ORIGINAL PAPER

THE EFFECT OF ARTIFICIAL INOCULATION WITH SELECTED FUSARIUM STRAINS ON NUTRITIONAL QUALITY AND ENSILING PROCESS OF BT MAIZE

VLIV UMĚLÉ INOKULACE VYBRANÝMI KMENY FUSÁRIÍ NA NUTRIČNÍ HODNOTU A PRŮBĚH SILÁŽOVÁNÍ BT KUKUŘICE

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ABSTRACT

The objective of the study was to compare the nutritive value and mycotoxin content of maize forage and silage of near isogenic control MONUMENTAL (C) and Bt maize (MONSANTO, MON 810) that was either untreated (Bt) or artificially inoculated with Fusarium strains (I-Bt). The inoculation was made in the growing crop in milk stage of maturity. Plants were harvested at the soft dough stage of maturity and ensiled in microsilage tubes. The content of forage dry matter (DM) was 307.6 g/kg in C, 306.9 g/kg in Bt and 298.0 g/kg in I-Bt. All forages were positive for deoxynivalenol, aflatoxin, fumonisins and zearalenone (P>0.05). Content of DM was the lowest in I-Bt silage (285.5 g/kg) and differed significantly from C (296.7 g/kg) or Bt (303.7 g/kg, P<0.05). Content of crude protein (CP) was the lowest in I-Bt silage (79.0 g/kg) and differed significantly from C or Bt (85.7 or 81.9 g/kg, respectively, P<0.05). Silages Bt and I-Bt had lower pH (3.93 and 3.96, respectively) than silage C (4.02, P<0.05). Silage I-Bt tended to have a higher degree of proteolysis (9.18 %) measured as $N\text{-}NH_3$ (% of total N) than silages C or Bt (8.64 or 8.9 %, respectively, P>0.05). Lactic acid was predominant product of fermentation in all silages, however silage I-Bt tended to have lower content of lactic acid (20.96 g/kg) than C or Bt (24.76 or 23.82 g/kg, P>0.05). I-Bt silage contained lower levels of deoxynivalenol (602 ppb) than C or Bt silage (748 and 690 ppb, respectively, P<0.05). Content of fumonisins and zearalenone in C did not differ from I-Bt (P<0.05) but both were lower than in Bt (P<0.05). In conclusion, nutritional value a fermentation parameters of Bt silage were similar to C except of CP content and pH that was lower in Bt (P<0.05). I-Bt silage had lower content of DM, CP and fat than Bt silage (P<0.05). Controversially, concentrations of mycotoxins in I-Bt silage were lower than in Bt.

Key words: Bt maize, Fusarium spp., mycotoxins, nutritive value, ensiling process

ABSTRAKT

Cílem studie bylo porovnat nutriční hodnotu a obsah mykotoxinů kukuřičné řezanky a siláže u izolinie MONUMENTAL (C) a Bt kukuřice (MONSANTO, MON 810), která byla neošetřená (Bt) nebo uměle inokulovaná Fusariovými kmeny (I-Bt). Inokulace byla provedena na porostu ve stádiu mléčné zralosti. Kukuřice byla sklízena v mléčně voskové zralosti a zasilážována v mikrosilážních tubusech. Obsah sušiny (DM) v řezance byl 307,6 g/kg u C, 306,9 g/kg u Bt a 298,0 g/kg u I-Bt. Řezanka ve všech skupinách byla pozitivní na deoxynivalenol, aflatoxin, fumonisiny a zearalenon (P>0,05). Obsah DM byl nejnižší u I-Bt siláže (285,5 g/kg) a průkazně se lišil od C (296,7 g/kg) nebo Bt (303,7 g/kg, P<0,05). Obsah dusíkatých látek (CP) byl nejnižší u siláže I-Bt (79,0 g/kg) a průkazně se lišil od siláží C (85,7 g/kg) a Bt (81,9 g/kg, P<0,05). Siláže Bt a I-Bt měly nižší pH (3,93 resp. 3,96) než siláž C (4,02, P<0,05). U siláže I-Bt byla pozorována tendence k vyššímu stupni proteolýzy (9.18 %) měřené jako $N\text{-}NH_3$ (% z celkového N) než u siláží C nebo Bt (8,64 resp. 8,9 %, P>0,05). Kyselina mléčná byla predominantním produktem fermentace ve všech silážích, nicméně u siláže I-Bt byla tendence k nižšímu obsahu kyseliny mléčné (20,96 g/kg) než u C nebo Bt (24,76 nebo 23,82 g/kg, P>0,05). Siláž I-Bt obsahovala nižší hodnoty deoxynivalenolu (602 ppb) než C nebo Bt siláže (748 a 690 ppb, P<0,05). Obsah fumonisinů a zearalenonu u C se nelišil od I-Bt (P<0,05), ale obojí bylo nižší než u Bt (P<0,05). Na závěr, nutriční hodnota a fermentační parametry Bt siláže byly obdobné jako u C s výjimkou obsahu CP a pH, které bylo nižší u Bt (P<0,05). Siláž I-Bt měla nižší obsah DM, CP a tuku než siláž Bt (P<0,05). Kontroverzně, koncentrace mykotoxinů v siláži I-Bt byly nižší než u Bt.

Klíčová slova: Bt kukuřice, Fusarium spp., mykotoxiny, nutriční hodnota, proces silážování

INTRODUCTION

In warmer European regions the European maize borer (EMB, Ostrinia nubilalis) causes significant reductions in maize yield. Furthermore, it has been demonstrated that damage resulting from insect infestation provides preferential sites for the penetration of the fungi, and some insects can even operate as vectors of mycotoxigenic fungi [24, 30]. Maize hybrids genetically enhanced to express proteins that are native to the Bacillus thuringiensis (Bt) bacterium, commonly referred to as Bt-maize hybrids, are resistant to damage caused by EMB infestation [16]. Published literature, reviewed by e. g. [3] has detected no consistent difference in feed intake or chemical composition of Bt maize compared to control or near isogenic hybrids. Reduction of the fungal infection level associated with reduced insect damage of Bt hybrids have been also suggested, but preliminary results are inconsistent [5, 22, 25, 28].

Thus, the objective of the study was to compare the nutritive value and mycotoxin content of maize forage and silage and to evaluate the fermentation process of near isogenic control (MONUMENTAL) and Bt maize (MONSANTO, MON 810) that was either untreated or artificially inoculated with selected Fusarium strains.

MATERIAL AND METHODS

In 2006 a field trial was conducted in the area of Ivanovice na Hané, Czech Republic. The experimental field of forage maize was divided into three areas of 10 m^2, two of them were sown with the Bt-hybrid MONSANTO (MON 810) which remained either untreated (Bt) or was artificially inoculated with the selected Fusarium strains (I-Bt). The third area was sown with the conventional maize hybrid MONUMENTAL (C). All hybrids were grown, harvested and ensiled under identical conditions. The inoculation was made in the growing crop in milk stage of maturity after harming the cobs 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, 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). Entire maize plants were harvested at the soft dough stage of maturity and ensiled in microsilage tubes (approximately 6,5 kg per tube, 6 tubes per treatment). Samples of forage were taken prior ensiling (3 samples per each forage). 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 from each tube were taken for subsequent analyses.

Analytical procedures

Samples of forage and silages were analyzed for the basal nutrients. Dry matter (DM) was determined after drying at 55°C for 2 days, 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 [1]. Neutral detergent fiber (NDF, with α-amylase) was estimated according to [33], ash-free acid detergent fiber (ADF) was estimated according [12].

The parameters characterizing the ensilage process and silage quality were determined from the aqueous silage extract prepared according [32]. 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 [7].

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 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 from silages 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 content of DM determined in C and Bt was

Table 1: Chemical composition (on a dry matter basis) and mycotoxins content of fresh maize forage of isoline (C), untreated Bt-hybrid (Bt) and artificially inoculated Bt-hybrid (I-Bt)

Tabulka 1: Chemické složení (v sušině) a obsah mykotoxinů čerstvé kukuřičné řezanky isolinie (C), neošetřeného Bt-hybridu (Bt) a uměle inokulovaného Bt-hybrid (I-Bt)

Parameters	Units	C	Bt	I-Bt	SEM
Dry matter	g/kg	307.6	306.9	298.0	1.959
Ash	g/kg	43.4	43.5	44.6	0.566
Crude protein	g/kg	76.1	73.4	73.7	0.158
Fat	g/kg	28.1	28.0	28.6	0.532
Crude fiber	g/kg	182.9	192.2	195.3	4.547
Acid detergent fiber	g/kg	212.2	214.7	213.2	2.449
Neutral detergent fiber	g/kg	414.9	417.2	410.4	1.585
Content of mycotoxins					
DON[1]	ppb	786.4	667.1	426.3	114.398
FUM[1]	ppb	25.0	16.67	91.9	53.276
AFL[1]	ppb	0.33	0.50	0.17	0.136
ZON[1]	ppb	63.9	46.3	47.10	16.432

[1]DON-deoxynivalenol, FUM-fumonisins, AFL-aflatoxin, ZON-zearalenon

[1]DON-deoxynivalenol, FUM-fumonisins, AFL-aflatoxin, ZON-zearalenon

Table 2: Nutritional value (on a dry matter basis) and mycotoxins content of maize silages prepared from control isoline (C), untreated (Bt) or artificially inoculated Bt-hybrid (I-Bt)

Tabulka 2: Nutriční hodnota (v sušině) a obsah mykotoxinů kukuřičné siláže připravené z kontrolní isolinie (C), neošetřeného (Bt) nebo uměle inokulovaného Bt-hybridu (I-Bt)

Parameters	Unit	C	Bt	I-Bt	SEM
Dry matter	g/kg	296.7 ^{ab}	303.7 ^{a}	285.5 ^{b}	4.832
Ash	g/kg	44.6	42.9	43.2	0.637
Crude protein	g/kg	85.7 ^{a}	81.9 ^{b}	79.0 ^{c}	0.655
Fat	g/kg	32.1 ^{a}	32.1 ^{a}	29.9 ^{b}	0.582
Crude fiber	g/kg	183.6	179.9	185.2	0.462
Acid detergent fiber	g/kg	205.4	205.4	211.3	4.789
Neutral detergent fiber	g/kg	362.8	352.9	359.3	6.160
Content of mycotoxins					
DON[1]	ppb	748.4 ^{a}	689.9 ^{a}	602.0 ^{b}	27.781
FUM[1]	ppb	52.6 ^{a}	161.4 ^{b}	74.5 ^{a}	18.298
AFL[1]	ppb	0.92 ^{ab}	1.33 ^{a}	0.68 ^{b}	0.177
ZON[1]	ppb	54.2 ^{a}	75.75 ^{b}	49.42 ^{a}	4.035

^{a,b,c} means in the same row followed by the different superscripts differ significantly (P<0.05)

[1]DON-deoxynivalenol, FUM-fumonisins, AFL-aflatoxin, ZON-zearalenon

^{a,b,c} hodnoty označené ve stejném řádku rozdílnými indexy se průkazně liší (P<0,05)

[1]DON-deoxynivalenol, FUM-fumonisins, AFL-aflatoxin, ZON-zearalenon

higher than in I-Bt (P<0.05). Content of other nutrients was not affected by the treatment (P>0.05). Forages of all groups were positive for DON, AFL FUM and ZON. Although non-significant, artificially inoculated Bt hybrid (I-Bt) had lower content of DON, and ZON and higher content of FUM in comparison to C or Bt forage (P>0.05).

Nutritional value of silages and characteristics of fermentation process is presented in Table 2 and 3. Content of DM, CP and fat was the lowest in I-Bt silage and differed significantly from C or Bt (P<0.05). Content of other nutrients was not affected by the treatment (P>0.05). Silages Bt and I-Bt had lower pH than silage C (P<0.05). Silage I-Bt tended to have a higher degree of proteolysis measured as $N\text{-}NH_3$ (% of total N) than silages C or Bt (P>0.05). Lactic acid was predominant product of fermentation in all silages, however silage I-Bt tended to have lower content of lactic acid than C or Bt (P>0.05). Content of acetic acids differed significantly among groups (P<0.05). Contents of butyric acid were

Table 3: Characteristics of fermentation process of silages prepared from control isoline (C) and untreated (Bt) or artificially inoculated Bt maize (I-Bt)
Table 3: Charakteristika fermentačního procesu siláže připravené z kontrolní isolinie (C) a neošetřené (Bt) nebo uměle inokulované Bt kukuřice (I-Bt)

Parameters	Unit	C	Bt	I-Bt	SEM
pH	-	4.02[a]	3.93[b]	3.96[b]	0.018
free acidity	mg KOH/100g	2274.3	2230.4	2208.9	26.319
Ammonia NH_3	g/kg	0.43	0.43	0.4	0.0159
Ammonia α-amino groups	g/kg	1.57[a]	1.52[a]	1.25[b]	0.0362
Proteolysis $^{N\text{-}NH3\ /\ total\ N}$	%	8.64	8.9	9.18	0.372
acetic acid	g/kg	13.45[a]	9.94[b]	12.0[c]	0.256
lactic acid	g/kg	24.76	23.82	20.96	1.688
ethanol	g/kg	5.7[a]	6.63[b]	5.97[ab]	0.249

[a,b,c] means in the same row followed by the different superscripts differ significantly (P<0.05)
[a,b,c] hodnoty označené ve stejném řádku rozdílnými indexy se průkazně liší (P<0,05)

below detection limit. Content of ethanol in C was lower than in Bt (P<0.05) and similar to that determined in I-Bt (P>0.05).

I-Bt silage contained lower levels of DON than C or Bt silage (P<0.05). Content of FUM and ZON in C did not differ from I-Bt (P<0.05) but both were lower than that determined in Bt (P<0.05). Similarly, higher levels of AFL were found in Bt in comparison to I-Bt (P<0.05).

DISCUSSION

Maize forage

In the present experiment the nutrient content of C and Bt forage did not differ significanly (P>0.05). This is in agreement with e. g. [6, 22] that measured the nutrient contents and did not find any significant differences in composition between the MON 810 hybrids and their near-isogenic controls. The content of DM determined in C and Bt was higher than in I-Bt (P<0.05). Content of other nutrients was not affected by the treatment (P>0.05).

Forages of all groups were positive for DON, AFL, FUM and ZON. Natural occurence of these mycotoxins in C or in Bt hybrids is in agreement with literature [15, 25, 29]. Although non-significant, there was a tendency to lower levels of mentioned mycotoxins in Bt maize forage in our experiment. Similar findings were also described in literature where Bt maize, depending on the severity of other impacts such as weather conditions, often had significantly reduced mycotoxin levels compared to non-Bt isolines [22, 25, 26]. The reduction in mycotoxins content is related especially to fumonisins levels because the insects that are controlled by Bt maize are important in predisposing plants to infection by F. verticillioides and F. graminearum. Based on the field trials, Bt maize has been shown to have significantly lower fumonisin levels than non-Bt isolines, especially when insect damage from EMB is high [15, 25, 26]. Similarly, [28] found that under high EMB pressure, the level of DON was reduced by 88% in Bt hybrids compared with non-Bt isolines while non-significant difference was observed where EMB pressure was low. Furthermore, in a central European field study, the association between EMB damage and DON concentrations was not consistent across years [20]. Although non-significant, I-Bt had lower content of DON, and ZON and higher content of FUM in comparison to C or Bt forage (P>0.05). This discrepancy was probably caused by a warm weather during the artificial inoculation that did not allow to inoculated Fusarium strains to fully develop on harmed maize plants.

Maize silage

Nutritional value of C and Bt silages did not differ except of CP that was higher in C compared to Bt (P<0.05). This is in agreement with published studies reviewed recently by e. g. [2] or [10]. On the other hand, higher content of DM and lignin in Bt silage in comparison to non-transgenic maize were reported by [9] and [27], respectively. The lack of consistent and significant composition differences would support the speculation that the insertion of genes in the events tested did not alter nutrient composition of maize silage. Although Bt and I-Bt silages had lower pH than C (P<0.05), in general parameters of fermentation process found in our experiment were close to values published by [18, 21 or 31].

All silages were positive for DON, FUM, AFL and ZON. This results confirm the earlier studies e. g. [4, 23, 29] that a mixture of toxins can be present simultaneously in a wholeplant maize sample both at harvest and after ensiling indicating an at least partial stability of these substances during fermentation. I-Bt silage contained lower levels of DON than C or Bt silage ($P<0.05$). Content of FUM and ZON in C did not differ from I-Bt ($P<0.05$) but both were lower than that determined in Bt ($P<0.05$). Similarly, higher levels of AFL were found in Bt in comparison to I-Bt ($P<0.05$). Contents of ZON and DON analysed in our experiment were higher than those reported by [11].

View on the persistence of Fusarium spp. and their mycotoxins in silages during the ensiling is not consistent. [13] described the inability of Fusarium to persist in ensiled maize. Similarly, [8] or [18] supposed that in a good quality silage is further mould growth and mycotoxin development unlikely. On the other hand, [17] detected species belonging to Penicillium, Fusarium and Aspergillus genera after two to three months of storage thus they supposed that the spectrum of fungal species present in silage varies with the duration of storage. Furthermore, studies focused on the stability of fusarium mycotoxins formed prior harvest during the ensiling process are inconsistent. According to [8], [14] or [21] acidic and anaerobic conditions reduce levels of ZON and DON already present before ensiling. On the other hand, [18, 19] described that levels of ZON or DON remained unchanged during fermentation.

CONCLUSION

Nutritional value of Bt silage was similar to its near isogenic control except of crude protein content that was lower in Bt ($P<0.05$). Bt silage had lower pH and lower content of acetic acid than control, other characteristics of fermentation process were similar. In Bt silage, there was slightly lower content of deoxynivalenol (non-significant) but higher content of fumonisins and zearalenone ($P<0.05$) in comparison to control. Artificially inoculated Bt silage had lower content of dry matter, crude protein and fat than Bt silage ($P<0.05$). Controversially, concentrations of mycotoxins in silage prapared from inoculated Bt maize were lower than from untreated Bt hybrid.

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