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Mycotoxins in conserved forage

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Spoilage of food and feed caused by filamentous fungi involves nutrient and energy losses and the risk of contamination with mycotoxins. Forages containing mycotoxins may not only impair the health of domestic animals thus inducing economic losses but also of men through carry-over in the food chain (Krogh, 1989).

Since detoxification processes of contaminated products are scarcely available, efforts have to be aimed at prevention of fungal growth, which is the basis of toxin formation.

To develop strategies for restriction of fungi and toxin contamination, a profound knowledge of risk factors in plant production and conservation is required.

Conditions for fungal growth and toxin formation

The development of hyphal fungi is dependent on certain environmental conditions, but limiting values vary in a wide range of tolerance. Nutritive contents of the substrate, water activity, temperature, pH and the gaseous composition of the atmosphere are the main influencing parameters on fungal growth (Table 1).

Filamentous fungi are able to use a wide variety of nutrients (carbohydrates, proteins, lipids, pectin et al.), whereas carbohydrate-rich, especially sugar containing substrates may be prefered (Böhm, 1989).

At water activities below 0.65, fungal growth is generally inhibited (Lacey, 1989). Optimal values of water activity range in the area of 0.8 to 0.95.

The range of temperature allowing fungal development varies between slight subzero up to 60°C. Optimal values usually range between 20-35°C.

Most fungi prefer pH values of about 4.5-6.5, but lower pH down to 2.0 or higher values up to 8 are also tolerated by some species (Böhm, 1989).

Hyphal fungi are regarded as obligate aerobes but there are species able to grow under microaerophilic conditions (Magan et al., 1984). With many fungi, the concentration of atmospheric oxygen have to be decreased below 0,14% before linear growth is considerably reduced. The development of some species may be increased at raising carbon dioxide concentrations up to 10%, whereas above 15% CO_2 growth usually becomes more and more inhibited. But there are species tolerating high CO_2 values. For example *Penicillium roqueforti* is known to grow up to 80% CO_2 providing there is at least about 4% of oxygen available (Lacey, 1989).

Table 1: Conditions for the development of most filamentous fungi

	Minimum	Optimum	Maximum
Water activity	0.65	0.8 - 0.95	0.99
Temperature (°C)	-2.0	20 - 35	60
pH	2.0	4.5 - 6.5	8.0
Oxygen (%)	0.14	>2	
Carbon dioxide (%)		<10	>15

In general, besides of some basic limits excluding growth totally, the tolerance to a certain factor, which is depending mutually on the other influencing parameters, may result in the development of predominant fungal species at different environmental commodities.

Requirements for the formation of mycotoxins are more close compared to conditions allowing growth (Northolt et al., 1982). For instance, higher concentrations of nitrogen may have inhibitory effects on mycotoxin production (Orvehed et al., 1988). Certain trace metals are known to increase mycotoxin formation of toxigenic fungi (Lillehoj et al., 1974). A deficiency of such components may result in restriction or even prevention of toxin enrichment.

Pre- and post-harvest fungal contamination

The colonization of plant material by microorganisms starts with the cultivation in the field and is influenced by complex ecological factors like climatical conditions, physical and chemical parameters of the soil, the atmosphere and the plant itself.

Although fungal infection is possible throughout the plant's growth, the probability of invasion with hyphal fungi is enhanced at senescence and seed ripening. The composition of the microflora of the mature plant changes with harvest and the environmental alteration in conservation and storage. However, pre- and post-harvest fungal contamination cannot be separated strictly, because so-called "field fungi" are recognized to develop either in storage and vice versa (Lacey, 1989).

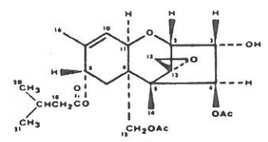
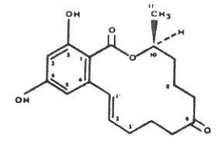
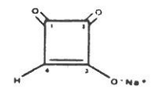
Mycotoxins of the genera *Fusarium*, *Aspergillus* and *Penicillium*

At present the deuteromycete genera of *Fusarium*, *Aspergillus* and *Penicillium* are regarded as most important producers of mycotoxins in food and feed. Fusaria are common plant saprophytes and pathogens thus occuring frequently in the "field" environment, whereas aspergilli and penicillia are regarded as typical "storage" fungi due to their tolerance to lower water activities (Lacey, 1989).

Mycotoxins are secondary metabolites of low molecular weight and of largely variing chemical structures.

Some important of about 400 so far identified mycotoxins and their biological activities are presented in more detail in the Tables 2, 3 and 4.

Table 2: Chemical structure and biological effects of mycotoxins produced by Fusarium

Toxin	Trichothecenes	Zearalenone	Moniliformin
Structural formula	 T-2 toxin	 Zearalenone	 Moniliformin
Deri-vatives	HT-2 toxin Deoxinivalenol Diacetoxyscirpenol et al.	Zearalenol Curvularin Monorden et al.	
Producing species	Fusarium graminearum F. culmorum F. nivale F. poae F. tritinctum Trichotnecium roseum Trichoderma viride et al.	Fusarium graminearum F. nivale F. tritinctum F. culmorum F. moniliforme F. equiseti F. avenaceum et al.	Fusarium moniliforme F. graminearum F. fusarioides
Biological activity	neurotoxic dermatotoxic hemorrhagic teratogenic antibiotic	estrogenic antibiotic carcinogenic ?	hemorrhagic cytotoxic

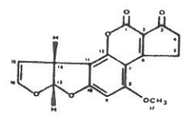
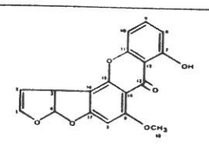
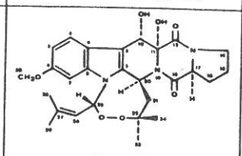
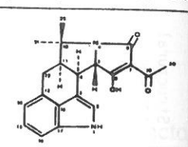
Among the mycotoxins predominatly produced by fusaria (Table 2), the trichothecenes represent an important and large group of about 100 today known derivatives (Gilbert, 1989). Neurotoxicity, dermatotoxicity and hemorrhagia are severe effects of these substances. Besides several fusaria there are also other genera like *Trichothecium* and *Trichoderma* producing trichothecenes as well.

Other important mycotoxins of fusaria are the zearalenone and related compounds of special estrogenic effects. A carcinogenic potential of zearalenone is at the time discussed controversially (Kuiper-Goodman et al., 1987).

Moniliformin induces hemorrhagic and cytotoxic effects in animals. Recent findings suggest that it may frequently appear in co-occurrence with trichothecenes and zearalenone in food and feed (Jelinek et al., 1989).

Important mycotoxins produced by aspergilli (Table 3) are the aflatoxins, sterigmatocystin, the fumitremorgins and cyclopiazonic acid (Moss, 1989). The aflatoxins derive mainly from just two species, *Aspergillus flavus*, and *Aspergillus parasiticus* and exhibit strong hepatotoxic, carcinogenic and immunosuppressive properties. The information available on aflatoxins, which were the first discovered mycotoxins, exceeds that for all other mycotoxins combined (Jelinek et al., 1989). In Germany, limiting concentrations in food and feed have been only established for the aflatoxins.

Table 3: Chemical structure and biological effects of mycotoxins produced by Aspergillus

Toxin	Aflatoxins	Sterigmatocystin	Fumitremorgins	Cyclopiazonic acid
Structural formula	 Aflatoxin B_1	 Sterigmatocystin	 Verruculogen	 Cyclopiazonic acid
Derivatives	Aflatoxin B_2, B_3, G_1, G_2, M_1, M_2 et al.	Dihydroxysterigmatocystin, Aspertoxin et al.	Fumitremorgin A, B, C, TR-2 et al.	Cyclopiazonic acid imine et al.
Producing species	Aspergillus flavus A. parasiticus	Aspergillus versicolor A. nidulans A. flavus et al.	Aspergillus fumigatus A. caespitosus Penicillium paraherquei P. janthinellum et al.	Aspergillus flavus A. versicolor Penicillium cyclopium et al.
Biological activity	hepatotoxic carcinogenic cytotoxic hemorrhagic immunosuppressive	hepatotoxic nephrotoxic carcinogenic	tremorgenic	neurotoxic hepatotoxic nephrotoxic carcinogenic

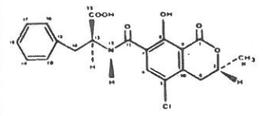
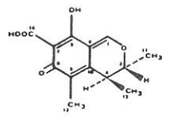
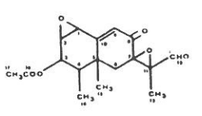
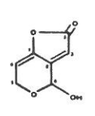
As the structural formula indicates, sterigmatocystin is related to the aflatoxins and is probably a precursor, but the acute toxicity is less than compared to aflatoxin B_1 (Terao, 1983).

Other important mycotoxins produced by aspergilli, but also by penicillia and other genera, are potent tremorgenic substances like verruculogen that belongs to the group of the fumitremorgins. Cyclopiazonic acid, which is produced by many strains of *Aspergillus flavus*, impairs the nervous system, liver and kidney and has carcinogenic properties.

Some mycotoxins, which are mainly produced by species of *Penicillium* (Jelinek et al., 1989) are presented in Table 4.

During the last decade, special attention has been drawn on the Ochratoxin A due to its severe nephrotoxic effects and its carcinogenic, immunosuppressive and teratogenic properties (Röschenthaler, 1984). In European commodities, *Penicillium verrucosum* is regarded as main producer of ochratoxin A.

Table 4: Chemical structure and biological effects of mycotoxins produced by Penicillium

Toxin	Ochratoxins	Citrinin	PR toxin	Patulin
Structural formula	 Ochratoxin A	 Citrinin	 PR toxin	 Patulin
Derivatives	Ochratoxin B, C. 4-Hydroxyochratoxin A	Decarboxycitrinin Ascochitin et al.	Eremofortin A, B, C	Isopatulin Penicillic acid Asciadiol
Producing species	Penicillium verrucosum P. palitans Aspergillus ochraceus A. melleus et al.	Penicillium citrinum P. viridicatum P. expansum P. notatum Aspergillus terreus et al.	Penicillium roqueforti	Penicillium expansum P. patulum P. claviforme P. roqueforti P. cyclopium Aspergillus clavatus Byssochlamys nivea et al.
Biological activity	nephrotoxic immunosuppressive carcinogenic teratogenic antibiotic	nephrotoxic carcinogenic teratogenic mutagenic antibiotic	hepatotoxic nephrotoxic mutagenic carcinogenic ?	carcinogenic hemorrhagic hepatotoxic nephrotoxic antibiotic

As a result of German monitoring studies, ochratoxin A has been recognized to occur frequently in samples of human blood, milk and kidney (Bauer et al., 1987). The origin of these intoxications is not yet clearly identified, as representative data on the occurrence of ochratoxin A in food are missing so far.

Citrinin is frequently co-detected with ochratoxin A (Jelinek et al., 1989) and has nephrotoxic, carcinogenic, teratogenic, mutagenic and antibiotic properties.

PR toxin is one of the most acutely toxic compound to be formed by *Penicillium roqueforti* (Scott, 1984). Degenerative changes were observed in the liver and kidney. PR toxin is a mutagenic agent, but it is not yet confined whether it is a carcinogen.

Patulin is a metabolite of several *Aspergillus* and *Penicillium* species including *Penicillium roqueforti*. Patulin is highly toxic against several animal tissues, especially liver, kidney, but also spleen, lung and brain. It also exhibits strong antibiotic effects (Engel et al., 1984).

Toxicity of mycotoxins

It was shown, that main target tissues directly impaired by mycotoxins are the nervous system, reproductive organs, kidney and liver.

Lethal concentrations of the strongest known mycotoxins to vertebrates range in the area of several mg/kg body weight, which is comparable to the acute toxicity of strychnine (Table 5).

Table 5: Acute toxicity of mycotoxins

Agent	LD_{50} /oral in mg/kg body weight	Animal
Aflatoxin B1	7.2	Rat
Patulin	30.0	Mouse
Ochatoxin A	20.0	Rat
T-2 toxin	3.8	Rat
Fusarenon-X	4.4	Rat
For comparison: Strychnine	7.5	Rat

Reference: Frank, 1990

Besides definite clinical symptoms, also indirect effects like reduced efficiency or increased susceptibility to diseases have to be taken into consideration. In food products special attention has to be focused on mycotoxins exhibiting carcinogenic, mutagenic and immunosuppressive properties, which generally cannot be regarded as harmless even at low concentrations towards the ng/kg range.

Methods for the analysis of mycotoxins

Due to their dangerous potential great efforts have been undertaken to develop effective and sensitive detection procedures. Today, modern methods on the basis of chemical/physical and immunological reactions are available for the determination of some important mycotoxins even in the range down to ng/kg (Chaytor et al., 1982; Lee et al, 1984; Tanaka, 1985)

However, main problems limiting analytical throughput are the restricted commercial availability of standard substances, difficulties with sample purification, high apparative and personal employment resulting in high costs for analysis.

Occurrence of mycotoxins in forage conservation

In the following, an overview is given on mycotoxins, which are known or can be expected to be of importance in the silage fermentation process.

There is very limited information available about mycotoxins that have been determined in silages of practical scale.

In Table 6 data derived from maize silages of whole plants, kernels and corn cob mix are presented.

Table 6: Occurrence of toxigenic fungi and mycotoxins in maize silage

Ensiled constituents	Toxigenic fungi (dominating species)	Number of samples n	Toxin positive samples n / %	Detected mycotoxin	Toxin concentration mg / kg	Reference
Whole plant	Byssochlamys nivea Aspergillus (fumigatus) Penicillium (roqueforti) Fusarium Trichoderma viride	25	14 / 56	Patulin	1.5 - 40	Escoula, 1977
		24	1 / 4	Zearalenone	0.042	Lepom, 1989
				Zearalenone	0.005	Gedek, 1983
	Penicillium roqueforti Byssochlamys / Paecilomyces Aspergillus (fumigatus) Trichoderma (harzianum)	37				Frevel et al., 1985
		14	1 / 7	Zearalenone		Lengauer et al., 1981
Kernels	Penicillium Aspergillus Fusarium Trichoderma Byssochlamys	20	3 / 15	T -2 toxin HT-2 toxin Zearalenone	0.44 0.20 0.05	Thalmann, 1986
Corn Cob Mix		198	17 / 9	Diacetoxyscirpenol Deoxynivalenol Zearalenone	0.88 - 1.5 0.21 0.025- 0.5	Thalmann, 1986
	Penicillium roqueforti	9				Frevel et al., 1985

Detected mycotoxins were zearalenone and several trichothecenes in concentrations of about 5 to approximately 900 µg/kg in about 4-15% of investigated samples. However, it cannot be excluded, that these contaminations might originate from fungal infections of growing maize during cultivation, as the producing *Fusarium* species are known to develop preferably under field conditions.

The patulin has been shown to be actively produced during the process of ensiling. Relatively high concentrations of patulin were detected up to 40 mg/kg.

Among the filamentous fungi identified *Penicillium roqueforti* and *Aspergillus fumigatus* seem to be dominating toxigenic species in maize silages.

Mycotoxins found in grass and undefined silages as well as fresh grass and hay are summarized in Table 7.

Again several trichothecenes and zearalenone were detected in concentrations within the µg/kg range. In hay treated with formic acid several hundred µg aflatoxins/kg were enriched. It was frequently reported that an application of formic acid enhances the risk of *Aspergillis flavus* infection and aflatoxin formation. As a consequence, formic acid has been recently prohibited in Sweden for use as a preservative of high moisture grain (Petterson et al., 1989).

In hay sterigmatocystin was occasionally found in low concentrations of about 40 µg/kg.

Concerning the identified fungal species no significant differences were observed compared to the findings in maize silage.

Table 7: Occurrence of toxigenic fungi and mycotoxins in silage, grass and hay

Forage	Toxigenic fungi (dominating species)	Number of samples	Toxin positive samples n / %	Detected mycotoxin	Toxin concentration mg / kg	Reference
Grass silage	Penicillium roqueforti Byssochlamys/Paecilomyces Aspergillus (fumigatus)	65				Frevel et al., 1985
				Zearalenone	0.009	Gedek, 1983
Silage (not defined)	Penicillium (roqueforti) Aspergillus (fumigatus) Paecilomyces varioti Trichoderma viride Fusarium moniliforme	260				Gedek, 1981
Grass/hay		19	4 / 20	T -2 triol T -2 toxin HT-2 toxin	0.65 0.2 - 0,3 0.2	Thalmann, 1986
Hay (treated with formic acid)	Aspergillus (flavus) Penicillium Trichothecium roseum Fusarium Trichoderma			Aflatoxin B_1 Aflatoxin G_1	0.51 - 0.67 0.18 - 0.37	Clevström et al., 1981
Hay		157	1 / 0,6	Sterigmatocystin	0.04	Buckle, 1983

It can be expected that the spectrum of mycotoxins that might occur in silages exceeds considerably the small number of just mentioned substances. This presumption is supported by investigations, where mycotoxins were enriched in experimental silages after inoculation with toxigenic fungi (Table 8). There is evidence that byssochlamic, mycophenolic and penicillic acids as well as PR toxin may be produced within the process of silage making.

Table 8: Production of mycotoxins in experimental silages and hay after inoculation with toxigenic fungi

Forage	Inoculant	Produced mycotoxin	Toxin concentration mg/kg	Reference
Maize silage, kernels "aerobic conditions"	Fusarium graminearum	Zearalenone	1.5 - 35	Escoula, 1979
Maize silage, whole plant	Byssochlamic nivea	Patulin Byssochlamic acid	0 - 42.5 0 - 34.3	Escoula, 1975
Maize silage, whole plant	Penicillium roqueforti	Patulin Mycophenolic acid Penicillic acid PR toxin	15.1 3.6 3.1 2.1	Amend et al., 1986
Hay	Aspergillus versicolor	Sterigmatocystin	0.7 - 2.0	Lepom et al., 1988 a

In addition, there have been several investigations testing the potency for mycotoxin formation of fungi in synthetic and complex media, that have been isolated from different silages and hay (Table 9).

Table 9: Mycotoxin production of isolated fungi from silages and hay in synthetic and complex media

Forage	Isolated fungi	Total number of isolates	Number of toxin producing isolates	Produced mycotoxin	Reference
Silages of grass, maize, rye, barley	Paecilomyces niveus	26	26	Patulin	Hacking et al., 1981
Silages of grass, maize, sugar beet leaves	Penicillium roqueforti	34	34	PR toxin	Gedek et al., 1981
	Penicillium verrucosum	28	9	Penicillic acid	
	Aspergillus fumigatus	13	6	Fumitremorgin B + Fumitremorgin C + Verruculogen + TR-2	
			7	Fumitremorgin C + TR-2	
	Aspergillus flavus	10	9	Kojic acid	
			1	Cyclopiazonic acid + Kojic acid	
Hay, mostly acid treated	Aspergillus flavus	9	9	Aflatoxin B_1 Aflatoxin B_2	Clevström et al., 1981
			5	Aflatoxin G_1 Aflatoxin G_2	
Hay	Aspergillus versicolor	9	9	Sterigmatocystin	Lepom et al., 1988 b

High percentages of isolated *Paecilomyces*, *Penicillium* and *Aspergillus* species were capable of producing patulin, PR toxin, penicillic acid, tremorgenic mycotoxins like fumitremorgins, verruculogen and TR-2, as well as kojic and cyclopiazonic acid, aflatoxins and sterigmatocystin.

Tasks of interest in research on mycotoxins in forage conservation

Due to the limited current knowledge, research on mycotoxins in forage conservation should be considerably enhanced to elucidate the potential risk of silage making. The following tasks are emphazised to be of special interest:

1) Representative studies should be carried out on the occurrence of important mycotoxins from fusaria, aspergilli and penicillia in grass and maize silages produced in practice to get basic knowledge on actual contamination dimensions.Thereby it would be of great advantage to check the harvested material for mycotoxin contamination before ensiling, in order to find out toxins originating from the field.
2) Laboratory and pilot scale experiments should clarify the risk of different ensiling techniques under variation of environmental conditions thus influencing fungi and toxin contamination. The whole phase of ensiling including time during unloading should be investigated. Additional attention should be focused on possible biochemical conversion of mycotoxins during ensiling. As a result, either less or enhanced toxic metabolites and derivatives may be detected.

3) Feeding experiments should be considerably intensified to get better knowledge about transmission and metabolic transformation of mycotoxins in silage fed livestock. Main problem to clarify is a possible enrichment of toxic substances in animal organs and fluids that are used in food production thus reaching human beings. A very interesting question will be to elucidate the metabolic capabilities of the rumen microflora for degrading mycotoxins. Thereby in-vitro systems simulating the rumen environment might be of great efficacy.

Ochratoxin A and zearalenone in growing and ensiled maize

In 1989 investigations were started at the Institute of Grassland and Forage Research, Braunschweig-Völkenrode, on the occurrence of mycotoxins in forages. The object of these investigations was to enhance knowledge about risk factors in forage cultivation and conservation that might increase fungi and mycotoxin enrichment (Oldenburg et al., 1989).

Special attention has been drawn on the occurrence of two mycotoxins, the ochratoxin A and the zearalenone in growing and ensiled maize. Both mycotoxins may reach human beings by carry-over and should be kept as low as technological feasible in food and feed (Bauer et al., 1987; Mirocha et al., 1981).

In the following some results of these experiments are presented.

The maize crops assigned for the ensiling experiment were analysed before harvesting for ochratoxin A and zearalenone contamination. As sample material whole plants or cobs from field areas of half a hectare were collected in intervals of 7 to 14 days from flowering to harvest. Both mycotoxins were quantitatively determined by enzyme-linked immunosorbent assay (Dietrich, 1989). The detection limits of these method range down to about 1 μg/kg.

Figure 1: Ochratoxin A in maize

location Völkenrode

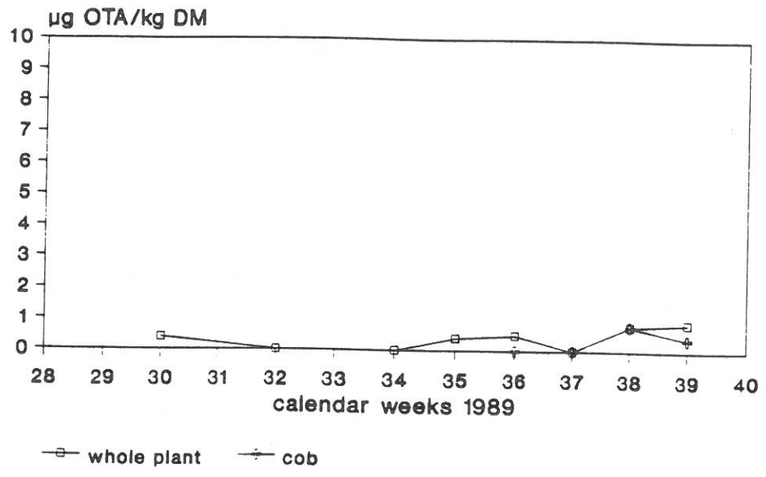
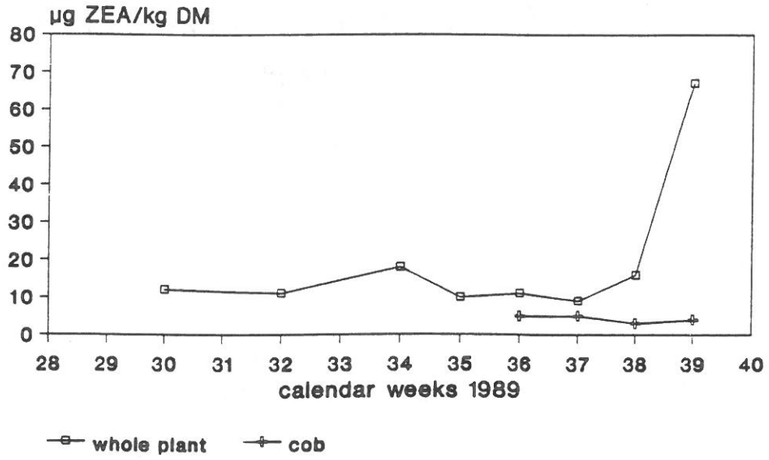


Figure 2: Zearalenone in maize

location Völkenrode



In growing maize, amounts of ochratoxin A varied in the area of detection limit (Figure 1), so that definite positive samples were not identified during cultivation of the maize.

By contrast, zearalenone was detected throughout the observed cultivation period in amounts of 10-20 μg/kg DM, when suddenly the zearalenone content increased in the week before harvest (Figure 2).

Thus the maize was precontaminated with zearalenone in a concentration of about 100 μg/kg DM before ensiling has been started.

The ensiling experiment was carried out by use of a laboratory silo equipment. After harvest, whole plants of maize were chopped to a length of about 5 mm, treated with biological or chemical additives and filled in portions about 750 g into the silos. As additives a chemical salt mixture on the basis of formate, benzoate and metabisulfite and an inoculant of lactic acid bacteria were applied. Furthermore different atmospheric conditions were maintained during three months of storage.

The results of gas tight stored maize are shown in Figure 3. Within a certain variation zearalenone content did not change significantly and was not influenced by the applied different additives. A decomposition of zearalenone was not observed at anaerobic conditions of ensiling.

In a second trial silos were sealed after one day of storage in order to promote initial fungal growth (Figure 4). An increase of zearalenone content was observed in all silos in the first days of storage. The highest value was detected in inoculated material after 90 days of ensiling.

When silos were daily aerated with about 200 mg O_2 per kg DM (Figure 5), there was a clear increase in zearalenone concentration in the chemically untreated and inoculated silages. Multiple values of zearalenone concentrations compared to the freshly harvested material were observed.

The chemical additive obviously inhibited the formation of zearalenone in spite of aeration, so that the zearalenone content remained constant until the end of storage.

Figure 3: Zearalenone in maize silage

gas tight storage

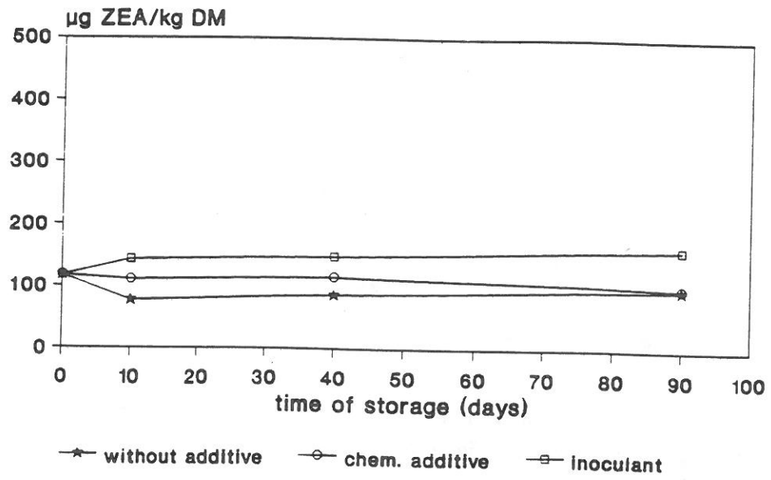


Figure 4: Zearalenone in maize silage

after day one gas tight storage

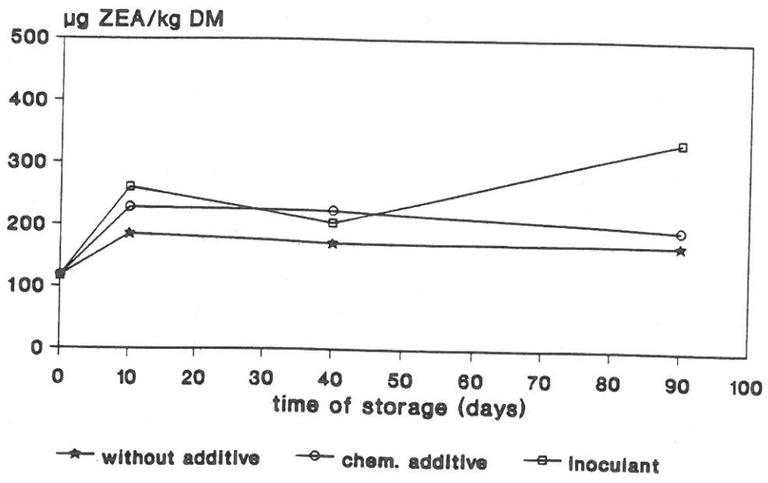
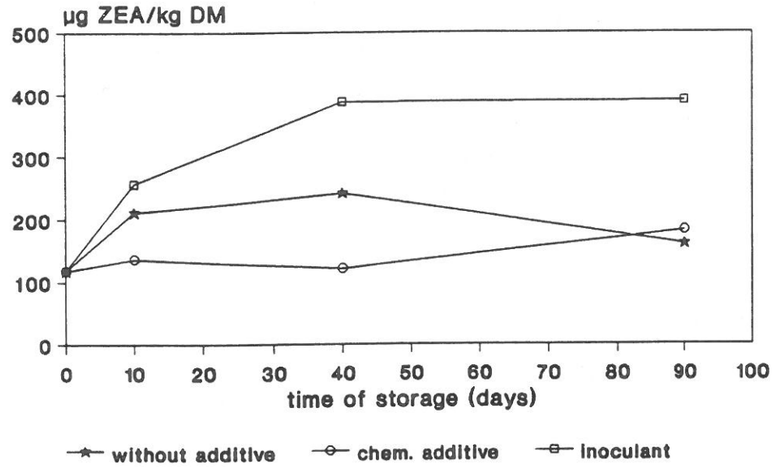


Figure 5: Zearalenone in maize silage

aeration 200 mg O_2/kg DM/day



In all silage samples investigated ochratoxin A again ranged within the area of detection limit and may be of minor importance in silage making.

These results have to be regarded as preliminary attempt to get better knowledge about the significance of ochratoxin A and zearalenone in growing maize and maize silage. It is not possible at the time to deduce general conclusions from the results of just one year of investigation.

Intensive and systematic long-term field and conservation experiments might give an answer how far modern production techniques may result in mycotoxin enrichment and which strategies could be applied in practice to reduce the risk of mycotoxin contamination in food and feed.

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