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Mini review

Pesticide use and mycotoxin production in *Fusarium* and *Aspergillus* phytopathogens

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Abstract

The major mycotoxigenic species of *Fusarium* and *Aspergillus* phytopathogens have been identified in this review. Since fungicides are widely used to control crop diseases caused by these fungi, it is pertinent to assess efficacy with respect to mycotoxin production. In both laboratory studies with pure cultures of phytopathogens and field trials with crop plants, the overall evidence concerning the effectiveness of fungicides is contradictory and in certain cases somewhat unexpected. In particular, at sub-lethal doses of a number of fungicides including carbendazim, tridemorph, difenoconazole and tebuconazole with triadimenol, mycotoxin production from *Fusarium* phytopathogens may increase. Furthermore, the efficacy of propiconazole and thiabendazole in the control of deoxynivalenol production from *F. graminearum* is not consistent. Evidence has been presented to suggest, for the first time, that fungicide-resistance in *F. culmorum* may be accompanied by a more persistent pattern of mycotoxin production. The limited evidence on the effects of fungicides on mycotoxin production in *Aspergillus* species is also conflicting. Under laboratory conditions, miconazole and fenpropimorph have been shown to increase aflatoxin production from *A. parasiticus*. Moreover, fenpropimorph increased production of the more toxic aflatoxin B_1. Since fungal infection of plant products is often preceded by insect damage, there is interest in the effectiveness of insecticides to reduce infestation, infection and mycotoxin contamination. Additionally, insecticides may be effective in their own right, causing a direct effect on mycotoxin synthesis. The bulk of the evidence relates to effects on aflatoxin (AF) components B_1, B_2, G_1 and G_2. Under laboratory conditions, AFB_1 production was most resistant to inhibition by insecticides, followed by AFG_1, AFG_2 and AFB_2. This pattern of inhibition was particularly consistent for the organophosphorus insecticides. In one field study, Bux and carbaryl were considerably more effective than naled in reducing AFB_1 contamination of maize kernels. It is concluded that if pesticide control is to be more effective in the future, additional criteria may be required in developing evaluation protocols for candidate compounds. In particular, the issue of fungicide-resistance in relation to mycotoxin production needs to be addressed in a concerted programme of research. Additionally, the potential of breeding and selecting cultivars resistant to disease caused by toxigenic fungi needs to be exploited in a parallel search for an environmentally acceptable solution to the question of mycotoxin contamination of plant products.

Abbreviations: NEO – neosolaniol; DAS – diacetoxyscirpenol; DON – deoxynivalenol; 3-ADON – 3-acetyl DON; 15-ADON – 15-acetyl DON; NIV – nivalenol; ZEN – zearalenone; FB_1, FB_2, FB_3 – fumonisin mycotoxins B_1, B_2, B_3; AFB_1, AFB_2, AFG_1, AFG_2 – aflatoxins B_1, B_2, G_1, G_2; OA, OB – ochratoxins A, B; CS, RS – control and resistant strains of *Fusarium culmorum*; YES – yeast extract-sucrose; PDA – potato dextrose agar; IMI – International Mycological Institute; PCR – polymerase chain reaction.

Introduction

Economic losses arising from crop diseases caused by phytopathogenic fungi are principally associated with yield reductions. However, crop quality and safety may also be adversely affected, undermining both consumer confidence and profitability to the producer. An important feature of safety centres on the extent of contamination of plant-derived foods and animal feed with the secondary metabolites of phytopathogenic fungi, particularly mycotoxins. These compounds are endowed with toxic properties towards humans and other animals, causing a wide range of acute and chronic effects collectively known as mycotoxicoses. Some mycotoxins bear structural analogy with or act as host-specific or non-specific phytotoxins (D'Mello and Macdonald, 1998). In chemical terms mycotoxins are a diverse group of compounds produced by an equally wide range of fungi. However, the synthesis of a particular mycotoxin is invariably confined to a small number of fungal species and may even be strain-specific. Many plant pathogenic species of *Fusarium*, *Aspergillus*, *Alternaria* and *Penicillium* produce the most important mycotoxins of concern in human and animal health (D'Mello et al., 1997a; Smith, 1997; Panigrahi, 1997; Abramson, 1997). Following infection with these fungi, seeds, nuts and fruit regularly become contaminated with mycotoxins which may directly or indirectly enter the human food chain. Forages infected with certain species of *Acremonium*, *Phomopsis* and *Pithomyces* may also contain mycotoxins associated with disorders in farm animals, particularly ruminants (D'Mello and Macdonald, 1997). Mycotoxins commonly occur in the spores of fungi, including those of *Stachybotrys* and *Alternaria* and inhalation therefore represents another route of entry into the body, adding an environmental health dimension to the risks posed by these substances.

Although mycotoxins have been linked with many human and animal disorders, it is their carcinogenic potential which has evoked particular concern (Smith, 1997). In addition, recent findings implicating mycotoxins in neurotoxic, hepatotoxic and immunosuppressive conditions have sustained the impetus for monitoring and remediation. There is now ample evidence indicating that certain mycotoxins are associated with reproductive dysfunction in animals but the implications for human fertility have yet to be addressed (D'Mello and Macdonald, 1997). Since the contamination of primary foods and animal feeds with mycotoxins represents an unacceptable hazard to human and animal health, measures are in force in many countries to monitor and regulate levels of these compounds (van Egmond and Dekker, 1996).

The synthesis of mycotoxins is determined by an elaborate array of factors, broadly classified into physical, biological and chemical, and by interactions involving these factors. Time, environmental temperature, humidity and physical damage caused by insect infestation are primary factors which interact in complex ways to induce mycotoxin synthesis. In general the fungi associated with disease in cereal plants and grain legumes are also endowed with the capacity to produce the major mycotoxins. Consequently, when pesticides are used to arrest the predisposing factors of insect infestation and fungal disease, the implications for mycotoxin production need to be considered.

In this review we compare the overall efficacy of fungicides and insecticides to control mycotoxin production in *Fusarium* and *Aspergillus* fungi, taking into account recent work on fungicide resistance, persistence of critical mycotoxins and alternative strategies for control of these mycotoxins. Particular attention is given to *Fusarium* and *Aspergillus* fungi due to the economic and health implications of their respective mycotoxins. In addition, virtually all studies comparing the effectiveness of pesticides to control mycotoxins have been conducted with these two genera of fungi, and a review is now opportune.

Toxigenic species of *Fusarium* and *Aspergillus*

The major toxigenic species of these genera are presented in Table 1. The natural occurrence of mycotoxins from *Fusarium* species is conventionally associated with temperate foods, since these fungi require somewhat lower temperatures for growth and mycotoxin production than the aflatoxigenic *Aspergillus* species described below. Extensive data exist to indicate the global scale of contamination of cereal grains with a number of *Fusarium* mycotoxins (Scott, 1989), of which the most important for human and animal health are the trichothecenes, zearalenone, moniliformin and the fumonisins (D'Mello et al., 1997a). The trichothecenes are subdivided into four basic groups, with types A and B representing the most important components. The synthesis of the two types of trichothecenes appears to be characteristic for a particular *Fusarium*

Table 1. Toxigenic species of *Fusarium* and *Aspergillus*

Fungal species	Mycotoxins[1,2]
F. culmorum	Deoxynivalenol (DON), 3-acetyl DON, 15-acetyl DON, nivalenol (NIV), fusarenon X (FX), Zearalenone (ZEN)
F. graminearum (*Gibberella zeae*)	DON, 15-ADON, NIV, FX, ZEN
F. sporotrichioides	T-2 toxin, HT-2 toxin, neosolaniol (NEO), diacetoxyscirpenol (DAS), FX, ZEN
F. poae	T-2 toxin, HT-2 toxin, NIV, DAS, FX
F. moniliforme (*Gibberella fujikuroi*)	Fumonisins, moniliformin, fusarin C
F. oxysporum	Moniliformin, wortmannin, fusaric acid, sambutoxin
F. sambucinum	Sambutoxin
A. flavus	Aflatoxins, cyclopiazonic acid
A. parasiticus	Aflatoxins
A. ochraceus	Ochratoxins

[1] Type A trichothecenes: T-2 toxin, HT-2 toxin, NEO, DAS.
[2] Type B trichothecenes: DON, 3-acetyl DON, 15-acetyl DON, NIV, FX.

species (Table 1). A common feature of many *Fusarium* species is their ability to synthesise zearalenone (ZEN), and its co-occurrence with certain trichothecenes raises important issues regarding additivity and/or synergism in the aetiology of mycotoxicoses in humans and animals. The secondary metabolism of *F. moniliforme* is also important in this respect since it is capable of producing at least three mycotoxins: the fumonisins (FB_1, FB_2 and FB_3), moniliformin and fusarin C. Certain strains of two *Aspergillus* species are capable of synthesising a family of related mycotoxins comprising: aflatoxin B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1 and AFG_2 respectively; Smith, 1997). Another species of *Aspergillus* produces ochratoxins A and B (OA and OB), a property shared with certain *Penicillium* fungi.

Role of *Fusarium* and *Aspergillus* fungi in phytopathology

Toxigenic fungi were considered to belong to two groups: 'field' (or pathogenic) and 'storage' or (saprophytic) but this distinction is perhaps over-simplistic and possibly counter-productive since virtually all the major toxigenic fungi in Table 1 are common pathogens of economically important plants. Scab of wheat, also known as fusarium head (ear) blight, has been caused by *F. graminearum* in epidemics in Canada and USA (Wiersma et al., 1996; Schaafsma et al., 1993; Miller et al., 1985) and by *F. culmorum* in Poland (Perkowski et al., 1996) and the Netherlands (Snijders and Perkowski, 1990). Other *Fusarium* species, e.g. *F. poae* and *F. avenaceum*, have also been implicated and Sturz and Johnston (1983) cautioned that initial colonisation by these fungi prior to ear emergence may encourage subsequent infection by end-of-season species such as *F. graminearum* and *F. culmorum*. Other cereal diseases have been associated with some of these pathogens. Thus, crown rot of wheat is attributed to *F. graminearum* (Wildermuth and McNamara, 1994) and ear rot of maize is linked with *F. graminearum* and *F. moniliforme* (Schaafsma et al., 1993).

Although *A. flavus* and *A. parasiticus* are commonly associated with the production of aflatoxins during storage the ultimate source of these fungi is likely to be soil from fields previously used for maize and other crops (Shearer et al., 1992). In addition, however, *A. flavus* has been implicated in aspergillus ear and kernel rot of maize (Campbell and White, 1995a).

A relationship between fungal infection and mycotoxin production was confirmed in the studies of Brown et al. (1995) who showed a direct correlation between colonisation with *A. flavus* and AFB_1 contamination of maize kernels. Susceptible genotypes of maize yielded grain with high levels of AFB_1 compared to kernels from resistant varieties.

Efficacy of fungicides

Since fungicides are widely used to control crop diseases, it is pertinent to consider the effects of these agents on mycotoxin production. In both laboratory studies with pure cultures of pathogens and field trials with crop plants the resulting evidence concerning the effectiveness of fungicides is contradictory and in certain cases somewhat unexpected. In a number of instances, fungicide concentrations tested in laboratory studies were in excess of maximum solubility levels in aqueous media and, therefore, the interpretation of results is not straightforward.

Effects on Fusarium *mycotoxins*

Hasan (1993) showed that dicloran, iprodione and vinclozolin each inhibited DAS and ZEN synthesis in *F. graminearum*, but efficacy depended upon dosage and the mycotoxin in question. Dicloran eliminated DAS production at 500 μg/ml, but only 250 μg/ml was sufficient to prevent ZEN synthesis. Vinclozolin arrested DAS production at 250 μg/ml whereas levels up to 500 μg/ml markedly reduced but failed to eliminate ZEN. With all these fungicides, mycelial growth was reduced but not totally inhibited, even at 500 μg/ml. These results may be compared with earlier observations indicating greater efficiency of maneb (at 50 μg/g) in preventing ZEN production in maize kernels inoculated with *F. graminearum*. In a concurrent series with a broth culture of this fungus, growth virtually ceased on addition of maneb at 50 μg/ml medium (Draughon and Churchville, 1985).

A comprehensive series of separate experiments with benomyl, thiabendazole, prochloraz, tebuconazole, tridemorph and fenpropimorph indicated that the two morpholine fungicides had no appreciable effects on the production of 3-ADON by *F. graminearum* in pure culture (Matthies and Buchenauer, 1996). The remaining fungicides inhibited 3-ADON synthesis to the same extent or marginally more than mycelium growth provided that a level of 1 μg/ml medium was used for benomyl, thiabendazole or tebuconazole and 0.005 μg/ml for prochloraz. Tebuconazole (0.1 μg/ml) induced an almost 4-fold increase in 3-ADON production, whilst another triazole, difenoconazole (0.1 μg/ml), increased 3-ADON production in *F. culmorum* cultures maintained at 25°C but not when incubated at 11°C (D'Mello et al., 1997b). Radial growth at 25°C was unaffected by this level of fungicide. At 1 μg/ml, difenoconazole reduced growth by 9% at 25°C but 3-ADON synthesis was inhibited altogether.

Increased mycotoxin production following exposure to sub-lethal doses of fungicides has also been demonstrated with *F. sporotrichioides*. Moss and Frank (1985) observed that low concentrations of tridemorph (6–8 μg/ml) enhanced growth marginally, but consistently, yet considerably inhibited synthesis of T-2 toxin and DAS. However, at tridemorph levels of 30–50 μg/ml, growth was reduced by 50% but T-2 toxin production was stimulated five-fold. In addition, sub-lethal concentrations of carbendazim influenced production of T-2 toxin and other mycotoxins; the effects were dependent upon fungicide dose and temperature (Placinta et al., 1996). At 25°C, carbendazim (5 μg/ml) markedly enhanced T-2 toxin concentrations, and also induced smaller increases in NEO production without affecting radial growth. However, in replicate cultures transferred to 11°C, carbendazim caused progressive reductions in both mycotoxins. At the highest levels of carbendazim (10 μg/ml) synthesis of these mycotoxins and ZEN was reduced to low but measurable levels irrespective of incubation temperature. These observations correlated with lower fungal growth. Thiabendazole (100 μg/ml) on the other hand, was highly effective as a suspension applied to maize in inhibiting growth and T-2 toxin production in *F. tricinctum* (Gabal, 1987).

Several field trials conducted to assess the effect of fungicides on mycotoxin control have yielded conflicting results. Draughon and Churchville (1985) reported that maneb prevented ZEN production in maize following inoculation of corn ears with *F. graminearum* 20 days after silk development, whereas grain from untreated ears accumulated ZEN up to 168 mg/kg. These results correlated well with their laboratory findings on the effectiveness of maneb. By contrast, Suty et al. (1996) reviewed data showing that following natural *Fusarium* infection of wheat, products containing tebuconazole markedly reduced DON levels in the grain. The carbendazim precursor fungicide, thiophanate-methyl, was investigated over three seasons in 1982, 1983 and 1987 in order to determine the effects on the incidence of fusarium ear blight in wheat and barley and the extent of contamination of kernels with DON and NIV (Ueda and Yoshizawa, 1988). Fungicide application at ear emergence and at flowering reduced disease damage to the crop, and mycotoxin contamination of the harvested grain decreased to non-detectable

levels in a few instances. The levels of DON in grain were highly correlated with NIV concentrations irrespective of fungicide applications. However, seasonal variations were observed and thiophanate-methyl was ineffective, in terms of mycotoxin control, for one cultivar of wheat and barley in the 1983 harvest. Further evidence of variable efficacy depending upon fungicide type and time of application were provided by the studies of Boyacioglu et al. (1992) who examined the effects of triadimefon, propiconazole and thiabendazole on DON in a wheat crop inoculated with *F. graminearum*. Triadimefon reduced DON concentrations in grain by 65% when the fungicide was applied prior to inoculation, but the effectiveness increased to 78% when triadimefon was applied 2 days after inoculation. For propiconazole applied at the same times, efficiency was 34 and 78% respectively, but for thiabendazole, efficacy of DON control was highest (83%) when applied prior to inoculation and lower (16%) when applied 2 days after inoculation. Thiabendazole had no effect on fungal infection at either application stage.

The opposite end of the spectrum, as regards fungicide efficacy under field conditions, is represented by the observations of Milus and Parsons (1994) who examined the effects of a wide range of fungicides on the control of fusarium ear blight of wheat and DON contamination of harvested grain. Disease incidence and DON concentrations in grain remained totally unaffected by fungicide applications. It is possible that differences in the timing and number of inoculations and fungicide applications as well as environmental factors may have contributed to the discrepancy between these observations and those of Boyacioglu et al. (1992). However, the theme of increased mycotoxin production has emerged from another source (Gareis and Ceynowa, 1994). In their studies, application of Matador (a mixture of tebuconazole and triadimenol) to winter wheat inoculated with *F. culmorum* reduced head blight but increased NIV content of harvested grain 16-fold. Thus, results of both laboratory studies and field trials show that some fungicides may reduce but not eliminate trichothecene production and at worst mycotoxin synthesis may indeed be enhanced.

Effects on Aspergillus *mycotoxins*

The limited evidence on fungicide efficacy in the regulation of *Aspergillus* mycotoxins is also conflicting. The studies reviewed here all refer to laboratory assessments using isolates cultured in different media or on cereal grains. Substantial reductions in AFB_1 contamination was reported by Gabal (1987) on treatment of whole maize with thiabendazole, but fungicide level was an important factor determining effectiveness. Rama Devi and Polassa (1984) showed that carbendazim, added as Bavistin, stimulated growth of *A. flavus* and *A. ochraceus* when added to a liquid medium at levels of up to 100 μg/ml, with AFB_1 and ochratoxin production remaining at control values, but at levels of 2–3 mg/ml both growth and mycotoxin production were totally inhibited. The low solubility of carbendazim in aqueous media (8 μg/ml at pH 7) makes these results difficult to explain. However, the effect of fungicide on mycotoxin production may not correspond proportionately with growth inhibition. For example, El-Kady et al. (1993) observed that at relatively low levels (10 μg/ml) of a carboxin-captan fungicide mixture, growth of *A. flavus* in a liquid medium was reduced by only 4% after 12 days of incubation, but aflatoxin synthesis was suppressed by 100%. At the same concentration of a mixture of the protectant organophosphorus fungicide, tolclofos-methyl, with thiram (Rizolex-T) fungal growth marginally increased, AFB_1 and AFB_2 remained at control levels, but AFG_1 and AFG_2 production decreased by at least 50%.

Although the fungicides cited above were effective to varying degrees in their mycotoxin-inhibiting potential, other fungicides are known to enhance total aflatoxin production and alter the relative proportions of the component aflatoxins. Sub-inhibitory levels of miconazole stimulated total aflatoxin production in *A. parasiticus* on four different substrates (Buchanan et al., 1987). Peptone-mineral salts substrate promoted low levels of aflatoxin, but a 4-fold increase in synthesis of the mycotoxin occurred in the presence of miconazole (0.1 μM). This theme was confirmed and developed further in the studies of Badii and Moss (1988). Incubation of *A. parasiticus* for 8 days in a liquid medium containing fenpropimorph (250 μg/ml) stimulated AFB_1 and AFG_1 production to 267 and 367 μg/flask from control levels of 75 and 167 μg/flask respectively for similar rates of fungal growth. The ratio of the two mycotoxins had shifted in favour of the more toxic AFB_1. Other studies have confirmed the persistence of AFB_1 synthesis in the presence of fungicide (Fernando and Bean, 1986). In different isolates of *A. parasiticus* and *A. flavus*, tricyclazole reduced production of all four aflatoxins in a

dose-related manner but detectable levels of AFB_1 persisted even at the highest concentration (100 μg/ml).

Pre-harvest occurrence of aflatoxins in peanuts is common (Anderson et al., 1996) and the fungi responsible have also been associated with aspergillus ear rot in maize (Campbell and White, 1995a). However, the effects of fungicides in the control of aflatoxin contamination have not been investigated in field trials. Consequently, it is not possible to corroborate the laboratory findings reviewed above with field data. It is likely that future research on the control of mycotoxins from *Aspergillus*, and indeed *Fusarium*, species will focus on the development of disease-resistant genotypes of crop plants. Such an approach would be consistent with the aims of good environmental practice and, in addition, might address the issue of fungicide resistance in plant pathogenic fungi.

Fungicide resistance

Both laboratory and field studies have underlined the ineffectiveness of some fungicides in controlling mycotoxin production by *Fusarium* and *Aspergillus* phytopathogens. Indeed, there is some evidence that fungicide use may be associated with increased synthesis of critical mycotoxins, and it is conceivable that fungicide resistance might contribute to increases in mycotoxin production. In the first study of its kind, D'Mello et al. (1997c) investigated the production of 3-ADON in a difenoconazole-resistant strain of *F. culmorum*. Resistance was developed in our laboratory using the following procedure. A freeze-dried culture of *F. culmorum* 309344 from IMI was re-suspended in Ringers solution and grown on potato dextrose agar (PDA) in 9 cm Petri dishes which were then incubated at 25°C until growth was established. Peripheral plugs from these colonies were used to prepare 5d-old cultures also on PDA. A plug was removed from these 5d-old cultures and placed centrally on to 20 ml PDA containing difenoconazole at 100 μg/ml. The fungicide was added as Plover (Ciba Agriculture), containing 250 g active ingredient/l. Prior to addition, Plover was diluted in ethanol. Several inoculated plates prepared in this manner were incubated at 25°C for 21 days, after which time they were stored at 4°C for approximately 11 months. These cultures were designated as 'resistant' for the purposes of our investigations since on further exposure to difenoconazole, these cultures had superior growth in comparison with the original control cultures. Control ('sensitive') cultures were prepared and stored in an identical process, except that equivalent volumes of ethanol without fungicide were added to the PDA prior to inoculation. After storage, control and resistant cultures were used to prepare 5 d-old colonies on PDA alone for control cultures and on PDA + difenoconazole at 100 μg/ml for resistant strains. Plugs were removed as before and placed centrally on PDA containing difenoconazole at 0, 0.1, 100 and 200 μg/ml to obtain a control and resistant series in factorial combination. Inoculated Petri dishes were incubated at 25°C for 8, 15 or 22 days. Following treatment, the resistant strain (RS) continued to synthesize 3-ADON even when difenoconazole levels of 100 and 200 μg/ml media were used. In contrast, the control strain (CS) of *F. culmorum* failed to produce the mycotoxin at either of these two concentrations of difenoconazole. These differences between RS and CS cultures were apparent at each of the three times of experimental observation (8, 15 and 22 days of incubation). Statistical tests indicated that two of these specific differences were significant ($P < 0.05$), on days 8 and 15 for the 100 μg/ml level of fungicide addition.

Further definition of these strain differences emerged in an extended study (D'Mello et al., 1998) designed to examine the pattern of 3-ADON production in CS and RS cultures over a period of 57 days, using lower levels of difenoconazole so that mycotoxin synthesis could be determined at equivalent levels of growth for both strains. In the earlier study (D'Mello et al., 1997c), the RS isolates exhibited superior growth relative to the CS cultures. That this objective was achieved is apparent from a comparison of the growth and 3-ADON results in the second study (D'Mello et al., 1998). In particular, it was clear that at 21 days, there were no significant differences ($P > 0.05$) in colony diameters between the two strains following exposure to difenoconazole at 1, 2 and 4 μg/ml, but 3-ADON production (Table 2) only occurred in RS cultures, albeit at relatively low levels. Similarly, at 43 days equivalent growth was attained by both strains on exposure to the fungicide at 6 and 10 μg/ml media, but 3-ADON synthesis was only apparent with RS isolates. Further analysis of variance showed that these values for the RS cultures were significantly different from the zero values recorded for CS ($P < 0.05$), reflecting previous findings by D'Mello et al. (1997c). Moreover, there was a significant interaction ($P < 0.01$) involving strain, time and difenoconazole level in the study of D'Mello et al. (1998). Whereas, relative to basal values, the 1 μg/ml addition of difenoconazole significantly

Table 2. Effects of incubation time and difenoconazole concentrations on 3-acetyl deoxynivalenol (3-ADON) production in control (CS) and fungicide-resistant strains (RS) of *Fusarium culmorum*[1]

Difenoconazole level (μg/ml)	Incubation time (days)											
	7		14		21		28		43		57	
	CS	RS	CS	RS	CS	RS	CS	RS	CS	RS	CS	RS
	3-ADON in culture extracts (mg/ml)											
0	1.4	1.4	6.8	13.1	10.6	10.7	6.7	5.6	11.2	5.2	10.8	10.3
1	0	0	0	0	0	0.3	2.9	1.8	2.9	4.2	5.6	8.8
2	0	0	0	0	0	0.2	0.8	0	2.2	1.4	5.9	1.0
4	0	0	0	0	0	0.6	0.6	0.4	2.0	2.9	7.1	1.2
6	0	0	0	0	0	0	0	0	0	0.3	0.9	1.5
10	0	0	0	0	0	0	0	0	0	0.2	1.0	1.4
LSD ($P < 0.05$)	3.03											

[1] From D'Mello et al. (1998).

depressed 3-ADON production in CS cultures at 43 and 57 days ($P < 0.01$ or better), this level of fungicide failed ($P > 0.05$) to reduce mycotoxin levels in RS cultures at the same stages of the experiment (Table 2). Some anomalous results have, however, appeared in this study. Thus, the depression in 3-ADON production was greater with the RS than with the CS cultures at 57 days when both were subjected to the 2 μg/ml addition of difenoconazole (Table 2). However, this discrepancy does not invalidate the main thrust of the observations concerning 3-ADON production in the two strains of *F. culmorum* (D'Mello et al., 1997c, 1998).

The production of trichothecene mycotoxins in *Fusarium* species occurs in a systematic process of oxygenations and esterifications. There is particular interest in the role of esterases not only in biosynthesis but also in the metabolism of trichothecenes in animals. It is theoretically possible to use esterases to distinguish between different species, toxigenic groups and races of *Fusarium*. That such an approach is feasible has been indicated by the recent results of Baayen et al. (1997) who reported that isolates from different vegetative compatibility groups of *F. oxysporum* had unique esterase profiles. In a second experiment, D'Mello et al. (1998) demonstrated that total esterase production increased progressively over time and at higher levels for RS from day 14 onwards to the extent that by day 35 the difference between RS and CS was significant ($P < 0.05$). Although the increase with time occurred in both strains, this enhancement appeared significantly earlier (at 14 days) for RS but delayed (until 21 days) for the CS cultures ($P < 0.05$). It is premature to attempt to correlate total esterase production with 3-ADON concentrations since such comparisons would only be valid for the specific esterase isoenzymes involved in trichothecene biosynthesis. With this objective in mind, further studies are now in progress to examine the profile differences for esterase isoenzymes in the two strains, but the potential for diagnostic use is clear if the results of the second experiment of D'Mello et al. (1998) are considered in conjunction with those of Baayen et al. (1997). These results are of practical importance since *F. culmorum* has been associated with fusarium head blight and mycotoxin contamination of wheat kernels (Snijders and Perkowski, 1990). Although difenoconazole is not intended for the control of fusarium head blight, the efficacy of this fungicide towards *Septoria tritici* and rust diseases and its relative persistence implies that any residues may affect the secondary metabolism of other fungi. *Fusarium* species commonly occur on ears in the field even in the absence of overt symptoms of disease (D'Mello et al., 1993). Furthermore, it is possible that there may be cross-resistance with other fungicides such as epoxiconazole, tebuconazole, thusilazole or prochloraz. Consequently, our results may have more universal implications for mycotoxin production in *Fusarium* species which have developed resistance to demethylation inhibitors in general. Although this would be of more practical significance, as yet there appears to be no evidence of resistance to demethylation inhibitors in field populations of *Fusarium culmorum*.

On the basis of the results presented by D'Mello et al. (1997c, 1998) there appears to be substantive evidence

that difenoconazole-resistance in *F. culmorum* may accelerate the onset of 3-ADON production following further exposure to the fungicide. In addition, although difenoconazole-induced suppression of 3-ADON synthesis does occur, this is less pronounced in RS than in CS cultures. New data also demonstrates consistently higher production of esterases in RS cultures, but the significance for toxigenicity and pathogenicity needs to be addressed.

Effects of insecticides

Since fungal infection of grain, nuts and fruit is often preceded by physical damage caused by insect invasion, much effort has been expended on the potential of insecticides to reduce infestation, infection and, therefore, mycotoxin contamination from 'storage' fungi such as the *Aspergilli*. Such a strategy offers the advantage of dual-function insecticides, potentially contributing to lower overall pesticide use. However, insecticides may be effective in their own right. In pure culture studies with *Aspergillus parasiticus*, uncomplicated by insect infestation, dichlorvos, landrin, malathion and diazinon significantly inhibited production of AFB_1 in a dose-dependent manner (Draughon and Ayres, 1981). AFB_1 inhibition was greater than the reduction in growth of the fungus; with dichlorvos at 100 mg/l of culture broth, fungal growth declined by 29% whereas AFB_1 synthesis was reduced by 99%. At this concentration, diazinon increased mycelial growth by 19% but AFB_1 production was still reduced by 23%. At 100 mg/l, naled inhibited growth totally, but not at 10 mg/l at which level it precipitated a 68% reduction in AFB_1 synthesis, but dichlorvos was still more effective, causing a 92% inhibition of AFB_1 production. AFB_2 levels were reduced below detection levels in all insecticide-treated cultures. At sub-lethal levels of insecticide, dichlorvos was more effective than naled in reducing production of AFG_1 and AFG_2. In general, AFB_1 synthesis was most resistant to inhibition by insecticides, followed by AFG_1, AFG_2 and AFB_2. This pattern of inhibition was particularly consistent for the organophosphorus insecticides (Draughon and Ayres, 1981).

In field studies, naled reduced AFB_1 concentrations in harvested kernels after application to a maize crop which had been artificially inoculated with *A. parasiticus* (Draughon et al., 1983). However, Bux and carbaryl were considerably more effective than naled, whereas in laboratory studies with both cultures naled exhibited higher efficacy. In the uninoculated maize crop, Bux and carbaryl were again highly effective in reducing natural production of AFB_1 while naled was virtually inactive.

Production of OA and OB by *A. ochraceus* in yeast extract-sucrose (YES) medium and in maize kernels can be reduced in a dose-dependent manner by application of dichlorvos at levels of up to 300 mg/l broth or per kg corn (Wu and Ayres, 1974). Mycelial growth, measured in YES cultures, remained at 80% of control values at the highest level of dichlorvos addition but OA and OB levels were reduced to, respectively, 21 and 11% of control values.

Naled has also been found to be effective in reducing ZEN levels in pure cultures of *Fusarium graminearum* in liquid media or on maize kernels when the insecticide has been applied as a liquid preparation or as fumigant at concentrations of 30 and 100 μl/l (Berisford and Ayres, 1976). Production of ZEN was completely inhibited only when naled was applied prior to inoculation of the culture media. When applied to 12-day or older cultures, naled did not inhibit ZEN synthesis but in 3 to 9-day cultures ZEN production was reduced by 45–92% when the insecticide was applied at 10–100 μl/l.

Biocontrol

Lack of confidence with respect to the efficacy of pesticides in the control of mycotoxin contamination, particularly following fusarium head blight induction (Milus and Parsons, 1994), has led to a quest for alternative methods. There is increasing optimism that exploitation of disease resistance in different plant genotypes offers considerable potential as an environmentally acceptable technique to control mycotoxin contamination of primary foodstuffs, and the limited evidence is encouraging. Among early studies, the evidence of Miller et al. (1985) marked a defining point in the application of this strategy. A single isolate of *F. graminearum* was used to experimentally infect spring wheat, rye and triticale cultivars. In genotypes resistant to infection, low kernel concentrations of DON (mean, 0.6 mg/kg) were recorded, while in susceptible cultivars much higher values (mean, 10.2 mg/kg) were found. Miller et al. (1985) suggested that resistant cultivars may be endowed with factors that prevent synthesis and/or promote degradation of the mycotoxin. It is also conceivable that resistant cultivars may simply prevent growth of the pathogen. Indeed, the following

evidence is indicative of resistance directed at the fungus itself rather than at the synthesis of mycotoxins. Consequently, reduced infection can be correlated with lower disease incidence which in turn may lead to reduced mycotoxin levels in grain of resistant genotypes. Marked effects were reported by Snijders and Perkowski (1990) who investigated the relationship between head blight induced by three pathotypes of *F. culmorum* in ten wheat genotypes and DON levels in harvested grain. Head blight incidence caused by the most virulent strain of the pathogen ranged from 2% for one wheat genotype to 62.5% for another, with the values for DON being 4.6 and 37.0 mg/kg grain, respectively. Overall, for the three pathotypes and ten genotypes, there was a linear correlation between incidence of head blight and DON contamination of grain. Work in Canada has further underlined genotype differences in fusarium head blight susceptibility and mycotoxin contamination of wheat (Wong et al., 1995). Canadian cultivars susceptible to the disease caused by *F. culmorum* had DON levels ranging from 17 to 121 mg/kg grain, while the same cultivars infected with *F. graminearum* yielded grain with DON concentrations in the range 6–53 mg/kg as well as higher levels of 15-ADON. In contrast, Chinese cultivars infected with these pathogens produced grain with DON levels of 0–10 and 0–6 mg/kg, respectively and low levels of NIV.

Investigations with fusarium ear rot of maize indicate the potential for wider application of resistant hybrids as a means of reducing mycotoxin contamination of plant products (Schaafsma et al., 1993). In one study, a resistant maize hybrid artificially inoculated with *F. graminearum* yielded kernels with DON concentrations ranging from 0.4 to 80.4 mg/kg, while in kernels from a susceptible line values of 1.3–313 mg/kg were found. Levels for ZEN were 0–0.1 mg/kg in the resistant hybrid and 0–8.4 in the susceptible cultivar. These results correlated with differences in ear rot ratings for the two hybrids.

It has been suggested that the most effective control of aspergillus ear and kernel rot in maize centres on the use of genetically resistant hybrids (Campbell and White, 1995b). This approach should also result in reduced contamination of kernels with aflatoxins. Evidence from plot experiments with artificially inoculated maize plants tends to justify the validity of such a strategy. In one study, susceptible lines yielded kernels with AFB_1 contents of up to 943 μg/kg, whereas resistant hybrids had around half the level of ear rot and AFB_1 levels typically in the range 3–21 μg/kg. Screening peanut genotypes for resistance to *Aspergillus* fungi is also being undertaken with a view to reducing mycotoxin contamination of kernels (Anderson et al., 1996).

Another method of biocontrol, based on the use of atoxigenic fungi, deserves mention as it amplifies ecological interactions with the potential for exploitation in the future. Garber and Cotty (1997) showed that co-inoculation of developing cotton bolls with a toxigenic and an atoxigenic strain of *A. flavus* reduced sclerotia formation and synthesis of aflatoxins. Lower toxicity may be expected not only from reduced aflatoxin production but also from decreased levels of other sclerotial metabolites.

Diagnostic issues

Conventional methods for the detection of toxigenic fungi are based on a combination of culturing isolates on specialised nutrient media to induce sporulation followed by microscopic examination of spores and conidia for characteristic morphological features. Such procedures are laborious, time-consuming and sometimes inconclusive. Consequently, there is considerable interest in the application of molecular biology techniques for rapid and positive identification of toxigenic fungi and already the polymerase chain reaction (PCR) has been shown to offer potential to accurately detect and identify several cereal pathogens directly from plant material. A laboratory-based PCR method has recently been developed to identify *F. poae*, *F. culmorum*, *F. graminearum*, *F. avenaceum* and *Michrodochium* (*Fusarium*) *nivale* in cereal grains. Indeed, a PCR diagnostic test for *Fusarium* on wheat is available from ADGEN at SAC (Stevenson, 1997). PCR methodology requires just 48 h to provide accurate results, whereas conventional procedures may take up to 21 days to yield somewhat ambiguous results. As presently designed, the PCR method is based on electrophoresis, but there is potential to develop alternative multi-well plate techniques to facilitate kit-based applications for use under field conditions.

Another method with some potential for species identification involves esterase profiling (Baayen et al., 1997). Although attention has focused on *F. oxysporum* and *A. parasiticus*, based on this evidence, there is now scope for investigating esterase patterns in other toxigenic species of *Fusarium*. D'Mello et al. (1998)

have correlated total esterase production with fungicide insensitivity in *F. culmorum*, but more recent unpublished data from our laboratory indicate that species differences may also be of diagnostic value. *F. sporotrichioides*, with the confirmed capacity to yield T-2 toxin, HT-2 toxin, NEO and DAS under our conditions, contained at least six different esterase isoenzymes, whereas *F. culmorum*, which produces only 3-ADON had just two esterase bands.

Conclusions

Evidence has been presented which highlights the overall ineffectiveness of pesticides to control mycotoxin production in *Fusarium* and *Aspergillus* fungi. In retrospect this conclusion is not entirely inconsistent with the notion that fungicides were designed for use against diseases and not necessarily against the mycotoxins associated with some of these diseases. If chemical control is to succeed in the future, additional criteria may need to be introduced into evaluation protocols for candidate pesticides. An invidious feature of fungicide application at sub-lethal doses emanates from persistence of mycotoxin production, occasionally at elevated levels. For example, increased persistence and changes in the pattern of 3-ADON production have been observed in a strain of *F. culmorum* resistant to the fungicide, difenoconazole. Development of fungicide resistance occurs readily in *Fusarium* phytopathogens but the effects on mycotoxin production need to be addressed in a more deliberate and systematic manner, given these findings on 3-ADON. Of the aflatoxins, AFB_1 production occurs more persistently in the presence of sub-lethal doses of fungicides and insecticides. This is of particular concern in view of the potency of AFB_1 as a carcinogen. Nevertheless, the concept that insecticides may exert a dual role in both insect and mycotoxin control may need further exploration to enable overall reductions in pesticide use. However, in terms of an environmentally acceptable alternative, there is considerable potential in breeding or selecting cultivars of crop plants which are resistant to those fungal diseases that are associated with mycotoxin contamination.

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