

A Note on Silage from Genetically Modified Maize Tested for Biological Activity

T. Twardowski^{1*}, A. Potkański², S. Pruszyński³, K. Adamczewski³

¹Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12/14, 61-704 Poznań, Poland

²August Cieszkowski Agricultural University, Department of Animal Nutrition and Feed Management, Wołyńska 33, 60-637 Poznań, Poland

³Institute of Plant Protection, Miczurina 20, 60-318 Poznań, Poland

Received: 13 December, 2002

Accepted: 31 March, 2003

Abstract

Forage from genetically modified (GM) maize in two consecutive years (1999 and 2000) was ensiled in bins of 120 l volume in two combinations: with formic acid (85%) and without. In the samples of ensilage material GM maize, basic parameters have been determined such as pH and dry matter. The determination of biological activity of the components of translational apparatus in model translation systems showed the complete inactivation of biological activities. In addition, degradation of nucleic acids in examined silages was discovered.

Keywords: GM maize, ensilage, biological activity, model translation

Introduction

Genetically modified crop plants are grown more and more widely all over the world. Genetic modifications of traits of forage plants allow farmers improved cost effectiveness of their cultivation by, for example, increasing their resistance to herbicides.

GM technology is used to increase crop yield, improve food quality and reduce the environmental impact of agriculture, amongst other things fosters the use of less toxic agrochemicals [1]. Up to now, no significant differences in nutritional assessment between feeds from isogenic and transgenic hybrids could be demonstrated [2].

Food and feed production by modern biotechnology methods have raised many controversial issues. Unfortunately, in many cases health and safety aspects have not been crucial in formulation of law, but the key aspects

were arguments of ethical and philosophical nature [3]. The problem of food and feed products derived from GMOs has not been solved either by Polish or EU legislative systems.

In Poland, these difficult problems are covered by three legal acts: about GMO, about feed and about food [4a,b,c]. All of them are in accordance with the recent EU legislation presented in Directive 2001/18/EC and related norms.

Based on information concerning food and feed published by the International Life Science Institute [5], the fundamental data concerning DNA and peptides in GM food and feed can be summarized as follows:

- All DNAs and peptides [including rDNA and peptides as the products of expression of rDNA] are composed of the same 4 nucleotides and 20 amino acids, respectively;
- Recombinant DNA technologies change neither the chemical properties of DNA nor peptides;

*Corresponding author

- DNA and rDNA are hydrolyzed according to the same kinetics;
- DNA [either rDNA] are not toxic [at standard consumption];
- DNA [either rDNA] is neither allergenic nor immunogenic according to available data;
- DNA [either rDNA] is not incorporated into genome of vertebrates during consumption;
- Consumption of GM food or feed does not change the total amount of consumed DNA or proteins.

Maize is one of the basic raw materials used to produce silage in Poland. The objective of these investigations was to estimate if ensiling genetically modified maize could affect its characteristics and utilization as a feed. Based on general knowledge, it is hard to expect any significant difference between GM and non-GM plants in terms of food and feeding. However, the public discussion concerning safety and nutritional value has raised several questions.

In this set of experiments, we wanted to compare the transgenic and non-transgenic maize in consideration of the effect of ensiling on degradation and, further on, biological activity of DNA and proteins. In future studies feeding experiments with rats will be performed.

Materials and Methods

We used maize XO896 JL (Pioneer) genetically modified for LibertyLink[®] system (Aventis). Within this system, herbicide Liberty 200 SL containing 20% of active compound – glufosinate has been used. In this system the biologically active compound is phosphinothricine [(HOCH₂)₃CNHCCO₂]₃P. This compound is imbibed by all green parts of the plant and inhibits the activity of glutamine synthase, an enzyme responsible for ammonia detoxification. The inactivation of glufosinate is performed through acetylation towards N-acetyl-phosphinothricin. This process is catalyzed by phosphinothricine acetyltransferase. The enzyme was isolated from bacteria. It is worth noting that the activity of the system is mainly located in green parts of the plant.

For our experiments the LibertyLink system genetically modified maize (GM) and isogenic maize were available. Forage from genetically modified (GM) and non GM maize plants in two consecutive years (1999 and 2000) was ensiled in containers of 120 l volume in two treatments: with a preserving agent (85% formic acid) and without it. We ensiled the whole maize plant harvested in the wax stage of grain. The material was cut by scissors into small pieces 1–2 cm length, all grains were damaged (cracked) in the mixer. The silos were opened after 60 days and silage was immediately analyzed. Each treatment was ensiled in three replications.

Dry matter, pH and basic nutrients were determined in all tests of all silage. Basic feed constituents were determined according to Weenden methods; dry matter using dried method in 60°C, crude protein by Kjeldahl method, crude fiber by Henneberg-Stohman method using 1.25%

H₂SO₄ and 1,25% KOH, crude ash by burning the feed in a furnace at 550–650°C, ether extract by Soxhlet method and pH using a pH-meter Elster (P-731). N-free extractiveness was calculated subtracting from 100 the total amounts of the remaining determined chemical constituents.

The means concerning chemical composition of silage were compared by Duncan's test at P≤0.01 and P≤0.05 (SAS 1990).

The *in vitro* translation system and separated components of this system have been isolated from: GM maize, "classical" (non modified) and yellow lupine (standard material in our laboratory). Lysate for *in vitro* translation was prepared from lupine seeds according to Gwózdź and Deckert (1989). The standard *in vitro* translation assay was performed in a volume of 25 µl and contained: 20 mM Hepes-KOH, pH 7.6, 110 mM potassium acetate, 3.2 mM magnesium acetate, 2 mM ATP, 0.4 mM GTP, 10 mM creatine phosphate, 0.1 mM spermine, 0.07 unit of creatine phosphokinase, 1 mM DTT, 40 µM mixture of amino acids (depleted of methionine or leucine), 185 kBq ³⁵S-methionine (specific activity 37 TBq/mmol) or 148–370 kBq ³H-leucine (specific activity 4.4–7.0 TBq/mmol) and 175–220 µg of lupine seeds lysate. As a message, 0.5–2.0 µg of lupin poly A RNA was used. The *in vitro* translation assay was performed at 27°C for 60 min. The activity was measured by TCA precipitation of small (3–5 µl) aliquots of translation reaction mixture [6, 7].

The ribosomes and elongation factors EF1, EF2 were isolated from lupine seeds, independent isolations and different batches of plant material, according to the procedure elaborated earlier in our laboratory. Binding of aminoacylated [¹⁴C]Phe-tRNA^{Phe} to the poly U programmed ribosomes was carried out in 50 µl of 50 mM Tris HCl pH 7.5 buffer containing 60 mM KCl, 5 mM MgCl₂, 3 mM DTT, 0.1 mM GTP, 10 ng poly U and various amount of [¹⁴C]Phe-tRNA^{Phe} (75 cpm/pmole). 0.3 A₂₆₀ units of ribosomes from wheat germ and 10 µg elongation factor EF1. Incubation time was 5 min. at 37°C. The reaction was stopped by adding ice-cold 10 mM Tris HCl pH 7.6 buffer containing 80 mM KCl and 10 mM mgCl₂. The reaction mixture was filtered under vacuum through a Millipore nitrocellulose filter (0.45 µm) and washed three times with the same buffer. The radioactivity bound to the filter was determined by scintillation counting.

Polyphenylalanine synthesis was carried out in 50 mM Tris HCl pH 7.5 buffer containing 100 mM KCl, 8 mM MgCl₂, 3 mM DTT, 0.1 mM GTP, 10 ng of poly U and various of [¹⁴C]Phe-tRNA^{Phe} (75 cpm/pmole), 0.3 A₂₆₀ units of ribosomes from wheat germ and 10 µg of EF1. The mixture was incubated 5 min. at 37°C. After that time 1 ml of 10% TCA was added and reaction was further incubated 15 min. at 90°C. The samples were then placed on ice for 3 min. and filtered through the fiber glass filters, washed with 5% TCA and radioactivity was determined by scintillation counting. We prepared the [³H]–Phe–tRNA and [¹⁴C]–Phe–tRNA as well as the polyacrylamide gel electrophoresis of nucleic acids according to standard procedures. Gel electrophoresis was

Table 1. Dry matter, pH and basic nutrients of silages from GM and non GM maize (1999).

	GM maize		Non GM maize		Significance		
	control	with FA	control	with FA	M	C	M*C
dry matter (g/kg)	328.8	319.9	329.5	330.0	**	*	*
pH	3.67	3.62	3.71	3.72	NS	*	NS
g/1000 g DM							
crude protein	92.8	93.2	96.5	97.0	NS	NS	NS
crude fiber	249.5	253.1	250.3	250.0	NS	NS	NS
ether extract	31.1	29.6	30.8	31.6	NS	NS	NS
N-free extractiveness	585.0	567.9	584.9	586.1	NS	NS	NS
crude ash	88.9	97.1	89.0	88.1	**	NS	NS

Table 2. Dry matter, pH and basic nutrients of silages from GM and non GM maize (2000).

	GM maize		Non GM maize		Significance		
	control	with FA	control	with FA	M	C	M*C
dry matter (g/kg)	240.9	248.5	245.6	251.3	*S	*	*
pH	3.59	3.51	3.54	3.57	NS	*	NS
g/1000 g DM							
crude protein	73.7	82.9	80.1	81.7	NS	*	NS
crude fiber	271.5	267.8	275.6	269.8	NS	NS	NS
ether extract	26.9	27.0	27.2	26.9	NS	NS	NS
N-free extractiveness	500.2	520.3	512.3	518.2	**	NS	*
crude ash	90.0	92.1	92.3	90.1	NS	NS	NS

FA – silage with addition of formic acid (85%), 4 ml/kg of fresh material, M – material (GM and nonGM), C- way of conservation (with FA and without FA), NS- non significant, * differences significant for and $P \leq 0.05$, ** differences significant for $P \leq 0.01$

on 20% gel with acrylamide to bisacrylamide ratio 30:1, in the presence of 0.1 M Tris non-adjusted, 8M urea, 0.1 M boric acid, 20 mM EDTA, 1% ammonium persulphate and 0.1% TEMED. Preelectrophoresis was run for 1 h at 500 V and samples were preincubated at 90°C for 2 min. Two A_{260} units were loaded per column. The electrophoresis was done for 7 h at 1200-1500 V. The gel was stained in 0.1% toluidine blue, 40% methanol, 10% acidic acid for 10 min. and destained with water.

For a long time we used the protein biosynthesis system derived from yellow lupine seeds as a model [a “standard”] in our laboratory. The routine use of this system allowed a detailed characterization of the conditions for optimal activity of the system. The activity of others and new systems we refer to the standard of this laboratory.

In our tests we checked:

- the integrity of nucleic acids after ensilage in comparison to genetically modified and non modified maize,
- biological activity of these systems in protein biosynthesis *in vitro*,

- activity in model tests of elongation of polypeptide synthesis in poly U system.

The conditions for testing the biosynthetic activity on ribosomes: the activity was measured by determination the specific [^3H] AcPhe-tRNA] binding and polymerization to poly-[Phe]_n on the the plant ribosomes. The assays [6, 8] were performed under the saturation level of Phe-tRNA on poly-U programmed ribosomes and in the presence of elongation factors 1 and 2 [6].

We also determined the integrity of nucleic acids before and after ensiling of both genetically modified and standard maize. The activity in translation system (polypeptide synthesis) in the homogenous plant poly-U system was assayed.

Results

In both treatments the maize silages were well fermented with pH ranging from 3.5-3.8 and dry matter – from 25-32% (Table 1 and 2). The chemical composition

Table 3. Determination of biological activity in model translation.

System prepared from:	A Phe-tRNA binding (% of remaining activity)	B poly(Phe) synthesis (% of remaining activity)
A 1. lupine	100	100
B 2. fresh GM maize 3. GM maize silage without formic acid (60 days) 4. GM maize silage with formic acid (60 days)	110 0 0	95 0 0
C 2. fresh GM maize 3. GM maize silage without formic acid (60 days) 4. GM maize silage with formic acid (60 days)	107 0 0	98 0 0
D 2. fresh GM maize 3. GM maize silage without formic acid (60 days) 4. GM maize silage with formic acid (60 days)	99 0 0	105 0 0

In panels A to D the following materials have been tested: A – lupine seeds (taken as reference material and as 100 % activity), B – GM maize, year 1999, C – GM maize, year 2000, D – non GM maize, year 1999 and 2000. 100% activity for lupin was equivalent for 5880 cpm in binding test and 5140 cpm in polymerization assay, the value of the blank sample did not exceed 300 cpm (about 5%); GM – genetically modified; Phe-tRNA - phenylalanine-transfer RNA; poly(Phe) – polyphenylalanine.

of the initial material had a decisive effect on the silage chemical composition. No distinct changes in the chemical composition of experimental silage was observed in the result of the ensiling process. Maize silage was characterized by chemical composition typical for the wax stage at which the plants were harvested, i.e. relating high dry matter which in 1999 ranged from 32-34%, while in 2000 – only 25% because of much lower precipitation and a slightly earlier harvest.

The obtained silages were characterized by a high pH ranging from 3.5 to 3.7, irrespective of the year of experiment and whether the ensiled material was genetically modified (GM) or not. No significant differences in silage chemical composition were recorded between GM and non GM maize. In 1999 experimental silage was characterized by mean crude protein content ranging from 93-97 g/kg DM, while in 2000 this was lower ranging from 73-83 g/kg DM.

As expected, N-free extractiveness was highest in experimental maize silage ranging from 500-586 g/kg DM. Crude fiber content fluctuated from 250-275 g/kg DM. Silage produced in 1999 were found to contain slightly more N-free extractiveness and less crude fiber. The application of the FA chemical additive to ensile maize did not effect chemical composition or pH in either of the experimental years.

On polyacrylamide gel electrophoresis we found the disintegration of the structure of nucleic acids. Fig. 1 shows polyacrylamide gel analysis at denaturing conditions. On column 1 and 2 the plant material before and after ensiling, respectively, was loaded. In the case of ensilage material (#2) the destruction of nucleic acids is evident. This can be attributed to the hydrolysis at acid conditions at elevated temperature during the ensiling

process. In the performed tests of biological activity (protein *in vitro* synthesis and model system for polypeptide elongation in poly-U coded ribosome), a zero level of silage material activity was observed (Table 3). These observations were in good correlation with the observed hydrolysis of nucleic acids. It is evident in our opinion that acidic conditions of ensiling hydrolyses the nucleic acids and desintegrated nucleic acids can't support protein biosynthesis.

Discussion

GM crops are currently commercialized and two main genetic transformations are herbicide tolerance and insect protection. Soybean and maize are the two main crops into which they have been introduced. There are many experiments around the world dedicated to the formation of new transgenic varieties of plants. However, till now, economically most significant are GM plants' herbicide resistance. The data from many studies showed that the GMO crops are not only equivalent in composition, but also similar in digestibility and feeding value for livestock [1, 9].

The protein biosynthesis (synthesis of polypeptide chains) is a vital process for all living organisms. In *in vitro* system we are able to simulate the native conditions in order to synthesize the native as well model peptides. The synthesis occurs on ribosome programmed with natural messengers (or on a model mRNA – poly-U) and is catalyzed by elongation factors EF1 and EF2. The aminoacyl-tRNA is a source of amino acid. The model system elaborated in our laboratory based on the activity of the most significant components: ribosomes, messenger RNA, transfer tRNAs and elongation factors. All the

components are equally important for the efficiency of the entire system. It is evident that the inability of protein biosynthesis represents inactivity of the components; such a system is unable to produce any proteins. However, the basic building “blocks” – amino acids and nucleotides, are unchanged and suitable as basic components of food or feed. The hydrolyzed proteins and nucleic acids are characterized by zero level activity in functional tests, but they can be effectively used as a source of components for food and feed.

In the experiments with genetically modified sugar beets and maize in which the glufosinate tolerant (*Pat*) gene was inserted, the authors [10] did not observe differences in their studies with ruminants and pigs due to the genetic manipulation in crude nutrients, digestibility and the energetic feeding value in comparison to the corresponding non-transgenic hybrids. In other studies, the authors [11] confirmed the substantial equivalence of the transgenic Bt corn and the corresponding non-transgenic hybrid Cesar on such parameters of nutrition physiology as digestibility and energy content for poultry, pigs and ruminants. In earlier and continuing experiments with GMO of the 1st generation at Braunschweig [2], no signif-

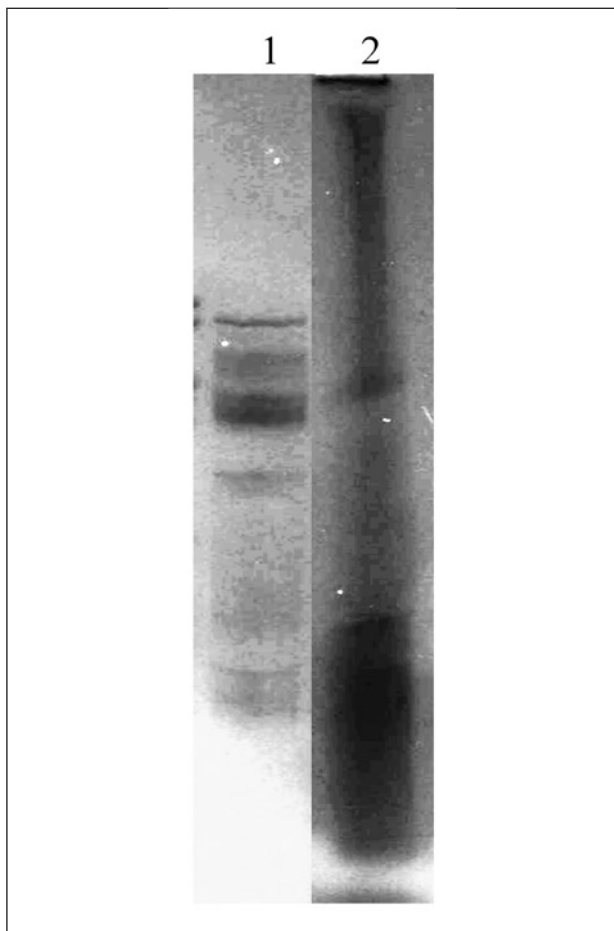


Fig. 1. Polyacrylamid gel electrophoresis analysis of the integrity of nucleic acids before (1) and after (2) ensilage (details in the text).

icant differences in nutritional assessment between feeds from isogenic and transgenic plants of the 1st generation have been found (e.g. maize grain, maize silage, sugar beet, sugar beet leaves silage, full fat soybeans). Furthermore, the so-called substantial equivalence of transgenic hybrids could be demonstrated, and recombinant plant DNA – constructs were not detectable in animal tissue.

The results in our experiments carried out in two consecutive years (1999-2000) showed that during the ensilage of GM and non GM maize biological activity assayed in model translation either with or without the addition of formic acid indicated the entire disintegration of nucleic acids. The chemical composition of maize silage depended on the season and harvesting time, without any effect of genetic modifications.

The observed differences in our experiments between analyzed samples (Table 1 and 2) are small and not significant. We consider them biologically irrelevant. What is more, we expect the energy content and feeding value to be the same, without any effect of the transgenesis process.

There are detailed and solid research data concerning genetically modified feeds in animal nutrition [10, 11]. In these reports data concerning BT corn have been presented. However, there is no evidence for expecting any differences between several different GM plants, e.g. between BT corn and LibertyLink[®] system modified corn. In 2001, over 50 mio hectares of genetically modified plants were cultivated. In terms of commercialization, systems LibertyLink[®] of Aventis (glufosinate resistant) and RoundupReady[®] (glyphosate resistant) are the most significant. The tendency of 10% per year increase of GM plant area has remained stable for the last three years. We shall expect the same in the near future, so the economic significance of GM food and feed will enlarge, proportionally. With these GM plants several field trials and laboratory analysis have been performed [12, 13, 14, 15 and references cited therein]. In genetic engineering of plant genome the *PAT* gene is used. This gene is responsible for the expression of enzyme acetyltransferase phosphinotricine. This enzyme inactivates glufosinate by acetylation. Glufosinate {phosphinotricine: $(\text{HOCH}_2)_3\text{CNHCCH}_2\text{CO}_2\text{P}$ } is universal herbicide working through the inhibition of activity of glutamate synthetase, and finally blocking detoxification of the plant from ammonia. Glufosinate is well known in agriculture under commercial names Basta, Ignite, Challenge and more.

However, as far as we are concerned, that coding genes in transgenic hybrids from GMO could be transferred to microbes and eventually increase antibiotic resistance in humans, so that it can usefully search for this possibility that might lead to the fate of recombinant plant DNA. However, it occurs in a greater rate in the digestive tract. In our experiment, ensilage of maize in laboratory conditions have appeared to be satisfactory, as well.

It was shown in many studies that plant material after consumption is rapidly degraded in both monogastric and ruminant digestive systems. The possibility that

genes from the plant system will be incorporated to the consumers' genome has never been proved, so it can be formulated as very low. This probability factor is further reduced by several orders of magnitude by decomposition of plant material through ensilation. It is also worth mentioning that no more than 0.00001% of DNA is modified out of the total amount of DNA in the genetically modified plant material. The substantial equivalence of GM and "classical" crops and is evident. The conventional methods of feed preparation (and food) guarantee fragmentation of the DNA chain. Temperature, pH and time are important factors in the food and feed preparation process.

There have been several objections concerning the presence of antibiotic resistance markers in genetically modified plants. In our opinion, such objections are insignificant, particularly in feed preparation. First of all in recent procedures the antibiotic markers are not used any more (but they could be present in older GM varieties). Secondly, the concentration of eventual product of expression is at a much lower concentration than the amount of antibiotics used routinely in animal breeding procedures (as additives to the feed). Most probably, the DNA including antibiotic resistance marker gene will be completely hydrolysed during feed preparation (ensilaging). If the gene will be active during and after digestion procedure the probability of incorporation into ruminant genome is extremely low; at least there has not been a single report about such a recombination. Another possibility is introduction of this new gene into the bacterial genome, which is not excluded, but once again it is very low. Even if the horizontal gene transfer will occur, the separated question should be the expression of this gene [3].

Conclusions

The analysis of biological activity in this model research allowed the determination of complete inactivation and degradation of nucleic acids in examined silages. We assumed that silage from genetically modified maize cannot pose any hazards and can be safely used as feed for farm animals. As a consequence of hydrolysis of nucleic acids occurred during the ensilage we detected zero level of activity of these crucial components in standard tests of protein biosynthesis. We know that hydrolyzed nucleic acids and proteins are not dangerous for our health, but valuable as components of the diet.

References

1. PHIPPS R.H., BEEVER D.E., New technology: Issues relating to the use of genetically modified crops. *J. Anim. Feed Sci.* **9**, 543, **2000**.
2. FLACHOWSKY G., AULRICH K., Zum Einsatz Gentechnisch veränderte Organismen (GVO) in der Tierernährung. Übers. *Tierernährg.* **29**, 45, **2001**.
3. "Towards strategic vision of life sciences and biotechnology; consultation document" CEC, 4.9.2001, COM (2001) 454.
4. Legal Act, 22 June 2001a, About genetically modified organisms [Dz. U., 25 July 2001, Nr 76, pos. 811].
Legal Act, 23 August 2001b, About feed [Dz. U., 25 October 2001, Nr 123, pos. 1350].
Legal Act, 11 May 2001c, About food [Dz. U., 22 June 2001 r., Nr 63, pos. 634].
5. International Life Science Institute Conference, 26–28 June, **2000**, Limelette, Belgium.
6. BOHUN E., TWARDOWSKI T. The α -sarcin domain of large ribosomal RNA is a strategic fragment for plant ribosome function. *J. Plant Physiol.* **143**, 650, **1994**.
7. PULIKOWSKA J., WOJTASZEK P., KORCZ A., MICHALSKI Z., CANDRESSE T., TWARDOWSKI T. Immunochemical properties of elongation factors 1 from wheat germ origin. *Eur. J. Biochem.* **171**, 131, **1988**.
8. TWARDOWSKI T., LEGOCKI A. B. Purification and some properties of EF2 from wheat germ. *Biochemia et Biophysica Acta.* **324**, 171, **1973**.
9. FLACHOWSKY G., AULRICH K. Nutritional assessment of feeds from genetically modified organism. *J. Anim. Feed Sci.* **10**, Suppl. 1, 181, **2001**.
10. BÖHME H., AULRICH K., DAENICKE R., FLACHOWSKY G. Genetically modified feeds in animal nutrition: 2nd communication: Glufosinate tolerant sugar beets (roots and silage) and maize grains for ruminants and pigs. *Arch. Anim. Nutr. Vol.* **54**, 197, **2001**.
11. AULRICH K., BÖHME H., HALLE I., FLACHOWSKY G., Genetically modified feeds in animal nutrition 1st communication: Bacillus Thuringiensis (Bt) corn in poultry, pig and ruminant nutrition. *Arch. Anim. Nutr.* **54**, 183, **2001**.
12. BERZSENYI Z., KOPACSI T., ARENDAS T., BONIS P., LAP D.Q. Three-years experiences on the efficacy and selectivity of glufosinate-ammonium in transgenic maize. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz. Sonderheft XVI*, 391-399, **1998**.
13. TROMAS J., LEDOUX P. Interet du glufosinate en ammonium desherbages du mais, ANPP-Dix Septieme Conference du Columa, Journées Internationales sur la Lutte Contre les Mauvaises Herbes, Dijon, 9-11 Decembre 1988, 815-822.
14. ADAMCZEWSKI K., SKRZYPCZAK G., LISOWICZ F., BUBNIEWICZ P. Aktualne problemy ochrony kukurydzy w Polsce. *Zesz. Probl. Post. Nauk Roln.* **450**, 63, **1997**.
15. ADAMCZEWSKI K., PRACZYK T., BUBNIEWICZ P., PIETRYGA J., Rośliny transgeniczne odporne na herbicydy w doświadczeniach Instytutu Ochrony Roślin. *Prog. Plant Protection. Post. Ochr. Roślin.* **39** (1), 231, **1999**.