SESSION 5B

MYCOTOXINS AND ASSOCIATED FOOD QUALITY AND SAFETY ISSUES

Chairman & Session Organiser:	Dr Anton J Alldrick *Campden & Chorleywood Food Research Association, UK*
Platform Papers:	5B-1 to 5B-4
Poster Papers:	P5B-5 to P5B-8

Fusarium mycotoxins in UK wheat production

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ABSTRACT

The European Commission has set maximum levels for fusarium mycotoxins in unprocessed cereals and cereal foodstuffs intended for human consumption. Legislation for deoxynivalenol and zearalenone will be introduced in July 2006. A five-year investigation was started in 2001 to assess the level of fusarium mycotoxin contamination in UK wheat production and the extent to which agronomic factors such as variety, crop rotation, land cultivation and fungicide application could affect this contamination. Each year c. 300 samples of wheat from fields of known agronomy were collected and analysed for ten trichothecenes and zearalenone. Results from the first four years have shown that the incidence of mycotoxins was generally low. Only five mycotoxins (deoxynivalenol, nivalenol, HT2, T2 and zearalenone) were detected in more than 5% of the samples tested. Of these deoxynivalenol was the predominant mycotoxin found, although the results are from selected samples rather than a stratified survey and therefore may not accurately represent the true UK situation. Preliminary statistical analysis of the four year dataset has shown that maize as previous crop in combination with minimum cultivation are important agronomic factors for deoxynivalenol contamination. Results from the completed project will be used to determine "Good Agricultural Practice" to minimise mycotoxin contamination of wheat.

INTRODUCTION

Fusarium mycotoxins are produced on cereal grains by many *Fusarium* species during fusarium head blight infection. The predominant mycotoxins found in cereals are the trichothecenes (which include deoxynivalenol (DON, also known as vomitoxin), nivalenol (NIV), HT2 and T2) and zearalenone (ZEAR). These mycotoxins are produced mainly in the field, although levels can increase further during grain storage under adverse conditions

The European Commission has set maximum levels for fusarium mycotoxins in unprocessed cereals and cereal foodstuffs intended for human consumption. Legislation for DON and ZEAR will be introduced in July 2006 (Anon., 2005). Maximum limits for DON and ZEAR in unprocessed wheat will be 1250 and 100 ppb (parts per billion), respectively. Lower limits are set for flour and finished cereal products. Legislation for HT2 and T2 is expected to follow. Previous one year surveys of fusarium mycotoxins have shown that levels are generally low within the UK (Turner *et al.*, 1999; Prickett *et al.*, 2000). However, the effects of different seasons and crop management on mycotoxin levels in UK wheat are unknown.

The main aim of this project was to determine the effects of agronomic factors on fusarium mycotoxin levels in UK wheat grain over a five year period (2001 – 2005). The results of the first four years of the project are detailed in this paper (2001-2004). Results from the completed project will be used to advise growers on "Good Agricultural Practice" to reduce fusarium mycotoxins during wheat production.

MATERIALS AND METHODS

Three hundred grain samples were collected at each harvest by crop consultants and growers. An equal number of samples were requested from each region: South, East, Midlands, North, Scotland and Northern Ireland.

An even number of grain samples were requested from each of the following categories; wheat left untreated at T3 (Zadoks' Growth Stage 59-69, Zadoks *et al.*, 1974), wheat sprayed with triazole, wheat sprayed with strobilurin (discontinued after 2002 due to anti-resistance legislation), wheat sprayed with a combination of triazole and strobilurin at T3 and organic wheat. Three kilogram grain samples were collected from at least ten points within the field at harvest. Samples were ripple divided and 500 g of grain was retained for visual assessment. The remaining 2.5 kg was milled using a 1 mm screen in a ZM100 Ultra Centrifugal Mill (Retsch, Haan, Germany). Milled samples were mixed in a tumbler mixer (Pascall, Crawley, UK) for 5 minutes before three 200 g laboratory sub-samples of flour were removed. One of these was sent to each analytical laboratory for mycotoxin analysis, the third was kept as an archive at -20°C. Agronomy data detailing the source, previous crop, cultivation, variety, intended use and fungicide inputs was collected for each grain sample.

Samples were analysed by RHM Technology, High Wycombe, UK by GC-MS analysis for ten trichothecenes (Limit of Quantification = 10 ppb) and by Central Science Laboratory, York, UK by HPLC analysis for ZEAR (Limit of Quantification = 5 ppb). The trichothecenes analysed were deoxynivalenol (DON), nivalenol (NIV), 3-acetylDON, 15-acetylDON, fusarenone X, T2 toxin, HT2 toxin, diacetoxyscirpenol (DAS), neosolaniol and T2 triol.

Statistical analysis was performed on Genstat 7 (Lawes Agricultural Trust, Harpenden, UK). Values of DON below the limit of quantification (10 ppb) were imputed as 5 ppb. DON values were log10 transformed to stabilise the variance. The data sets from the first four years were combined and significant agronomic factors were selected using a stepwise model selection ANOVA. Temporal (year) and spatial (region) factors were forced into the model. All other factors were placed in the model in chronological order in which they occur in the season. For each significant factor the back-transformed predicted mean and 95% confidence limits were calculated.

RESULTS

Incidence of fusarium mycotoxins in UK wheat in the first four years of the project (2001-2004) were generally low with only four mycotoxins detected in more than 5% of samples tested. The predominant mycotoxins found are detailed in Table 1. Data for HT2 and T2

were pooled as future legislation will be based on the combined concentration of HT2 and T2. HT2 was the major component of this data.

Table 1. Mycotoxin content of UK wheat at harvest (2001-2004, 1298 samples).

			Mycotoxin concentration (ppb)				
	%>10ppb[1]	%>LL[2]	Mean	Median	90th%	95th%	Max
DON	85	2.6	227	38	336	716	20333
NIV	68	NLI	28	17	67	98	430
HT2+T2	55	NLS	11	<20	29	42	214
ZEAR	41	3.6	20	<5	30	75	1292

[1]Percentage of samples above the limit of quantification for trichothecenes (10 ppb). [2]Percentage of samples above the legal limit to be introduced in July 2006 for unprocessed wheat (1250 ppb DON and 100 ppb ZEAR). NLI, no legal limit intended; NLS, no legal limit set (to be introduced 2007-2008). Means are based on an imputation of 1.67 (0.83 for ZEAR) for all samples below the limit of quantification (10 ppb; 5 ppb for ZEAR).

The incidence and concentration of fusarium mycotoxins in UK wheat were similar in each year tested. The vast majority of samples were below the current EU proposed maximum limits of 1250 ppb for DON and 100 ppb of ZEAR in unprocessed wheat. The concentration of DON found in UK wheat from 2001 to 2003 was generally low (mean of 140 ppb) compared to levels found in other European countries and elsewhere in the world (Anon., 2001). In 2004, however, the mean DON content was 469 ppb. It should be noted that these results are from selected samples and are not a stratified survey thus the mean values may not accurately represent the true UK mean values.

The most noticeable differences in the four years were higher incidence of HT2+T2 in 2003 and higher concentrations of DON and ZEAR in 2004. In two out of the four years, 2001 and 2004, ZEAR exceeded the forthcoming limits in more samples than DON.

Statistical analysis of the data from the first four years showed that year, region, previous crop, cultivation and varietal fusarium head blight resistance score had significant effects on the concentration of DON in wheat samples. There were significant interactions between year and region, and between previous crop and cultivation. The interaction of year and region indicate the importance of climate on mycotoxin production. Regional differences indicated that DON contamination decreased northwards (Figure 1). This was probably due to climate variation in the regions (some *Fusarium* pathogens prefer warmer conditions). Ploughing following maize or cereals reduced DON contamination of wheat (Figure 2). However, ploughing failed to provide the same benefit following non-cereal crops. An inverse relationship existed between fusarium head blight resistance rating and DON content of grain samples for winter wheat cultivars (Figure 3). Although, there is no available UK score for fusarium head blight resistance of Petrus, a German variety, it is known to have a good resistance to fusarium head blight (Buerstmayr *et al.*, 2004). DON content of spring wheat varieties was similar to DON content of winter wheat varieties with disease resistance ratings of six and seven.

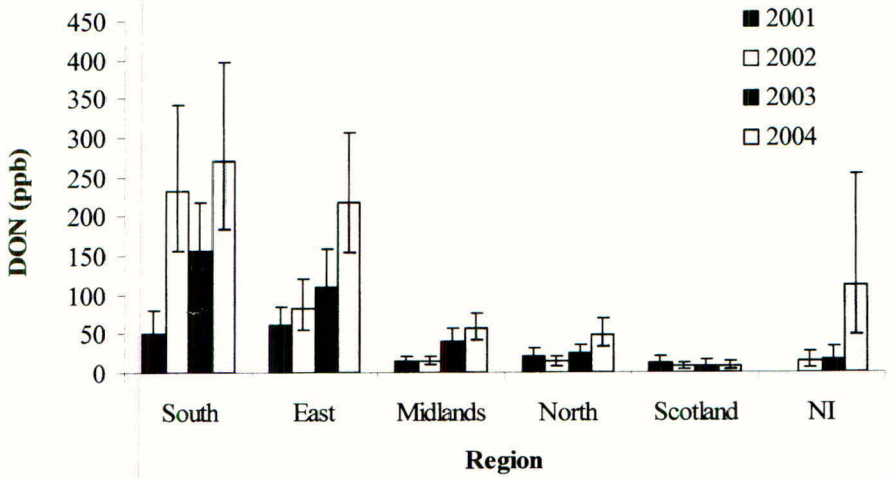

Figure 1. DON contamination of wheat by region for each year. Samples were not obtained from Northern Ireland (NI) in 2001. Bars represent 95% confidence limits.

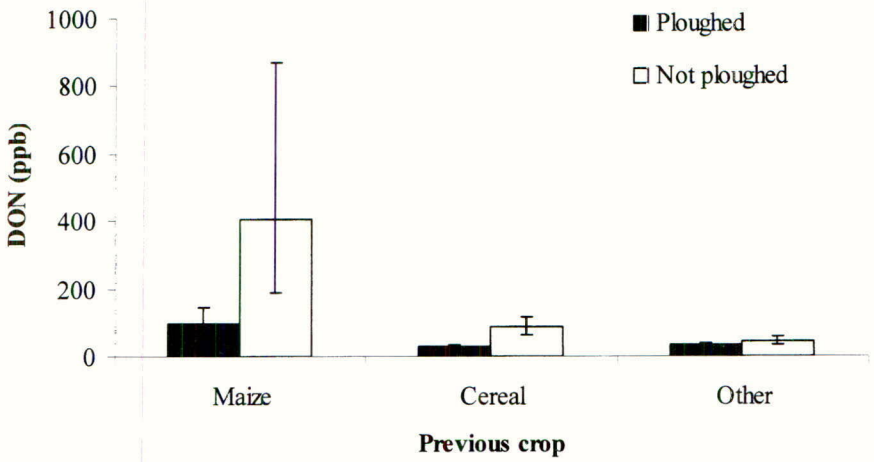

Figure 2. Effect of cultivation and previous crop on DON contamination of wheat. Bars represent 95% confidence limits.

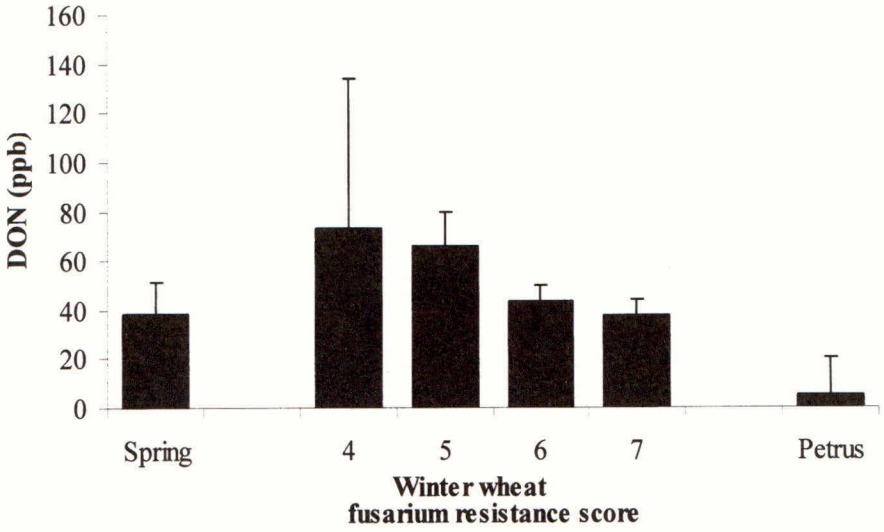

Figure 3. DON content of samples grouped by Fusarium ear blight resistance rating. Bars represent 95% confidence limits.

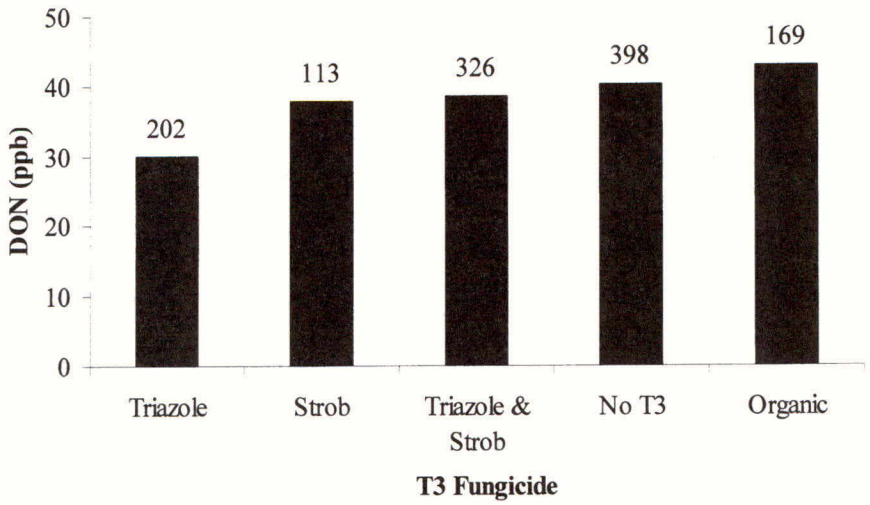

Figure 4. DON content of wheat samples grouped by T3 fungicide use. Number above each column represents sample size for that regime.

There was no significant difference for DON content in harvested grain between organic and conventional systems or between fungicide regimes at T3 (Figure 4). This maybe due to

grouping fungicides together as triazoles and strobilurins and/or the low rates at which fungicides were used on the field. T3 fungicides were further divided to distinguish samples which had received a triazole registered for fusarium ear blight control. Analysis still showed no significant difference between T3 regimes. This is probably due to the low number of samples which received these chemicals at optimum rates and timings.

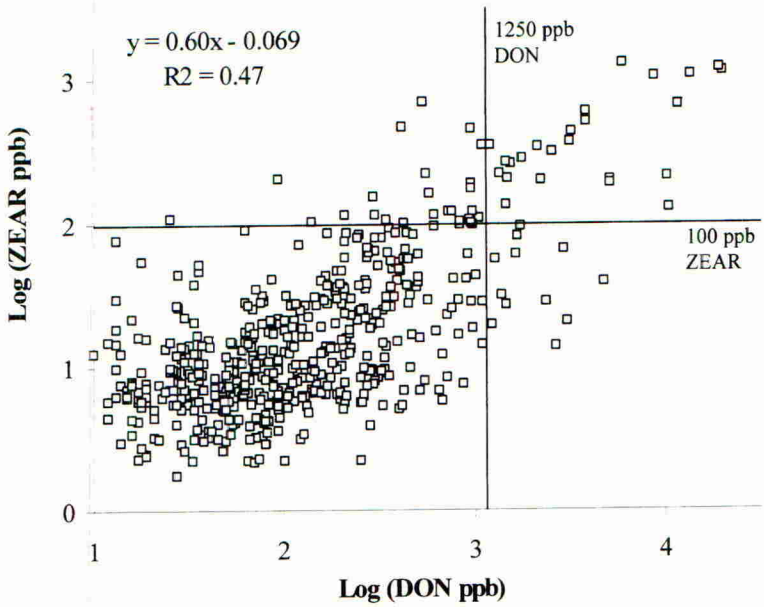

Figure 5. Relationship between concentration of ZEAR and DON detected in samples above the limits of quantification in 2001-2004. Lines show forthcoming limits for DON and ZEAR in unprocessed wheat.

Although, ZEAR occurred at a lower frequency than DON above 10 ppb, it exceeded the legislative limit more frequently than DON (Table 1). Regression analysis revealed a significant positive relationship between ZEAR and DON concentration, although, half of the samples which exceeded the forthcoming limit for ZEAR were below the limit for DON (Figure 5).

DISCUSSION

Fusarium head blight pathogens infect cereals primarily at anthesis and the weather conditions during this period are important in relation to the incidence and severity of the disease (Birzele, 2002). Similarly, the production and abundance of fusarium mycotoxins can also vary between seasons and across regions. DON was the predominant mycotoxin detected in UK wheat samples with an incidence above the limit of quantification of 85% and a mean concentration of 227 ppb. However, ZEAR exceeded the forthcoming

legislative limit more frequently than DON, because of its lower limit of 100 ppb, although the results of this study are from selected samples rather than a stratified survey and therefore may not accurately represent the true UK situation. Evidence from this project indicates that zearalerone maybe of more concern in some years than DON and as such it is important that this mycotoxin is monitored and the factors affecting its concentration in harvested wheat are determined.

The presence of significant interaction between previous crop and cultivation indicated the importance of host crop debris in the life-cycle of fusarium head blight pathogens. When wheat crops were grown following a host of *Fusarium* spp. such as maize or cereals, levels of DON were higher. Particularly high DON concentrations were found where maize was the previous crop since it is an alternative host to *Fusarium graminearum* which is known to be a potent DON producer. However, DON levels were significantly lower in wheat crops following a host of *Fusarium* where ploughing was practised in comparison to wheat crops following a host but with minimum cultivation.

Winter wheat varieties with higher resistance ratings to fusarium head blight also had lower DON levels. Furthermore, the presence of lower DON content in the limited number of Petrus samples indicated the good potential for the use of varietal resistance for the reduction of fusarium mycotoxin accumulation in wheat. The failure of T3 fungicides to reduce DON in the sampled commercial crops was probably due to the large number of different fungicides used within each category and the use of less than optimum rates and/or timing in the majority of samples. Results from replicated field trials have shown that metconazole, prothioconazole or tebuconazole used at half or full rate can significantly reduce DON levels in harvested grain (Nicholson *et al.*, 2003). Previous studies using a low number of wheat samples from conventional and organic crop regimes have failed to show a consistent difference between the two (Birzele *et al.*, 2002; Champeil *et al.*, 2004). This study, however, has benefited from a larger number of organic samples and results indicate that there is no significant difference for DON concentration between organic and conventional wheat samples in the UK.

Results from similar studies in Germany (Obst *et al.*, 2000) and France (Barrier-Guillot *et al.*, 2004) have shown similar effects of agronomy on DON contamination of wheat. Analysis of all 1500 samples over five years will provide a clear picture of fusarium mycotoxin levels in UK wheat over a range of different seasons and will allow major statistical analysis of all agronomic factors. Results will aid the cereal industry to prepare for EU legislation on the maximum permissible levels of fusarium mycotoxins in cereal grains and products. Simultaneously, results will also be used to advise growers of "Good Agricultural Practice" to minimise fusarium mycotoxin levels in UK wheat production.

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Ecology and control of ochratoxin in grapes and dried vine fruits

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ABSTRACT

The population dynamics of mould contamination of Corinth currants and sultanas during drying was investigated in Greece in 2004. This showed that the total fungal populations and frequency of isolation was changed with time during the 10-14 day drying period. Altitude above sea level affected the final contamination levels, especially with the mycotoxigenic *Aspergillus carbonarius*. The main species isolated were *Aspergillus* section *Nigri* and *A.carbonarius*, yeasts, *Penicillium* species and occasionally *Cladosporium*, *Alternaria* and *Botrytis* species. The highest frequency of isolation and of ochratoxin (OTA) was isolated from currants/sultanas at sea level and the lowest concentration at the highest altitude (600-1000m). Potential for control of *A.carbonarius* and OTA production by isolates from currants/sultanas using Sodium metabisulphite and controlled atmospheres were evaluated. This showed that potential for control of growth required 750-1000 ppm *in vitro* on a grape juice medium, regardless of environmental conditions. However, >500 ppm was effective for OTA control.

INTRODUCTION

The production of raisins is a traditional cultivation in Greece and mainly two types of raisins are cultivated, the golden raisins called sultanas and the black Corinth raisins (currants). The latter have taken their name of the region of Corinth where this type first grew, the name currant, also, is probably a corruption of the word Corinth. There is a big industry of dried fruits aiming both at the internal and most importantly at the external markets.

Currants are made from the black Corinth grapes. The grapes are harvesting by hand in August and September and placed in the sun to dry. The drying process lasts for about 7-14 days depending on the weather and from time to time they are turned and swept into heaps, until completely dried, the growers then sort out the dried product (Dekanea, 2005). For the sultanas the process is similar except that often potassium carbonate is added during drying for enhancing the desiccation process. At the end of drying the currants/sultanas are taken to industry, quality levels are measured and, if necessary, insect infestation is controlled using methyl bromide or in some cases CO_2 and then the product is stored before processing. The raisins are usually stored in areas with humidity not >60% and a temperature of 12-18°C. Before package the raisins are checked for ochratoxin (OTA). If they are free of OTA and

have the quality levels that are desired the final product is packaged in paper cartons or polyethylene bags.

The highest OTA content, among grapes and its derivatives, has been measured in dried vine fruits (MAFF, 1997) with > 40 μg/kg. Macdonald *et al.* (1999) found a maximum level of 53.6 μg/kg in black dried vine fruits (currants). Magnoli *et al.* (2004) did a survey of black and white dried vine fruits from Argentinian markets. OTA was detected in 67.7% of the black and 84.2% of the white dried vine fruits with mean levels of 6.3 and 4.42 ng/g, respectively. The highest concentration was found in a black dried vine fruit sample (14 ng/g). Another recent survey took place in Sweden for two years (Möller and Nyberg, 2003) found a range of < 0.1-19.0 μg kg^{-1} in 1999/2000, with a median concentration of 0.9 μg/kg. In 2001/2002 the range was <0.1-34.6 μg/kg with a median of 0.2 μg/kg. In the second study, which was based on nearly the same brand and products, the median OTA was much lower. The results from the first study were similar to those found in the UK by MacDonald *et al.* (1999) and in Greece by Stefanaki *et al.* (2003).

There is little knowledge of the fungal population dynamics and diversity during the drying process. The conditions which allow preferential dominance of the mycotoxigenic *A.carbonarius* have not been previously identified. The objectives of this study were to examine the effect of natural drying regimes on the ecological changes in fungal community structure at different altitudes in drying raisins in two areas of Greece (Peleponisa and Crete). The potential of using sodium metabisulphite to control growth and OTA production was also examined *in vitro*.

MATERIALS AND METHODS

Sampling area

Samples of drying currants were obtained from the region of Aeghio in northeast Peloponnese, Greece, a characteristic production region of black raisins (Corinth currants) with the distinctive name of "Vostizza". Sultanas were obtained from different areas in Crete. Three replicate vineyards from different drying areas were chosen during the 2004 season to represent three different altitudes, sea level, 300-500 m, and 600-1000 m. Just prior to harvest, and after 4, 8 and just prior to storage 1-2 kg samples were taken at random.

Mycological analysis of grapes and currants

To determine the mycoflora on grapes before and during sun drying of raisins two identification media were used: Malt extract agar (MEA, Oxoid Ltd) that was made up as directed and MEA + glycerol a modified water activity level of 0.95 (MEA95). In both media a small amount of chloramphenicol was added prior to sterilization at 121°C for 15 minutes, to inhibit bacterial growth. From each sample 10 g were taken and suspended in 90 ml diluent (sterile-distilled water + 0.5 g agar + 0.005% Tween 80) and homogenized in a Colworth Stomacher 200 (Seword Ltd) for 10 min. The mixture was then serially diluted. From each dilution, 0.2 ml was spread using a sterile glass spreader onto the surface of both media (four replicates of each). All transfers were made with an automatic pipette and disposable sterile tips. After 7 days the numbers of colonies in the serial dilution method were counted and reported as colony-forming units (CFU)/gram of sample.

Ten berries per bunch were randomly selected and directly plated onto MEA and MEA95. The berries were aseptically cut in half before plating in Petri plates. All the Petri plates were incubated at 25°C. Fungal identification of black *Aspergilli* was carried out to species level only for *A. carbonarius*, while the others isolates were grouped in the *A. niger* aggregate. The aim was to identify and differentiate the percentage *A. carbonarius* out of the *A. niger* aggregate and the total mycoflora

OTA production ability of isolates of *A. carbonarius*

Representative strains of *A. carbonarius* were single point inoculated on 50% coconut cream agar CCA (Dyer and McCammon, 1994). The plates were incubated for 7 days at 25°C. The reverse side of each plate were observed under long-wave ultraviolet (UV) light (365nm) for the characteristic blue-green fluorescence produced by ochratoxin A. After 7 days incubation the plates were destructively examined by exposure to 25% ammonia. The plates were left for 1 h and then under UV light. OTA has an intense violet fluorescence.

Ochratoxin analysis of grapes and dried vine fruits

The detection of OTA concentration in grapes and currants was performed by HPLC, following the methodology proposed by Zimmerli and Dick (1996). Injections of 50 μl standard or samples were injected into an HPLC system consisting of a Perkin Elmer 200, equipped with an ISS 200 sampling system and a Perkin Elmer LC 420 fluorescence detector set at 333nm excitation and 470 nm emission. A Spherisorb Excel ODS2 (250x4.6mm; 5μm) column was employed with a mobile phase of acetonitrile-water-acetic acid (57:41:2) at a flow rate of 1.00 ml min^{-1}. The limit of detection was estimated as 0.2 μg/kg.

In vitro* ecological studies on isolates of *A. carbonarius

Six isolates of *A. carbonarius* were used in this study. Three from currants/sultanas respectively, based on those showing very high fluorescence in the screening assay. For comparison an isolate (isolate DM) from wine grapes with known OTA producing ability was also used (Mitchell *et al.*, 2004).

Effect of a_w and sodium metabisulphite (NaMBS) on growth and ochratoxin production

Grape juice agar medium (GJM) was used as the basal medium throughout the in vitro studies. The medium was prepared by mixing 25% (v/v) supermarket long life red grape juice and 2.5% agar (Oxoid, UK technical agar no.3) in distilled water. The medium after the water activity and NaMBS adjustment was sterilized at 121°C for 15 min, cooled to approximately 50°C and poured into sterile 90 mm diameter sterile plastic Petri plates.

Adjustment of the medium a_w to the required treatment was done by adding D(+) glucose (Sigma) to the basal medium. The a_w of this basic medium was 0.985, determined with a Humidat Sprint IC II thermoconstanter (Novasina, Zurich, Switzerland). Two more a_w treatments was used: 0.965 and 0.93. Those were obtained by adding 18.73 and 50.35g D(+) glucose 100/g medium, respectively. The pH of all treatments was modified to 4.2 using a buffer solution. A 100ml solution of this buffer was made by mixing 55.90 ml of 0.1 M citric acid $C_6H_8O_7 \cdot H_2O$ (AnalaR®) and 44.10 of 0.2 M sodium phosphate Na_2HPO_4 (Sigma). Sodium metabisulphite ($Na_2S_2O_5$; NaMBS; BDH Chemicals Ltd, Poole, England) was used as

source for the SO_2. Six concentrations were used: 0, 100, 250, 500, 750 and 1000 ppm. The Petri plates of each treatment were centrally inoculated with a small loop of spore suspension (10^6 spores/ml). All experiments were carried out with 4 replicates per treatment and incubated at 25°C.

RESULTS

Population dynamics of fungi on drying vine fruits

Figure 1 shows an example of the changes in fungal populations in a field site for currants at sea level for the four main component species on media at 0.95 a_w. This shows that just prior to harvesting and drying there were differences between dominant populations. At sea level yeasts, *Penicillium* spp. and *Aspergillus* section *Nigri* were isolated in about log_{10} 3 CFUs/g grapes. At the other two altitudes no *A. carbonarius* populations were isolated from the pre-harvest grapes (data not shown). Just prior to harvest, at the low altitude, *A.carbonarius* and *A. niger* group represented about 50% each with Log_{10} 4-5 CFUs/g of the final fungal load. At higher altitudes this was about 30% as *Penicillium* spp. were also present. Figure 2 shows the effect of altitude on the mean frequency of isolation of different species from sultanas during drying based on direct plating onto agar medium modified to 0.95 a_w. This shows that there were a higher frequency of *A.carbonarius* isolated from drying sultanas at sea level than at higher altitude. Contamination with OTA was higher at sea level than at higher altitude with the range being 2.83-11.38 and 1.81-1.62 µg/kg currants respectively (Dekanea, 2005).

Representative isolates *A. carbonarius* were monitored under UV light for the characteristic fluorescence that ochratoxin A produce, using the coconut cream agar and NH_4 solution. Out of the 43 isolates examined from currants 32 (74.4%) produced characteristic fluorescence. For sultanas a total of 45 isolates were examined and 58% were found to be OTA producers.

In vitro control using Sodium metabisulphite

Figure 3 shows the effect of treatments on the growth rate (mm/day) of some of the isolates examined in this study. The use of NaMBS significantly affected the growth rate of all isolates although high concentrations were required for control. Figure 3 shows the efficacy of NaMBS concentrations required for controlling growth of an isolate from sultanas. For more than 50% inhibition concentrations at least 500 ppm NaMBS was required. For complete inhibition, up to 1000 ppm was required for all isolates (6) examined, regardless of the a_w level over the experimental period. For all *A. carbonarius* isolates growth was stimulated by 100-250 ppm NaMBS. All strains had an optimal a_w for growth at 0.985 in the presence of 100 ppm NaMBS. However, there was little difference in relative growth rates at 0.985 and 0.965 a_w. Comparisons between the isolates showed that similar growth patterns occurred under the treatment tested. There was also little difference between isolates from drying vine fruits and from grapes for wine production.

The effect of NaMBS x a_w interactions showed that between 500-750 ppm was required for effective control of OTA production by isolates, regardless of whether from currants or sultanas. Table 1 shows the LD_{50} and LD_{90} values for control of OTA production using NaMBS. This shows that at 0.985 aw at least 650-700 is required for 90% inhibition of OTA. However at the lower a_w levels 400-600 was adequate for 90% inhibition of OTA production.

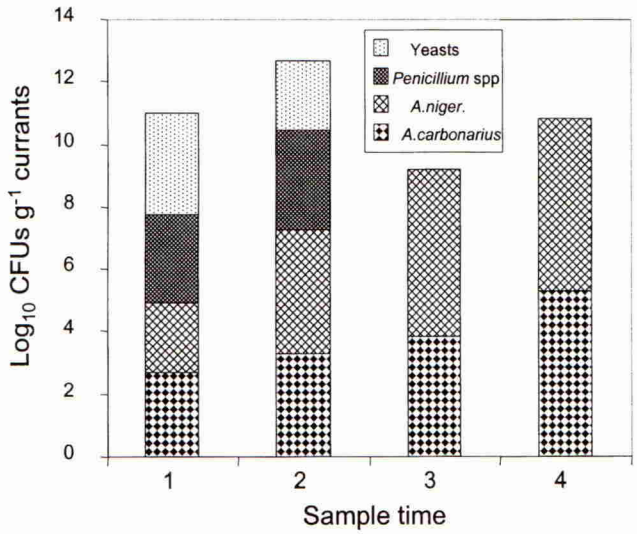

Figure 1. Changes in dominant fungal species/genera on currants prior to harvest to storage at sea level on malt extract agar (0.95 a_w). Key: 1, pre-harvest; 2, 4 days of drying; 3, 8 days drying; 4, end of drying, prior to storage.

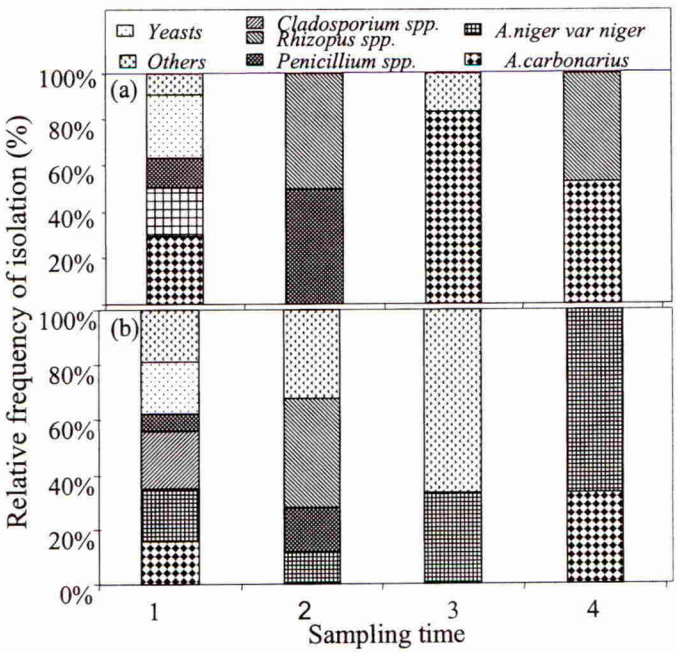

Figure 2. Relative frequency of isolation of different species from direct plated drying sultanas from (a) sea level and (b) 600-800 meters above sea level.

Key: 1, pre-harvest; 2, 4 days of drying; 3, 8 days of drying; 4, end of drying, prior to storage.

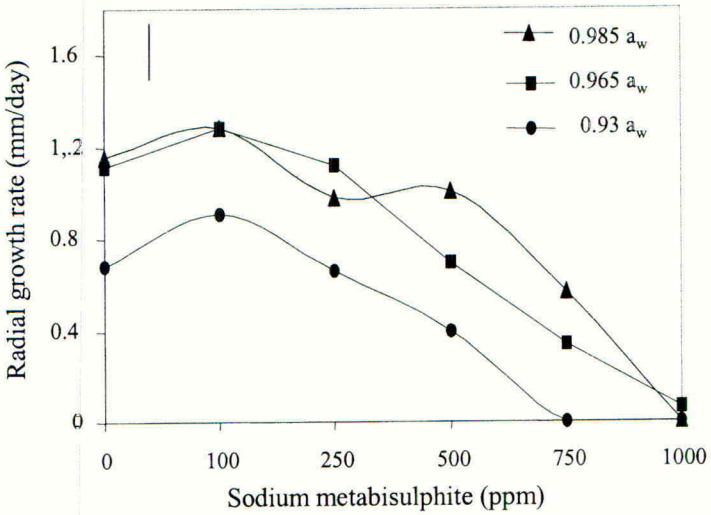

Figure 3. Effect of sodium metabisulphite (ppm) on mycelial growth of a isolate of *A.carbonarius* from sultanas at three different water activity levels at 25oC on a white grape juice medium. Bar indicates LSD (P=0.05).

Table 2. The LD_{50} and LD_{90} concentrations of sodium metabisulphite required for inhibiting ochratoxin A production by three isolates of *A.carbonarius* isolated from currants and grown on red grape juice medium at 25°C.

	NaMBS (ppm)	
	LD_{50}	LD_{90}
Water activity	**Strain DM**	
0.985	600	690
0.965	170	620
0.93	420	480
	Strain 119	
0.985	345	640
0.965	200	465
0.93	400	450
	Strain 127	
0.985	550	735
0.965	185	430
0.93	235	400

DISCUSSION

This study has shown that the incidence of *A. carbonarius*, which is considered to play the major role in ochratoxin A contamination of grapes and dried vine fruits although other black

Aspergilli are also an important component. Generally, the final *A.carbonarius* and *A.niger* aggregate species were present at up to log 3-4 CFUs/gram currants/sultanas at the end of the drying period, especially in fields at sea level. It is known that the spores of black *Aspergilli* are present in vineyard soil in high concentrations (Kazi *et al.*, 2003). Moreover, Battilani *et al.* (2003) demonstrated that *Aspergilli* section *Nigri* are present on wine grapes early in the growing season and that their population increased during later grape maturity. The present study shows that the population of section *Nigri* does gradually increase during drying of grapes for raisin production resulting in almost all being contaminated just prior to storage. Currants and sultanas have a high sugar content which becomes more concentrated as they dry. Thus they may provide a selective nutritional substrate which is conducive to growth of black *Aspergilli* and other such xerotolerant spoilage fungi. There were differences which depended on altitude above sea level for both currants and sultanas. There are certain differences in the meteorology between fields at sea level and those at high altitude. The latter, has cooler mean temperatures and lower relative humidities.

The increase in the populations of *A. carbonarius* during drying could be due to the fact that the drying area is usually on the ground and the currants/sultanas are more exposed to contamination for quite a long period. This coupled with the sugar content could predispose them to colonisation. Furthermore, contamination can be worse if growers do not remove any heavily mouldy bunches. No previous studies have shown the progress of black *Aspergilli* populations and colonisations during drying of raisins. In Australia, Leong *et al.* (2004) studied the black *Aspergilli* populations during drying and demonstrated an increase during the initial stages of drying with a slight decrease in the latter stage. However, in their study rainfall was an important factor. In the present study there was no rainfall during the sampling period.

Although *A. carbonarius* was present on fresh grapes no ochratoxin was detect in these samples. The occurrence of ochratoxigenic fungi does not necessarily mean OTA production occurs (Battilani *et al.*, 2003). Besides, it is known that the optimum conditions for OTA production are different than those for *Aspergilli* growth, at least under *in vitro* conditions (Mitchell *et al.,* 2004). Similarly, OTA producing fungi from the *A.niger* group have not always been isolated when OTA has been found to contaminate samples (Sage *et al.*, 2002). It is well established from previous studies that OTA naturally occurs on grapes and raisins. However, it is difficult to compare our results with those from the literature because previous studies have used raisins purchased from the market and so have been industrially processed and packaged. They thus often have a lower black *Aspergilli* population. However, MacDonald *et al.* (1999) reported higher OTA concentration (53.6 µg/kg) in such samples while others reported lower concentrations (Stefanaki *et al.*, 2003; Magnoli *et al.*, 2004).

The present study examined the efficacy of sodium metabisulpite to control germination, growth and ochratoxin A production of *A. carbonarius* strains. For complete mycelial growth inhibition a concentration of between 750-1000 ppm was required regardless of the a_w level used. The ochratoxin production appeared to be inhibited by up to 750 ppm. At 0.95 a_w the concentration required for inhibition was lower (500 ppm). However, at lower concentrations (100, 250 ppm) *A. carbonarius* growth was stimulated. In the literature there are few studies to examine the effect of SO_2 on the growth of other fungi but to our knowledge this is the first studies where *A. carbonarius* strains have been considered. However, *A.niger* was effectively controlled in onions by $S0_2$ at 1% (v/v) exposure for 72 hrs (Tamizharasi and Narasimham, 1992).

Generally, the information collected from previous studies suggests that the threshold SO_2 concentration may vary considerably between fungi. Those differences could be due to the fact that several factors influence the efficacy of SO_2. Particularly, the tolerance of *Penicillium* species to high concentrations of SO_2 has been suggested to be due to their ability to actively transport the SO_2 into the mycelia. In the present study it is possible that a percentage of SO_2 was absorbed and bound to the glucose substance reducing its antifungal activity.

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Estimation of the uncertainty of sample processing for the analysis of fumonisin FB1 in maize

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ABSTRACT

Mycotoxins are toxic materials produced by various fungi that grow on several food and feed commodities before harvest and/or during storage. They may be present in very high concentrations in confined small portions of the stored commodity and have a patchy distribution. For this reason, large bulk samples have to be taken and analysed for toxin content with quantifiable uncertainty. On the other hand, large samples are neither easily nor economically transported and analysed in the laboratory, therefore the bulk sample must be reduced in size to obtain a test portion that is of suitable size, and is representative of the average concentration of the contaminants in the sample with quantifiable uncertainty. The present paper describes one study carried out at the FAO/IAEA laboratory for the estimation of the uncertainty of subsampling for fumonisin FB1 determination in maize samples. It was estimated that the combined relative standard uncertainty (CV_{SP}) of sample processing of 25 g analytical portion was equal to 0.36.

INTRODUCTION

According to the definitions introduced by Hill and Reynolds (Hill et al.1999), sample preparation is the procedure used, if required, to convert the laboratory sample into the analytical sample by removal of parts (soil, stones, bones,...) not to be analysed. Sample processing is the procedure (e.g. cutting, grinding, mixing) used to make the analytical sample acceptably homogeneous with respect to the analyte distribution, prior to removal of the analytical portion. In the case of maize samples, the processing procedure includes sub-dividing and grinding the maize kernels.

Sample processing is very important in mycotoxins analysis because of the uneven distribution of the toxins. A reliable and representative result can only be achieved if the variations, in the analyte levels in the commodity, are eliminated through an effective homogenization step of a properly collected bulk sample. In other words, efforts must be made to ensure both an appropriate and representative sampling step and an efficient processing step.

As an example, the sampling plan for aflatoxins in peanuts (FAO, 1993) recommends taking 20 kg bulk sample and analysis of its representative 100 g portion. This size of bulk sample, however, is difficult to process at the laboratory. The present paper describes a study carried out at the FAO/IAEA laboratory for the estimation of the uncertainty of subsampling for fumonisin FB1 determination in maize samples.

MATERIALS AND METHODS:

Due to the fact that grinding a whole bulk sample of 20 kg is not a very practical laboratory operation, we tested the applicability and efficiency of a multi stage procedure.

The procedure consisted of

- thorough mixing of 20 kg naturally contaminated maize grains in a concrete mixer,
- subdivision into 2 x 10 kg portions, using a sample divider
- further mixing and sub-division into 5 x 2 kg portions
- further mixing and sub-division into 2 x 1 kg portions
- grinding of 1 kg portion
- thorough mixing and sub-dividing into 25 g and 150 g test portions with a sample divider
- extraction of the test portion and HPLC analysis of the maize extract

The multistage procedure is presented in figure 1.

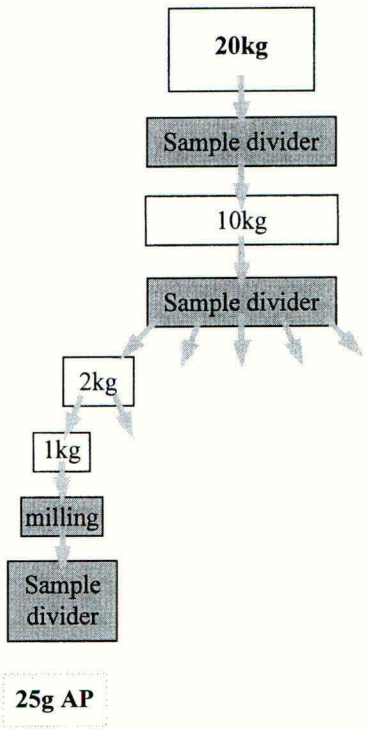

Figure 1: Scheme of sample processing and withdrawal of analytical portions

The analysis of fumonisin FB_1 in the naturally contaminated maize sample was carried out by the analytical procedure described by Visconti (Visconti et al. 1994) based on extraction with methanol/ water 3:1 v/v, cleanup on strong anion exchange (Sax) cartridges and reversed phase HPLC analysis with fluorescence detection of the FB1 after derivatization with ortho-phthalaldehyde (OPA). Table 1 contains a list of equipment used.

Table 1: List of equipment used

STEPS OF THE ANALYSIS	EQUIPMENT
Sample preparation and processing	Sample mixer (concrete mixer 80 l capacity); Cemotec 1090 Sample Mill.
Withdrawal of subsamples and analytical portions	In-house sample divider; 1 and 3 digit balance
Sample extraction	Rotary shaker: CERTOMAT®
Clean-up of sample	SPE 12 port vacuum manifold , SAX cartridge
Concentration-evaporation of samples	Concentration workstation; Turbovap®, Zymark
HPLC analysis	Reversed-phase LC Nova pak C18, 3.9x150 mm column, C18 column guard, Waters® 474 Scanning Fluorescence detector, 335 nm (Ex), 440 nm (Em)

Statistical treatment of data

Each step of the analytical procedure contributes to the total uncertainty of the result (expressed as relative standard deviation or CV) according to the following equation (Ambrus, 2004):

$$CV_R = \sqrt{CV_S^2 + CV_{SP}^2 + CV_A^2} \qquad \text{(equ. 1)}$$

Where CV_R is the uncertainty of result, CV_S the uncertainty of sampling, CV_{Sp} the uncertainty of sample (preparation) processing and CV_A the uncertainty of analysis. The analytical phase may include, for instance, the extraction, cleanup, evaporation, derivatisation and instrumental determination.

CV_L is the uncertainty of the laboratory phase (combined uncertainty of sample processing and analysis) and is described by equation:

$$CV_L = \sqrt{CV_{SP}^2 + CV_A^2} \qquad \text{(equ.2)}$$

From equation (2) the following formula for the uncertainty of sample processing can be derived:

$$CV_{SP} = \sqrt{CV_L^2 - CV_A^2} \qquad \text{(equ.3)}$$

RESULTS AND CONCLUSIONS:

Table 2 presents the results of FB1 content from duplicate analysis of 150 g analytical portions obtained by sub-division of each 10 times 1 kg portions (make reference to figure 1).

Table 2: FB1 content of duplicate analysis of 150 g analytical portions

1 kg portion	Code	R1 (mg/kg)	R2 (mg/kg)	Mean (mg/kg)	Reldif^2
1	1/AP1	1.86	1.96	1.91	0.002652
	1/AP2	2.20	2.25	2.23	0.000510
	1/AP3	2.30	2.07	2.18	0.011816
	1/AP4	2.27	1.99	2.13	0.017301
	1/AP5	2.21	2.13	2.17	0.001405
2	2/AP1	2.94	2.99	2.97	0.000257
	2/AP2	1.15	1.58	1.36	0.096113
	2/AP3	2.70	2.64	2.67	0.000583
	2/AP4	2.29	2.32	2.31	0.000098
	2/AP5	2.00	2.83	2.41	0.117893
3	3/AP1	2.27	1.96	2.11	0.022043
	3/AP2	2.31	2.45	2.38	0.003411
	3/AP3	2.35	2.75	2.55	0.024920
	3/AP4	2.55	2.57	2.56	0.000103
	3/AP5	2.43	2.52	2.47	0.001359
4	4/AP1	2.41	2.79	2.60	0.021112
	4/AP2	2.39	2.44	2.42	0.000306
	4/AP3	2.49	2.43	2.46	0.000633
	4/AP4	2.31	2.50	2.40	0.006150
	4/AP5	2.78	1.80	2.29	0.181713
5	5/AP1	3.13	2.60	2.87	0.033525
	5/AP2	2.99	3.02	3.01	0.000082
	5/AP3	3.02	2.91	2.96	0.001342
	5/AP5	3.00	2.88	2.94	0.001788
6	6/AP1	3.31	3.10	3.20	0.004308
	6/AP2	5.02	4.72	4.87	0.003727
	6/AP3	4.47	3.00	3.73	0.153974
	6/AP4	2.76	2.92	2.84	0.003257
	6/AP5	3.57	3.52	3.54	0.000194
7	7/AP1	3.13	3.39	3.26	0.006252
	7/AP2	3.61	3.67	3.64	0.000278
	7/AP3	3.20	3.01	3.11	0.003781
	7/AP4	3.54	4.34	3.94	0.041491
	7/AP5	2.33	2.30	2.31	0.000087
8	8/AP1	2.63	2.66	2.64	0.000137
	8/AP2	2.47	2.66	2.57	0.005601
	8/AP3	2.33	2.63	2.48	0.015096
	8/AP4	2.29	2.28	2.28	0.000004
	8/AP5	2.30	2.81	2.55	0.040559
9	9/AP1	2.55	3.16	2.86	0.044479
	9/AP2	2.60	2.53	2.56	0.000754
	9/AP3	2.34	2.54	2.44	0.006904
	9/AP4	2.75	2.79	2.77	0.000217

	9/AP5	2.61	2.48	2.55	0.002812
10	10/AP1	2.76	1.99	2.38	0.105777
	10/AP2	2.51	2.62	2.57	0.001907
	10/AP3	2.23	2.38	2.30	0.004202
	10/AP4	2.33	2.64	2.49	0.015696
	10/AP5	2.10	2.32	2.21	0.009778
	Sum				1.018
		CV_A			0.102
		CV_L		0.215	

The results in table 2 were statistically elaborated and used to establish the within laboratory reproducibility CV_A value. In the present study CV_A was calculated using the following formula (Miller et al, 2002):

$$CV_A = \sqrt{\frac{\sum d^2}{2n}} \qquad \text{(equ.4)}$$

where d is the relative difference between replicates, (d= (R1-R2)/ mean residue), and n is the number of duplicate test portions measured. CV_A includes the contributions of the operations performed from the cleanup to the final HPLC determination, and it provides information on the average reproducibility of these procedures for the concentration range covered.

As it shown in table 2, CV_A=10.2%. This is the best estimate available for the reproducibility of the measurements and can be used for the estimation of the combined uncertainty of the results.

Table 2 also shows the combined variability of the laboratory phase (CV_L) of the average FB1 content of the 150 g test portions withdrawn from 1 kg sub-samples. The uncertainty of sample processing for the 150 g analytical portion was estimated by applying equation (3).

The uncertainty of sample processing ,CV_{SP}, for the 150 g analytical portions is equal to 0.203 and is representative of the sample processing uncertainty at the 1 kg level.

As 25 g analytical portions represent the usual amount analysed for mycotoxins at the analytical laboratory, the study also included the testing of the homogeneity of the milled sample at 25 g level.

Analytical portions of 25 g were withdrawn from each 1 kg portions. Table 3 presents the FB1 concentrations found in 25 g analytical portions from each 1 kg portion (make reference to figure 1) .
The average residue content of 150 g analytical portion was compared to the FB1 content of the corresponding 25 g analytical portion using a paired t-test. The test revealed no significance difference between the two sets of data. However the average FB1 content for the 150 g APs was a better estimate for the 1 kg portion as it was derived from five times duplicate (5X2) measurements.

The uncertainty of sample processing (CV_{SP}) of 25 g test portions, was estimated by applying equation (3) to the replicate FB1 measurement of 25 g test portion, where CV_L= 0.37 and CV_A=0.102. CV_{SP} amounted to 0.36 and it represents the estimate of the combined uncertainty of sample processing of 25 g analytical portion withdrawn from 1 kg of milled maize sub-sampled from 2 kg whole grain maize, which in turn is obtained by subdividing 10 kg maize into 2 kg portions using a sample divider.

Table 3: FB1 content of duplicate analysis of 25 g analytical portions

1 kg sample	FB1 content in 25g AP
1	2.42
2	4.87
3	-
4	2.33
5	1.51
6	3.17
7	2.39
8	2.83
9	1.86
10	2.27
CV_L	0.37
CV_{SP}	0.36

For a given commodity, different mills will provide different degrees of particle size. The smaller the particle size, the more comminuted the sample material and the more homogenous the distribution of the mycotoxin. Since the efficiency of sample processing may vary from sample to sample, and from equipment to equipment, its regular control should be included in the internal quality control programme of the laboratory.

As a conclusion, the present study provides a strong warning signal that the grinding and mixing of 1 kg sub-samples and the subsequent sub-dividing and analysis of 25 g analytical portion should be carried out with utmost care.

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Fungicide targeting on ripening ears for improved control of *Fusarium* ear blight and the mycotoxins deoxynivalenol and nivalenol

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ABSTRACT

Studies were carried out to compare different spray systems for improved targeting of fungicides on the ears of ripening wheat during anthesis. Wind tunnel experiments identified a range of possible nozzle types which could be applied in the field. In two contrasting seasons (wet and dry) the efficacy of the best spray treatments were compared in field experiments where wheat was inoculated with *Fusarium culmorum* prior to fungicide applications with amistar+folicur or prosario. These showed that in a wet year (2003) targeting improved control of ear blight by the fungicides. Generally there was higher contamination with nivalenol (NIV) than with deoxynivalenol (DON). In the dry year there was some correlation between *Fusarium* contaminated grain and spray nozzle treatments. Generally, the pre-orifice flat fan nozzle and the conventional flat fan nozzle at 45° angled backwords were the best treatments. Ear blight and trichothecene contamination were less in 2004 when the environmental conditions during the critical anthesis period was very dry. Taqman PCR and full trichothecene analyses confirmed the presence of *F. culmorum*, with contributions from other species such as *F. avenaceum* and *F. graminearum* and the absence of other trichothecenes.

INTRODUCTION

Fusarium infection is of concern because of impacts on crop yield and quality and the concomitant contamination with trichothecene mycotoxins, particularly deoxynivalenol (DON) and nivalenol (NIV) which are produced under conducive environmental conditions during anthesis (Magan *et al.*, 2005). Suppression of *Fusarium* ear blight is partially achieved by the application of fungicides. Previous studies have suggested that sub-optimal concentrations of fungicides may stimulate DON production especially at intermediate grain moisture contents (Magan *et al.*, 2002; Ramirez *et al.*, 2004; Jennings and Kohl, 2004). It has been suggested that it is critical that the correct concentrations of fungicides can reach the ripening ear at the beginning of anthesis for the best chance of control of ear blight and DON contamination. However, examination of the most effective spray systems for targeting applications on the ears have not been examined previously in the UK. This study examined a range of different spray systems for efficacy and relative control of ear blight and DON/NIV in UK cereals over a period of two years.

MATERIAL AND METHODS

Wind tunnel experiments

Wind tunnel experiments were carried out using tray grown plants at anthesis and spraying with both tracer dyes and fungicide mixtures to determine coverage with different spray systems on the front, back and total ear. The tests also enabled the quantification of relative amounts of fungicides on the front and back of ears (Powell *et al.*, 2004).

A series of experiments were done to examine deposits of fungicide and trace dyes on coverage of ears of ripening wheat plants in a wind tunnel. Conventional flat fan, 10° angled air induction nozzle, two conventional flat fan nozzles at 45°, one forward and one back (twin cap), wide angle hollow cone coarse spray, conventional flat fan nozzle 45° backword fine spray, conventional flat fan 45° medium spray, wide angle hollow cone fine spray, pre-orifice flat fan and 10° angled air induction nozzle were examined. All were used at 150 l/ha except the final treatment which was at 100 l/ha.

Field trials

Field trials have been conducted for two years with 4 spray nozzle treatments (Conventional flat fan (F110-03), 10° angled air induction nozzle (Amistar), pre-orifice flat fan nozzle (F110-04) and a fine hollow cone nozzle (D2-53). Three fungicides used were: 0.3 l Amistar + 0.3 l/ha Folicur; 1.2 l/ha Prosario; 0.6 l/ha Prosario. There were all applied at the beginning of anthesis. A full factorial experiment was carried out and the treatment plots were sprayed with a spore suspension of *F.culmorum* (10^5 spores/ml) three days prior to fungicide application. Misting was carried out for these three days to aid establishment of the infection. 100 ears per plot were collected two weeks after spraying and immediately prior to harvest for ear blight assessments and analysis of DON and NIV. These were analysed using the method described by Ramirez et al. (2004). Some samples were also examined for full trichothecene analysis using GC-MS. Taqman PCR was also employed to examine the relative amounts of DNA in the final grain samples in 2003 and 2004 (Waalwijk, 2002).

RESULTS

Wind tunnel experiments

Figure 1 shows the ear deposits on the front and back parts of ears from the different application systems tested. The differences in deposits were generally very similar. There was a suggestion that the highest deposits were achieved using the wide angle hollow cone nozzle (WRW2) and the F110-015 and F110-04 nozzles angled backwords. Many of the application systems resulted in a similar distribution on both sides of the ears.

Field trials

Figure 2 shows the effect of different nozzle systems tested and fungicide applications on *Fusarium* ear blight two weeks after application of inoculum and fungicides during anthesis. This shows that two nozzle treatments (the pre-orifice flat fan nozzle and the conventional flat fan nozzle at 45° angled backwords) were better than the other two tested. The Prosario fungicide was also much more effective, especially at the normal application rate.

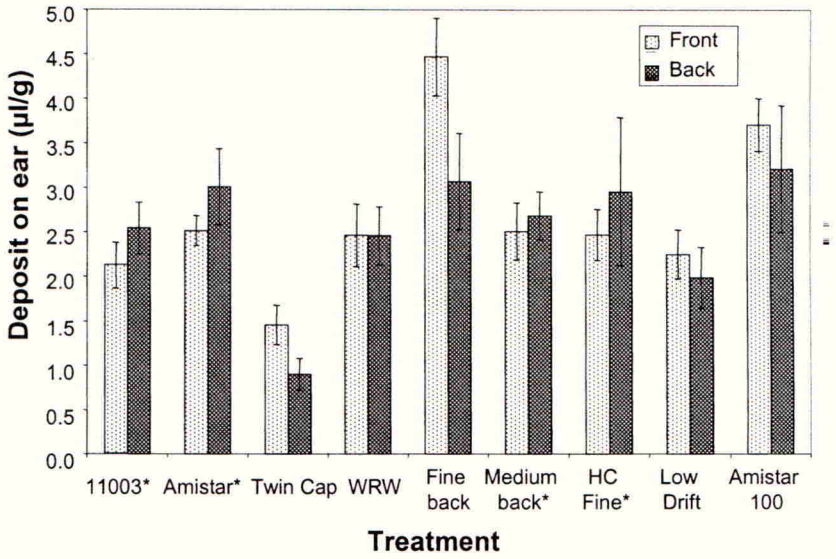

Figure 1. Comparison of total deposits on front and back of ears (ul/g) from different application systems in the wind tunnel experiments (* = application systems evaluated in the field trials in 2003 and 2004).

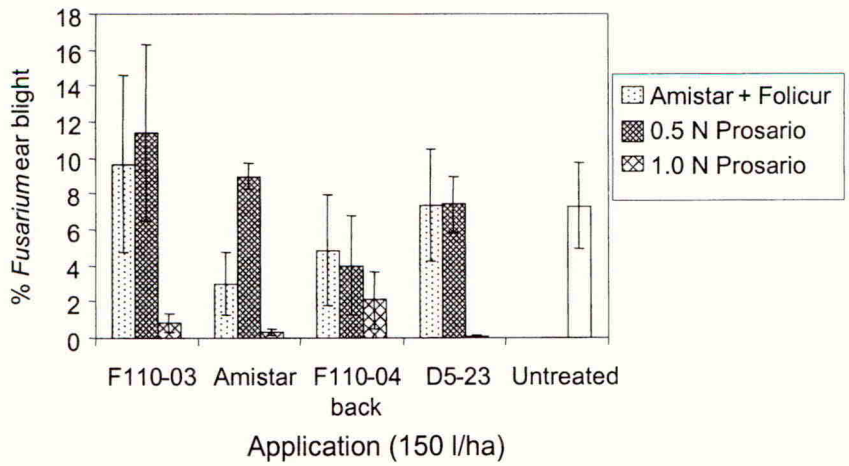

Figure 2. Effect of some treatments on relative *Fusarium* ear blight where different spray nozzles and fungicides were employed. Bars indicate standard error. For key to treatments see Materials and Methods section.

Figure 3 shows the effect of treatments on relative amounts of biomass of *F. culmorum* present in the harvested grain. The highest amount using quantitative PCR was present in the control. The least was present in the nozzle treatments which were most effective and again confirms the effectiveness of Prosario at the normal application rate.

The effect of treatments on DON and NIV in 2003 is shown in Figure 4. In both 2003 and 2004 GC-MS showed that these were the only trichothecenes present. There was lower DON than NIV levels. Generally, based on these results there was very little effect of nozzle targeting on relative amounts of NIV present in the harvested grain. There was some effect of the Prosario fungicide at the normal rate of application with some nozzle treatments but there was no particular trend. In 2004 the experiment showed that there was much less *Fusarium* ear blight during anthesis than in 2003 making infection and establishment very poor. Thus both *Fusarium* ear blight symptoms and DON/NIV levels were very low, with a high isolation of *F. avenaceum, F. graminearum* and *F. poae* as well as *F.culmorum*. There were some differences between treatments in relation to actual pink grains present in harvested grain but these were not statistically significant.

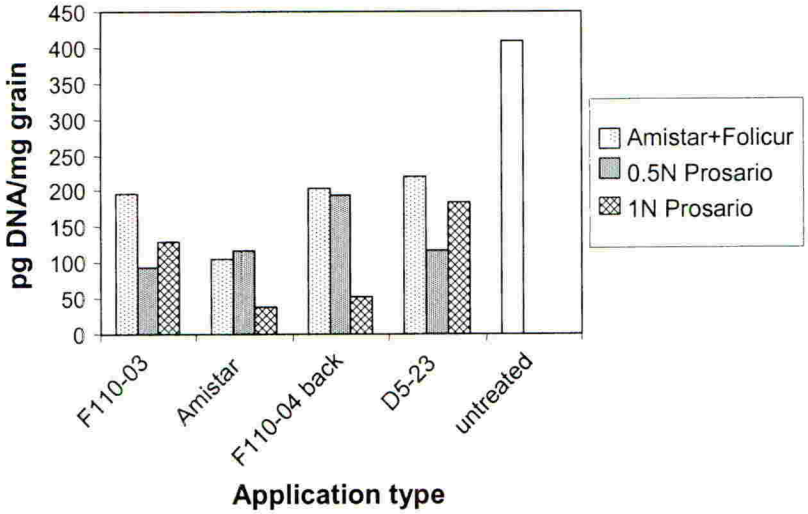

Figure 3. The mean concentration biomass of *F. culmorum* in grain treated with different spray systems and different fungicides in 2003.

DISCUSSION

The deposit distribution on the front and the back of the ear for the four application systems evaluated in the field trials, suggested a slight trend for a higher deposit on the back of the ear compared to the front for all treatments. The deposit patterns also suggested that there was a slightly higher deposit on the front or the back of the ear from the Amistar nozzle and this was the application system that tended to give the highest yields in the field trial for the three fungicide treatments evaluated (Powell *et al.*, 2004).

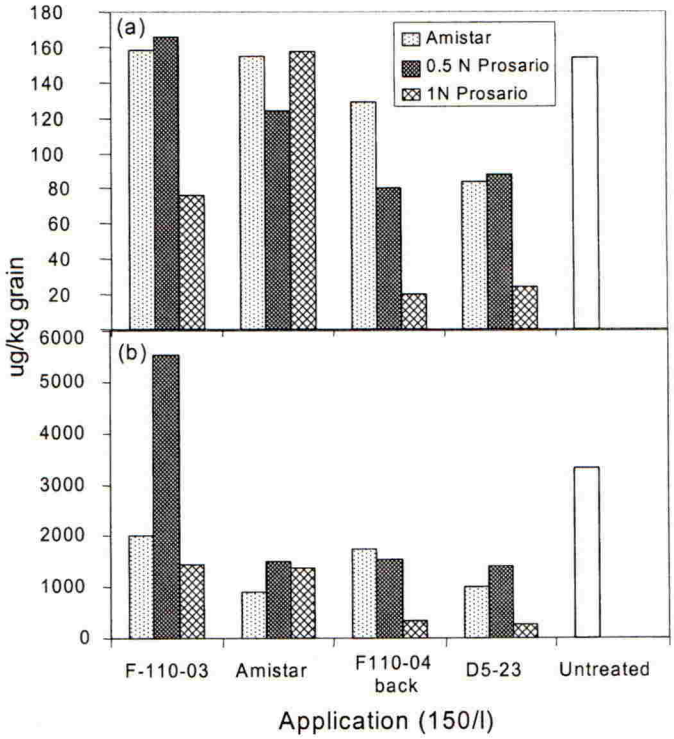

Figure 4. Effect of spray treatments and fungicide applications on content of (a) DON and (b) NIV in harvested wheat grain in 2003. The data is mean of GC-MS analyses.

Of the fungicide treatments, the full dose of Prosario resulted in the lowest ear blight infection and the highest resulting grain yield. This was irrespective of application system used suggesting that when fungicide performance was very good application choice was less important. When fungicide performance was less robust more variation was apparent between the application systems. However, the Amistar nozzle tended to result in the lowest ear blight infection.

The use of quantitative PCR confirmed some of the differences in ear blight obtained during flowering in the harvested grain. The effective control of *F. culmorum* biomass in the Amistar and F110-04 flat fan nozzle treatments showed that with an effective fungicide, control is very good. There is some information that sub-optimal applications of fungicides can result in a stimulation of mycotoxin production (Magan *et al.*, 2002). This is partly borne out here by the application of a 50% of the normal rate of application of Prosario. This was not very effective regardless of spray nozzle type used. This suggests that it is important to use fungicides at the recommended rates for ensuring that some control of ear blight is achieved and more importantly, no opportunity exists for stimulation of mycotoxin contamination by physiologically stressing the pathogen where it is still able to partially colonise the ripening

grain. The *F. culmorum* isolate used appears to be a high NIV as well as a DON producer. The levels of DON were low in all treatments when compared with that of NIV. We know that under optimum humidity and temperature conditions *F. culmorum* isolates can produce high amounts of DON and NIV within 10-15 days (Hope *et al.*, 2003). Further recent studies on grain suggests that environmental conditions conducive to DON production by *F. culmorum* and *F. graminearum* are slightly different and depends on prevailing temperature and microclimate conditions at the initiation of anthesis.

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The effect of tillage on *Fusarium* infection and mycotoxins on barley and oats

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ABSTRACT

Direct drilling and conventional soil tillage with autumn ploughing were compared in a field trial during 2003-2004. Oat and barley cultivars were sown using both methods and the development of *Fusarium* infection in ears and panicles studied during kernel development from ear emergence until harvested and dried grain. Direct drilling increased the infection of *F. avenaceum* and *F. poae* compared to other *Fusarium* species, but it did not have any effect either on *F. culmorum* or *F. graminearum*. In 2004, *F. langsethiae* was often found in the early samplings after oat panicle emergence, and high contamination of T-2 and HT-2 toxins was observed in harvested oat grain. In 2003, this species was found very rarely and these toxins were almost absent in the grain.

INTRODUCTION

Direct drilling without ploughing or with reduced tillage has increased in popularity during recent years in Finland. Leaving plant residues on the soil surface can, however, increase plant diseases on cereals. *Fusarium* species that cause head blight and form mycotoxins have raised problems in Central Europe when cultivation methods were changed (Bailey & Duczek, 1996, Yi *et al*, 2001). In Northern Europe, oats and barley are the main cereal crops and problems with changing practices have been studied during recent years in Norway (Henriksen, 1999). The present study is the first attempt to find out whether cultivation practices have any influence on the *Fusarium* infection and mycotoxin contents of cereals in Finland.

Infection of *Fusarium* species is strongly influenced by weather conditions and *Fusarium* infection rates and mycotoxin contamination vary from year to year. Crop debris is the main source of *Fusarium* inocula and inoculum production is dependent on rainfall and temperature. Warm and moist conditions favour infection during ear emergence and anthesis of wheat and barley (Xu, 2003), but little is known of infections on oats (Langseth & Elen, 1996). The distribution of *Fusarium* species as ear blight pathogens varies in cereal production in Europe because of their different requirements. The most important species are *Fusarium graminearum*, *F. culmorum* and *F. avenaceum*. Species not as pathogenic but also toxigenic include *F. poae*, *F. tricinctum* and *F. sporotrichioides*. *F. graminearum* is the most important producer of deoxynivalenol (DON) in the warmer areas in Europe, while *F. culmorum* is the predominant toxin forming species in cooler regions. In northern areas, *F. poae* is a common species producing nivalenol (NIV) and *F. sporotrichioides* forms T-2/HT-2 toxins (Bottalico & Perrone, 2002). In Norway, *F. langsethiae* which forms T-2/HT-2 toxins, has been the most important mycotoxin producer during recent years (Kosiak *et al*, 2003).

This new species is closely related to *F. sporotrichioides* (Torp & Nirenberg, 2004). *F. avenaceum* is the most common species infecting grain in the cool conditions of Finland (Eskola *et al*, 2001).

The aim of this study was to find out whether there are any differences in the *Fusarium* infection and mycotoxin contens on barley and oats in Finnish conditions when they are continuously cultivated without tillage compared to use of conventional tillage with autumn ploughing.

MATERIALS AND METHODS

Field trial in Jokioinen

The field trial at MTT Plant Protection in Jokioinen was started in 2003. In the first year the preceding crop for all the plots was barley and in the second year it was oats. Autumn ploughed and direct drilled areas were kept in the same places in both years.
Malting barley and food oat cultivars were used in the trial. In 2003, the oat cultivars used were 'Veli', 'Aslak' and 'Roope' and the barley cultivars 'Saana' and 'Scarlett'. In 2004, the cultivars were 'Roope', 'Freja', 'Veli' and 'Belinda' for oats and 'Saana', 'Scarlett', 'Barke' and 'Annabell' for barley. The seed were treated with carboxin+imazalil (Täyssato).

Sampling, starting at ear and panicle emergence, was done every two weeks from all plots. Randomly chosen twenty ears or panicles per plot were sampled for investigation. To isolate *Fusarium* fungi, two kernels of each ear (panicle) were taken for incubation. Harvest was carried out in 2003 at the end of August, barley cv 'Scarlett' was harvested later, on 5th of September. In 2004, harvesting started on barley cv 'Saana' on 25th of August and continued until 15th of September, when the late oat cultivars were harvested. In 2003, no lodging occurred, but severe lodging was observed at the beginning of August in 2004. Only unlodged cereal stands were harvested for analysis. The harvested grain was dried and samples were taken from both raw and cleaned grain (2-mm sieve) for *Fusarium* and mycotoxin analyses.

Fusarium and mycotoxin analysis

The kernels and grains were incubated on agar medium containing pentachloronitrobenzene (PCNB) (Nash & Snyder medium, Nelson *et al,* 1983) at room temperature (22°C) and the growing hyphae were isolated on potato dextrose (PDA) medium for identification. The *Fusarium* cultures were identified microscopically.

Deoxynivalenol (DON), diacetylscirpenol (DAS), 3-acetyldeoxynivalenol (3-Ac-DON), fusarenon-X (F-X), nivalenol (NIV), T-2 and HT-2 were analysed and quantitated using standard methods (Pettersson & Langseth, 2002). Trichothecenes were analysed from grain samples with GC-MS and zearalenone with HPLC (Romer Labs Methods: Zearalenone HPLC MycoSep^{TM} 226 Method). Trichothecenes were analysed from cleaned grain in 2003, in 2004 from both raw and cleaned grain. Zearalenone was analysed from altogether 75 samples in 2003 and 10 samples in 2004 chosen by their DON content and *F. culmorum/F. graminearum* infection.

Weather conditions

The growing season in 2003 started late; temperatures were low and there were frequent rains during May. The cool period continued until mid-July and was followed by a three-week period of high temperatures reaching up to 30° C. During the first days of August the temperatures decreased and the weather turned cooler and rainy. In 2004, early spring was very warm and the sowing conditions were good. The cool period starting in early May retarded plant development. Heavy rains occurred at the end of June and beginning of July and again at the end of July and August. Temperatures above 25°C occurred only at the beginning of May and in the first week of August. The first temperatures below 0°C were measured on 25th of August and the first night frost on 9th of September.

RESULTS AND DISCUSSION

Fusarium infection in developing kernels and harvested grain

Cool and rainy early summers did not favour inoculum production of *Fusarium* in the debris, which needs warm weather. The high temperatures during July in 2003 were optimal for *F. culmorum* and *F. graminearum* infections (Xu, 2003), but lack of rain and humidity inhibited infections. In the first samplings after ear emergence only few infections were detected in the kernels. F. *poae* (Fig. 1) and *F. sporotrichioides* were the earliest colonisers of kernels while *F. culmorum* and *F. graminearum* were very rarely found in the early samplings (Fig. 2). Most of the earliest *Fusarium* infections were on direct drilled plots. Infections increased rapidly when there was rain in the beginning of August. Especially *F. avenaceum* colonised developing grain during a couple of weeks before harvest. It was the main species detected in direct drilled plots. *F. poae* infections increased on both oats and barley, especially in direct drilling. *F. graminearum* infection was most often found on cv Scarlett and cv Roope both in direct drilled and ploughed plots.

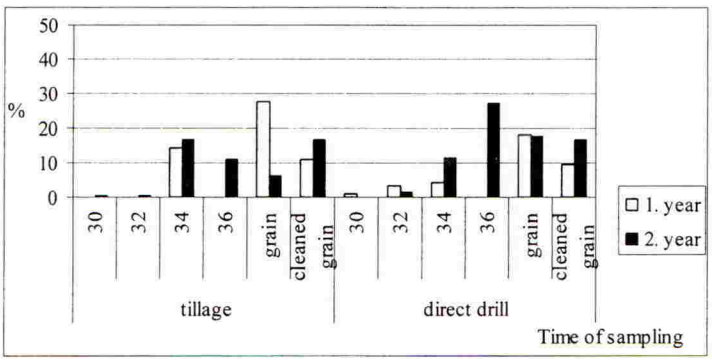

Figure 1. Development of *Fusarium poae* infection in oats (cv Veli) on tilled and direct drilled trial plots from ear emergence (time=week) until harvest and harvested grain

In 2004, when the humidity was high, *Fusarium* infections were detected in kernels already at the first sampling. The first species found was *F. langsethiae* which had previously very seldom been detected in Finland. In this trial, the species was quite common on oat cultivars in the early samplings (Fig. 3), but less often found on barley. *F. langsethiae* has been reported to be common in Norway. It is related to *F. sporotrichioides* and the species is also a producer of T-2 and HT-2 mycotoxins (Thrane *et al*, 2004). At the beginning of sampling, *F. langsethiae* was most abundant on oat cultivars growing on normally tilled plots.

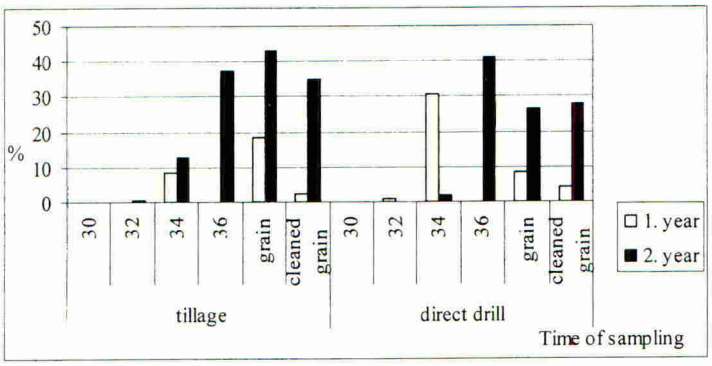

Figure 2. Development of *Fusarium culmorum* infection in barley (cv Scarlett) on tilled and direct drilled trial plots from ear emergence (time=week) until harvest and harvested grain

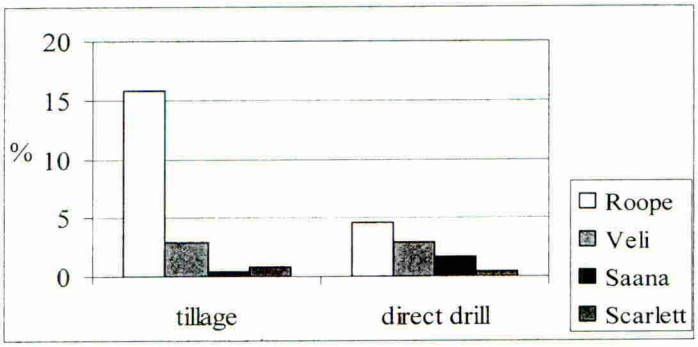

Figure 3. *Fusarium langsethiae* in kernels of oats (cv Veli and Roope) and barley (cv Saana and Scarlett) in the first sampling in 2004 (2nd year)

F. sporotrichioides was detected in low percentages throughout the sampling period, but the species did not become more abundant towards harvest. *F. poae* was detected in early samplings and it soon infected all cultivars but was most common on oat cultivars and barley cv Saana at harvest time. *F. poae* seemed to become more prevalent earlier in direct drilled than in tilled plots (Fig. 1). *F. avenaceum* was detected at early sampling in very low percentages and the species became more abundant in mid-August. It was the dominant Fusarium species in direct drilled plots at the end of the growing season.

The DON producers *F. culmorum* and *F. graminearum* started to increase at the end of August after heavy rainfalls. *F. culmorum* infection increased rapidly on barley just before harvest in September (Fig. 2). Cultivar Saana was harvested earlier and its contamination was lower. Direct drilling seemed to decrease *F. culmorum* infection both on oats and barley during the growing season.

Direct drilling increased *F. avenaceum* in the grain of both oats and barley and decreased infections of other *Fusarium* species detected at the same time. The effect was best seen on oat cv Roope. Cultivar Veli behaved in a different way; *F. avenaceum* increased, but the other *Fusarium* species are not reduced. On barley cv Saana the effect was similar to that of oats, *F. avenaceum* increased in direct drilling. *F. culmorum* was, however, in 2004 nearly as common in barley grain as *F. avenaceum*. *F. langsethiae* was rarely detected in harvested grain.

Fusarium mycotoxins in harvested grain

In 2003, the mycotoxin contents were low in the samples. The DON contents were below 400 µg/kg, the highest values were detected on oat cv Roope and 'Veli'. NIV and T-2/HT-2 were detected in a few samples, but the contents were low. No zearalenone was detected (limit of detection 20 µg/kg). In 2004, the DON contents in the grain were quite low, although the infection rates of *F. culmorum/F. graminearum* were high. The highest DON content, 800 µg/kg, was detected in uncleaned grain of barley cv Barke. Cleaning reduced the DON content of barley, the highest value being 410 µg/kg ('Barke'). In direct drilling the DON contents were lower in uncleaned grain. Barley cultivars other than cv Barke had lower DON contents and cleaning did not have much effect on the results. The highest DON content in uncleaned oat grain, 400 µg/kg, occurred on 'Roope', and cleaning reduced it to 300 µg/kg. DON contamination is considered to be higher on oats than on barley but, according to Norwegian results, oats and barley cultivated in similar conditions both contained the same contents of DON (Langseth & Elen, 1996). The trial years were mostly cool and, especially in 2004 very rainy. In earlier studies conducted in Finland the mycotoxin contents of oats were very low after cool and rainy growing seasons (Eskola *et al*, 2001; Hietaniemi *et al*, 2004).

Nivalenol (NIV) produced by *F. sporotrichioides* and *F. poae* (Thrane *et al*, 2004) was detected in nearly all barley samples analysed in 2004. The highest NIV contents were detected on cv Saana. *F. poae* infections were high on oats and NIV was detected in nearly all samples. HT-2 toxin was detected in most of the analysed oat samples and many grain samples also contained T-2 toxin. The T-2/HT-2 contents on uncleaned oat grain were exceptionally high. *F. sporotrichioides* and *F. langsethiae* are producers of the toxins (Thrane *et al*, 2004), and *F. langsethiae* was detected at an early stage of grain development of oats. Cleaning removed poorly developed grains and decreased the toxin contents of all cereals. The zearalenone contents were very low.

The two-year period of the field trial was too short to show any differences between cultivation methods in *Fusarium* infection or mycotoxin contents of the grain. The DON contents were higher on barley than on oats, but there was great variation between cultivars and years. Direct drilling seemed to reduce the DON contents, but opposite results were also obtained. The cool periods of the growing seasons did not favour *Fusarium* infection and mycotoxin production. *F. langsethiae*, a toxin producer that can be a problem in oat production, was an exception. The trial will be continued for long-term effect of reduced tillage on grain quality and safety.

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Use of antioxidants and essential oils for controlling mycotoxins in grain

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ABSTRACT

A total of 23 essential oils and 5 antioxidants were screened for control of growth and mycotoxin production by *Fusarium culmorum, F.graminearum* (deoxynivalenol, DON) and *Penicillium verrucosum* (ochratoxin, OTA). Of these, three essential oils (bay, clove and cinnamon oil) and two antioxidants (propyl paraben and hydroxymethylanisole) were found to be particularly effective in controlling growth in the range 50-200 ppm at 15 and 25°C over the range 0.995 to 0.955 water activity (a_w). More detailed studies were carried out to examine the efficacy of interactions between water activity (a_w) (0.955, 0.995), temperature (15°C and 25°C) and these control treatments (100ppm; 500ppm) on growth and both DON and OTA on irradiated wheat grain. Higher concentrations of the control treatments (500 ppm) were needed to reduce or inhibit growth significantly on wheat grain when compared to that in vitro. Growth *F. culmorum* and *P.verrcucosum* were significantly affected by temperature, a_w and antifungal treatment. At 100 ppm concentration the essential oils/antioxidants stimulated growth under some of the experimental conditions. Growth was significantly inhibited by 500ppm concentration. Cinnamon and clove essential oils were the most effective inhibitors of growth regardless of temperature or a_w level used. The control treatments had a variable effect on the production of mycotoxins. BHA and Clove oil inhibited DON and OTA production at both 100 and 500 ppm concentration on wheat grain.

INTRODUCTION

The contamination of cereals with trichothecenes and ochratoxin (OTA) occur predominantly pre- and post-harvest, caused by *Fusarium* ear blight and *Penicillium verrucosum* respectively (Magan & Olsen, 2004). Poorly dried grain at harvest can be rapidly colonised by *Fusarium* species, which have been considered as an intermediate group in terms of environmental tolerance. Thus, treatment of moist grain post-harvest could be one way of inhibiting *Fusarium* colonisation and prevent contamination with deoxynivalenol and nivalenol (DON/NIV). *P. verrucosum* is predominantly responsible for OTA in northern Europe where cooler damp harvesting conditions exists.

Recent studies have attempted to determine the efficacy of extracts from selected plants as antimicrobal and antifungal agents. Some studies have shown that specific essential oils can control spore germination and growth of spoilage fungi. For example, found that oregano and thyme essential oils inhibited the growth of *Aspergillus niger*, *A. ochraceus* and *A. flavus* (Magan *et al.*, 2004). Antioxidants have also been demonstrated to inhibit fungal growth and some are already used as preservatives in food. Thompson (1993) studied the effects of esters

of p-hydroxybenzoic acid (paraben) on the growth of three mycotoxigenic fungi. Butyl and propyl parabens were the most effective, completely inhibiting mycelial growth at 1.0-2.0 mM.concentration. However, the impact of environmental factors were not considered in these previous studies. Recent studies have shown that potential exists for controlling *Fusarium* section Liseola and fumonisin production over a range of environmental conditions (Magan *et al.*, 2004).

The overall objectives of this study were to (a) screen 23 essential oils and 6 antioxidants for inhibiting growth of *F. culmorum, F. graminearum* and *P. verrucosum* in vitro; (b) efficacy of the best treatments for controlling DON and OTA production under different environmental conditions; and (c) examine control of mycotoxin production in inoculated wheat grain under different water availability and temperature conditions.

MATERIALS AND METHODS

Fungal species and isolates

The *F. culmorum* (isolate98WW4.5FC) and *F. graminearum* (Chemotype 1, IBST1003) isolates used were isolated were all from UK wheat grain. Both isolates had a known history of mycotoxin production (Lacey *et al.*, 2000; Jennings *et al.*, 2000). Three *P. verrucosum* isolates (OTA11) were studied.

In vitro screening of essential oils and antioxidants

The *in vitro* screen consisted of a two-stage process. An initial screen was carried out to identify promising compounds. Subsequently, detailed studies were only carried out with the best treatments in vitro on milled wheat agar and in situ on wheat grain. The initial screen included testing 23 essential oils (basil, bay, lime, rosemary, cinnamon leaf, pine sylventris, orange, peppermint yakima, ginger, marjoram, eucalyptus, clove, lemongrass, grapefruit, sweet fennel, mandarin red, lemon sicilian, thyme, basil linalol, aniseed, nutmeg, spearmint and sage (F.D. Copeland & Sons, Ltd., London)) and six antioxidants (propyl gallate, propyl paraben (PP), butylated hydroxy anisole (BHA), propionic acid, benzoic acid and butylated hydroxytoluene (BHT, Sigma)).

For essential oils this was based on a zone of inhibition assay; and for the antioxidant screen inhibition of mycelial extension was measured. The basic medium for both methods was a 2% milled wheat agar. The a_w of the agar was modified to 0.995 a_w by the addition of glycerol (Dallyn, 1978). The inoculum used was a spore suspension at 10^6 ml, obtained from 7 day old plate cultures (MEA) of all species and isolates. The results from the initial screen demonstrated that only three essential oils and two antioxidants had potential for control of *F. culmorum, F. graminearum* and *P. verrucosum*.

A detailed study of the efficacy of clove, bay and cinnamon oils and the antioxidants BHA and propyl paraben were screened at 50, 100, 200 and 500 ppm on a 2% milled wheat agar at three a_w levels (0.995, 0.985 and 0.955) at 15 and 25°C. The essential oils and antioxidants were dissolved in 5 ml of methanol and then added to the molten media to produce the final concentrations. The control was the basic medium to which 5 ml of methanol only was added. Plates were inoculated centrally with 10^6 spores/ml of *F. culmorum, F. graminearum* and *P. verrucosum* obtained from a 7 day old plate culture (MEA) using a 1µl sterile loop. Plates of

the same a_w were sealed in plastic bags and incubated at 15°C or 25°C. Colony diameters were measured at regular intervals by taking two readings at right angles to each other. Experiments were carried out with two replicates of all treatments and repeated once.

In situ study

Irradiated wheat grain (12kGr) with retained germinative capacity was used as the in situ substrate. The a_w of the grain was adjusted to the desired level by the addition of sterile distilled water. The volume of water (ml/g grain) required to adjust the a_w was calculated from a moisture absorption isotherm. The isotherm was created using grain from the same batch as that used for the experiment. Irradiated wheat grain was weighed into sterile flasks. Essential oil or antioxidant was dissolved in 5ml of methanol to result in a final concentration of either 100ppm or 500ppm in the grain (w/w), with the control treated with methanol only. This solution was then incorporated into the sterile water to be used for the a_w adjustment to 0.995 a_w (=30% moisture content) and 0.955 a_w (22% moisture content). This was then added to the flasks. The flasks were allowed to equilibrate at 4°C for 96 hours, with periodic shaking. 24 hours before inoculation, the grain from the flasks was poured into Petri plates to provide a monolayer of wheat grain (approx. 20g/plate). Samples of grain were taken to check the accuracy of the a_w using an Aqualab water activity meter (Decagon Inc, Washington State, USA).

Plates were inoculated centrally with a loop (1 µl) of a 10^6/ml spore suspension of the appropriate *Fusarium* species. Inoculated plates of the same a_w were then placed in a sealed container including beakers with 250mls of a water/glycerol solution at the same a_w as the treatment to help maintain equilibrium relative humidity of the chamber. Four replicates per treatment were incubated at 15°C and 25°C for 30 days and experiments repeated once. The colony diameters were measured daily or as required for the 30 day incubation period. The temporal mycelial extension rates were plotted and the linear regression of the slope used to determine the growth rates (mm/day).

Mycotoxin analysis

At the end of the incubation period the samples were analysed for DON/NIV using HPLC system and UV-light and OTA was determined using HPLC and fluorescence detector. The methods have been detailed elsewhere (Hope et al., 2005; Cairn-Fuller et al., 2005).

RESULTS

Effect of essential oils and antioxidants on growth

Of the 23 essential oils tested only those of clove, bay and cinnamon leaf inhibited *F. culmorum* and *F. graminearum*. Clove produced the smallest zones of inhibition. The methanol control did not produce any zones of inhibition. Of the antioxidants only BHA and propyl paraben significantly ($P > 0.05$) inhibited growth (results not shown). BHT did exhibit fungicidal properties, but these were not significant. None of the antioxidants stimulated growth of *F. culmorum* or *F. graminearum* isolates used in this initial screen. Table 1 shows the LD_{50} values of the best treatments for inhibiting growth of *F. culmorum* and *F.graminearum* on wheat based media in relation to different a_w x temperatures. This shows

that BHA was the best antioxidant and clove and cinnamon oils the best essential oil treatments over a range of conditions over which these species can grow and produce trichothecenes. Growth of *F. culmorum* and *F. graminearum* was completely inhibited by essential oil/antioxidant concentrations of ≥500ppm. Significant inhibition of growth occurred at 100ppm concentration when compared to untreated controls. Temperature and a_w levels affected the efficacy of the essential oils and antioxidants. For example, cinnamon oil at 200ppm inhibited growth under all conditions except 0.985 a_w, and 25°C. Clove and bay essential oils stimulated growth, which was particularly evident at low concentrations. However, this stimulation did not always occur at the same environmental conditions. For example, bay oil, at all a_w levels, resulted in a significant stimulation of growth at 25°C but not at 15°C. This demonstrates the importance of including an environmental matrix approach in screening for new control agents. BHA and propyl paraben were the most effective at inhibiting growth *in vitro*, under most of the environmental conditions tested. Overall, Cinnamon oil was the best test treatment for inhibiting growth of *F. culmorum* and *F. graminearum*.

Figures 1 shows the effects of the essential oils on inhibition of spore germination and growth of *P. verrucosum*. Of the essential oils screened, bay leaf (west Indian), cinnamon leaf, clove bud, clove leaf (cleaned), lemongrass and thyme were found to be the most effective at controlling spore germination and mycelial growth of *P. verrucosum*. These essential oils all gave mean radial zones of inhibition >5mm. Based on this cinnamon, clove leaf (cleaned) and thyme were chosen for more detailed study.

Effect of temperature, a_w levels, essential oils/antioxidants on mycotoxin production *in situ*

Figure 2 shows the effect of the treatments on DON production by *F. culmorum* and *F. graminearum* on irradiated wheat grain. Significant control of DON was achieved by the antioxidant and essential oils, especially at 500ppm, at both temperatures and a_w levels tested.

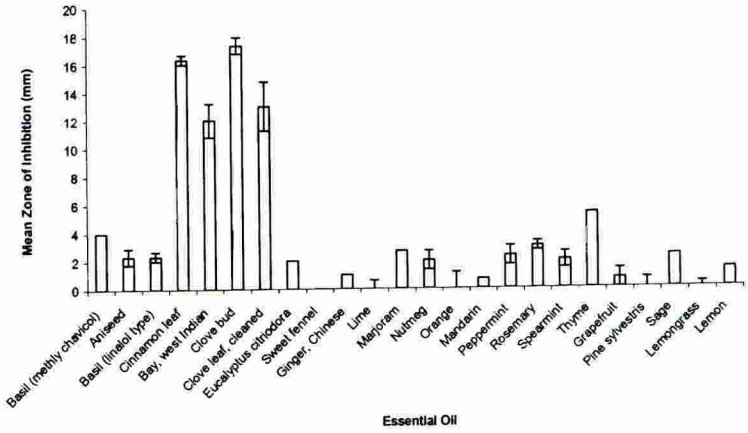

Figure 1. Zones of inhibition of *Penicillium verrucosum* (OTA11) when exposed to various essential oils diluted 1:10 methanol after 48 hours at 25°C on a wheat-based substrate at 0.995 a_w. Bars indicate standard errors of mean.

500ppm of bay leaf oil was most effective at inhibiting DON production under all the temperatures and a_w levels tested. However, DON production was stimulated above the levels of the untreated control when cinnamon oil or propyl paraben was used at 500ppm, at 25°C and 0.955 a_w. At 100ppm the treatments were unable to significantly reduce mycotoxin production and in many cases stimulated production. ANOVA analysis showed that all of the experimental variables and their interactions had a significant effect on DON production.

Figure 3 shows the concentrations (ppb) of OTA produced by the two ochratoxigenic species at different a_w x temperature conditions. It is noteworthy that no OTA was produced by *P. verrucosum* at 0.90 a_w even after 56 d growth on the milled wheat substrate at both temperatures in the control, or with the essential oil treatments. Significantly more OTA was produced at 25°C than 15°C for this species particularly in media containing 50 ppm and 150 ppm of the essential oils. OTA production by *P. verrucosum* was shown to be largely unaffected by temperature. Cinnamon leaf oil was shown to be the most effective treatment for inhibiting OTA production by this species. OTA production by *P. verrucosum* was stimulated with the addition of 50 ppm of all three essential oils tested at 0.95 and 0.99a_w at 15°C and 0.99 a_w at 25°C. However at 500 ppm effective control was achieved with most treatments.

3.3 Effect of environmental factors and essential oils/antioxidants on growth rates *in situ*

Figure 1 shows the effect of treatments on growth of *F. culmorum* and *F. graminearum,* on wheat grain. At 100 ppm, regardless of the environmental factors, control of growth of *F. culmorum* and *F. graminearum* was often not more than 50% on wheat grain. At least 500 ppm of the treatments were required for >75% control of growth under the environmental conditions examined (Figure 2). Therefore, treatments were less effective on irradiated wheat grain than

Table 1. The LD_{50} values of essential oils and antioxidants for control of growth of *F. culmorum* and *F. graminearum*

	F. culmorum					
Temperature (°C)	15			25		
Water activity	**0.995**	**0.98**	**0.955**	**0.995**	**0.98**	**0.955**
Treatments						
Bay leaf	135	120	150	310	210	240
BHA	50	25	75	75	80	50
Cinnamon oil	105	90	105	30	140	100
Clove oil	110	110	105	190	120	190
Propyl paraben	75	50	100	100	90	25
	F. graminearum					
Bay leaf	160	150	105	280	340	375
BHA	60	60	20	60	290	110
Cinnamon oil	110	110	40	115	130	150
Clove oil	110	105	40	140	95	110
Propyl paraben	60	55	40	100	125	75

on milled wheat agar, with higher concentrations of the treatments required to attain the same levels of control. The best treatment for controlling growth was clove essential oil, regardless of a_w, temperature or *Fusarium* species. ANOVA tests showed that a_w, temperature, treatment, and concentration had a significant effect on growth of both species ($P > 0.05$). Interactions between the experimental variables were also found to be statistically significant. Changes in treatment concentration and a_w elicited the largest effects on growth rate of all the experimental factors.

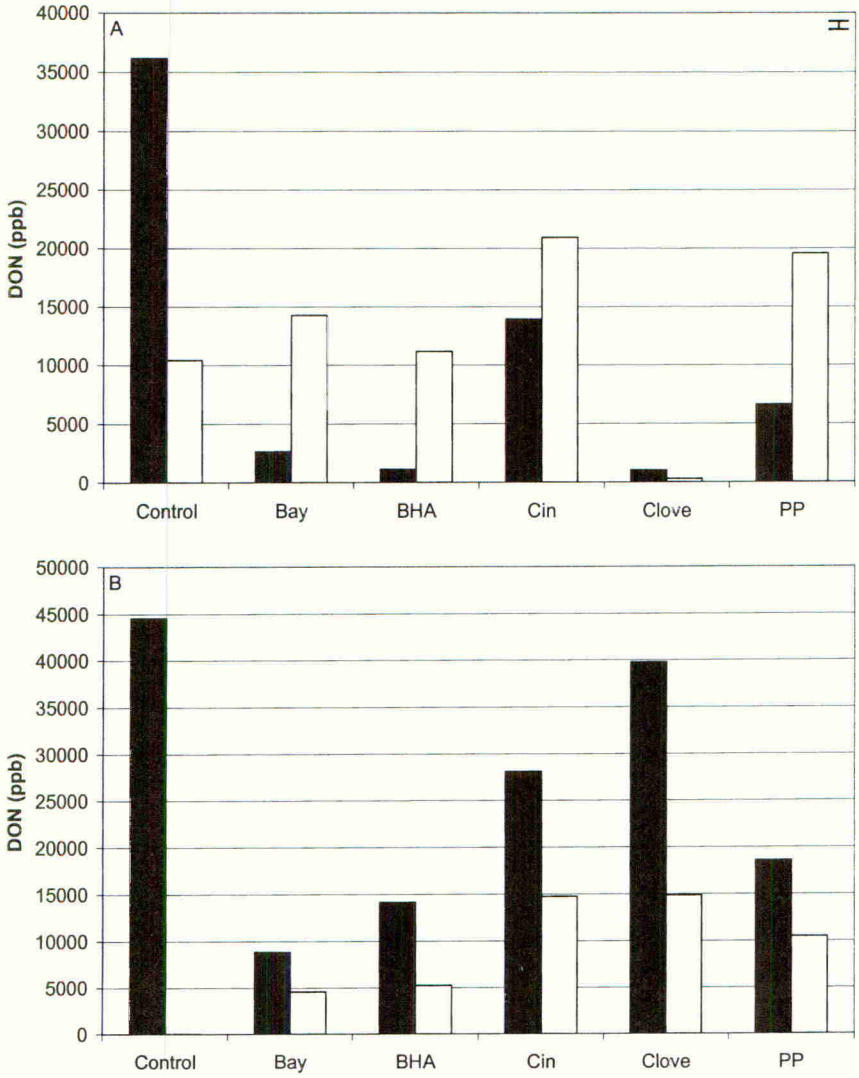

Figure 2. DON production by *F. graminearum* on irradiated wheat grain at (A) 0.995 a_w and (B) 0.955 a_w treated with 100 ppm of different essential oils or antioxidants. Plates were incubated at 25°C (black) and 15°C (white bars). Key to treatments: Bay, bay leaf oil; BHA, butyl hydroxyanisole; Cin, cinnamon oil; Clove, clove essential oil; PP, propyl paraben.

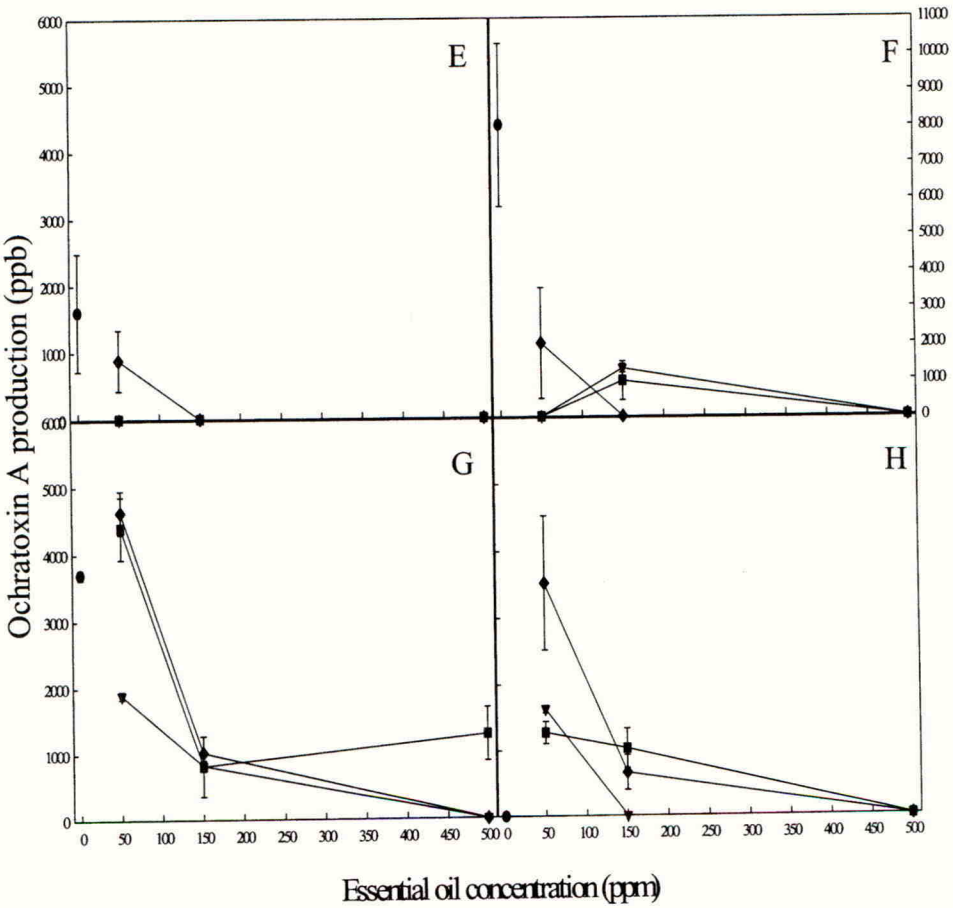

Figure 3. Effect of various concentrations of essential oils cinnamon leaf, clove bud and thyme on ochratoxin A production by *Penicllium verrucosum* (OTA11) at (A) 0.95 a_w at 15°C (B) 0.99 a_w at 15°C (C) 0.95 a_w at 25°C (D) 0.99 a_w at 25°C respectively. Key to treatments: •, control; ▼, cinnamon leaf; ■, clove leaf (cleaned); ●, thyme.

DISCUSSION

This study has shown that only a few essential oils and antioxidants are effective in controlling the growth of the *Fusarium species* and *P. verrucosum*. The efficacy of these control treatments were however affected by changes in environmental factors. Of particular importance was the fact that while low concentrations (50-100 ppm) were effective in vitro, much higher concentrations (500 ppm) were required to control growth on wheat grain with retained germinative capacity. This suggests that care is needed in extrapolating from in vitro to in situ studies. However, the results obtained from the *in situ* study showed that the best essential oils/antioxidants can significantly reduce growth as well as mycotoxin production

across a range of conditions similar to those encountered in grain during drying. Therefore, it is possible that application of essential oils/antioxidants could provide an important tool in the control of *Fusarium* species preharvest, and *P. verrcosum* post-harvest on wheat grain and more importantly mycotoxin formation at the critical control points in the chain of cereal production.

The modes of action of essential oils are largely unknown. However, there is some information in the literature towards this end. In a study of Egyptian essential oils by Farag *et al.* (1987) it was concluded that there was a relationship between the chemical structure of the main component of an essential oil and its antimicrobal activity. The inhibitory effect of the oils was generally attributed to the presence of an aromatic nucleus containing a polar functional group. This is a similar structure to that of phenols and chlorophenols, which are widely used in disinfectants. The presence of phenolic OH groups able to form hydrogen bonds with the active sites of target enzymes was thought to increase antimicrobal activity. BHA and propyl paraben were effective inhibitors of mycelial growth under some conditions. This is similar to the findings of Thompson *et al.* (1992). The mechanism of action for the parabens which produce inhibition of mycelial growth in toxigenic fungi is not clear. However, it is known that the effectiveness of the parabens increases with an increase in the chain length of the ester group (Thompson 1993). Khan *et al.* (2001) suggested that propyl paraben and BHA worked mainly at the cell membrane eliminating the pH component of the protomotive force and affecting energy transduction and substrate transport. BHA has also been shown to have a direct effect on the mitochondrial electron chain of trypanosomes thus inhibiting respiration (Khan, 2001). The economics of the use of these compounds needs to be examined in more detail.

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Occurrence of *Fusarium* mycotoxins in Czech wheat in 2003-2004

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ABSTRACT

The deoxynivalenol content, presence of kernels infected by *Fusarium* spp. pathogens and the frequency of kernels visually infected by *Fusarium* head blight in wheat intended for human consumption were monitored in 2003-2004. About 1,000 samples provided by growers from various regions of the Czech Republic were analysed each year. For the samples from 2004 with the deoxynivalenol content above the limit of detection (0.20 $mg.kg^{-1}$), the zearalenon content was also assessed. In 2003 and 2004, deoxynivalenol was detected in 4.0 and 4.5 % of the examined samples at the maximum values of 5.1 $mg.kg^{-1}$ and 18.3 $mg.kg^{-1}$, respectively. The values of the zearalenon content were low; values above the limit of detection of the method used (0.05 $mg.kg^{-1}$) were found for 9 samples only and the maximum value was 0.12 $mg.kg^{-1}$. Significant correlations were found between the deoxynivalenol content, percentage of kernels infected by *Fusarium* spp. pathogens and the frequency of visually scabby kernels.

INTRODUCTION

Fusarium head blight or scab of wheat is one of the most damaging diseases on cereals in regions with mild and humid climatic conditions (Cook, 1981). It can be caused by up to 17 various species of the *Fusarium* genus (Parry *et al.*, 1995). The most frequent species in Europe are *Fusarium graminearum*, *F. culmorum*, and *F. avenaceum* (Logrieco & Botalico, 2001). In the Czech Republic, the most frequent pathogen infecting heads was *F. graminearum* followed by *F. poae* and *F. culmorum* (Tvarůžek, 2003). The basic toxins produced by *F. graminearum* are deoxynivalenol (DON), nivalenol, and zearalenon (ZEA).

With regard to clearly adverse effects of *Fusarium* mycotoxins on both human and animal health, limits for their maximum acceptable amounts in cereals and final products for human consumption or feeds have been adopted in a number of countries during several past years. Commission Regulation (EC) No 856/2005, which sets maximum limits for the content of *Fusarium* mycotoxins in cereals for human nutrition and in some products from them, will come into force in the European Union from 1 July 2006. One of major issues related to practical applications of this regulation is the large number and costs of analyses. If the selection for analyses were carried out randomly, there would be a danger of not catching the samples with a high content of mycotoxins due to their variable frequencies. A reasonable approach seems to be the assessment of the mycotoxin content in cereals based on a reliable analysis of their occurrence risk. To create bases and develop methodologies for evaluation of contamination risks, the occurrence of mycotoxins has to be monitored in a representative set of samples in a longer time series and knowledge of potential relationships between visually assessable grain characteristics and the mycotoxin content must be obtained and analysed.

The objectives of the study were i) to determine a level of contamination by some *Fusarium* mycotoxins in wheat produced for food consumption in the Czech Republic and ii) to observe the relationship between the percentage of visually scabby kernels, kernels infected by *Fusarium* spp. pathogens, and the DON and ZEA content.

MATERIALS AND METHODS

The samples of wheat provided by growers from various regions of the Czech Republic without any post-harvest treatments were used for analyses. First, they were evaluated for the number of VSK. The other parameters, such as the DON content and percentage of infected kernels (IK) by *Fusarium* spp. pathogens, were then assessed in the samples containing more than two (in 2003) or five (in 2004) VSK in 100 g. In 2004, the samples with the DON presence were also analysed for the ZEA content. The immunochemical assay ELISA with the limit of detection (LOD) for DON of 0.20 $mg.kg^{-1}$ and for ZEA of 0.05 $mg.kg^{-1}$ was used for analyses. The infection of kernels by *Fusarium* spp. pathogens (IK) was assessed using a paper roll test described by Tvarůžek *et al.* (2004). VSK evaluation was based on kernel colour (pink, chalky white or pale grey) and shape (thin or shrivelled). The samples from 2003 with the DON content > 1.20 $mg.kg^{-1}$ were also analysed for the content of this mycotoxin separately in the kernels determined as VSK and healthy ones.

RESULTS

In 2003, a total of 1,000 samples of wheat were screened for VSK, of which 69 samples containing two and more scabby kernels were selected. The DON content higher than 0.20 $mg.kg^{-1}$ (detected maximum value 5.10 $mg.kg^{-1}$) was found in 39 of these samples including 18 cultivars. The *Fusarium* spp. pathogens on kernels (IK) were found in all of these 39 samples at the frequency from 2 to 57 %. The percentage of VSK ranged from 0.17 – 5.91 %. A highly significant correlation was found between the DON content and IK (r=0.63 at p=0.01), and even closer correlation was found between the DON content and VSK (r=0.81 at p=0.01) (Table 1).

Table 1. Correlation coefficients between deoxynivalenol and zearalenon content, VSK and IK in wheat (** significant at p=0,01, ns non-significant, ne not evaluated)

		DON	ZEA	VSK	IK
DON	2003	X	ne	**	**
	2004		ns	**	**
ZEA	2003	ne	X	ne	ne
	2004	ns		ns	ns
VSK	2003	**	ne	X	**
	2004	**	ns		**
IK	2003	**	ne	**	X
	2004	**	ns	**	

In 2004, a total of 1,063 wheat samples were visually estimated, of which 50 samples were selected containing five and more VSK. The DON content higher than 0.20 $mg.kg^{-1}$ (detected maximum value 18.30 $mg.kg^{-1}$) was found in 48 of these samples including 22 cultivars. The IK were present in all samples with the proved DON presence in this year too. The frequency of IK ranged from 2 to 99 %. Percentage of VSK was found at the frequency from 0.26 to 11.36 %. The relationship was highly significant between the DON content and IK ($r=0.59$ at $p=0.01$) as well as between the DON content and VSK ($r= 0.92$ at $p=0.01$) (Table 1).

The ZEA content was measured for 36 samples from 2004 with the DON content from 0.20 $mg.kg^{-1}$ to 18.30 $mg.kg^{-1}$. The content above the LOD was found in 9 samples with the values ranging from 0.051 to 0.124 $mg.kg^{-1}$. No significant correlations were found either between the DON and ZEA content or between IK and ZEA content or between VSK and ZEA content (Table 1).

The DON content in kernels selected as VSK from a total number of 16 samples in 2003 averaged 56.1 $mg.kg^{-1}$ (the range for individual samples was between 45.60 and 91.60 $mg.kg^{-1}$) and in the remaining healthy kernels 0.8 $mg.kg^{-1}$ (< 0.20 – 2.10 $mg.kg^{-1}$).

DISCUSSION

Fusarium mycotoxins are widely distributed within the food chain of the EU (Anonymous 2005). The critical factors that affect their occurrence in cereals are climatic conditions and agronomic practices. Thus, the proportion of production contaminated by these mycotoxins varies in individual countries and regions as well as in crop years. The reported frequency of mycotoxin presence in cereals is also influenced by the limit of detection for a used method, therefore it seems more useful to compare the percentage of samples showing values above a certain limit, for instance, 1.25 $mg.kg^{-1}$ for deoxynivalenol, which is given by Commission Regulation (EC) No 856/2005. The presented monitoring of wheat in the Czech Republic included 1.6 and 3.3 % of such samples in 2003 and 2004, respectively. The maximum values found may also be affected by a size of the examined set and the method of sample selection for analyses, it means whether these samples are selected on the basis of a certain risk analysis or randomly selected. Since their frequency is rather low, the samples with high mycotoxin values may be omitted in the case of a smaller set examined. The analyses of vast sets are restricted by high costs. In the presented monitoring, an appropriate approach showed to be visual prescreening of a large number of samples and then mycotoxin analyses of only the samples with visually scabby kernels even if there was some suspicion only. Symptoms of visual infection can be difficult to estimate in some cases and they can also be affected by growing conditions. Zhang & Jin (2004), for instance, indicate that the variability in grain colour and size can be caused, besides *Fusarium* spp., by environmental stress, which hampers distinguishing the types of damage and data interpretation.

Separate analyses of deoxynivalenol in kernel fractions evaluated as VSK and healthy ones for the samples with the content > 1.20 $mg.kg^{-1}$ confirmed that the total toxin content was particularly due to the strongly infected and well visually detectable kernels.

In both years, the significant relationship was found between IK and DON content as well as between VSK and DON content. Liu *et al.* (1997) found for *Fusarium culmorum* a highly significant correlation between percentage of kernels visually estimated as strongly infected and the DON content, whereas this relationship was not significant for the kernels visually

estimated as slightly and medium-infected. In both years, the relationship between DON and VSK was closer than that between DON and IK. Zhang & Jin (2004) came to a similar conclusion, when they had not found any relationship between the presence of the *Fusarium* pathogens on kernels and the DON content, however they had found significant correlation between the DON content and visual estimation of infected kernels.

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Determination of deoxynivalenol in baby food and relevant cereals with two different commercial screening tests

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ABSTRACT

Deoxynivalenol (DON, vomitoxin) is found in abundance in various cereal crops and belongs to the trichothecenes, a group of mycotoxins produced by the Fusarium moulds EU legislation will specify maximum limits for the presence of DON in a variety of foodstuffs including babyfood. The permitted level for babyfood will be 100µg/kg^{-1}. This study assesses two different test systems, an ELISA screening test and immunoaffinity columns with high-pressure liquid chromatography (HPLC) for determination of deoxynivalenol in baby food and cereal samples.

INTRODUCTION

The mycotoxin, deoxynivalenol, commonly known as vomitoxin, is a natural toxin produced by several moulds of the genus *Fusarium,* in particular, *Fusarium graminearum* and *F. culmorum*. It is found in various cereals including wheat, corn, barley and rye and is the most reported mycotoxin after aflatoxin. Deoxynivalenol can have serious effects on both humans and animals due to consumption of contaminated cereals. As a result, the European Commission intends to introduce legislative limits for cereals and baby food to prevent and reduce the levels of Deoxynivalenol in the foodchain. Since the use of adequate sampling and validated methods of analysis are essential for the development of any standard for mycotoxins, provisions concerning sampling and analysis will be introduced at a later stage.

This study evaluates two test systems for the determination of deoxynivalenol in a variety of cereals and also in baby foods, which have been spiked at 50ppb and/or 100ppb deoynivalenol:

RIDASCREEN DON ELISA screening test
DONPREP immunoaffinity columns + UV-HPLC

EXPERIMENTAL

Methodology for immunoaffinity columns with HPLC

Extraction

200ml of deionised water/HPLC grade water was added to 25g of ground sample (plus 8g of PEG for baby food analysis) in a blender jar and blended at high speed for 2 minutes. Alternatively it is possible to extract the toxin from the sample by shaking for 30 minutes. Immediately after blending or shaking, a minimum of 20ml of the supernatant was filtered through Whatman No 113 filter paper. The sample was mixed again and filtered through microfibre filter paper.

Immunoaffinity chromatography

The immunoaffinity columns (IACs) were allowed to reach ambient temperature before use. The immunoaffinity column was firmly attached to a 10ml glass syringe barrel using an adaptor and 2ml of the extract (equivalent to 0.25g of sample) was passed through at a flow rate of 1 drop/second or under gravity. The column was washed with 5ml of HPLC grade water at the same flow rate. Using a syringe pump unit, air was flushed through the column to remove the last few drops of water. 1.5ml of HPLC Grade methanol (i.e. eluant) was passed through the column at a flow rate of 1 drop per second and collected in a collection tube. Air was passed through the column to collect the last drops of methanol. The glass tube was then placed in an evaporating system and blown down to dryness. The eluate was reconstituted in 1ml of mobile phase and mixed for 30 seconds using a vortex mixer. Finally 100µl of the reconstituted eluate (equivalent to 0.025g of sample) was injected onto the UV-HPLC System.

HPLC conditions:

Column:	C18 deactivated reverse phase packing material
Mobile Phase:	94:3:3 v/v/v HPLC grade water:methanol:acetonitrile
Flow Rate:	1ml/min
Oven Temperature:	40 Degrees C
UV Detection:	218nm

Methodology for screening using the ELISA kit

Extraction

A representative sample (according to accepted sampling techniques) was ground and thoroughly mixed prior to proceeding with the extraction procedure. A cereal sample weighing 5g was mixed with 25ml of distilled water (5g of sample with 50ml of distilled water for babyfood) and shaken vigorously for 3 minutes. The sample was then centrifuged for 10 minutes at 3000g (RT) and the supernatant used for analysis in the ELISA.

ELISA method

50ul of sample or standard was pipetted into the microplate wells, followed by 50ul of enzyme conjugate. The next step was to add 50ul of DON antibody and incubate at 30 minutes at room temperature. The plate was then washed 3 times with wash buffer and 2 drops of substrate were added, the plate was left for 15 minutes at room temperature in the dark for the reaction to occur. Two drops of stop solution were added and the absorbances read at 450nm using a plate reader.

RESULTS AND DISCUSSION

Table 1 Comparison of IACs with HPLC and the ELISA screening test in cereals

Various cereals were tested for deoxynivalenol using columns and with UV-HPLC and the ELISA test.

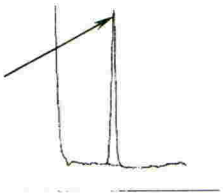
Commodity	IAC with HPLC	ELISA test
Wheat (CRM 396)	Not Tested	<LOD
Wheat	159	200
Oats (P42)	6	<LOD
Oats (B)	22	31
Maize	414	667
Rye	30	52
Malted Barley (h1b)	14	24

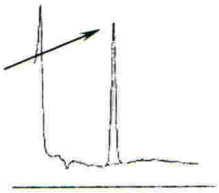
Table 2 Comparison of IACs HPLC and the ELISA screening test in babyfood

Commodity	IAC with HPLC			ELISA test	
	Blank Sample	Spikes at 50ppb % Recovery	Spikes at 100ppb % Recovery	Blank Samples	Spikes at 100ppb % Recovery
Milk pap with rice, wheat, yogurt, fruits	<LOD	101%	94%	<LOD	115%
Milk pap with rice, maize, banana	<LOD	Not Tested	Not Tested	<LOD	123%
Porridge, apple	<LOD	86%	96%	<LOD	97%
Milk pap with rice, maize, apple, carrot	<LOD	108%	102%	<LOD	135%
Milk pap with rice, wheat, fruit	33ppb	85%	84%	45ppb	129%
Porridge, wheat, rice, barley	<LOD	107%	101%	<LOD	110%
Milk pap with rice, chocolate, nuts	<LOD	88%	87%	<LOD	110%
Wheat, porridge, chocolate	<LOD	110%	100%	<LOD	115%
Cereal mix with apple and blueberry	<LOD	90%	86%	<LOD	Not Tested
Muesli	<LOD	92%	85%	<LOD	Not Tested

Immunoaffinity columns used with HPLC and an ELISA test were used to test a selection of cereal-based babyfood containing dried fruit and chocolate. In this case all, but one of the samples were found to be blank or below the limit of detection (LOD). Samples were then spiked at 50ppb and 100ppb according to the proposed legislative limit for DON in babyfood and re-analysed to check for recoveries.

HPLC traces for IACs with cereals (Fig 1) and baby food (Fig 2)





With regard to sample analysis the LOD for the IACs with HPLC is equivalent to 6ppb and for the ELISA is equivalent to 18.5ppb for cereals and 37ppb for babyfood.

CONCLUSIONS

In this study the IACs with HPLC were found to give recoveries of >80% for cereal and also for baby food samples spiked at 50 μg kg^{-1} and 100 μg kg^{-1}. For both sample types the HPLC traces were found to be clean with little background interference even with highly complex and coloured baby food samples. Although sample extraction with water was found to be suitable for the analysis of cereals, water with the addition of polyethylene glycol (PEG) was found to give better recoveries with the columns for more complex baby food samples. Using immunoaffinity columns for analysing baby food has many advantages. These include concentration of very low levels of toxin and efficient clean-up of the sample resulting in better sensitivity and less background interference with complex food samples.

The ELISA was found to be a fast microplate assay (45 min incubation time) and to be suitable for multiple sample analysis. In this study, the limit of detection was found to be 18.5ppb for cereals and 37ppb for babyfood and the majority of the samples analysed were found to be below the limit of detection of the test. Samples spiked with Deoxynivalenol at a 100 μg kg^{-1} level were found to give recoveries of > 80 % using the ELISA.

Both test systems were found to be suitable for analysis of a variety of baby food samples and cereals, in accordance with the proposed European legislative limits.

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POSTER SESSION 5PC

PESTICIDE FORMULATION TECHNOLOGY AND ADJUVANT SCIENCE

Session Organiser: Dr Hans de Ruiter
SURfaPLUS R&D, Wageningen, The Netherlands

Poster Papers: P5C-1 to P5C-3

Phytotoxicity and adjuvancy of lactate esters in 2,4-D based agrochemical formulations

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ABSTRACT

Application of n-propyl lactate, n-butyl lactate and 2-ethylhexyl lactate on tomato seedlings did not result in phytotoxic effects when applied at 0.05 and 0.5% (*wt/wt*). The results of this study show that lactate esters can strongly enhance the performance of the herbicides 2,4-D-dimethylamine (salt) and 2,4-D-2-ethylhexyl (ester) on *Chenopodium album* (fat-hen).

INTRODUCTION

Lactate esters are used as 'green' solvents in many industries. In the agrochemical industry there is also a tendency towards the use of 'green' solvents due to environmental and safety issues. Lactates are known to show good compatibility with 'natural' substrates such as wood, leather or fibres. Lactate esters may enhance the foliar penetration of agrochemicals by serving as a solvent in the drop deposit. A second option is that the solvents themselves penetrate into the waxy leaf cuticle and enhance the mobility of the chemicals in the cuticle. The solvents also reduce surface tension so that an influence of both wetting and spreading will be possible. The solvents used in this study are n-propyl lactate (NPL), butyl lactate (BL) and 2-ethylhexyl lactate (EHL). Table 1 provides an overview on some relevant physical properties of these esters.

Table 1. Physical properties of some lactate esters

Lactate ester	Solubility in water (% (*wt/wt*) at 20 °C)	Viscosity (cP at 20 °C)	Vapour pressure (mbar at 20 °C)	Surface tension (mN/m at 25 °C)	Flash point ISO 2719 (°C)
NPL	100	3.3	1	30.4	69
BL	4.5	3.9	0.4	29.5	79
EHL	0.03	7.7	0.02	29.8	113

The aim of this study is to compare different lactate esters on their bio-enhancement potential in 2,4-D dimethylamine salt (DMA) and 2-ethylhexyl ester (2-EH) formulations. Such adjuvancy can be beneficial in the development of more cost-effective and environmentally friendly formulations. To complete the set of data, the phytotoxicity of these solvents was measured.

MATERIALS AND METHODS

Plant material

Adjuvant studies were performed on fat-hen (*Chenopodium album* L.), while phytotoxicity studies were performed on tomato.

Tomato seeds (cv. Astona F1) were germinated under the same conditions as used for fat-hen (see below). At 12-14 days after germination the tomato seedlings were transferred (one plant per pot) to 11-cm-diameter plastic pots filled with a mixture of sand and humic potting soil (Soil no. 12, Colent, Lent, The Netherlands) (1:2 by volume). The pots were placed on sub-irrigation matting that was wetted daily with a nutrient solution. The tomato plants were brought to the greenhouse with the same conditions regarding temperature and relative humidity regime. Additional artificial light was provided when necessary. The tomato seedlings were treated 26-28 days after the seeds were sown. Then, the tomato plants had 4 unfolded leaves and were 16 cm tall.

Fat-hen was grown in a growth chamber under 14 h of light, at 18/12 (±0.5)°C (day/night) temperature, and in 70/80 (±5)% (day/night) relative humidity. Light was provided by high-pressure mercury lamps and fluorescent tubes to give 70 W/m2 (PAR) at leaf level. After emergence, the fat-hen plants were thinned to two plants per pot. Fat-hen was treated at the four-leaf stage. The fresh weight of fat-hen was measured 21 days after treatment (21 DAT).

Application of treatment solutions

The solutions are applied with an air-pressured laboratory track sprayer having 1.2-mm nozzles fitted with a perforated (0.6 mm) whirling pin and delivering 200 litre/ha at 303 kPa.

Phytotoxicity

The reference product used for phytotoxicity studies was 'Arkopal N 080' (nonylphenol polyoxyethylene (8) from Clariant). The solvents and reference product were included at 0.05 (*v/v*) and 0.5% (*wt/v*) respectively. The treatment solutions were shaken just before application to ensure a homogeneous distribution of the products. It was checked before, that a homogeneous distribution was obtained also with the more lipophilic solvents like 2-ethylhexyl lactate ester. Leaves were inspected visually at 3-4 days after treatment (DAT) and a phytotoxicity rating (1 to 5) was recorded. The scale used for the phytotoxicity rating was: 1 = no discoloration or necrosis to 5 = complete necrosis of the wetted plant surface plus loss of leaf turgor. A score of 4 was given when there was complete necrosis without loss of leaf turgor. The fresh weight of the aerial plant parts was measured 7 DAT.

Adjuvancy

The solvents n-propyl lactate, n-butyl lactate and 2-ethylhexyl lactate ('PURASOLV' NPL, -BL and –EHL, respectively) all supplied by PURAC Biochem, The Netherlands, were added to

the herbicides or applied alone (NPL) at a concentration of 0.5% (*v/v*). The carrier was demineralized water.

A sub-optimal rate of the herbicides, giving a 0-20% growth reduction without solvents, was used to demonstrate the adjuvant effects. We applied 2,4-D 2-EH (solvent-free EW formulation; 'Lentemul D' 450 g a.e. /litre) and 2,4-D DMA (SL formulation; 'Spritz Hormin 500' g a.e. /litre) both supplied by Nufarm, Austria.

Experimental design and data analysis

The phytotoxicity experiments were conducted as a randomized complete block with four replicates on two separate occasions. The variance between treatments was very low over the experiment as there was little phytotoxicity. The adjuvancy experiments were also conducted with four replicates in a completely randomized design on two separate occasions. The data were subjected to analysis of variance using the statistical package 'Genstat' Release 6.1; Rothamsted Experimental Station. The means of treatment were compared according to Fisher's LSD (0.05). LSD (0.05) is the Least Significant Difference between mean values at the 5% level.

RESULTS AND DISCUSSION

Phytotoxicity

The visual assessment and the fresh weight measurement (Table 2) demonstrated that most treatments were not phytotoxic and looked the same as the untreated plants. Alone, the reference product at 0.5% (*wt/v*) demonstrated a low but clearly visible level of phytotoxicity. This was not reflected in a reduction of fresh weight. Fresh weight levels in the second experiment were higher than in the first experiment because the second experiment was conducted in April and the first experiment in March when less light is available for growth.

EHL is the most lipophilic solvent and, as a consequence, may penetrate into both the lipophilic leaf cuticle and the lipophilic cell membrane. The observation that phytotoxic effects are not observed at the 0.5% (*v/v*) level indicates that either the penetration of EHL into the plant or its interaction with the cell membrane is rather limited at this concentration. Application of the solvents and the reference product at the somewhat unrealistic concentration of 5%, demonstrated severe phytotoxic symptoms from the reference product but EHL. NPL and BL were not phytotoxic at 5% (*v/v*).

This study provides indirect evidence that lactate esters exert their activity on the leaf surface. This can be beneficial in situations where any risk on plant phytotoxicity has to be excluded (ornamentals, fruit, vegetables etc.). Application of EHL may result in phytotoxic effects in situations where accumulation of solvents may occur.

Table 2. Phytotoxicity (means of four replicates) of solvents and reference product

No.	Product	Conc. (%) a	Phytotoxicity visual assessment b Experiment 1	Phytotoxicity visual assessment b Experiment 2	Phytotoxicity fresh weight (g) Experiment 1	Phytotoxicity fresh weight (g) Experiment 2
1	Untreated	-	1.0	1.0	8.6	10.5
2	Demin water	-	1.0	1.0	8.1	9.6
3	NPL	0.05	1.0	1.0	9.1	8.2
4	NPL	0.5	1.0	1.0	7.9	10.0
5	BL	0.05	1.0	1.0	9.0	10.0
6	BL	0.5	1.0	1.0	8.5	10.2
7	EHL	0.05	1.0	1.0	7.6	9.9
8	EHL	0.5	1.0	1.0	8.1	10.3
9	Arkopal N-080	0.05	1.0	1.0	8.3	10.7
10	Arkopal N-080	0.5	2.3	2.5	8.1	9.6

a *v/v* for PL, BL and EHL solvents and *wt/v* for Arkopal N-080
b 1-5 rating; 1 = no phytotoxic effects, 5 = necrosis and loss of leaf turgor;

Adjuvancy

NPL enhanced the performance of 2,4-D DMA salt (Figure 1; Table 3).

Table 3. Influence of PL, BL, EHL solvents on 2,4-D efficacy when tested on fat-hen as fresh foliar weight (g), (mean of 4 replicates)

No.	Herbicide	Adjuvant 0.5% *v/v*	Fat-hen Means (g)
1	Untreated	-	13.53
2	-	NPL	15.23
3	2,4-D DMA[1]	-	9.70
4	2,4-D DMA	NPL	5.53
5	2,4-D 2-EH [2]	-	11.16
6	2,4-D 2-EH	NPL	9.01
7	2,4-D 2-EH	BL	8.11
8	2,4-D 2-EH	EHL	6.53
	LSD (0.05)		1.3

[1] dimethylamine salt of 2,4-D (500 g a.e. /litre); dose 0.3 mM (equiv. to 13.3 g a.e. /ha at 200 litres/ha).
[2] 2-ethylhexyl ester of 2,4-D (450 g a.e. /litre); dose 0.1 mM (equiv. to 4.4 g a.e. /ha at 200 litres/ha).

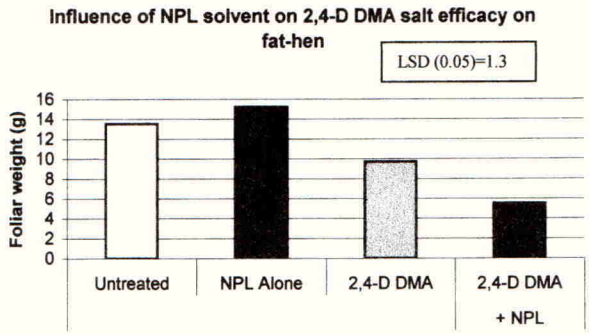

Figure 1. Influence of NPL on 2,4-D DMA salt efficacy

NPL, BL and EHL also enhanced the performance of 2,4-D 2-EH (Figure 2; Table 3). EHL was significantly better than NPL and tends to be better than BL in enhancing efficacy of 2,4-D 2-EH. We did not observe phytotoxic symptoms caused by the solvents applied.

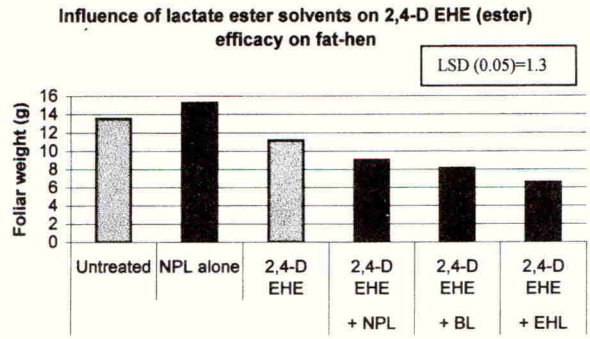

Figure 2. Influence of lactate ester solvents on 2,4-D 2-EH efficacy

These efficacy data do not reveal the exact mechanism(s) involved in the activity of the PURAC solvents. Below, a discussion is started based on the data so far. Fat-hen is somewhat wettable. The similarity in surface tension of the various solvents (Table 1) and the differences in performance between NPL with the DMA salt and the 2-EH ester (Figure 2; Table 3) indicate that it is not just a matter of improved retention of spray solution on the foliage of fat-hen. An adjuvant that increases foliar uptake may serve as a solvent for the active ingredient in the visually dry drop deposit or may enhance the permeability of the leaf cuticle or provide both functions. Penetration of the adjuvant into the leaf cuticle is required to fulfil the latter function.

Based on studies with other carbohydrate-based adjuvants and the structure of the lactate esters, it seems likely that the lactate esters will not or hardly move into the waxy leaf cuticle. This means that the action of the lactate ester solvents is most likely located on the leaf surface. We suggest that they improve the availability of the active ingredient for uptake. This is reasonable for the solid DMA salt of 2,4-D. The 2,4-D 2-EH (ester) is a liquid and very lipophilic.

The concentration of the 2,4-D ester in the treatment solution is 0.15 g a.e. /litre - much lower than the 5 g/litre (approximately) of the solvent. It is suggested that the greater amount of solvent provides a more intimate contact between ester and the leaf surface. That the EHL solvent (as the most lipophilic solvent) is the most effective one with 2,4-D 2-EH agrees with this view. More sophisticated experiments with isolated leaf cuticles and spray retention measurements are required to explain in detail the mechanism(s) involved with the lactate ester solvents.

NPL applied at 0.5% (*v/v*) did not reduce growth of fat-hen (Table 3; Figures 1, 2). In the other experiments with herbicides we also did not observe local leaf necrosis caused by BL or EHL. This preliminary observation agrees with the suggestion above that the lactate esters will not or hardly move into the leaf cuticle and subsequently to the plant tissue.

CONCLUSIONS

Application of the solvents NPL, BL and EHL on tomato seedlings did not result in phytotoxic effects up to 0.5% (*wt/wt*).

This study has shown that lactate esters can strongly enhance the performance of 2,4-D DMA salt and 2,4-D 2-EH (ester) on fat-hen.

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Adjuvant improves performance of pre-emergence residual herbicides in cereals

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ABSTRACT

Previous studies have reported that adjuvants can enhance the performance of pre-emergence residual herbicides in a range of broadleaved crops by reducing spray drift, improving spray deposition and reducing leaching. This paper reports a trial investigating the effect of the adjuvant HM 9679-A on the efficacy of five pre-emergence herbicide treatments applied via four nozzle types on the control of blackgrass (*Alopecurus myosuroides*). The addition of HM 9679-A to each of the five herbicide treatments significantly reduced the number of blackgrass plants assessed 43 days after application. This effect was maintained after the application of a standard post emergence herbicide. The number of blackgrass heads/m^2 recorded in the following spring, 273 days after the pre emergence treatment, was reduced in comparison to the herbicide alone.

INTRODUCTION

Blackgrass (*Alopecurus myosuroides*) is a widespread weed in England and in 2002 it was estimated that there were over 1000 confirmed cases of herbicide resistance spread across 30 counties in England (WRAG, 2003). Enhanced metabolism resistance is widespread affecting many cereal herbicides (e.g. isoproturon, pendimethalin and flupyrsulfuron-methyl), and target site resistance to "fops" and "dims" is increasing and remains a potential threat to ALS inhibitors (e.g. mesosulfuron). High levels of control can be achieved only by a combination of cultural practices and the use of an integrated herbicide programme. A key principle is to treat blackgrass at an early growth stage to reduce the effects of enhanced metabolism resistance, and one important component is the use of a pre-emergence herbicide. The combination of increased blackgrass resistance, the trend towards earlier establishment using reduced tillage and the introduction of flufenacet based herbicides has resulted in increased use and it is currently estimated that 0.5 million hectares of winter wheat are treated with pre-emergence herbicides annually (Market research data from 2005, BASF, *pers. comm.*). The efficacy of pre-emergence herbicides can be affected by a number of environmental factors, for example, spray drift, poor deposition over the soil surface, volatilisation from the soil surface and leaching of the active ingredient below the active zone. The addition of an adjuvant can improve their performance by reducing spray drift, improving spray deposition and reducing leaching (McMullan *et al.*, 1998). Previous studies have demonstrated that the addition of the adjuvant 'Grounded' can enhance the performance of pre-emergence herbicides when applied in maize and a range of broad-leaved crops (McMullan *et al.*, 1998; Thomas & Morgan, 2001). In this study the effect of this adjuvant when applied in mixture with a range of pre-emergence herbicides via four different nozzle types in cereals was investigated.

MATERIALS AND METHODS

Winter wheat (cv. Robigus), was drilled to a depth of 25-30mm on 16 September 2004 at 250 seeds/m^2, and subsequently rolled to create a fine firm seedbed. The following herbicide treatments were applied:

1) Untreated
2) 'Alpha Trifluralin 48EC' (480 g/litre trifluralin) at 2 litres/ha
3) 'Crystal' (flufenacet/pendimethalin 60/300 g/litre) at 4 litres/ha + trifluralin at 2 litres/ha
4) 'Stomp 400' (pendimethalin 400 g/litre) at 5 litres/ha
5) 'Liberator' (flufenacet/diflufenican 240/100 g/litre) at 0.6 litres/ha
6) 'AUK 10560' (experimental product) + trifluralin at 2 litres/ha

Each treatment was applied either alone or in conjunction with 0.2 litres/ha 'Grounded' (a proprietary blend of $C_8 - C_{12}$ aliphatic hydrocarbons, hexahydric alcohol ethoxylates, and $C_{18} - C_{20}$ alkanoic acids, hereafter coded HM 9679-A) using a Martin Lishman sprayer mounted on an ATV via a range of nozzles detailed in Table 1. Application date was on 22 September 2004, 6 days after drilling.

Table 1. Application details of nozzles used to apply pre-emergence herbicides

Nozzle	Specification	Application volume (litres/ha)	Spray quality	Pressure (kPa)
Flat fan	04 F110	200	Medium	200
Lo-Drift	02 F110	100	Medium	200
Air inclusion	02 F110	100	Medium	200
Variable pressure	02 F110	100	Medium	200

Each plot measured 3m x 3m and was replicated five times. The trial was located near Maidwell, Northampton on a clay soil. Resistance testing in 2004 established that the field blackgrass population had enhanced metabolism resistance, R? to "fop" herbicides and RR to pendimethalin as defined by the Weed Resistance Action Group classification. The efficacy of the pre-emergence treatments on the control of blackgrass was assessed on 4 November 2004 (43 DAT), by counting the number of blackgrass plants in five 0.1m^2 quadrats per plot, and then calculating the mean plants/m^2.

All plots were then oversprayed with a post emergence application of 0.4 kg/ha 'Atlantis WG' (mesosulfuron-methyl/iodosulfuron-methyl-sodium 30/6 g/kg) + 2 litres/ha trifluralin + 1.0 litres/ha 'BioPower' (an adjuvant containing 6.7% *wt/wt* 3,6-dioxaeicosylsulphate sodium salt and 20.2% *wt/wt* 3,6-dioxaoctadecylsulphate sodium salt), applied on 23 March 2005 via a flat fan nozzle in 200 litres/ha spray volume. Subsequently, the number of blackgrass heads in five 0.1m^2 quadrats per plot was recorded on 22 June 2005 (273 days after the pre-emergence treatments, 91 days after the post emergence application) and the number of heads/m^2 calculated.

RESULTS

Germination of blackgrass was rapid in the mild wet autumn, and in the untreated plots a mean of 1780 plants/m^2 was recorded in the November assessment (Table 2). With the exception of trifluralin 2.0 litres/ha applied via a variable pressure nozzle, all pre-emergence herbicides

significantly reduced the number of blackgrass plants/m^2 assessed 43 days after application. Factorial analysis was used to compare pre-emergence herbicide performance (Table 3). There was a clear hierarchy in the performance, with pendimethalin-based treatments (either alone or in combination with flufenacet + trifluralin) giving significantly the greatest reduction in blackgrass numbers. The next most effective treatment was flufenacet/diflufenican, which in turn gave a significantly lower number of blackgrass plants/m^2 in comparison with either the experimental tank mixture (AUK 10560 + trifluralin) or trifluralin (Table 3). The addition of the adjuvant HM 9679-A tended to enhance the performance of the pre-emergence herbicides. The adjuvant effect was statistically significant in specific herbicide-nozzle combinations (Table 2). For example when applied via a flat fan nozzle, use of the adjuvant reduced the blackgrass population from 600 to 375 plants/m^2 with trifluralin and from 145 to 70 plants/m^2 with the mixture of flufenacet/pendimethalin + trifluralin (Table 2). In contrast, there was no clear effect when applied with pendimethalin alone. The effect of the adjuvant on overall herbicide performance was significant (Table 3).

Table 2. The effect of pre-emergence herbicide, adjuvant and nozzle type on the number of blackgrass plants/m^2. Assessed 43 DAT. LSD (p =0.05) = 354.9. Statistical analysis was carried out on the log-transformed data

Treatment (dose rate product litres/ha)	Flat Fan (04F110) 200litres/ha	Lo-Drift (LD02F110) 100litres/ha	Air inclusion (BBJ02F110) 100litres/ha	Variable Pressure (VP02F110) 100litres/ha
Untreated	1780.0 a			
Trifluralin (2.0)	600 bc	702.5 bc	750.0 bc	775.0 ab
Trifluralin + HM 9679-A (2.0 + 0.2)	375.0 b-h	532.5 bc	505.0 bcd	305.0 c-j
Flufenacet/pendimethalin + trifluralin (4.0 + 2.0)	145.0 g-m	182.5 e-i	145.0 h-m	60.0 mn
Flufenacet/pendimethalin + trifluralin + HM 9679-A (4.0 + 2.0 + 0.2)	70.0 k-n	85.0 k-n	65.0 lmn	75.0 lmn
Pendimethalin (5.0)	100.0 i-n	77.5 k-n	80.0 k-n	60.0 n
Pendimethalin + HM 9679-A (5.0 + 0.2)	50.0 n	92.5 j-n	90.0 k-n	217.5 e-l
Flufenacet/diflufenican (0.6)	315.0 c-j	237.5 c-j	450.0 b-e	275.0 b-i
Flufenacet/diflufenican + HM 9679-A (0.6 + 0.2)	215.0 d-k	77.5 k-n	140.0 h-m	155.0 f-m
AUK10560 + trifluralin (2.0)	355.0 b-h	412.5 b-f	440.0 b-e	440.0 b-e
AUK10560 + trifluralin (2.0) + HM 9679-A (0.2)	270.0 b-i	387.5 b-g	255.0 c-l	300.0 b-h

In this trial the effect of nozzle type on the performance of the pre-emergence herbicide was not significant in relation to the number of blackgrass plants/m^2 recorded in November.

Table 3. Factorial analysis on blackgrass plants/m^2 assessed on 43 DAT. Means followed by the same letter do not significantly differ, ns = not significant

Factor	Blackgrass plants/m^2	Log LSD (p=0.05)
Pre-emergence herbicide (product litres/ha)		LSD = 0.16
Trifluralin (2.0)	568.1	2.70 a
Flufenacet/pendimethalin + trifluralin (4.0 + 2.0)	103.4	1.96 d
Pendimethalin (5.0)	95.9	1.91 d
Flufenacet/diflufenican (0.6)	233.1	2.28 c
AUK 10560 + trifluralin (2.0)	357.5	2.54 b
Pre-emergence adjuvant		LSD = 0.10
No adjuvant	330.1	2.36 a
+ adjuvant	213.1	2.20 b
Nozzle / water volume (litres/ha)		LSD = 0.14
Flat fan (200)	249.5	2.26 ns
Lo-Drift (100)	278.8	2.30 ns
Air inclusion (100)	292.0	2.31 ns
Variable pressure (100)	266.2	2.25 ns

The effect of the performance of the pre-emergence herbicide as a component of an integrated blackgrass control programme, was assessed by overspraying the trial with a standard post emergence herbicide mixture, and then assessing the heads/m^2 recorded in June (Table 4). The blackgrass population in the untreated was high at 2400 heads/m^2 and applying only a post emergence application of mesosulfuron-methyl/iodosulfuron-methyl-sodium + trifluralin + 'Biopower' reduced this to 411 heads/m^2 equivalent to 83% control. All the pre-emergence treatments applied in sequence with a post-emergence spray application, significantly reduced the number of heads/m^2 in comparison to the post-emergence only treatment, and control ranged from 88 – 98%.

Factorial analysis was used to compare performance of the herbicides, adjuvant and nozzle type (Table 5). A pre-emergence application of flufenacet/pendimethalin gave significantly the greatest reduction in blackgrass heads/m^2 and this equated to 97% control. The development combination (AUK 10560 + trifluralin), trifluralin or pendimethalin alone all gave similar levels of control and were significantly better than the flufenacet/diflufenican (Table 5). The addition of HM 9679-A improved the levels of control of blackgrass achieved, reducing the number of heads/m^2 recorded from 236.0 to 130.6 when applied in combination with trifluralin via a flat fan nozzle (Table 4), and from 262.8 to 114.2 with flufenacet/diflufenican via an air inclusion nozzle.

Overall the addition of the adjuvant HM 9679-A to pre-emergence herbicides significantly reduced the number of blackgrass heads recorded (Table 5). In this trial the performance of the flat fan, Lo-Drift and air inclusion nozzles overall was similar, and significantly better than the variable pressure nozzles in terms of the number of blackgrass heads/m^2 assessed in June (Table 5).

Table 4. The effect of pre-emergence herbicide, adjuvant and nozzle type on the number of blackgrass heads/m^2. The percentage control is given in parenthesis. All treatments (including the untreated) received a post emergence herbicide treatment. Assessment on 22 June 2005 (91 DAT with the post-emergence herbicide). LSD (p=0.05) = 83.8

Treatment (litres/ha)	Flat Fan (04F110) 200litres/ha	Lo-Drift (LD02F110) 100litres/ha	Air inclusion (BBJ02F110) 100litres/ha	Variable Pressure (VP02F110) 100litres/ha
Untreated (no post emergence)	2400			
Untreated	411.4 a (83)			
Trifluralin (2.0)	236.0 bcd (90)	90.6 l-p (96)	156.8 d-m (93)	226.2 b-f (91)
Trifluralin + HM 9679-A (2.0 + 0.2)	130.6 h-p (95)	119.8 i-p (95)	130.8 h-p (95)	182.8 c-j (92)
Flufenacet/pendimethalin + trifluralin (4.0 + 2.0)	63.8 op (97)	99.8 j-p (96)	73.4 m-p (97)	65.6 nop (97)
Flufenacet/pendimethalin + trifluralin + HM 9679-A (4.0 + 2.0 + 0.2)	59.4 p (98)	97.6 k-p (96)	67.0 nop (97)	86.0 l-p (96)
Pendimethalin (5.0)	128.8 l-p (95)	105.8 i-p (96)	137.0 g-p (94)	184.8 c-i (92)
Pendimethalin + HM 9679-A (5.0+0.2)	99.6 j-p (96)	112.8 i-p (95)	178.2 d-k (93)	180.0 c-k (93)
Flufenacet/diflufenican (0.6)	218.8 b-g (91)	281.0 b (88)	262.8 bc (89)	236.6 bcd (90)
Flufenacet/diflufenican + HM 9679-A (0.6 + 0.2)	213.0 b-h (91)	162.8 d-l (93)	114.2 i-p (95)	229.4 b-e (90)
AUK10560 + trifluralin (2.0)	145.6 f-o (94)	130.4 h-p (95)	121.4 i-p (95)	151.4 e-m (94)
AUK10560 + trifluralin (2.0) + HM 9679-A (0.2)	129.6 h-p (95)	121.8 i-p (95)	141.0 g-p (94)	147.6 e-n (94)

Table 5. Factorial analysis on blackgrass heads/m^2 assessed on 22 June 2005 (273 DAT with the pre-emergence herbicides). Means followed by the same letter do not differ significantly

Factor	Blackgrass heads/m^2 LSD (P=0.05)
Pre emergence herbicide (product litres/ha)	LSD = 29.7
Trifluralin (2.0)	159.2 b
Flufenacet/pendimethalin + trifluralin (4.0 + 2.0)	76.6 a
Pendimethalin (5.0)	140.9 b
Flufenacet/diflufenican (0.6)	214.8 d
AUK 10560 + trifluralin (2.0)	136.1 b
Pre emergence adjuvant	LSD = 18.8
No adjuvant	155.8 a
+ adjuvant	135.2 b
Nozzle / water volume (litres/ha)	LSD = 26.5
Flat fan (200)	142.5 a
Lo-Drift (100)	132.2 a
Air inclusion (100)	138.3 a
Variable pressure (100)	169.0 b

DISCUSSION

The addition of the adjuvant HM 9679-A to a range of pre-emergence herbicides significantly improved the control of blackgrass. The number of blackgrass plants recorded in the autumn was reduced in comparison to the herbicide alone, and this effect was still apparent when the number of heads/m^2 was assessed in the following June, 273 days after application. Previous studies in a range of broad-leaved crops have also reported improvements in the performance of trifluralin, pendimethalin and other herbicides when applied with HM 9679-A (McMullan *et al.*, 1998; Thomas & Morgan, 2001). Improving the performance of the pre-emergence component of the herbicide programme, has some important implications. Firstly, the selection pressure exerted on the subsequent post-emergence herbicide is reduced as the number of weed plants per unit area is reduced. Secondly, a reduction in the number of post-emergence herbicides or the selection of lower cost option may be possible. Finally, this has ramifications on the population dynamics of blackgrass. Typically 98% control is required to maintain or start to reduce the blackgrass population, and as this trial demonstrates in high-pressure sites this level of control is difficult to achieve. The addition of HM 9679- A to the pre-emergence herbicide to enhance activity can, in conjunction with an integrated control strategy, enable this level of control to be achieved, reducing the blackgrass population in future years.

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Amido propyl amines – new adjuvant class for agrochemicals

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ABSTRACT

Amido propyl amines (APAs) are a new chemistry class explored to meet the demand of increased efficacy and improved environmental properties of agrochemical formulations. For a series of various amido propyl amines based surfactants, physical and chemical properties have been determined and are shown to be dependant on the pH environment. The adjuvancy has been investigated using glyphosate and strobilurin as active ingredients. The efficacy of APA derivatives was determined in glass house trials. The studies showed excellent adjuvant properties for both glyphosate and azoxystrobin, compared to standard products on the market. Furthermore, it is shown that APAs have excellent compatibility with high loading glyphosate formulations.

INTRODUCTION

Due to consumer's interest and agricultural policies, there is a huge incentive for the agrochemical industry to develop formulations with increased efficacy and improved environmental profiles. One way of meeting these demands is to further explore the use of adjuvants in pesticide formulations. The use of fatty amine based surfactants as adjuvants for foliar pesticides, particularly hydrophilic herbicides like glyphosate and paraquat, is well known. However, the use of adjuvants to enhance the performance of fungicides and insecticides is less explored.

This paper will describe a new type of adjuvant explored to meet the demand of increased efficacy and improved environmental profile. It has been shown by other authors that the amine functionality in an adjuvant is important for adsorption to the foliar, the interaction with the plant cuticle and the penetration of the active ingredient (Coret & Chamel, 1993). The amido propyl amines are believed to possess these properties and, at the same time, offer environmental and compatibility benefits.

MATERIALS AND METHODS

Three different amido propyl amines (APA) based on C8/C10 fatty acid (APA C8/10), coco fatty acid (APA C), commercial available as 'Adsee C-80/W', and soy fatty acid (APA S) have been synthesised by Akzo Nobel Surfactants AB, Stenungsund, Sweden. Dimethyl amino propylamine was converted into the alkyl amide by condensation with the different fatty acids. Polyethoxy (15) tallow amine (TA 15) and a commercial available phosphate ester (PhE) were used as model adjuvant for comparative purposes in the glyphosate tests.

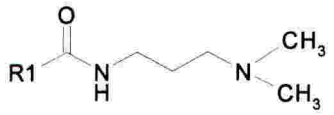

Figure 1. Amido propyl amines (APA) are fatty acid compounds with N,N-dimethyl-1,3 propanediamine. R1 can be C8/C10, coco or soy fatty acids

Equilibrium surface tension was measured by the Du Noüy ring method, with a Sigma 70KSV instrument. Contact angle measurement was made using a FTÅ 200 instrument, equipped with a video camera and an image analysis software, on a hydrophobic surface consisting of 'Parafilm PM-992' (American Can Company). As a reference, equilibrium surface tension of distilled and deionized water is 72 mN/m and contact angle on Para film after 60 s equals 102°. The concentration of surfactant used for contact angle and surface tension measurements, has been 0.1% and the contact angle after 60 s is given. All measurements were done at a temperature of 20°C and a humidity of 53%. The water used was distilled and deionized. Glacial acetic acid or phosphoric acid (Merck) was used to adjust the pH.

All surfactants were blended with glyphosate [N-(phosphonomethyl)glycine] as the isopropylamine salt, (62% *wt/wt*, Monsanto) for compatibility tests and greenhouse trials. Three different concentrations of the surfactant were used, 6, 10 and 15% of active content respectively, while the glyphosate was kept at a concentration of 30% a.e.. The formulations had a pH between 5 and 6. The biological efficacy was tested on pot-grown couch grass (*Elymus repens)*, winter oilseed rape (*Brassica napus*) cv. Apex and Mediterranean ryegrass (*Lolium rigidum*). The formulations were sprayed at 0.20, 0.40 and 0.75 kg a.e./ha on *B. napus* and *E. repens*. A lower dose range was used on the L. rigidum, 0.08, 0.15 and 0.25 kg a.e./ha. The treatments were applied at a spray pressure of 210 kPa delivering 200 liters/ha. There were three replicates of each treatment. Damage as a % compared with the best untreated was assessed on the three species at 11, 19 and 30 days after spraying. The test was performed by Castan Consultants, Bristol, UK.

The fungicide trial was performed as curative treatment of wheat infected with *Mycosphaerella graminicola (Septoria tritici).* Surfactant (0.25%) was added to the commercially available 'Ortiva' (azoxystrobin 250 g/litre SC) from Syngenta which was sprayed at 62.5 g a.i./ha with a water volume of 200 litres/ha. The pH of the spray liquid was between 8 and 10. The biological efficacy was expressed as the degree of plant infections, i.e. leaf necrosis, in %. The test was performed by Surfaplus and Plant Research International, Wageningen, Netherlands.

RESULTS

Surface chemistry properties and different pH

As can be seen from Figure 1, the chemistry of amido amines will be sensitive to pH and protonation. At high pH, the molecule will be nonionic, but at lower pH the amine will protonate and be more cationic. This process could be followed by monitoring the viscosity versus pH (Figure 2). Protonation starts close to pH 8 and below pH 6, the molecules are predominately protonated. The protonation degree of the molecule will have a huge impact on the physical properties such as adsorption properties and solubility properties. Most natural

surfaces (such as a leaf surface) are negatively charged and a cationic adjuvant will adsorb more readily to such a surface.

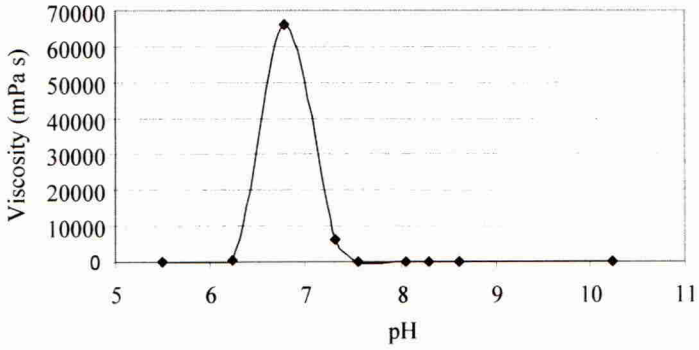

Figure 2. The pH versus viscosity for APA C. Phosphoric acid is used to lower the pH

The influence of pH is clearly seen in the surface chemistry properties (Table 1) and the data confirms that protonation takes place. Solubility will increase upon protonation, as seen for APA C and APA S. CMC (critical micelle concentration) is higher for cationic surfactants (at pH=5) compared to nonionic (at pH = 10). As can be seen, surface tension and wetting properties will also be influenced by pH. The data suggests that specific interactions are taking place between the surface and the surfactants, most likely due to different types of aggregates formed at different pH.

Table 1. Surface Chemistry properties for APAs at different pH

Alkyl chain	Surface tension (mN/m)		CMC (g/litre)		Contact Angle (°, 60 sec)		Water Solubility	
	pH =5	pH=10	pH =5	pH=10	pH =5	pH=10	pH =5	pH=10
C 8/10	28	27	1.0	0.4	86	49	Yes	Yes
Coco	32	27	0.05	0.05	65	41	Yes	Disp
Soya	39	29	0.03	0.03	73	43	Yes	No

Glyphosate compatibility

All three APAs tested in this study have an extremely good compatibility with isopropylamine (IPA) - glyphosate salt. A standard glyphosate adjuvant, such as the tallow amine 15 EO, suffers from a salting out effect in most electrolyte solutions that is related to the cloud point phenomena of ethylene oxide containing surfactants (Holmberg *et al.*, 2003). Thus, increasing the glyphosate concentration in a formulation containing an ethoxylated surfactant, will lead to incompatibility between the adjuvant and the glyphosate – the formulation will phase separate. The sensitivity towards electrolyte salts can be monitored by measuring the temperature at which the formulation becomes cloudy upon heating (cloud point). The APA surfactants on the

contrary, contain no ethylene oxide and have no cloud point. They have been shown to be very insensitive to high salt concentrations and could thus be used in highly concentrated glyphosate formulations. Figure 3 shows the effect on cloud point between different IPA-glyphosate formulations containing two different adjuvants, APA C or TA 15. APA C gives a stable and clear formulation at all concentrations up to 470 g/litre a.e. while a formulation containing TA 15 will be separated at 40°C in 470 g/litre formulation.

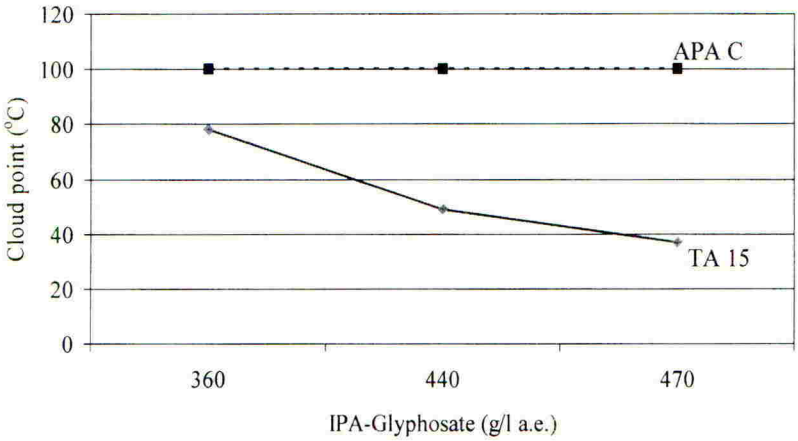

Figure 3. Compatibility between IPA-glyphosate and two different surfactants; TA 15 and APA C. Surfactant concentration is 10% in all formulations

Green house trials - Glyphosate

Several formulations have been tested at different concentrations in green house trials. Figure 4 shows the comparison between the three APAs at low surfactant concentration (6%) and low glyphosate rate concentration (0.20 kg a.e./h for *B. napus* and *E. repens*, 0.08 kg a.e./h for *L. rigidum*) . As can be seen, the coco based APAs are outperforming both the soy and C8/10 derivatives. It is also ranked over the reference TA 15 for *B. napus* and *E. repens*, but this was not statistically significant. All APA derivatives are superior over the commonly used phosphate ester (PhE).

Furthermore, the study showed that the APA C can be diluted to low concentrations, while maintaining the bioefficacy (Figure 5). This opens possibilities for efficient high loading formulations. An additional advantage is the shortened burn down time seen in Figure 5. The plant damage at 11 DAT seen in the formulations based on APA C compared to TA 15 is significantly higher. However, at high spray concentrations and at high surfactant loadings, no differences are seen between the APA C and TA 15 after 30 DAT. The advantage is at low surfactant concentrations and at low spray concentrations.

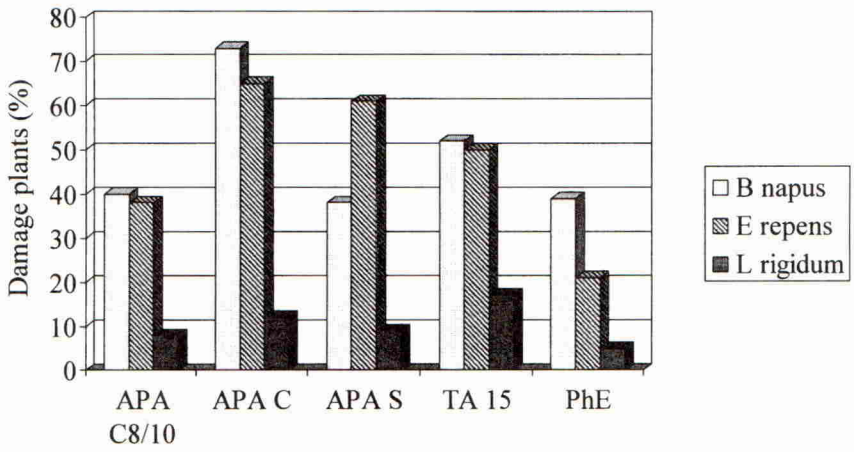

Figure 4. Green house trials with IPA-Glyphosate and three different APA derivatives. TA 15 and PhE are used as references

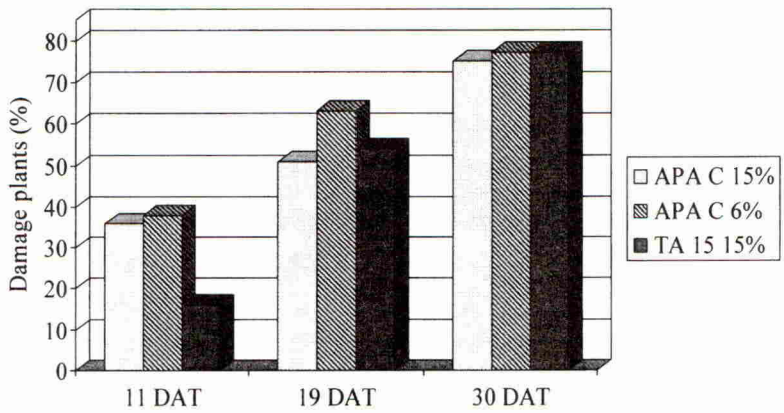

Figure 5. Green house tests with IPA-Glyphosate on *B. napus* with two different surfactants. Glyphosate application rate 0.40 kg a.e./ha.

When surfactants are used as adjuvants in agrochemical formulations, a number of different surfactant properties contribute to the final result and act together both in a positive and negative manner. Multivariate data analysis has been used in earlier studies and has showed that high surface tension and low CMC are some of the most important parameters for a good glyphosate adjuvant (Strandberg *et al.*, 2004). Thus, the data in Table 1 suggest that both the soy and coco based derivatives should be good adjuvants for glyphosate, at pH close to 5. This was confirmed in the green house trials. It seems that the APA C delivers the right balance between wetting, adsorptivity and penetration enhancement at low concentrations to become a next generation adjuvant for glyphosate.

Green house trials – Strobilurin

Short-chained APA showed an extremely good adjuvancy on the performance of azoxystrobin, increasing the fungicidal efficacy by over 50% (Figure 6). The most hydrophilic APA derivative, giving the lowest surface tension, showed to be the best adjuvant for the water insoluble fungicide. The efficacy seems to be directly related to the length of the surfactant alkyl chain. The efficacy of 'Ortiva' was increased by the addition of all APAs.

The azoxystrobin 'Ortiva' formulations have a pH above 8. This means that the character of the surfactant will be more nonionic in the fungicide formulation compared to cationic in the case of glyphosate. The data suggest that a water insoluble systemic fungicide needs a small, water soluble adjuvant that reduces the surface tension and enables good contact between the strobilurin particles and the plant, such as the C8/10 APA.

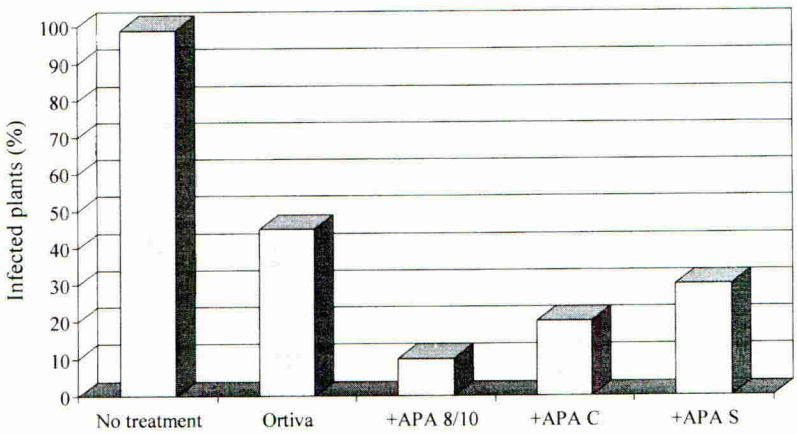

Figure 6. Efficacy of the strobilurin based formulation 'Ortiva' on *Mycosphaerella graminicola* infected wheat. The different APA derivatives were added to the Ortiva treatment solution.

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