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Fungal growth in silages of sugarbeet press pulp and maize

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SUMMARY

Fungal spoilage of animal feed silage occurs frequently. In spoiled silage of sugarbeet press pulp sampled in The Netherlands during the period 1986–90, 40% of the samples were infested by *Penicillium roquefortii*. Other fungi of health significance included *Aspergillus fumigatus* (8% of all samples) and *Byssochlamys* spp. (4% of all samples). *P. roquefortii* is also the dominant spoilage mould in maize silage. However, no PR-toxin could be detected in 25 lumps of *P. roquefortii*-infested maize silage, although one lump contained a fluorescent substance, with an R_f-value close to that of PR-toxin. This silage sample was not mutagenic, but had a cytotoxic effect towards *Salmonella typhimurium* in the Ames test. All *P. roquefortii*-infested lumps contained fluorescent fungal metabolites which were absent in samples taken at 5 cm distance from the corresponding lumps in the silage heaps. It is recommended that lumps of fungal-infested silage are removed before feeding the silage to cattle.

INTRODUCTION

Sugarbeet pulp, a residue of the beet sugar extraction process, is a high-energy animal feed ingredient. About one-third (i.e. 600000 tonnes) of the yearly sugarbeet pulp production in The Netherlands is produced as sugarbeet press pulp with a dry matter (DM) content of 22%. Approximately 30% of this press pulp is used as fresh pulp and 70% is preserved as silage. Maize is specifically grown as a fodder crop in The Netherlands, with an estimated yearly production of 10 million tonnes, most of which is chopped and ensiled immediately after harvest. Usually, ensiling is carried out in heaps of 1–1·5 m height which have been compressed by tractor, and covered with two sheets of 0·15 mm thick polythene, or one sheet weighted down with a 10–20 cm layer of soil.

Quite often distinct fungal growth and sporulation can be recognised when opening the heap for use. This fungal growth is indicated by blue, green, orange, red, yellow or white layers, lumps or surface discolorations. Questions from the farmers prompted us to investigate the nature of the fungi occurring in sugarbeet and maize silages, with a view to assessing the possible risk of mycotoxicoses.

MATERIALS AND METHODS

During the years 1986–89, a total of 120 samples of sugarbeet press pulp silage showing discoloration were received from Dutch farmers. During the period December 1989 to April 1990, maize silage samples from 14 farms were tested specifically for blue-green *Penicillium roquefortii*-infested lumps. Samples of *c.* 1 kg were taken from 25 lumps, from silage at 5 cm and 50 cm away from the lump. Particulars of the silages including covering material, maize cultivar, dry matter content, particle size, density and feeding rate were recorded.

Penicillium roquefortii strain SP1165, able to produce PR-toxin, was kindly provided by L. Leistner, Bundesforschungsanstalt für Fleischforschung, Kulmbach, Germany, as a reference culture.

Samples of silage with visible fungal growth were suspended in twice their weight of sterile peptone-physiological salt solution containing 5 g/l peptone (Oxoid L34) and 8·5 g/l NaCl, streaked on three plates of malt extract agar (Oxoid CM59) and incubated at 25 °C until colonies became visible. Representative colonies were isolated and purified on the same medium. Identification to genus or species level was based on microscopic morphology.

A 20 g finely ground sample was extracted with ethylacetate and dichloromethane, purified on a kieselgel column, chromatographed on alufoil kieselgel 60 HPTLC plates (catalogue number 5633.0001, Merck, Darmstadt, Germany) and viewed under a 366 nm UV light source according to Amend & Müller (1986). PR-toxin producing *Penicillium roquefortii* strain SP1165, and PR-toxin (Sigma P4771) were used as reference materials.

A 100 g sample was extracted according to the method of Filtenborg *et al.* (1983). The Ames test was carried out according to Ames & Maron (1982) using *Salmonella typhimurium* tester strains TA98 and TA100, with and without the addition of S9 liver homogenate.

RESULTS

From a total of 120 samples of clearly mould-infested silage of sugarbeet press pulp, the major fungal species isolated was *Penicillium roquefortii* (Table 1). Other fungi, in order of occurrence, were *Mucor* spp., *Neurospora* spp., *Aspergillus fumigatus*, *Geotrichum candidum*, *Monascus ruber*, *Byssochlamys* spp. and *Trichoderma* spp. *P. roquefortii* has been divided into two chemotypes (Frisvad 1988). In pure culture on laboratory media, chemotype I is able to produce PR-toxin, roquefortin C and mycophenolic acid. Chemotype II can produce patulin, penicillic acid, roquefortin C, mycophenolic acid and botryodiploidin. Pure cultures of *A. fumigatus* can produce a variety of mycotoxins including fumigaclavines, fumitoxins, fumitremorgens, gliotoxin and verruculogen; this genus is also an opportunistic pathogen which has been associated with mycotic abortion in cattle (Counter 1973). *Byssochlamys* spp. can produce patulin and byssochlamic acid among others. *Trichoderma viride* can produce trichodermin (Frisvad 1988).

Table 1. *Fungi isolated from mouldy silages of sugarbeet press pulp*

Identification	Percentage of total (n = 120)
Penicillium roquefortii	40
Mucor spp.	23
Neurospora spp.	11
Aspergillus fumigatus	8
Geotrichum candidum	8
Monascus ruber	4
Byssochlamys spp.	4
Trichoderma spp.	2

The occurrence of mycotoxins in sugarbeet press pulp silage was not investigated. As *P. roquefortii* was also the most frequent spoilage mould in maize silage, we investigated the presence of PR-toxin, which is regarded as the most acutely toxic metabolite of *P. roquefortii* (Schoch *et al.* 1984). The particulars of the maize silage heaps with a clearly visible presence of *Penicillium roquefortii* are listed in Table 2.

Of the 25 samples from the centre of lumps of *P. roquefortii*-infested silage, none contained PR-toxin. However, one sample (14–1A) contained an unidentified fluorescent substance with an R_f-value close to that of PR-toxin, possibly a metabolite or degradation product. It was demonstrated that this was not PR-toxin by spiking the sample with reference PR-toxin.

Table 2. *Characteristics of maize silage piles sampled on farms in The Netherlands*

Heap no.	Location	Number of samples	Maize cultivar	Dry matter content (%)	Covering*	Particle size†	Density‡	Rate of use (m/week)
1	Waspik	2	Slavis	29	PP	1	1	1·00
2	Rijsbergen	2	Presta + Sogetta	28–33	PP	3	3	1·00
3	Heesbeen	2	Sonia	27–32	PS	1	1	–
4	Nieuwleusen	1	LG2080	32	PS	2	2	0·90
5	Wezep	2	Splenda	29	PP	3	3	1·50
6	Goor	2	Brutus	35	PP	3	1	1·50
7	Rietmolen	2	–	31	PS	2	1	1·00
8	Hellendoorn	2	–	32	PS	2	2	1·50
9	Mariënheem	2	Sonia	27	PS	3	2	0·80
10	Woudenberg	2	Brutus	28	PS	2	3	1·25
11	Varsseveld	1	Brutus	34	PS	1	1	0·80
12	Balkbrug-I	2	LG20 + 180	30	PS	2	2	0·80
13	Balkbrug-II	1	Sonia	30	PP	2	1	1·00
14	Dalfsen	2	Brutus	35	PS	1	1	1·00

* Covering: PP = two polyethylene sheets; PS = one polyethylene sheet covered with soil.
† Particle size: 1 (good) = < 7 mm; 2 (satisfactory) = 7–10 mm; 3 (poor) = > 10 mm.
‡ Density: 1 (good) = > 200 kg DM/m^3; 2 (satisfactory) = 170–200 kg DM/m^3; 3 (poor) = < 170 kg dm/m^3.

Table 3. *Results of Ames test on selected samples of* P. roquefortii-*infested maize silage*

Sample	Dose* (ml extract)	Number of revertants (triplicates)											
		TA98			TA98+S9			TA100			TA100+S9†		
14–1A	0	25	15	21	33	28	23	89	86	77	61	70	106
	0·5	19	23	15	18	19	21	7	8	17	51	32	30
	1·0	17	14	23	23	19	16	4	4	5	19	19	28
14–2A	0	25	15	21	33	28	23	89	86	77	61	70	106
	0·5	19	17	31	10	17	15	87	78	84	85	84	79
	1·0	24	22	18	22	13	16	85	81	82	82	83	89
14–1B	0	32	32	33	37	41	43	115	122	127	143	145	137
	0·5	25	33	21	33	37	31	117	119	132	125	137	139
	1·0	35	37	31	25	42	38	121	123	115	153	151	135

* Dose: 0·5 ml extract corresponds with 2 g silage; 1 ml extract corresponds with 4 g silage.
† S9: liver homogenate.

This compound was absent in the corresponding samples 14–1B (taken at 5 cm distance from the lump containing sample 14–1A) and 14–1C (taken at 50 cm from the affected lump).

The Ames test was carried out on samples 14–1A (suspect), 14–1B (at 5 cm distance from the suspect lump) and 14–2A (a similar lump from the same silage heap). No mutagenesis was observed, but sample 14–1A was significantly cytotoxic to *S. typhimurium* strain TA100 (Table 3) at a level corresponding with 2 g silage.

DISCUSSION

The predominant fungi associated with visible growth and spoilage in sugarbeet press pulp silage were *P. roquefortii*, *Mucor* spp. and *Neurospora* spp. Gedek *et al.* (1981) also found, in decreasing order of occurrence, *P. roquefortii* (39% of all samples), Mucorales (16%), *Aspergillus fumigatus* (7%), *Cladosporium* spp. (3%), *Monascus purpureus* (1·5%), *Paecilomyces varioti* (1·5%), *Scopulariopsis brevicaulis* (1·2%), *Trichoderma viride* (0·8%) and *Fusarium moniliforme* (0·8%) in 260 samples of maize silage.

In maize silages, attention was given only to the occurrence of *P. roquefortii*. In general, all silages were well-covered and well-kept. At the time of opening, the upper surface showed no signs of fungal growth. Almost all of the blue-green lumps were found 20–80 cm beneath the upper surface. In some silages, the blue-green material was present as a layer. The occurrence of *P. roquefortii* in this area might be the consequence of more favourable environmental conditions such as higher temperature compared to the lower temperature at the upper surface, and lesser density of the silage compared to that at the bottom of the heaps. Similar stratification was also observed in unsealed silos with moist barley (Lacey 1971) where lack of oxygen was suggested as a factor limiting growth in the deeper layers. Possibly a tolerance to increased concentrations of CO_2 might contribute to the dominating occurrence of *P. roquefortii*. Silages with *P. roquefortii* represented a range of maize cultivars, so there was no reason to suspect a varietal effect. Spoiled silages did not differ in DM content from the mean of all silages (32·6% DM); the particle size (chopping rate) was not visually different from that of sound silages. However, in many of the spoiled silages the rate of use was lower than that usually recommended (i.e. $\geqslant$ 1·5 m/week for silages with a soil top cover, or $\geqslant$ 2 m/week for silages without soil top cover). We assume therefore that the *P. roquefortii* spoilage is produced when the rate of use is too slow, and that fungal growth may progress into the silage heap starting from the surface where silage is cut away for use. The unloading rate was also a major factor influencing fungal growth in moist barley (Lacey 1971).

Although none of the blue-green lumps showed the presence of PR-toxin, one lump contained a fluorescent substance which might have been closely related to PR-toxin.

Amend & Müller (1986) found significantly lower levels of PR-toxin in maize silage compared to pure cultures. They ascribed this discrepancy to the instability of PR-toxin in the presence of proteins, amino acids and amines. Also pH values < 3 destabilize PR-toxin.

In the Ames test, PR-toxin is mutagenic and cytotoxic to the liver and human cell lines (Schoch *et al.* 1984). In this study, silage sample 14–1A was not mutagenic, but it had a cytotoxic effect on *Salmonella typhimurium* TA100. A blue-green *P. roquefortii* lump from the same silage heap did not show this behaviour. This apparent heterogeneity could be associated with the fact that only chemotype I of *P. roquefortii* is able to produce PR-toxin (Frisvad 1988). It is also possible that PR-toxin is first produced, and subsequently metabolized as was

observed by Wei & Liu (1978). They found that of four tested strains of *P. roquefortii*, all were able to produce PR-toxin in pure culture, but the level of PR-toxin fell to zero 1–2 days after having reached its maximum. Also, Gedek *et al.* (1981) observed that all of 34 isolated strains were able to produce PR-toxin, whereas only two isolates produced roquefortin. In principle, it thus is possible that *P. roquefortii* leaves no detectable traces of PR-toxin but that toxin degradation products remain in the silage; no data are available on the toxicity of such degradation products. All samples taken from the centre of lumps contained fluorescent substances which were not present in fresh maize and which therefore were probably formed by *P. roquefortii*. In samples taken 5 and 50 cm away from lumps, such fluorescent compounds were absent. We conclude therefore that these metabolites do not diffuse outside the lumps of spoiled maize silage. The same conclusion can be drawn with regard to the substance responsible for the cytotoxic effect.

Although PR-toxin was absent, there is a certain risk of toxicity associated with the consumption of silage lumps containing *P. roquefortii*. Toxic effects of *P. roquefortii*-infested maize silage in milk cattle included feed refusal, reduced rumen fermentation activity, intestinal inflammation, mastitis and abortion (Vesely *et al.* 1981; Häggblom 1990). Since it appears that *P. roquefortii* metabolites do not migrate outside the infested material, it is recommended that clearly (blue-green) fungal-infested silage is removed before feeding silage to cattle.

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