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IDENTIFICATION AND QUANTIFICATION OF FUNGI IN GRASS-LEGUMINOUS SILAGE

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Abstract: Objective of the research was to determine the presence, total count and species of fungi in samples of grass-leguminous silages depending on the botanical composition of mixture, nitrogen fertilization and crop utilization phase. Most of identified fungi species are of *Fusarium* genus (80-91%). In analyzed samples of silage total fungi count in average for three year period of research (2005-2007) was 2.31-3.09 log10 CFU g^{-1} and it varied depending on the investigated factors. Factor of type of mixture had significant effect on fungi count in first two investigation years, whereas nitrogen fertilization caused variations in fungi count in the first and third investigation year. Cutting in early utilization phases statistically significantly influenced total fungi count only in one investigation year (2006).

Key words: fungi, silage, grass-legume mixtures, nitrogen fertilization

Introduction

Ensiling is a special form of preservation of fodder livestock feed based on series of complex chemical and microbiological processes. Production of high quality grass and leguminous silage is the best way of their utilization.

Quality of silage is measured by certain chemical-fermentative parameters, but it is very important to determine the quality in regard to presence of harmful micro-organisms, moulds or fungi, yeasts and clostridia. Fungi are eukaryotic micro-organisms. They develop in silage where oxygen is present. They most often belong to following genera: *Penicillium, Fusarium, Aspergillus, Mucor, Trichoderma, Byssochlamys (Đorđević et al., 2003; Amigot et al., 2006)*. *Seglar (2003)* and *Bočarov-Stančić et al. (2005*) distinguish two groups of fungi: field fungi which develop during vegetation, infect the vegetative mass, and in silage they can be found only if oxygen is present (*Fusarium* spp.) and storage fungi which are introduced to silage in the form of spores with particles of soil (*Mucor, Penicillium, Aspergillus* and *Monilia* spp.).

Fungi not only diminish the value of livestock feed, but also have negative effect on health of animals and humans. In animals they cause respiratory disturbances, abnormal rumen fermentation, decrease of reproduction functions, decrease of productivity, kidney damage, skin and eye irritations *(Gotlieb, 2002)*. Total fungi count above 10^9 CFU kg^{-1} in livestock feed can induce such disorders. Therefore, total fungi count is another indicator of the quality of forage *(Di Constanzo et al., 1995)*.

Objective of the research was to establish if type of mixture, nitrogen nutrition and phase of utilization of plants have influence on incidence and fungi count in silage, if they disrupt the quality of silage and if such silage can be used in livestock nutrition, i.e. if the total fungi count exceeds the value allowed by adequate Rulebook.

Materials and Methods

For the purpose of solving the investigation objective the trial was carried out in the Institute for Animal Husbandry, in period 2005 to 2007, in two phases: in field and laboratory conditions. Field trial was done in 4 repetitions, with elemental parcel size of 10 m^2.

Trial was carried out according to model of poly-factorial trial 4×4×2. Investigated factors were: mixture (pure alfalfa crop-M, mixture of alfalfa and cocksfoot -S1, mixture of alfalfa, cocksfoot and tall fescue -S2, mixture of alfalfa, cocksfoot, tall fescue and sainfoin -S3), then fertilization with nitrogen (0, 70, 140, 210 kgN ha^{-1}) and utilization phase (stage of buttonization-F1, stage of 50% of plant blooming-F2). Fertilization was done twice, at the beginning of vegetation, first ½ of nitrogen (in the stage of beginning of intensive plant growth) and the second ½ of nitrogen after the first cut.

For preparation of silage material from the second cut was used in order to register the effect of total nitrogen fertilization and avoid the impact of increased presence of weed in the first cut on quality of ensiled material. Dry mass was ensiled in experimental containers, micro-silo of volume of 10 dm^3. For higher quality of fermentation, before filling of dishes material was treated using microbiological preparation Sil-All^{4x4}. Sampling of silage for microbiological analysis was done 90 days after closing of containers.

In microbiological analysis the presence of fungi was concluded with special focus on potentially toxigenic genera: *Fusarium*, *Aspergillus* and *Penicillium*. Total of 96 samples in two repetitions were analyzed using standard methods. Identification of genera was done based on morphological characteristics of fungi described by *Mihajlović (1983), Muntañola-Cvetković (1990)* and *Watanabe (1994)*. Isolation of special fungi genera was done by sowing of samples on selective media for fungi (moulds).

Results of microbiological analysis of total fungi count in silage, using mathematical method of logarithm, transferred into log10 CFU g^{-1}, and subsequently processed using non-parameter statistics Kruskal-Wallis (ANOVA), test of median significance testing *(Stat. Soft, STATISTICA 6, 2001)*.

Results and Discussion

In Table 1 the frequency of isolated fungi genera from investigated silage samples is given, regardless of the type of mixture and nitrogen fertilization, as well as their average percentage presence for three year period.

Table 1. Frequency of fungi genera in investigated samples of alfalfa and grass-leguminous silages (2005-2007) in different investigation stages

Genus of fungi	Frequency (%)	
	Phase of utilization F1	Phase of utilization F2
Acremonium	2	-
Alternaria	2	3,6
Aspergillus	10	1,8
Fusarium	80	91
Mucor	-	3,6
Paecylomices	2	-
Penicillium	2	-
Rhizopus	2	-

The highest presence in F1 utilization phase, as well as F2, was established for species of genus *Fusarium* with 80 and 91%, respectively. Other species were present in lower percentage, in phase F1 species from following genera: *Aspergillus* (10%), *Rhizopus* (2%), *Paecylomices* (2%), *Penicillium* (2%), *Acremonium* (2%) and *Alternaria* (2%), and in F2: *Mucor* (3.6%), *Alternaria* (3.6%) and *Aspergillus* (1.8%).

According to *Đorđević et al. (2003)***,** microbiological population of alfalfa silage consisted of following species belonging to genera: *Acremonium*, *Botryotrichum, Mucor*, *Strepromyces*, *Penicillium*, *Cladosporium*. In investigations of microbes in silages all over Europe, *Chadd (2004) (citat acc. Adamović et al., 2005)* concluded that the most frequent are fungi of genera *Penicillium*, *Aspergillus* and *Fusarium,* which is similar to our results.

In Table 2, the total fungi count in investigated silage samples is presented according to investigated factors for three year period.

Kruskal-Wallis test showed that there was significant difference ($p=0.0374$) between silages from different mixtures in 2005 for total fungi count. Silage from pure alfalfa crop had less fungi compared to other mixtures, 2.57 log10

CFU g^{-1}. The highest fungi count was established in silage from mixture of alfalfa, cocksfoot and tall fescue (S2) - 3.38 log10 CFU g^{-1}, followed by mixtures S3 and S1. This occurrence could be explained by lower content of fermentable sugars in alfalfa *(Bruno et al., 1998)*.

Test showed high significance (p=0.0235) in silages in 2005 depending on the fertilization. Increase of the quantity of nitrogen fertilizer caused gradual increase of fungi count in silages from 2.54 to3.4 log10 CFU g^{-1}. Although there are very few studies of the influence of N fertilization on total fungi count in silage, there are numerous studies done on other cultures. So, according to data presented by *Lemmens et al. (2004), Heier et al. (2005), Krnjaja et al. (2009)* fertilization significantly increased number of *Fusarium* species in wheat, whereas in corn it had no significant effect *(Krnjaja et al., 2008)*.

Table 2. Total fungi count in analyzed silage samples (log10 CFU g^{-1})

Treatments*	Total fungi count		
	Investigation year		
	2005	2006	2007
Mixture (S)			
S1	3.13	2.02	3.00
S2	3.38	3.90	3.36
S3	3.27	1.39	2.64
M	2.57	1.94	2.24
Fertilization N (N)			
0	2.54	1.97	1.81
70	2.95	2.06	3.13
140	3.12	2.01	3.06
210	3.74	3.22	3.24
Utilization phase F			
F1	3.17	1.76	2.77
F2	3.00	2.86	2.85
Average	3.09	2.31	2.81
H_S (3,n=96)	8.4619*	20.5345**	7.1483
p_s	0.0374	0.0001	0.0673
H_N (3,n=96)	9.4827*	5.3411	9.3085*
p_n	0.0235	0.1485	0.0255
H_F (3,n=96)	0.3254	4.9465*	0.6433
p_f	0.5684	0.0261	0.7998

H_S - Kruskal-Wallis test for effect of mixture; H_N - Kruskal-Wallis test for effect of N; H_F - Kruskal-Wallis test for effect of utilization phase.

In 2006, very significant differences (p=0.0001) in total fungi count were established between silages from different mixtures, whereas utilization phase showed significance at the level of p=0.0261. The highest total fungi count was established like in preceding year in silage samples of mixture of alfalfa, cocksfoot and tall fescue (S2) 3.90 log10 CFU g^{-1}, whereas samples of silage from other

mixtures and pure alfalfa crop had significantly lower total fungi count of 1.39 log10 CFU g^{-1} (S3) to 2.02 log10 CFU g^{-1} (S1).

Later cutting had significant effect on increase of total fungi count in silage. So, fungi count in samples of silage cut in phase F1 was 1.76 log10 CFU g^{-1}, and in F2 2.86 log10 CFU g^{-1}.

In the last investigation year of silage preparation the only statistically significant difference ($p=0.0255$) occurred between fertilization treatments, whereas remaining two factors showed no statistically significant effect on total fungi count in silage. Total fungi count in control treatment differed significantly compared to fertilization treatments, where considerably uniform mould count was established. Fungi count in control was 1.81 log10 CFU g^{-1}, whereas in nitrogen treatment it was from 3.06 to 3.24 log10 CFU g^{-1}.

In microbiological analysis of silage prepared from grass *Cynodon dactylon* and treated with biological inoculants fungi count slightly lower compared to our research was established - 1.73 to 2.37 log10 CFU g^{-1} *(Adesogan et al., 2004)*. In investigation of alfalfa silages *Amigot et al. (2006)* obtained fungi count of up to 6 x 10^5 CFU kg^{-1} which is lower value compared to value obtained in our research. However, in alfalfa silage prepared in bales, total fungi count was higher compared to obtained results and in average was 1,5 x 10^5 CFU g^{-1} *(O'Brien et al., 2007)*. According to Rulebook on maximum allowed quantity of harmful substances in livestock feed *(Službeni list SFRJ, 1990),* the highest fungi count can not exceed 300 000 g^{-1}. Obtained data in our study don't exceed the limit, so it can be concluded that based on this parameter the investigated silage samples were of good, i.e. satisfactory quality.

Conclusion

Based on research of the effect of type of mixture, nitrogen fertilization and utilization phase of plants on incidence and fungi count in silage, it can be concluded that the type of mixture as a factor had significant effect in the first two years of investigation. The highest total fungi count in both years of 3.38 and 3.90 log10 CFU g^{-1} was recorded in mixture of alfalfa, cocksfoot and tall fescue. Nitrogen fertilization significantly increased the total fungi count in the first and third investigation year: from 2.54 to 3.74 and from 1.81 to 3.24 log10 CFU g^{-1}. Contrary to previous two investigated factors, utilization phase exhibited significant effect only in 2006 because delay in cutting increased total fungi count from 1.76 to 2.86 log10 CFU g^{-1}. The most present were fungi species of genus *Fusarium* with 80.0 and 91.0%, respectively. Obtained results on total fungi count in silage do not exceed maximum allowed value of 300 000 g^{-1}, which means that silage, based on this parameter, is of good quality and can be used in livestock nutrition.

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Identifikacija i kvantifikacija gljiva u travno-leguminoznoj silaži

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Rezime

Cilj istraživanja je bio da se odredi prisustvo, ukupan broj i vrste gljiva u uzorcima silaža travno-leguminoznih smeša u zavisnosti od botaničkog sastava smeše, đubrenja azotom i faze iskorišćavanja useva. Većinom su identifikovane vrste gljiva iz roda *Fusarium* (80-91%). U ispitivanim uzorcima silaže ukupan broj gljiva u proseku za trogodišnji period istraživanja (2005-2007) je bio od 2.31-3.09 log10 CFU g^{-1} i varirao je u zavisnosti od ispitivanih faktora. Faktor vrsta smeše je značajno uticao na broj gljiva u prve dve godine istraživanja, dok je đubrenje azotom prouzrokovalo variranja u broju gljiva u prvoj i trećoj godini istraživanja. Kosidba u ranijim fazama iskorščivanja statistički značajno je uticala na ukupan broj gljiva samo u jednoj godini istraživanja (2006).

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