Biotechnology in Animal Husbandry 20 (1- 2), p 101 - 108, 2004
Publisher: Institute for Animal Husbandry, Belgrade-Zemun

ISSN 1450-9156
UDC: 632.35

# INCIDENCE OF FUSARIUM SPECIES ON RED CLOVER SEED[1]

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*Abstract:* Phytopathological investigation of seed of three commercial red clover cultivars – K9, K17 and NS Kolubara, and two pre-treatments of seeds – rinsing and surface disinfection of seeds ($T_1$) and surface disinfection of seeds ($T_2$) have determined that frequency of species within the genus *Fusarium* varied from 0 to 13%. By use of $T_1$ pre-treatment the presence of *Fusarium* species on the seed was observed in neither of examined cultivars. In this pre-treatment the presence of 1% *Penicillium* spp. was observed on seed of cultivar K17 only, while the seeds K9 and NS Kolubara were non-infected. By use of $T_2$ pre-treatment, the frequency of *Fusarium* species varied from 1 to 13%, depending on cultivar. From the seeds of all three examined cultivars, *Fusarium verticillioides* (2-13%) and *F. proliferatum* (2-6%) were isolated and from the seed of cultivar K9, *F. oxysporum* (4%), *F. solani* (2%) and undetermined *Fusarium* species (3%) were isolated, while *F. subglutinans* (1%) was isolated only from the seed of NS Kolubara. Besides *Fusarium* species, by application of pre-treatment $T_2$, in all examined cultivars the species from genera *Cladosporium* (6-14%), *Alternaria* (6-7%) and *Acremonium* (1-5%) were isolated, and *Penicillium* spp. (1%) from the seed of cultivar K17. The results obtained indicate that frequency of mycobiota on the seed of red clover depended more on the seed pre-treatment than on the examined cultivars.

Considering the significant differences gained in health state of seed by application of $T_1$ and $T_2$ pre-treatments, further investigations should be directed towards the development of method which would show real health state of seed, that is, natural contamination of seed. In this case it is the use of pre-treatment $T_1$.

*Key words:* Fusarium spp., incidence, red clover (Trifolium pratense L.), seed infection.

## *Introduction*

Seed of grown plants represents favourable substrate for development of numerous phytopathogene and saprobe fungus. Degree of change, accordingly, depends on type of seed, pathogens and environmental factors (*Milošević et al., 1995*). Symptoms expressed on red clover seed and seed of other legumes, caused by pathogen organisms, are manifested as seed rot, reduction of seed germination, damping-off, and post emergence seedling root-rot (*Tyler et al., 1956; Schmithenner, 1964; Hanckok, 1983*). Seed coat, cotyledons, young shoots and leaf primordia of red clover and alfalfa could be invaded by hyphae pathogen fungus from the

[1] Original scientific paper – Presented on poster section of the 7. International Symposium "Modern trends in Livestock production", Belgrade, 30.09. to 3.10. 2003.

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genus *Fusarium* (*Chi et al., 1964*). Red clover, alfalfa, sweetclover, and Ladino white clover were extremely susceptible to some species of Pythium when inoculated at the time of seeding or within 1 day thereafter (*Halpin and Hanson, 1958*).

According to the method *Kilpatrik et al. (1954a)*, *Fusarium* spp. have been isolated from diseased red clover roots more frequently than any other fungi (*Kilpatrick et al., 1954b; Fulton and Hanson, 1960*). The most frequent being *Fusarium moniliforme* Sheldon., *F. oxysporum Schlect*, *F. solani (Mart.)* App. & Wr. and *F. roseum* Lk. From other species of fungi usually the species from the genera *Gliocladium, Rhizoctonia, Pythium, Phoma, Penicillium, Trichoderma, Alternaria, Aspergillus, Mucor, Chaetomium and other Pythium* spp. were most commonly isolated from very young plants of red clover (*Kilpatrick et al., 1954b; Fulton and Hanson, 1960*).

During 1997. in the area of Kruševac town severe wilting of red clover on the surfaces meant for the production of fodder and seeds was observed. Disease was caused by the infection of root and tracheomycosis by *Fusarium* species (*Urošević et al., 1999*). Obtained isolates of this species have expressed pathogenicity to red clover (80%), alfalfa (63), white clover (67%), soybean (40), beans (23%), peas (17%), lupin (13%) and fodder peas (7%). According to authors' description regarding morphological properties of fungi it could be concluded that it was *Fusarium oxysporum*.

In Serbia there are little data on agens of red clover, especially of certain species of the genus *Fusarium*. Also, as there is little routine experience on their identification, and in seed health testing the of commercial red clover cultivars only the genus Fusarium, but not the species, is being determined. Considering the importance of use of healthy seeding material, and in order to determine the significance of certain species of the *Fusarium* genus within etiology of red clover seed diseases, in this paper the study on the nature of infection of the seed of commercial red clover cultivars most often grown in Serbia was carried out.

## *Material and Methods*

Investigations were carried out in Laboratory for Phytopathological Research in the Maize Research Institute in Zemun Polje. As initial material in this research seed of three red clover cultivars (K9, K17 and NS Kolubara), of the 2001 harvest was used.

For isolation of fungi from red clover seed the method of nutrient media was used with seed pre-treatment. In first pre-treatment ($T_1$) seed was rinsed for 4 hours in running tap water, surface disinfected for 5 min. in 1% sodium hypochlorite solution (NaOCl), rinsed three times in sterile water and dried between two filter papers. In second pre-treatment ($T_2$) surface disinfections with of seed was carried out. Of each cultivar 100 seeds were examined being distributed in 10 Petri dishes, 10 seeds per dish, were seeded to water agar and incubated on room temperature.

After 10 days colonies developed around seed were analysed microscopically and for further identification sample of colony was transferred on two medias: synthetic low nutrient media (SNA) with 2 x 4 cm sterile filter paper (*Nirenberg and O'Donnell, 1998*) in Petri dishes of diameter of 9 cm, and on media containing of 2% agar and 5 sterile carnation leaves (CLA) in Petri dish of 6 cm diameter (*Fisher et al., 1982*).

Determination of *Fusarium* species was carried out by colony inspection *in situ* and native preparations prepared from fungus cultures grown on SNA and CLA nutrient media (*Nelson et al., 1983; Burgess et al., 1994*).

*Results*

Microscopic observation of fungus colonies isolated from red clover seed has determined smaller differences in regard to infection of seed between investigated cultivars than in different pre-treatments (Table 1). In pre-treatment $T_1$ no infection of seed by the species from *Fusarium* genus was observed except for the presence of 1% *Penicillium* spp. on seed of cultivar K17. By application of pre-treatment $T_2$, *Fusarium* species were isolated from 1 to 13%, depending on tested cultivar. *Fusarium verticillioides* (Sacc.) Nirenberg (syn. *Fusarium moniliforme*) was determined in high percentage (13%) on seed of cultivar K17, relatively high percentage on seed of cultivar NS Kolubara (5%) and in low percentage on seed of cultivar K9 (2%). Presence of *F. proliferatum* (Matsushima) Nirenberg was determined in relatively high percentage on seed of cultivar K9 (6%) and in low percentage on seed of cultivar NS Kolubara (3%) and K17 (2%). From seed of K9 cultivar some other species of *Fusarium* genus were isolated and they are *F. oxysporum* (4%), *F. solani* (2%) and *Fusarium* spp. (3%), and *F. subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas (1%) from the seed of cultivar NS Kolubara.

In seed pre-treatment $T_2$, besides *Fusarium* species, the species from genera *Acremonium, Alternaria, Cladosporium and Penicillium* were isolated as well. By application of this pre-treatment the most often isolated species were those from genus *Cladosporium*, and that in high percentage on seed of cultivar NS Kolubara (14%) and K9 (12%) and in relatively high percentage on seed of cultivar K17 (6%). Relatively high percentage of presence of *Alternaria* spp. was determined on seed of all investigated cultivars (6-7%). Presence of *Acremonium* spp. was established in relatively high percentage on seed of cultivar NS Kolubara (5%) and in low percentage on seed of cultivars K9 and K17 (1%), while species from *Penicillium* genus (1%) was established only on seed of cultivar K17.

By application of pre-treatment $T_1$ the interior (natural) seed contamination was established and with the application of pre-treatment $T_2$ the surface infection of seed of red clover was established (*Lević, personal communication*).

*Table 1. Frequency (%) of Fusarium, Acremonium, Alternaria, Cladosporium and Penicillium species on seed of various red clover cultivars (Trifolium pratense L.)*
*Tabela 1. Učestalost (%) Fusarium, Acremonium, Alternaria, Cladosporium i Penicillium vrsta na semenu različitih sorti crvene deteline (Trifolium pratense L.)*

| Cultivar/ Sorta | Intensity of seed infection/Intenzitet infekcije semena (%) | | | |
|---|---|---|---|---|
| | Pre-treatment/ Pretretman T | | Pre-treatment/ Pretretman T | |
| | Fungus type/ Tip gljivice | % | Fungus type/ Tip gljivice | % |
| K9 | -* | 0 | Acremonium spp. | 1 |
| | | | Alternaria spp. | 7 |
| | | | Cladosporium spp. | 12 |
| | | | Fusarium spp. | 3 |
| | | | Fusarium verticillioides | 2 |
| | | | Fusarium oxysporum | 4 |
| | | | Fusarium proliferatum | 6 |
| | | | Fusarium solani | 2 |
| K17 | | | Acremonium spp. | 1 |
| | | | Alternaria spp. | 6 |
| | | | Cladosporium spp. | 6 |
| | | | Fusarium proliferatum | 2 |
| | | | Fusarium verticillioides | 13 |
| | Penicillium spp. | 1 | Penicillium spp. | 1 |
| NS Kolubara | -* | 0 | Acremonium spp. | 5 |
| | | | Alternaria spp. | 7 |
| | | | Cladosporium spp. | 14 |
| | | | Fusarium proliferatum | 3 |
| | | | Fusarium subglutinans | 1 |
| | | | Fusarium verticillioides | 5 |

-* pathogens were not found/nisu pronađeni patogeni

## *Discussion*

Many diseases of red clover (*Trifolium pratense L.*) are limiting the yield, quality and longevity of crops of this important fodder plant for animal feeds. One of the major problems in the production of red clover is the establishment and maintenance of stands. Lack of persistence is a major problem. Several factors are involved, one of which is the root and crown disease complex. Stand losses are most conspicuous in the second year, but also occur commonly in the first. In the humid areas of the United States, few red clover fields remain productive beyond the second year after seeding (*Kilpatrick et al., 1954a; Moody et al., 1967*).

Studies on mycobiota of seed of red clover cultivars most often grown in Serbia have shown that, in natural conditions, they are, depending on cultivar, infected by the species

from *Fusarium* genus up to 13%, the frequency of *F. verticillioides* being very high therein. According to literature references *F. moniliforme* (syn. *F. verticillioides*), *F. oxysporum* and *F. solani* could be considered as potential inducers of disease in red clover. Mentioned species have proved their pathogenicity in regard to root of red clover and other hosts when inoculated at the tip of root (*Leath and Kendall, 1978*).

In laboratory tests on red clover seedlings isolates *F. oxysporum, F. solani and F. roseum* Link have caused a disease whose intensity ranged from 23 to 95%. Isolates of *Chaetomium, Mucor, Aspergillus, Alternaria, Penicillium, and Trichoderma* were in general either nonpathogenic or only weakly pathogenic of red clover seedlings (*Kilpatrick et al., 1954b*). Results of the tests made in greenhouse show that all the isolates of *Pythium debaryanum* Hesse tested and some of the isolates of *F. oxysporum, F. roseum, and F. solani* were very pathogenic on red clover seedlings while some isolates *Rhizoctonia, Phoma and Gliocladium* were moderately pathogenic (*Kilpatrick et al., 1954b*).

Symptoms incited by pathogenic isolates included pre-emergence killing, post-emergence damping-off, stunting, and rotting of the roots and hypocotyls (*Kilpatrick et al., 1954b*). Many pathogenic isolates of *F. oxysporum and Phoma* spp. caused post-emergence damping-off. The most pathogenic isolates of *F. oxysporum* caused a severe necrosis of the upper part of the primary root and lower part of the hypocotyl. Also, in these parts of root dark brown to black necrosis was caused by pathogenic isolates from *Phoma and Rhizoctonia* species. When the rotting was severe the seedlings would fall to the ground and die. *F. solani* caused a severe dark brown to black decay of the entire root system. The pathogenic isolates of *F. roseum* caused a brown rot of the roots and lower part of the hypocotyls. *Gliocladium roseum* Bainier caused a dark brown rot of the roots and frequently of the lower part of hypocotyl. The most severe type of preemergence killing was caused by *P. debaryanum*.

In our country *Todorov et al. (1995)* have established that wilting of red clover is caused by *F. oxysporum* Schl. f. sp. *medicaginis* (Weimer) Snyder & Hansen, while *Radović et al. (1999)* observed that genotypes of red clover which have proved resistant to fusaric acid - toxin, have also proved resitant to *F. oxysporum f. sp. medicaginis.*

Obtained results indicate the significance of rinsing of seed ($T_1$) in elimination of presence of fungi on seed. This measure contributes to improvement of health condition of red clover seed. In practice, however, there is no example confirming the actual application of this or similar method of preventive protection of seed from infection. One of the reasons is insufficient knowledge of the role of seed as carrier of some pathogen fungi that are important from the economical aspect, including species of Fusarium genus, as well as economical and practical importance.

## *Conclusions*

Results obtained on the basis of conducted research indicate that:

Two different seed pre-treatments, which mutually differed in 4 h rinsing of seed ($T_1$) and non-rinsing of seed ($T_2$) before surface-sterilisation have brought about. By application of pre-treatment $T_1$ in all tested cultivars the presence of *Fusarium* species from 1 to 13% was observed. *Fusarium verticillioides* and *F. proliferatum* were isolated from seed of all tested cultivars, while *F. oxysporum, F. solani, F. subglutinans* and *Fusarium* spp. were isolated from the seed of one cultivar only. Also, the fungi from genera *Acremonium, Alternaria,*

*Cladosporium and Penicillium* were isolated.

Considering the significant differences gained in health state of seed by application of $T_1$ and $T_2$ pre-treatments, further investigations should be directed towards the development of method which would show real health state of seed, that is, natural contamination of seed. In this case it is the use of pre-treatment $T_1$.

## UČESTALOST FUSARIUM VRSTA NA SEMENU CRVENE DETELINE

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*Rezime*

Fitopatološkim ispitivanjima semena tri komercijalna kultivara crvene deteline - K9, K17 i NS Kolubara, i dva pretretmana semena - ispiranje i površinska dezinfekcija semena ($T_1$) i površinska dezinfekcija semena ($T_2$), utvrđeno je da je učestalost vrsta iz roda *Fusarium* varirala od 0 do 13%. Primenom $T_1$ pretretmana nije utvrđeno prisustvo *Fusarium* vrsta na semenu ni u jednog ispitivanog kultivara. U ovom pretretmanu je samo na semenu kultivara K17 ustanovljeno prisustvo 1% *Penicillium* spp., dok je seme K9 i NS Kolubara bilo bez zaraze. Primenom $T_2$ pretretmana, učestalost *Fusarium* vrsta je varirala od 1 do 13%, zavisno od kultivara. Iz semena sva tri ispitivana kultivara izolovane su *Fusarium* verticillioides (2-13%) i *F. proliferatum* (2-6%) dok su iz semena kultivara K9 izolovane *F. oxysporum (4%), F. solani (2%)* i nedeterminisane *Fusarium* vrste (3%), a *F. subglutinans* (1%) jedino iz semena NS Kolubara. Pored *Fusarium* vrsta, primenom pretretmana kod svih ispitivanih kultivara, izolovane su vrste iz rodova *Cladosporium (6-14%), Alternaria (6-7%) i Acremonium (1-5%)*, a *Penicillium spp. (1%)* iz semena kultivara K17. Dobijeni rezultati ukazuju da je učestalost mikobiota na semenu crvene deteline više zavisila od pretretmana semena nego od ispitivane sorte.

S obzirom da su konstatovane značajne razlike u zdravstvenom stanju semena primenom $T_1$ i $T_2$ pretretmana, buduća istraživanja trebalo bi usmeriti u pravcu razvoja metode koja pokazuje realno zdravstveno stanje semena, odnosno prirodnu zarazu semena. U ovom slučaju to je primena pretretmana $T_1$.

*Ključne reči:* crvena detelina *(Trifolium pratense L.), Fusarium* spp., infekcija semena, učestalost.

*Literature*

1. BURGESS, L. W., SUMMERELL, B.A., BULLOCK, S., GOTT, K.P., BACKHOUSE, D. (1994): Laboratory Manual for *Fusarium* Research. Fusarium Research Laboratory, Department of Crop Sciences, University of Sydney and Royal Botanic Gardens, Sydney, pp. 133.
2. CHI, C.C., CHILDERS, W.R., HANSON, E.W. (1964): Penetration and subsequent development of three *Fusarium* species in alfalfa and red clover. Phytopathology 54: 434-437.

3. FISHER, N. L., BURGESS, L.W., TOUSSOUN, T.A., NELSON, P.E. (1982): Carnation leaves a substrate and for preservating cultures of *Fusarium* species. Phytopathology 72: 151-153.
4. FULTON, N.D., HANSON, E.W. (1960): Studies on root rots of red clover in Wisconsin. Phytopathology 50: 541-550.
5. HALPIN, J. E., HANSON, E. W. (1958) : Effect of age of seedlings of alfalfa, red clover, Ladino white clover, and sweetclover on susceptibility to Pythium. Phytopathology 48 (9): 481-485.
6. HANCOCK, J.G. (1983): Seedling diseases of alfalfa in California. Plant Disease 67: 1203-1208.
7. KILPATRICK, R.A., HANSON, E.W., DICKSON, J.G. (1954a): Root and crown rots of red clover in Wisconsin and the relative prevalence of associated fungi. Phytophathology 44: 252-259.
8. KILPATRICK, R.A., HANSON, E.W., DICKSON, J.G. (1954b): Relative pathogenicity of fungi associated with root rots of red clover in Wisconsin. Phytophathology 44: 292-297.
9. LEATH, K.T., KENDALL, W.A. (1978): *Fusarium* root rot of forage species: pathogenicity and host range. Phytopathology 68: 826-831.
10. LEVIĆ, J. Personal communication.
11. MILOŠEVIĆ, M., KONSTANTINOVIĆ, B., ZLOKOLICA, M., DRAGANIĆ, M. (1995): Miko ora semena pšenice, ječma i kukuruza. Zaštita bilja 46 (3) 213: 221-227.
12. MOODY, A.R., KILPATRICK, R.A., RICH, A.E. (1967): Effect of cutting height on development of root rot in clover. Plant Disease Reporter 51: 826-828.
13. NELSON, P.E., TOUSSOUN, T.A., MARASAS, W.F.O. (1983): *Fusarium* Species, an Illustrated Manual for Identification. The Pennsylvania State University Press, University Park and London, pp. 133.
14. NIRENBERG, H., O'DONNELL, K. (1998): New *Fusarium* species and combinations within the *Gibberrella fujikuroi* species complex. Mycologia 90: 434-458.
15. RADOVIĆ, J., UROŠEVIĆ, B., LUGIĆ, Z., NEŠKOVIĆ M. (1999): *In vitro* selekcija crvene deteline (*Trifolium pratense L.*) na otpornost prema *Fusarium oxysporum* f. sp. *medicaginis*. Program i izvodi saopštenja: 91 str. XIII simpozijum Jugoslovenskog društva za fiziologiju biljaka. Beograd.
16. SCHMITTHENNER A. F. (1964): Prevalence and virulence of *Phytophtora, Aphanomyces, Pythium, Rhizoctonia, and Fusarium* isolated from diseased alfalfa seedlings. Phytopathology 54: 1012-1018.
17. TYLER, L. J., MURPHY, R. P., MACDONALD, H. A. (1956): Effect of seed treatment on seedling stands and on hay yields of forage legumes and grasses. Phytopathology 46: 37-44.
18. TODOROV, D., STOJŠIN, V., PETROVIĆ, R., MILIJIĆ S. (1995): Paraziti i štetočine krmnog bilja i njihovo suzbijanje. Zbornik rezimea: 45-46 str. Drugo jugoslovensko savetovanje o zaštiti bilja. Vrnjač21. ka Banja, 30. oktobar – 3. novembar 1995.
19. UROŠEVIĆ, B., TOMIĆ, Z., RADOVIĆ, J. (1999): *Fusarium* sp. prouzrokovač23. uvelosti crvene deteline. Zaštita bilja 50 (4): 311-318.