PROJECT REPORT No. 289

PRACTICAL GUIDELINES TO MINIMISE MYCOTOXIN DEVELOPMENT IN UK CEREALS, IN LINE WITH FORTHCOMING EU LEGISLATION, USING THE CORRECT AGRONOMIC TECHNIQUES AND GRAIN STORAGE MANAGEMENT

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by

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ABSTRACT

Ochratoxin A is a mycotoxin produced by *Penicillium verrucosum,* a mould that occurs widely in poorly stored cereals in the UK and its occurrence is associated with storage of damp grain. Evaluation of the risk posed by this mycotoxin to human health indicates that its presence in cereals and other food products must be minimised. To this end the European Community have introduced maximum permitted limits for this mycotoxin of 5 µg/kg and 3 µg/kg for whole cereal grains and processed products respectively. The aim of this study was to examine whether different levels of field fungi on cereal grains entering storage affect the growth of the storage fungi and the development of mycotoxins (principally but not exclusively ochratoxin A) post-harvest.

Field trials were carried out in Kent, Hampshire, the Cotswolds and Yorkshire to ensure that the study covered a range of soil types, geographical and climatic locations. At each site 2 malting barley varieties 2 bread-making wheat and 2 feed wheat were grown in 2000 and 2001. At each site and for each variety, 24 m x 120 m plots were established and all received 'standard' commercial applications of fertilisers, insecticides, growth regulators and herbicides. Plots were divided into four sub-plots and different fungicide regimes applied to each sub plot. At harvest, grain was transferred undried into 0.5 tonne bags, stored under cover and monitored for moisture content and temperature for 28-42 days. Samples taken at harvest and during the storage period were examined for moulds and for ochratoxin A.

The fungicide application regime used had little effect on the composition or amounts of field moulds, in particular sooty moulds, entering storage. Two scenarios were put forward; either field moulds were able to establish themselves on the ear after the activity of the T3 fungicide spray had diminished or mixing of spores in the combine had a levelling effect on spore numbers. Either scenario suggests that trying to manipulate levels of field moulds entering the store to reduce mycotoxin development is not a viable option. Neither *P. verrucosum* nor ochratoxin A were found in any of the grain samples at harvest. Cereals are put at risk from ochratoxin A (or other mycotoxins) under fairly well defined combinations of temperature and grain moisture content but its actual development is determined by other factors that are strongly indicated but not proven. It is concluded that prediction cannot be made on the basis of grain moisture and temperature alone. There is a strong indication that grain may become contaminated with *P. verrucosum* during or shortly after harvest through the presence of this fungus in handling equipment or the store environment.

Results from this project confirm that the most effective method for preventing ochratoxin A formation in stored grain is the rapid dying of moist grain, to moisture content below 15%, before storage.

SUMMARY

Introduction

The presence of mycotoxins in cereals reduces the quality of grain, is likely to be a risk to human and animal health, and causes economic loss through their effect on livestock production. The moulds associated with grain are generally divided into ‘field’ and 'storage fungi'. Field fungi such as *Fusarium*, *Cladosporium* and *Alternaria* species infect the ear during growth of the crop. These organisms appear to 'die out' after harvest as they are overgrown by ‘storage’ fungi such as *Aspergillus* and *Penicillium* species during storage. In the UK the important mycotoxins formed in grain by field fungi include trichothecenes (e.g. deoxynivalenol, nivalenol, HT2 toxin and T2 toxin) and zearalenone (all produced by *Fusarium* species).

Aflatoxins are the most important fungal toxins worldwide and together with ochratoxin A are the only storage mycotoxins for which legislation currently exists within the EC. Earlier legislation for aflatoxins has recently been widened to include cereals although these toxins are rarely produced under UK conditions. Recently, maximum permissible levels of 5 ppb in raw cereals and 3 ppb in processed products have been set for ochratoxin A. Surveys of stored UK cereals show that a small but persistent percentage of samples examined exceed this level (2-3%). This represents a large tonnage of grain and a potential serious economic loss. The food and brewing industries are increasingly demanding high quality cereals for food and drink products and require grain conforming to the statutory limits set. There is a major incentive for the UK Cereal Industry to minimise ochratoxin A and other mycotoxins in grain to enable it to remain competitive with other member states within the EU. Clearly there is an urgent need to understand the factors that encourage mycotoxin formation both pre and post harvest as this may provide a means of minimising this problem.

It has been known for many years that grain must be stored dry under good hygienic conditions to prevent mould growth, insect invasion and to maintain quality. Compliance with Good Agricultural practice (GAP) will address most of these requirements. The continued occurrence of ochratoxin A in grain suggests that either GAP is not always rigidly followed or that all the factors involved in the formation of ochratoxin A are not fully understood.

The time between harvest and the reduction of grain moisture to a safe level is of vital importance and ambient air ventilation or hot air dryers are commonly used. If grain can be dried within a few hours a significant risk of ochratoxin A formation only arises on those occasions when damp grain has to be stored before drying e.g. because the capacity of the dryers is exceeded. Under such circumstances it is vital that the wettest grain, at greatest risk from toxin production, is given priority.

The continued occurrence of ochratoxin A in UK grain must imply that either grain is not/cannot always be dried quickly enough or that stored grain is not managed effectively. If these situations are unavoidable it is important that all factors that affect the potential formation of ochratoxin A are fully understood and sound advice is available on how to minimise this risk. There is evidence to suggest that some practices in the field, including fungicide application, may affect the extent of the formation of storage mycotoxins through its effect on field micro-flora. This may be one reason why it has been difficult to predict the extent of mycotoxin problem formation during storage.

Current guidelines for safe storage of cereals may not be specific enough to avoid or minimise mycotoxin occurrence. The aim of this study was to examine the effects of different fungal infections resulting from common fungicide regimes, on the development of storage organisms and ochratoxin A production. In addition, different storage situations were produced to assess more widely the relationship between the growth of storage moulds, mycotoxins (not exclusively restricted to ochratoxin A) and storage conditions. The Report discusses any changes necessary to current guidelines and means of effectively disseminating the information relevant to this problem.

Materials & Methods

On farm studies

Field trials were carried out on Velcourt managed farms in Kent (Dover), Hampshire (Braishfield), the Cotswolds (Stowell Park) and Yorkshire (Settrington) to ensure the study covered a range of soil types, geographical and climatic locations. At each site 2 malting barley varieties (Pearl and Halcyon), 2 bread-making wheat (Hereward and Malacca) and 2 feed wheat (Consort and Claire) were grown for 2 successive years (2000 and 2001). At each site and for each variety, plots 24 m x 120 m were established and all received 'standard' commercial applications of fertilisers, insecticides, growth regulators and herbicides. The main plots were divided into four sub-plots 24 m x 30 m (labelled A-D) and different fungicides regimes applied to each sub plot. For wheat these were T1 and T2 applications of Opus (a.i. epoxiconazole 125g/l) + Amistar(a.i. azoxystrobin 250g/l) at ¼ and ¾ rate with and without an ear wash (T3) of Folicur (a.i. tebuconazole 250 g/l). For barley T1 and T2 applications were Landmark (a.i. epoxiconazole +kresoxim-methyl both 125g/l) at ¼ and ¾ rate with and without an ear wash of Folicur.

<u>Sampling</u>

Just prior to harvest a random sample of 200 ears (20 ears from 10 points within a plot) was taken from each wheat and barley sub-plot. The grain from each sub-plot was harvested at moisture contents in the range 18-25 % and 500 kg placed in a plastic 'hessian-like' bag. All bags were stored under cover without any drying.

Grain samples (approximately 2 kg) were taken from each bulk bag at harvest and then after 4, 7, 14 and 28 days storage in the first year of the trial. In the second year selected bags were also sampled after 42 days

storage. The sample was obtained by combining ten 200 g sub-samples taken at random from through out the bulk to ensure that the 2 kg sample was representative of the bulk. In the second year of the trial the sampling of the bulk was carried out using a multi-level grain-sampling spear (comprising 3 compartments, one at the top, one in the centre and one near the bottom of the spear). Three sub-samples were taken using the spear, one from the centre of the bag and the other two from positions diagonally opposed to each other in the outer part of the bag. These were combined to give the 2 kg bulk sample required. At each sample date care was taken to avoid sampling from the outside edge of the bag or from the same position within the bag. Samples were returned to the laboratory without delay for mould identification and counting and for ochratoxin A analysis.

Grain temperature in each 500 kg sample bag was recorded at sampling using an industry standard temperature probe and the moisture content of the composite 2 kg samples checked using calibrated moisture meters.

Mould enumeration and ochratoxin A analysis

Mould counts and species identification were carried out by well-established standard methods, including use of a medium optimised for *P .verrucosum*Analysis for ochratoxin A was carried out using a fully validated HPLC method based that provides reliable quantitative results. Estimation of errors in acquiring the samples from the bulk grain and in mould counting was carried out by taking 10 replicate 2 kg samples drawn from selected samples. Pre-harvest grain was not analysed for ochratoxin A as no potential ochratoxin A producing fungi were isolated from these samples.

Studies on experimental laboratory plots

At CSL 2 plots (12 m x 9 m) of winter wheat cv. Charger were drilled at a seed rate of 320 kg/ha in October 1999. Each main plot was subsequently divided into four sub-plots (3 m x 9 m). At the third node detectable growth stage (GS 33) the two main plots were treated with either a ¼ rate or a ¾ rate fungicide application of Landmark (active ingredients epoxiconazole 125 g/l and kresoxim methyl 125 g/l). Plants were inoculated in June 2000 at mid-anthesis (GS 65). Three of the four sub-plots in each main plot were spray inoculated with 1.5 l of a suspension containing 10^4 conidia per ml of either *Alternaria alternata* (isolate 764), *Cladosporium hebarum* (isolate 183) or an equal mixture of the two isolates. The fourth plot was sprayed with an equal volume of water. Through the use of mist irrigation the humidity around the ear was maintained at 70 % for five days post inoculation in order to aid ear infection.

Plots were harvested at grain moisture content of about 22 % at the end of August. A sample of 200 ears per sub-plot was taken just prior to harvest and the grain removed using a Hege 16 laboratory thresher (Hans-Ulrich Hege GmbH & Co., Germany). The remainder of the grain harvested from each plot (approximately 25 kg) was stored in sealed potato sacks at 20˚C. A representative 200 g sample was taken from each sack at 4, 7 and 14 days. All grain samples were analysed using methods used in the main storage experiment.

Results and discussion

Effect of fungicide treatment on mould counts prior to and immediately after harvest

Moulds isolated from grain and chaff samples taken immediately prior to harvest comprised mainly of *Cladosporium* and *Alternaria* species (sooty moulds). Some *Penicillium* species were present but no *P. verrucosum* was detected in these pre-harvest samples. Fungi occurred in approximately the same proportions on both the chaff and grain with, in each case, *Cladosporium* species being the predominant moulds isolated. However, mould levels on the chaff were generally a factor of about 10 higher than those found on grain.

Table 1 summarises the total moulds infecting wheat at harvest in 2001. Results in 2000 and for barley were, in general, similar. Log_{10} values for mould counts are shown for each experimental treatment. In total 128 samples of wheat and 64 of barley were examined over the 2 years. A mean value is calculated for each site to indicate the total level of fungal infection. Results were similar to the pre-harvest samples, the fungal species isolated at harvest being almost exclusively the sooty moulds with some *Fusarium* species present. Again *Cladosporium* species were present at higher levels than *Alternaria*.

The study was set up to examine the extent to which levels of field fungi may affect the production of toxins, particularly ochratoxin A, in store. Each situation resulted in a set of 4 adjacent plots treated identically except for the application of fungicide. Errors in sampling and mould counting were estimated so that a difference in value of greater than $\log_{10}$ 0.7 between any 2 matched samples is considered to indicate a significant effect.

In both years, no clear effect could be related to the original fungicide treatments, neither did different cultivars have significantly different flora. Some differences related to site so that barley at Dover suffered a much higher level of infection in 2000 than in 2001 although different fungicide treatments produced the same result in each sample in the set.

Table 1: Total mould counts for species of *Alternaria, Cladosporium* and *Fusarium* determined in wheat at harvest, 2001.

Site	Variety	Mould counts log_{10} CFU/gram, for each fungicide treatment				
		A	B	C	D	Mean value*
Stowell Park	Hereward	4.93	4.71	4.78	4.82	
	Malacca	5.09	5.17	5.12	5.16	4.92
	Consort	4.51	5.01	5.02	4.82	
	Claire	4.69	4.90	4.96	4.08	
Braishfield	Hereward	5.26	5.26	5.38	5.39	
	Malacca	5.51	5.24	5.38	5.49	5.36
	Consort	5.29	5.51	5.08	5.19	
	Claire	5.51	5.32	5.27	5.40	
Settrington	Hereward	5.12	5.19	5.09	4.65	
	Malacca	5.27	5.14	5.13	5.19	5.16
	Consort	5.22	5.15	5.35	5.16	
	Claire	5.16	4.98	5.24	5.14	
Dover	Hereward	4.97	4.99	5.08	5.00	
	Malacca	4.83	5.08	4.86	4.96	5.00
	Consort	5.08	4.91	5.05	5.15	
	Claire	5.07	4.98	4.98	4.88	

*= log_{10} of average of each mould count for the site

The absence of variation in sooty mould levels resulting from the differential use of fungicides may have several explanations. Sooty moulds are saprophytes, which colonise the senescing ear late in the growing season, at a time when fungicidal activity has ended. Surface sterilisation of some samples (data not presented) indicated that all sooty mould counts were a result of external rather than internal infection of the grain. Separation of grain from chaff during the harvesting process will disturb spores, which will circulate in the combine and adhere to the grain surface. This mixing of spores in the combine may have a levelling effect on spore numbers thus hiding any differences, which may have been achieved through the differential use of fungicide. Differences in other ear pathogens, such as powdery mildew, brown and yellow rust, may have been achieved through using the different fungicide regimes. Such pathogens would not have been accounted for in this study as they were not assessed for in the field, also as they are obligate pathogens would not have grown on agar plates and therefore counts could not have been carried out.

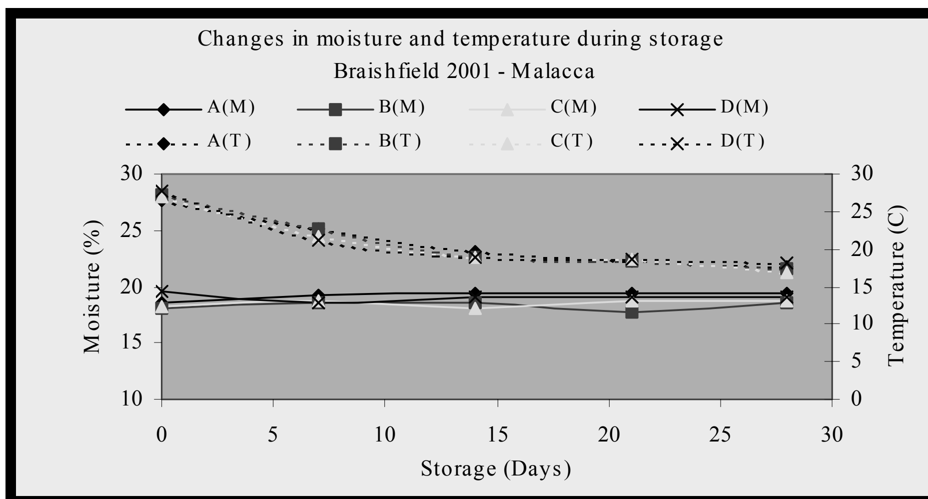
Levels of *Fusarium* found in both years were low. Recent research has shown that for optimum control of *Fusarium* species on the ear, fungicides need to be applied during flowering within two to three days of the *Fusarium* inoculum arriving on the ear. This study followed current commercial practice by applying an ear wash at GS 59/60.

Change in mould flora during storage

It is generally accepted that ochratoxin A is nearly always a post-harvest problem. To establish whether or not there are on-going effects in store that relate back to the levels of field moulds entering the store it is vital that the 4 samples in each set are grown on the same soil, receive common agricultural input and are exposed to the same climatic conditions. Once grain is transferred to the 0.5 tonne-scale storage the most important

parameters that need to be monitored are temperature and the moisture content of the grain in the bags. In 2000 the moisture content of each grain sample and its temperature immediately after harvest was recorded but not then subsequently monitored during storage. In 2001, a comprehensive record of the moisture content of each stored batch of grain and its temperature was carried out on each farm. This showed how closely each set of samples were matched in terms of temperature and moisture and also served to indicate as early as possible those samples that heated. Having this information and the estimate of variability expected due to sampling, mould counts and chemical analysis, made it possible to determine whether any differences in subsequent mould or mycotoxin formation related to the nature of the original fungicide applications. Typical data for temperature and moisture content are shown in figure 1, in which temperature and grain moisture content is plotted against time for the 28 day storage period.

Figure 1: Record of temperature and moisture content of Malacca wheat stored at the Braishfield site in Year 2001



A, B, C, D= different fungicide treatments, M = moisture content, %, T= Temperature ºC

Dotted lines are temperature, continuous lines are moisture content

Results show that in 2001 all the sets of stored wheat were well matched so that any difference in the rate or amount of mould development or ochratoxin A formation could be related to the level of field mould entering the store. The sets of stored barley at Braishfield and Dover were also well matched. Differences were seen between samples at Stowell Park and Settrington because high initial moisture content of the barley at harvest caused grain heating at variable rates in each bag. The aim to produce different conditions was thus achieved as grain at moisture contents between 18 and 24% at different temperatures and levels and combination of moulds were produced.

Immediately after harvest the most commonly found 'storage' fungi were *Penicillium* species. However, these were always at very low levels in comparison to the 'field' fungi and *P. verrucosum* was not isolated from any sample at this time. The absence of *P. verrucosum* in grain from the field is consistent with the absence of ochratoxin A in freshly harvested grain. Storage of cereals with moisture contents above 15% is

likely to result in a rise in the levels of storage moulds present. The way this occurs will depend on the actual moisture content, temperature of storage and the fungal species present, with the fungal profile changing with time. Here, by 28 days storage *Penicillium* and other storage fungi had in general increased considerably. Grain in 2001 was harvested at higher moisture content than in 2000, thus the development of *Penicillium* species occurred earlier and built up to higher levels. All grain at Stowell Park and Dover stored above 20% moisture content developed *P. verrucosum* by 28 days (Table 2).

Table 2: *P. verrucosum* and ochratoxin A content (µg/kg) of wheat samples stored for 28 days - 2001

Moisture content, % range	Mean temp. 28days	Variety	Ochratoxin A (OA) µg/kg and *P. verrucosum* (PV) counts, log_{10} for fungicide regime used							
			A		B		C		D	
			OA	PV	OA	PV	OA	PV	OA	PV
			Stowell Park, 28 days storage							
24-24.5	19	Hereward	<0.2	3.2	**0.2**	4.0	<0.2	3.7	<0.2	4.0
21.5-23.5	16	Malacca	**5.2**	3.8	<0.2	3.3	**0.7**	4.5	**0.6**	3.4
22.5-24.5	21	Consort	<0.2	3.6	<0.2	4.7	**37**	4.8	<0.2	4.0
22.5-24.5	18	Claire	**0.8**	4.8	<0.2	4.4	**0.4**	3.7	<0.2	2.7
			Braishfield, 28 days storage							
18-20	16.5	Hereward	<0.2	-	<0.2	-	<0.2	-	<0.2	-
18-19.5	17.5	Malacca	<0.2	-	<0.2	-	<0.2	-	<0.2	-
17.5-19	16.5	Consort	<0.2	-	<0.2	-	<0.2	-	<0.2	-
18-19.5	16.5	Claire	<0.2	-	<0.2	3.7	<0.2	-	<0.2	-
			Setterington, 28 days storage							
19-20	15	Hereward	<0.2	2.0	<0.2	-	<0.2	-	<0.2	2.2
18.5-19.5	15	Malacca	<0.2	-	<0.2	-	<0.2	-	<0.2	-
19.5-20	15	Consort	<0.2	-	<0.2	-	<0.2	1.7	<0.2	-
19-19.5	15	Claire	<0.2	-	<0.2	-	<0.2	-	<0.2	-
			Dover, 28 days storage							
19-20.5	16	Hereward	**0.6**	4.4	**0.4**	5.5	**3.2**	3.0	<0.2	4.5
18-19.5	14.5	Malacca	<0.2	-	**2.5**	-	<0.2	2.9	<0.2	-
19-20.5	16	Consort	**1.4**	4.6	<0.2	3.8	<0.2	4.3	**0.3**	4.6
18.5-20.5	15	Claire	0.2	3.2	<0.2	2.7	**0.4**	3.0	<0.2	3.7
			Dover, 42 days storage							
	14.5	Hereward	**4.0**	5.7	**4.9**	5.7	**0.5**	4.4	**2.7**	4.7
	13.5	Malacca	**0.6**	3.5	**0.4**	3.7	<0.2	3.0	<0.2	4.5
	15	Consort	**8.1**	4.9	**0.5**	4.3	**21.9**	4.7	**22.8**	4.6
	13.5	Claire	**0.3**	4.6	**0.3**	3.4	**1.8**	5.0	<0.2	3.9

* A = ¼ rate T1 + T2; B = ¼ rate T1, T2 + T3; C = ¾ rate T1 + T2; D = ¾ rate T1, T2 + T3

Levels of storage fungi after 28 days storage showed no correlation with the different fungicide regimes used but were more influenced by the harvest temperature and moisture content of the grain. The largest increases in *Penicillium* species were seen where grain was stored with an initial moisture content between 18 and 24 %, and where the initial storage temperature was between 15 and 22°C. *Penicillium* moulds, including *P.*

verrucosum, favour lower temperatures so that grain up to 25% moisture at a temperature between 10 and 25°C is at most risk from the development of ochratoxin A.

In general, *P. verrucosum* was at levels much lower than the other *Penicillium* species and there was generally a lag phase before it appeared. This suggests that the initial inoculum of *P. verrucosum* was very low and as no *P. verrucosum* was isolated from the field, that the grain became contaminated either during the harvest or on entering the store. Possible sources of inoculum suggested are the combine, spores in grain dust, ventilation systems or residues remaining in stores and handling equipment. Thus good hygiene between harvests may also help reduce ochratoxin A.

Where the harvest moisture content of barley was greater than 24 % and the initial storage temperature of grain was between 18-24°C, rapid heating of the grain occurred and under such circumstances it was the *Aspergillus* species which dominated. At Settrington in 2001, barley was harvested at a moisture content of about 27 % and grain temperature of 18°C. By 21 days temperature had risen in one storage bag to over 60°C and only *Aspergillus* species were isolated. Species found included several important mycotoxin-producing moulds such as *A. flavus* (aflatoxins), *A. nidulans* (sterigmatocystin) and *A. fumigatus*. When harvest moisture contents were between 15 and 18 % no one type of storage mould predominated, however *Eurotium* and *Wallemia* species were, often visible by 28 days. *Wallemia sebi* has been reported as a possible source of mycotoxins.

Formation of ochratoxin A during storage

Little ochratoxin A was detected in 2000 because wheat and barley were too dry at harvest, this was consistent with the absence or low amounts of *P.verrucosum* detected. In 2001, wheat ranged from 18 to 24.5% moisture content at harvest and at these levels significant problems with ochratoxin A would be expected during storage. No ochratoxin A was detected in barley because samples were either too wet or too dry at harvest. Samples that were too wet resulted in heating and the pre-dominance of thermophilic mould species. Conditions of storage at Braishfield, Settrington and Dover would not be expected to lead to rapid development of ochratoxin A although the grain would be at risk in the long term if not dried. Grain from Stowell Park should however present a high risk. In practice, ochratoxin A only developed in wheat at Stowell Park and Dover. *P.verrucosum* was always found in samples containing ochratoxin A but some samples contained the mould without ochratoxin A. At Dover in 2001 both *P. verrucosum* and ochratoxin A increased between 28 and 42 days. Storage conditions at Stowell Park and Dover were very different and yet the frequency of ochratoxin A and amount of infection with *P. verrucosum* was similar. In contrast, the moisture content and temperature conditions at Stowell Park, Braishfield and Settrington were quite similar although ochratoxin was formed in some samples at Stowell Park but not at Braishfield. This suggests that development of ochratoxin A is also site related or dependent on a factor that has not been identified in this study. The difficulty in predicting whether or not ochratoxin will develop, even when storage conditions favour its production, has been a common theme running through research studies over recent years. One

factor often discussed in this context is the origin and point of infection with *P. verrucosum* and that could at least in part be crucial and could help to explain this unpredictability.

General discussion

In the years studied, the application of fungicide during cereal growth in the field had little effect on the amount or composition of field moulds present on grain at harvest, which in turn had no demonstrable effect on the development of storage moulds or the formation of ochratoxin A. Most of the field moulds present on the samples studied were *Alternaria* and *Cladosporium. Fusarium* infection was low both in 2000 and 2001. Finding no-effect means that post-harvest factors need not be considered when developing new fungicides or application regimes to control pre-harvest disease and mycotoxin formation.

This study identifies 3 situations that can occur during storage of cereals. Firstly when grain is below 16-17% moisture content it presents a very small risk of mycotoxins developing in store at any temperature as long as it is then effectively managed. Because within a large bulk the moisture content recorded is the average value, grain for storage should be at no higher than 14-15% to provide a sufficient safety margin. Secondly, grain of 18-25% moisture is at high risk from storage mould growth and mycotoxin development. The risk will also depend partly on temperature and, in general, development of mould will become slower once temperatures fall below 15˚C although *Penicillium* moulds, including *P. verrucosum,* favour lower temperatures. Thus grain between 18 and 25% moisture at any temperature between 10˚ and 25˚C is at risk from the development of ochratoxin A. Thirdly, if temperatures rise above 25˚C, *Aspergillus* species tend to dominate so that important mycotoxin species such as *A. flavus* (aflatoxins), *A. nidulans* (sterigmatocystin) or *A. fumigatus* (range of less well studied mycotoxins) may develop.

The development of *P. verrucosum* and ochratoxin A is clearly difficult to predict and to eliminate or minimise ochratoxin A it is essential to dry grain to 15% or below as soon as possible after harvest and ensure that the whole bulk of grain remains at or below this moisture throughout storage. Whether or not grain clearly at potential risk will develop ochratoxin A, or how quickly, is difficult to predict. This work and other studies suggest that there are other factors involved such that a confident forecast cannot be made on the basis of grain moisture and temperature alone. One question that remains unanswered is at what point the grain becomes infected with spores of *P. verrucosum*. This mould appears to be closely associated with the storage situation including grain handling machinery and storage structures. Dust and old grain residues are thus likely reservoirs of mould spores and it is suggested that scrupulous hygiene in handling equipment, ventilation systems and stores is vital to reduce the infection of fresh grain. As *P. verrucosum* is closely associated with dust it is possible that air movement, including ventilation systems used for on floor aeration of hot air drying of grain might assist in this inoculation process.

Conclusions and recommendations

This project, in part, aimed to bridge a gap between field and store in respect of establishing if differing levels of field fungi resulting from different fungicide regimes would subsequently affect the development of storage fungi and hence the development of toxins such as ochratoxin A. No effects on the levels of field moulds or subsequent mycotoxin formation in store could be attributed to different fungicide treatments although the 2 seasons were not ideal as 2000 was very dry and in 2001 there was very little disease pressure.

Even under precisely controlled conditions it is difficult to predict whether grain potentially at risk from the formation of ochratoxin A will actually become contaminated or indeed how quickly. A confident forecast of ochratoxin A contamination of grain cannot be made on the basis of grain moisture and temperature alone, other factors must be involved. Evidence is accumulating that the source and time at which the grain becomes 'inoculated' with *P. verrucosum* must be important. It is clear that there was little or no ochratoxin A producing fungi in the field as no *P. verrucosum* was isolated at harvest. A rapid, simple to use diagnostic test specific for *P. verrucosum* could provide a better indication of whether cereals were at risk.

Sources of inoculum may be contaminated combines, trailers, augers, grain stores etc. that have not been cleaned and contain left over contaminated grain from the previous harvest. A more rigorous hygiene programme at the beginning and during harvest may help to reduce sources of *P. verrucosum* inoculum and may also give more time in bottle neck situations at drying before the development of ochratoxin A. However more research is needed in the areas of sources of infection, the control of these points of infection and the production of ochratoxin A in bottle neck situations.

In summary, this study does not suggest any relationship between fungicide regime, effect of mould levels at harvest and subsequent development of ochratoxin A in store. Cereals are put at risk from ochratoxin A by fairly well defined combinations of temperature and grain moisture content but its actual development is determined by other factors that are strongly indicated but not proven. Recommendations for grain storage should include a section specifically devoted to ochratoxin A.

TECHNICAL REPORT

Introduction

The presence of mycotoxins in cereals reduces the quality of grain, is likely to be a risk to human and animal health, and causes economic loss through their effect on livestock production. The moulds associated with grain are generally divided into 'field' and 'storage fungi'. Field fungi such as *Fusarium*, *Cladosporium* and *Alternaria* species infect the ear during growth of the crop. These organisms appear to 'die out' after harvest as they are overgrown by 'storage' fungi such as *Aspergillus* and *Penicillium* species during storage (Christenson and Kaumann, 1969). In the UK the important mycotoxins formed in grain by field fungi include trichothecenes (e.g. deoxynivalenol, nivalenol, HT2 toxin and T2 toxin) and zearalenone (all produced by *Fusarium* species).

Aflatoxins are the most important fungal toxins worldwide and together with ochratoxin A are the only storage mycotoxins for which legislation currently exists within the EC. Earlier legislation for aflatoxins has recently been widened to include cereals although these toxins are rarely produced under UK conditions. Recently, maximum permissible levels of 5 ppb in raw cereals and 3 ppb in processed products have been set for ochratoxin A. Surveys of stored UK cereals (Scudamore *et al.,* 1999; Prickett *et al*., 2000) show that a small but persistent percentage of samples examined exceed this level (2-3%). This represents a large tonnage of grain and a potential serious economic loss. The food and brewing industries are increasingly demanding high quality cereals for food and drink products and require grain conforming to the statutory limits set. There is a major incentive for the UK Cereal Industry to minimise ochratoxin A and other mycotoxins in grain to enable it to remain competitive with other member states within the EU. Clearly there is an urgent need to understand the factors that encourage mycotoxin formation both pre and post harvest as this may provide a means of minimising this problem.

It has been known for many years that grain must be stored dry under good hygienic conditions to prevent mould growth, insect invasion and to maintain quality. Compliance with Good Agricultural practice (GAP) will address most of these requirements. The continued occurrence of ochratoxin A in grain suggests that either GAP is not always rigidly followed or that all the factors involved in the formation of ochratoxin A are not fully understood.

Through a HACCP like approach the major Critical Control Point for the prevention of ochratoxin A formation has been identified as the rapid and effective drying of grain at harvest (FSA 1999, unpublished report). The time between harvest and the reduction of grain moisture to a safe level is of vital importance. Grain can be dried through the use of ambient air ventilation or hot air dryers. In general the use of ambient air ventilation has been shown to work well (Scudamore and Wilkin, 1999) provided the store and drying equipment are well maintained, the grain is not excessively wet or the air temperature not too low. As this is

a relatively slow method of drying grain, the upper layers within a stack can remain wet for a considerable length of time and may be at the risk from ochratoxin A formation for the longest. Hot air drying is a more rapid and efficient method of drying grain to a safe storage moisture content. As grain can be dried within a few hours a significant risk of ochratoxin A formation only arises on those occasions when damp grain has to be stored before drying e.g. because the capacity of the dryers is exceeded. Under such circumstances it is vital that the wettest grain, at greatest risk from toxin production, is given priority.

The continued occurrence of ochratoxin A in UK grain must imply that either grain is not/cannot always be dried quickly enough or that stored grain is not managed effectively. If these situations are unavoidable it is important that all factors that affect the potential formation of ochratoxin A are fully understood and sound advice is available on how to minimise this risk. There are sufficient citings in the scientific literature (e.g. Mislivec *et al*. 1988, Ramakrishna *et al*. 1993) to suggest that some factors in the field prior to harvest may affect the extent of the formation of storage mycotoxins although this possibility has not been fully investigated and may be one reason why it is very difficult to predict the likely extent of the mycotoxin problem during storage. A current HGCA study is investigating the effects of fungicides on *Fusarium* Ear Blight, mycotoxin accumulation and ways to optimise its control. This concentrates on *Fusarium* infection and the field interactions but the effect of fungicide treatment on field fungi might subsequently influence how storage fungi and associated mycotoxins develop. If changes in fungicide application regimes subsequently affect the formation of field or storage mycotoxins it is important to be fully aware of the potential effects of such changes.

Current guidelines for safe storage of cereals may not be specific enough to avoid or minimise mycotoxin occurrence. The aim of this study was to examine the effects of different fungal field infections resulting from common fungicide regimes, on the subsequent development of storage organisms and ochratoxin A production. In addition, whether or not fungicides have any indirect post harvest effects, the different storage situations generated in the course of this study presents the opportunity to assess more widely the relationship between the growth of storage moulds, mycotoxins (not exclusively restricted to ochratoxin A) and storage conditions. The Report discusses any changes necessary to current guidelines and means of effectively disseminating the information relevant to this problem.

Materials & Methods

On farm studies

Field trials were carried out on Velcourt managed farms in Kent (Dover), Hampshire (Braishfield), the Cotswolds (Stowell Park) and Yorkshire (Settrington) to ensure the study covered a range of soil types, geographical and climatic locations. At each site 2 malting barley varieties (Pearl and Halcyon), 2 bread-making wheat varieties (Hereward and Malacca) and 2 feed wheat varieties (Consort and Claire) were grown for 2 successive years (2000 and 2001). At each site and for each variety, plots were established 24 m x 120 m, these plots all received 'standard' commercial applications of fertilisers, insecticides, growth regulators and herbicides. The main plots were divided into four sub-plots 24 m x 30 m (labelled A-D) and different fungicides regimes applied to each sub plot as detailed in Table 1a and 1 b. Details of the trial plan are shown in Appendix A.

<u>Harvest</u>

Just prior to harvest a random sample of 200 ears (20 ears from 10 points within a plot) was taken from each wheat and barley sub-plot. The grain from each sub-plot was harvested at moisture content in the range 18-25 % and 500 kg placed in a plastic 'hessian-like' bag. All bags were stored under cover but without any drying.

<u>Sampling</u>

Grain samples (2 kg) were taken from each bulk bag at harvest and then after 4, 7, 14 and 28 days storage in the first year of the trial. In the second year selected bags were also sampled after 42 days storage. In order to ensure that the 2 kg sample was representative of the bulk this was obtained by combining ten 200 g sub-samples taken at random from through out the bulk.

In the second year of the trial sampling of the bulk was carried out using a multi-level grain-sampling spear (comprising 3 compartments, one at the top, one in the centre and one near the bottom of the spear). Three sub-samples were taken using the spear, one from the centre of the bag and the other two from positions diagonally opposed to each other in the outer part of the bag. These were combined to give the approximately 2 kg bulk samples required. At each sample date care was taken to avoid sampling from the outside edge of the bag or from the same position within the bag.

Grain temperature in each 500 kg sample bag was recorded at the time of sampling using an industry standard temperature probe and the moisture content of the individual composite 2 kg samples checked using calibrated moisture meters.

To avoid changes in mould and toxin levels during the transportation of grain samples they were sent for analysis as soon as possible after each sampling time. On arrival at the analytical laboratory (CSL) the

samples were split; half for mould analysis (stored at 4°C before processing) and the other portion for toxin determination (stored at -30°C).

Table 1a: Details of fungicide applications to wheat plots

Treatment	Fungicide rate	Fungicide and timing	
		T1 and T2	T3
A	1/4	Opus (a.i. epoxiconazole 125g/l) + Amistar (a.i. azoxystrobin 250g/l)	No application
B	1/4	Opus (a.i. epoxiconazole 125g/l) + Amistar (a.i. azoxystrobin 250g/l)	Folicur (a.i. tebuconazole 250 g/l)
C	3/4	Opus (a.i. epoxiconazole 125g/l) + Amistar (a.i. azoxystrobin 250g/l)	No application
D	3/4	Opus (a.i. epoxiconazole 125g/l) + Amistar (a.i. azoxystrobin 250g/l)	Folicur (a.i. tebuconazole 250 g/l)

T1 = 2nd node detectable; T2 = flag leaf ligule just visible; T3 = end of heading

Table 1b: Details of fungicide applications to barley plots

Treatment	Fungicide rate	Fungicide and timing	
		T1 and T2	T3
A	1/4	Landmark (a.i. epoxiconazole +kresoxim-methyl both 125g/l)	No application
B	1/4	Landmark (a.i. epoxiconazole + kresoxim-methyl both 125g/l)	Folicur (a.i. tebuconazole 250 g/l)
C	3/4	Landmark (a.i. epoxiconazole + kresoxim-methyl both 125g/l)	No application
D	3/4	Landmark (a.i. epoxiconazole + kresoxim-methyl both 125g/l)	Folicur (a.i. tebuconazole 250 g/l)

T1 = 2nd node detectable; T2 = flag leaf ligule just visible; T3 = end of heading

Estimation of sampling error

Estimation of the sampling errors to be expected in acquiring the samples from the bulk grain was carried out in year two of the project. Ten replicate 2 kg samples were drawn from the bulk bag of barley cultivar Pearl stored for 4 days at Braishfield and from the wheat cultivar Consort stored for 28 days at Stowell Park.

Mould counts were carried out for each replicate sample using the standard counting method used throughout the study and the total error would comprise that due to sampling and that inherent in mould counting.

Mould enumeration

For mould analysis 40 g taken from each grain sample was weighed directly into a stomacher bag, 360 ml of 0.1 % bacteriological peptone (Unipath) water was added and the grain soaked for 30 minutes. After soaking, the grain was stomached for 1 minute and the resulting suspension labelled as the 10^{-1} dilution. This suspension was serially diluted down to 10^{-6}. For each dilution, two DG18 agar plates (31.5 g commercial dehydration formulation; 220 g glycerol; 50 mg chloramphenicol; 1000 ml distilled water) were labelled and 0.1 ml dilution suspension pipetted onto the agar surface. The inoculum was spread over the surface of the agar and incubated at 25°C for 10 days. Colonies on each plate were identified and counted.

Ochratoxin A analysis

The whole sample of each grain portion stored for ochratoxin A analysis was milled and mixed thoroughly. The remainder was then re-frozen and stored for future reference. Analysis for ochratoxin A was carried out using a fully validated HPLC method based on that tested by Scudamore and MacDonald 1998 that provides fully quantitative results. Pre-harvest grain was not analysed for ochratoxin A as no potential ochratoxin A producing fungi were isolated from these samples. The total error would comprise that due to sampling and that inherent in ochratoxin A analysis.

25 g of ground sample was extracted in 100 ml of acetonitrile:water (60:40). The mixture was blended using a homogeniser for 3 minutes and filtered the through filter paper by gravity. 5 ml of the filtrate was added to 55 ml phosphor buffered saline solution (PBS). This was transferred ≥ 55 ml in to a pre-labelled plastic sample tube, and place on an ASPEC (automated sample preparation equipment) for automated immunoaffinity column clean-up and analysis.

The immunoaffinity clean-up column was conditioned with PBS, the sample added (50 ml), the column washed with PBS and then air-dried. Ochratoxin A was eluted with 2% acetic acid in methanol. The eluate was diluted with water before HPLC analysis.

A Spherisorb ODS2 HPLC column (25cm x 4.6mm) was used, with an inline filter, C18 guard column or equivalent and a mobile phase consisting of acetonitrile:water:acetic acid (99:99:2), flowing at 1 ml/minute. The product was detected using fluorescence with excitation set at 333nm and emission at 477nm.

Studies on experimental laboratory plots

At CSL 2 plots (12 m x 9 m) of winter wheat c.v. Charger were drilled at a seed rate of 320 kg/ha in October 1999. Each main plot was subsequently divided into four sub-plots (3 m x 9 m).

At the third node detectable growth stage (GS 33) the two main plots were treated with either a ¼ rate or a ¾ rate fungicide application of Landmark (active ingredients epoxiconazole 125 g/l and kresoxim methyl 125 g/l).

Plants were inoculated in June 2000 at mid-anthesis (GS 65). Three of the four sub-plots in each main plot were spray inoculated with 1.5 l of a conidial suspension containing 10^4 conidia per ml of either *Alternaria alternata* (isolate 764), *Cladosporium hebarum* (isolate 183) or an equal mixture of the two isolates. The fourth plot was sprayed with an equal volume of water. Through the use of mist irrigation the humidity around the ear was maintained at 70 % for five days post inoculation in order to aid ear infection.

Plots were harvested at grain moisture content of about 22 % at the end of August. A sample of 200 ears per sub-plot was taken just prior to harvest and the grain removed using a Hege 16 laboratory thresher (Hans-Ulrich Hege GmbH & Co., Germany). The remainder of the grain harvested from each plot (approximately 25 kg) was stored in sealed potato sacks at 20˚C. A representative 200 g sample was taken from each sack at 4, 7 and 14 days. All grain samples were analysed using methods used in the main storage experiment.

Results and discussion

Effect of fungicide treatment on mould counts prior to and immediately after harvest

Moulds isolated from grain and chaff samples taken immediately prior to harvest comprised mainly of *Cladosporium* and *Alternaria* species (the sooty moulds). Some *Penicillium* species were present but no *P. verrucosum* was detected in these pre-harvest samples. The fungi occurred in approximately the same proportions on both the chaff and grain with, in each case, *Cladosporium* species being the predominant moulds isolated. However, mould levels on the chaff were generally higher than those found on grain by a factor of about 10. Because a large amount of data was collected a specimen set considered to be representative is provided in Appendix B

A summary of the total moulds infecting wheat and barley grains at harvest in 2000 and 2001 are presented in tables 2-5 and should be considered together with data in Appendix B. Log_{10} values for mould counts are shown for each experimental treatment, in total 128 for wheat and 64 for barley. In addition, a mean value is calculated for each site to indicate the total level of fungal infection. Values for yeasts are not included. At harvest results were similar to the pre-harvest samples, the fungal species isolated being almost exclusively the sooty moulds with some *Fusarium* species present. Again *Cladosporium* species were present at higher levels than *Alternaria*.

The study was set up to provide a snap shot of the extent to which levels of field fungi may affect the production of toxins, particularly ochratoxin A, in store. Each situation resulted in a set of 4 adjacent plots treated identically except for the application of fungicide. Past experience of mould counting techniques led to the conclusion that a difference of 2-5 times in mould count would be needed to indicate a significant effect. This would equate to a $\log_{10}$ value of 0.3-0.7. In addition there would be a second source of error associated with sampling. Attempts at testing that this value was appropriate were made and this is described in detail in Appendix C. The calculations and logic support that a difference in value greater than $\log_{10}$ 0.7 between any 2 matched samples is a realistic figure to indicate a significant effect.

Table 2: Effect of fungicide on total mould counts for *Alternaria, Cladosporium* and *Fusarium* species determined from wheat grain at harvest (2000).

		Mould counts log_{10} CFU/gram, for each fungicide treatment				
Site	Variety	A	B	C	D	Mean value*
Stowell Park	Hereward	5.18	4.96	4.74	5.02	
	Malacca	5.02	4.99	5.30	4.95	4.95
	Consort	4.81	4.86	4.56	4.62	
	Claire	4.79	5.08	4.77	4.93	
Braishfield	Hereward	5.08	5.08	5.30	5.08	
	Malacca	5.40	5.39	5.30	5.35	5.30
	Consort	5.36	5.27	4.96	5.33	
	Claire	5.30	5.45	5.31	5.48	
Settrington	Hereward	4.98	5.03	5.16	5.03	
	Malacca	5.10	5.26	5.13	5.09	5.23
	Consort	5.05	5.08	5.15	4.86	
	Claire	5.23	5.30	5.31	5.82	
Dover	Hereward	4.83	4.99	5.04	4.82	
	Malacca	5.23	4.81	5.04	4.97	4.98
	Consort	4.91	4.98	5.10	5.13	
	Claire	5.04	4.81	4.95	4.63	

*= log_{10} of average of each mould count for the site

Table 3: Effect of fungicide on total mould counts for *Alternaria, Cladosporium* and *Fusarium* species determined from barley grain at harvest (2000).

		Mould counts log_{10} CFU/gram, for each fungicide treatment				
Site	Variety	A	B	C	D	Mean value
Stowell Park	Pearl	4.60	4.63	5.02	4.55	4.66
	Halcyon	4.54	4.43	4.70	4.48	
Braishfield	Pearl	5.18	5.02	5.18	5.11	5.03
	Halcyon	5.15	4.90	4.84	4.96	
Settrington	Pearl	5.02	5.04	4.96	4.93	5.05
	Halcyon	5.16	5.23	5.06	4.88	
Dover	Pearl	6.04	5.98	6.10	6.22	6.0
	Halcyon	6.11	6.16	5.38	5.04	

Table 4: Effect of fungicide on total mould counts for *Alternaria, Cladosporium* and *Fusarium* species determined from wheat grain at harvest (2001).

Site	Variety	Mould counts $\log_{10}$ CFU/gram, for each fungicide treatment				Mean value*
		A	B	C	D	
Stowell Park	Hereward	4.93	4.71	4.78	4.82	
	Malacca	5.09	5.17	5.12	5.16	4.92
	Consort	4.51	5.01	5.02	4.82	
	Claire	4.69	4.90	4.96	4.08	
Braishfield	Hereward	5.26	5.26	5.38	5.39	
	Malacca	5.51	5.24	5.38	5.49	5.36
	Consort	5.29	5.51	5.08	5.19	
	Claire	5.51	5.32	5.27	5.40	
Settrington	Hereward	5.12	5.19	5.09	4.65	
	Malacca	5.27	5.14	5.13	5.19	5.16
	Consort	5.22	5.15	5.35	5.16	
	Claire	5.16	4.98	5.24	5.14	
Dover	Hereward	4.97	4.99	5.08	5.00	
	Malacca	4.83	5.08	4.86	4.96	5.00
	Consort	5.08	4.91	5.05	5.15	
	Claire	5.07	4.98	4.98	4.88	

*= $\log_{10}$ of average of each mould count for the site

Table 5: Effect of fungicide on total mould counts for *Alternaria, Cladosporium* and *Fusarium* species determined from barley grain at harvest (2001).

Site	Variety	Mould counts $\log_{10}$ CFU/gram, for each fungicide treatment				Mean value
		A	B	C	D	
Stowell Park	Pearl	4.94	4.89	5.06	4.92	4.95
	Halcyon	5.07	4.95	4.87	4.84	
Braishfield	Pearl	5.26	4.85	4.90	4.81	4.81
	Halcyon	4.58	4.69	5.07	4.62	
Settrington	Pearl	4.69	4.93	5.09	4.85	5.00
	Halcyon	4.99	5.00	5.18	5.06	
Dover	Pearl	4.95	4.98	4.97	4.74	4.95
	Halcyon	4.77	5.11	4.98	5.02	

In planning this study it had been anticipated that the use of 4 different fungicide regimes would have resulted in differences in fungal counts or different proportions of the species present in grain at harvest. However, in the 2 years of the experiment, 32 sets of data relating to different fungicide treatment were obtained for wheat, but the only difference in count exceeding a value of $\log_{10}$ 0.7 within any set of 4 fungicide treatments was for Claire wheat grown at Stowell Park in 2001 (Table 4). Even this difference can be discounted because an atypically low *Cladosporium* count was obtained for the sample taken at harvest. Subsequent counts in samples taken after 4 and 7 days storage were substantially higher although it is highly unlikely that amounts of *Cladosporium* would have increased during storage under the existing conditions. In the same way, 16 sets of data were obtained for barley with the same absence of any significant effect on mould composition or levels. The absence of any clear effects on moulds at harvest was unexpected.

The absence of any major variation in sooty mould levels may have several explanations. Sooty moulds are saprophytes, which colonise the senescing ear. As a result they tend to colonise the ear late in the growing season, at a time when fungicidal activity may have been lost. Surface sterilisation of some samples (data not presented) indicated that all sooty mould counts were a result of external rather than internal infection of the grain. Separation of grain from chaff will disturb spore on the chaff, these would then be circulated in the combine and settle on grain surface. This action could serve to negate any differences, which may have been achieved through the differential use of fungicide. Differences in other ear pathogens, such as powdery mildew, brown and yellow rust, may have been achieved through using the different fungicide regimes. However these would not have shown up as they were not assessed in the field and would not have shown up on agar plates as they are obligate pathogens.

Mean mould counts for wheat and barley showed overall infection at a site to be similar for both years (Tables 2-5). The only exception was for the barley at Dover where infection was much higher in 2000. There were slight differences between sites with sooty mould levels in wheat highest at Braishfield and Settrington and lowest at Stowell Park and Dover, however these were probably not statistically significant. Levels in barley at Dover in 2000 were about $\log_{10}$ 1.0 (10 times higher) than at any of the other sites.

Levels of *Fusarium* species isolated from grain were low in both years at all sites (Table 6). As with the sooty moulds fungicide appeared to have little or no effect on the levels of *Fusarium* species isolated. Unlike the sooty moulds the main reason for the lack of control of *Fusarium* species was the incorrect timing of fungicide application. Recent research has shown that for optimum control of *Fusarium* species on the ear, fungicides need to be applied during flowering within two to three days of the *Fusarium* inoculum arriving on the ear (GS 65 : HGCA project No 2067). This study followed current commercial practice by applying an ear wash at GS 59/60.

Table 6: Mould counts for *Fusarium* species determined from wheat and barley grain at harvest (2001).

Site	Variety	Mould counts $\log_{10}$ CFU/gram, for each fungicide treatment				Mean variety	Mean all values
		A	B	C	D		
Stowell Park	Pearl	3.47	3.69	4.47	4.30	4.15	3.97
	Halcyon	3.30	3.30	-	4.00	3.67	
	Hereward	2.54	2.74	-	3.39	2.92	
	Malacca	3.00	2.30	2.30	3.39	2.98	2.97
	Consort	3.30	3.47	-	3.00	3.17	
	Claire	2.30	2.69	3.17	2.47	2.79	
Braishfield	Pearl	-	-	-	-	-	-
	Halcyon	-	-	2.30	3.00	-	
	Hereward	3.00	3.39	3.17	2.39	3.11	
	Malacca	3.30	3.47	3.47	3.39	3.41	3.31
	Consort	2.69	2.69	3.30	-	3.18	
	Claire	3.69	2.39	2.69	2.69	3.19	
Settrington	Pearl	2.69	3.00	3.17	3.17	3.05	3.05
	Halcyon	-	3.00	3.39	3.00	3.05	
	Hereward	4.00	4.17	3.60	2.69	3.86	
	Malacca	3.39	2.17	3.47	4.30	3.80	3.95
	Consort	4.00	3.39	4.00	4.17	3.97	
	Claire	4.17	4.30	4.00	3.69	4.09	
Dover	Pearl	2.00	2.00	-	-	-	-
	Halcyon	3.00	3.17	3.00	2.00	3.06	
	Hereward	4.74	5.17	5.00	4.17	4.85	
	Malacca	4.69	4.17	4.77	4.60	4.61	4.74
	Consort	4.74	4.00	4.81	5.19	4.85	
	Claire	5.00	4.30	4.00	4.17	4.56	

Differences between varieties

The mean mould count from each site for each variety for each of the 4 treatments received is given in tables 7 and 8. Hereward had the lowest mould counts in both 2000 and 2001 although the difference was so small as to be statistically insignificant. Both barley varieties appeared to have similar levels of moulds but the levels were different for the 2 years.

Table 7: Effect of variety effects on total mould counts for *Alternaria, Cladosporium* and *Fusarium* species determined from wheat and barley grain at harvest (2000).

	Mould counts $\log_{10}$ CFU/gram, for each fungicide treatment, per variety				
Variety	A	B	C	D	Mean
			WHEAT		
Hereward	5.04	5.02	5.10	5.00	5.04
Malacca	5.21	5.17	5.21	5.12	5.16
Consort	5.08	5.07	4.99	5.06	5.06
Claire	5.13	5.21	5.14	5.44	5.25$
Mean	5.12	5.12	5.12	5.19(5.10)$	
			BARLEY		
Pearl	5.54	5.48	5.60	5.68	5.58
Halcyon	5.60	5.63	5.07	4.88	5.41
Mean	5.57	5.56	5.41	5.45	

$= discarding very high *Cladosporium* count at Settrington

Table 8: Effect of variety effects on total mould counts for *Alternaria, Cladosporium* and *Fusarium* species determined from wheat and barley grain at harvest (2001).

	Mould counts $\log_{10}$ CFU/gram, for each fungicide treatment, per variety				
Variety	A	B	C	D	Mean
			WHEAT		
Hereward	5.09	5.08	5.13	5.06	5.09
Malacca	5.24	5.16	5.16	5.24	5.21
Concert	5.11	5.21	5.15	5.10	5.14
Claire	5.20	5.08	5.14	5.08	5.13
Mean	5.17	5.14	5.15	5.13	
			BARLEY		
Pearl	4.99	4.91	5.01	4.84	4.95
Halcyon	4.89	4.96	5.04	4.92	4.96
Mean	4.94	4.94	5.03	4.88	

Comparison of mean mould counts at harvest, for both wheat and barley, indicated that the use of different fungicide regimes, i.e. 3/4 rate and 1/4 rate with and without an ear wash application, had not produced different levels of 'field' fungi entering store. However, the levels of moulds and their composition did vary from site to site.

Change in mould flora during storage

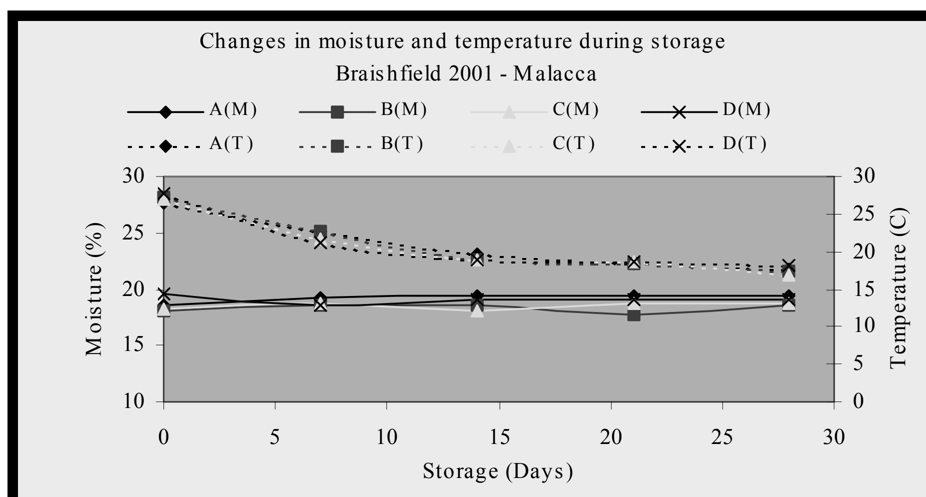
Results from this study and limited evidence from other reports confirm that ochratoxin A is nearly always a post-harvest problem. To establish whether or not there are on-going effects in store that relate back to the fungicide regime applied in the field it is vital that the 4 samples in each comparative set representing the different fungicide applications are treated in the identical manner, i.e. are grown on the same soil, receive common agricultural input and are exposed to the same climatic conditions. This was achieved for each set of samples prior to and at harvest. Once grain is transferred to the 0.5 tonne storage bags kept under cover the important parameters that need to be monitored are temperature and the moisture content of the grain in the bags. External factors such as ventilation and avoidance of direct sunlight must be kept similar for each bag.

Temperature and moisture content determination during storage and their relevance

In 2000 the moisture content of each grain sample and its temperature immediately after harvest was recorded but for most of the samples these factors were not then subsequently monitored during storage. This led to the possibility that effects identified could have been due to changes resulting from different temperatures or moisture contents in individual bags rather than from different levels of field moulds entering the store. To remove this uncertainty in 2001, a comprehensive record of the moisture content of each stored batch of grain and its temperature was carried out on each farm. This showed how closely each of the 4 differently treated batches of grain in each set were matched in terms of temperature and moisture and also served to indicate when heating of the grain developed. The availability of this information considered together with the estimate of variability expected from sampling, mould counting and chemical analysis, made it possible to determine any differences in subsequent mould or mycotoxin formation that were due to the original fungicide applications. An example of the data for temperature and moisture content is shown in graphical form in figure 1 while the complete records for the other 31 sets are provided in Appendix D. Temperature and grain moisture content is plotted against time for the 28 day storage period.

In 2001 sets of stored wheat within each site and between fungicide regimes were well matched so that difference in mould development or ochratoxin A formation could be related the level of field mould entering the store. The sets of stored barley at Braishfield and Dover were also well matched. Differences were seen between sites for example at Stowell Park and Settrington high initial moisture content of the barley at harvest caused grain heating. The aim to produce different conditions was thus achieved as grain at moisture contents between 18 and 24% at different temperatures and levels and combination of moulds were produced.

Figure 1: Record of storage temperature and moisture content in Malacca wheat stored at Braishfield in 2001.



A, B, C, D= different fungicide treatments, M = moisture content, %, T= Temperature ºC

Dotted lines are temperature, continuous lines are moisture content

Monitoring of temperature and grain moisture content thus showed that a number of different storage conditions were achieved, as was originally planned. Careful control and monitoring of conditions thus allowed the maximum chance of detecting any effects due to field application of fungicides and also provided the opportunity to study the formation of moulds and ochratoxin A over a range of conditions in a simulated storage situation.

Examination of grain immediately after harvest for the presence of 'storage' fungi indicated that *Penicillium* species were the most commonly found being present in most samples. However, these were always at very low levels in comparison to the 'field' fungi; *P. verrucosum* was not isolated from any sample. These Penicillia were too variable and present at too low levels to relate to fungicide application. The absence of *P. verrucosum* in grain sampled at harvest is consistent with findings elsewhere which has also shown that *P. verrucosum* is rarely, if ever, present in samples taken at harvest. A result of this is that ochratoxin A is extremely unlikely to occur in freshly-harvested grain.

Storage of cereals with moisture content above approximately 15% is likely to result in a rise in the levels of storage moulds present. The way this occurs will depend on the moisture content, storage temperature and the fungal species present. Table 9 shows the total for *Penicillium* species, excluding *P. verrucosum,* after 28 days storage during 2000 for wheat at the Dover site. Samples where *P. verrucosum* was present are shown in bold type. In most cases the *Penicillium* levels were considerably higher than those found at harvest. *P.*

verrucosum was only detected in Malacca wheat treated with fungicide regimes C and D. However, the findings were consistent with the moisture content and temperature that fell from 22˚C at harvest to about 18˚C by 28 days. There was no clear indication that any specific fungicide treatment had affected the development of Penicillia in store. Temperature and moisture content were similar for the 4 storage bags. Raw data for this and the other 3 sites in 2000 are given in Appendix D.

Table 9: *Penicillium* counts (excluding *P. verrucosum*) from wheat grain after 28 days storage for matched sets of 4 fungicide treatments (Dover, 2000).

	Total *Penicillium* mould counts, $\log_{10}$			
	Hereward (18.4)*	Malacca (18.0)*	Consort (18.4)*	Claire (18.5)*
		DOVER		
A	5.54	4.18	5.30	4.74
B	5.48	4.70	4.70	none
C	5.41	**4.60**	5.60	5.18
D	5.90	**4.30**	5.56	5.42

* = Mean moisture content for A-D, % at harvest. Samples in **bold** type also contained *P. verrucosu*m

Table 10 presents the equivalent data for wheat for 2001 obtained on all 4 sites. Except for some of the barley each set of stored samples was closely matched for temperature and moisture content throughout the 28 days of storage as discussed earlier. However, because grain was generally harvested at higher moisture than in 2000, the development of *Penicillium* species was earlier and built up to higher levels. In addition all the grain at Stowell Park and Dover stored above 20% moisture content also had developed *P. verrucosum* by 28 days. Similar to the field fungi, the use of different fungicide regimes did not produce consistent differences in levels of storage fungi entering store. Equally, differences seen in levels of storage fungi after 28 days storage were not related to the different fungicide regimes. These seemed to be more related to the harvest temperature and moisture content of the grain.

The largest increases in *Penicillium* species were seen where grain was stored with an initial moisture content between 18 and 24 %, and where the initial storage temperature was between 15 and 22°C. This was seen particularly in grain stored from winter wheat crops at Stowell Park and Dover in 2001 (Table 10). A similar set of storage condition may have occurred in wheat stored at Settrington in 2000 (Table 9) as high levels of *Penicillium* species were isolated, however it is difficult to verify this as grain storage temperatures were not recorded in 2000. *Penicillium* moulds, including *P. verrucosum*, favour lower temperatures so that grain up to 25% moisture at a temperature between 10 and 25C is at most risk from the development of ochratoxin A.

Table 10: *Penicillium* counts (excluding *P. verrucosum*) from wheat grain after 28 days storage for matched sets of 4 fungicide treatments on 4 sites (2001).

	Total *Penicillium* mould counts, log_{10}			
	Hereward	Malacca	Consort	Claire
		BRAISHFIELD		
A	4.39 (19.2)*	4.47 (18.5)*	5.00 (18.2)*	5.39 (19.4)*
B	3.30	4.77	5.00	5.10
C	3.69	3.00	2.69	**5.36**
D	3.69	4.30	3.39	4.93
		STOWELL PARK		
A	**5.65** (24.2)*	**5.06** (23.0)*	**5.69** (24.4)*	**5.50** (23.7)*
B	**5.59**	**4.97**	**5.63**	**5.69**
C	**5.47**	**5.38**	**5.73**	**5.71**
D	**5.36**	**5.47**	**5.64**	**5.81**
		SETTERINGTON		
A	**4.17** (19.3)*	4.17 (19.0)*	4.17 (19.7)*	3.14 (19.2)*
B	4.69	4.39	4.00	4.65
C	4.00	4.39	**4.30**	4.09
D	**4.74**	4.17	4.30	4.00
		DOVER		
A	**5.91** (20.5)*	5.74 (18.5)*	**6.00** (20.5)*	**5.82** (20.5)*
B	**5.74**	5.67	**6.04**	**5.67**
C	**5.90**	**5.49**	**5.83**	**5.65**
D	**5.91**	5.59	**5.90**	**5.72**

* = Mean moisture content for A-D, % at harvest. Samples in **bold** type also contained *P. verrucosu*m

In general, where *P. verrucosum* was isolated from grain, it was found at levels much lower than the other *Penicillium* species. This, in addition to the long lag phase generally associated with the appearance of *P. verrucosum,* suggests that the initial inoculum of *P. verrucosum* was very low. There are two possible points at which grain may become contaminated by *P.verrucosum*, one during harvesting and the other on entering the store. If the combine is over-wintered still containing residues of grain then these will absorb moisture and eventually become mouldy. If this is not removed before the start of harvest, it will provide a source of inoculum which could contaminate freshly harvested grain as it passes through the combine. A second possible source for contamination of grain by *P. verrucosum* spores may be the grain dust derived from residues of previously handled stored grain with spores being spread through the ventilation systems used for aeration and drying of grain or from residues remaining in stores and handling equipment. Thus ensuring good hygienic procedures between harvests in addition to drying may also help reduce contamination of grain by ochratoxin A.

Where the harvest moisture content of barley was greater than 24 % and the initial storage temperature of grain was between 18-24°C, rapid heating of the grain occurred and under such circumstances it was the *Aspergillus* species which dominated. At Settrington in 2001, barley was harvested at a moisture content of about 27 % (Table 10) with an initial grain temperature of 18°C. Grain temperature after 21 days storage had

risen in one storage bag to over 60°C and only *Aspergillus* species were isolated. Among the *Aspergillus* species found were several important mycotoxin producing moulds including *A. flavus* (aflatoxins), *A. nidulans* (sterigmatocystin) and *A. fumigatus* (this is a human pathogen and produces a range of less studied mycotoxins).

When harvest moisture contents were between 15 and 18 % no one type of storage mould predominated, however *Eurotium* and *Wallemia* species were, in general, isolated more frequently after 28 days in this moisture range. Work elsewhere has identified *Wallemia sebi* as a possible source of mycotoxins (Moss 1993). Grain at the lower end of this moisture range is at very small risk from mycotoxins developing in store at any temperature. However, this is an average moisture content and within a large bulk of grain there will be a much wider distribution of moisture. Storage at this moisture content leaves too small a margin should transfer of moisture occur over a long period so that storage moisture no higher than 14-15% is important to provide a sufficient safety margin. This is backed up by this study because grain harvested and stored at 15 % moisture content (Table 9) showed no increase in moulds even after 28 days storage.

Formation of ochratoxin A during storage

Chemical analysis was carried out using the HPLC method described earlier. This is a well-studied and validated method and is accepted for government-supported surveillance and considered suitable for statutory control purposes with typical operational parameters already established. The error due to analysis was considered small in relation to sampling and mould counting.

In 2000, very little ochratoxin A was detected mainly because grain, both wheat and barley, was too dry at harvest. This was consistent with the absence or low amounts of *P.verrucosum* detected. In 2001, wheat ranged from 18 to 24.5% moisture content at harvest and at these levels significant problems with ochratoxin A would be expected during storage. Table 11 shows the results obtained for ochratoxin A in wheat after 28 days storage for all samples and in addition after 42 days at Dover. Mould counts for *P. verrucosum* are attached alongside each analytical result. Earlier studies suggest that the conditions of storage at Braishfield, Settrington and Dover would not lead to rapid development of ochratoxin A although the grain would be at risk in the long term if not dried. Grain from Stowell Park would present a high risk. In the event, ochratoxin A only developed in wheat at Stowell Park and Dover. *P. verrucosum* was found in all samples containing ochratoxin A but some samples contained the mould without ochratoxin A. At Dover in 2001 both *P. verrucosum* and ochratoxin A increased between 28 and 42 days storage. However there was no significant different in *P. verrucosum* counts or in ochratoxin A occurrence or concentration that could be attributed to the original different fungicide regimes.

Storage conditions at Stowell Park and Dover were very different and yet the frequency of ochratoxin A and levels of *P. verrucosum* were similar. Conversely, even though the moisture content and temperature conditions at Stowell Park, Braishfield and Settrington were similar ochratoxin A was formed in some

samples at Stowell Park but not at Braishfield. This suggests that development of ochratoxin may be dependent on a factor that has not been specifically identified in this study. The difficulty in predicting whether or not ochratoxin A will develop, even when storage conditions favour its production, has been a common theme running through research studies over recent years. One factor often discussed as crucial in this context is the point at which *P. verrucosum* contaminates grain. Discovering this could help this the unpredictability.

Table 11: Ochratoxin A content (µg/kg) of wheat samples stored for 28 days - 2001

Moisture content, % range	Mean temp. 28days	Variety	Ochratoxin A (OA) µg/kg and *P. verrucosum* (PV) counts, $\log_{10}$ for fungicide regime used							
			A		B		C		D	
			OA	PV	OA	PV	OA	PV	OA	PV
			Stowell Park, 28 days storage							
24-24.5	19	Hereward	<0.2	3.2	**0.2**	4.0	<0.2	3.7	<0.2	4.0
21.5-23.5	16	Malacca	**5.2**	3.8	<0.2	3.3	**0.7**	4.5	**0.6**	3.4
22.5-24.5	21	Consort	<0.2	3.6	<0.2	4.7	**37**	4.8	<0.2	4.0
22.5-24.5	18	Claire	**0.8**	4.8	<0.2	4.4	**0.4**	3.7	<0.2	2.7
			Braishfield, 28 days storage							
18-20	16.5	Hereward	<0.2	-	<0.2	-	<0.2	-	<0.2	-
18-19.5	17.5	Malacca	<0.2	-	<0.2	-	<0.2	-	<0.2	-
17.5-19	16.5	Consort	<0.2	-	<0.2	-	<0.2	-	<0.2	-
18-19.5	16.5	Claire	<0.2	-	<0.2	3.7	<0.2	-	<0.2	-
			Setterington, 28 days storage							
19-20	15	Hereward	<0.2	2.0	<0.2	-	<0.2	-	<0.2	2.2
18.5-19.5	15	Malacca	<0.2	-	<0.2	-	<0.2	-	<0.2	-
19.5-20	15	Consort	<0.2	-	<0.2	-	<0.2	1.7	<0.2	-
19-19.5	15	Claire	<0.2	-	<0.2	-	<0.2	-	<0.2	-
			Dover, 28 days storage							
19-20.5	16	Hereward	**0.6**	4.4	**0.4**	5.5	**3.2**	3.0	<0.2	4.5
18-19.5	14.5	Malacca	<0.2	-	**2.5**	-	<0.2	2.9	<0.2	-
19-20.5	16	Consort	**1.4**	4.6	<0.2	3.8	<0.2	4.3	**0.3**	4.6
18.5-20.5	15	Claire	0.2	3.2	<0.2	2.7	**0.4**	3.0	<0.2	3.7
			Dover, 42 days storage							
	14.5	Hereward	**4.0**	5.7	**4.9**	5.7	**0.5**	4.4	**2.7**	4.7
	13.5	Malacca	**0.6**	3.5	**0.4**	3.7	<0.2	3.0	<0.2	4.5
	15	Consort	**8.1**	4.9	**0.5**	4.3	**21.9**	4.7	**22.8**	4.6
	13.5	Claire	**0.3**	4.6	**0.3**	3.4	**1.8**	5.0	<0.2	3.9

[*] A = ¼ rate T1 + T2; B = ¼ rate T1, T2 + T3; C = ¾ rate T1 + T2; D = ¾ rate T1, T2 + T3

Inoculated field plots

In small experimental plots at the CSL site, no differences in contamination of grain by either *Cladosporium* or *Alternaria* species were detected at harvest between inoculated and control plots. There are two possible explanations for this. Firstly mixing of spores during combining, as described earlier or through incorrect timing of the inoculum. Inoculation of plots occurred at mid flowering (GS65), however, sooty mould development takes place on the senescing ear. Thus inoculum may have arrived at the ear too early, with

spores not surviving the intervening period between inoculation and natural ear colonisation by the sooty mould fungi. With hindsight a more appropriate time for inoculation of sooty mould species might have been GS85.

There was no *P. verrucosum* isolated and hence no ochratoxin A was detected, in any of the grain samples from either sooty mould inoculated or control plots during storage. However, other *Penicillium* species increased significantly in the grain stored from plots both inoculated and control plots treated with ¾ rate Landmark at GS33 when compared to those from the plot treated with ¼ rate Landmark (see Appendix E). As there were no obvious differences in the level of field fungi or moisture content between the two fungicide treatments it is difficult to explain this difference.

General Discussion

In the years studied, the application of fungicide during cereal growth in the field had little effect on the amount or composition of field moulds present on grain at harvest, which in turn had no demonstrable effect on the development of storage moulds or the formation of ochratoxin A. Most of the field moulds present on the samples studied were *Alternaria* and *Cladosporium*. *Fusarium* infection was low both in 2000 and 2001. Finding no-effect means that post-harvest factors need not be considered when developing new fungicides or application regimes to control pre-harvest disease and mycotoxin formation.

This study identifies 3 situations that can occur during storage of cereals. Firstly when grain is below 16-17% moisture content it presents a very small risk of mycotoxins developing in store at any temperature as long as it is then effectively managed. Because within a large bulk the moisture content is the average value, grain for storage should be at no higher than 14-15% to provide a sufficient safety margin. Secondly, grain of 18-25% moisture is at high risk from storage mould growth, in particular *Penicillium* species, and mycotoxin development. The risk will also depend partly on temperature and in general development of mould will become slower once temperatures fall below 15˚C although *Penicillium* moulds including *P. verrucosum* favour lower temperatures. Thus grain between 18 and 25% moisture at any temperature between 10˚ and 25˚C is at risk from the development of ochratoxin A. Thirdly, if temperatures rise above 25˚C, *Aspergillus* species tend to dominate so that important mycotoxin producing species such as *A. flavus* (aflatoxins). *A. nidulans* (sterigmatocystin) or *A. fumigatus* (range of less studied mycotoxins) may develop.

The development of *P. verrucosum* and ochratoxin A in cereals is clearly difficult to predict and so to eliminate or minimise ochratoxin A it is essential to dry grain to 15% or below as soon as possible after harvest and ensure that the whole bulk of grain remains at or below this moisture throughout storage. Whether or not grain at potential risk will develop ochratoxin A, or how quickly, is difficult to predict. This work and other studies suggest that there are other factors involved such that a confident forecast cannot be made on the basis of grain moisture and temperature alone. One question that remains unanswered is at what

point the grain becomes infected with spores of *P. verrucosum*. This mould appears to be closely associated with the storage situation including grain handling machinery and storage structures. Dust and old grain residues are thus likely reservoirs of mould spores and it is suggested that scrupulous hygiene in handling equipment, ventilation systems and stores is vital to reduce the infection of fresh grain. *As P. verrucosum* is closely associated with dust it is possible that air movement, including ventilation systems used for on floor aeration of hot air drying of grain might assist in this inoculation process.

Conclusions and recommendations

Generally in mould ecology studies, the field and storage situations have been studied in isolation thus ignoring any possible dynamic interactions that may be taking place. This project, in part, aimed to bridge that gap between field and store in respect of establishing if differing levels of field fungi would subsequently affect the development of storage fungi and hence the development of toxins such as ochratoxin A. This was to be achieved through the use of differential fungicide regimes to manipulate levels of field fungi on the ear and thus subsequently on grain. However, no significant differences in the levels of field fungi, in particular the sooty moulds were seen between the various fungicide regimes used. Following on from this, there appeared to be no detectable influence on the development of storage fungi that could be related to levels of field fungi entering the store. However, subtle changes may not have been picked up and would need to be the subject of a further and separately designed experiment.

The development of sooty moulds on the ear after a fungicide has ceased to be effective and possible mixing of spores in the combine may both serve to balance the levels of field fungi entering the store. Thus it would seem that the use of fungicides to prevent later development of storage fungi through manipulation of field fungi is not appropriate at least in the 2 seasons studied in this project. These 2 seasons were not helpful in maximising any differences, as year 1 was very dry and in year 2 there was very little disease pressure.

This project has once again reiterated the importance of effective and efficient drying of grain in order to reduce the chances of contamination of grain by ochratoxin A. The formation of mycotoxins post-harvest is based on grain moisture content, temperature, the fungi present and the time it is held under unsatisfactory conditions. To eliminate or minimise the development of ochratoxin A it is thus essential to dry grain quickly to 15% or below as soon as possible after harvest. It is then vital to ensure by constant monitoring that the whole bulk of grain remains at or below this moisture throughout storage and that even small pockets of damp grain are avoided. High concentrations of ochratoxin may develop in these small damp areas and this could contaminate a much greater tonnage of grain when moved or sold.

It has shown that even under precisely controlled conditions it is difficult to predict whether grain potentially at risk from the formation of ochratoxin A contamination will actually become contaminated or indeed how quickly. This and other studies suggest that a confident forecast of ochratoxin A contamination of grain can

not be made on the basis of grain moisture and temperature alone, other factors must be involved. Evidence is accumulating as other factors are understood or eliminated that the source and time at which the grain becomes 'inoculated' with *P. verrucosum* must be important. It is clear that there was little or no ochratoxin A producing fungi in the field as no *P. verrucosum* was isolated from that source in this project and confirms the data presented in other studies. A rapid, simple to use diagnostic test specific for *P. verrucosum* could provide a better indication of whether cereals were at potential risk through the presence of this organism but the time of application of the test would need to be carefully targeted.

Potential sources of inoculum may come from contaminated combine, trailers, augers, grain store etc. that have not been cleaned and contains left over contaminated grain from the previous harvest. A more rigorous hygiene programme at the beginning and during harvest may help to reduce sources of *P. verrucosum* inoculum and may also give more time in bottle neck situations at drying before the development of ochratoxin A. However more research is needed in the areas of sources of infection, the control of these points of infection and the production of ochratoxin A in bottle neck situations.

In summary, this study does not suggest any relationship between fungicide regime, effect of mould levels at harvest and subsequent development of ochratoxin A in store. Cereals are put at risk from ochratoxin A by fairly well defined combinations of temperature and grain moisture content but its actual development is determined by other factors that are strongly indicated but not proven. Recommendations for grain storage should include a section specifically devoted to ochratoxin A..

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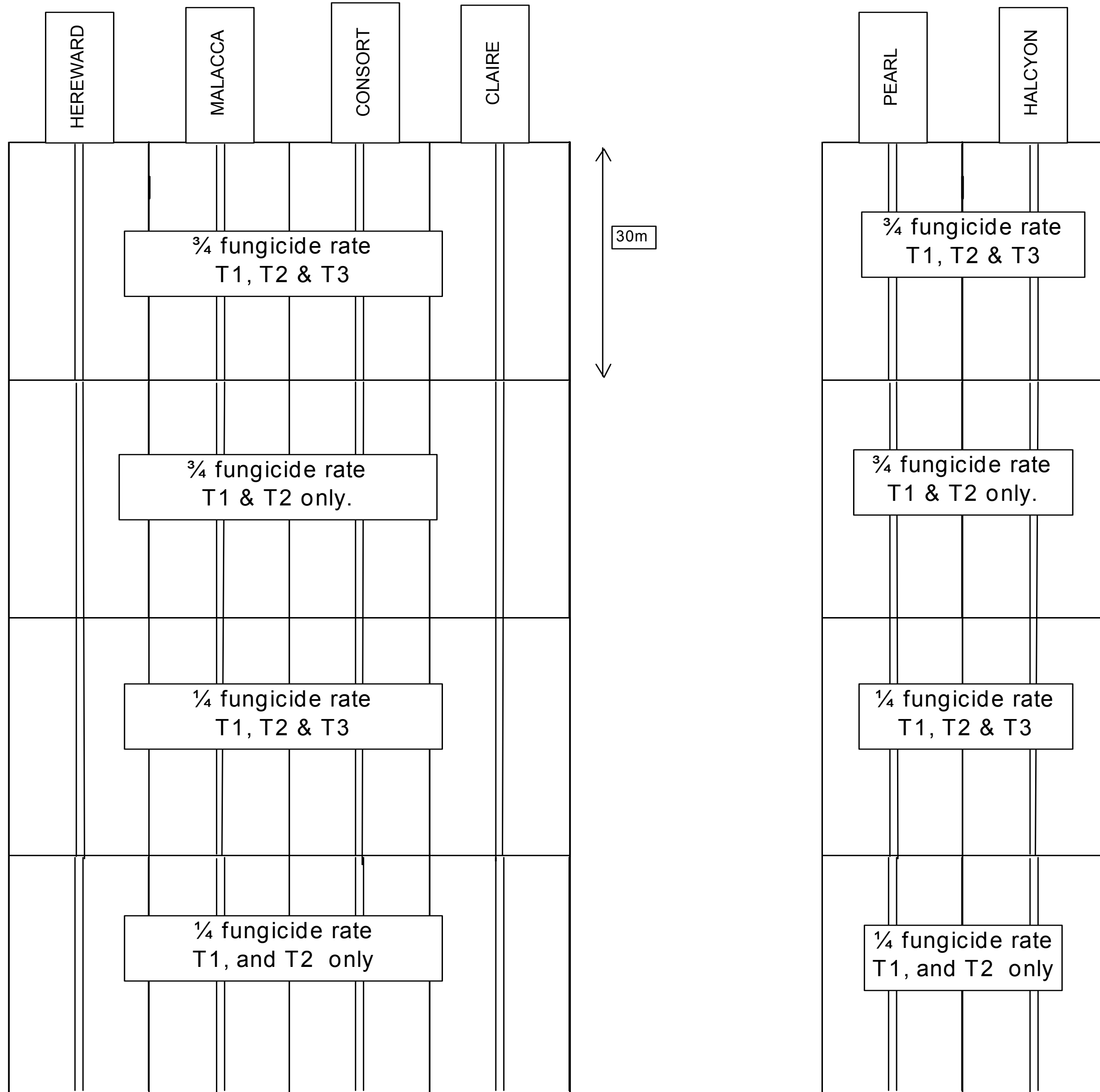
APPENDIX A PLOT LAYOUT DIAGAM

Trial ref. no. Pt2000-04, Pt2001-04 and Pt2002-04

Locations

Setterington, Stowell, Dover, Braishfield.



T1 = GS 31
T2 = GS39
T3 = GS 59

T1 = GS 31
T2 = GS39 (not GS 49)
T3 = GS 59

T1 AND T2 FUNGICIDE MIX TO BE AS PER FARM - IE. STROBILURIN+TRIAZOLE
T3 FOLICUR

APPENDIX B DETAILED MOULD COUNTS AND SPECIES PRESENT FROM PRE-HARVEST TO END OF STORAGE EXPERIMENTAL PERIOD- SPECIMEN DATA SET

Plate counts for barley samples 2001 – Dover

Variety	Fungicide regime*	Species isolated	Log $_{10}$ colony counts – days after harvest					
			0	4	7	14	21	28
Pearl	A	*Alternaria alternata*	4.30	-	4.60	4.17	3.69	3.00
		Cladosporium spp.	4.84	-	4.77	4.39	3.69	3.17
		Fusarium spp.	2.00	-	3.00			
		Epicoccum nigram	2.00	-				
		Penicillium spp.		-	2.00	4.74	5.09	5.46
		Aspergillus candidus		-				2.00
		Aspergillus versicolor		-				
		Eurotium spp.		-	3.00	2.69	3.17	4.17
		Wallemia sp.		-			3.74	
		Yeasts	5.64	-	5.32	4.30	4.00	3.69
		MC %	19.6	-	20	19.7	19.2	17.6
		TEMP.	24.6	-	20.2	21.2	26.5	15.7
	B	*Alternaria alternata*	4.60	-	4.39	3.69	4.00	3.00
		Cladosporium spp.	4.74	-	4.84	3.84	4.17	4.00
		Fusarium spp.	2.00	-	3.17	3.00		
		Epicoccum nigram		-				
		Penicillium spp.	2.00	-	2.30	4.47	4.97	5.45
		Aspergillus candidus		-				
		Aspergillus versicolor		-				
		Eurotium spp.		-	3.3.	4.00	4.30	4.30
		Wallemia sp.		-				
		Yeasts	5.44	-	5.57	4.17	4.00	4.17
		MC %	19.5	-	19.9	19.6	19.1	18.2
		TEMP.	24.6	-	20.3	21	26.1	15.4

* A = ¼ rate T1 + T2; B = ¼ rate T1, T2 + T3; C = ¾ rate T1 + T2; D = ¾ rate T1, T2 + T3

Plate counts for barley samples 2001 – Dover

Variety	Fungicide regime*	Species isolated	Log 10 colony counts – days after harvest 0	4	7	14	21	28
	C	*Alternaria alternata*	4.39	-	4.11	3.30	3.30	3.00
		Cladosporium spp.	4.87	-	4.65	4.00	4.00	3.30
		Fusarium spp.		-				
		Epicoccum nigram		-				
		Penicillium spp.	3.00	-	3.17	4.30	4.81	4.97
		Aspergillus candidus		-				
		Aspergillus versicolor		-				
		Eurotium spp.		-	2.00	3.69	4.00	4.30
		Wallemia sp.		-				4.17
		Yeasts spp.	5.62	-	5.74	4.17	4.00	3.69
		MC %	19.5	-	19.6	19.3	18.6	18.1
		TEMP.	24.6	-	20.3	22	25.6	16.8
Pearl	D	*Alternaria alternata*	4.00	-	4.00	4.00	4.00	3.69
		Cladosporium spp.	4.65	-	4.47	4.00	4.17	3.54
		Fusarium spp.		-				
		Epicoccum nigram	3.00	-				
		Penicillium spp.		-	2.00	4.17	4.00	4.84
		Aspergillus candidus		-				
		Aspergillus versicolor		-				
		Eurotium spp.		-	3.00		2.00	3.69
		Wallemia sp.		-			3.77	4.60
		Yeasts	5.71	-	5.88	4.65	4.39	3.69
		MC %	19	-	19.3	19.1	17.8	16.2
		TEMP.	24.6	-	21	21.9	25.1	14.6

* A = ¼ rate T1 + T2; B = ¼ rate T1, T2 + T3; C = ¾ rate T1 + T2; D = ¾ rate T1, T2 + T3

Plate counts for barley samples 2001 – Dover

Variety	Fungicide regime*	Species isolated	Log $_{10}$ colony counts – days after harvest					
			0	4	7	14	21	28
Halcyon	A	*Alternaria alternata*	4.39	-	4.17	3.69	4.17	4.00
		Cladosporium spp.	4.54	-	4.81	4.39	4.39	3.69
		Fusarium spp.	3.00	-	3.00	3.00		
		Epicoccum nigram		-				
		Penicillium spp.		-	2.00	3.00	4.17	4.3
		Aspergillus candidus		-				
		Aspergillus versicolor		-				2.69
		Eurotium spp.		-	2.30		1.69	2.69
		Wallemia sp.		-			4.00	4.00
		Yeasts	5.67	-	5.67	4.60	4.17	3.69
		MC %	19	-	19.3	18.5	18.5	17.1
		TEMP.	24.6	-	16.7	19.8	23.8	16.2
	B	*Alternaria alternata*	4.77	-	4.39	4.17	4.17	3.69
		Cladosporium spp.	4.84	-	4.60	4.30	4.17	3.47
		Fusarium spp.	3.17	-				
		Epicoccum nigram		-		3.00		
		Penicillium spp.		-	3.00	3.39	4.39	4.69
		Aspergillus candidus		-				
		Aspergillus versicolor		-				2.00
		Eurotium spp.		-	2.00			3.00
		Wallemia sp.		-				4.17
		Yeasts	5.63	-	5.57	4.39	4.30	4.00
		MC %	18.7	-	19.1	18.3	19.7	15.1
		TEMP.	24.6	-	17.9	19.8	21	14.8

* A = ¼ rate T1 + T2; B = ¼ rate T1, T2 + T3; C = ¾ rate T1 + T2; D = ¾ rate T1, T2 + T3

Plate counts for barley samples 2001 – Dover

Variety	Fungicide regime*	Species isolated	Log $_{10}$ colony counts – days after harvest					
			0	4	7	14	21	28
Halcyon	C	*Alternaria alternata*	4.54	-	4.47	3.74	4	3.69
		Cladosporium spp.	4.77	-	4.97	3.69	3.69	3.39
		Fusarium spp.	3.00	-	3.00			
		Epicoccum nigram		-				
		Penicillium spp.		-	3.00	4.17	4.00	4.6
		Aspergillus candidus		-				
		Aspergillus versicolor		-				
		Eurotium spp.		-	3.00		2.17	3.00
		Wallemia sp.		-				3.69
		Yeasts	5.61	-	5.75	4.00	4.65	4.17
		MC %	18.3	-	19.1	18.3	16.7	16
		TEMP.	24.6	-	18.9	20.5	23.9	16.6
	D	*Alternaria alternata*	4.39	-	4.17	3.69	3.69	
		Cladosporium spp.	4.90	-	4.92	4.00	3.69	3.00
		Fusarium spp.	2.00	-	3.07	3.30		
		Epicoccum nigram		-		2.69		
		Penicillium spp.		-	3.00	2.90	4.17	4.47
		Aspergillus candidus		-				
		Aspergillus versicolor		-				3.69
		Eurotium spp.		-	2.00		3.69	4.02
		Wallemia sp.		-				3.84
		Yeasts	5.91	-	5.94	4.17	4.00	4.17
		MC %	18.1	-	18.9	17.9	17.4	15.7
		TEMP.	24.6	-	19.4	20.2	24.8	16.3

* A = ¼ rate T1 + T2; B = ¼ rate T1, T2 + T3; C = ¾ rate T1 + T2; D = ¾ rate T1, T2 + T3

Plate counts for wheat samples 2001 – Dover

Variety	Fungicide regime*	Species isolated	Log$_{10}$ colony counts – days after harvest						
			Pre-harvest		0	14	21	28	42
			Chaff	Grain					
Hereward	A	*Alternaria alternata*	5.04	3.69	4.30	4.00			
		Cladosporium spp.	6.31	5.14	4.84	4.60	4.39	4.47	4.6
		Fusarium spp.	4.74	3.69	3.39	3.00			
		Epicoccum nigram		3.00	3.00				
		Penicillium spp.	5.26	3.95	3.65	5.56	5.81	5.91	6.39
		Penicillium verrucosum				3.17	4.00	4.39	5.69
		Aspergillus versicolor							
		Eurotium spp.							
		Yeast spp.	4.81	4.00	3.69				
		MC %			20.8	19.2	21.2	19.5	19.3
		TEMP.			15.5	14.2	14.9	16.0	13.7
	B	*Alternaria alternata*	4.97	3.90	4.30				
		Cladosporium spp.	6.28	4.95	4.87	4.87	4.60	4.39	4.30
		Fusarium spp.	5.17	3.30	3.54	4.00			
		Epicoccum nigram	4.00						
		Penicillium spp.	4.54	3.00	3.17	5.29	5.67	5.74	6.32
		Penicillium verrucosum				3.39	4.47	5.53	5.69
		Aspergillus versicolor				2.69		3.69	
		Eurotium spp.				2.69			
		Yeast spp.	4.39	3.69	4.39	4.17			
		MC %			20.8	19.0	20.9	19.4	19.2
		TEMP.			15.3	14.3	14.2	15.7	14.4

* A = ¼ rate T1 + T2; B = ¼ rate T1, T2 + T3; C = ¾ rate T1 + T2; D = ¾ rate T1, T2 + T3

Table 4. Plate counts for wheat samples 2001 – Dover (cont)

Variety	Fungicide regime*	Species isolated	Log $_{10}$ colony counts – days after harvest						
			Pre-harvest		0	14	21	28	42
			Chaff	Grain					
Hereward	C	*Alternaria alternata*	4.97	3.95	4.30	4.00			
		Cladosporium spp.	6.29	4.84	4.90	4.69	4.74	4.54	4.17
		Fusarium spp.	5.00	4.00	4.17	2.69			
		Epicoccum nigram							
		Penicillium spp.		3.39	3.74	5.53	5.86	5.90	6.34
		Penicillium verrucosum				3.65	3.30	3.00	4.39
		Aspergillus versicolor							
		Eurotium spp.							
		Yeast spp.	4.47	4.00	4.00				
		MC %			20.8	19.0	21.0	18.8	19.6
		TEMP.			15.4	14.1	14.4	15.8	15.7
	D	*Alternaria alternata*	5.45	4.54	4.60				
		Cladosporium spp.	5.99	5.06	4.77	4.60	4.60	4.00	4.17
		Fusarium spp.	4.17		3.17	1.69			
		Epicoccum nigram							
		Penicillium spp.	5.02	3.39	3.30	5.41	5.81	5.91	6.86
		Penicillium verrucosum					3.60	4.54	4.69
		Aspergillus versicolor				2.69			5.60
		Eurotium spp.							
		Yeast spp.	4.39	4.39	4.47	3.69			
		MC %			19.5	19.3	20.7	19.1	19.0
		TEMP.			15.2	13.3	14.3	15.5	13.9

* A = ¼ rate T1 + T2; B = ¼ rate T1, T2 + T3; C = ¾ rate T1 + T2; D = ¾ rate T1, T2 + T3

Plate counts for wheat samples 2001 – Dover (cont)

Variety	Fungicide regime*	Species isolated	Log 10 colony counts – days after harvest						
			Pre-harvest		0	14	21	28	42
			Chaff	Grain					
Malacca	A	*Alternaria alternata*	5.16	3.69	3.87	3.30	3.69	3.60	3.69
		Cladosporium spp.	6.25	4.95	4.77	4.39	4.60	4.39	4.17
		Fusarium spp.	4.69	3.00	3.30	2.69	2.69		
		Epicoccum nigram		1.69	3.00				
		Penicillium spp.	4.47	2.69	3.69	4.54	5.41	5.74	6.02
		Penicillium verrucosum							3.54
		Aspergillus candidus							
		Aspergillus versicolor							
		Eurotium spp.		1.69	1.69	1.69			
		Wallemia spp.							3.69
		Yeast spp.	4.54	4.00	4.00	3.00			4.00
		MC %			18.5	18.4	19.7	17.9	19.1
		TEMP.			14.6	13.0	13.0	14.3	13.3
	B	*Alternaria alternata*	5.43	3.69	3.69		2.69		
		Cladosporium spp.	5.96	5.07	5.06	4.60	4.54	4.00	3.30
		Fusarium spp.	4.17	3.54	3.00				
		Epicoccum nigram	4.17	2.39	3.00				
		Penicillium spp.	4.60	2.00	3.77	4.84	5.34	5.67	5.90
		Penicillium verrucosum					3.39		3.69
		Aspergillus candidus							
		Aspergillus versicolor							
		Eurotium spp.					2.69		3.30
		Wallemia spp.							4.74
		Yeast spp.	5.07	4.17	3.69				
		MC %			18.5	18.3	20.0	18.0	18.9
		TEMP.			14.6	13.3	13.3	15.0	13.4

* A = ¼ rate T1 + T2; B = ¼ rate T1, T2 + T3; C = ¾ rate T1 + T2; D = ¾ rate T1, T2 + T3

Plate counts for wheat samples 2001 – Dover (cont)

Variety	Fungicide regime*	Species isolated	Log $_{10}$ colony counts – days after harvest						
			Pre-harvest		0	14	21	28	42
			Chaff	Grain					
Malacca	C	*Alternaria alternata*	4.97	3.30	3.47	2.69	2.00		
		Cladosporium spp.	6.09	4.47	4.69	4.17	4.00	4.47	4.17
		Fusarium spp.	4.77	4.17	4.30			3.00	
		Epicoccum nigram							
		Penicillium spp.	4.17		3.77	4.65	5.56	5.49	5.87
		Penicillium verrucosum				2.69	2.00	2.90	3.00
		Aspergillus candidus							3.00
		Aspergillus versicolor							3.39
		Eurotium spp.					2.69		3.00
		Wallemia spp.							
		Yeast spp.	4.17		3.69				
		MC %			18.5	19.4	19.8	18.3	19.8
		TEMP.			14.7	13.4	13.3	14.8	12.9
	D	*Alternaria alternata*	5.34	3.69	3.69				
		Cladosporium spp.	6.08	4.87	4.92	4.17	4.00	4.47	4.47
		Fusarium spp.	4.60	2.74	3.47		2.69		
		Epicoccum nigram							
		Penicillium spp.	4.77	2.30	3.65	4.92	5.51	5.59	5.65
		Penicillium verrucosum							4.47
		Aspergillus candidus							
		Aspergillus versicolor							3.00
		Eurotium spp.						2.69	
		Wallemia spp.							
		Yeast spp.	4.60	3.69	3.54				
		MC %			18.5	18.4	20.0	18.0	18.2
		TEMP.			14.6	13.6	13.2	14.8	13.9

* A = ¼ rate T1 + T2; B = ¼ rate T1, T2 + T3; C = ¾ rate T1 + T2; D = ¾ rate T1, T2 + T3

Plate counts for wheat samples 2001 – Dover (cont)

Variety	Fungicide regime*	Species isolated	Log $_{10}$ colony counts – days after harvest						
			Pre-harvest		0	14	21	28	42
			Chaff	Grain					
Consort	A	*Alternaria alternata*	5.46	3.00	3.47				
		Cladosporium spp.	6.24	5.04	4.95	4.39	4.47	4.17	4.54
		Fusarium spp.	4.74	2.69	2.69				
		Epicoccum nigram		2.00	2.69				
		Penicillium spp.	4.81	3.84	4.00	5.76	5.77	6.00	6.64
		Penicillium verrucosum				3.00	3.69	4.60	4.90
		Aspergillus versicolor							5.00
		Eurotium spp.							
		Wallemia spp.							
		Yeast spp.	4.54						
		MC %			20.6	19.6	21.5	19.4	20.2
		TEMP.			16.0	13.7	13.9	16.2	14.9
	B	*Alternaria alternata*	5.40	3.69	3.60				
		Cladosporium spp.	5.97	4.92	4.87	4.30	3.84	3.00	
		Fusarium spp.	4.00	1.69	3.39				
		Epicoccum nigram	4.17						
		Penicillium spp.	4.87	3.84	3.69	5.66	5.76	6.04	6.43
		Penicillium verrucosum					3.39	3.81	4.30
		Aspergillus versicolor							
		Eurotium spp.		1.69					
		Wallemia spp.						3.69	
		Yeast spp.	4.97						
		MC %			20.9	19.3	21.5	19.3	19.6
		TEMP.			15.5	13.8	14.2	15.7	15.5

* A = ¼ rate T1 + T2; B = ¼ rate T1, T2 + T3; C = ¾ rate T1 + T2; D = ¾ rate T1, T2 + T3

Plate counts for wheat samples 2001 – Dover (cont)

Variety	Fungicide regime*	Species isolated	Log $_{10}$ colony counts – days after harvest						
			Pre-harvest		0	14	21	28	42
			Chaff	Grain					
Consort	C	*Alternaria alternata*	5.54	4.00	4.17				
		Cladosporium spp.	6.21	5.13	4.97	4.39	4.47	4.54	4.39
		Fusarium spp.	4.81	3.00	3.60	2.69			
		Epicoccum nigram	4.00						
		Penicillium spp.	5.00	4.00	4.30	5.59	5.89	5.83	6.49
		Penicillium verrucosum					3.84	4.30	4.69
		Aspergillus versicolor							3.00
		Eurotium spp.							
		Wallemia spp.							
		Yeast spp.	4.54						
		MC %			21.6	19.1	21.4	19.2	19.9
		TEMP.			16.0	13.9	14.4	15.7	14.6
	D	*Alternaria alternata*	5.38	4.17	4.30				
		Cladosporium spp.	6.25	5.04	5.07	4.47	4.17	4.17	4.00
		Fusarium spp.	5.19	3.65	3.60	3.00			
		Epicoccum nigram	4.00						
		Penicillium spp.	4.30	3.30	3.77	5.61	5.88	5.90	6.21
		Penicillium verrucosum				4.00	4.30	4.65	4.65
		Aspergillus versicolor						4.00	4.30
		Eurotium spp.					3.69	3.47	3.00
		Wallemia spp.							
		Yeast spp.	4.47						
		MC %			18.7	19.0	20.6	19.9	19.6
		TEMP.			16.3	14.7	14.2	16.3	14.2

* A = ¼ rate T1 + T2; B = ¼ rate T1, T2 + T3; C = ¾ rate T1 + T2; D = ¾ rate T1, T2 + T3

Plate counts for wheat samples 2001 – Dover (cont)

Variety	Fungicide regime*	Species isolated	Log $_{10}$ colony counts – days after harvest						
			Pre-harvest		0	14	21	28	42
			Chaff	Grain					
Claire	A	*Alternaria alternata*	5.26	4.00	4.17	4.00			
		Cladosporium spp.	6.27	5.04	5.00	4.81	4.65	4.60	4.47
		Fusarium spp.	5.00	3.69	3.47				
		Epicoccum nigram							
		Penicillium spp.	5.00	2.69	3.90	4.95	5.44	5.82	6.38
		Penicillium verrucosum				1.69	3.00	3.17	4.60
		Aspergillus candidus							
		Aspergillus versicolor							
		Wallemia spp.							
		Yeast spp.	4.87	4.00	4.30	4.17	4.39	4.00	
		MC %			21.0	18.3	20.6	18.7	19.3
		TEMP.			15.5	13.6	13.4	15.0	13.6
	B	*Alternaria alternata*	5.50	4.17	4.00	2.69			
		Cladosporium spp.	6.29	4.92	4.92	4.77	4.65	4.39	4.17
		Fusarium spp.	4.30						
		Epicoccum nigram	4.17	2.00	3.17				
		Penicillium spp.	4.00	3.69	3.77	5.00	5.39	5.67	5.84
		Penicillium verrucosum						2.74	3.39
		Aspergillus candidus							3.69
		Aspergillus versicolor							3.69
		Wallemia spp.						3.00	4.00
		Yeast spp.	4.90	3.69	3.84	4.47	4.39		
		MC %			19.0	19.2	19.9	19.0	18.9
		TEMP.			15.3	13.3	13.3	14.9	14.6

* A = ¼ rate T1 + T2; B = ¼ rate T1, T2 + T3; C = ¾ rate T1 + T2; D = ¾ rate T1, T2 + T3

Plate counts for wheat samples 2001 – Dover (cont)

Variety	Fungicide regime*	Species isolated	Log $_{10}$ colony counts – days after harvest						
			Pre-harvest		0	14	21	28	42
			Chaff	Grain					
Claire	C	*Alternaria alternata*	5.46	3.69	3.74	3.69			
		Cladosporium spp.	6.34	4.92	4.95	4.39	4.65	4.69	4.54
		Fusarium spp.	4.00	2.69	3.00	2.69			
		Epicoccum nigram							
		Penicillium spp.	5.21	2.69	3.30	5.13	5.30	5.65	5.87
		Penicillium verrucosum					3.17	3.00	5.00
		Aspergillus candidus							
		Aspergillus versicolor							
		Wallemia spp.							
		Yeast spp.	4.97	3.69	3.77	3.69	4.39		
		MC %			18.7	18.5	20.5	18.8	19.0
		TEMP.			15.0	13.1	12.9	14.6	13.6
	D	*Alternaria alternata*	5.66	3.69	3.54	2.69	3.69		
		Cladosporium spp.	6.23	4.90	4.84	4.30	4.17	4.00	4.47
		Fusarium spp.	4.17	3.69	3.47	2.69	3.69		
		Epicoccum nigram		2.69	2.69				
		Penicillium spp.	4.00	1.69	3.00	5.02	5.38	5.72	5.60
		Penicillium verrucosum					3.00	3.69	3.77
		Aspergillus candidus							
		Aspergillus versicolor							
		Wallemia spp.							
		Yeast spp.	4.77						
		MC %			19.2	19.1	20.7	18.3	18.7
		TEMP.			14.7	13.7	13.7	15.0	13.7

* A = ¼ rate T1 + T2; B = ¼ rate T1, T2 + T3; C = ¾ rate T1 + T2; D = ¾ rate T1, T2 + T3

APPENDIX C SOURCES OF VARIABILITY AND ESTIMATION

The results of any field-based experiments are affected by the compounded errors or variability in measurements inherent at each stage in the study. Some of these factors are difficult to control, e. g. climate. Within the laboratory, these variables are easier to minimise although not to eliminate. Thus in the current study it was vital to assess the effect of the principle sources of variability and agree the criteria by which a significant experimental effect might be judged. A number of the possible variables were minimised on each of the 4 farm sites by closely matching each set of plots for position, fertiliser, and herbicide and insecticide inputs while any variation in climate should be avoided because plots were adjacent to each other. However, this still leaves several important aspects of the study for which the variability is unknown or difficult to control. The main variables were the collection of representative samples before and at harvest and during storage, the moisture content and temperature of grain during storage, mould counting and chemical analysis.

Each of these factors were thus assessed as far as possible and/or monitored during the current work. Results from the first year (2000) failed to show any clear pattern or correlation with fungicide use. If any effects that had occurred were small in comparison to the errors due to some of these factors, these effects could have been masked. Thus it was vital to attempt to quantify these errors. Some of the data from 2000 was thus examined retrospectively in an attempt to estimate the variation due to sampling error and mould counting. In the second year (2001) further studies were carried out to confirm the initial tentative findings and to obtain more data.

Combined error in grain sampling and mould counting

In 2000 a number of the stored grain samples were relatively dry so that any change in mould levels present at harvest would be slow during storage. Because under these circumstances the field fungi present at harvest will neither be overgrown by rapidly developing storage moulds, nor will themselves increase to any significant extent this presents an opportunity to study the errors due to sampling and the mould counting technique. Selection of those samples from the bags that had been stored over a period of 28 days containing grain at 17% moisture content or less and in which no significant growth of storage fungi had occurred, approximated to a set of replicate samples being taken. These data were used to estimate the combined variation due to sampling and counting of the field-derived moulds. A summary of this information is presented in Table C1 for wheat and barley. The mould counts are the total for all field fungi, almost exclusively *Alternaria, Cladosporium* and *Fusarium* species. A-D are the different fungicide regimes used. The % standard deviation for each individual storage situation ranged from 7.0% to 54.3% with means from 25.1% to 34.2% for each set of 4 cereal samples. There was little difference between wheat and barley and the mean variation for all 20 sets of data was 30.1%. This suggested that the combined error for sampling and for mould

counting was about 30%. As small changes in mould distribution and amounts might have during 28 days the actual error could less.

In a pre-planned study in the 2nd year, 2 sets (one barley and one wheat) of 10 replicate samples were taken from stored bags of grain shortly after harvesting and after 28 days storage. Each sample was prepared by combining 10 sub-samples in the Report. These samples were used to complement the information obtained in 2000. Results are summarised in Table C2. Mould counts were carried out using duplicate sets of agar plates for each sample. These values indicate the combined error in mould counting and in sampling and were about 41%. This is a little higher than was estimated above from the 2000 results. There was much less error in measurement of the total *Penicillium* counts (% s. d. 20%). Limited evidence suggests that the error was less when the levels of fungi were higher.

Errors involved in mould counting alone

Table C3 gives the results calculated for each pair of plate counts for each set of 10 replicates, the mean fungal count, the standard deviation and the % standard deviation for *Alternaria, Cladosporium* and total field fungi after 4 days storage and for total *Penicillium* after 28 days. This data represent the error due mould count only. For *Alternaria*, the mean counts obtained for the 10 replicate sets ranged from 2.5 to 25 $x10^3$ cfu/g, for *Cladosporium* 30 to 135.8 cfu/g, for total field moulds 45 to 138.5 cfu/g and for total *Penicillium* after 28 days storage 385 to 786 cfu/g. Mean % s. d. for the pairs in each sets of 10 replicates were respectively 25.65 (range 0 to 46.91%), 32.49 (range 16.21 to 46.99%), 26.47 (range 0.42 to 41.46%) and 8.9 (1.59 to 21.02%). Classical mould counting techniques have been used for many years although information on the quantitative performance of these procedures is difficult to find in the literature. It is generally considered to be semi-quantitative at the best. Here, the error in mould counting has been shown to be surprisingly smalll especially when mould counts were above a level of $\log_{10}$ 5.0.

Summary of error in mould determination and ochratoxin A analysis

Total error are greater than those that can be attributed to mould counting alone of ochratoxin A analysis alone. The results indicate that the relative errors due to sampling and to mould counting are in general of the same order. Thus when attempting to determine whether effects are significant in this study the following criteria are adopted; mould counts below 10^4 are too unreliable and can be dismissed for this purpose. The combined error due to sampling and mould counting has a % s. d. of no greater than 50% when mould counts are between 10^4 and 10^5 ($\log_{10}$ values of 4.0 and 5.0) and as low as 20% at the 10^6 ($\log_{10}$ value of 6.0). For mould counts the maximum variation obtained was 45 to 138.5 $x10^3$ (a factor of 3 for total field moulds) and 385 to 786 (a factor of 2 for total *Penicillium*).

Table C1: Estimation of the variability attributable to sampling error and mould counting using replicate samples taking during a 28 day storage period in 2000 using grain of less than 17% moisture content.

Grain sample and Site	Approximate Moisture content, %	Days	Total mould count for field fungi, x10^3 A	B	C	D	Mean
		0	130	206	105	210	
Barley, Pearl		4	130	260	162	270	
		7	170	165	210	300	
Braishfield	15	14	240	230	200	180	
		28	127	140	210	220	
		mean	159.4	200.2	177.4	236	
		sd	48.44	48.37	45.04	48.27	
		sd, %	30.38	24.17	25.39	20.45	25.1
		0	122	70	80	190	
Barley, Halcyon		4	140	130	340	190	
		7	110	160	230	280	
Braishfield	15	14	180	125	250	270	
		28	46	146	292	141	
		mean	119.6	126.2	238.4	214.2	
		sd	48.94	34.31	98.107	59.10	
		sd, %	40.92	27.19	41.15	27.59	34.2
		0	105	110	136.5	85	
Barley, Pearl		4	36	80.2	75.15	91	
		7	130	57	105	76	
Settrinton	15	14	66.1	60	140	80	
		28	41	90	57	80	
		mean	75.62	79.44	102.73	82.4	
		sd	40.86	21.95	36.70	5.77	
		sd, %	54.03	27.63	35.72	7	31.1
		0	115	133	211	90	
Wheat, Malacca	17	4	165	110.1	55	70	
		14	75	110	151.5	70	
Stowell Park		28	50	145	100	74	
		sd	50.23	17.42	67.20	9.52	
		mean	101.25	124.52	129.38	76	
		sd, %	49.61	13.99	51.94	12.53	32.01
		0	285	341.5	260	285	
Wheat, Malacca	17	4	230	135	160	255	
		7	315	175	165	280	
Braishfield		14	285	120	100	210	
		28	250	129	145	225	
		mean	273	180.1	166	251	
		sd	33.28	92.65	58.46	33.05	
		sd, %	12.19	51.44	35.22	13.17	28
Wheat/barley	%, sd overall	mean					30.1

Taking this into account and applying an additional 'safety' factor of x2 suggests that a difference in numerical mould count of about x5 ($\log_{10}$ 0.7) would indicate a significant effect due to different fungicide applications if all other factors are controlled. For ochratoxin A determination, error due to the analysis is small so the principle source of variation will be in sampling.

Table C2: Estimation of the variability attributable to sampling error and mould counting using 10 replicate samples after 4 and 28 day storage period in stored wheat and barley.

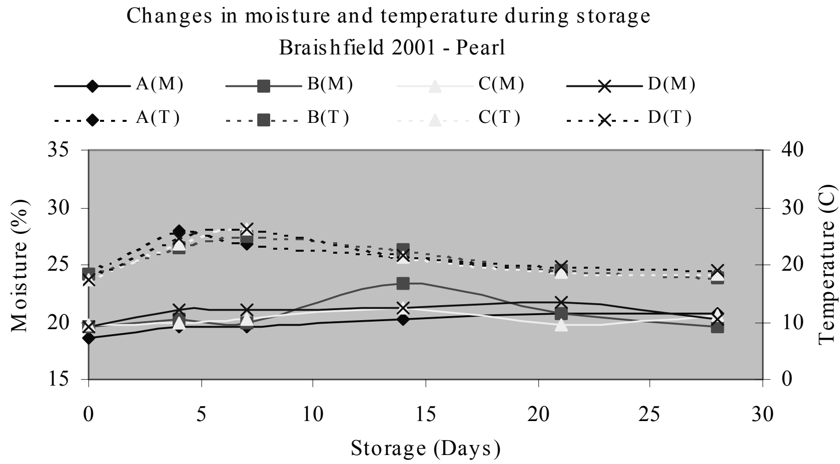
function	Mould counts, colony forming units/g x 10^3			
	Alternaria, 4 day	*Cladosporium,* 4 day	Total field moulds, 4 days	*Penicillium,* 28 days
Mean count	12	65	77	597
Count range	2.5-24.5	28-134	42-141	385-786
sd	5.85	32.66	33.09	118.28
% sd, mean value	48.79	42.93	41.38	19.82

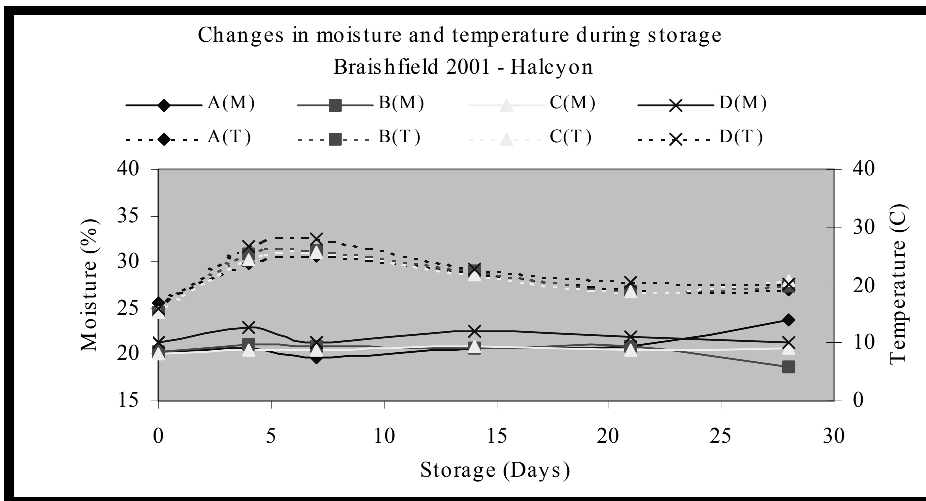
Table C3: Variability of mould count using duplicate counts

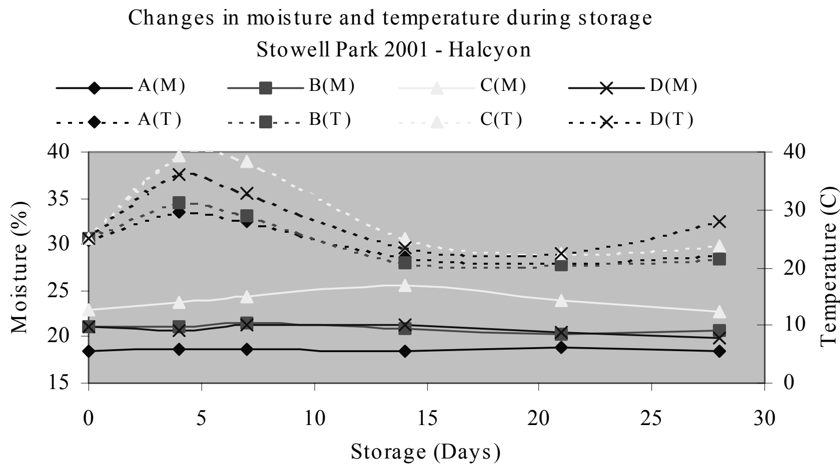
Mould	function	Replicate number of duplicate mould counts, colony forming units/g x10^3										sd, %
		1	2	3	4	5	6	7	8	9	10	Mean
Alternaria		10	20	10	10	20	2	6.	20	30	10	
		10	10	10	20	10	3	7	10	20	10	
	sd	0	7.036	0	7.036	7.036	0.728	0.743	7.036	7.248	0	
	mean	10	15	10	15	15	2.5	6.5	15	25	10	
	sd, %	0	46.91	0	46.91	46.91	28.56	11.34	46.91	29.00	0	25.65
Cladosporium		71	40	40	50	50	71	120	50	71	100	
		89	20	30	40	100	40	151	30	129	50	
	sd	12.961	14.043	6.795	7.290	35.270	21.906	22.040	14.086	41.019	35.270	
	mean	80	30	35	45	75	55.5	135.8	40	100	75	
	sd, %	16.21	46.99	19.43	16.21	46.99	39.62	16.23	35.08	41.1	46.99	32.49
Total field moulds		81	60	50	60	70	73	126	70	101	110	
		99	30	40	60	110	43	151	40	149	60	
		12.961	21.079	6.795	0.255	28.235	21.178	17.833	21.121	33.899	35.270	
		90	45	45	60	90	58	138.5	55	125	85	
		14.41	47	15.11	0.42	31.35	36.64	12.84	38.31	27.17	41.46	26.47
Penicillium		603	479	646	759	398	525	617	575	661	537	
(28days storage)		661	562	759	813	372	617	631	603	724	398	
	sd	41.012	58.690	79.903	38.184	18.385	65.054	9.900	19.799	44.548	98.288	
	mean	632	520.5	702.5	786	385	571	624	589	692.5	467.5	
	sd, %	12.98	11.28	11.37	4.86	4.77	11.39	1.59	3.36	6.43	21.02	8.9

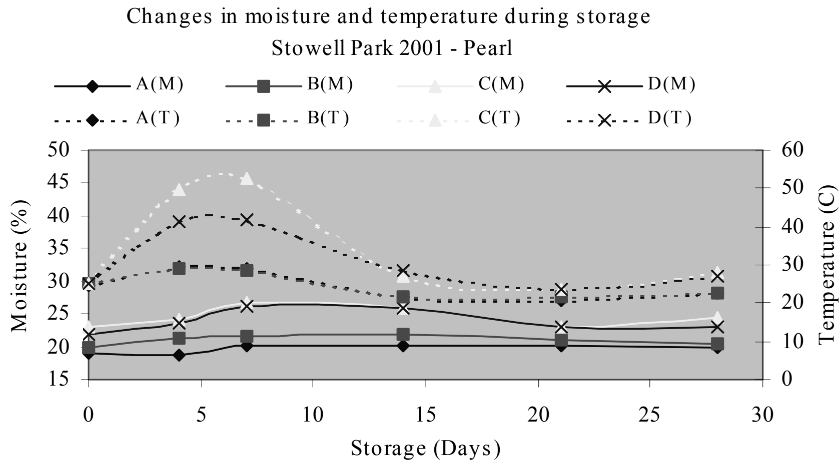
APPENDIX D
RECORD OF TEMPERATURE AND MOISTURE CONTENT DURING STORAGE

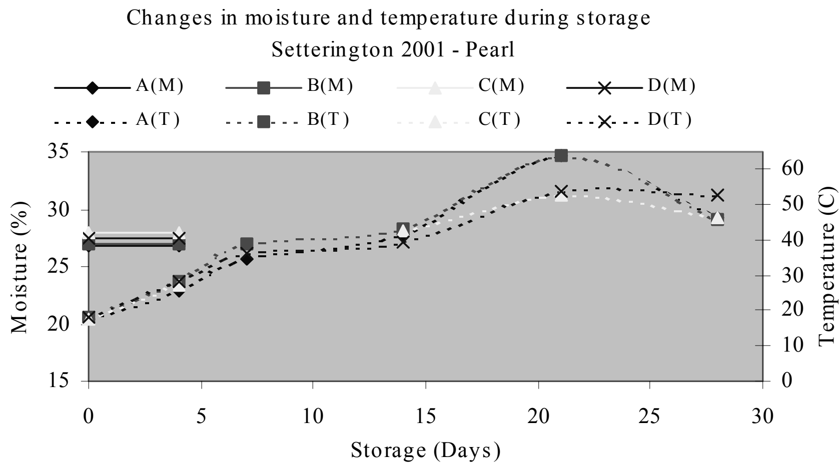
BARLEY

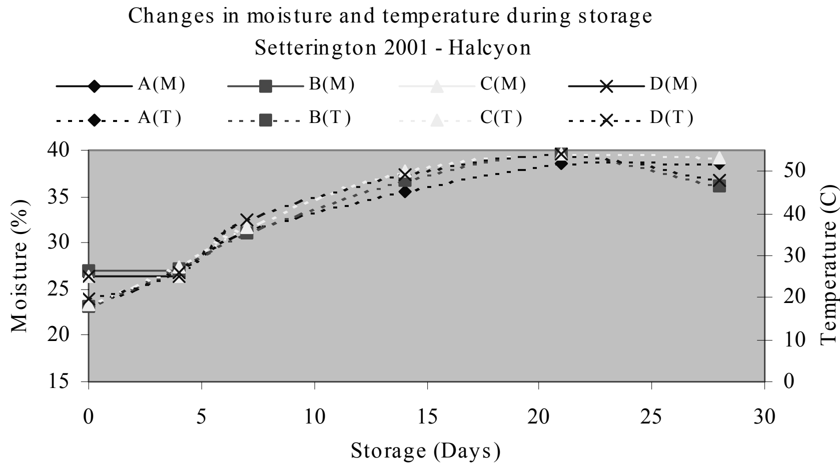


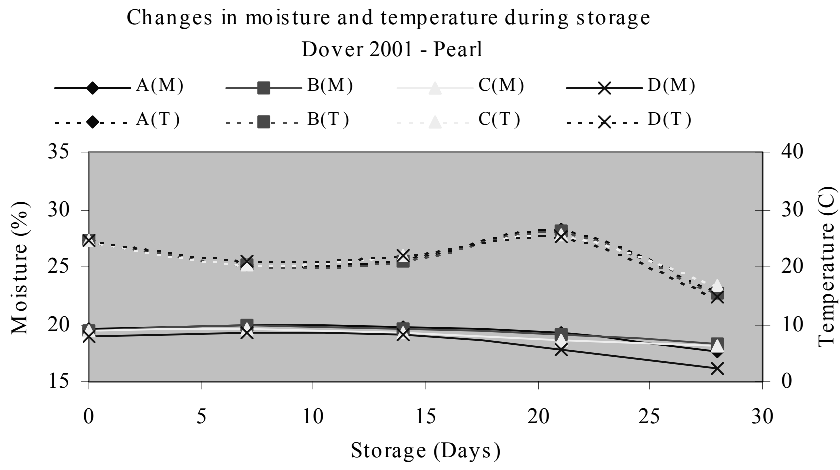


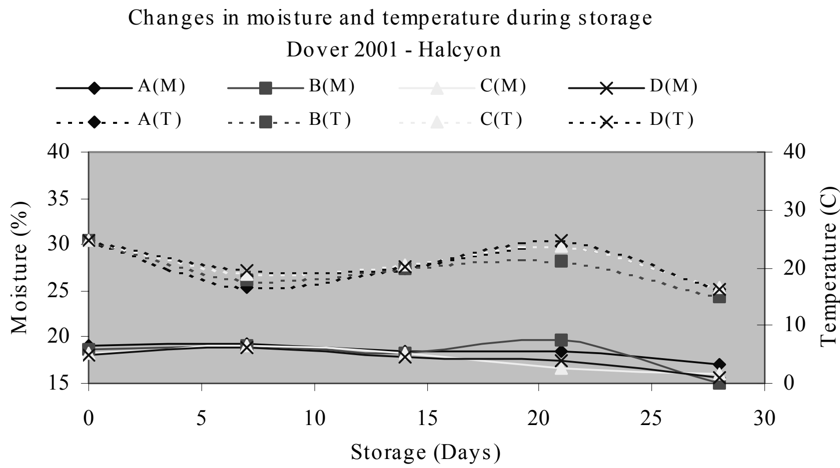



Changes in moisture and temperature during storage
Stowell Park 2001 - Pearl
A(M)
B(M)
C(M)
D(M)
A(T)
B(T)
C(T)
D(T)
Moisture (%)
Temperature (C)
Storage (Days)


Changes in moisture and temperature during storage
Setterington 2001 - Pearl
A(M)
B(M)
C(M)
D(M)
A(T)
B(T)
C(T)
D(T)
Moisture (%)
Temperature (C)
Storage (Days)

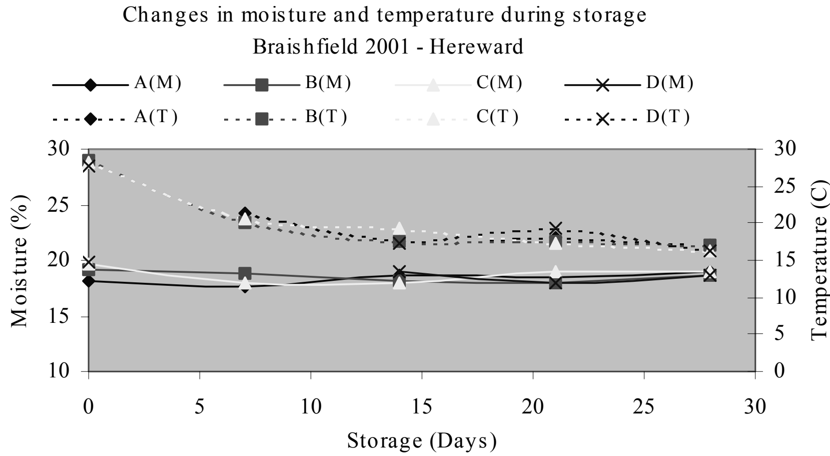

Changes in moisture and temperature during storage
Setterington 2001 - Halcyon
A(M)
B(M)
C(M)
D(M)
A(T)
B(T)
C(T)
D(T)
Moisture (%)
Temperature (C)
Storage (Days)

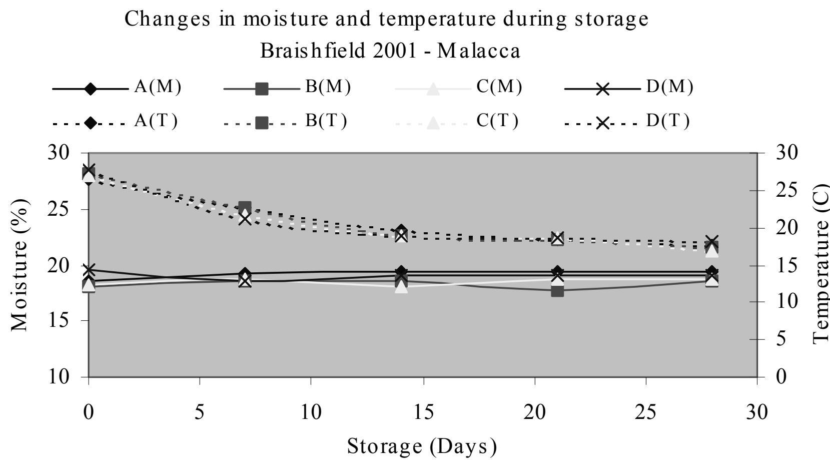

Changes in moisture and temperature during storage
Dover 2001 - Pearl
A(M)
B(M)
C(M)
D(M)
A(T)
B(T)
C(T)
D(T)
Moisture (%)
Temperature (C)
Storage (Days)

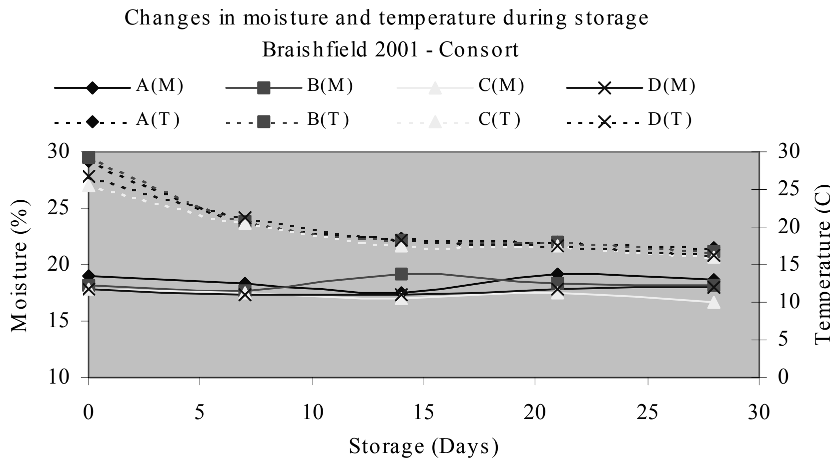

Changes in moisture and temperature during storage
Dover 2001 - Halcyon
A(M)
B(M)
C(M)
D(M)
A(T)
B(T)
C(T)
D(T)
Moisture (%)
Temperature (C)
Storage (Days)

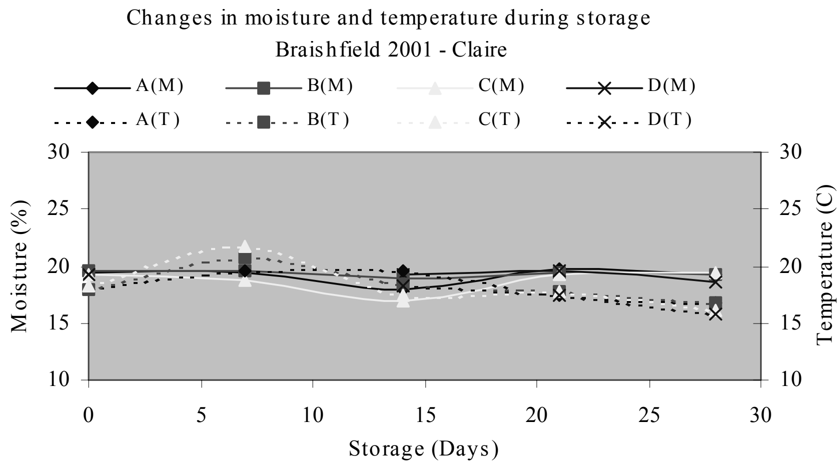
APPENDIX D
RECORD OF TEMPERATURE AND MOISTURE CONTENT DURING STORAGE

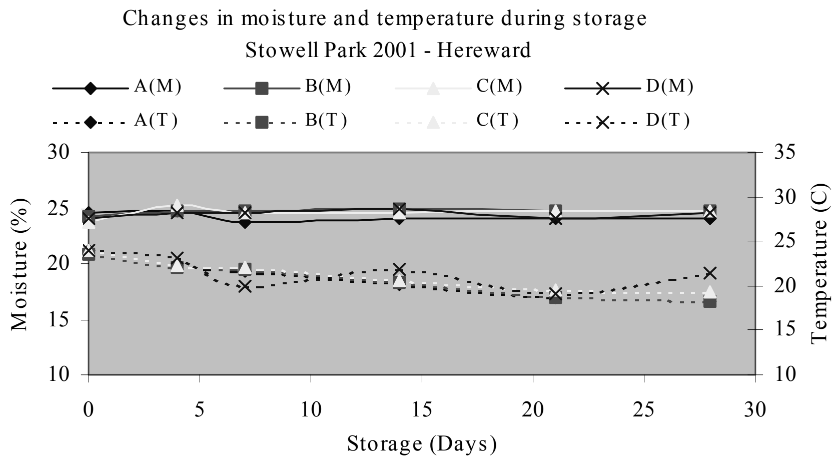
WHEAT

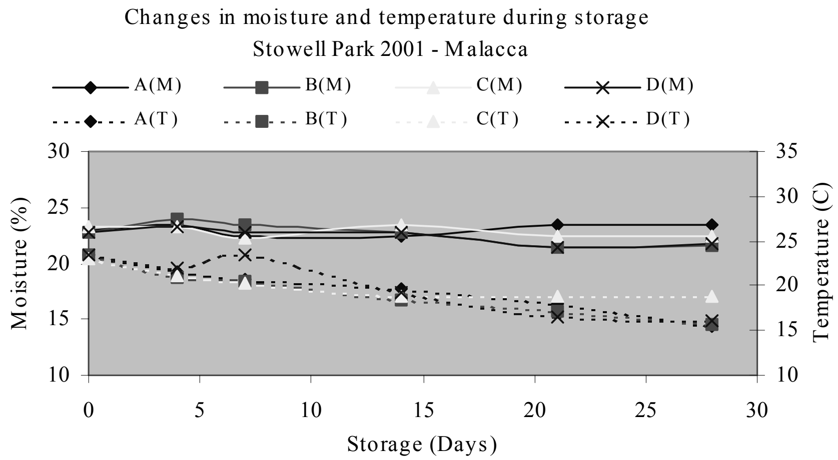


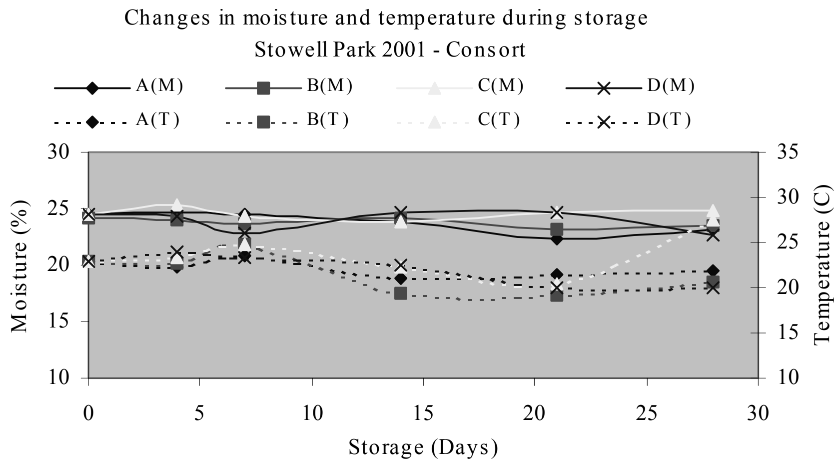


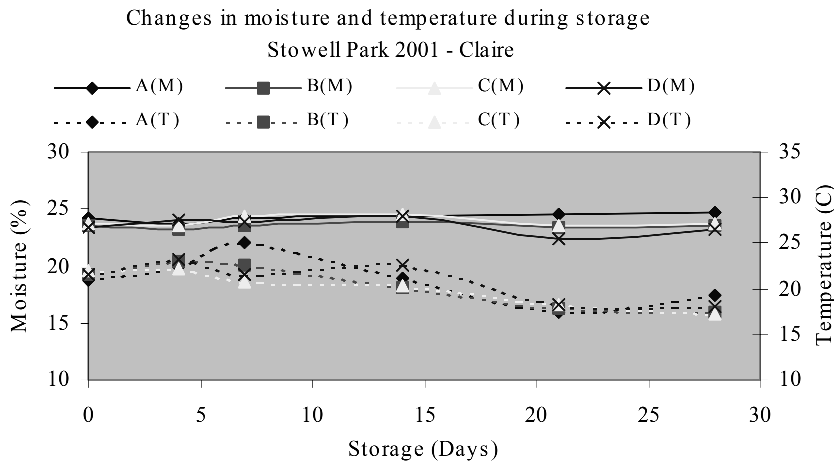


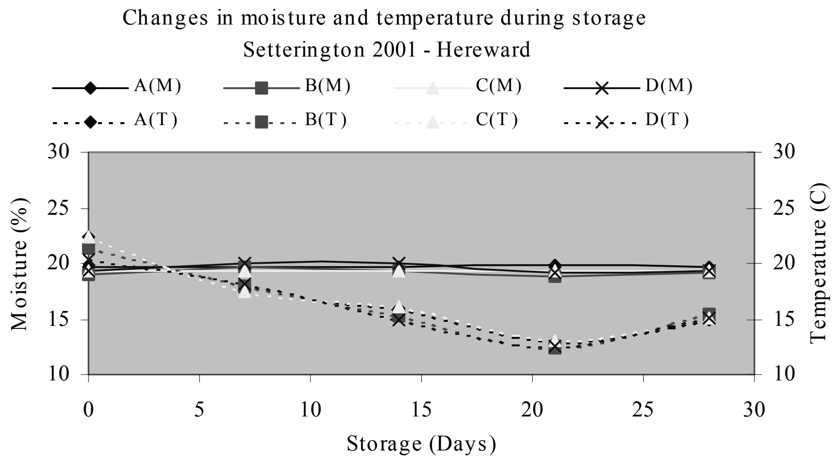

Changes in moisture and temperature during storage
Braishfield 2001 - Claire
A(M)
B(M)
C(M)
D(M)
A(T)
B(T)
C(T)
D(T)
Moisture (%)
Temperature (C)
Storage (Days)

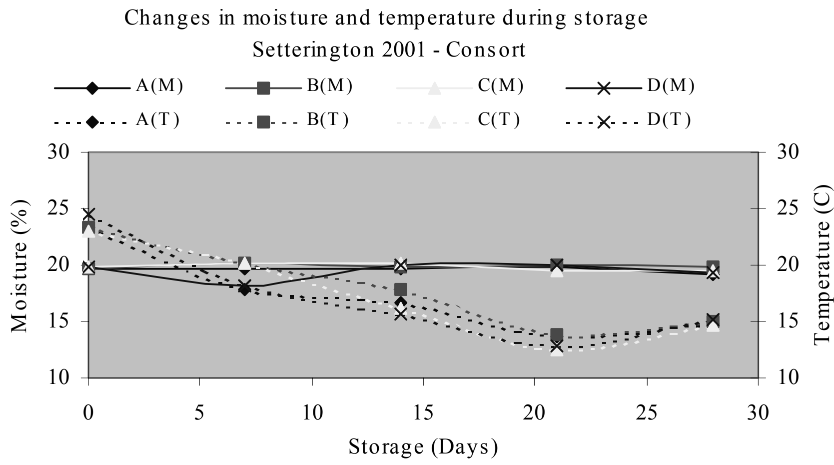

Changes in moisture and temperature during storage
Stowell Park 2001 - Hereward
A(M)
B(M)
C(M)
D(M)
A(T)
B(T)
C(T)
D(T)
Moisture (%)
Temperature (C)
Storage (Days)

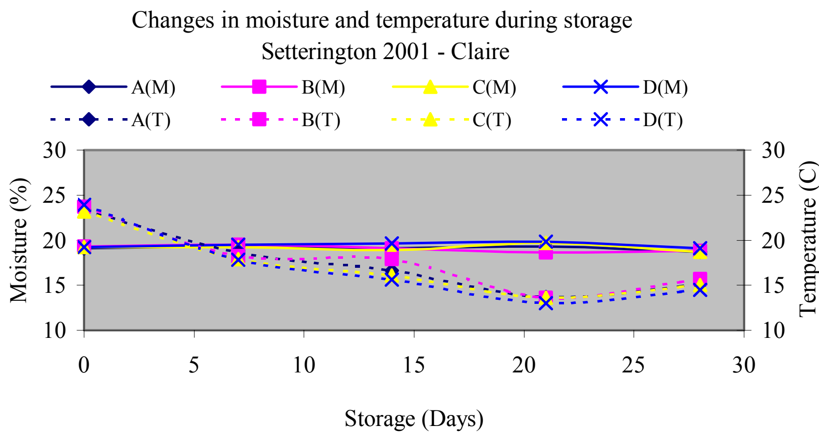

Changes in moisture and temperature during storage
Stowell Park 2001 - Malacca
A(M)
B(M)
C(M)
D(M)
A(T)
B(T)
C(T)
D(T)
Moisture (%)
Temperature (C)
Storage (Days)

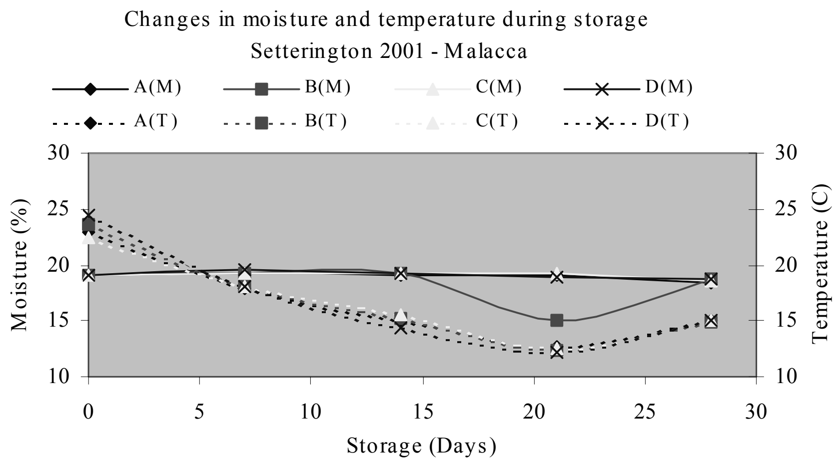

Changes in moisture and temperature during storage
Stowell Park 2001 - Consort
A(M)
B(M)
C(M)
D(M)
A(T)
B(T)
C(T)
D(T)
Moisture (%)
Temperature (C)
Storage (Days)
30
25
20
15
10
35
0
5

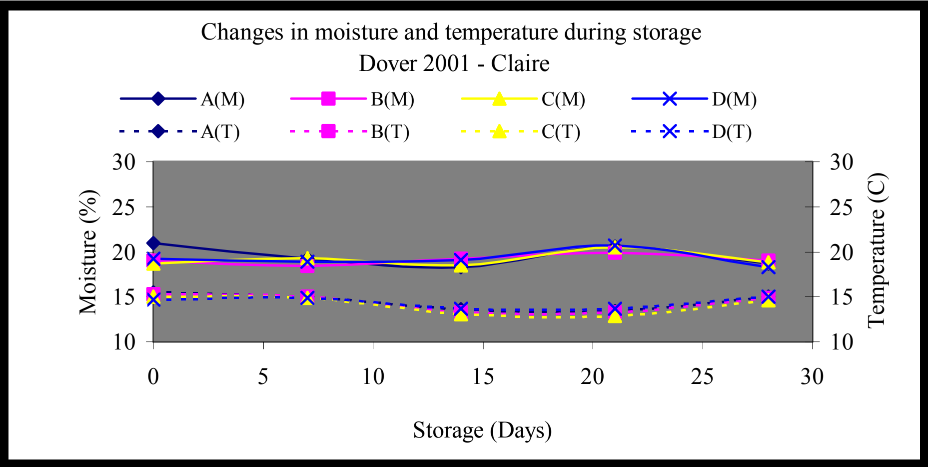

Changes in moisture and temperature during storage
Stowell Park 2001 - Claire
A(M)
B(M)
C(M)
D(M)
A(T)
B(T)
C(T)
D(T)
Moisture (%)
Temperature (C)
Storage (Days)
30
25
20
15
10
35
0
5


Changes in moisture and temperature during storage
Setterington 2001 - Hereward
A(M)
B(M)
C(M)
D(M)
A(T)
B(T)
C(T)
D(T)
Moisture (%)
Temperature (C)
Storage (Days)
30
25
20
15
10
0
5


Changes in moisture and temperature during storage
Setterington 2001 - Consort
A(M)
B(M)
C(M)
D(M)
A(T)
B(T)
C(T)
D(T)
Moisture (%)
Temperature (C)
Storage (Days)


Changes in moisture and temperature during storage
Setterington 2001 - Claire
A(M)
B(M)
C(M)
D(M)
A(T)
B(T)
C(T)
D(T)
Moisture (%)
Temperature (C)
Storage (Days)


Changes in moisture and temperature during storage
Setterington 2001 - Malacca
A(M)
B(M)
C(M)
D(M)
A(T)
B(T)
C(T)
D(T)
Moisture (%)
Temperature (C)
Storage (Days)


Changes in moisture and temperature during storage
Dover 2001 - Claire
A(M)
B(M)
C(M)
D(M)
A(T)
B(T)
C(T)
D(T)
Moisture (%)
Temperature (C)
30
25
20
15
10
0
5
10
15
20
25
30
Storage (Days)

APPENDIX E MOULD COUNTS AND SPECIES FOR CSL EXPERIMENTAL PLOTS

Table 1. Plate counts for HGCA CSL plots 2000 (1/4 rate fungicide application).

Inoculum	Species isolated	Colony counts after harvest (days) log CFU/g		
		4	7	14
Control	*Alternaria* spp.	4.4	4.0	3.7
	Aureobasidium pullulans	4.0		
MC=21.7%	*Cladosporium* spp.	5.0	5.0	4.6
Temp=20˚ C	*Epicoccum nigram*	2.7		
	Fusarium spp.	3.5	4.4	4.5
	Penicillium spp.		4.8	4.0
	Eurotium spp.			2.7
	Sporobolomyces roseus	4.8	4.4	4.2
	Yeasts	5.4	4.9	4.8
Cladosporium	*Alternaria* spp.	4.0	4.3	4.3
	Aureobasidium pullulans		4.2	
	Cladosporium spp.	4.9	4.4	4.8
	Fusarium spp.	4.4	4.0	4.3
	Penicillium spp.		3.9	3.7
	Wallemia sebi		3.7	
	Eurotium spp.			2.0
	Sporobolomyces roseus	5.3		4.8
	Yeasts	5.3	5.0	4.9
Alternaria	*Alternaria* spp.	4.2	4.4	3.7
	Aureobasidium pullulans	3.7		
	Cladosporium spp.	5.0	5.0	4.2
	Epicoccum nigram	2.0		
	Fusarium spp.	4.4	4.0	4.2
	Aspergillus spp.	1.7		
	Penicillium spp.		3.7	4.4
	Sporobolomyces roseus	4.6	4.8	4.0
	Yeasts	5.0	4.9	4.5
Mixed inoculum	*Alternaria* spp.	4.7	4.3	4.2
	Aureobasidium pullulans	2.7	2.7	3.5
	Cladosporium spp.	5.5	4.6	4.7
	Fusarium spp.	4.7	4.5	4.2
	Penicillium spp.	4.2	4.0	4.4
	Wallemia sebi		3.7	
	Sporobolomyces roseus	5.2	4.8	4.0
	Yeasts	5.6	4.9	4.6

Table 2. Plate counts for HGCA CSL plots 2000 (3/4 rate fungicide application).

Inoculum	Species isolated	Colony counts after harvest (days) log CFU/g		
		4	7	14
Control	*Alternaria* spp.	4.3	4.3	4.3
	Aureobasidium pullulans	3.7	4.0	
MC=21.7%	*Cladosporium* spp.	4.7	4.7	4.9
Temp=20˚ C	*Fusarium* spp.	4.4	4.5	4.4
	Penicillium spp.	2.5	4.0	5.0
	Eurotium spp.		2.3	2.5
	Sporobolomyces roseus	4.6	4.0	4.4
	Yeasts	5.6	5.0	5.3
Cladosporium	*Alternaria* spp.	4.4	4.2	4.0
	Aureobasidium pullulans		4.0	
	Cladosporium spp.	4.8	4.9	4.8
	Fusarium spp.	4.2	4.2	
	Penicillium spp.	2.7	4.5	5.0
	Penicillium verrucosum			2.7
	Wallemia sebi			
	Eurotium spp.			
	Sporobolomyces roseus	4.5	4.0	4.0
	Yeasts	5.4	5.0	4.7
Alternaria	*Alternaria* spp.	4.3	4.2	4.2
	Aureobasidium pullulans			
	Cladosporium spp.	4.8	5.0	4.8
	Epicoccum nigram			
	Fusarium spp.	4.4		4.5
	Eurotium spp.		2.0	2.0
	Penicillium spp.	2.7	4.2	5.0
	Sporobolomyces roseus	3.7	4.0	3.7
	Yeasts	5.4	5.1	4.3
Mixed inoculum	*Alternaria* spp.	4.4	4.2	3.0
	Aureobasidium pullulans	4.0		
	Cladosporium spp.	5.0	4.8	4.5
	Fusarium spp.	3.5	4.5	4.0
	Penicillium spp.		4.5	5.0
	Penicillium verrucosum			1.7
	Eurotium spp.		2.7	
	Sporobolomyces roseus	4.8	4.2	4.5
	Yeasts	5.4	4.7	4.0