Journal of Food Protection, Vol. 59, No. 12, 1996, Pages 1311–1317

Colonization of Barley Grain by *Penicillium verrucosum* and Ochratoxin A Formation in the Presence of Competing Fungi

NANNAPANENI RAMAKRISHNA,[1†] JOHN LACEY,[1]* and JOHN E. SMITH[2]

[1]*IACR—Rothamsted, Harpenden, Herts. AL5 2JQ, U.K.; and* [2]*Department of Bioscience and Biotechnology, University of Strathclyde, Glasgow, G1 1XW, U.K.*

(MS# 96-20: Received 7 February 1996/Accepted 23 April 1996)

ABSTRACT

Colonization of barley grain by *Penicillium verrucosum* and the formation of ochratoxin A were studied, both in pure culture and when paired with *Aspergillus flavus, Fusarium sporotrichioides,* and *Hyphopichia burtonii,* at 20° and 30°C and at 0.97, 0.95 and 0.90 a_w over a 3-week period. Grain colonization was assessed on the basis of visible molding, seed infection, and numbers of CFU and by observing hyphal extension on the grain surface by scanning electron microscopy. Ochratoxin A concentrations were assayed by enzyme-linked immunosorbent assay using a monoclonal antibody. Germination of *P. verrucosum* spores was unaffected by the presence of other species. However, seed infection under most conditions was markedly decreased, relative to pure culture, by the presence of *A. flavus* and *H. burtonii,* but only slightly by *F. sporotrichioides.* The number of CFU of *P. verrucosum* was only slightly decreased in the presence of other species under most conditions. Generally, production of ochratoxin A by *P. verrucosum* was inhibited, sometimes significantly, in the presence of *A. flavus* and *H. burtonii,* but was changed only slightly by the presence of *F. sporotrichioides.* There was occasionally temporary enhancement in ochratoxin A production with all species during the 3-week incubation period.

Key words: *Penicillium verrucosum,* ochratoxin A, fungal competition, barley grain

Penicillium verrucosum is important in stored grain because of its ability to produce ochratoxin A, a nephrotoxin known also to be carcinogenic and mutagenic. Barley grain is commonly colonized by toxigenic *P. verrucosum* together with various species of *Aspergillus, Fusarium,* and yeasts *(1, 8). Fusarium* spp. are generally considered to be field fungi with a high water requirement for growth, although some can sometimes grow in stored grain, while *P. verrucosum* and *Aspergillus* spp. are typical storage species that are able to thrive at relatively low water activities (a_w). Different fungi colonizing stored grain grow at different rates that are determined by their individual responses to the temperature and water activity of the substrate. Little is known of how the presence of other species affects the colonization of cereal grains by *P. verrucosum* and subsequent ochratoxin A production, nor how these interactions are affected over a period of time by environmental conditions, especially temperature and water activity. Cuero et al. *(5)* reported that ochratoxin A production by *P. verrucosum* in maize was enhanced by the presence of *Fusarium graminearum* or *Aspergillus niger,* while Mislivec et al. *(10)* found that *A. ochraceus,* another producer of ochratoxin A, was inhibited by *A. flavus* in broth cultures.

This paper describes colonization of barley grain by *P. verrucosum* and subsequent ochratoxin A production in the presence of *A. flavus, F. sporotrichioides,* and *H. burtonii* at different temperatures and water activities over a period of time.

MATERIALS AND METHODS

Source of barley grain and elimination of initial grain microflora

Barley grain harvested on Rothamsted Farm, with an initial water content of 13 to 14%, was exposed to 12 kGy of gamma irradiation at 25°C from a ^{60}Co source. This dose was sufficient to eliminate all fungi, bacteria, and yeasts on or within the grain without affecting seed germination *(14).* The gamma-irradiated barley grain was either used immediately or stored for up to 2 months at 4°C.

Adjustment of the water activity of gamma-irradiated grain

Water activity was adjusted to 0.97, 0.95, or 0.90 a_w (27.4, 24.4, and 20.2% water content, respectively) by aseptically adding calculated amounts of sterile distilled water to the gamma-irradiated grain in sterile 1-liter flasks. The wetted grain was then equilibrated for 5 days at 4°C with shaking by hand several times a day. The quantities of water necessary to produce different water activities were calculated using the equation $v = [w(a - b)/(100 - a)] - I$, where v is volume of water to be added (ml), w is weight of grain to be treated (g), a is the water content required in the grain (%), b is the initial water content of the grain (%) and **I** is the volume of water to be added subsequently with the inoculum.

* Author for correspondence. Tel: +44 1582 763133; Fax: +44 1582 760981; E-mail: john.lacey@bbsrc.ac.uk

† Present address: Department of Food Science, University of Arkansas, Fayetteville, AR 72701, USA

Sources of isolates

Penicillium verrucosum Dierkx (C2706), *Fusarium sporotrichioides* (Peck) Wollenw. (C2661) and *Hyphopichia burtonii* (Boidin et al.) von Arx and van der Walt (anamorph *Candida chodatii* (Nechitch) Berkhout) (C2699) were obtained from Rothamsted Experimental Station culture collection. *Aspergillus flavus* Link ex Fr. (IMI 102556) was obtained from the University of Strathclyde.

Inoculum preparation

All species were maintained in culture on 10 g of autoclaved barley grain containing 30% water in 28-ml Universal bottles at 25°C. Spore and/or cell suspensions of fungi and yeast were prepared by shaking 4 to 5 colonized grains from 14-day old cultures in 10 ml of sterile distilled water containing 0.05% Tween-20. Spore and/or cell suspensions containing 20% glycerol could be stored at 20°C for 5 days without loss of viability. Concentrations of spore and/or cell suspensions were adjusted to 10^6 spores and/or cells ml^{-1} using hemocytometer.

Determination of Penicillium verrucosum *hyphal extension on grain surface*

Individual gamma-irradiated barley grains with 0.97, 0.95 or 0.90 a_w were aseptically point inoculated with 2 µl of spore suspension containing 250 spores and/or cells of either one or two species, and placed in desiccators with their internal atmosphere maintained at humidities corresponding to the a_w of the grain at either 20 or 30°C, as described by Ramakrishna et al. *(15)*. Spores of the three species of filamentous fungi studied and cells of *H. burtonii* were recognized by their distinctive morphological features. Germination was determined by the production of germ tubes and the lengths of the longest hyphae growing from 20 germinating spores were measured for as long as the source spore could be identified by using scanning electron microscopy *(15)*.

Grain inoculation with one or two species in microporous bags

Gamma-irradiated barley grain, equilibrated to near 0.97, 0.95 or 0.90 a_w, was transferred aseptically to microporous bags (20 by 14 cm, Valmic®, 0.3 µm pore size, from Van Leer U.K. Ltd) *(3)* to a weight of either 10 or 40 g per bag. The grain was then inoculated with spore suspensions of *P. verrucosum,* either alone or together with spores of *A. flavus, F. sporotrichioides,* or *H. burtonii.* Grain was inoculated with 0.1 ml of suspension of each of two species (each containing 10^6 spores and/or cells ml^{-1}) per 10 g of grain for paired cultures, or with 0.1 ml each of sterile distilled water and *P. verrucosum* spore suspension per 10 g of grain for single culture. The uninoculated control was treated with 0.2 ml sterile distilled water per 10 g of grain instead of inoculum. The microporous bags were then sealed and the inoculum was uniformly mixed throughout the grain by vigorously shaking by hand the microporous bag for 1 min. Finally the grain was spread in a thin layer for incubation. The entire single experiment utilized 144 microporous bags containing either 10 or 40 g of grain, resulting in four different inoculation combinations ($n = 4$) by three water activities ($n = 3$) by two temperatures ($n = 2$) by three incubation periods ($n = 3$) in two replicates ($n = 2$). The grain in the microporous bags was incubated in a computer-controlled humidifier, multigas incubator (NAPCO Model 7300) at 20 or 30°C and at 97, 95, or 90% relative humidity for up to 21 days.

Assessment of Penicillium verrucosum *colonization*

Colonization of grain by *P. verrucosum* was assessed as visible growth, seed infection, and CFU after 7, 14, and 21 days incubation, using the total contents of bags containing 10 g of grain.

Visible growth. Visible mycelium of *P. verrucosum* on grain was assessed using an arbitrary 1 to 5 scale: 1, negligible *P. verrucosum* growth; 2, *P. verrucosum* growth just visible; 3, patches of *P. verrucosum* growth readily visible on most grains; 4, thin layer of *P. verrucosum* growth all over the grain surface of most grains; and 5, heavily sporulating *P. verrucosum* colonies on all grains.

Seed infection. The entire 10 g of grain from one microporous bag was transferred to a sterile Petri dish, wetted with ethanol for 1 min, and surface sterilized with 10% sodium hypochlorite solution for 5 min to remove surface contamination. One hundred grains were plated onto malt extract agar in 9-cm diameter Petri dishes, with 10 grains per plate, and incubated at 25°C for 6 days before the percentage of grains infected by each species was recorded.

CFU. The entire 10 g of grain from a second microporous bag was soaked for 20 min in 90 ml of 0.1% dilute Difco agar within the microporous bag itself, prior to stomaching for 10 min in a Colworth Stomacher (A.J. Seward Ltd., London). The microporous bag containing grain was carefully placed inside two other stomacher bags before maceration. A logarithmic dilution series down to $1:10^9$ was prepared using 0.2% agar. Two replicates of 0.1-ml aliquots each from four different dilutions of the stomached sample were aseptically transferred to malt extract agar plates and spread uniformly with a spreader. Plates were incubated for 6 days at 25°C before colonies of individual species were counted for calculating the number of CFU g^{-1} of grain.

Determination of ochratoxin A formation

The production of ochratoxin A in barley by *P. verrucosum* in single and paired cultures was determined after 7, 14, and 21 days of incubation by monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) *(13)*. Two microporous bags, each containing 40 g of barley grain, were separately extracted with 200 ml of acetonitrile = 0.5% KCl = 6% H_2SO_4 (89:10:1) for 3 min in a Waring blender. The grain extract was filtered through Whatman no. 41 filter paper and diluted 1:100 with Tris HCl buffer for the ELISA. Ochratoxin A was determined by indirect competitive ELISA using protocols described in Ramakrishna et al. *(13)*. Each standard solution or grain extract was replicated in two wells in a microtiter plate, using 100 µl per well. Standard curves for ochratoxin A were prepared by plotting absorbance at 450 nm against log concentration of pure toxin. Concentrations of ochratoxin A in grain extracts were interpolated from the standard curve in order to calculate their concentrations in the original samples. Thus ochratoxin A concentration (ng g^{-1}) is calculated as ochratoxin A concentration in the sample extract (ng ml^{-1}) times the dilution factor times the sample extract volume (ml) per sample weight (g).

Statistical analysis of data

Analyses of variance were performed on the ochratoxin A production data, using a log transformation to stabilize the variance. Since the seed infection and CFU data were unreplicated, no statistical analysis was possible. Correlations were formed between the variables seed infection, CFU, and toxin production, under the four inoculation treatments (*P. verrucosum* alone and *P. verrucosum* plus each of the other three fungi) in order to study the relationship between the variables at the different water activities and temperature regimens used.

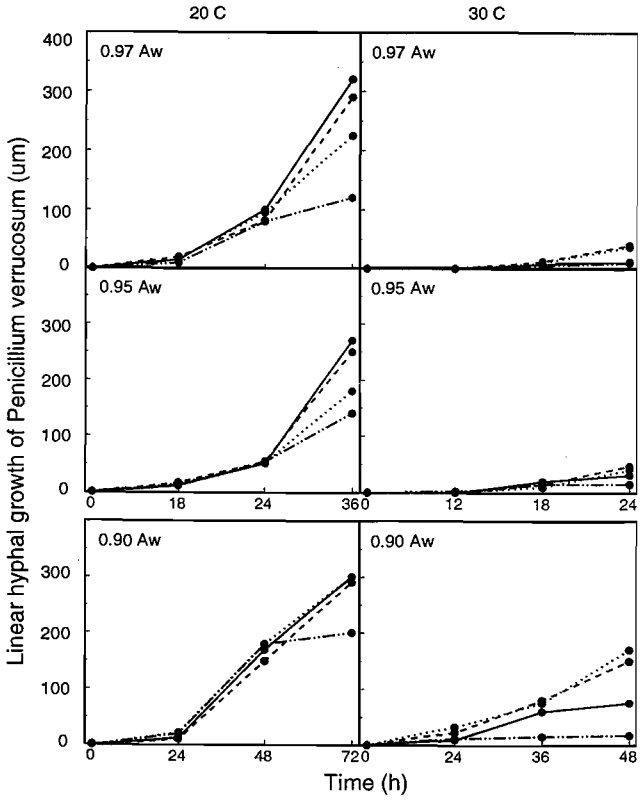

FIGURE 1. *Linear growth of* Penicillium verrucosum *on the surface of barley grain when alone (----) or in the presence of* Aspergillus flavus *(—),* Fusarium sporotrichioides *(. . . .) or* Hyphopichia burtonii *(.. ..) at different a_w and temperatures.*

RESULTS

Effects of fungal interactions on Penicillium verrucosum *colonization*

Spore germination and hyphal growth. On the surface of the barley grain germ tubes were produced from 40 to 60% of the spores of *P. verrucosum, A. flavus,* and *F. poae,* and from 85 to 95% of cells of *H. burtonii,* irrespective of temperature and a_w and whether the species were growing alone or were paired with another species. *P. verrucosum* grew faster than *A. flavus* at all a_w at 20°C, especially after 18 or 24 h of incubation, and was unaffected by the presence of *A. flavus* during this period (Fig. 1). At 30°C, the growth of *P. verrucosum* was prevented by *A. flavus* and *H. burtonii* at all a_w after 24 h. *P. verrucosum* grew much more slowly than *F. sporotrichioides* at all a_w at 30°C but was unaffected by its presence during the 24-h incubation period due to the small amount of hyphal branching by *F. sporotrichioides.* At 20°C at all a_w of all the fungi interacting in paired culture, *F. sporotrichioides* affected the growth of *P. verrucosum* least over the 36-h incubation period. In spite of identical growth rates, the growth of *P. verrucosum* was restricted by *H. burtonii* at all a_w at 20°C after 24 h of incubation while *P. verrucosum* grew much more slowly than *H. burtonii* at 30°C and was inhibited by it after 24 h, irrespective of a_w.

Visible growth. Visible growth of *P. verrucosum* increased as a_w increased and temperature decreased, but differences between treatments were less marked after 14 days than earlier. Visible growth of *P. verrucosum* was decreased under all conditions by competition with *A. flavus*

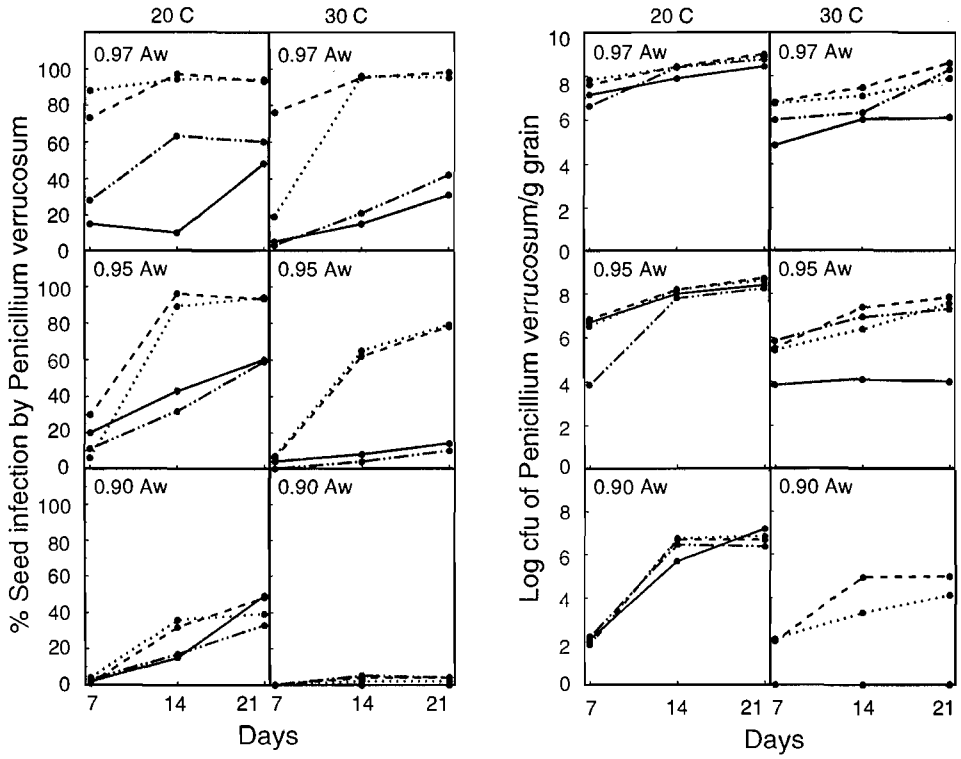

FIGURE 2. *Colonization of barley grain by* Penicillium verrucosum *when grown alone (----) or in the presence of* Aspergillus flavus *(—),* Fusarium sporotrichioides *(. . . .) or* Hyphopichia burtonii *(.. ..) at different a_w and temperatures.*

or *H. burtonii* and it failed to grow at 30°C in the presence of *A. flavus*, regardless of a_w. Visible growth of *P. verrucosum* appeared unaffected by the presence of *F. sporotrichioides* at 20°C and 0.97 or 0.95 a_w, but it was decreased under other conditions.

Seed infection. Seed infection by *P. verrucosum* increased with increasing a_w and decreasing temperature (Fig. 2). Almost 100% of the grains were infected at both 20 and 30°C and 0.97 a_w and at 20°C and 0.95 a_w. Seed infection decreased to about 80% at 30°C and 0.95 a_w and to 50% at 20°C and 0.90 a_w. However, fewer than 5% of the grains were infected at 30°C and 0.90 a_w. Seed infection by *P. verrucosum* was decreased by the presence of *A. flavus* or *H. burtonii* under all conditions, except at 20°C and 0.90 a_w, where seed infection by *P. verrucosum* in the presence of *A. flavus* was initially slower but then caught up with that in pure culture. *P. verrucosum* was little affected by *F. sporotrichioides* under any of the conditions tested, except that infection by *P. verrucosum* was initially slower at 30°C and 0.97 a_w and at 20°C and 0.95 a_w than when growing alone.

CFU. P. verrucosum populations increased with increasing a_w and decreasing temperature (Fig. 2). There was a rapid increase in CFU during the first week of incubation but the increase was then slower to 21 days. Generally, CFU of *P. verrucosum* were slightly decreased by fungal competition under most conditions. Marked decreases in *P. verrucosum* CFU were only noted in competition with *A. flavus* at 30°C at all a_w.

Effects of fungal interactions on ochratoxin A production by P. verrucosum

Figure 3 shows ochratoxin A production by *P. verrucosum* both when grown alone and in the presence of *A. flavus, F. sporotrichioides,* or *H. burtonii* at 20 and 30°C and at 0.97, 0.95, or 0.90 a_w.

No competition. Production of ochratoxin A was significantly greater at 20°C than at 30°C and increased with increasing a_w. The concentration of toxin in the grain at 20°C was more than 600 times greater at 0.97 a_w (125 μg g^{-1}) than at 0.90 a_w (0.2 μg g^{-1}). Ochratoxin A concentrations increased significantly with time at both incubation temperatures and 0.97 or 0.95 a_w but not at 0.90 a_w where there was little change in concentration after 7 days of incubation at either temperature.

In the presence of A. flavus. At 30°C and 0.97 a_w, production of ochratoxin A by *P. verrucosum* was almost completely inhibited by competition with *A. flavus* (Fig. 3), resulting in only 3 to 6 ng of ochratoxin A per g, compared with 4,300 ng of ochratoxin A per g when *P. verrucosum* was growing alone. At 30°C and 0.95 a_w, little ochratoxin A was produced during the first 7 days of competition with *A. flavus* and none was detected after 21 days of incubation. Competition decreased ochratoxin A production significantly at 20°C and 0.97 a_w and there was some decrease also at 20°C and 0.95 a_w but the difference was not significant. At 0.90 a_w, toxin concentrations were slightly greater in paired than in single culture at both 20 and 30°C after all periods of incubation, although toxin concentration possibly declined slightly after 2 weeks of incubation in the presence of *A. flavus.*

In the presence of F. sporotrichioides. Changes in ochratoxin A production in the presence of *F. sporotrichioides* differed with temperature and a_w (Fig. 3). Concentrations were decreased significantly in competition with *F. sporotrichioides* at 30°C and 0.97 a_w but the rate of toxin accumulation at 20°C and 0.97 a_w was only slightly less than in pure culture. Toxin production was unchanged by competition at 30°C and 0.95 a_w after 7 days but then declined, while at 20°C and 0.95 a_w toxin production was slightly greater than in single culture after 14 days, but the difference was not significant. Ochratoxin A production at 20°C and 0.90 a_w was less than in single culture in the presence of *F. sporotrichioides,* while it was slightly increased after 14 days at 30°C and 0.90 a_w, but then declined and did not differ significantly from that of single culture.

In the presence of H. burtonii. Ochratoxin A production was generally decreased by competition with *H. burtonii*

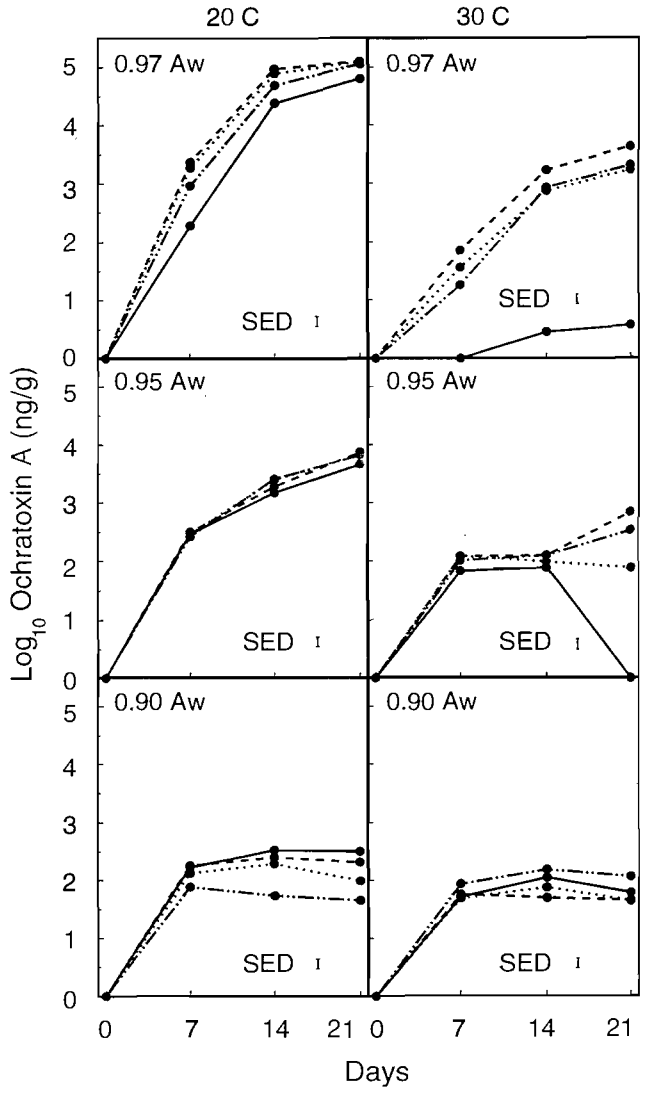

FIGURE 3. *Ochratoxin A production in barley by* Penicillium verrucosum *when grown alone (----) or in the presence of* Aspergillus flavus *(—),* Fusarium sporotrichioides *(. . . .) or* Hyphopichia burtonii *(.. ..) at different a_w and temperatures.*

TABLE 1. *Correlation coefficients for ochratoxin A production in single and paired cultures at different temperatures, over the range of 0.90 to 0.97 a_w, against* Penicillium verrucosum *seed infection or CFU*

Parameters[c]	Temp. (°C)	Correlation coefficient[a] Single or paired cultures[b] Pv alone	Pv + Af	Pv + Fs	Pv + Hb
SI vs OA	20	0.697	−0.196	0.437	0.725
	30	0.938	0.931	0.950	0.819
CFU vs OA	20	0.601	−0.473	0.465	0.557
	30	0.814	0.711	0.851	0.855

[a] Critical value for 95% significance test with $n = 9$ is 0.582. A larger value indicates significant correlation between variables.
[b] Pv, *Penicillium verrucosum;* Af, *Aspergillus flavus;* Fs, *Fusarium sporotrichioides;* Hb, *Hyphopichia burtonii.*
[c] SI, seed infection (%); OA, ochratoxin A production (log ng g^{-1}); CFU, log CFU g^{-1}.

(Fig. 3) except at 30°C and 0.90 a_w, where it was significantly greater than in pure culture. Slight stimulation was also noted at 20°C and 0.95 a_w, but only after 14 days of incubation.

Correlation analyses

When changes in ochratoxin A production by *P. verrucosum* are compared with seed infection or CFU in mixed fungal cultures (Tables 1 to 3), decreased colonization generally led to decreased toxin production, possibly due to loss of substrate. Correlation analyses indicate significant correlations between indices of colonization and ochratoxin A formation, especially when *P. verrucosum* was growing alone and in paired cultures at 30°C (Table 1). Except for interactions with *H. burtonii,* seed infection by *P. verrucosum* was more highly correlated with toxin production than CFU but the difference was small. At 20°C, correlation

TABLE 2. *Correlation coefficients for ochratoxin production in single and paired cultures at different water activities over the range of 20 to 30°C against* Penicillium verrucosum *seed infection or CFU*

Parameters[c]	a_w	Correlation coefficient[a] Single or paired cultures[b] Pv alone	Pv + Af	Pv + Fs	Af + Hb
SI vs OA	0.97	0.742	−0.003	0.160	0.044
	0.95	0.271	−0.844	0.125	−0.299
	0.90	−0.718	−0.560	−0.593	0.636
CFU vs OA	0.97	0.197	−0.650	−0.254	0.208
	0.95	0.236	−0.852	−0.252	0.361
	0.90	−0.299	−0.696	−0.389	0.902

[a] Critical value for 95% significance test with $n = 9$ is 0.81. A larger value indicates significant correlation between variables.
[b] Pv, *Penicillium verrucosum;* Af, *Aspergillus flavus;* Fs, *Fusarium sporotrichioides;* Hb, *Hyphopichia burtonii.*
[c] SI, seed infection (%); OA, ochratoxin A production (log ng g^{-1}); CFU, log CFU g^{-1}.

TABLE 3. *Correlation coefficients for* Penicillium verrucosum *seed infection against CFU at different temperatures and water activities*

Temp (°C)	Correlation coefficient[a] Single or paired cultures[b] Pv alone	Pv + Af	Pv + Fs	Pv + Hb
20	0.879	0.674	0.818	0.889
30	0.869	0.776	0.830	0.521
a_w				
0.97	0.679	0.554	0.638	0.942
0.95	0.969	0.927	0.856	0.592
0.90	0.797	0.889	0.925	0.867

[a] Critical value for 95% significance test for different temperatures, with $n = 9$, is 0.582 and for different water activities, with $n = 6$, is 0.81. A larger value indicates significant correlation between variables. Seed infection, %; CFU, log CFU g^{-1}.
[b] Pv, *Penicillium verrucosum;* Af, *Aspergillus flavus;* Fs, *Fusarium sporotrichioides;* Hb, *Hyphopichia burtonii.*

coefficients for both *P. verrucosum* seed infection and CFU with ochratoxin A production in interactions with *A. flavus* and *F. sporotrichioides* were all small and not significant. The only significant correlations between seed infection or CFU and ochratoxin formation at different a_w were inverse correlations in paired cultures with *A. flavus* (Table 2). Except for *P. verrucosum* growing alone at 0.90 a_w and in paired culture with *H. burtonii* at 0.95 a_w, seed infection and CFU all correlated significantly at 0.95 and 0.90 a_w (Table 3). By contrast, the only significant correlation between seed infection and CFU at 0.97 a_w was for paired culture with *H. burtonii.*

DISCUSSION

P. verrucosum showed four patterns of colonization of barley grain, based on seed infection and CFU assessments in different environmental conditions over a period of time. These could be explained in terms of linear growth rates of *P. verrucosum* hyphae observed on the grain surface by SEM. The patterns generally depended upon the competing species involved: (i) *P. verrucosum* percent seed infection and number of CFU were unaffected by competition; (ii) *P. verrucosum* percent seed infection and number of CFU increased initially more slowly during competition than in pure culture; (iii) *P. verrucosum* seed infection and CFU were markedly decreased by competition; and (iv) *P. verrucosum* seed infection was markedly decreased but its sporulation was unaffected during competition.

Under some conditions, the amount of visible growth correlated well with seed infection or CFU, especially during the early stages of colonization. However, when fungal growth was extensive, differences in visible molding could not be distinguished. Although neither seed infection nor CFU measure fungal biomass, they were the only methods that could be used to assess separately the growth of different species in mixed cultures. Chemical methods based on ergosterol *(16)* or chitin *(7)* allow quantitative

assay only of total fungal growth, while an ATP assay does not distinguish between grain and microbial ATP *(9)*. Different fungi could perhaps be distinguished by immunological methods *(2, 6, 11, 12)* or by molecular methods, but species-specific antibody probes still have to be developed for such ecological studies.

As with colonization, different patterns of ochratoxin A production could be distinguished in paired cultures relative to single culture. The five different patterns recognized were (i) ochratoxin A production in paired culture changed little from that in pure culture; (ii) ochratoxin A production increased more slowly in paired than in single culture but did not differ significantly after three weeks of incubation; (iii) ochratoxin A production was markedly less in paired culture than in single culture during all incubation periods; (iv) ochratoxin A production, although it increased initially, decreased subsequently to less than in pure culture; and (v) ochratoxin A production increased initially but differed little from pure culture after three weeks of incubation.

Although the amount of ochratoxin A produced sometimes correlated with measures of growth, this was not always so. Generally, with longer incubation periods, ochratoxin A formation in barley grain was decreased, irrespective of the a_w, temperature, and species involved in competition. For example, ochratoxin A production by *P. verrucosum* was decreased at 20°C and 0.97 or 0.95 a_w by competition with *A. flavus* but was almost completely inhibited at 30°C and 0.97 a_w. The small amount of toxin formed initially at 30°C and 0.95 a_w had disappeared after three weeks of incubation, while toxin production at 0.90 a_w was initially greater than in pure culture at both temperatures but decreased with longer incubation periods. Enhanced production of ochratoxin A was sometimes only temporary. Production was decreased by *H. burtonii* under all conditions, except for a temporary increase at 20°C and 0.95 a_w and 30°C and 0.90 a_w. By contrast, both aflatoxin and ochratoxin A production in maize were reported to be enhanced by *H. burtonii* at all a_w and temperatures tested *(4, 5)*. However, observations in these experiments were made only after 14 days of incubation so that changes in production before or after this time would have been missed, although it is also possible that interactions might have different effects on different substrates. Unless the experiment materials are sampled at regular intervals, accurate conclusions cannot be drawn on the changing levels of ochratoxin A in stored grain ecosystems. Ideally, experiments should also have been conducted at temperatures intermediate between 20 and 30°C, but this would have required a large amount of further work that was not possible within the scope of the present study.

Measurement of colonization using seed infection or CFU gave little indication of ochratoxin production in barley grain in paired cultures although these measures might be helpful if *P. verrucosum* was growing alone. Correlations between seed infection or CFU and ochratoxin A concentration were most often significant when *P. verrucosum* was growing in pure culture and the effects of a_w and incubation period were compared at one temperature. There was no consistent pattern in the occurrence of significant correlations in paired cultures. The relationship between seed infection or CFU and ochratoxin production at different temperatures and with different incubation periods at constant a_w rarely gave a significant relationship and then it was inverse. Seed infection and CFU were significantly correlated in most culture combinations at 20 and 30°C and at 0.95 and 0.90 a_w but rarely at 0.97 a_w.

It is evident that the mycotoxin concentration in a sample of grain is continuously fluctuating and is determined by a combination of effects, whether fungi are alone or in the presence of other species. There may simultaneously be stimulation, inhibition, and degradation of ochratoxin A in different parts of a grain bulk and at different times in any one part. The final concentration of toxin is thus an integration of all these changes, although colonization by *Penicillium verrucosum*, especially at higher temperatures, may give a good indication of the likely level of contamination with ochratoxin A.

ACKNOWLEDGMENTS

We thank Ms. S. J. Welham for statistical analyses. N. R. thanks the Association of Commonwealth Universities for the award of a Commonwealth Scholarship. IARC-Rothamsted is grant-aided by the Biotechnology and Biological Science Research Council.

REFERENCES

1. Clarke, J. H., and S. T. Hill. 1981. Mycofloras of moist barley during sealed storage in farm and laboratory silos. Trans. Br. Mycol. Soc. 77:557–565.
2. Clarke, J. H., A. D. MacNicoll, and J. A. Norman. 1986. Immunological detection of fungi in plants, including stored cereals. Int. Biodeterior. Suppl. 22:123–130.
3. Cuero, R. G., J. E. Smith, and J. Lacey. 1985. A novel containment system for laboratory-scale solid particulate fermentations. Biotechnol. Lett. 7:463–466.
4. Cuero, R. G., J. E. Smith, and J. Lacey. 1987. Stimulation by *Hyphopichia burtonii* and *Bacillus amyloliquefaciens* of aflatoxin production by *Aspergillus flavus* in irradiated maize and rice grains. Appl. Environ. Microbiol. 53:1142–1146.
5. Cuero, R. G., J. E. Smith, and J. Lacey. 1988. Mycotoxin formation by *Aspergillus flavus* and *Fusarium graminearum* in irradiated maize grains in the presence of other fungi. J. Food Prot. 51:452–456.
6. Dewey, F. M., M. M. MacDonald, and S. I. Phillips. 1989. Development of monoclonal-antibody-ELISA, -DOT-BLOT and -DIP-STICK immunoassays for *Humicola lanuginosa* in rice. J. Gen. Microbiol. 135:361–374.
7. Donald, W. W., and C. J. Mirocha. 1977. Chitin as a measure of fungal growth in stored corn and soybean seed. Cereal Chem. 54:466–474.
8. Hill, R. A., and J. Lacey. 1983. Factors determining the microflora of stored barley grain. Ann. Appl. Biol. 102:467–483.
9. Jarvis, B., and M. C. Easter. 1987. Rapid methods in the assessment of microbial quality; experiences and needs. J. Appl. Bacteriol. Symp. Suppl. 63:115S–126S.
10. Mislivec, P. B., M. W. Trucksess, and L. Stoloff. 1988. Effect of other toxigenic mould species on aflatoxin production by *Aspergillus flavus* in sterile broth shake culture. J. Food Prot. 51:449–451.
11. Notermans, S., C. J. Heuvelman, R. R. Beumer, and R. Maas. 1986. Immunological detection of moulds in food relation between antigen production and growth. Int. J. Food Microbiol. 3:253–261.
12. Notermans, S., C. J. Heuvelman, H. P. Van Egmond, W. E. Paulsch, and J. R. Besling. 1986. Detection of mould in food by enzyme-linked immunosorbent assay. J. Food Prot. 49:786–791.
13. Ramakrishna, N., J. Lacey, A. A. G. Candlish, J. E. Smith, and I. A.

Goodbrand. 1990. 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.

14. Ramakrishna, N., J. Lacey, and J. E. Smith. 1991. Effect of surface sterilization, fumigation and gamma irradiation on the microflora and germination of barley seeds. Int. J. Food Microbiol. 13:47–54.
15. Ramakrishna, N., J. Lacey, and J. E. Smith. 1993. Effects of water activity and temperature on the growth of fungi interacting on barley grain. Mycol. Res. 97:1393–1402.
16. Seitz, L. M., D. B. Sauer, R. Burroughs, H. E. Mohr, and J. D. Hubbard. 1979. Ergosterol as a measure of fungal growth. Phytopathology 69:1202–1203.