6 reps, and pathogenicity of fungal isolates in relation to the control was tested using Dunette's test. After 14 days re-isolation of pathogen was done.

Key words: *Leptosphaeria maculans*, *Leptospaeria biglobosa*, pathogenicity, pycnospores, seeds, stem canker

INTRODUCTION

Oilseed rape (*Brassica napus* var. *oleifera* L.) is one of the four most important oilseed plants in the world (Marinković et al. 2007). Rapeseed is parasited by growing number of pathogenic fungi: *Plasmodiophora brassicae* Wor. *Peronospora parasitica* (Pers.) Fr. *Alternaria brassicae* (Berk.) Sacc, *Leptosphaeria maculans* (Desm.) Ces. de Not, *Leptosphaeria biglobosa* Shoem and Brun, *Sclerotinia sclerotiorum* (Lib.) de Bary, *Botrytis cinerea* Pers, *Erisiphe cruciferarum* Opix ex Junell and some others. All the above types, depending on the climate and other factors, may result in minor or major damage to crops. Blackleg and stem canker is economically the most important disease of oilseed rape in Europe, Australia and North America (Fitt et al. 2006; Gosende

et al., 2003; Howlett et al. 2001) This disease is caused by two species of plant pathogenic fungi of the genus *Leptosphaeria*, *L. maculans* (Desm.) Ces. de Not, anamorphic stadium: *Phoma lingam* (Tode ex Fr.) Desm and *L. biglobosa* Shoem and Brun. Both types are present in all continents (Anon 2004 loc cit. Fitt et al. 2006). Pathogen on oilseed rape cause symptoms of cotyledon stage until ripening crops (Mitrovic and Trkulja.2010). At the root, stem and upper stem parasite causes the symptoms of canker, while on the leaf and pod occurs in the form of spots. When sowing infected seeds in presence of soil moisture pycnidia burst and release piknospore that can infect the hypocotyl or the symptoms manifest themselves in the form of spot of the cotyledons (Barbetti & Khangura 2000 cit. Loc. West et al. 2001). Within the spots on cotyledons new pycnidia are created. These pycnidia in favorable conditions released pycnospores that raindrops and insects transfer to the new plant. Pycnospore germinate in infectious hyphae and over stoma or wound to infect plant tissue. Pycnospores role in the epidemiology of the disease in Western Europe is minimal but it is very important in Western Australia (West et al. 2001). It was found that in Australia, infection of seedlings (cotyledon stage) except ascospores can cause fungus and piknospore (Barbetti & Khangura 2000 cit. Loc. West et al. 2001). The infected seeds can also be a source of inoculum for the initial infection of cotyledons. These infections result in the appearance of spots circular in shape with a large number of pycnidia formed in the early stages of plant development (Sylvester-Bradley & Makepeace 1985). The possibility of transferring pathogens seeds of cabbage, oilseed rape, radish and other *brassicas* is probably due to that the fungus is present in all continents (West et al. 2001).

MATERIALS AND METHODS

Isolation of fungi and obtaining pure cultures

Infected plants of oilseed rape were collected during 2009 /2010 in the region of Vojvodina. The infected plant parts (root, stem, upper stem, leaf, flower, pod, seeds) with certain symptoms of disease were used for isolation of fungi. Fragments of the diseased tissue were immersed in a 3% solution of sodium hypochlorite for a period of 3-5 minutes, and then were washed with distilled water, and naturally dried in a controlled environment. After drying, the affected tissue fragments were applied to the culture medium of potato dextrose agar (PDA) (Difco Detroit USA) which was previously poured into Petri dishes. To prevent bacterial growth, the substrate was added 50 mg of streptomycin sulfate (Galenika Belgrade, Serbia) per liter. Petri dishes seeded with such a substrate are placed in a thermostat at a temperature of 25 ± 1 ° C. After 10 to 15 days was observed the formation of pycnidia and pycnospora under binocular. Pycnospore, which are released in the form of droplets of pycnidia are transferred to the tip of the needle into the plastic tube which was previously added 2 ml of sterile water. Prepared conidial suspension was poured into Petri dishes. After 48 hours of germination of conidia was observed under the binocular. Germinated conidia, together with part of the nutrient medium, were transferred to PDA medium and placed in a thermostat at 25 ± 1 º C in order to obtain pure cultures of the fungus. In this way was isolated 119 strains of the fungus, while the present studies the following isolates were used: C-5, L-5, K-7, LJ-3, S-11, St-1, GS-3 and Lm as a reference isolate (*L. maculans*) followed by K-113, K-115 and Lb as a reference isolate (*L. biglobosa*). Reference isolates originate from the Centre for Agricultural Studies, Rothamsted, UK.

Disinfection of seeds

The seeds were disinfected by dipping in a 3% solution of sodium hypochlorite (NaOCl) for 3 to 5 min, and then were washed with tap water and dried at room temperature under controlled conditions. The success of disinfection was tested on PDA nutrient medium. In two Petri dishes was applied disinfected seed of cultivar Quinta. As a control were used not disinfected seeds. In each Petri dish were placed 5 seeds. Thus prepared Petri dishes were placed in a thermostat at 25^0C ± 1^0C in the dark. After 15 days was carried out visual and microscopic review of seed, nutrient medium and seedlings.

Spreading of pathogens by seed

In order to prove the way of spreading parasites, the test is done on spreading of the parasite by seeds. The suspension of pycnospores was prepared as follows: Each isolate was applied to a PDA nutrient medium with three replications. After 10-15 days on the basis a binocular reviews in Petri dishes was added 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 release of piknospora. In prepared suspension of pycnospora and mycelium is submerged seed of rapeseed cultivar Quinta. The submerged seed was kept at 20 º C ± 1 º C and 12 h photoperiod for 48 h. By 5 inoculated seeds were then sown in plastic containers with cell diameter 4 x 5 cm filled with compost. After planting, plastic containers were kept at 25 º C ± 1 º C and 12h photoperiod. The appearance of symptoms was assessed after 7 and 14 days after emergence of the plants. Ratings were made with + which meant visible symptoms on cotyledons or hypocotyl and – what referred to healthy plants. The control variety seeds were soaked in distilled water. The experiment was set to 6 replicates and pathogenicity of fungal isolates in relation to the control was tested using Dunette's test (Table 1). After 14 days was performed re-isolation pathogen.

RESULTS AND DISCUSSION

Number of infected (failed) and healthy plants was the main criterion for assessing the level of pathogenicity (transfer of parasite seed) of tested isolates. After 7 days, all the isolates (Lm, C-5, L-5, K-7, LJ-3, S-11, St-1, GS-3) are shown at specific pathogenicity of rapeseed plants (Fig. 1.2). The pathogenicity was manifested in the number of seedlings, the number of lodged plants, leaf spot cotyledon and hypocotyl (Fig. 1) and (Fig. 2). At L.b (reference isolate *L. biglobosa*) K-113 and K-115 strains after 7 days was not observed pathogenicity on oilseed rape plants. Number of seedlings in these isolates was similar to the control treatment (Graph.1). Even after 14 days (Lb, K-113 and K-115 isolates) was not observed occurrence of symptoms on plants (Graph. 2)

Table 1. Pathogenicity of isolates compared to the control application (Dunette's test).
Tabela 1. Patogenost izolata u poređenju sa kontrolom (Danetov test).

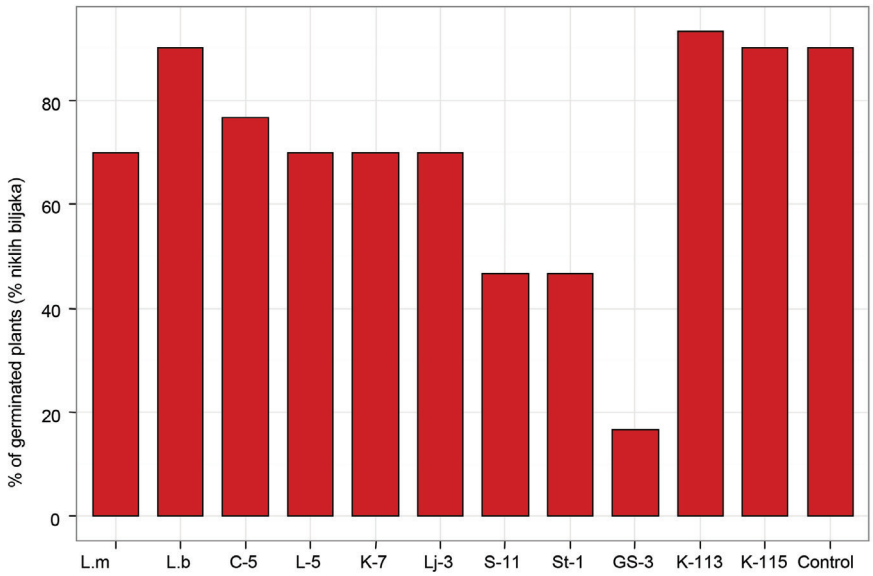
Isolates (Izolati)	% of germinated plants after 7 days (% niklih biljaka posle 7 dana)	% of healthy plants after 14 days (% zdravih biljaka posle 14 dana)
L.m	ns[a]	*
L.b	ns	ns
C-5	ns	**
L-5	ns	**
K-7	ns	**
Lj-3	ns	**
S-11	**	**
St-1	**	**
GS-3	**	**
K-113	ns	ns
K-115	ns	ns

ns- not significant; *$P < 0.05$; **$P < 0.01$
(ns- nije značajno)
[a]all comparisons were done with *arcsin* transformated data
([a]sva poređena rađena sa *arcsin* transformisanim podacima)

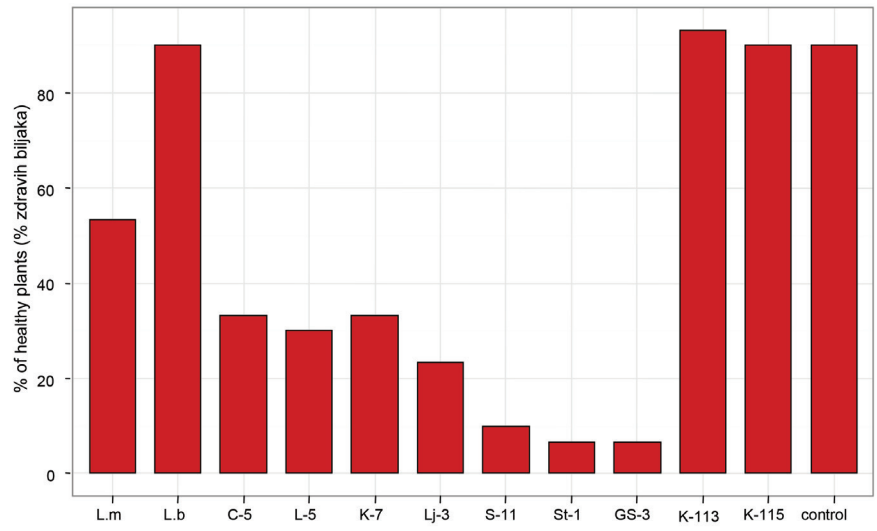
Fig.1. Symptoms in oilseed rape plants originated from seeds inoculated with L. maculans.
Slika 1. Simptomi na biljkama uljane repice kao posledica zaraženosti semena gljivom L. maculans.

Fig.2. Symptoms on the hypocotyl and cotyledons of rapeseed plants caused from infected seed with *L. maculans* (left: infected plants, right: healthy plant).
Slika 2. Simptomi na hipokotilu i kotiledonima biljaka uljane repice kao posledica zaraženosti semena gljivom *L. maculans* (le vo: zaražene biljke, desno zdrave biljke).

Limiting factor in the production of rapeseed, which in recent years expanding the production regions of our country, are a great number of plant pathogenic fungi. Among the many diseases, stem canker is an economically important disease of oilseed rape in the world (Fitt et al., 2006), and probably in the near future with us. The parasite causes the symptoms on cotyledon and growth stages until maturity (Petrie, 1979, Paul and Rawlinson, 1992). The fungus is naturally maintained by pycnidia, mycelia and pseudotecija (Williams, 1992). On the basis of performed tests all isolates: Lm, C-5, L-5, K-7, LJ-3, S-11, St-1, GS-3 caused the symptoms of the disease on rapeseed seedlings except isolates of Lb, K -113, and K-115, which means that they cannot be transmitted by seed of rapeseed (Graph.1). After 14 days, the percentage of dead plants, arising from infected seed has increased among most of the isolates, which agrees with the results of Bonman et al. (1981). The appearance of symptoms of the hypocotyl and cotyledons infected seeds, indicate that the parasite may be transmitted in this way (Petrie 1979b, 1992 Hall, Wood and Barbetti, 1977a). Wood & Barbetti (1977a) reported that in Western Australia, the percentage of infected seeds ranges from 0.1-0.2%, while in Canada it ranges up to 5% (Hall et al. 1996). Gabrielson (1983) reported that the seed can be infected up to 18%. In addition to oilseed rape pathogen is transmitted by other *brassica* (Gugel & Petrie 1992). The presence of *L. maculans* and *L. biglobosa* in the world where rape is grown and on all continents can be interpreted as the ability to transfer them to the seed of this plant (West et. al 2001). The frequent occurrence of symptoms on cotyledons and hypocotyl in Australia,



Graph. 1. Pathogenicity of isolates on the oilseed rape plants after 7 days.
Grafik 1. Patogenost izolata na biljkama uljane repice posle 7 dana.



Graph. 2. Pathogenicity of isolates on the oilseed rape plants after 14 days.
Grafik 2. Patogenost izolata na biljkama uljane repice posle 14 dana.

despite the ascospores is associated with frequent contamination of seeds (Barbetti and Khangura cit. Loc. West et al. 2001, West et al., 2001). Although infections are on rare seeds, can be very important in the spread of pathogens into new regions (Jacobsen & Williams, 1971), or the spread of the fungus within the same and neighbouring regions (Bonman et al., 1981). In experiments in Canada, frequent infections seeds initially caused a leaf spot on plants and later pathogen caused stem canker (Hall et al. 1996). These statements confirm our examination of the possibility of transmission of the fungus by the seed.

CONCLUSION

Isolates C-5, L-5, K-7, LJ-3, S-11, St-1 and GS-3 caused symptoms after 7 and 14 days, whereas occurrence of symptoms at K-113 and K-115 was not observed. The results obtained in these studies provide new information on pathogenic fungi of stem canker on rapeseed in Serbia, which contributes to a better understanding of the epidemiology of the disease and easier control. Based on the conducted research it can be concluded that the pathogenic fungus *L. maculans* and *L. biglobosa* can be transmitted also by rapeseed seed.

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MOGUĆNOST PRENOŠENJA *LEPTOSPHAERIA MACULANS* I *LEPTOSPHAERIA BIGLOBOSA* SEMENOM ULJANE REPICE

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REZIME

Rak stabla (crna noga) je najznačajnije oboljenje uljane repice širom sveta. Bolest mogu prouzrokovati dve vrste patogenih gljiva iz roda *Leptosphaeria*: *Leptosphaeria maculans* (Desm.) ces. i de Not, anamorf *Phoma lingam* (Tode. Fr.) Desmas, koja prouzrokuje rak stabla i crnu nogu na korenu i *Leptospaeria biglobosa* Shoem and Brun, koja prouzrokuje simptome slične raku ali na gornjim delovima stabla obično prouzrokujući manje štete. Ozbiljnije štete mogu nastati u zemljama sa višim letnjim temperaturama. Patogene gljive se iz jedne oblasti u drugu prenose askosporama i zaraženim semenom. Da bi se utvrdili načini prenošenja parazita testirana je mogućnost prenošenja semenom. Dezinfikovano seme uljane repice, kultivara Quinta je potopljeno u suspenziju piknospora. Ovakvo seme je držano na 20ºC ± 1ºC i 12h fotoperiodu tokom 48 sati. Posle setve seme je preneto u plastične posude a temperatura čuvanja je bila 25ºC ± 1º C i fotoperiod od 12h. U ovom eksperimentu korišćeni su sledeći izolati: C-5, L-5, K-7, LJ-3, S-11, St-1, GS-3 i Lm kao referentni izolat (*L. maculans*) kao i K-113, K-115 i Lb kao referentni izolat (*L. biglobosa*). Ocena prisustva simptoma je rađena posle 7 i 14 dana od nicanja. Sa znakom + su se obeležavale biljke sa vidljivim simptomima na kotiledonima ili hipokotilu a znakom – biljke bez pojave simptoma. Posle 7 dana izolati Lm, C-5, L-5, K-7, LJ-3, S-11, St-1 i GS-3 su izazvali simptome bolesti na biljkama uljane repice. Patogenost se ogledala kroz broj oštećenih klijanaca, lisnih pega i povijenih kotiledona. Kod izolata Lb (referentni izolat *L. biglobosa*) K-113 i K-115 posle 7 i 14 dana nije primećena patogenost na biljkama uljane repice. Kontrolna varijanta semena je potapana u destilovanu vodu. Ogled je postavljen u 6 ponavljanja, i patogenost izolata gljive u odnosu na kontrolu je izražena Danetovim testom. Posle 14 dana izvedena je ponovna izolacija patogena.

Ključne reči: *Leptosphaeria maculans*, *Leptospaeria biglobosa*, patogenost, piknospore, seme, rak stabla

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