EFFECTS OF INTERACTIONS BETWEEN WATER ACTIVITY, TEMPERATURE AND TOXIGENIC FUNGI ON FUNGAL COLONISATION AND MYCOTOXIN PRODUCTION IN BARLEY

Nannapaneni RAMAKRISHNA[1], John LACEY[1] and John E. SMITH[2]

1, A.F.R.C. Institute of Arable Crops Research, Rothamsted Experimental Station, Plant Pathology Department, Harpenden, Herts AL5 2JQ, UK.

2, Department of Bioscience and Biotechnology, University of Strathclyde, Glasgow G1 1XW, UK.

Abstract

Barley grain was inoculated with *Aspergillus flavus*, *Penicillium verrucosum* and *Fusarium poae*, either individually or in pairs and incubated at different a_w and temperatures. Germination of spores on the grain surface was observed by scanning electron microscope and colonisation was assessed by direct and dilution plating. Aflatoxin B_1, T-2 toxin and ochratoxin A were assayed by enzyme-linked immunosorbent assay. All fungi tested germinated when another species was present, whether in contact or slightly apart, on the grain surface. Colonisation by *A. flavus* was decreased by *P. verrucosum* at 20°C but not at 30°C while that of *P. verrucosum* was decreased by *A. flavus* at both temperatures. Growth of *F. poae* was inhibited by *A. flavus* and *P. verrucosum* under all environmental conditions. Aflatoxin B_1 production by *A. flavus* was decreased by *P. verrucosum* under most conditions but was enhanced by *F. poae* at 20°C/0.97a_w and at 30°C/0.90a_w. *A. flavus* decreased Ochratoxin A production by *P. verrucosum* except at 0.90 a_w and increased T-2 toxin production at 20°C/0.95a_w but not at 20°C/0.97a_w. Interaction between *F. poae* and *P. verrucosum* decreased ochratoxin A production but enhanced T-2 toxin production.

Introduction

Grain may be colonised by microorganisms at all stages of its development before harvest and during storage. Inoculum of a wide range of species can be present on the grain surface, often in close proximity. When the propagules of the microorganisms germinate, different species compete for space and substrate. How they interact with one another will determine which species survive.

Colonisation of barley by *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. is common (Hill and Lacey, 1983). Some species produce mycotoxins but it is not known how their mycotoxin production is affected by the presence of other species. In maize before harvest, *Aspergillus flavus* infection and aflatoxin contamination can be decreased by the presence of *Fusarium moniliforme* (Wicklow *et al.*, 1988) and *Penicillium oxalicum* (Ehrlich

et al., 1985) while in stored grain it may be enhanced by *Hyphopichia burtonii* and *Bacillus amyloliquefaciens* (Cuero *et al.*, 1987). In some conditions, ochratoxin A production by *P. viridicatum* and zearalenone production by *F. graminearum* were also enhanced by the presence of other filamentous fungi in maize (Cuero *et al.*, 1988). However, the relationship between fungal growth and mycotoxin production during interspecific interactions has never been determined.

We report here studies of the early growth of interacting fungi on grain surfaces by scanning electron microscopy, and their subsequent colonisation of the grain, assessed by direct and dilution plating. Mycotoxin production in barley by *A. flavus, P. verrucosum* and *F. poae* were assayed in single and paired cultures in different environments.

Materials and methods

Preparation of spore suspensions

Spore suspensions of *A. flavus, P. verrucosum* and *F. poae* were prepared from 14 day-old-cultures on autoclaved barley grain, incubated at 25 °C, by mixing 4-5 colonised grain in sterile distilled water containing 0.05% Tween-20. Concentrations were adjusted to 10^6 spores ml^{-1} and used to prepare inoculum containing 2.5 x 10^5 spores ml^{-1} by mixing 100 μl of stock suspensions of one or two fungi with distilled water in a 96 well microtitre plate. Thus 2 μl of suspension contained 250 spores of each fungus either alone or paired with another species.

Inoculation of grain

Barley grains, sterilized with 12 kGy gamma irradiation (Ramakrishna *et al.*, 1990b), were adjusted to 0.97, 0.95 or 0.90 a_w (water contents 27.4%, 24.4% and 20.2% respectively) by adding sterile water. Individuals grains of different a_w were aseptically transferred to compartmentalized Petri dishes (Sterilin; 25 wells, each 2 cm square x 2 cm deep), and placed dent side down leaving the flat side exposed. Individual grains were then point inoculated on the flat side with 2 μl of single or mixed spore suspension.

Gamma irradiated barley grains (10 or 40 g) adjusted to different a_w were also aseptically transferred to bags made of microporous film (Valmic^R, Vanleer UK Ltd) and inoculated with suspensions containing 10^6 spores ml^{-1} of toxigenic *A. flavus, P. verrucosum, F. poae*, either alone or in pairs, with 0.1 ml of each per 10 g grain.

Controlled environment incubation

Petri dishes containing inoculated grain were incubated at 20° or 30 °C in desiccators containing glycerol solution of the same a_w. Concentrations of glycerol required to give 0.97, 0.95 or 0.90 a_w were respectively, 1.6, 2.75 and 5.5 molal (Dallyn and Fox, 1980). Microporous bags containing inoculated grain were incubated at 20 °C or 30 °C in a controlled environment incubator (NAPCO 7300) at 97%, 95% or 90% relative humidity for 14 days.

Scanning electron microscopy of fungal growth on the grain surface

Every 12 h, up to 96 h, one grain from each inoculation treatment was fixed for 24 h in formaldehyde vapour in a desiccator. Grains were then mounted onto aluminum stubs, gold coated with a sputter coater (EMScope, Model AE 1231) and examined by scanning electron microscope (Hitachi S450). The lengths of the longest hyphae from 20 germinating spores of *A. flavus, P. verrucosum* and *F. poae* growing alone or with another

species were measured at magnifications of 100, 1000 or 10000 times.

Assessment of grain colonisation by fungi

Colonisation of barley grain by *A. flavus, P. verrucosum* and *F. poae* in single and paired cultures was determined after 14 days incubation by direct and dilution plating of 10 g samples.

Estimation of mycotoxin production in single and paired cultures

Production of aflatoxin B_1, T-2 toxin and ochratoxin A in grain, by toxigenic *A. flavus, P. verrucosum* and *F. poae* respectively, growing alone or with another species, was determined after 14 days incubation by enzyme linked immunosorbent assay (ELISA) (Ramakrishna *et al.* 1990a) using 40 g samples in two replicates.

Statistical analysis

Analyses of variance were performed on results of ELISA assays of toxins using $\log_{10}$ transformed values.

Results

Spore germination of interacting fungi on the grain surface

Conidia of *A. flavus* or *P. verrucosum* and micro-conidia of *F. poae* have distinct shapes and ornamentations that enables them to be identified by scanning electron microscopy. The ornamentation of *A. flavus* conidia is lobate-reticulate and of *P. verrucosum* micro-verrucose and both conidia are globose. *F. poae* micro-conidia are irregularily ridged and obovate in shape.

When inoculated in pairs onto the grain surface, all species germinated in the presence of all others and in all environments tested, producing a germtube which grew linearly on the grain surface. At 20 °C, spores of all three species germinated within 18 h at 0.97 and 0.95 a_w but at 0.90 a_w, *A. flavus* germinated only after 36 h but the other species within 24 h. At 30 °C, *A. flavus* germinated within 6 h at 0.97 and 0.95 a_w but took 12 h at 0.90 a_w while *P. verrucosum* germinated only after 18 h at 0.97 or 0.95 a_w and *F. poae* after 12 h. Both *P. verrucosum* and *F. poae* germinated within 24 h at 30 °C/0.90a_w. Germination was unaffected, even when the spores of the interacting fungi were in contact, and 40-60% spores of all species germinated whether alone or paired with another species. However, only <10% of *A. flavus* spores germinated at 20 °C/0.90a_w.

Growth and mycotoxin production of interacting fungi

Growth and mycotoxin production of *A. flavus, P. verrucosum* and *F. poae* in single and paired cultures are shown in Tables I-III.

***Aspergillus flavus* and *Penicillium verrucosum*:** At 20 °C, linear growth of *P. verrucosum* on the grain surface was much faster than that of *A. flavus* and was unaffected by its presence. Growth of *A. flavus* was restricted by *P. verrucosum* within 36 h at 20 °C and 0.97 or 0.95 a_w and after 96 h at 0.90 a_w. At 30 °C at all a_w, linear growth of *A. flavus* was unaffected by *P. verrucosum* and that of *P. verrucosum* was almost prevented by the presence of *A. flavus* after 24 h at 0.97 or 0.95 a_w but only after 48 h at 0.90 a_w. Seed infection and cfu of *P. verrucosum* were decreased by the presence of *A. flavus* under all conditions but especially at 30 °C while those of *A. flavus* were unaffected by *P. verrucosum* at

30°C/0.97a_w or 30°C/0.95a_w but were decreased under other conditions as compared to single cultures of *A. flavus* and *P. verrucosum* (Tables I and II)

Aflatoxin B_1 production by *A. flavus* was decreased by competition with *P. verrucosum* under all conditions except at 30°C/0.95 a_w where production compared to single culture, was slightly increased (Table I). Ochratoxin A production by *P. verrucosum* was significantly decreased in competition with *A. flavus* at 0.97 and 0.95 a_w at both temperatures but at 0.90 a_w, production was increased (Table II).

***Aspergillus flavus* and *Fusarium poae*:** On the grain surface at 20°C, *F. poae* grew faster than *A. flavus* at all a_w but neither species was affected by the presence of the other. However, at 30°C, *A. flavus* grew much faster than *F. poae* with no difference from pure culture, while growth of *F. poae* was always decreased by *A. flavus*. *A. flavus* seed infection and cfu were unaffected by *F. poae* but those of *F. poae* were decreased by *A. flavus* (Tables I and III).

More aflatoxin B_1 was produced in competition with *F. poae* at 20°C/0.97a_w and at 30°C/0.90a_w but less under all other conditions compared to single cultures of *A. flavus* (Table I). T-2 toxin production was decreased by *A. flavus* at 20°C/0.97a_w but was significantly increased at 20°C/0.95 a_w (Table III). No T-2 toxin was produced by *F. poae* at 30°C or at 0.90 a_w.

Table I. Growth and aflatoxin B_1 production by *Aspergillus flavus* (*Af*) in single and paired cultures on barley grain.

Assessments		*Af* alone		*Af* in the presence of: *P. verrucosum*		*F. poae*	
	A_w	20°	30°C	20°	30°C	20°	30°C
Linear growth[1]	0.97	200	750	42	800	170	700
on grain surface	0.95	100	600	14	500	110	550
(μm)	0.90	5	40	7	65	6	50
Seeds[2]	0.97	100	100	92	99	100	100
infected (%)	0.95	100	97	53	98	98	100
	0.90	8	99	10	59	9	80
Log colony[2]	0.97	7.6	7.7	7.7	7.7	8.5	7.4
forming units	0.95	7.9	7.7	5.2	7.0	7.6	7.1
	0.90	3.0	6.1	2.0	4.4	3.1	5.7
Aflatoxin B_1[2,3]	0.97	1.8	3.6	1.6	3.2	2.0	3.6
(Log ng/g)	0.95	1.6	3.4	1.0	3.5	1.2	3.3
	0.90	1.5	1.6	1.5	1.4	1.2	1.9

1, Linear growth recorded by SEM after 36 h at 20°C or after 24 h at 30°C.
2, Seed infection, cfu and mycotoxin production determined after 14 days incubation.
3, s.e.d ± 0.14

Table II. Growth and ochratoxin A production by *Penicillium verrucosum* (*Pv*) in single and paired cultures on barley grain after 14 days at different a_w and temperatures.

Assessments		*Pv* alone		*Pv* in the presence of: *A. flavus*		*F. poae*	
	A_w	20°	30°C	20°	30°C	20°	30°C
Linear growth[1]	0.97	290	40	300	12	225	37
on grain surface	0.95	250	47	270	31	180	40
(μm)	0.90	70	23	80	10	65	33
Seeds[2]	0.97	97	95	10	15	94	96
infected (%)	0.95	96	62	43	8	89	65
	0.90	32	4	15	0	36	2
Log colony[2]	0.97	9.4	8.5	8.9	6.0	9.4	8.0
forming units	0.95	8.6	8.4	8.7	4.1	8.6	7.4
	0.90	6.7	4.9	5.7	0.0	6.7	3.4
Ochratoxin A[2,3]	0.97	5.0	3.2	4.4	0.5	4.9	2.9
(Log ng/g)	0.95	3.3	2.1	3.2	1.9	3.4	2.0
	0.90	2.4	1.7	2.5	2.0	2.3	1.9

1 and 2, see Table I. 3, s.e.d ± 0.16

Table III. Growth and T-2 toxin production by *Fusarium poae* (*Fp*) in single and paired cultures on barley grain after 14 days at differen a_w and temperatures.

Assessments		*Fp* alone		*Fp* in the presence of: *A. flavus*		*P. verrucosum*	
	A_w	20°	30°C	20°	30°C	20°	30°C
Linear growth[1]	0.97	300	350	300	45	290	300
on grain surface	0.95	220	225	105	60	190	200
(μm)	0.90	15	17	15	5	11	11
Seeds[2]	0.97	91	24	14	11	1	7
infected (%)	0.95	10	4	3	0	0	0
Log colony[2]	0.97	5.6	2.0	0	0	0	0
forming units	0.95	2.5	1.9	0	0	0	0
T-2 toxin[2,3]	0.97	3.2	0.0	3.1	0	3.4	0
(Log ng/g)	0.95	2.7	0.0	3.0	0	3.2	0

1 and 2, see Table I. 3, s.e.d ± 0.08

Penicillium verrucosum* and *Fusarium poae: At 20°C, growth rates of both species on the grain surface were similar at 0.97 or 0.95 a_w but *P. verrucosum* grew faster than *F. poae* at 0.90 a_w. Growth of both *P. verrucosum* and *F. poae* were only slightly less when mixed than in pure culture at 0.97 and 0.95 a_w and 20°C. At 30°C at both 0.97 and 0.95 a_w, *F. poae* grew much faster than *P. verrucosum* but neither species grew much less than in pure culture. However, seed infection and cfu of *F. poae* were decreased by *P. verrucosum* but those of *P. verrucosum* were little affected (Tables II and III).

Ochratoxin A production was decreased significantly by competition with *F. poae* except at 20°C/0.95a_w and at 30°C/0.90a_w where it was slightly enhanced (Table II). T-2 toxin production by *F. poae* was significantly increased by *P. verrucosum* at 20°C/0.97a_w and 20°C/0.95a_w (Table III). No T-2 toxin was produced by *F. poae* at 30°C nor at 0.90 a_w in either single or paired cultures.

Discussion

Interspecific interactions on grain may occur during spore germination, growth and spore production. There was no inhibition of spore germination in the presence of other fungi on the grain surface and all spores germinated to give a linear germtubes. Growth rates differed with species and environmental conditions. Growth of *A. flavus* was much faster at 30°C than at 20°C and *P. verrucosum* faster at 20°C. Initial growth of *F. poae* was relatively unaffected by temperature at all a_w. Although *F. poae* spores germinated at 0.90 a_w, growth was extremely slow. *F. poae* seed infection and cfu could not be detected at 0.90 a_w, even after 14 days incubation.

When *A. flavus* and *P. verrucosum* were inoculated together, their relative growth rates under different environmental conditions determined which species would colonise most of the available surface area on the grain. Both species produced extensive hyphal growth under favourable conditions which restricted the growth of competing species on the grain surface. Colonisation of grain by both species was decreased in paired cultures under most environmental conditions. Usually decreased seed infection by *P. verrucosum* was accompanied by decreased cfu production, but at 20°C/0.97a_w or 20°C/0.95a_w, cfu were unaffected although seed infection was decreased by 50-90% in the presence of *A. flavus*. *F. poae* grew faster than both *P. verrucosum* and *A. flavus* at 20°C, but growth of these species was little affected by *F. poae* in mixed infections. *F. poae* produced fewer hyphae than the other two species and this may have been insufficient to prevent growth of the competing species. Interacting hyphae of all three species always appeared normal without any structural changes suggesting that toxic substances are not involved, at least in the early stages of competition.

Mycotoxin production was significantly affected by interactions between a_w, temperature, incubation period and competing fungi. Decreased colonisation by *A. flavus* and *P. verrucosum* in mixed cultures resulted in decreased production of both aflatoxin B_1 and ochratoxin A under most environmental conditions. Ochratoxin A production was almost completely inhibited in the presence of *A. flavus* at 30°C/0.97a_w and was significantly decreased in mixed culture at 30°C/0.95a_w than single cultures of *P. verrucosum*. Although growth of *P. verrucosum* was decreased by *A. flavus* at 30°C/0.90a_w, ochratoxin A production in paired culture was twice that of *P. verrucosum* grown alone. At 0.90 a_w, aflatoxin B_1 production was either decreased or unaffected in the presence of *P. verrucosum* but was enhanced in the presence of *F. poae* although only at 30°C. Previously, Cuero *et al.* (1988) had observed enhancement of both aflatoxin B_1 and

zearalenone production in paired fungal cultures at 0.90 a_w but inhibition at 0.98 a_w, irrespective of temperature and competing species.

T-2 toxin production was significantly enhanced by competition with *P. verrucosum*, although growth of *F. poae* was decreased. Growth of *A. flavus* was not affected by *F. poae*, but aflatoxin B_1 production was decreased at 20°C/0.95 and enhanced at 20°C/0.97a_w while the reverse occurred with T-2 toxin production by *F. poae* in the presence of *A. flavus*. Fabri *et al.* (1984) have found that T-2 toxin could stimulate aflatoxin B_1 production by *A. flavus* in liquid culture. Although T-2 toxin was produced by *F. poae* at 20°C/0.97 and 0.95a_w, aflatoxin B_1 production was enhanced only at 0.97 a_w and decreased at 0.95 a_w in the presence of *F. poae* compared to production in pure cultures of *A. flavus*.

Thus, growth and mycotoxin production of individual species during fungal interactions was significantly influenced by environmental conditions, but our results do not suggest a single mechanism that may be operating. There could be several chemical substances that might mediate mycotoxin enhancement or degradation during fungal competition, but their effects may differ with differing environmental conditions and incubation periods, perhaps because their concentrations change.

References

Cuero, R., Smith, J.E., and Lacey, J. (1987) Stimulation by *Hyphopichia burtonii* and *Bacillus amyloliquefaciens* of aflatoxin production by *Aspergillus flavus* in irradiated maize and rice grains. *Appl. Envi. Microbiol.* 53, 1142-1146.

Cuero, R., Smith, J.E., and Lacey, J. (1988) Mycotoxin formation by *Aspergillus flavus* and *Fusarium graminearum* in irradiated maize grains in the presence of other fungi. *J. Food Prot. 51*, 542-456.

Dallyn, H and Fox, A. (1980) Spoilage of materials of reduced water activity by xerophyllic fungi. In *Microbial Growth and Survival in Extremes of Environment* (ed. G.H. Gould and J.E.L. Corry), Society of Applied Bacteriology Technical Series No 15. London, UK.

Ehrlich, K., Ciegler, M.K, and Lee, L. (1985) Fungal competition and mycotoxin production on corn. *Experientia* 41, 691-693.

Fabbri, A.A., Panfill, G., Fenelli, C. and Visconti, A. (1984) Effect of T-2 toxin on aflatoxin production. *Trans Br. Mycol. Soc.* 83, 150-152.

Hill, R. A. and Lacey, J. (1983) Factors determining the microflora of stored barley grain. *Ann. Appl. Biol.* 102, 467-483.

Ramakrishna, N., Lacey J., Candlish, A.A.G., Smith, J.E. and Goodbrand, I.A. (1990a) Monoclonal antibody-based enzyme linked immunosorbent assay of aflatoxin B_1, T-2 toxin and ochratoxin A in barley. *J. Assoc. Off. Anal. Chem* 73, 71-76.

Ramakrishna, N., Lacey J. and Smith, J.E. (1990b) Effect of surface sterilization, fumigation and gamma irradiation on the microflora and germination of barley seeds. *International J. Food Microbiol* (In Press).

Wicklow, D.T., Horn, B.W., Shotwell, O.L., Hesseltine, C.W., and Caldwell, R.W. (1988) Fungal interference with *Aspergillus flavus* infection and aflatoxin contamination of maize grown in a controlled environment. *Phytopathology* 78, 68-74.

INTERACTIONS ENTRE L'EAU, LA TEMPERATURE ET LES CHAMPIGNONS TOXINOGENES SUR LA PRODUCTION DES MYCOTOXINES DANS L'ORGE

N. RAMAKRINSHNA (1), J. LACEY (1) et J.E. SMITH (2)

(1) AFRC, Institute of Arable Crops Research
Rothamsted Experimental Station, Harpenden,
Herts AL5 2JQ, U.K.
(2) Department of Bioscience and Biotechnology,
University of Strathclyde, Glasgow G1 1XW, U.K.

RESUME

Aspergillus flavus, *Penicillium verrucosum*, *Fusarium poae* et la levure, *Hyphopichia burtonii*, ont été inoculés à de l'orge, individuellement, OUpar deux et incubés à 0,97, 0,95 ou 0,90 a_w et 30 ou 20 C. La contamination des grains a été mesurée par dénombrements de germes par dilution et par dépot direct sur milieu. La production d'aflatoxine B1 (AFB1), de toxine T-2 (T2) et d'ochratoxine A (OA) a été mesurée à la fois dans les cultures fongiques simples ou mixtes après 7, 14 et 21 jours d'incubation par dosage ELISA. La colonisation par *A. flavus* a été réduite par la compétition avec *P. verrucosum* à toutes les a_w et à 20 C, mais n'a pas été modifiée à 30 C, tandis que celle de *P. verrucosum* diminuait fortement dans sa compétition avec *A. flavus* dans la plupart des conditions d'incubation. La croissance de *F. poae* a été affectée par la compétition avec toutes les autres espèces. La production d'AFB1 par *A. flavus* a été nettement diminuée en compétition avec *P. verrusocum* dans la plupart des milieux d'incubation mais s'est trouvée nettement augmentée en compétition avec *F. poae* à 0,97 a_w et à 20 C. La production d'OA par *P. verrusocum* a nettement diminué en compétition avec *A. flavus* à 0,97 ou 0,95 a_w à la fois à 20 et à 30 C, mais s'est trouvée légèrement augmentée à 0,90 a_w aux deux températures. L'interaction entre *F. poae* et *P. verrucosum* a donné une diminution de la production d'OA mais a augmenté la production de T2. Lorsque *A. flavus* a été mis en compétition avec *F. poae* à 20 C, la production de T2 a diminué à 0,97 a_w mais a été légèrement augmentée à 0,95 a_w. Les taux de AFB1, T2 et OA ont été nettement diminués lorsque les champignons toxinogènes ont été mis en compétition avec *H. burtonii* dans la plupart des conditions d'incubation.