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Effects of ensiling of *Bacillus thuringiensis* (Bt) maize (MON810) on degradation of the crystal 1Ab (Cry1Ab) protein and compositional quality of silage

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The study investigated the degradation of the Bt protein (Cry1Ab) in Bt maize during ensiling and chemical composition of the silage. Two laboratory studies were conducted at the University of Fort Hare. One *Bacillus thuringiensis* (Bt) maize cultivar (DKC80-12B) and its isoline (DKC80-10) in the 2008/2009 study and two Bt maize cultivar (DKC61-25B and PAN6Q-321B) and their isolines (DKC61-24 and PAN6777) in the 2009/2010 study, were ensiled for 42 days and analyzed for ash-free dry matter, Cry1Ab protein, neutral detergent fiber, acid detergent fiber, acid detergent lignin and crude protein. Silage from two of the Bt maize varieties had lower ash-free dry matter than their near-isolines, while variety DKC61-25B was similar to its near-isoline. At least, 60% of the initial Cry1Ab protein concentration remained in the silage. Bt maize had higher neutral and acid detergent fiber than its isolines before ensiling and remained constant after ensiling in 2008/2009, but was neither influenced by ensiling nor variety in 2009/2010. Crude protein and acid detergent lignin in Bt maize silages were similar to those in silages derived from their near-isolines. The findings suggested that Bt maize will result in silage with similar chemical composition to that of non-Bt maize but a large proportion of the Cry1Ab protein persists.

Key words: Ash-free dry-matter, *Bacillus thuringiensis* (Bt) maize, crystal 1Ab (Cry1Ab) protein, fiber, lignin, silage.

INTRODUCTION

The increasing cultivation of yellow *Bacillus thuringiensis* (Bt) maize, with the MON810 event and possibly other events in the future, will increase its use as animal feed in South Africa (Vermeulen et al., 2005). There are public concerns over human, animal and environmental health effects due to the incorporation of *B. thuringiensis* genes into crop plants. One of the concerns is the possible toxicity of gene products (Bertoni and Marsan, 2005). The genetically modified organism (GMO) threshold level (<1%) specified for labeling food and feed by the EU (Saeglitz and Bartsch, 2003) could have implications on using Bt maize as animal feed, particularly in South

Africa, which relies on European markets. Guertler et al. (2010) reported that the Bt protein (Cry1Ab) was present in faecal, blood and milk but not urine samples of dairy cows fed with Bt maize (MON810) diets. The low degradation of the Cry1Ab protein by ruminal microorganisms may suggest a possible negative effect of the protein on the microbes. Maize silage constitutes an important part of the diet of beef and dairy cattle in South Africa (Meeske et al., 2000), and the silage making process, involving fermentation, could be useful in reducing activity of the Cry1Ab protein in Bt maize plant material.

During feed processing the Cry1Ab protein could undergo biochemical processes including some degradation (Hupfer et al., 1999). Mechanical disruption of cell walls and membranes during chopping, microbial activity, anaerobic fermentation and subsequent low pH, during ensiling, have been associated with degradation of the Bt

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maize-specific DNA and Cry1Ab protein (Hupfer et al., 1999; Lutz et al., 2006). Rauschen and Schuphan (2006), however, reported that silage making did not result in a decrease in activity of the Cry1Ab protein in Bt maize (MON810). Lutz et al. (2006) reported that the Cry1Ab protein could still be detected after 61 days of ensiling Bt maize with the transformation event 176. Wiedemann et al. (2007) reported no effects of silage from Bt maize (Bt 176), on six ruminal bacterial strains. Hence, there could be differences in the effects of Bt maize feed as a result of differences in transformation events (Bt 11, Bt 176 and MON810) which have different promoters although the result is the same Cry1Ab protein. It is, therefore, essential to determine the effects of silage making on activity of the Cry1Ab protein in Bt maize (MON810) varieties commonly grown in South Africa.

In addition to concerns of contents of Cry1Ab in feed, Bt maize has also been reported to have higher lignin content than corresponding isogenic lines, which could affect digestibility of the feed. Saxena and Stotzky (2001) reported 33 to 97% more total lignin in Bt maize cultivars than the isolines, whereas Folmer et al. (2002) reported slight differences. Some studies on silage making did not find any consistent trends in the lignin content of Bt and non-Bt isolines maize silage (Tarkalson et al., 2007; Jung and Sheaffer, 2004). Moreover, Aulrich et al. (2001) and Barrière et al. (2001) reported no significant differences in the quality of silages from Bt maize (Bt 176) and the respective isoline in terms of crude nutrients, organic matter and NDF digestibility. However, no information could be accessed in the literature on the feeding value of silage from Bt maize with the MON810 event. Differences observed among the different studies may be attributed to cultivar differences and environmental conditions under which the maize was grown. It is therefore also essential to determine the effects of genetic modification on silage quality from locally grown Bt maize varieties in South Africa.

For risk assessment, locally cultivated Bt maize (MON810) varieties should be investigated for potential effects on nutritional value of their silage relative to their non transgenic isolines. The general objective of this study was to determine the effects of ensiling Bt maize on concentration of the Cry1Ab protein and chemical composition of the resulting silage.

MATERIALS AND METHODS

This study was carried out using commercially grown Bt maize varieties and their near-isogenic lines, which were produced at the University of Fort Hare Research Farm (South Africa) in 2008/2009 and 2009/2010 seasons.

Experimental maize material

In 2008/2009, a commercially available Bt maize (DKC80-12B)

variety and its near-isogenic line (DKC80-10) were grown. Roundup was applied in all plots two weeks before planting and the maize was planted on 8th February, 2008 at 40 000 plants ha^{-1}. The treatments were replicated three times in an RCBD with 6 m × 18 m plots. The inter-row and in-row spacing were 0.9 m and 0.27 m, respectively. Fertilizer (2:3:4 (30)) (N: P: K) was applied at planting at a rate of 25 kg N ha^{-1}. Lime ammonium nitrate (28% N) was applied at six weeks after planting at 50 kg N ha^{-1}. The plots were hoe-weeded at six weeks after planting and supplementary irrigation was applied when required. At the three-quarter milk-line stage, samples of the maize hybrids were collected by cutting whole-maize plants at 30 cm above the ground. The plant material was then used in the silage making experiment.

In the 2009/2010 season, another field study was set up with different maize varieties. The treatments applied were two Bt maize varieties, both with event MON810 (PAN6Q-321B and DKC61-25B) and their isolines (PAN6777 and DKC61-24). The management and harvesting of the crop were as described for the 2008/2009 experiment.

Silage making

The plant materials were chopped manually to <10 mm using cane knives (2008/09) or shredded (< 10 mm) with an electric shredder (Viking GE 103 shredder, Abbey Garden Sales, UK) (2009/10). A representative sample of about 700 g was ensiled in 1 L glass jars used as mini-silos. The material was compacted and consolidated leaving no headspace and sealed with lids that did not allow exchange of gases. Three replicates per treatment for each of the sampling intervals were prepared to allow for destructive sampling. The jars were stored at 24 to 28°C in the dark (Filya, 2004). Destructive sampling was done for fresh non-ensiled material and silages after 2, 4, 8, 15 and 42 days of ensiling for the 2008/09, whereas for 2009/2010 only initial samples and those after 8 and 42 days of ensiling were collected. The samples were stored at -18°C before they were oven-dried at 45°C to constant weight and ground to pass through a 2 mm sieve and stored in zip-lock bags.

Chemical analysis

The samples were then tested for ash-free dry matter (AFDM) as described by Okalebo et al. (2002) and for Cry1Ab protein using a commercially available ELISA kit (Bt-Cry1Ab/1Ac ELISA protein, Agdia, Elkhart, IN). Determination of the quantity of the Cry1Ab was done using an RT-2100C microplate reader (Rayto Life And Analytical Science Co., Ltd) at a wavelength of 450 nm. A standard curve was determined from the negative and positive control at concentrations 0, 2.5, 5, 10 and 20 ng Cry1Ab proteins and the curve equation was used to calculate concentrations of the proteins in the samples. Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were analyzed using the Filter Bag Technique (ANKOM[200]) as described in the AOAC (AOAC, 1984). The LECO Truspec Nitrogen Analyzer was used to analyze nitrogen (N) in silage on a dry matter basis using the Dumas Combustion method (AOAC, 1990). The crude protein (CP) was then calculated from the N contents in the silage samples.

Statistical analysis

Data of AFDM, Cry1Ab protein, NDF, ADF, ADL and CP were subjected to two-way ANOVA (variety and ensiling time) using GenStat Release 7.22 DE (Lawes Agricultural Trust, 2008) and separation of means was done using the LSD at $p < 0.05$.

Table 1. F-probabilities for AFDM, Cry1Ab protein and quality parameters of maize silages.

Source of Variation	AFDM (%)	Cry1Ab (ng g^{-1})	NDF (%)	ADF (%)	ADL (%)	CP (%)
Silage from 2008/2009 season						
Ensiling (E)	0.001*	0.734^{ns}	0.001*	0.001*	0.001*	0.070^{ns}
Variety (V)	0.001*	N/A	0.001*	0.023*	0.136^{ns}	0.246^{ns}
E × V	0.001*	N/A	0.085^{ns}	0.771^{ns}	0.067^{ns}	0.913^{ns}
Silage from 2009/2010 season						
Ensiling (E)	0.092^{ns}	0.001*	0.081^{ns}	0.691^{ns}	0.672^{ns}	0.074^{ns}
Variety (V)	0.025*	0.027*	0.480^{ns}	0.254^{ns}	0.087^{ns}	0.107^{ns}
E × V	0.575^{ns}	0.019*	0.068^{ns}	0.250^{ns}	0.096^{ns}	0.441^{ns}

*Significance at p = 0.05; N/A - not applicable because only one Bt maize variety was used in the 2008/2009 experiment; ns - non significant at p = 0.05 and ND = not determined. AFDM, NDF, ADF, ADL and CP were expressed as percentages of total dry matter.
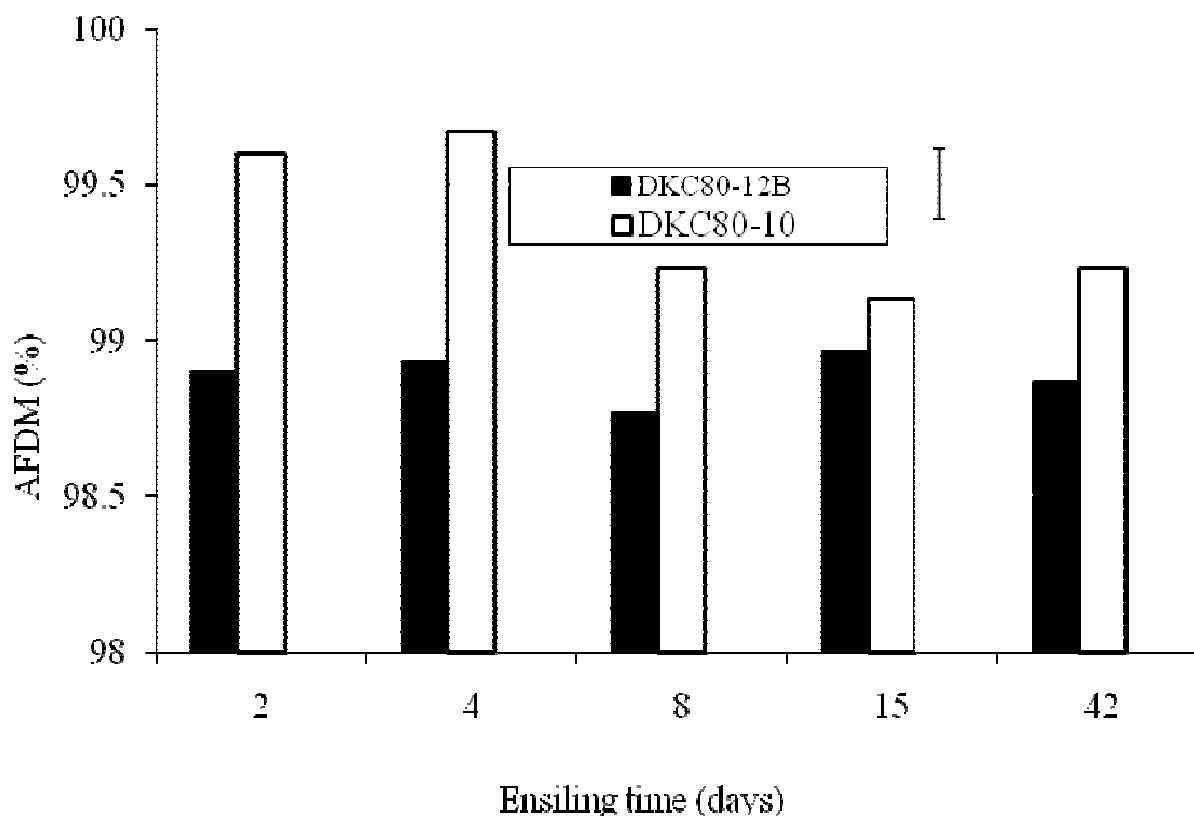

Figure 1. Ash-free dry matter of Bt and non-Bt silages as affected by ensiling of the 2008/2009 maize material. Error bar represents the least significant difference (LSD) at p < 0.05. AFDM was expressed as percentages of total dry matter.

RESULTS

AFDM, Cry1Ab protein, NDF, ADF, ADL and CP in silage from 2008/2009 maize

Dry matter, based on AFDM, was dependent on interaction effects of variety and ensiling, in the maize material produced in 2008/2009 season (Table 1). DKC80-12B silage had lower AFDM compared to DKC80-10 (Figure 1). Ensiling had an inconsistent effect on AFDM over time. Further more, there was no consistent degradation of Cry protein during the ensiling period (Table 2).

It was also observed that concentrations of NDF and

Table 2. Changes in Cry1Ab protein and quality parameters of silages during ensiling of the 2008/2009 maize material.

Time (day)	0	2	4	8	15	42	LSD (p<0.05)
Cry1Ab protein (ng g^{-1})[#]	297.0	332.3	317.4	304.3	319.0	308.8	52.86
NDF (%)	65.8	55.2	55.7	57.0	57.7	55.6	2.29
ADF (%)	33.5	25.5	25.1	26.7	26.9	25.7	1.89
ADL (%)	8.7	ND	6.2	5.1	5.2	4.3	1.74
CP (%)	13.4	13.3	13.2	14.9	14.2	14.7	1.44

ND - not determined; [#]Cry1Ab was only for DKC80-12B, whereas all other parameters were averaged cross varieties (insignificant interaction effects). AFDM, NDF, ADF, ADL and CP were expressed as percentages of total dry matter.

Table 3. Quality parameters of silage from 2008/2009 Bt and non-Bt maize hybrids.

Variety	NDF (%)	ADF (%)	ADL (%)	CP (%)
DKC80-12B	59.0	27.8	5.5	14.2
DKC80-10	56.7	26.6	6.3	13.7
LSD(p = 0.05)	1.32	1.08	1.10	0.83

AFDM, NDF, ADF, ADL and CP were expressed as percentages of total dry matter; concentrations were averaged across all ensiling periods (insignificant interaction effects). NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; CP, crude protein.

Table 4. Quality parameters of silage from Bt and non-Bt maize hybrids of the 2009/2010 maize materials.

Variety	AFDM (%)	NDF (%)	ADF (%)	ADL (%)	CP (%)
DKC61-25B	94.86	54.7	28.2	3.31	8.84
PAN6Q-321B	94.92	57.6	30.7	3.62	8.09
DKC61-24	94.88	54.2	26.5	3.10	9.40
PAN6777	95.74	53.9	27.1	2.44	7.90
LSD(p = 0.05)	0.648	3.84	4.52	0.925	1.341

ND - not determined; AFDM, NDF, ADF, ADL and CP were expressed as percentages of total dry matter. Concentrations were averaged across all ensiling periods (insignificant interaction effects). AFDM, ash-free dry matter; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; CP, crude protein.

ADF in maize material produced in the 2008/2009 season declined within 2 days of ensiling and remained constant for the rest of the period (Table 2). Higher NDF and ADF content was found in Bt than non-Bt maize silages (Table 3). ADL of the maize was initially high (8.7%) and declined to 4.3% within the 42 days (Table 2). There were no differences in ADL observed between Bt and non-Bt maize (Table 3). Crude protein was not influenced by ensiling or type of variety.

AFDM, Cry1Ab protein, NDF, ADF, ADL and CP in silage from 2009/2010 maize

In the maize material produced in 2009/2010 season, AFDM was influenced only by variety. Silage from PAN6777 had higher AFDM than DKC61-25B, DKC61-24 and PAN6Q-321B (Table 4). Silage from the Bt maize cultivars had similar NDF and ADF contents to those of

their isogenic lines, which ranged from 53 to 58% and 26 to 31%, respectively (Table 4). PAN 6Q-321B had higher ADL content than its near-isoline (PAN6777), whereas DKC61-24 had higher CP than PAN6777 (Table 4).

There was also an interaction effect between ensiling and varieties on contents of the Cry1Ab protein (Table 5). Concentration of the Cry1Ab protein decreased significantly with ensiling (Table 5) such that at the end of the ensiling period, the Cry1Ab protein in DKC61-25B and PAN6Q-321B had decreased to 72.1 and 60% of the initial levels, respectively. DKC61-25B had significantly higher Cry1Ab content than PAN6Q-321B.

DISCUSSION

Non-Bt maize silages had higher AFDM content except for DKC61-24 in the second study which had similar percent of AFDM to both Bt maize silages. The reason for

Table 5. Cry1Ab protein concentration in Bt maize varieties during ensiling.

Variety	Concentration of Cry1Ab (ng g^{-1})		
	0 day	8 day	42 day
DKC61-25B	366.3	336.0	264.2
PAN6Q-321B	364.7	335.9	218.6
LSD(p = 0.05)		23.49	

this effect was not clear. These results suggest that the MON810 event reduces the AFDM in the maize and the resultant silage.

Results from both experiments also suggested that ensiling Bt maize resulted in a large proportion of the initial Cry1Ab concentration remaining in the silage. Whereas Cry1Ab protein remained relatively unchanged throughout the ensiling process in the 2008/2009 study, the protein degraded to at least 60% of its original amount as a result of ensiling in the 2009/2010 study. The differences could be due to differences in chemical composition of the materials ensiled in the two experiments. For example, the initial lignin content in the maize materials of the first experiment was >8% whereas it was <4% in the second experiment. These results are in contrast with the findings of Lutz et al. (2006), who reported a marked degradation of Cry1Ab protein to 23.5% during 61 days of ensiling Bt-176 maize. Folmer et al. (2002) also reported significant degradation of the Cry1Ab protein to trace amounts in <10 days for two Bt-maize (event Bt11) silages. Lutz at al. (2006) suggested that degradation of the Cry1Ab protein is dependent on low pH and bacterial composition of silages. It is important to state that the concentrations of Cry1Ab protein measured in this study were much lower than reported in other studies (Nguyen and Jehle, 2007; Rauschen and Schuphan, 2006). The difference could be explained by the lower sensitivity of the ELISA procedure used in this study, which was a modification of a procedure to determine presence or absence of the protein. Based on results from both experiments, silage-making may not be a useful strategy to inactivate the Cry1Ab protein in Bt maize (MON810).

NDF, ADF, CP and DM have been used to determine digestibility coefficients (Hunt et al., 1993). NDF is the insoluble fiber in feed that comprises of hemicelluloses, cellulose and lignin (Van Soest et al., 1991). This insoluble fiber is a requirement for normal rumen function in ruminants and NDF is considered a better index for estimating the fiber requirement of animals than ADF. Significant decline in NDF and ADF, in the 2008/2009 maize material was in agreement with Li et al. (2010) and Hunt et al. (1993), who reported declines in NDF and ADF in rice straw and whole maize, respectively, as a result of ensiling. The decline could be a result of hydrolysis of cell walls by lactic acid bacteria (Li et al., 2010). However, this effect was not evident in the second

experiment with the 2009/2010 maize, where the NDF and ADF contents were lower. Although, the two experiments showed different results in terms of NDF and ADF, in each of the experiments both fiber parameters followed similar trends. In the first study, DKC80-12B had significantly higher NDF and ADF than DKC61-25. Consistently, higher NDF content for Bt11 maize silages have been reported in the literature (Jung and Sheaffer, 2004). The insertion of the cry genes into the plant genome appears to increase the fiber content of Bt maize silages. On the other hand, there were no differences between Bt and non-Bt silages in the second experiment, which was in agreement with Aulrich et al. (2001) and Barrière et al. (2001) who reported no significant differences in feeding value of silages from Bt maize (Bt 176 event) varieties and their respective isolines. Differences in these findings can not only be explained by transformation events but also by other parameters such as the chemical composition of the parent maize varieties. In a previous study, Hunt et al. (1993) con-cluded that there were differences in the chemical composition among different hybrids. However, Meeske et al. (2000) did not find any differences between NDF and ADF of silages of 21 different conventional maize hybrids.

Moreover, higher ADL content in PAN6Q-321B than its near-isogenic non-Bt silage in the second experiment (with 2009/2010 maize) was in agreement with the findings of Jung and Sheaffer (2004), who reported slightly higher lignin content in Bt maize silage than in the near-isoline hybrids. However, similarities in ADL between Bt and non-Bt silages from DKC maize varieties (DKC80-12B and DKC80-10 in the first experiment, and between DKC61-25B and DKC6124 in the second experiment), suggested that lignin content was not increased by genetic modification (MON810 event). Effect of genetic modification (MON810) on lignin content appears to depend on the background genetic material.

The higher lignin content in fresh non-ensiled maize than in the silages, in the first experiment, was in agreement with Hunt et al. (1993), who reported higher lignin content in fresh samples compared to ensiled samples. Ensiling thus has an influence in the degradation of lignin in silages which could result in improved digestibility of silages.

In addition, similar CP in silage made from Bt maize hybrids to that from their parent plants, suggested that

incorporation of the cry genes into the maize plant genome does not alter the CP content of the silages from Bt maize (MON810). Murdoch (1962) classified feeding value of silage into high, medium and low, based on CP content. The CP contents of 14.18% (Bt silage) and 13.70% (non-Bt silage) meant that the silages from the first experiment were of medium feeding value (12 to 15% CP), while those from the second experiment (<9.5% CP) had a low feeding value.

These findings therefore suggest that modification of maize with *B. thuringiensis* gene (event MON810) did not essentially influence the compositional quality of silage, although, the Pannar Bt maize silage had higher levels of ADL, while a significant proportion of the initial concentration of the Cry1Ab remained in Bt maize silage.

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REFERENCES

AOAC (1984). Official methods of analysis (14th Edition). Association of Official Analytical Chemists, Arlington, Virginia.

AOAC (1990). Official Methods of Analysis- Protein (Crude) in Animal Feed: Combustion Method (15th Edition). Association of Official Analytical Chemists: Arlington, Virginia.

Aulrich K, Böhme H, Daenicke R, Halle I, Flachowsky G (2001). Genetically modified feeds in animal nutrition. 1st com: *Bacillus thuringiensis* (Bt) corn in poultry, pig and ruminant nutrition. Arch. Anim. Nutr. 54: 183-195.

Barrière Y, Vérité R, Brunschwig P, Surault F, Emile JC (2001). Feeding value of corn silage estimated with sheep and dairy cows is not altered by genetic incorporation of bt176 resistance to *Ostrinia nubilalis*. J. Dairy Sci. 84: 1863-1871.

Bertoni G, Marsan PA (2005). Safety Risks for Animals Fed Genetic Modified (GM) Plants. Vet. Res. Commun. 29: 13-18.

Filya I (2004). Nutritive value and aerobic stability of whole crop maize silage harvested at four stages of maturity. Anim. Feed Sci. Technol. 116: 141- 150.

Folmer JD, Grant RJ, Milton CT, Beck J (2002). Utilization of Bt corn residues by grazing beef steers and Bt corn silage and grain by growing beef cattle and lactating dairy cows. J. Anim. Sci. 80: 1352-1361.

Hunt CW, Kezar W, Hinman DD, Combs JJ, Loesche JA, Moen T (1993). Effects of hybrid and ensiling with and without a microbial inoculant on the nutritional characteristics of whole-plant corn. J. Anim. Sci. 71: 38-33.

Hupfer C, Mayer J, Hotzel H, Sache K Engel KH (1999). The effect of ensiling on PCR-based detection of genetically modified maize. Eur. Food Res. Technol. 209: 301-304.

Jung HG, Sheaffer CC (2004). Influence of Bt transgenes on cell wall lignification and digestibility of maize stover for silage. Crop Sci. 44: 1781-1789.

Lawes Agricultural Trust (2008). GenStat Release 7.22 DE Reference Manual, VSN International, Helmel, Hempstead, Hertfordshire, UK.

Li J, Shen Y, Cai Y (2010). Improvement of fermentation quality of rice straw silage by application of bacterial inoculant and glucose. Asian-Aust. J. Anim. Sci. 23(7): 901-906.

Lutz B, Wiedemann S, Albrecht C (2006). Degradation of transgenic Cry1Ab DNA and protein in Bt-176 maize during the ensiling process. J. Anim. Physiol. Anim. Nutr. 90: 116-123.

Meeske R, Basson HM, Pienaar JP, Cruywagen CW (2000). A comparison of the yield, nutritional value and predicted productiom potential of different maize hybrids for silage production. S. Afr. J. Anim. Sci. 30: 18-21.

Murdoch J (1962). Making and Feeding Silage (2nd Edition). Farming Press (Book) Ltd, Ipswich.

Nguyen HT, Jehle JA (2007). Quantitative analysis of seasonal and tissue-specific expression of Cry1Ab in transgenic maize MON810. J. Plant Dis. Prot. 114(2): 82-87.

Okalebo JR, Gathua KW, Woomer PL (2002). Laboratory Methods of Soil and Pant Analysis: A Working Manual (2nd Edition). TSBF–KARI and SACRED Africa, Nairobi, Kenya.

Rauschen S, Schuphan I (2006). Fate of the Cry1Ab protein from Bt-maize MON810 Silage in biogas production facilities. J. Agric. Food Chem. 54: 879-883.

Saeglitz C, Bartsch D (2003). Regulatory and associated political issue with respect to Bt transgenic maize in the European union. J. Invert. Pathol. 83: 107-109.

Saxena D, Stotzky G (2001). Bt corn has a higher lignin content than non-Bt corn. Am. J. Bot. 88: 1704-1706.

Tarkalson DD, Kachman SD, Knops JMN, Thies JE, Wortmann CS (2007). Decomposition of Bt and Non-Bt Corn Hybrid Residues in the Field. Nutr. Cycl. Agroecosyst. 80: 211-222.

Van Soest PJ, Robertson JB, Lewis BA (1991). Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74: 3583-3597.

Vermeulen H, Kirsten JF, Doyer TO, Schönfeldt HC (2005). Attitudes and acceptance of South African urban consumers towards genetically modified white maize. Agrekon, 44: 118-137.

Wiedemann S, Gürtler P, Albrecht C (2007). Effect of feeding cows genetically modified maize on the bacterial community in the bovine rumen. Appl. Environ. Microb. 73(24): 8012-8017.