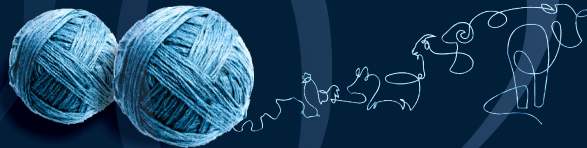


13th
INTERNATIONAL
SYMPOSIUM

MODERN
TRENDS
IN LIVESTOCK
PRODUCTION



P R O C E E D I N G S

6 - 8 October 2021, Belgrade, Serbia

Institute for Animal Husbandry
Belgrade - Zemun, SERBIA

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INTERNATIONAL
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**MODERN
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6-8 October 2021, Belgrade, Serbia

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FUSARIUM SPP. AND DEOXYNIVALENOL CONTAMINATION OF RYEGRASS SEEDS

**Vesna Krnjaja¹, Violeta Mandić¹, Zorica Bijelić¹, Slavica Stanković²,
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Abstract: The aim of this study was to evaluate fungal infection, with a special focus on *Fusarium* spp. and deoxynivalenol (DON) presence, as a participant in Fusarium head blight (FHB) pathogenesis in two cultivars of Italian ryegrass K-13 and K-29. A total of 24 seed samples were collected during harvest in 2019.

By mycological analyses, *Acremonium* spp., *Alternaria* spp., *Fusarium* spp. and non-sporulating species (*Mycelia sterilia*) were isolated on the seeds of both cultivars of Italian ryegrass. Among *Fusarium* spp., four species, *F. graminearum*, *F. poae*, *F. proliferatum* and *F. subglutinans* were identified in cultivar K-13 and three species, *F. graminearum*, *F. poae* and *F. subglutinans*, in cultivar K-29. *F. graminearum* and *F. poae* were identified as FHB pathogens, of which *F. graminearum* was dominant in both cultivars with 20.5% (cultivar K-13) and 32% (cultivar K-29) compared to *F. poae* which was present in both cultivars with a frequency of 0.5%. The frequency of DON positive samples was 100%. A statistically significant difference in DON level was found between the two tested cultivars, with a higher DON level in cultivar K-29 (5334.33 $\mu\text{g kg}^{-1}$) compared to cultivar K-13 (4738.58 $\mu\text{g kg}^{-1}$).

The obtained results indicate that two *Fusarium* species, *F. graminearum* and *F. poae*, were FHB pathogens, with *F. graminearum* as the predominant species in both cultivars of Italian ryegrass. High DON levels (>3000 $\mu\text{g kg}^{-1}$) in the tested seed of Italian ryegrass indicate on potentially significant participation of DON in FHB pathogenesis, as well as a potential risk for the quality seed production, feed safety and the food chain in general. In Serbia, this is the first report about *Fusarium* infection and DON presence in ryegrass seed.

Key words: *Fusarium* spp., deoxynivalenol, Italian ryegrass

Introduction

Italian ryegrass (*Lolium multiflorum* Lam.) is annual and also biennial or short-lived perennial bunchgrass. It is a high-yielding and high-quality fodder crop native to southern Europe. It is an integral part of pasture for hay and silage production. Its high nutrition, digestibility, and palatability, conduces to the diet of ruminants (dairy, sheep) (Simić *et al.*, 2005; 2012). This crop grows best between 20 and 25°C and requires a cool climate with high rainfall and well-drained soils. It has the fastest growth and regeneration and produces a lot of forage in a short time (Delić *et al.*, 2012; Kostrzevska *et al.*, 2020). It is sensitive to strong frosts and quite sensitive to drought, and longer droughts can bring it very low yields (Simić *et al.*, 2005).

In recently year, due to global warming, the occurrence of pathogenic and potentially toxigenic fungi from *Fusarium*, *Aspergillus* and *Penicillium* genera has become more frequent on cereal grains and other grasses (Silva *et al.*, 2014; Torres *et al.*, 2019). In addition, agro-ecological and environmental conditions in Serbia are favorable to growth of these fungal species on cereal grains, with *Fusarium* species as predominant (Lević, 2008). Members of *Fusarium graminearum* species complex (FGSC) cause Fusarium head blight (FHB) disease of small grains and several grasses, including annual ryegrass (Machado *et al.*, 2015). *F. graminearum* is the most common pathogen which cause FHB of small grains. The first symptoms of FHB appear after flowering at anthesis. Spikelets are bleached, and with development of mycelium, the disease spreads throughout the head (spike), causing shrivelled seeds (Francesconi *et al.*, 2019). *F. graminearum*-infected seeds have poor germination with slow emergence and can cause seedling blight disease (Wiewióra, 2012). *F. graminearum* is a hemibiotroph, with the biotrophic (short last from 24 to 32 h after infection) and necrotrophic phase (the pathogen grows and extends within the head through the vascular bundles and parenchyma) (Bai *et al.*, 2002; Goswami and Kistler, 2004; Trail, 2009).

In addition to causing FHB disease, *Fusarium* species produce a wide range of toxic secondary metabolites (mycotoxins), which are serious constraints for feed/food production (Richardson *et al.*, 1996). The occurrence of these fungal metabolites in food/feed poses health risks to the consumers (Haidukowski *et al.*, 2005). Deoxynivalenol (DON), primarily produced by *F. graminearum*, is the most associated with FHB. It belongs sesquiterpene epoxides, namely trichothecenes. The most commonly DON found in grain/seed from FHB-diseased cereal and grass heads (Gunupuru *et al.*, 2017). Negative effects of DON ingestion by experimental animals are manifested as acute (emesis) and chronic poisoning (anorexia, growth retard, neurotoxicity, immunotoxicity as well as reproduction disorders. Likewise, DON can be associated with human gastroenteritis (Pestka, 2010). Referring to

participation of DON in FHB pathogenesis, it also has the ability to be virulent and facilitates the spread of fusariosis within the head. Like FHB, DON may cause premature bleaching of head and leaves. DON-producing *F. graminearum* strains can suppress programmed cell death (apoptosis) in the leaves and support biotrophic and necrotrophic phases of *Fusarium* infection (Diamond *et al.*, 2013). According to Bushnell *et al.* (2010), DON-producing strains inhibited the formation of the wall thickenings, allowing the pathogen to spread in the head.

According to European Regulations EC No. 1126/2007 amended the Regulation (EC) No. 1881/2006 and EC No. 576/2006, the maximum limits of DON level are 1250 $\mu\text{g kg}^{-1}$ in unprocessed cereal other than durum wheat, oats and maize and 8000 $\mu\text{g kg}^{-1}$ in unprocessed cereal and cereal products except in maize by-products intended for animal feeding, respectively. Quality and healthy seeds are a prerequisite for high yields of ryegrass as well as for safe feed production. The high yield of seed and fodders is correlated with the genetic potential of the selected varieties (Veljjević *et al.*, 2016). Grass (cereal) cultivars have different types of resistance to FHB (from I to V resistance types), there is no unsusceptible genotype. Types I and II are the most described. Type I resistance is resistance to initial infection, type II is resistance to spread of pathogen within the head. Other types include resistance to seed infection (type III), tolerance to FHB and DON (type IV), and resistance to DON accumulation (Type V) (Mesterházy, 1995; Boutigny *et al.*, 2008). In addition to growing resistant cultivars, crop rotation, fertilization, tillage, and the application of fungicides and biological agents can also significantly reduce *Fusarium* seed infestation (Shah *et al.*, 2018).

Since the quality and healthy seed is important for ryegrass, livestock and feed production, the main aim of this work was to identify and quantify *Fusarium* spp. associated with ryegrass seeds, as well as to determine DON levels in seed samples of two ryegrass cultivars and to assess its potential risk in FHB pathogenesis.

Materials and Methods

A total of 24 seed samples of two commercial Italian ryegrass cultivars (12 samples of cultivar K-13 and 12 samples of cultivar K-29) were analyzed by mycological and toxicity tests. About 1 kg of ryegrass intended for seed production were randomly taken during the 2019 harvest season. The area of tested ryegrass cultivars was on about one hectare and located on the experimental fields of Institute for Animal Husbandry, Belgrade-Zemun in Serbia.

The each of 24 samples was divided into two sub-samples, so, it was 12 sub-samples per cultivar. In mycological analyses, per each cultivar, the first 12 seed sub-samples were pooled in one representative sample. To evaluate *Fusarium*

spp., 600 seeds per cultivar from representative samples were analyzed. First, the seeds were surface-disinfected for 3 minutes with 1% sodium hypochlorite, washed twice with sterilized distilled water and dried on filter paper. Then, the seeds were plated, 10 seeds per 90 mm Petri-dishes with Potato Dextrose Agar (PDA), and incubated at room temperature during 7-10 days. Based on microscopic observations, *Fusarium* species were identified using fungal keys of *Burgess et al. (1994)* and *Leslie and Summerell (2006)*. Incidence of fungal species was presented as percentage values in pooled seed samples.

For mycotoxicological analyses, a total of 24 samples, 12 sub-samples per cultivar, were ground in an analytical mill (IKA A11, Staufen, Germany). Using the moisture analyzer (OHAUS MB35, USA), the moisture content of tested samples was determined. ELISA assay for determining DON levels was done according to the manufacturer's instructions Celer Tecna® ELISA kits. The limit of detection for DON was 40 µg kg⁻¹.

Data were statistically analyzed using the independent-samples T-test (IBM SPSS Statistic 20). Pearson's correlation coefficients between DON levels and moisture contents were determined.

Results and Discussions

The results of mycobiota isolated from the seed of two Italian ryegrass cultivars, K-13 and K-29, are shown in Table 1. The fungal species isolated were *Acremonium* spp., *Alternaria* spp., *F. graminearum*, *F. poae*, *F. proliferatum*, *F. subglutinans* and non-sporulating fungi (Mycelia sterilia) on the seed of both cultivars, except on seed of cultivar K-29, in which *F. proliferatum* was not identified. Among isolated species, *Alternaria* spp. was the most present with 70% and 61.67% on the seed of K-13 and K-29 cultivar, respectively. Considering incidence of *Fusarium* species, *F. graminearum* was the most common at both cultivars, with higher incidence on the seed of cultivar K-29 (32%) than cultivar K-13 (20.50%). *F. graminearum* is followed by *F. subglutinans* which was isolated in a higher percent on the seed of cultivar K-13 (7.50%) compared to cultivar K-29 (2%). A low incidence of *F. poae* (0.50%) was found on the seed of both cultivars, as well as the incidence of *F. proliferatum* on the seed of cultivar K-13 (0.17%). In a similar study, *Wiewióra (2012)* has established *Alternaria alternata* as the most common species on the seed of perennial ryegrass and identified 10 *Fusarium* species of which *F. avenaceum* and *F. solani* were the most frequent. Further, the same autor stated that the most species such as *Alternaria* spp., *Epicoccum* spp., *Septonema* spp. and *Penicillium* spp. were saprophytes or weak parasites, while *Drechslera* spp., *Fusarium* spp., *Phoma* spp., *Curvularia* spp. and *Bipolaris* spp. were presented as pathogenic species. Similar results were reported by *Varga and*

Fischl (2005) on the ryegrass seeds and *Pathak and Zaidi (2013)* on wheat seeds. According to reported data of *Torres et al. (2019)*, the major FHB pathogens include FGSC members and related species such as *F. avenaceum*, *F. culmorum* and *F. poae*. Similarly, in this study, *F. graminearum* and *F. poae* identified as FHB pathogens from which *F. graminearum* was more presence than *F. poae* on the seed of both tested cultivars. *Machado et al. (2015)* have identified three FGSC species, *F. graminearum*, *F. asiaticum* and *F. cortaderie* from diseased ryegrass spikes and confirmed their pathogenicity to ryegrass. It was the first report of FGSC members as head blight pathogens of ryegrass (*L. multiflorum* L.) in Brazil. The results of this study were also the first report of *Fusarium* spp. incidence on the seed of two Italian ryegrass cultivars (K-13 and K-29) in Serbia.

Table 1. Incidence of fungal species on the seed of two tested ryegrass cultivars

Fungal species	Incidence (%)	
	Cultivar of ryegrass	
	K-13	K-29
<i>Acremonium</i> spp.	0.50	0.83
<i>Alternaria</i> spp.	70	61.67
<i>Fusarium graminearum</i>	20.5	32
<i>Fusarium poae</i>	0.50	0.50
<i>Fusarium proliferatum</i>	0.17	0
<i>Fusarium subglutinans</i>	7.50	2
<i>Mycelia sterilia</i>	0.83	3

By mycotoxicological assays, DON was found in all tested seed samples of two ryegrass cultivars, respectively the incidence of DON positive samples was 100%. It has been established the statistically significant higher mean level of DON on the seed of cultivar K-29 (5334.33 $\mu\text{g kg}^{-1}$) compared to cultivar K-13 (4738.58 $\mu\text{g kg}^{-1}$). There were no statistically significant differences between means of moisture contents of seeds in tested cultivars (Table 2). By investigating the participation of DON in FHB pathogenesis, *Bai et al. (2002)* have stated that DON production had a significant role in the spread of FHB within a spike. According to the reports of *Diamond et al. (2013)*, relatively low DON levels might inhibit plant programmed cell death (PCD), while high DON levels might induce cell death. So, DON-producing *F. graminearum* strains with low and 10,000 $\mu\text{g kg}^{-1}$ DON levels might contribute to biotrophic and necrotrophic phases development in *Fusarium* infection leading to FHB disease symptoms (*Gunupuru et al., 2017*). In Brazil, the first report of DON-producing *F. graminearum* isolated from ryegrass spikes, as well as their pathogenicity on annual ryegrass reported by *Machado et al. (2015)*. In this study, high DON levels in all seed samples of both

cultivars indicating that there was potentially risk of isolated *F. graminearum* strains in FHB pathogenesis.

Table 2. Means of DON level and moisture content in tested seed samples of two ryegrass cultivars

Item	DON level ($\mu\text{g kg}^{-1}$)		Moisture content (%)
	Mean \pm S.D.	Range	Mean \pm S.D.
Cultivar K-13	4738.58 \pm 244.17 ^a	3157 – 5586	11.74 \pm 0.32
Cultivar K-29	5334.33 \pm 147.26 ^b	4382 – 5886	11.30 \pm 0.53
Level of significance	*	-	ns

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$ level; *, ** - significant at the 0.05 and 0.01 probability levels; ns - not significant.

Temperature, moisture and relative humidity are the most important environmental factors influencing the development of FHB and therefore DON accumulation in cereal grains (Wegulo, 2012). Weak positive correlations were found for levels of DON with moisture contents on seeds of tested cultivars K-13 ($r = 0.348$) and K-29 ($r = 0.380$) (data not presented). Similarly, positive correlations between moisture-related variables with biological variables such as DON accumulation in wheat cultivars were established by Cowger *et al.* (2009), Kriss *et al.* (2010) and Hernandez Nopsa *et al.* (2012).

Conclusion

This study presents a natural occurrence of fungal infection and DON mycotoxin in the seed of two ryegrass cultivars, K-13 and K-29, as a participant in FHB pathogenesis. *Acremonium* spp., *Alternaria* spp., *Fusarium* spp. and non-sporulating fungal species (*Mycelia sterilia*) were isolated at both cultivars. Among *Fusarium* spp., *F. graminearum*, *F. poae*, *F. proliferatum*, and *F. subglutinans* and *F. graminearum*, *F. poae*, and *F. subglutinans* were identified on the seed of cultivars K-13 and K-29, respectively. *F. graminearum* and *F. poae* were identified as FHB pathogens, with *F. graminearum* as predominant in both cultivars. All tested seed samples were DON positive. DON level in cultivar K-29 was statistically significant higher (5334.33 $\mu\text{g kg}^{-1}$) than in K-13 (4738.58 $\mu\text{g kg}^{-1}$). High DON levels ($>3000 \mu\text{g kg}^{-1}$) in tested seed samples were indicated on potential significant participation of DON in FHB pathogenesis. Both ryegrass cultivars were susceptible to FHB pathogens. These results indicate on importance of health of seeds, especially for ryegrass seeds production as well as forage production. Ryegrass as the weed may also be potentially a source of inoculum for FHB epidemics in cereal crops.

***Fusarium* spp. i deoksinivalenol kontaminacija semena italijanskog ljulja**

Vesna Krnjaja, Violeta Mandić, Zorica Bijelić, Slavica Stanković, Milica Nikolić, Tanja Vasić, Nikola Delić

Rezime

Cilj rada bio je da se oceni gljivična infekcija sa specijalnim fokusom na *Fusarium* spp. i prisustvo deoksinivalenola (DON) kao učesnika u patogenezi fuzarioze klasa (FHB) kod dve sorte italijanskog ljulja, K-13 i K-29. Ukupno 24 uzoraka semena sakupljeno je tokom žetve u 2019. godini. Mikološkim analizama izolovane su *Acremonium* spp., *Alternaria* spp., *Fusarium* spp. i nesporulišuce vrste (*Mycelia sterilia*) na semenu obe ispitivane sorte italijanskog ljulja. Među *Fusarium* spp., identifikovane su četiri vrste, *F. graminearum*, *F. poae*, *F. proliferatum* i *F. subglutinans*, kod sorte K-13 i tri vrste, *F. graminearum*, *F. poae* i *F. subglutinans*, kod sorte K-29. *F. graminearum* i *F. poae* su identifikovane kao FHB patogeni, od kojih *F. graminearum* je dominantnija kod obe ispitivane sorte sa 20,5% (sorta K-13) i 32% (sorta K-29) u odnosu na *F. poae* koja je bila prisutna kod obe sorte sa učestalošću od 0,5%. Učestalost DON pozitivnih uzoraka bila je 100%. Utvrđena je statistički značajna razlika u koncentraciji DON između dve ispitivane sorte, s tim da je utvrđena veća koncentracija DON kod sorte K-29 (5334,33 $\mu\text{g kg}^{-1}$) u odnosu na sortu K-13 (4738,58 $\mu\text{g kg}^{-1}$). Dobijeni rezultati ukazuju da su dve *Fusarium* vrste, *F. graminearum* i *F. poae*, FHB patogeni, s tim da je *F. graminearum* preovladjujuća vrsta kod obe ispitivane sorte italijanskog ljulja. Visoke koncentracije DON mikotoksina (>3000 $\mu\text{g kg}^{-1}$) u ispitivanim uzorcima semena italijanskog ljulja ukazuju na potencijalno značajno učešće DON u FHB patogenezi, kao i na potencijalni rizik za proizvodnju kvalitetnog semena, bezbednost hrane za životinje i lanca ishrane uopšte. U Srbiji, ovo je prva objava o *Fusarium* infekciji i prisustvu DON u semenu italijanskog ljulja.

Ključne reči: *Fusarium* spp., deoksinivalenol, italijanski ljulj

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