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Australian Journal of Agricultural Research

VOLUME 53, 2002

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Aust. J. Agric. Res., 2002, **53**, 1317–1326

Production of zearalenone, deoxynivalenol, nivalenol, and acetylated derivatives by Australian isolates of *Fusarium graminearum* and *F. pseudograminearum* in relation to source and culturing conditions

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Abstract. Australian isolates of *Fusarium pseudograminearum* (*Fp* = *F. graminearum* Group 1) and *F. graminearum* (*Fg* = *F. graminearum* Group 2) can produce mycotoxins including zearalenone (ZEA), 4-deoxynivalenol (DON), and nivalenol (NIV). *Fp* isolates from wheat and barley tillers in southern Queensland all produced ZEA and DON in culture, and one typical isolate also produced 3-acetyldeoxynivalenol. Most *Fg* isolates from wheat and sorghum grains in southern Queensland produced ZEA and DON and one typical isolate also produced 15-acetyldeoxynivalenol. *Fg* isolates from maize plants in northern Queensland were all ZEA and NIV producers, which was consistent with previous reports, and they also produced high concentrations of acetyl-nivalenols. ZEA and either DON or NIV production by cultures derived from different conidia (and ascospores in *Fg* isolates) varied by 4–18-fold for ZEA and 2–4-fold for DON/NIV production, and there were significant negative correlations between ZEA and either DON or NIV, indicating a common controlling process. The pattern of ZEA production was quite different between *Fp* and *Fg*, with ZEA production being relatively delayed in *Fg*. After 7 days incubation at 28°C on maize meal, one *Fp* isolate produced 49 mg ZEA/kg, but in both DON-producing and NIV-producing isolates of *Fg*, ZEA concentrations after 7 days were <1 mg/kg. ZEA and DON were produced on sorghum and combined wheat–barley grains as well as maize meal, although there were trends for maize meal to be more productive, probably due to greater surface area or different gaseous exchange. Low temperature incubation of a *Fg* DON-type isolate increased ZEA production, but did not affect either a *Fg* NIV-type isolate or a *Fp* isolate. Relationships between these patterns of mycotoxin production, pathogenicity, and implications for crop contamination are discussed.

Additional keywords: Gibberella zeae, *G. coronicola*, head scab, crown rot, head blight, phytotoxicity.

Introduction

Until recently, the pathogen causing crown rot and head scab in winter cereals and stalk and cob rot of maize was known as *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schw.) Petch). In 1977, Francis and Burgess (1977) distinguished 2 populations within *F. graminearum* and these were designated Groups 1 and 2. The Group 1 population was associated with crown rot of winter cereals, rarely formed perithecia in nature, and did not form perithecia in culture. In contrast, the Group 2 population was normally associated with stalk and cob rot in maize and head scab of winter cereals and formed perithecia abundantly in nature and in culture. In 1999, Aoki and O'Donnell (1999*a*) indicated that the two populations should be regarded as separate species and the Group 1 population was described as *F. pseudograminearum* O'Donnell & T. Aoki. They subsequently were able to produce the teleomorph of *F. pseudograminearum* and described it as *Gibberella coronicola* T. Aoki & O'Donnell (Aoki and O'Donnell 1999*b*).

The distribution of *F. graminearum* (*Fg*) and *F. pseudograminearum* (*Fp*) is related to the distribution of their hosts, and in turn, host distribution is controlled by climate (particularly the amount of summer rainfall). *Fp* is more common in drier areas where it causes crown rot of winter cereals. *Fg* is more prevalent in wetter, coastal areas where maize is grown. Occasional outbreaks of head scab in wheat (caused by *Fg*) are usually related to wet springs.

Sexual reproduction in *Fg* (Wolf and Mirocha 1977) is affected by the metabolite zearalenone (ZEA), with perithecial production inhibited both in the absence of ZEA, and in the presence of concentrations above a given

 10.1071/AR02041 0004-9409/02/121317

optimum. We demonstrated (Blaney and Dodman 1988) that the absence of perithecial production by *Fp* is not due to absence of ZEA production, nor (apparently) to inhibition by very high ZEA concentrations. Subsequently, Windels *et al.* (1989) found no correlation between perithecial production and ZEA production in isolates grown on rice culture or agar media. However, considering that perithecial production and ZEA production might occur at different times and in different parts of any given substrate, we postulated that the pattern of ZEA production might differ between isolates in different media and culturing conditions.

The role of trichothecenes produced by *Fg* and *Fp* is also of interest. Recent studies have confirmed that deoxynivalenol (DON), nivalenol (NIV), and their acetylated derivates are phytotoxic to the wheat coleoptile (Eudes *et al.* 2000). Liu *et al.* (1999) concluded that in wheat cultivars, resistance to head scab was positively correlated to level of resistance to cell membrane injury of embyronic roots and buds by DON, and Mesterhazy *et al.* (1999) found close but varying relationships between DON content and head scab severity, with the variation due to tolerance to infection. Consequently, differences in trichothecene production by *Fp* and *Fg* might be relevant to their respective modes of infection. We previously examined the production of ZEA, DON, and NIV in culture by Queensland isolates of *Fg* and *Fp* (Blaney and Dodman 1988) and reported for the first time that all *Fp* isolates produced ZEA and DON, that most *Fg* isolates from southern Queensland produced ZEA and DON but a couple produced ZEA and NIV, and that all *Fg* isolates from maize grown on the tablelands of northern Queensland produced ZEA and NIV.

Mycotoxins produced in grains by *Fg* can cause disease in humans and domestic animals. This is rare in Australia, but the oestrogenic syndrome caused by ZEA in pigs has been reported (Blaney *et al.* 1984), as has the feed refusal and vomiting syndrome caused in pigs by DON (Moore *et al.* 1985) and NIV (Williams and Blaney 1994). Some of this feed refusal syndrome has been attributed to acetylated derivatives of DON and NIV, which are more toxic to animals than DON and NIV (Sato and Ueno 1977). The 3- and 15-acetyl isomers of DON have some geographical separation in other parts of the world; for example, the 3-isomer is more common in Japan (Yoshizawa and Jin 1995), whereas the 15-isomer is more common in North America (Mirocha *et al.* 1989). The identity of Australian isomers has not been reported.

In the investigation reported here, our objectives were: (*i*) to determine the degree of variation in mycotoxin production in cultures derived from single conidia and ascospores of different isolates; (*ii*) to compare the effects of substrate, time, and temperature on mycotoxin production by different types of isolates in laboratory culture; (*iii*) to investigate differences in the pattern of ZEA and trichothecenes produced by *Fg* and *Fp*; (*iv*) to further clarify the extent of the geographic separation of DON/NIV chemotypes, and postulate an hypothesis for this; and (*v*) to identify acetylated trichothecenes produced and discuss the implications for mycotoxin contamination of Australian grains.

Materials and methods

Isolates of Fg *and* Fp

The source of isolates and the isolation techniques were described (Blaney and Dodman 1988). Isolates selected for the study were: 1713 (typical of *Fp* isolates from wheat stalks, southern Qld); B1 (high-DON-producing *Fg* isolate from wheat seed, southern Qld); B62 (moderate-DON-producing *Fg* isolate from wheat seed, southern Qld); 1669 (high-NIV-producing *Fg* isolate from wheat spikelet, southern Qld); and B56 (NIV-producing *Fg* isolate from maize, northern Qld). Five *Fp* isolates (S273, S279, S280, S281, S282) were freshly isolated prior to culture from barley tillers in southern Qld, 6 *Fg* isolates (B103–B108) were freshly isolated from maize stalks in northern Qld, and 2 *Fg* isolates (S305 and S321) were obtained from sorghum grain in southern Qld.

Single-spore procedures and mycotoxin production

The development of cultures from single conidia has been described previously (Blaney and Dodman 1988). Cultures from single ascospores were obtained by removing perithecia produced on carnation-leaf agar (Tio *et al.* 1977), washing them briefly in a few drops of sterile water on a sterile microscope slide, and then crushing the perithecia to eject the ascospores. The ascospores were then washed onto the surface of water agar in a Petri dish and incubated at 25°C for 16 h. Single germinated ascospores were located under a dissecting microscope and checked under the compound microscope to verify that they were ascospores and not conidia. They were then picked off with a needle and transferred to potato-dextrose agar (PDA) for propagation.

Maize-meal cultures were inoculated with plugs from the PDA cultures as previously described (Blaney and Dodman 1988) and a similar procedure was used for cultures on wheat–barley (1 : 1) or sorghum grain. Wherever cultures were replicated, this was done by taking replicate plugs of the PDA culture originating from a single spore. In some instances, the cultures were shaken every 2–3 days to separate the grains. They were incubated in the dark for various times, on different substrates, and at different temperatures, and then stored at –20°C until assayed.

Mycotoxin assays

Cultures were extracted and assayed for ZEA, DON, NIV, and derivatives by 2-dimensional thin-layer-chromatography (TLC) as described previously (Blaney and Dodman 1988). The acetyl DON derivatives were distinguished by TLC on silica gel plates developed in ethyl actetate–hexane (3 : 1, v/v) (Miller *et al.* 1983), in which system the Rfs (Rf = ratio of chromatographic mobility relative to solvent front) of DON, 15-acetyl DON, and 3-acetyl DON were 0.18, 0.38, and 0.54, respectively.

In addition, acetyl derivatives of DON and NIV were quantified along with DON and NIV in cultures by high performance liquid chromatography (HPLC), using a C-18 column operated at 35°C and a methanol : water solvent program changing from 8 : 92 to 30 : 70 over 20 min. Detection was by UV absorbance at 224 nm.

Fractions of the HPLC eluate stream were collected for confirmation of identity of the acetyl derivatives by capillary gas chromatography-mass spectrometry (GC-MS) (Blaney and Dodman 1988).

Table 1. Mycotoxin production (range and median, mg/kg, based on original dry substrate weight of 50 g) in maize meal after 4 weeks at 28°C by single-conidial cultures of *F. pseudograminearum* (*Fp*) and *Fusarium graminearum* (*Fg*), and single-ascospore cultures of *Fg* deoxynivalenol- and *Fg* nivalenol-producers

Isolate	Zearalenone	Deoxynivalenol	Nivalenol	Correlation (*r*) ZEA *v.* DON/NIV
1713 (*Fp*)[A]	183–3300 (813)	16–28 (24)	<2	–0.404
B1 (*Fg*)[A]	117–1180 (303)	630–1450 (1224)	<2	–0.800*
B1 (*Fg*)[B]	203–1230 (401)	167–478 (335)	<2	–0.655*
B62 (*Fg*)[B]	216–2300 (1230)	70–198 (121)	<2	–0.645*
1669 (*Fg*)[B]	3.5–12.2 (7.2)	<2	85–210 (126)	–0.711*
B56 (*Fg*)[B]	0.6–3.0 (1.5)	<2	7–34 (23)	–0.528

*$P < 0.05$.
[A]Single-conidial cultures, $n = 12$. [B]Single-ascospore cultures, $n = 10$.

Experimental design

Experiment 1

The first experiment examined production of ZEA, DON, and NIV in cultures derived from several spores of different isolates, incubated for 4 weeks at 28°C on maize meal. Inocula were: (a) 12 single-conidial cultures each of a typical *Fp* isolate (1713) and a virulent *Fg* DON-chemotype (B1); (b) 10 single-ascospore cultures each of B1 and another *Fg* DON-chemotype (B62); and (c) 12 single-ascospore cultures each of 2 *Fg* NIV-chemotypes (southern Qld, 1669; and northern Qld, B56).

Experiment 2

This experiment compared mycotoxin production by 5 *Fp* isolates (S273, S279, S280, S281, and S282) freshly isolated from barley tillers. Replicate cultures of the 5 isolates were also grown on a mixture of wheat–barley (1 : 1) grain for 4 weeks. Further replicates of 2 of the isolates were grown on wheat–barley grain with the culture shaken briefly every 3–4 days to separate the grains.

Experiment 3

Two isolates (S305 and S321) of *Fg* from sorghum grain were grown on maize meal for 4 weeks at 28°C. Replicate cultures were grown on sorghum grain under similar conditions. Further replicates of one isolate were grown on maize and sorghum for 2 weeks each at 28°C and 15°C.

Experiment 4

This experiment was in 2 parts. Firstly, we measured the rate of production of ZEA and DON by *Fp* isolate 1713, by incubation of replicate maize-meal cultures for either 1, 2, 3, or 4 weeks at 28°C; and secondly, we compared this with 2 weeks at 28°C followed by 2 weeks at 15°C. All of these cultures were derived from a single conidium.

Experiment 5

This experiment was also in 2 parts. Firstly, we measured the rate of ZEA, DON, and acetyl DON by a high-DON producing *Fg* isolate (B1), following incubation of replicate cultures for either 1, 2, 3, or 4 weeks at 28°C on maize meal; and secondly, we compared incubation for 4 weeks at 28°C with incubation for 2 weeks each at 28°C and 15°C, but using replicate cultures derived from a different conidium to the first part.

Experiment 6

This experiment examined the rate of production of ZEA, NIV, and acetyl NIV by northern Qld isolates B103-108, by incubation of replicate cultures for 1, 2, 3, or 4 weeks at 28°C.

Experiment 7

This experiment examined the rate of mycotoxin production by one northern Qld isolate (B104) by incubating replicate cultures for 1, 2, 3, or 4 weeks on maize meal at 28°C or for 2 weeks each at 28°C and 15°C.

Results

By visual inspection, mycelial growth in all cultures commenced within a few days of incubation, and reached a maximum by about 2 weeks.

Experiment 1

This experiment aimed to determine the degree of variation in mycotoxin production between cultures derived from different spores. The isolates had been selected to represent typical examples of different type isolates in regard to mycotoxin production in culture (Blaney and Dodman 1988). The results are in Table 1. Mycotoxin production by different single-spore cultures varied by about 4–18-fold for ZEA but only 2–4-fold for DON/NIV. This extent of variation in mycotoxin production between conidial cultures was not greatly different than between ascospore cultures for isolate B1. There were negative correlations between ZEA and either DON or NIV production and these correlations were statistically significant in isolates producing higher concentrations of these mycotoxins. This inter-spore variation was much greater than the variation between replicate cultures derived from single spores, as observed in subsequent experiments.

Experiment 2

This experiment further investigated the comparatively lower DON production by *Fp* in culture compared with *Fg* that we reported previously (Blaney and Dodman 1988). Fresh isolates were used in case the DON-production potential of *Fp* isolates was reduced in storage. We also grew the isolates on wheat–barley grains, as these plants are the usual hosts of *Fp* isolates, and we had previously found high DON production by one *Fp* isolate in a shaken wheat–barley

Table 2. Mycotoxin production (mg/kg) by *Fusarium pseudograminearum* isolates after 4 weeks at 28°C in either maize meal or still or shaken wheat–barley grain cultures

Maize meal and wheat–barley cultures from each isolate were inoculated with a plug from an agar culture derived from a single spore of each of those isolates

Isolate	Substrate	Culture conditions	Zearalenone	Deoxynivalenol
S273	Maize meal	Still	860	3.5
	Wheat–barley	Still	177	2.5
S279	Maize meal	Still	169	6.1
	Wheat–barley	Still	8	<0.1
S280	Maize meal	Still	250	6.1
	Wheat–barley	Still	118	1.4
	Wheat–barley	Shaken	610	1.0
S281	Maize meal	Still	497	26
	Wheat–barley	Still	51	0.7
S282	Maize meal	Still	2500	7.0
	Wheat–barley	Still	172	0.8
	Wheat–barley	Shaken	510	<0.1

culture, the medium used to prepare inoculum for crown rot infection trials.

The results are in Table 2. Five fresh *Fp* isolates produced moderate ZEA concentrations and low DON concentrations, which is similar to *Fp* (Group 1) isolates examined previously (Blaney and Dodman 1988). There was consistency of results across the 5 isolates for more mycotoxin production on maize meal than on wheat–barley grain; on maize meal, ZEA production (mg/kg) was 169–2500 (mean 855) and DON production (mg/kg) was 3.5–26 (mean 10); and on wheat–barley, ZEA production was 8–177 (mean 105) and DON production was <0.1–2.5 (mean 1.1). Shaking the wheat–barley cultures improved ZEA production with 2 isolates. These results should be viewed in light of the extent of variation seen between replicate cultures in Expts 4, 5, and 7, which was much less than the variation between cultures originating from different spores as shown in Expt 1.

Table 3. Mycotoxin production (mg/kg) by *Fusarium graminearum* isolates from sorghum grain, grown on maize meal and sorghum grain under different conditions

Maize meal and sorghum cultures from each isolate were inoculated with a plug from an agar culture derived from a single spore of each of those isolates

Isolate	Substrate	Culture conditions	Zearalenone	Deoxynivalenol
S305	Maize	4 wk, 28°C	1060	50
	Sorghum	4 wk, 28°C	690	126
S321	Maize	4 wk, 28°C	440	17
	Sorghum	4 wk, 28°C	850	67
S321	Maize	2 wk, 28°C; 2 wk, 15°C	3300 1050	0.7 13
	Sorghum	2 wk, 28°C; 2 wk, 15°C		

Experiment 3

The intent of this experiment was to determine if 2 isolates of *Fg* from sorghum grain were DON or NIV producers, and if mycotoxins could be produced on sorghum grain. The results are in Table 3. Both isolates produced substantial concentrations of ZEA and DON both on sorghum grain and maize meal. There was a trend for incubation at 28°C and 15°C each for 2 weeks to increase ZEA at the expense of DON production on both grain substrates compared with 4 weeks at 28°C, but this was not confirmed by sufficient replication.

Experiment 4

This experiment aimed to measure the relative rate of production of ZEA and DON over 4 weeks by a typical

Table 4. Mycotoxin production (mg/kg) by replicate cultures derived from a single conidium of isolate 1713 of *Fusarium pseudograminearum* in maize meal for different periods

Order of presentation of results from replicate cultures is the same for ZEA, DON, and acetyl DON. Analysis of variation based on log transformation with back-transformed means in parentheses. Parts A and B were analysed separately, and in each part, means followed by the same letter are not significantly different at $P = 0.05$

Culture time and temperature	Zearalenone	Deoxynivalenol	3-Acetyldeoxynivalenol
		Part A	
1 wk 28°C	9, 213, 61 (49)a	16, 4, 11 (9)a	43, 7, 24 (19)a
2 wk 28°C	2820, 231, 2566 (1187)b	17, 27, 16 (19)b	8, 41, 13 (16)a
3 wk 28°C	1968, 2793, 638 (1519)b	23, 13, 15 (16)ab	29, 7, 15 (14)a
4 wk 28°C	1263, 379, 705 (696)b	40, 32, 33 (35)c	17, 19, 20 (19)a
		Part B	
4 wk 28°C	560, 935 (724)a	26, 23 (24)a	NA
2 wk each 28/15°C	765, 445 (583)a	28, 17 (22)a	NA

NA, Not assayed.

Table 5. Mycotoxin production (mg/kg) by replicate cultures derived from two conidia of isolate B1 of *Fusarium graminearum* in maize meal for different periods

Analysis of variation based on log transformation with back-transformed means in parentheses. Means followed by the same letter are not significantly different at $P = 0.05$. Cultures from different conidia were statistically analysed separately

Culture time and temperature	Zearalenone	Deoxynivalenol	15-Acetyldeoxynivalenol
		Conidium A	
1 wk 28°C	0.3, 0.3, 0.3 (0.3)a	270, 505, 379 (372)a	325, 415, 360 (365)a
2 wk 28°C	12, 23, 17 (17)b	511, 1623, 1357 (1040)b	290, 337, 270 (298)a
3 wk 28°C	113, 61, 108 (91)c	1530, 1623, 1101 (1398)b	60, 62, 134 (79)b
4 wk 28°C	106, 148, 59 (97)c	1503, 1177, 1934 (1507)b	22, 22, 50 (29)c
		Conidium B	
4 wk 28°C	414, 621, 486 (500)b	408, 355, 478 (411)a	NA
2 wk each 28/15°C	1971, 1071, 1114 (1330)a	252, 294, 434 (318)a	NA

NA, Not assayed.

isolate of *Fp*, and to identify any acetylated derivatives produced. The results are in Table 4. Production of ZEA showed a tendency to peak after about 2–3 weeks at 28°C. This isolate also produced 3-acetyl DON in relatively constant amounts. DON slowly increased until the fourth week. Incubation for Weeks 3 and 4 at 15°C did not increase mycotoxin concentrations significantly.

Experiment 5

This experiment aimed to measure the relative rate of production of ZEA and DON over 4 weeks by a typical isolate of *Fg*, and to identify any acetylated derivatives produced. The results are in Table 5. Isolate B1 produced large amounts of acetyl-deoxynivalenol, confirmed as 15-acetyl DON. Production of trichothecenes paralleled length of incubation, with 15-acetyl DON peaking after 1 week and DON after 2–4 weeks. However, ZEA production was minimal after 1 week, low after 2 weeks, but then increased sharply until weeks 3–4. Incubation at 28°C and 15°C each for 2 weeks significantly increased ZEA production compared with 4 weeks at 28°C, which was similar to the trend in Expt 3 with another *Fg* isolate, but in contrast to Expt 4 with *Fp* isolate 1713.

Experiment 6

The aim of this experiment was to determine whether several fresh isolates of *Fg* from northern Qld were all NIV-types as reported previously (Blaney and Dodman 1988), to examine the rate of mycotoxin production, and to identify any acetylated derivatives produced. The results are in Table 6. All isolates produced NIV, 4-acetyl NIV, and (probably) 4,15-diacetyl NIV. On our 2-dimensional TLC system, these compounds were identified by their reaction with aluminium trichloride, coincidence with reference compounds, and their Rf values, in the first and second dimension, respectively, which were as follows: NIV, 0.07 and 0.09; 4-acetyl NIV, 0.44 and 0.31; diacetyl NIV, 0.62 and 0.45. Concentrations were estimated by visual comparison with standards and were roughly similar to those we found for NIV and 4-acetyl NIV by HPLC, which are reported in Table 6. We were able to positively identify 4-acetyl NIV (Fusarenon X) by GC–MS.

Table 6. Mycotoxin production (mg/kg) by *Fusarium graminearum* isolates from northern Queensland on maize meal for different periods at 28°C

Maize meal and sorghum cultures from each isolate were inoculated with a plug from an agar culture derived from a single spore of each of those isolates

Isolate	Period (week)	Zearalenone	Nivalenol	4-Acetylnivalenol[A]
B103	1	0.7	7	14
	2	8	109	95
	3	9	195	204
	4	16	259	164
B104	1	0.4	15	42
	2	0.4	57	63
	3	47	250	206
	4	100	396	194
B105	1	0.3	7	29
	2	30	23	47
	3	48	86	156
	4	45	210	144
B106	1	0.7	8	10
	2	0.4	34	51
	3	15	55	102
	4	26	193	144
B107	1	0.6	7	7
	2	8	41	77
	3	27	81	93
	4	47	55	51
B108	1	0.5	4	5
	2	8	35	60
	3	38	75	138
	4	92	148	177

[A]Large amounts of diacetyl-nivalenol also present, estimated by TLC to be similar concentrations to acetyl NIV.

In our HPLC conditions, the 4-acetyl NIV produced a double peak (well separated from the presumed diacetyl-NIV), and we were unable to determine whether these were isomers or an artefact such as a methylation product. Both parts of the doublet produced an apparently identical pentafluoropropionate derivative on GC–MS and so they were collectively quantified as 4-acetyl NIV. The penta-fluoropropionate of our presumed diacetyl-NIV produced a similar mass spectrum to 4-acetyl NIV, but positive identification was not made as we lacked reference materials.

There was strong consistency across the isolates, in that ZEA production was minimal after 1–2 weeks, but then increased sharply until Weeks 3–4. The average ZEA production across the 6 isolates for Weeks 1, 2, 3, and 4, respectively, was 0.5, 9, 31, and 54 mg/kg. These results were somewhat similar to those with the *Fg* DON-type (B1) in Table 5. However, trichothecene production was much greater in the DON-type (B1) than in NIV-types of *Fg*, which in this experiment averaged 8, 50, 124, and 210 mg NIV/kg and 18, 66, 150, and 146 mg 4-acetyl NIV/kg for Weeks 1, 2, 3, and 4. After 4 weeks, the variation between isolates was of a similar order to the variation between spores in Expt 1, and greater than between replicates in Expt 7.

Experiment 7

The experiment examined the variation between replicate cultures of one northern Qld isolate as they grew for 4 weeks and to determine if low temperature incubation increased ZEA concentrations. The results are in Table 7. Production of ZEA and NIV by B104 was much less than by B104 in the previous experiment (these cultures were derived from a different conidium than that used in Expt 6) but the pattern was otherwise similar. Incubation at 28°C and 15°C each for 2 weeks did not increase ZEA concentrations compared with 4 weeks at 28°C, as occurred with the *Fg* DON-type isolate (B1, Table 5).

Discussion

Mycotoxin production by Fg *and* Fp *in cultures derived from different conidia and ascospores*

ZEA and either DON or NIV production by cultures derived from different conidia and ascospores (Table 1) varied by 4–18-fold for ZEA and 2–4-fold for either DON or NIV. The significant negative correlation that we found between ZEA and trichothecene production by conidial and ascospore cultures in *Fg* types seems to indicate a common controlling process, although they do not appear to share a biosynthetic pathway. This suggests to us that ZEA and trichothecenes both have important ecological roles in *Fg* and *Fp*, but that one is more important than the other at different stages in pathogenesis, growth, reproduction, or survival. The high variation in production between spores and isolates (far greater than the variation shown between replicate cultures) hints at a mechanism for allowing expression of diversity of mycotoxin production in response to different circumstances, with selection tending towards production of either ZEA or DON/NIV at the expense of the other. This is explored further below.

Effect of substrate, time, and temperature on mycotoxin production

Mycotoxin production in cultures is affected by substrate, moisture content, gaseous equilibrium, and time and temperature of incubation (Vesonder *et al.* 1982; Greenhalgh *et al.* 1983). The general conditions we employed are similar to those used for Canadian isolates of *Fg* by Greenhalgh *et al.* (1983), i.e. 28 days incubation on maize substrate at 28°C. This medium performed well with our isolates, but mycotoxins were also produced on wheat–barley grains and on sorghum grain, indicating that the nature of the medium is not critical for these fungi. Of course, living plants have additional defences to resist invasion, compared with sterilised grain. Maize meal was superior to wheat–barley grain for ZEA and DON production by *Fp*, but this could be due to surface area and gaseous environment as much as the grain itself. On maize medium, our *Fp* types consistently produced less DON than *Fg* types (Blaney and Dodman 1988 and present data), but we emphasise that *Fp* might perform very differently when growing in tillers. When invading a tiller, it might be the concentration applied at particular sites at the cellular level, rather than total production in culture, that is most important.

The acetyl derivatives of DON are precursors of DON and can be de-acetylated by either the fungal mycelium

Table 7. Mycotoxin production (mg/kg) by replicate cultures derived from a single conidium of northern Qld isolate B104 of *Fusarium graminearum*, in maize meal for different periods

Analysis of variation based on log transformation with back-transformed means in parentheses. Means followed by the same letter are not significantly different at $P = 0.05$

Culture time and temperature	Zearalenone	Nivalenol	4-Acetylnivalenol
1 wk 28°C	0.3, 1.3, 0.5 (0.6)a	2.6, 0.5, <0.2 (0.6)a	0.6, 3, 4 (2)a
2 wk 28°C	10, 10, 16 (12)b	8, 7, 5 (7)b	25, 16, 30 (23)b
3 wk 28°C	75, 25, 54 (47)c	24, 17, 11 (17)bc	30, 31, 28 (30)b
4 wk 28°C	65, 39, 50 (50)c	31, 27, 33 (30)c	6, 17, 32 (15)b
2 wk each 28/15°C	36, 53, 33 (40)c	6, 9, 16 (10)bc	16, 21, 18 (18)b

(Yoshizawa and Morooka 1975) or maize tissue. It is of considerable interest that our *Fp* isolate produced the 3-isomer and our *Fg* isolate the 15-isomer. Based on only one isolate of each, we cannot claim that this is a consistent relationship, but it certainly is worth further investigation. Our results with isolate B1 (Table 5) were consistent with 15-acetyl DON being the main precursor of DON. Production of 15-acetyl DON paralleled mycelial growth and DON accumulated in amounts consistent with a constant rate of de-acetylation. Production of 15-acetyl DON decreased markedly after 14 days at 28°C, leading to a greatly reduced rate of DON accumulation. These results are similar to those of Greenhalgh *et al.* (1983) with Canadian isolates. In comparison, the production of 3-acetyl DON (Table 4) by *Fp* appeared to remain relatively constant throughout the 4 weeks incubation.

The overall trichothecene production patterns of NIV-chemotypes from northern Queensland (Table 6) were different from those of the DON-chemotype B1 (Table 5). NIV, 4-acetyl NIV, and diacetyl NIV all accumulated in the cultures up to 28 days, although NIV usually predominated. Acetyl NIV (and probably diacetyl NIV) is considered a precursor of NIV. However, production of 4-acetyl NIV continued throughout the 4 weeks of incubation, as with 3-acetyl DON production by *Fp*, whereas production of 15-acetyl DON by B1 practically ceased after 2 weeks (Table 5). This slower and more constant rate of production of trichothecenes by our *Fp* isolate and NIV-type *Fg* isolate suggests a different pathogenicity pattern, but it is also possible that it was an artefact of our culturing conditions.

It has been suggested that ZEA production can be increased in response to cooler conditions. Mirocha and Christensen (1974) reported that ZEA production in *Fg* was maximised by incubation at 24–27°C for 7–14 days to develop sufficient fungal mycelium, followed by incubation at 12–14°C for 28–42 days. However, Naik *et al.* (1987) found that cold incubation increased ZEA production in only 1 of 5 Canadian isolates. ZEA production by our *Fg* isolate B1 was more than doubled by incubating at 28°C and 15°C for 14 days each, compared with 28 days at 28°C (Table 5), but one *Fg* NIV-type isolate from northern Queensland (Table 7) did not react in this way. Ambient temperatures in southern *v.* northern Qld grain-growing areas do not suggest any explanation for the difference. It seems that a sudden reduction in temperature triggers the switch from DON to ZEA production in some susceptible isolates.

ZEA, DON, and NIV production in relation to perithecial formation by Fg

Our results show a clear distinction between representatives of *Fp* and *Fg* isolates in respect to *rate* of ZEA production. After 7 days, *Fp* isolate 1713 (Table 4) had produced a mean of 49 mg ZEA/kg, which was 4% of the maximum attained after 14 days. In contrast, *Fg* DON and NIV chemotype isolates had produced <1 mg/kg after 7 days, which was <2% of the maximum attained after 28 days (Tables 5 and 7). This indicates that in *Fg* types, initial production of ZEA is inhibited, perhaps until after perithecial production is triggered by some change such as near-exhaustion of some key nutrient in the substrate. This ability to inhibit ZEA production is not required by *Fp* isolates, which do not produce perithecia in culture.

Eugenio (cited by Wolf and Mirocha 1977) found that ZEA production by ascospore cultures derived from a single perithecium of *G. zeae* varied widely, and was inversely related to the potential of each isolate to produce perithecia. An extrapolation from these results and ours suggests that production of perithecia might be positively correlated with trichothecene production, as well as negatively correlated with ZEA production in *Fg* types. As we have noted previously (Blaney and Dodman 1988), our *Fg* NIV-chemotypes from both northern and southern Queensland produced much less ZEA than did DON-chemotypes (both *Fp* and *Fg*). It has also been noted that NIV-chemotypes in northern Queensland are quite prolific perithecia producers, as discussed below.

Production of ZEA and DON by Fp *in southern Queensland*

These results support our previous investigation in that all *Fp* isolates produced ZEA and DON and none produced NIV. In our *Fp* isolates, production of ZEA was not inhibited during early mycelial growth, with the consequence that high concentrations accumulated in culture. Perhaps as a consequence of selection for high ZEA production, less DON tended to be produced. However, there was a striking exception to this pattern in a single isolate (1662), a virulent isolate of *Fp* kept for crown rot inoculation trials, which produced around 150 mg DON/kg and 1 mg ZEA/kg, both on maize meal (Blaney and Dodman 1988) and on a shaken wheat–barley culture (unpublished data). A correlation between trichothecene production and virulence in *Fg* has been noted (Al-Heeti and Smalley 1988; Desjardins *et al.* 1996; and others). Harris *et al.* (1999) suggested that trichothecenes (DON) act as virulence factors to enhance the spread of *Fg* on maize, since transgenic strains with the trichothecene-biosynthetic pathway blocked were still pathogenic, but less virulent than wild strains. From results presented here, we conclude that DON production has some relationship to virulence in *Fp* types as well, but that there are stronger environmental influences in the paddock that select for high ZEA production, and that low DON production does not greatly handicap *Fp* types in respect to infection of the crown in natural circumstances.

The very high ZEA concentrations produced by many of our *Fp* isolates suggest that ZEA could play an important role in these types, unrelated to sexual reproduction. *Fp* tends to grow in drier conditions, and ZEA might be more important in either survival or pathogenesis in the drier

environment. Research in China has shown that ZEA is also a plant hormone in wheat, maize, and several other plants; for example, Yao *et al.* (1990) showed that germination of seeds of wheat and other plants was inhibited by ZEA treatment, but that seedling growth was accelerated. Fu and Meng (1994) suggested that ZEA functioned as a growth regulator in wheat and Zhao and Meng (1999) showed that ZEA plays an important role in vernalisation of winter wheat, by promoting shooting. Perhaps ZEA production by *Fp* interferes with growth regulation by immature wheat plants and renders them more vulnerable to penetration of the fungal hyphae through tissues.

Production of ZEA and NIV by Fg *isolates in northern Queensland*

As with our previous investigations (Blaney and Dodman 1988), all additional and freshly harvested isolates from northern Queensland were NIV-chemotypes, whereas most isolates from southern Queensland were DON-types. The most obvious selection influence on the population of *F. graminearum* in northern Queensland has been the long period of maize breeding for resistance to ear- and stalk-rots. (One of the physical changes has been development of a very long and tight husk closure.) The maize hybrids used almost exclusively in this region were bred by Department of Primary Industries officers from 2 sources, obtained almost 40 years ago, and the breeding program has been closed since then. One of these sources (Atherton Tableland 1) is a composite of southern and central American lines (flint) obtained via CIMMYT in Mexico, and the other (AT2) is a composite of subtropical lines (dent) then available in southern Queensland. Some original maize introductions were only surface-sterilised with sulfuric acid, which was probably insufficient to prevent the introduction of *Fg* as an internal infection of the seed.

The climate features a persistently wet and often cool, maize growing and maturation period. Selection against disease has been a major goal of the breeding program, and this has eradicated many diseases. Resistance to fusarium ear rot is far greater than in commercial hybrids, which are tested annually in the region, but are quickly taken over completely by the pathogen (Ian Martin, pers. comm.). It would not surprise if the same selection process induced increased perithecial production in *Fg*, as discharge of ascospores from perithecia is the main source of infection. Potential for production of the phytotoxic trichothecenes by the fungus would also seem likely to increase, and this appears to be borne out by the results presented here, particularly in respect to the acetylated nivalenols, that in total exceeded NIV concentrations in most cultures.

However, this does not explain the preponderance of NIV-chemotypes of *Fg* (DON has been detected in maize in the region, but at very low frequency compared with NIV, unpubl. data). It seems likely that DON-types would regularly be introduced in commercial hybrids from southern Qld, but have not survived as well as NIV-types. Our most likely conclusion is that the breeding program has selected against DON-types (that is, DON-types are less virulent on maize than NIV-types). The toxicity of DON-types towards these hybrids is worth investigation.

Implications for mycotoxin contamination of field crops

In general, outbreaks of head scab in wheat are quite uncommon in Australia, mainly because dry conditions prevail during anthesis and because wheat is not rotated with maize to any significant extent. Our most prolific producer of DON (isolate B1) was from a small, school farm where maize and wheat were all grown in small quantities (Moore *et al.* 1985). It produced abundant perithecia and was apparently quite virulent. Repeated cycling of these crops might select for both high DON production and virulence, leading to greater contamination. Increased scab in coastal parts of New South Wales over the past few seasons has partly been due to this. It has also been suggested that sorghum might play a role in this problem as it is frequently rotated with wheat in drier areas. Our results support this by showing that 2 *Fg* isolates from sorghum grain were capable of production of both ZEA and DON in culture on either maize or sorghum.

Mycotoxin production in laboratory culture can be different than in field crops. In Australia, DON and ZEA have been found in wheat grain infected with *Fg* (Blaney *et al.* 1987*b*) but DON:ZEA ratios were typically in the range of 50:1, whereas isolates from these grains produced DON:ZEA ratios between 1:20 and 20:1 in maize-meal culture (Blaney and Dodman 1988). The reason for this appears to be that DON is produced early in the fungal invasion process, and that the dry conditions usually prevailing during wheat maturation and harvest in the northern Australian wheatbelt do not favour continued growth by the fungus and production of ZEA. This pattern could change if wet weather delayed harvest, when ZEA content could increase. The very few cases where ZEA has affected pigs have been associated with prolonged storage of high-moisture maize and sorghum (Blaney *et al.* 1984) on the Atherton Tableland or high-moisture wheat in southern Queensland (Moore *et al.* 1985). It is clear that the fungus did not require cold stress to produce copious amounts of ZEA in those instances.

Head scab is occasionally caused by *Fp* due to rain splash in very wet conditions in southern Queensland and northern New South Wales but has led to only minor mycotoxin contamination of wheat. Burgess *et al.* (1987) reported 0.6 mg DON/kg in a single sample in which 38% of grains were infected with *Fp*. By comparison, the sample from which our *Fg* B1 was taken contained 34 mg DON/kg and 6 mg ZEA/kg (Moore *et al.* 1985). Drying before higher concentrations of ZEA had time to be produced could also be

the reason why wheat and barley tillers infected with *Fp* contained roughly equal amounts of ZEA and DON (about 20–30 mg/kg in the basal 200 mm of stalk; Blaney *et al.* 1987*a*). Our results suggest that such samples should also be assayed for 3-acetyl DON, and also that further research should be conducted into the role of these mycotoxins in the pathogenesis of crown rot.

The maize breeding program in northern Queensland appears to have been moderately successful in terms of mycotoxin contamination as well as ear-rot resistance; NIV and ZEA concentrations in maize from individual crops rarely exceed 1 mg/kg (Blaney *et al.* 1986 and unpublished data). This is below the concentration that affects pigs (Williams and Blaney 1994), unless the grain is stored moist. Introduction of these resistant maize hybrids (without the pathogen) into other parts of Australia where maize is grown, could be advantageous in that it could reduce inoculum levels of *Fg* and perhaps help to reduce head scab in wheat.

Despite Australia's fortunate position in respect to a low prevalence of head scab compared with many other countries, it is clear that grain quality should be continually monitored. As some of the isolates examined produced large amounts of acetylated trichothecenes in culture, it is possible that these could also contaminate field crops and should be included in surveillance programs.

Acknowledgments

We thank Mr Tony Tyler, Mr Neale Obst, and Ms Sam Murray for technical assistance; Mr Mark Ramsey for the supply of *F. graminearum* isolates from northern Queensland; Mr Ian Martin for advice on maize breeding programs; Dr Graham Wildermuth for advice on head blight and crown rot incidence; and Dr Chris Moore for identification of several trichothecenes by capillary gas chromatography–mass spectrometry.

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Manuscript received 12 March 2002, accepted 20 August 2002