THE EFFECT OF ARTIFICIAL INOCULATION WITH *FUSARIUM* STRAINS ON NUTRITIVE VALUE OF MAIZE AND ENSILING PROCESS*

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The objective of the study was to determine the influence of artificial *Fusarium* contamination of maize on nutritive value and ensiling process. Silages were prepared from maize hybrid MONUMENTAL that was either untreated (C) or artificially inoculated with *Fusarium* strains (I) and from Bt–hybrid MONSANTO MON 810 (Bt). The inoculation was made in the growing crop at the end of flowering. Plants were harvested at soft dough stage of maturity and ensiled in microsilage tubes. Forage dry matter (DM) content differed significantly ($P < 0.05$), being 362.2 g/kg in C, 375.8 g/kg in Bt and 350.7 g/kg in I. Forage I contained higher levels of deoxynivalenol (DON), fumonisins (FUM) and aflatoxin (AFL) than forage C or Bt ($P < 0.05$). Content of DM of silages differed significantly, being 346.8 g/kg in C, 360.8 g/kg in Bt and 331.1 g/kg in I ($P < 0.05$). Silage I had higher content of DON and FUM ($P < 0.05$) than silage C and Bt.

maize; *Fusarium spp*.; mycotoxins; nutritive value; ensiling process

INTRODUCTION

Maize silage has been the main preserved forage fed to ruminants in many countries. Although in past, forage crops and silage had received little attention, mycotoxins in maize silage have been recently identified with dairy herd health problems during years with near ideal growing conditions and record maize yields. The mycotoxin content of the plant fractions other than the kernel is therefore significant to animal growth and health.

Fusarium species are common contaminants of maize (*Zea mays* L.). Although this fungus principally infects cereals, it can also be found on forages in the field and is thought to proliferate before harvest (Christensen et al., 1977). Depending on the species and environmental factors, *Fusarium spp*. are able to produce mycotoxins such as fumonisins, zearalenone, and trichothecenes, which may be dangerous to both human and animal health (Prelusky et al., 1994). The conditions associated with well preserved silage, i.e. low pH and anaerobiosis, are unfavourable for the growth of most moulds. Exclusion of air should ensure that only few fungi are able to grow or survive under these conditions. (Lepom et al., 1988). Most *Fusarium spp*. associated with maize and grass in the field, are aerobic and are unable to grow in silage so they do not appear to be a major contaminant in store (Burmeister et al., 1972). However, some “field” mycotoxins such as zearalenone have been reported to survive ensilage (Lepom et al., 1988).

The objective of the present study was to determine the effect of selected *Fusarium* strains artificially inoculated on conventional maize hybrid (MONUMENTAL) on nutritional value, fermentation process and mycotoxin content of silage subsequently prepared from this hybrid in comparison with untreated maize and Bt-hybrid.

MATERIAL AND METHODS

In 2005 a field trial was conducted in the area of Crop Research Institute, Prague-Ruzyně, Czech Republic. The experimental field of maize forage was divided into three areas of 10 m^2, two of them were sown with the conventional maize hybrid MONUMENTAL which remained either untreated (C) or was artificially inoculated with the selected *Fusarium* strains (I). The third area was sown with the Bt-hybrid MONSANTO (MON 810). All hybrids were grown, harvested and ensiled under identical conditions. The inoculation was made in the growing crop at the end of flowering after harming the cops and stalks with wire brush. Subsequently whole plants were infected with a prepared suspension of *Fusarium* strains (*F. verticillioides* – LS 10/04, *F. verticillioides* – LS 225/02, *F. verticillioides* – LS 104/03, *F. subglutinans* – LS 164/02,

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F. subglutinans – LS 14/04, *F. subglutinans* – LS 25/03, *F. graminearum* – LS 245/02, *F. graminearum* – LS 41/02, *F. graminearum* – LS 273/02, Collection of phytopathogenic fungi, Crop Research Institute, Prague-Ruzyně, CR). Entire maize plants were harvested at the soft dough stage of maturity (i.e. 7 weeks from inoculation) and ensiled in microsilage tubes (approximately 6.5 kg per tube, 5 tubes per treatment). Samples of forage were taken prior ensiling. Immediately after filling, the tubes were hermetically closed and fermented at 25 °C (± 1 °C) for 100 days. After the fermentation tubes were opened, the contents were mixed thoroughly and samples were taken for subsequent analyses.

Analytical procedures

Samples of forage and silages were analyzed for the basal nutrients. DM was determined by drying at 55 °C for 24 h, followed by milling through a 1 mm screen and drying for another 4 h at 103 °C. Content of crude protein (CP), crude fiber (CF), ash and fat were estimated according to AOAC (1984). Neutral detergent fiber (NDF, with α-amylase) was estimated according to Van Soest et al. (1991), ash-free acid detergent fiber (ADF) was estimated according to Goering and Van Soest (1970).

The parameters characterizing the ensilage process and silage quality were determined from the aqueous silage extract prepared according to Suzuki and Lund (1980). pH was determined using accurate pH meter. Aqueous silage extract acidity (free acidity) was determined after titration of 0.1 M KOH to pH = 8.5. Analysis of amino acid N ($N\text{-}NH_2$) was conducted by formol titration with 0.1 M KOH after addition of HCHO. Ammonia content was determined by the Conway microdiffusion method (Conway, 1962).

The volatile fatty acids (VFA) and alcohols were determined from aqueous extract using gas chromatography on CHROM-5 gas chromatograph (Laboratorní přístroje Praha, CR) fitted with glass column, packed with 80/120 Carbopack B-DA/4% CARBOWAX 20 M. The temperature gradient program was held at 85 °C for 2 min (methanol and ethanol) and increased to 147 °C at a rate of 7.5 °C /min, then increased to 180 °C at a rate of 15 °C/min for determination of other alcohols, volatile fatty acids and lactic acid. As an internal standard trimethylacetic acid was used, nitrogen was the carrier gas. Results were evaluated by program CSW 1.5 method with internal standard ISTD – 2.

From the obtained results the degree of proteolysis was calculated according to the following formula: degree of proteolysis (%) = $N\text{-}NH_3$ / total N

Commercially available quantitative ELISA assay kits Veratox (Neogen Corp., Lansing, MI, USA) were used for measuring the presence of *Fusarium* mycotoxins according to the manufacturer's instructions.

Statistical analysis

Data obtained in the experiment were analysed using one-way ANOVA of the Statgraphics 7.0 package (Manugistics Inc., and Statistical Graphics Corporation, Rockville, Maryland, USA).

RESULTS

The nutrient content of the fresh maize forages is given in Table 1. The highest DM content was determined in Bt (375.8 g/kg), intermediate in C (362.2 g/kg) and the lowest in I (350.7 g/kg). All values differ significantly ($P < 0.05$). The content of CP in C was higher than in I ($P < 0.05$) while CP content in Bt did not differe significantly from C or I ($P > 0.05$). Content of ash in C was similar to that determined in Bt and was lower than in I ($P < 0.05$). Content of CF in Bt was higher ($P < 0.05$) than those determined in C and I that

Table 1. Content of nutrients and mycotoxins in maize forage

Parameters	Unit	C	Bt	I	SEM
Dry matter	g/kg	362.1[a]	375.8[b]	350.7[c]	1.925
Ash	g/kg	47.0[a]	46.1[a]	55.6[b]	0.826
Crude protein	g/kg	65.9[a]	62.2[ab]	59.3[b]	2.975
Fat	g/kg	21.7	23.4	21.8	0.579
Crude fiber	g/kg	179.6[a]	195.3[b]	178.1[a]	2.975
Acid detergent fiber	g/kg	216.3	223.7	211.0	4.747
Neutral detergent fiber	g/kg	416.1	424.2	418.9	9.188
Content of mycotoxins					
DON[1]	ng/kg	37.8[a]	39.5[a]	55.6[b]	2.990
FUM[1]	ng/kg	ND[a]	2.5[a]	354.6[b]	40.941
AFL[1]	ng/kg	1.0[a]	1.1[a]	1.8[b]	0.148
ZON[1]	ng/kg	12.5	13.0	17.6	3.887

[a, b] means followed by the superscripts are significantly different ($P < 0.05$)
[1] DON – deoxynivalenol, FUM – fumonisins, AFL – aflatoxin, ZON – zearalenone
ND – not detected

Table 2. Nutritional value and mycotoxins content of silages prepared from control, Bt or artificially inoculated maize

Parameters	Unit	C	Bt	I	SEM
Dry matter	g/kg	346.8[a]	360.8[b]	331.1[c]	4.028
Ash	g/kg	49.2[a]	45.3[b]	57.2 [c]	0.969
Crude protein	g/kg	67.3	62.8	63.5	1.576
Fat	g/kg	26.8[a]	27.8[a]	22.1[b]	0.623
Crude fiber	g/kg	173.5[a]	176.1[a]	183.7[b]	2.071
Acid detergent fiber	g/kg	201.4	208.5	209.5	3.990
Neutral detergent fiber	g/kg	389.8[a]	425.9[b]	406.3[a]	9.930
Content of mycotoxins					
DON[1]	ng/kg	129.9[a]	127.4[a]	194.8[b]	10.916
FUM[1]	ng/kg	ND	ND	65.2	–
AFL[1]	ng/kg	2.2	2.3	2.95	0.277
ZON[1]	ng/kg	22.0	24.1	27.1	4.961

[a, b] means followed by the superscripts are significantly different ($P < 0.05$)
[1]DON – deoxynivalenol, FUM – fumonisins, AFL – aflatoxin, ZON – zearalenone
ND – not detected

Table 3. Characteristics of fermentation process of silages prepared from control, Bt or artificially inoculated maize

Parameters	Unit	C	Bt	I	SEM
pH	–	4.0	4.1	4.1	0.042
Free acidity	mg KOH/100g	1556.5[ab]	1337.7[a]	1581.7[b]	71.562
Ammonia NH_3	g/kg	0.3	0.3	0.3	0.010
Ammonia α-amino groups	g/kg	1.1	1.0	1.1	0.033
Proteolysis (N-NH_3 / total N)	%	6.7[a]	6.6[a]	8.0[b]	0.312
Acetic acid	g/kg	2.9[ab]	2.1[a]	3.1[b]	0.309
Butyric acid	g/kg	0.3	1.5	0.3	0.446
i-valeric acid	g/kg	0.2	0.2	0.1	0.108
Lactic acid	g/kg	34.8	23.8	25.1	4.047
Ethanol	g/kg	2.8	3.0	4.1	0.793

[a, b] means followed by the superscripts are significantly different ($P < 0.05$)

were similar. Other parameters (fat, ADF and NDF) were not affected by the treatment ($P > 0.05$). Forage from C and Bt plants was positive for deoxynivalenol (DON), aflatoxin (AFL), and zearalenone (ZON) while fumonisins (FUM) were only detected in small amounts in Bt. Artificially harmed and inoculated maize forage (I) was positive for all mentioned mycotoxins. Content of AFL, DON and FUM were higher in I forage in comparison to C and Bt ($P < 0.05$). I forage tended to have higher content of ZON than detected in C and Bt ($P > 0.05$).

Nutritional value of silages prepared from untreated (C), genetically modified (Bt) or artificially inoculated conventional maize (I) and characteristics of fermentation process is presented in Tables 2 and 3. Content of DM and fat differed significantly among groups ($P < 0.05$). Content of fat and CF in I was significantly different ($P < 0.05$) from values determined in C or Bt that were close. Content of NDF in Bt was higher than in C ($P < 0.05$), ADF and CP contents were not affected by the treatment ($P > 0.05$). The silage prepared from artificially harmed and subsequently inoculated maize (I) had higher content of DON and FUM ($P < 0.05$) in comparison to silage C and Bt. Levels of AFL and ZON tended to be higher in I than in C or Bt, results were not significantly different ($P > 0.05$).

After 100 d of ensiling in microsilage tubes average pH values of all silages were similar ($P > 0.05$). Silage I showed a higher degree of proteolysis measured as N-NH_3 (% of total N) than silage C or Bt ($P < 0.05$). Contents of lactic and butyric acids and ethanol were not influenced by the treatment ($P > 0.05$). Content of acetic acid, that was the highest in I, differed significantly from Bt ($P < 0.05$) but was similar to values determined in C ($P > 0.05$).

DISCUSSION

The composition of the suspension of *Fusarium* species that were inoculated on the maize plants was selected with regards to natural occurence of the strains. *F. verticilloides* (formerly *F. moniliforme*) is a specie that is almost ubiquitous in maize and belongs to one of the main species producing high yields of fumonisins (Cawood et al., 1991). *F. graminearum* is the major ZON-producing fungus that causes maize ear and stalk rots (Christensen et al., 1988). In addition to the fungi listed above, *Fusarium subglutinans* can also produce toxins in maize (Kostecki et al., 1997).

Maize forage

Nutritional value of Bt forage was similar to C. This is in agreement with the findings in other studies focused on the evaluation of Bt and its near isogenic conventional hybrids (e.g. Aumaitre et al., 2002; Flachowsky et al., 2007). These data suggest that genetically modified plants do not significantly differ in their nutritional value from those of the isogenic variety. Maize forage artificially inoculated with *Fusarium* strains (I) had lower content of DM, OM, and CP and higher content of ash than untreated maize plants ($P < 0.05$). Our findings are in agreement with widely observed phenomenon that mould growth can reduce the content of nutrients including vitamins and amino acids such as lysine in feedstuffs (e.g. Kao, Robinson, 1972). The energy value of feeds is usually reduced as a consequence of mould growth.

In the present study, forage material from the C and Bt plants was positive for DON and ZON. Low amounts of AFL were also determined, while FUM were only detected in small amounts in Bt. The natural occurrence of these mycotoxins in maize plants is in agreement with literature. E.g. Schollenberg et al. (2006) determined that maize plant material in Germany was naturally contaminated not only with DON and ZON but also with other *Fusarium* mycotoxins such as NIV or T-2 toxin. Similarly, in the study of Reid et al. (1996) the presence of DON in control plants with the identification of *F. graminearum*, confirmed natural infection of maize plants with this mould. In Poland, Visconti et al. (1990) found DON in both kernels and cobs of maize naturally infected by *F. graminearum.* The content of mycotoxins determined in our study was lower than reported by Oldenberg (1993) who demonstrated the presence of zearalenone in whole plants and parts of maize used for silage making at concentrations up to 300 μg/kg that was mainly accumulated at the end of the ripening process, with subsequent contamination of the silage. Similarly, the DON contents of forage maize in the study of Oldenburg and Hoeppner (2003) ranging between 0.22 and 12.9 mg/kg DM were higher than that in the present experiment. Zearalenone concentration found in the latter study was considerably lower than DON which is in agreement with our findings. Described variations in mycotoxins content are associated with factors such as fungal strain (Vesonder et al., 1982), microbial competition (Reid et al., 1999), and culture substrate (Greenhalgh et al., 1983). Furthermore, various responses in DON accumulation also have been observed in relation to maize hybrid (Atlin et al., 1983).

Artificially inoculated maize forage (I) was positive for all determined *Fusarium* toxins – DON, ZON, FUM and AFL. In contrast to C where FUM were not detected, in I forage FUM were prevalent (predominant) mycotoxins with an average value of 354.6 ng/kg. Whole plant mycotoxins content was lower than that determined in the study of Lepom et al. (1990) or Miller et al. (1983). However, they measured the mycotoxin concentration in the infected ears although they noted that toxins were found throughout the whole plant but mainly in the infected part. Interestingly, there are reports that the ear and kernels often have significantly lower concentrations of DON compared to the leaves and stalk (Di Menna et al., 1997; Lauren, Di Menna, 1999; Lew et al., 1997).

Maize silage

Differences in feeding value between C and I silages were similar to that of maize forage. Characteristics describing fermentation process in our experiment corresponded to values described by Steidlová and Kalač (2002) who evaluated fermentation process of 113 samples of silages prepared from maize hybrids commonly grown in the Czech Republic from 86 farm. A course of fermentation process of studied silages was not influenced by the inoculation of *Fusarium* species ($P > 0.05$) except of a higher degree of proteolysis in I ($P < 0.05$). This is in agreement with Mansfield et al. (2005) who did not observe any relationships between fermentation characteristics (pH and DM, ammonia, lactic acid, acetic, propionic, butyric, and isobutyric acid content) and DON incidence or DON levels in the samples. Similarly, Lepom et al. (1988) did not ascertain any relationships between the fermentation process and the toxin content of the silage.

Based on the observation of the inability of *Fusarium* strains to persist in ensiled maize, Golosov et al. (1967) suggested that they are not capable to survive the low oxygen content and low pH in silage. The optimal pH for growth of *F. verticillioides* is 7.0 (Marin et al., 1995), while the mean pH of C and I silages in our study was 4.01 and 4.06, respectively. The silage prepared from artificially harmed and subsequently inoculaced maize (I) had higher content of DON and FUM ($P < 0.05$) in comparison with silage made from untreated maize (C). Garon et al. (2006) during a 9-month-study in Normandy, France, found the recurrence of around 20 different species of fungi including *Fusarium verticillioides* in silage samples with average ZON content of 23–41 ppb and DON content of 100–213 ppb.

View on the persistence of *Fusarium spp*. and their mycotoxins in silages during the fermentation process is not consistent. LeBars and Escoula (1974) supposed that the spectrum of fungal species present in silage made from maize, sorghum and grass varies with the duration of storage because they detected species belonging to *Penicillium*, *Fusarium* and *Aspergillus* genera after two to three months of storage. On the other hand, Damaglou et al. (1984) or Lepom et al. (1988) supposed that in a good quality silage prepared under acidic anaerobic conditions further mould growth and mycotoxin development is unlikely. Furthermore, these conditions also tend to reduce levels of some mycotoxins already present at ensilement, as documented in the study of Hacking (1979) who found that ZON remained stable only for a very short time after ensiling or of Damaglou et al. (1984). However, in corn cob maize silage infected with *F. culmorum* studied over a 12 week period by Lepom et al. (1988) ZON levels remained unchanged for the whole period although no *F. culmorum* could be isolated by 11 days of the experiment. Further, in the subsequent experiments of Lepom et al. (1990) DON in naturally contaminated maize remained still present in silages, thus they concluded that the most probable way of contamination of maize silage with DON seems to be infection of maize plants by *Fusarium* species in the field. Similarly, the contamination of the maize silage with DON and ZON were also reported by Schollenberg et al. (2006) who assumed that the detection of different *Fusarium* toxins in silage indicates an at least partial stability of these substances during fermentation. In contrast to previous reports that DON concentration does not change after ensiling (Lepom et al., 1990), Mansfield et al. (2005) found that DON concentration was lower in ensiled samples compared with those collected at harvest but they did not find any effect of physical and chemical characteristics of ensiled samples on DON concentration. Therefore, they suggested that toxin reduction may be due to the activity of silage microflora. On the other hand, Joffe (1986) described that the *Fusarium* moulds associated with alimentary toxic aleukia grew prolifically at 25–30 °C without producing much mycotoxin, but at near-freezing temperatures, large quantities of mycotoxins were produced with minimal mould growth and thus supposed that the conditions most suitable for mould growth may not be the optimum conditions for mycotoxin formation.

CONCLUSIONS

Artificial inoculation of maize plants during growth with selected *Fusarium* strains resulted in increasing mainly in deoxynivalenol and fumonisins contents in comparison to control or Bt-hybrid ($P < 0.05$) while levels of zearalenone tended to be higher than in control or Bt-hybrid ($P > 0.05$) in both forages and silages, respectively.

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Vliv umělé inokulace *Fusariovými* kmeny na nutriční hodnotu kukuřice a průběh silážování.

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Cílem studie bylo stanovit vliv umělé kontaminace kukuřice *Fusariovými* kmeny na nutriční hodnotu a průběh silážování. Siláže byly vyrobeny z hybridu MONUMENTAL, který byl buď neošetřený (C), nebo uměle inokulovaný suspenzí Fusariových kmenů (I), a z Bt-hybridu MONSANTO MON 810 (Bt). Inokulace byla provedena na porostu kukuřice na konci květu. Kukuřice byla sklizena v mléčně voskové zralosti a zasilážována v mikrosilážních tubusech (5 tubusů na zásah). Obsah sušiny (DM) kukuřičné řezanky se průkazně lišil mezi skupinami a byl 362,2 g/kg u C, 375,8 g/kg u Bt a 350,7 g/kg u I ($P < 0,05$). Inokulovaná řezanka I obsahovala vyšší hladinu deoxynivalenolu (DON), fumonisinu (FUM) a aflatoxinu (AFL) než C nebo Bt ($P < 0,05$). Obsah DM siláží se průkazně lišil mezi skupinami a byl 346,8 g/kg u C, 360,8 g/kg u Bt a 331,1 g/kg u I ($P < 0,05$). Siláž I obsahovala vyšší hladiny DON a FUM než siláž C nebo Bt ($P < 0,05$).

kukuřice; *Fusarium spp*.; mykotoxiny; výživná hodnota; proces silážování

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