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MYCOPOPULATIONS OF ALFALFA SILAGE WITH PARTICULAR REWIEV ON TOXIGENIC *FUSARIUM* spp.

ABSTRACT: Mycological and mycotoxicological investigations of alfalfa samples (initial not fermented plant material, as well as silage obtained from unfaded and faded state of the same) were performed during the year 2003. Total of 14 fungal species, included in 11 genera, were identified during the present study. The most frequent moulds were *Chaetomium piluliferum* and their anamorph *Botryotrichum piluliferum* (83.3% and 66.7% respectively). Potentially toxigenic *Fusarium* spp. (*F. culmorum, F. semitectum* and *F. sporotrichioides*) were observed less frequently (from 16.7% to 33.0%) and only on initial not fermented alfalfa.

From the sample of alfalfa faded state contaminated with 0.25 mg/kg of diacetoxyscirpenol (DAS) and 1.28 mg/kg of zearalenone (ZEA) *F. semitectum* SL-B was isolated. The production of fusariotoxins by this strain was later on tested *in vitro* conditions. Different aeration treatments in semisynthetic medium with glucose (GPK) or sucrose (SPK), as well as cultivation on sterilized corn kernels (moisture content 47%) were used. The highest yield od DAS (64.0 mg/l) was obtained during submerged cultivation of *F. semitectum* SL-B in GPK (210 rpms, 3 days, room temperature). Production of T-2 toxin, but rather poor (0.08 mg/1), was detected only in SPK (150 rpms, 3 days, room temperature). ZEA was found exclusively after 25 days of cultivation on corn kernels at room temperature (21—25°C).

KEY WORDS: alfalfa, *Fusarium*, moulds, silage, toxicity

INTRODUCTION

Silage moulds can occur because of existence of pockets containing residual oxygen, or subsequent introduction of air into ensilaged material. Although this problem can be overcome by good pressurizing of the mass and its covering, superficial parts of silage, then those which are in contact with the walls of silo structures, as well as silage composed of more mature material which is harder to pressurize, represent potential places for contamination with

fungal species (Đ o r đ e v i ć et al., 2004). According to S e l g a r (2004), some silage moulds can also grow in conditions of low oxygen level and moderately low pH value. However, their survival is limited by competition with anaerobic bacteria. The reason of occurrence of fungi in silage is usually an increase in pH value which is due to consumption of lactic acid by yeasts which become active after the introduction of oxygen into a silo. *Candida* and *Hansenula* are microorganisms which consume lactic acid, and when their count exceeds 10^5 cells per gram of stock-cattle feed, increased mould activity occurs.

Two types of mycopopulation can cause deterioration of ensilaged material. The so-called "field fungi" which develop during the vegetation period in crops, infect either the grain or the forage, generally in conditions of high humidity (> 70%) and temperature variations (warm days and cold nights). According to S e l g a r ' s assertion (2004), most frequently they do not develop on stored silage because of low pH and lack of oxygen. However, this does not exclude presence of their toxic metabolites which biosynthesis can be initiated already in field conditions. In this respect, the most interesting ones are *Fusarium* species which are also connected with production of mycotoxins, or human and animal diseases, unlike other field mycopopulations, which cause only plant diseases (*Diplodia, Anthrachnose, Helminthosporium, Ustilago*).

Moulds which develop on stored feed — "storage fungi", usually do not attack crops before harvest. Spores of these moulds which originate in soil are brought into the silo together with stock-cattle feed. Dominant moulds in North America isolated from silage are: *Mucor, Penicillium, Aspergillus*, and *Monilla* (S e l g a r, 2004). Most of about twenty fungal species of this type, identified on ensilaged material in the USA, are thought not to have the ability of mycotoxins' biosynthesis. Even though *A. flavus* has been classified as a storage microorganism, in its case production of aflatoxins can take place already in field conditions.

MATERIALS AND METHODS

Fungal cultures. Isolation of fungal species has been carried out by standard mycological methods (M u n t a ñ o l a - C v e t k o v i ć, 1987). Culture identifications were carried out according to N e l s o n et al. (1983) for *Fusarium* species, whereas other moulds have been determined according to D o m s h et al. (1980) and E l l i s (1971). *Fusarium semitectum* SL-B has been isolated from a sample of unfaded alfalfa which contained 0.25 mg/kg of diacetoxiscirpenol (DAS), and 1.28 mg/kg of zearalenone (ZEA). The cultures have been kept on potato-dextrose agar (PDA) at 6°C. Prior to preparation of medium for testing the ability of biosynthesis of fusariotoxins, the isolates were subcultivated on PDA for the period of seven days at 27°C.

Cultivation types. A. GPK liquid medium (5% glucose + 0.1% yeast extract + 0.1% peptone, pH 5.3) 250/500 ml, rotatory laboratory shaker (210 rpm) during the period of three days at room temperature (21—25°C); **B**. GPK liquid medium 250/500 ml, rotatory laboratory shaker (150 rpm) during the

period of three days at room temperature (21—25°C); **C**. SPK liquid medium (5% saccharose + 0.1% yeast extract + 0.1% peptone, pH 5.3) 250/500 ml, rotatory laboratory shaker during the period of three days at room temperature (21—25°C); **D**. SPK liquid medium, 100/500 ml, stationary cultivation during 25 days at room temperature (21—25°C); **E**. sterile corn kernels (uncontaminated by mycotoxins, water content 47%) stationary cultivation during 25 days at room temperature (21—25°C).

Determination of fusariotoxins. Qualitative and quantitative ZEA determination in liquid media (**A—D**) was carried out by applying modified method of Pepeljnjak and Babić (1991). The modification consisted in adding 20% anhyd. Na_2SO_4 and silica gel to fungal culture filtrate during initial extraction of toxin with acetonitrile. The rest of the analysis has been carried out according to the given procedure. Identification of ZEA in corn kernels (**E**) was performed according to the *Regulations on sampling methods and methods of physical, chemical and microbiological analyses of fodder* (Official Gazette of SFRJ, No. 15/87).

Extraction of A type trihotecenes (T-2 toxin and DAS) was carried out in all cases with ethyl acetate, according to Romer et al. (1978), and TLC determination by Pepeljnjak and Babić (1991) method.

RESULTS AND DISCUSSION

The results of mycological study of alfalfa and silage are shown in Table 1.

During the present study, a total of 14 species were identified, namely, 11 fungal genera, of which the most frequent one was the species *Chaetomium piluliferum* and its anamorph *Bolriotrichum piluliferum* (83.3% and 66.7% respectively). *Bolriolrichum piluliferum*, according to the data provided by Domsh et al. (1980), is widely spread all around the world (soil, plant rhizosphere, animal excrement etc.). Optimal growth temperature for this mould is 25—30°C, and pH 5.5. In addition to these features, it is distinguished by the ability to dissolve starch, pectin, xylan, and carboxymethyl cellulose. Bearing in mind these features, as well as the fact that when *Bolriotrichum piluliferum* grows on straw it dissolves well cellulose and lignin, producing humus substances in the course, the presence of this anamorph and its teleomorph on faded alfalfa and alfalfa silage is only logical.

The highest number of fungal species (6) has been identified on unfaded alfalfa and silage obtained from it (**SL2** and **SL4**) independently of the type of treading. The majority of moulds identified on these three types of samples belonged to typical field mycopopulation, such as the genera *Alternaria, Cladosporium* and *Fusarium*. (Table 1).

In addition to other species of fungi identified during the present study, in Serbia and Montenegro there are listed: on ensilaged corn kernels — *Penicillium aurantiogriseum* (Bočarov-Stančić, 2003), on the silage of the whole corn plant *Aspergillus versicolor* (Djordjević et al., 2004), and on ensilaged soy grain *A. versicolor, F. oxysporum, F. subglutinanas*, and *Rhisopus nigricans* (Bočarov-Stančić, 2003).

Table 1. Identified fungal species on alfalfa and alfalfa silage

No.	Species	A	B	SL2	SL4	SL6	SL8
1.	*Acremoniella atra* (Fr.) Keissl.	—	—	+	+	—	+
2.	*Alternaria alternata* (Corda) Sacc.	+	—	—	—	—	—
3.	*Bolryolrichum piluliferum* Sacc. & March.	—	—	+	+	+	+
4.	*Chaetomium globosum* Kunze ex Steud.	—	—	+	—	—	—
5.	*C. piluliferum* J. Daniels	—	+	+	+	+	+
6.	*Cladosporium herbarum* (Pers.) Link ex Gray	+	—	+	—	—	—
7.	*Epicoccum purpurascens* Ehrenb. ex Sclecht.	+	—	—	—	—	—
8.	*Fusarium culmorum* (W. G. Sm.) Sacc.	—	+	—	—	—	—
9.	*F. semitectum* Berck & Rav.	+	+	—	—	—	—
10.	*F. sporolrichioides* Shreb.	+	—	—	—	—	—
11.	*Geotrichum candidum* Link ex Leman	—	—	—	+	—	—
12.	*Mucor racemosus* Fres.	—	—	+	+	+	+
13.	*Penicillium sp.*	—	—	+	—	—	—
14.	*Trichocladium opacum Ellis.* (Corda) Hughes	—	—	—	+	—	—
	TOTAL	6	3	6	6	3	4

Legenda: **A** — initial unfaded material;
B — initial faded material;
SL2 — silage from **A**, better treading;
SL4 — silage from **A**, weaker treading;
SL6 — silage from **B**, better treading;
SL8 — silage from **B**, weaker treading.

Potentially toxygenic *Fusarium* species (*F. culmorum, F. semitectum, F. sporolrichioides*) were determined only on initial, not ensilaged material, with 16.7 to 33.0%. Although K r n j a j a et al. (2004) did not find these species on the diseased alfalfa plants, except for *F. sporotrichioides*, but primarily *F. oxysporum, F. solani, F. equiseli*, and *F. proliferatum*, it is necessary to specially pay attention to them, considering the fact that these *Fusarium* species are potential producers of a larger number of mycotoxins and that they are connected not only to the occurrence of plant diseases, but also diseases in humans and animals (Table 2). In other words, it is known that mycotoxins: aflatoxin, deoxynivalenol, zearelenone, and fumonysins are usually diagnosed in silage (D j o r d j e v i ć et al., 2004; S e l g a r, 2004).

Table 2. Toxigenic potential of *Fusarium* spp. identified on alfalfa (literature data)

Species	*F. culmorum*	*F. semitectum*	*F. sporotrichioides*
	Type A trichotecenes		
1. 4-Acetoxyscirpendiol	—	a	—
2. Diacetoxyscirpenol	a, b	a	a
3. Monoacetoxyscirpenol	—	a	—
4. Neosolaniol	a, b	a	a
5. Scirpentriol	—	a	—
6. HT-2 toxin	—	—	a

7. T-2 tetraol	—	—	a, b
8. T-2 toxin	a	a	a
Type B trichotecenes			
9. 3-Acetyldeoxynivalenol	a	—	—
10. Deoxynivalenol	a, b	a	a
11. Diacetylnivalenol	a	a	a
12. Nivalenol	—	a	a
13. Nivalenol diacetate	—	a	—
14. Nivalenol monoacetat	—	a	a
Zearalcnone and derivatives			
15. Zearalenol	—	a	—
16. Zearalenone	a	a	a
Other fusariotoxins			
17. Butenolide	—	a	a

Legend: **a** — according to Marasas et al. (1984),
b — according to Ožegović and Pepeljnjak (1995).

Taking into account the data presented in Table 2, the culture *F. semitectum* SL-B, isolated from the sample of faded alfalfa which contained 0.25 mg/kg of DAS and 1.28 mg/kg of ZEA, was subjected to toxicological *in vitro* study. *F. semitectum* is a cosmopolite fungus, which is according to Domsh et al. (1980), most frequently isolated from tropical and subtropical regions of the world. This saprogenic soil microorganism can also be found on decaying vegetal material in the countries of temperate zone of Europe and North America. In our country, the species *F. semitectum* is quoted as causing agent of soy and aubergine (eggplant) seed diseases (Jovičević and Milošević, 1990), sunflower seed (Noory, 1983), wheat and barley seed (Bočarov-Stančić et al., 2000), and corn in field conditions (Lević et al., 2004). This mould is, according to Marasas et al., (1984), the only toxic species from the Arthrosporiela section, which is related to the following human and animal diseases: a) **degnala disease** in water buffalo and cattle in India and Pakistan, which is characterized by edematous swelling of legs, necrosis, and appearance of necrosed skin on extremities, and b) **human esophageal cancer** in the Chinese province Henan.

In the course of the present study, significant impact of cultivation condition on the biosynthesis of fusariotoxins through *F. semitectum* SL-B, that is, on the type of toxin and its yield was established (Table 3).

Table 3. The yield of fusariotoxins and basic cultivation parameters of *F. semilectum* SL-B *in vitro* conditions

Med.	Cult. type	pH	Microscopic characteristics	Toxin yield (mg/l od mg/kg)		
				DAS	T-2	ZEA
GPY	A	4.2	Exceptionally fatt, segmented and vacuolized hyphae with outstanding spherical deformations.	64.0	0	0
	B	4.0	Medium fatt, segmented and vacuolized hyphae, with periodical spherical deformations.	31.8	0	0
SPY	C	3.5	Loose mycelium. Fatt, segmented and vacuolized hyphae.	9.6	0.08	0
	D	3.0	Fatt, segmented and vacuolized hyphae with pearl like deformations.	0	0	0
C. K.	E	—	—	4.0	0	6.4

Legend: GPY — 5% glucose + 0,1% yeast extract + 0,1% peptone, pH 5.3;
SPY — 5% saccharose + 0.1% yeast extract + 0.1% peptone, pH 5.3;
C. K. — corn kernels (47% water content).

Stationary cultivation in semisynthetic liquid medium with saccharose (**D**) did not produce a positive result in the case of any of tested mycotoxins, whereas at submersed cultivation in the same medium (**C**), biosynthesis of only trichotecen of the A type (0.08 mg/l T-2 of toxin, and 9.6 mg/l of DAS) was achieved. The results obtained by the use of nutritive medium with glucose (**A** and **B**) also speak of the positive impact of aeration on DAS production. In both cases (Table 3) there are detected not only considerably higher yields of the same trichotecen than in SPK, but also two times higher when cultivating *F. semitectum* SL-B fungus in conditions of increased aeration (64.0 mg/l with respect to 31.8 mg/l). Although Marasas et al. (1984) most frequently propose PSC medium and Chapek's broth as media for studying toxigenicity of *Fusarium* spp. and the temperature of 25°C, we decided to use the liquid media mentioned above (SPK and GPK) in which we achieved satisfactory results, taking into account our previous studies (Bočarov-Stančić et al., 2004).

According to the data provided by literature (Ožegović and Pepeljnjak, 1995), *Fusarium* species begin to decay at the substrate humidity of 12—13%, whereas at 22—23% they multiply intensively. Lević et al. (2004) quote slightly higher values — minimum water contents which enable development of the representatives of the same genus are 18—19%. At the same time, this represents a limiting value for biosynthesis of mycotoxins in cereals, although better yields were, as a rule, obtained on natural substrates with a higher water content. In accordance with this, in the present study sterile, uncontaminated corn kernels with initial of 47% water content were used. Although literature quotes temperature stress as optimal condition for ZEA biosynthesis (a higher number of weeks at 25°C, which are followed by a couple of weeks at 10°C), Marasas et al. (1984), show that in the case of some *Fusarium* isolates higher yields can be obtained by cultivation at constant temperature. For all these reasons, in the present study of toxigenic potential

of *F. semitectum* SL-B was used for cultivation at room temperature (21—25°C) during four weeks. As Table 3 shows, in the given conditions (**E**), production of ZEA in the quantity of 6.4 mg/kg was verified. Relatively low yield of this mycotoxin obtained can be explained by weak potential for biosynthesis of *F. semitectum* strains in comparison with the main ZEA producers: *F. graminearum*, of which, according to Ožegović and Pepeljnjak (1995), even 93% of the strains are toxigenic, or *F. culmorum* with 63% of isolates as producers of the same fusariotoxin. DAS yields on the same substrate were also rather low (4.0 mg/kg), although according to Marasas et al. (1984), larger quantities of the same mycotoxin (up to 23.49 mg/kg) can be produced on cereals. Even though the same authors quote examples of biosynthesis of T-2 toxin which can develop on rice (30°C, seven days), or on ground white corn kernels (21 days, 15°C), during the present study it was not possible to detect this type A trichotecene in corn kernels used for cultivation (E).

CONCLUSIONS

On ensilaged material, despite the low pH value and lack of oxygen, certain types of field moulds and fungi characteristic of feed storing may develop, some of which are potential producers of mycotoxins.

On alfalfa and various types of its silage, the dominant types were pectinolitic, chemicellulolitic, and cellulolitic fungi — teleomorph *Chaetomium piluliferum* and its anamorph *Bolryotrichum piluliferum* (83.3% and 66.7%, respectively).

Typical field mycopopulation (*Alternaria, Cladosporium*, and *Fusarium*) were found mainly on the initial poorly areated material and the silage obtained from it.

From three potentially toxigenic *Fusarium* species isolated from alfalfa, with culture *F. semitectum* SL-B it was established that the biosynthesis of fusariotoxins *in vitro* conditions was favoured by: 1) DAS — increased aeration (210 rpm) and use of glucose as the C atom source; 2) T-2 toxin — saccharose as the source of carbon, and 3) ZEA — cultivation on the corn kernels and the temperature of 21—25°C.

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МИКОПОПУЛАЦИЈЕ СИЛАЖЕ ОД ЛУЦЕРКЕ, С ПОСЕБНИМ ОСВРТОМ НА ПРИСУСТВО ТОКСИГЕНИХ *FUSARIUM* ВРСТА

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Резиме

Током 2003. год. извршена су миколошка и микотоксиколошка истраживања узорака луцерке (непровенули, провенули полазни материјал и силажа од истих). Идентификовано је укупно 14 врста, односно 11 родова гљива од којих су са највећом учесталошћу забележени *Chaetomium piluliferum* и његов анаморф *Bolryotrichum piluliferum* (83.3 односно 66.7%). Потенцијално токсигене *Fusarium* spp. (*F. culmorum, F. semilectum* и *F. sporotrichioides*) уочене су знатно ређе (од 16.7 до 33.0%). Из узорка провенуле луцерке контаминиране са зеараленоном (ЗЕА) и диацетоксисцирпенолом (ДАС) изолован је сој *F. semitectum* SL-B чија је способност за биосинтезу фузариотоксина испитана у *in vitro* условима. Највећи принос ДАС-а (64.0 mg/l) је постигнут при субверзној култивацији у течној подлози са глукозом. Слаба производња Т-2 токсина (0.08 mg/kg) је добијена само у течној подлози са сахарозом. ЗЕА је нађен искључиво при култивацији на влажном стерилном зрну кукуруза (6.4 mg/kg).