Fungal infestation and mycotoxin contamination of South African commercial maize harvested in 1989 and 1990

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The incidence of seedborne fungi and contamination by their respective mycotoxins were determined in commercial South African maize harvested in 1989 and 1990 from the five major production areas. Differentiation was made between yellow and white maize as well as between the different commercial grades. The predominant fungus was Fusarium subglutinans, *followed closely by* F. moniliforme. *The mean levels of* F. moniliforme *were highest in the northern Orange Free State and the western Transvaal, whereas the levels of* F. subglutinans *were highest in the eastern Free State and eastern Transvaal In Natal, the mean level of* F. moniliforme *increased sharply in 1990 compared to 1989. Very few isolates of* Aspergillus flavus *and none of* A. parasiticus *were recovered. Fungal isolations and mycotoxin levels were higher in 1990 than 1989. The fumonisins, a group of mycotoxins produced by* F. moniliforme, *were more frequently detected and at higher levels than the mycotoxins moniliformin, deoxynivalenol and nivalenol. No zearalenone or aflatoxin was detected in the samples. Higher levels of* F. moniliforme *were found in yellow than in white maize, but white maize contained higher levels of fumonisin contamination than yellow maize.*

The ear-rot complex of maize (*Zea mays*) in South Africa is caused primarily by *Diplodia maydis* (Berk.) Sacc. [= *Stenocarpella maydis* (Berk.) Sutton], *Fusarium moniliforme* Sheldon, and *F. subglutinans* (Wollen. and Reinking) Nelson, Toussoun and Marasas, *F. graminearum* Schwabe [teleomorph: *Gibberella zeae* (Schw.) Petch] and *D. macrospora* Earle [= *S. macrospora* (Earle) Sutton].[1-4] All five of these fungi produce mycotoxins which are highly toxic to animals.[5-7] The mycotoxin(s) produced by *D. maydis*, which causes diplodiosis in sheep,[8] perinatal mortality in lambs[5] and weight reductions in poultry,[9] has not yet been chemically characterized.

Two other highly toxigenic fungi that occur sporadically in local maize, but are not ear-rot pathogens under local conditions, are *Aspergillus flavus* Link and *A. parasiticus* Speare.[10] Isolated outbreaks of aflatoxicoses in farm animals caused by aflatoxins, produced by these *Aspergillus* species, in improperly harvested and stored maize have been reported in South Africa.[11,12]

The fumonisins, a group of mycotoxins produced by *F. moniliforme*,[13,14] have been found to occur naturally in maize-based feeds associated with animal toxicoses.[15-17] Fumonisin B_1 (FB_1) has been shown to cause equine leukoencephalomalacia,[18] porcine pulmonary oedema[19] and liver cancer in rats.[20] The fumonisins are also associated with maize-based human foodstuffs.[21-24] It is, therefore, imperative that maize be analysed primarily for the fumonisins as maize is consumed as a staple foodstuff by millions of people in South Africa. The occurrence of *F. moniliforme* and the fumonisins, in addition to other fungi and mycotoxins, is reported here for the local maize crops of 1989 and 1990.

Materials and methods

Maize sampling. A total of 631 grain samples (each sample approximately 500 g) of commercial maize were analysed. The 1989 crop: these samples numbered 165 of white maize (WM) of three grades (WM1, WM2, WM3), and 147 of yellow maize (YM) of three grades (YM1, YM2, YM3). Samples were drawn from the five most important (80–90% total national crop) maize production areas in the country, namely, northern (NOFS) and eastern (EOFS) Orange Free State, western (WTVL) and eastern (ETVL) Transvaal, and Natal. The 1990 crop: there were 155 samples of WM and 164 of YM. This set of samples was again composed of the three grades of maize from the same production areas. Grading of local maize is based on parameters such as the visual assessment of the percentage of mouldy, discoloured and broken kernels, and the presence of foreign matter. The best quality maize is represented by the highest grades, namely, YM1 and WM1.

All the samples were taken from storage silos by sampling from the grain stream while 30 tonnes of maize was outloaded from the silo. A divider was used to obtain 500-g samples. The number of samples representing the different maize grades and production areas varied depending on the availability of the grades and the number of silos in each area. Relatively few third-grade samples were received for analysis.

Mycological analysis. All 631 samples (1989 and 1990) were analysed for fungal infestation. In the case of the 1989 samples, two subsamples of kernels (each ca. 100 g) from each sample were surface-disinfested for 1 min in a 3.5% sodium hypochlorite solution and rinsed twice in sterile water. One hundred kernels (5 kernels/plate) from one subsample were transferred to 1.5% malt extract agar (MEA) containing 150 mg novobiocin $litre^{-1}$, and 100 kernels from the other subsample (5 kernels/plate) transferred to a selective *Aspergillus flavus–A. parasiticus* agar medium (AFPA).[25] The 1990 samples were plated onto only MEA. The plates were incubated at 25°C in the dark for 5–7 days. All developing fungal colonies were identified and counted directly on the agar plates. Fungi considered important in this study were *F. moniliforme, F. subglutinans, F. graminearum, D. maydis, D. macrospora, A. flavus* and *A. parasiticus*.

Chemical analysis. The following selected mycotoxins were determined: fumonisins B_1 (FB_1), B_2 (FB_2) and B_3 (FB_3) produced by *F. moniliforme*;[26] deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) produced by F. graminearum;[7] moniliformin (MON) produced by *F. subglutinans*;[7] and aflatoxins (AFLA) produced by *A. flavus* and *A. parasiticus*.[11] Selected samples (1989 = 121; 1990 = 128) were analysed for FB_1 and FB_2, while FB_3 levels were also determined in the 1990 samples.[27] Five samples per area (where possible) were randomly selected within each of the first- and second-grade samples. All third-grade samples were analysed for the fumonisins. Selected samples (1989 = 41; 1990 = 55) were analysed for the other mycotoxins.[28-31] One sample per area was randomly selected within each of the first- and second-grade samples, whereas all third-grade samples were analysed for these five mycotoxins.

Analytical standards. DON, MON, FB_1, FB_2 and FB_3 were isolated within the MRC's Programme on Mycotoxins and Experimental Carcinogenesis. ZEA and AFLA were obtained from Makor Chemicals, Jerusalem. NIV was from Wako Chemicals, Tokyo. The identity and purity of each standard were assessed by either thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), capillary gas chromatography (GC) or ultra-violet (UV) spectroscopy.

Apparatus. Capillary GC separations were performed using a Carlo Erba Mega 5 300 gas chromatograph equipped with a split/splitless injector and either a 30-m x 0.32-mm i.d. DB-5, or a 25-m x 0.32-mm i.d. SE-30, fused silica capillary column. Compounds were detected with a ^{63}Ni electron capture detector (ECD) for NIV and DON determinations. HPLC separations were performed on either a 4-µm Nova-Pak C_{18}, or a 7-µm Phenomenex ODS 30 column, using a Waters Model 510 liquid chromatographic pump. Peak detection was performed using either a Hewlett-Packard Model 1040A diode array detector (DAD) or a Perkin-Elmer 650S fluorimeter. Data were collected using a Waters 745 module, and the levels of each toxin were calculated from individual toxin calibration curves. UV wavelength ranges and ratios were monitored using the HPLC/DAD system for HPLC verification purposes.

Statistical methods and data presentation. Statistical analyses of data were done by using the Statistical Analysis System (SAS) program package. Analyses were done on log(ln)-transformed data, but the original (untransformed) data are presented in the figures and tables. The mean mycotoxin levels given in this article represent the mean of the positive samples only, whereas the mean fungal levels are the mean of all the samples. Where applicable, mention is made in the results of significant statistical differences calculated at $P < 0.05$. Some differences are not given due to significant interactions between variables. The limited number (and randomness) of samples tested for the mycotoxins DON, NIV and MON did not allow for meaningful statistical analysis of those particular data.

Table 1. Mean incidence of fungi in maize samples from different production areas in South Africa.[a]

	Production areas				
	NOFS	EOFS	NATAL	WTVL	ETVL
1989 samples					
F. moniliforme[b]	17.0	4.4	9.8	15.7	6.6
F. subglutinans[c]	10.0b	18.4a	12.5b	11.5b	10.6b
F. graminearum[c]	1.1a	2.8a	3.7a	2.4a	1.7a
D. maydis[b]	9.5	3.2	3.9	10.6	5.1
1990 samples					
F. moniliforme[c]	14.0a	5.1b	13.9a	14.3a	7.3b
F. subglutinans[b]	13.8	17.6	12.5	10.2	19.6
F. *graminearum*[b]	0.8	1.1	2.3	1.2	4.2
D. maydis[c]	8.6b	7.9b	8.6b	12.0a	8.1b

[a]Mycological data based on 312 maize samples (165 white and 147 yellow) for 1989, and 319 samples (155 white and 164 yellow) for 1990.

[b]Statistical differences not given due to significant ($P < 0.05$) interactions between locations and other variables.

[c]Means in a row followed by different letters are significantly different at $P < 0.05$.

Results

The predominant fungus was *F. subglutinans*, with isolation frequencies (percentage of plated kernels) of 12.5% and 14.7% for 1989 and 1990, respectively. This was followed by *F. moniliforme* (11.2% and 10.8%), *D. maydis* (6.8% and 9.3%), and *F. graminearum* (2.3% and 1.9%). The fungi *D. macrospora* and *A. flavus* were isolated at very low frequencies (< 0.1%), whereas *A. parasiticus* was not isolated at all. Other fungal genera such as *Penicillium, Nigrospora, Altemaria* and *Acremonium* were also isolated, but were not relevant to this study.

In 1989, *F. moniliforme* was most frequent in NOFS (17.0%) and WTVL (15.7%) maize, but for 1990, maize from WTVL (14.3%), NOFS (14.0%) and Natal (13.9%) had the highest levels of infection (Table 1). The highest levels of *F. subglutinans* were recorded in EOFS maize in 1989 (18.4%), and in ETVL (19.6%) and EOFS (17.6%) maize in 1990. *F. graminearum* was isolated most f requently in maize from Natal (1 989: 3.7 %) and ETVL (1990: 4.2%), while the highest levels of *D. maydis* were isolated from WTVL (10.6%) and NOFS (9.5%) maize during 1989 and in 1990 from WTVL samples (12.0%).

Fusarium levels in maize of the different grades (Fig. 1) indicated no significant differences, for both years, between the grades for each species. Increased levels of seedborne *D. maydis* were associated with lower maize grades, but these differences between grades were significant only for the 1990 samples.

The fumonisins were the most frequently recorded mycotoxins, namely, 67.8% and 82.8% detectable contamination of the 1989 and 1990 samples, respectively. In general, fumonisin levels ranged from 0–7 020 ng g^{-1} for 1989 ($FB_1 + FB_2$) and 0–7 100 ng g^{-1} for 1990 ($FB_1 + FB_2 + FB_3$). Other mycotoxins (percentage of samples contaminated, maximum level) detected in the samples were MON (1989: 2.4%, 15 ng g^{-1}; 1990: 21.8%, 2 040

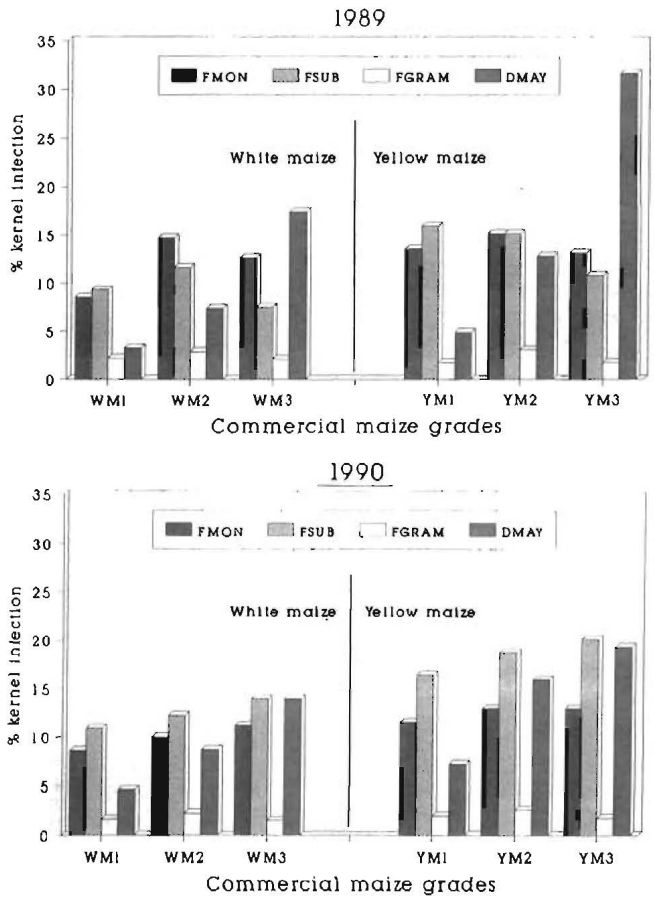

Fig. 1. Mean incidence of fungi in samples of the different commercial grades of South African maize.

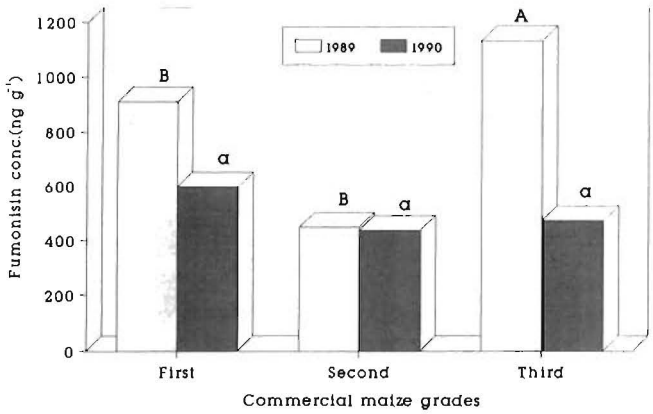

Fig. 2. Mean (positives) total fumonisin levels in samples representing the different commercial grades of South African maize (the white and yellow types combined).

ng g^{-1}), DON (1989: 43.9%, 925 ng g^{-1}; 1990: 72.7%, 1 830 ng g^{-1}), and NIV (1989: 4.9%, 70 ng g^{-1}; 1990: 70.9%, 370 ng g^{-1}). The mycotoxins ZEA (detection limit = 50 ng g^{-1}) and AFLA (detection limit = 0.5 ng g^{-1}) were not detected in any of the samples tested.

In 1989, the levels of FB_1 determined in NOFS maize were significantly ($P < 0.05$) higher than in the EOFS only, and FB_2 significantly higher in NOFS than in Natal and EOFS (Table 2). Results for 1990 indicated that samples from WTVL were more often contaminated with the fumonisins than those from the other areas, but the mean fumonisin levels (positive) were, however, highest in Natal and not in WTVL maize.

In agreement with the levels of *F. moniliforme* in maize of different grades, the mean levels of total fumonisins in the various maize grades (white and yellow types combined) indicated no clear patterns of difference for both years (Fig. 2). In 1989, the lowest grades (WM3 + YM3) of maize had significantly ($P < 0.05$) higher levels of fumonisin contamination than the higher grades, but this difference was not noted in the 1990 samples.

The mean fungal and fumonisin levels in samples of white and yellow maize are summarized in Fig. 3. Yellow maize samples had higher levels of *F. moniliforme* infection than the white, yet the mean levels of fumonisin were higher in white than yellow maize. The mean levels of *F. subglutinans* and *D. maydis* were also higher in yellow than white maize, but this difference did not apply to *F. graminearum*.

Mean levels of other mycotoxins detected indicated more contamination in 1990 than 1989 maize (Fig. 4). In 1989 only one out of 41 samples contained detectable MON at 15 ng g^{-1}, but in 1990 22% (12 out of 55) of the maize analysed contained MON ranging from 0–2 040 ng g^{-1}. The levels of DON ranged from

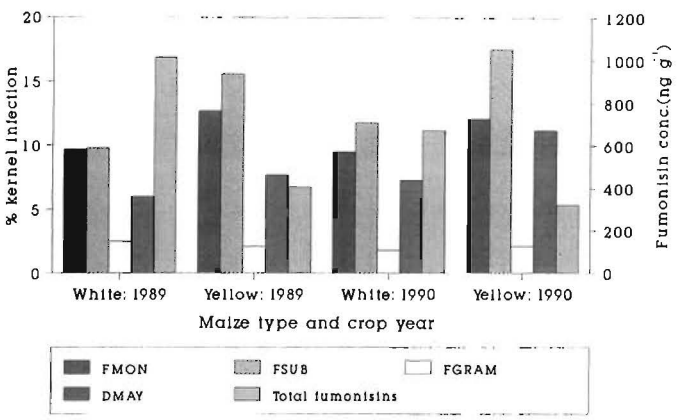

Fig. 3. Mean incidence of fungi and total fumonisin levels (positives) in samples of South African white and yellow maize.

Table 2. Fumonisin levels (ng g^{-1}) in samples of South African maize from different production areas.

		Production areas				
		NOFS	EOFS	NATAL	WTVL	ETVL
1989 crop						
FB_1	Positives[a]	24/28	5/20	14/21	20/28	18/24
	Range[b]	0–5 420	0–135	0–380	0–1 310	0–2 520
	Mean/pos[c]	1 104a	90b	182a	315a	712a
FB_2	Positives	15/28	1/20	6/21	13/28	14/24
	Range	0–1 600	0–115	0–330	0–695	0–870
	Mean/pos	526a	115c	126b	202ab	300ab
1990 crop						
FB_1	Positives	23/26	18/25	14/19	34/34	17/24
	Range	0–1 490	0–2 470	0–2 910	20–5 030	0–960
	Mean/pos	294ab	180b	547ab	412a	228b
FB_2	Positives	14/26	5/25	12/19	23/34	11/24
	Range	0–950	0–850	0–1 190	0–1 670	0–330
	Mean/pos	205ab	279b	291a	183a	125ab
FB_3	Positives	9/26	4/25	10/19	17/34	7/24
	Range	0–200	0–270	0–270	0–400	0–150
	Mean/pos	70ab	98b	88a	87ab	70ab

[a]Number of samples that tested positive out of *n* samples.

[b]Range of detection of the fumonisin in *n* samples.

[c]Mean level for the positive samples only. Means in a row followed by different letters are significantly different at $P < 0.05$.

0–1 830 ng g^{-1} in 1990 compared to 0–925 ng g^{-1} in 1989, with a 29% increase in the number of DON-positive samples. The number of samples contaminated with NIV increased from 5% (positives) in 1989 to 69% in 1990, with an increase in range from 0–70 ng^{-1} to 0–370 ng g^{-1}, respectively. Although NIV occurred only in EOFS and Natal samples in 1989, it was relatively evenly distributed in material from all areas in 1990. Natal consistently had the highest levels of DON, whereas ETVL had the highest levels of MON.

Discussion

The predominance of *F. subglutinans* over *F. moniliforme* in this investigation is in agreement with an earlier study,[2] but contrary to more recent findings from the same maize growing

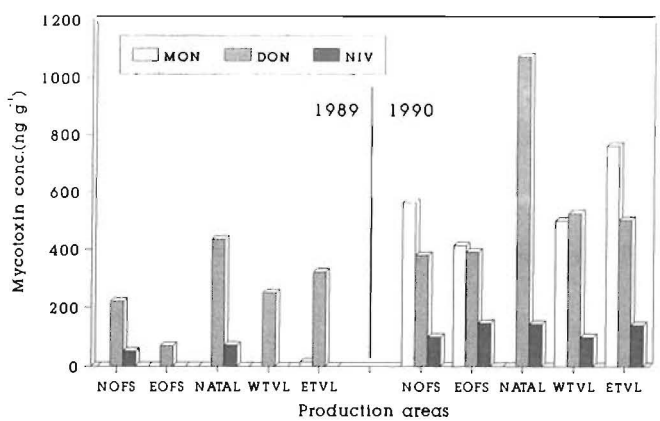

Fig. 4. Mean (positives) mycotoxin levels in samples of South African maize from different production areas.

areas.[3,4] The latter study[4] found that in maize harvested in 1988, 49.6% of the total fungal isolations were *F. moniliforme* and only 12.0% were *F. subglutinans*. This change in fungal incidence levels can be ascribed to variable climatic and environmental conditions in the growing areas. Of note in the present study are the relatively high levels of *F. subglutinans* in the known *F. moniliforme*-dominated areas of NOFS and WTVL,[4] and conversely the relatively high levels of *F. moniliforme* in Natal. These changes are also ascribed to variable climatic conditions.

Infection of maize by *F. moniliforme* and *F. subglutinans* appears to have had little or no effect on the visible quality of the grain, thereby not affecting the samples' grading. The only fungus to have been associated with lower grades is *D. maydis*. This may be linked to the fact that this pathogen is known to infect and kill the kernel embryo[32] and to significantly reduce kernel germination,[33] whereas the two *Fusarium* species seldom had negative effects on kernel germination.[33]

The fumonisins were the major mycotoxin contaminants of the maize samples, although relatively few samples had fumonisin levels above 1 000 ng g^{-1}, namely, 13.2% of the samples tested in 1989 and 9.4% in 1990. Maize production areas with the highest levels of fumonisin contamination in one year were not necessarily those with the highest levels the following year, as indicated by NOFS maize having the highest levels of fumonisins in 1989, but in 1990 WTVL and Natal samples were the most contaminated. The correlations between total fumonisin and *F. moniliforme* levels indicated statistical significance ($P < 0.05$) for both maize type and year (1989: white maize, $r = 0.40$; yellow maize, $r = 0.60$; 1990: white maize, $r = 0.42$; yellow maize, $r = 0.45$). Previous reports have found that such correlations could either be poor[22] or statistically significant ($P < 0.05$) for all the samples tested.[34]

Individual contamination levels of the fumonisins in the different grades of maize indicated that there was no consistent relationship between maize grades and fumonisin levels. Caution should, therefore, be exercised in making assumptions by visual observation of the level of fumonisin contamination based on the grade of the grain, as the high-quality grades, WM1 and YM1, do not necessarily imply low fumonisin levels. This is well illustrated by the fact that the 1989 sample with the highest levels of fumonisin contamination, namely FB_1 at 5 420 ng g^{-1} and FB_2 at 1 600 ng g^{-1}, came from a sample of WM1 from NOFS. This was again found in 1990 with a sample of WM1 maize from WTVL containing 5 030 ng g^{-1} FB_1, 1 670 ng g^{-1} FB_2, and 400 ng g^{-1} FB_3.

One possible explanation for the finding that *F. moniliforme* kernel infection levels were highest in yellow maize, whereas the amounts of the fumonisins were greatest in white maize, is that yellow maize is more susceptible to infection by *F. moniliforme*, but white maize is a better substrate for the production of the fumonisins. Previous studies have shown that yellow maize is more commonly infected with *F. moniliforme*, as well as *F. subglutinans* and *D. maydis*, than white maize.[3,4] Further studies need to address, in particular, the higher fumonisin levels in white maize as this type is the one most widely used as a human foodstuff in South Africa.

Contamination of South African maize with other *Fusarium* mycotoxins must not be discounted as unimportant. Although the levels of MON, DON and NIV were low in 1989, the amounts of these three mycotoxins increased sharply in the 1990 harvest. Of the 1990 samples analysed for DON (range: 0–1 830 ng g^{-1}) and NIV (range: 0–370 ng g^{-1}), very few had *F. graminearum* infection levels higher than 5% and none above 9%. Such a low fungal versus mycotoxin ratio is probably due to certain environmental and physiological (plant and pathogen) effects. Marasas *et al.*[35] reported the presence of DON (2 500 ng g^{-1}) in a sample of hand-selected, *Fusarium*-infected South African maize, but stated that these two mycotoxins had not been found at biologically significant levels in local maize intended for human or animal consumption. They concluded that this could probably be ascribed to the unfavourable climate for mycotoxin production in the main maize-growing areas and could also be due to the fact that commercially produced maize is stored at moisture levels below 14%, which does not allow for further mycotoxin production.

The mycotoxin MON was detected at a range of 0–2 040 ng g^{-1}, which was associated with *F. subglutinans* levels of 10–30%. Natural contamination of maize by MON has been reported from the Transkei at levels ranging from 0–1 410 ng g^{-1},[36] and natural infection of maize by toxigenic, MON-producing strains of *F. subglutinans* has also been reported for areas of the Transvaal.[2] It can be concluded that, with a few exceptions such as the DON levels in 1990 Natal maize, the contamination of commercial maize with MON, DON, NIV, ZEA and AFLA has so far posed little risk to human and animal health in this country.

This survey revealed that fumonisin contamination of South African maize was relatively low, especially when compared to levels detected in maize from the USA and some other countries.[37] Because of the increasing world-wide importance of the fumonisins and the probable introduction in the near future of regulatory measures aimed at this group of mycotoxins, it is important that local and imported maize be screened for the fumonisins on a routine basis. This would ensure that the quality of local maize remains of a high international standard and that no undue risks are posed to either human or animal health.

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Function estimation with neural nets

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We report on the use of multilayer networks as 'estimators' of functions, or their application to problems of nonlinear regression. A set of target functions with adjustable complexity is used. The results of empirical investigation into the extent to which function estimation is possible, and the relationship between the network parameters, are presented. This could provide the designer of a neural-based system with some intuition about the design choices to be made.

Background

The primary use of multilayer networks has been as classifiers, and impressive results have been reported in this respect (see e.g. refs 1–3). We regard the success of the classification networks as due to their superior interpolation abilities, compared to linear and other classifiers. More generally, multilayer networks can be viewed purely as interpolators or as estimators of functions, that is, they can also be applied to problems of nonlinear regression. Although this application of neural networks has been proposed by several researchers (see e.g. ref. 4), little is known about their practical capabilities. In this communication we investigate empirically the extent to which function estimation is possible, and also the relationship between the network parameters and the complexity of the function. Rather than compare neural networks with standard regression techniques, we have decided to use a set of target functions with adjustable complexity to gain an understanding of the 'absolute abilities' of such neural networks.

To obtain target functions of variable complexity, we used polynomials as the functions to be estimated. The hypothesis is that the complexity of the functions can be described by two parameters, namely the order of the polynomial and the dimensionality of the input space (i.e. the number of variables of which the polynomial is a function). This hypothesis is tested by simulations investigating the minimum error obtainable with a given network, the number of hidden units that are required for a given error and the number of training samples needed.

One of the aims of this investigation is to provide the designer of a neural-based system with some intuition of:

- the size of network (number of hidden units) required for a given error,
- the minimum error obtainable (with any size network) for a function with given complexity and dimension, and
- the number of training samples to be used.

Below we describe our experimental protocol, followed by the results obtained.

Experimental protocol and data

The network used in this investigation is a three-layered per-