Effects of Different Cereal and Oilseed Substrates on the Growth and Production of Toxins by *Aspergillus alutaceus* and *Penicillium verrucosum*

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Cereal substrates (corn and wheat) and oilseeds (peanut, rapeseed, and soybean) were evaluated for their ability to support the production of ochratoxin A (OA), ochratoxin B (OB), and citrinin by *Aspergillus alutaceus* (formerly *ochraceus*) and *Penicillium verrucosum* over different periods of time (7, 15, and 30 days). Peanut and soybean supported the production of fungal biomass (26 and 6 mg/g glucosamine, respectively), OA (345 and 243 μg/g, respectively), and OB (130 and 390 μg/g, respectively) by *A. alutaceus* better than other substrates after 30 days of incubation, while corn and wheat did not support OB production during this time period. *P. verrucosum* produced higher amounts of fungal biomass and OA on wheat (10 mg/g glucosamine and 97 μg/g OA) than other substrates after 30 days. Corn and wheat supported citrinin production by *P. verrucosum* (124 and 102 μg/g, respectively, after 30 days) while oilseeds did not. Thus, substrates in addition to type of fungi have a dramatic influence on the nature and amount of toxic metabolites produced. Furthermore, this appears to be the first report of OB production in oilseeds.

INTRODUCTION

Ochratoxigenic *Aspergillus* and *Penicillium* species are widespread in nature. *Aspergillus alutaceus* (formerly *A. ochraceus* Wilhelm, NRRL 3174) Wilhelm and *Penicillium viridicatum* Westling have been frequently isolated from cereals, oilseeds, vegetables, and feeds (Chu, 1974; Marquardt et al., 1990). Ochratoxin A (OA), a metabolite produced by the above fungi, has been encountered in various agricultural commodities (Krogh, 1987). OA together with citrinin has also been produced by *P. viridicatum* growing on grains (Damoglou, 1984). Studies have demonstrated the nephrotoxic effects of OA and citrinin in different species including farm livestock (Krogh, 1977). Furthermore, both OA and citrinin have been implicated in Balkan endemic nephropathy (Krogh, 1977). Ochratoxin B (OB), a much less toxic metabolite produced by *A. alutaceus*, has been encountered in barley (Krogh 1973), corn (Shotwell et al., 1971), bread (Visconti and Bottalico, 1983), and country-cured ham (Escher et al., 1973). Chu (1974) reviewed ochratoxin production by *A. alutaceus* and *P. viridicatum* on certain substrates sterilized prior to inoculation. Production of OA and citrinin by both *A. alutaceus* and *P. viridicatum* under laboratory conditions in cereals such as barley and wheat, but not oilseeds, has been reported (Harwig and Chen, 1974; Damoglou, 1984). Although toxin production under laboratory conditions is not directly comparable to that occurring under natural conditions, these observations are relevant to the problem of ochratoxin and citrinin production in nature. No study, however, has directly compared the production of these three toxins over time on cereal and oilseed crops. Also, the time course relationship between the production of these toxins and degree of fungal growth as estimated by the concentration of glucosamine has not been determined. Rotter et al. (1989) has recently demonstrated that the glucosamine content of cereal grains provides a good estimate of degree of fungal contamination. The current studies were carried out to better establish the nature of these interrelationships. The studies should also confirm the observation by Pitt (1987) that *P. verrucosum* Dierckx produces both OA and citrinin.

MATERIALS AND METHODS

Preparation of Inoculum. *P. verrucosum* Dierckx was isolated from a barley sample collected from a storage bin in Manitoba, and its identity was confirmed by the Biosystematics Research Institute, Ottawa, Canada; *A. alutaceus* (*A. ochraceus* Whilhelm, NRRL 3174) was obtained courtesy of Dr. C. W. Hesseltine, U.S. Department of Agriculture, Northern Regional Laboratory, Peoria, IL. The fungi were grown on potato dextrose agar slants with 3% NaCl at 28 °C for 2-3 weeks. The conidia were harvested following the addition of sterilized 0.05% Tween 80 in water and aseptic dislodgement with a sterile inoculating loop. The suspension had a concentration of approximately 10^6 conidia/mL.

Preparation of Samples. The selected substrates for the study were wheat (*Triticum aestivum* L.), corn (*Zea mays* L.), peanut (*Arachis hypogea* L.), soybean (*Glycine max* L.), and rapeseed (*Brassica napus* L.). Samples (60 g each) of the above cereals and oilseeds were dispersed into several 500-mL Erlenmeyer flasks. The moisture content of each sample was adjusted to 24-25% by the addition of the appropriate volume of distilled water. The moisture content was confirmed by the use of a moisture determination balance (Ohaus Corp., Florham Park, NJ). After being autoclaved at 121 °C for 15 min, sam-

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0021-8561/90/1438-1506$02.50/0

ples were inoculated with 2 mL each of conidial suspension under aseptic conditions. Uninoculated samples served as the controls. Treated samples in triplicate and the controls in duplicate were incubated in the dark at 28 °C for 7, 15, and 30 days, respectively. The experiment was terminated at the end of each incubation period by drying the samples at room temperature (25 °C) under a fume hood for 24 h. The dried samples were ground by using a Cyclotec 1093 sample mill grinder (Tecator, Hoganas, Sweden) with 1-mm screen prior to subsampling for analysis. Samples were stored in polyethylene bags at −20 °C until analyzed.

Estimation of Fungal Biomass, Ochratoxin A, Ochratoxin B, and Citrinin. Fungal biomass (glucosamine content) was measured according to the procedure described by Rotter et al. (1989). Ground samples (10 g each) for mycotoxin analysis were extracted with $CHCl_3$–0.1 M H_3PO_4 (20:1 v/v) and subjected to reverse-phase thin-layer chromatography (RPTLC) for cleanup. OA and OB were eluted from RPTLC coating material with methanol, and citrinin was eluted with methanol–acetonitrile–2-propanol (40:40:20 v/v/v) according to the procedure of Frohlich et al. (1988). The concentrations of OA and OB were determined by high-performance liquid chromatography (HPLC) using a HP 1046A programmable fluorescence detector (Hewlett-Packard Ltd.) according to the procedure of Josefson and Moller (1979). Citrinin concentration after HPLC was estimated at 326 nm by using a Waters tunable UV detector (Waters-Millipore Ltd.). The citrinin eluting buffer contained a 70:25:5 v/v/v mixture of acetonitrile–2-propanol (80:20 v/v), 1% formic acid, and 1 mM EDTA. Aliquots (20 μL) of the samples were injected into a 250 × 4.6 mm column containing 5-μm diameter C-18 bonded-phase adsorbant (Beckmann Inc., Altex Division); OA and OB were eluted at 50 °C at a flow rate of 1.5 mL/min, and citrinin was eluted at 22 °C at a flow rate of 1 mL/min.

Reference Standards. OA and citrinin reference standards were obtained from Sigma Chemical Co., St. Louis, MO. The OB reference standard was obtained from soybean infected with *A. alutaceus*. The contaminated beans (50 g) were extracted with 250 mL of chloroform plus 12.5 mL of 0.1 M phosphoric acid. The extract was evaporated to dryness, reconstituted with 10 mL of benzene in five parts of 2 mL each, and passed through a silica gel column (250 × 9 mm) that had been packed as slurry in chloroform and further equilibriated with benzene–acetic acid (9:1). OB was eluted from the column with benzene–acetic acid (9:1), and fractions were collected separately and checked for OB. Fractions containing OB were pooled and evaporated to dryness. The sample was reconstituted with 4 mL of methanol and further purified by passing through a C-18 reverse-phase column (230 × 9 mm, 30–50-μm particles) with 70% methanol in water. OB-containing fractions were pooled, evaporated to dryness, and reconstituted with an appropriate volume of methanol for the measurement of concentration using a molar absorption coefficient at 318 nm, $E = 6900$ (Cole and Cox, 1981). Spectrophotometric measurements were carried out on a Beckman DU-8 spectrophotometer. Confirmatory mass spectra were determined by using a Hewlett-Packard 5985B liquid chromatography mass spectrometry system equipped with negative-ion capability.

Statistical Analysis. As the analysis of variance (ANOVA) of data showed two-way and three-way interactions between time, substrate, and concentration of glucosamine, OA, OB, or citrinin, the data were further subjected to stepwise regression analysis to establish the relationship between fungal growth and toxin production over different incubation periods for the different substrates in the absence or the presence of the two species of fungi (Statistical Analysis System, 1986).

RESULTS

The original and the uninoculated control samples, in all cases, did not contain detectable levels of OA, OB, and citrinin and had very low concentrations of glucosamine (less than 0.2 mg of glucosamine/g of grain or oilseed). A preliminary study demonstrated that very little OA, OB, or citrinin was produced by the two fungal species on the different seeds prior to 5 days of incubation, whereas, as

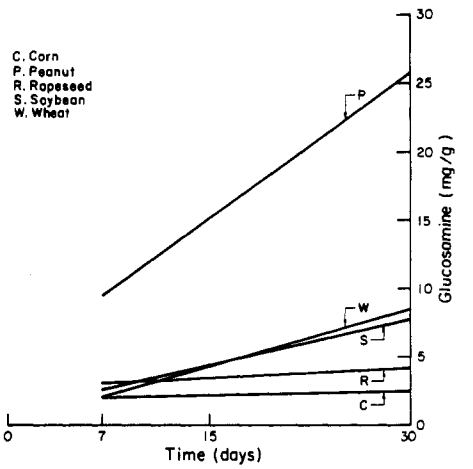

Figure 1. Growth (glucosamine concentration) of *A. alutaceus* on different substrates as determined by regression analysis. The concentration of glucosamine (Y, mg/g) on the different substrates is as follows: Y (corn) = 2.5240 + 0.0147 (day 30, NS), SE (b_1) = 0.0127; Y (peanut) = 25.8093 + 0.7106 (day 30)***, SE (b_1) = 0.0682; Y (rapeseed) = 4.3537 + 0.0543 (day 30)**, SE (b_1) = 0.0820; Y (soybean) = 7.5919 + 0.2015 (day 30)*, SE (b_1) = 0.0820; Y (wheat) = 8.3298 + 0.2560 (day 30)**, SE (b_1) = 0.0739. The regression equation is represented by the following: $Y = b_0 + b_1$ (day 30) − b_2 (day 30), where b_0 is the intercept, b_1 is the linear regression, and b_2 is the quadratic regression. SE (b_1) is the standard error for linear regression, and SE (b_2) is the standard error for quadratic equation. NS, not significant. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ for linear or quadratic regression.

Table I. Influence of Fungal Species and Substrate on OA, OB, and Citrinin Production (30 Days of Incubation)[a]

fungi and substrate	OA, μg/g	OB, μg/g	citrinin, μg/g	fungal biomass,[b] mg/g
A. alutaceus				
cereal substrates				
corn	74 ± 1	0.0	0.0	2.5 ± 0.1
wheat	72 ± 1	0.0	0.0	8.3 ± 0.1
oilseed substrates				
peanut	342 ± 2	132 ± 2	0.0	25.8 ± 0.1
soybean	243 ± 2	389 ± 5	0.0	7.6 ± 0.1
rapeseed	34 ± 1	19 ± 1	0.0	4.4 ± 0.2
P. verrucosum				
cereal substrates				
corn	21 ± 1	0.0	126 ± 1	3.6 ± 0.1
wheat	98 ± 1	0.0	102 ± 1	10.1 ± 0.1
oilseed substrates				
peanut	18 ± 1	0.0	0.0	6.4 ± 0.1
soybean	7 ± 1	0.0	0.0	6.4 ± 0.1
rapeseed	3 ± 1	0.0	0.0	7.0 ± 0.1

[a] Values ± SE are from data in Figures 1–6. Maximal values as calculated from the quadratic regression equations when substrates were incubated with *A. alutaceus* were as follows: soybeans, 260 μg of OA/g at 26 days; peanuts, 160 μg of OB/g at 22 days; rapeseed, 141 μgof OB/g at 19 days. Wheat incubated with *P. verrucosum* yielded a maximum concentration of citrinin of 120 μg/g at 23 days. [b] Fungal biomass as determined from glucosamine concentration.

is evident from data in Figures 1–6, there was considerable growth and toxin production by day 7. Fungal growth and toxin production between days 5 and 7 may therefore have occurred in a logrithmic manner. This was not evaluated.

Substrates varied greatly in their ability to support the growth and the corresponding production of OA and OB by *A. alutaceus*. Fungal growth between days 7 and 30 as determined by regression analysis was linear in all cases (Figure 1) and was significantly greater ($P < 0.001$) in peanut (25.8 mg/g at 30 days, Table I) than on all other substrates tested. Corn supported considerably less fungal growth (2.6 mg/g glucosamine) compared to wheat and peanut ($P < 0.001$). OA production by *A. alutaceus* on

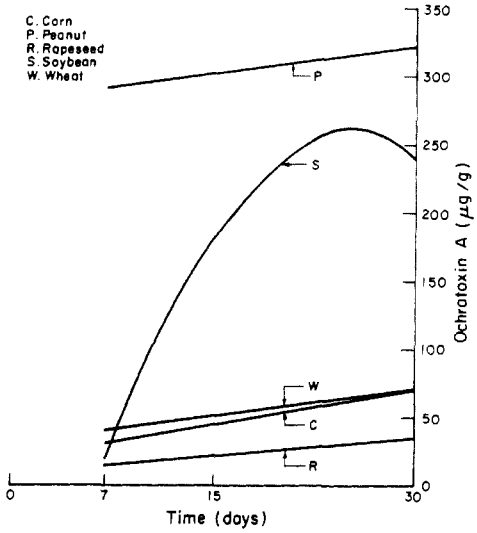

Figure 2. OA production by *A. alutaceus* on different substrates as determined by regression analysis. The concentration of OA (Y, μg/g) on the different substrates is as follows: Y (corn) = 73.7097 + 1.9146 (day 30)***, SE (b_1) = 0.2326; Y (peanut) = 342.2092 + 2.3397 (day 30, NS), SE (b_1) = 1.6788; Y (rapeseed) = 34.4086 + 0.8350 (day 30)**, SE (b_1) = 0.2264; Y (soybean) = 243.3567 − 7.0627 (day 30)* − 0.7237 (day 30)***, SE (b_1) = 2.4245, SE (b_2) = 0.1066; Y (wheat) = 72.3359 + 1.2538 (day 30)*, SE (b_1) = 0.5211. NS, not significant. *P < 0.05, **P < 0.01, and ***P < 0.001 for linear or quadratic regression. See Figure 1 for description of regression equation.

soybean showed a curvilinear trend, while it was linear on other substrates (Figure 2). Regression analysis indicated that maximal concentration of 260 μg of OA/g occurred at 26 days of incubation, which compares to the 30-day value of 243 μg of OA/g (Table I). Peanut after 30 days of incubation supported the highest production of OA (345 μg/g) exhibiting a nonsignificant upward trend between days 7 and 30 (P > 0.05). OA production on soybean increased in a curvilinear manner (P < 0.001) during 7–30 days of incubation with maximum calculated values being 260 μg of OA/g. Although rapeseed, corn, and wheat appeared to be poor substrates for OA production by *A. alutaceus*, they also supported considerable production of the toxin. Soybean, peanut, and rapeseed also supported the production of OB, while none was produced on corn and wheat (Figure 3). Soybean had significantly higher concentration of OB (389 μg/g, P < 0.001) than other substrates after 30 days of incubation (Table I). The maximum calculated productions of OB on peanut and rapeseed were 160 and 141 μg/g at 22 and 19 days, respectively. These concentrations particularly for rapeseed decreased dramatically by 30 days (Figure 3 and Table I). At 7 days of incubation only peanut supported OB production.

Growth of *P. verrucosum* showed significant (P < 0.01) curvilinear trends over time for all substrates (Figure 4). Wheat appeared to be a better substrate for the growth of the above fungus (10 mg/g after 30 days; P < 0.001) compared to other substrates. Corn differed significantly (P < 0.001) from the other substrates in having a low concentration of glucosamine after 30 days of incubation (3.6 mg/g, Table I). OA production by *P. verrucosum* over time was linear when the substrates were wheat, peanut, soybean, and corn, while it was curvilinear for rapeseed, which supported a relatively negligible amount of OA production after 15 and 30 days of incubation (Figure 5). Wheat after 30 days of incubation had a significantly higher concentration of OA (97 μg/g, P < 0.001) than all other substrates. Citrinin production over time by *P. verrucosum* on corn was linear, while wheat showed a significant curvilinear trend and reached a maximum calculated value of 120 μg/g at 23 days (Figure 6; Table I). Corn and wheat had almost the same concentration of citrinin after 30 days of incubation (124 and 102 μg/g, respectively). The three oilseeds did not support the production of citrinin by *P. verrucosum*.

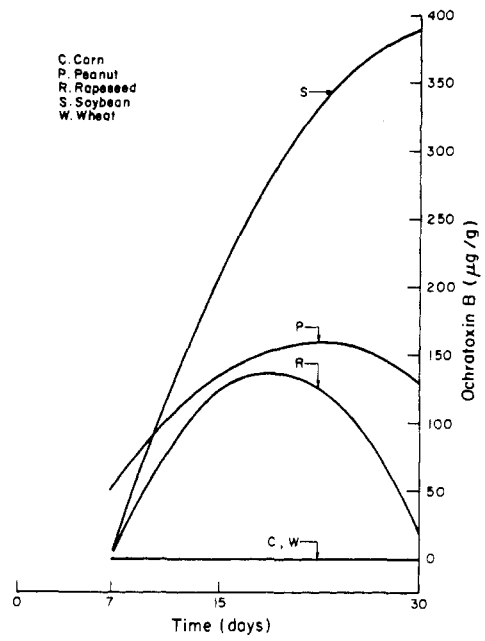

Figure 3. OB production by *A. alutaceus* on different substrates as determined by regression analysis. The concentration of OB (Y, μg/g) on the different substrates is as follows: Y (corn) = 0.00; Y (peanut) = 132.46 − 7.02 (day 30)* − 0.4487 (day 30)**, SE (b_1) = 2.2122, SE (b_2) = 0.0973; Y (rapeseed) = 19.1967 − 21.9474 (day 30)*** − 0.9905 (day 30)***, SE (b_1) = 1.4175, SE (b_2) = 0.0623; Y (soybean) = 389.3833 + 2.4770 (day 30) − 0.6284 (day 30)*, SE (b_1) = 4.7770, SE (b_2) = 0.2101; Y (wheat) = 0.00. *P < 0.05, **P < 0.01, and ***P < 0.001 for linear or quadratic regression. See Figure 1 for description of regression equation.

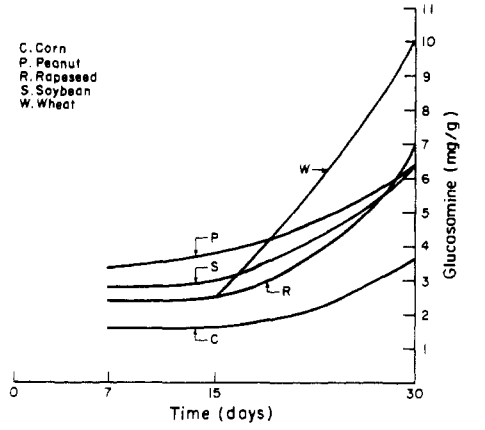

Figure 4. Growth (glucosamine concentration) of *P. verrucosum* on different substrates as determined by regression analysis. The glucosamine concentration (Y, mg/g) on the different substrates is as follows: Y (corn) = 3.6933 + 0.2375 (day 30)*** + 0.0065 (day 30)**, SE (b_1) = 0.0316, SE (b_2) = 0.0014; Y (peanut) = 6.4367 + 0.2741 (day 30)*** + 0.0062 (day 30)**, SE (b_2) = 0.0016; Y (rapeseed) = 7.0033 + 0.5141 (day 30)*** + 0.0136 (day 30)**, SE (b_1) = 0.0888, SE (b_2) = 0.0039; Y (soybean) = 6.37 + 0.3510 (day 30)*** + 0.0086 (day 30)**, SE (b_1) = 0.0412, SE (b_2) = 0.0018; Y (wheat) = 10.1167 + 0.7409 (day 30)*** + 0.0177 (day 30)**, SE (b_1) = 0.0843, SE (b_2) = 0.0037. *P < 0.05, **P < 0.01, and ***P < 0.001 for linear or quadratic regression. See Figure 1 for description of regression equation.

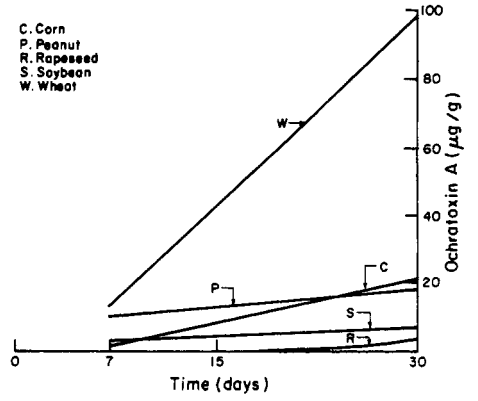

Figure 5. OA production by *P. verrucosum* on different substrates as determined by regression analysis. The OA concentration (Y, μg/g) on the different substrates is as follows: Y (corn) = 21.1057 + 0.8622 (day 30)***, SE (b_1) = 0.116; Y (peanut) = 17.8521 + 0.3127 (day 30)***, SE (b_1) = 0.0513; Y (rapeseed) = 3.2467 + 0.3576 (day 30)*** + 0.0094 (day 30)***, SE (b_1) = 0.0184, SE (b_2) = 0.0008; Y (soybean) = 7.1216 + 0.1655 (day 30)*, SE (b_1) = 0.0628; Y (wheat) = 98.0229 + 3.7057 (day 30)***, SE (b_1) = 0.5124. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ for linear or quadratic regression. See Figure 1 for description of regression equation.

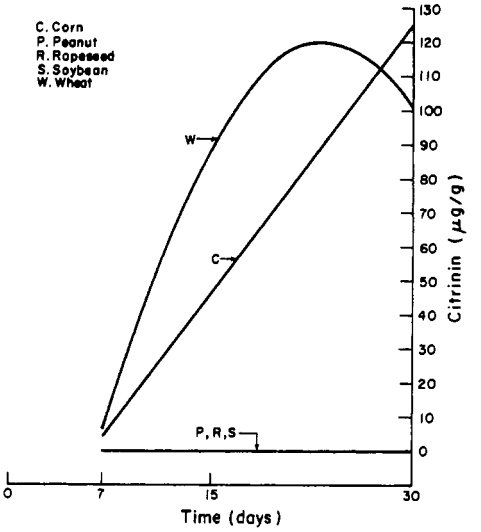

Figure 6. Citrinin production by *P. verrucosum* on different substrates as determined by regression analysis. The citrinin concentration (Y, μg/g) on the different substrates is as follows: Y (corn) = 125.5450 + 5.2572 (day 30)**, SE (b_1) = 1.2159; Y (peanut) = 0.00; Y (rapeseed) = 0.00; Y (soybean) = 0.00; Y (wheat) = 101.88 − 5.5829 (day 30)*** − 0.4258 (day 30)***, SE (b_1) = 0.3924, SE (b_2) = 0.0172. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ for linear or quadratic regression. See Figure 1 for description of regression equation.

DISCUSSION

The results of the current study clearly demonstrate that fungal biomass, OA, OB, and citrinin production by *A. alutaceus* and *P. verrucosum* is greatly influenced by the type of substrate, with the differences between the cereals and oilseeds being particularly pronounced. They also indicate that amount of fungal mass as determined by the concentration of glucosamine increased over the 7–30-day incubation period in a linear or curvilinear manner. Toxin production over this time period also generally tended to increase in a linear manner except for the production of OA by *A. alutaceus* on soybeans, OB with the same fungus on peanuts and rapeseed, and citrinin as produced by *P. verrucosum* on wheat. In these cases, maximum concentrations were reached prior to 30 days of incubation with the subsequent decrease in concentration of OB being very substantial for rapeseed after 22 days of incubation. Peanut and soybean supported a very high level of OA production by *A. alutaceus*, whereas wheat, corn, and rapeseed did not. Soybean was an excellent substrate for the production of OB by *A. alutaceus*, while peanut and rapeseed were relatively poor substrates. OB was not detected in corn and wheat. These results suggest that oilseed substrates such as peanut and soybean but not rapeseed when incubated with *A. alutaceus* support a much higher production of both OA and OB than the two cereal grains, wheat and corn. Similar comparisons have not been reported in the literature, although there have been several studies on the production of OA and OB on grains. Several authors have obtained high yields of OA from *A. alutaceus* grown on wheat with the values ranging from 1300 (Hesseltine et al., 1972) to 4000 μg/g (Lindenfelser and Ciegler, 1973). Trenk et al. (1971) reported OA production by *A. alutaceus* on chopped corn, polished rice, and wheat bran in the range 1500–1800 μg/g at optimal temperature and time. Soybean has also been reported to support OA production under laboratory conditions (Semeniuk et al., 1970) but the natural occurrence of OA in soybeans has not been observed.

The natural occurrence of OB in corn (Shotwell, 1971), barley (Krogh, 1973b), and moldy bread (Visconti and Bottalico, 1983) has been reported. It has been reported that both *A. alutaceus* and *P. viridicatum* could produce OB. *A. alutaceus* produced 2–4 μg/g OB on country-cured ham (Escher et al., 1973), while *P. viridicatum* produced 100 μg/g OB on pearled wheat (Hesseltine et al., 1972). In the current study peanut, soybean, and rapeseed supported OB production by *A. alutaceus*, whereas corn and wheat did not. The values for peanut and soybean obtained in this study were also much higher than those reported by other authors. The production of OB on grains and oilseeds, however, may not be harmful, as Stormer et al., (1985) have reported that OB, when administered together with OA to rats, reduces the toxic effects of OA.

In the current study the mycotoxins that were produced and the susceptibility of the five substrates to *P. verrucosum* were very different from that of *A. alutaceus*. Results with *P. verrucosum* demonstrated that wheat supported the greatest amount of fungal biomass production and corn the least amount. With regard to OA production wheat, by far, was the best substrate, although OA production by corn per unit biomass of fungi was also high. Peanut, soybean, and rapeseed supported an intermediate growth of the fungus, but produced relatively low amounts of OA. With regard to citrinin production, very high amounts were produced on the wheat and corn substrates and none on peanut, soybean, and rapeseed. Harwig and Chen (1974) also reported that both OA (273 μg/g) and citrinin (182 μg/g) were produced by *P. viridicatum* on ground wheat. There, nevertheless, has been some confusion about the production of citrinin and OA in the literature by *P. verrucosum* and *P. virdicatum* (Pitt, 1987). Recent studies by Pitt (1987) would suggest that *P. verrucosum*, but not *P. viridicatum*, produces the two compounds. The current studies in part support the observations of Pitt (1987).

All samples in this study were autoclaved under identical conditions to destroy endogenous fungi or spores. On the basis of a visual inspection autoclaving did not have a significant apparent effect on the physical structure of any of the different seeds. It may, however, have changed the susceptibility of the seed to infection. Incubation of non-treated grain samples that are spore-free would provide

a means of establishing the effect of sterilization treatments but probably would have resulted in the growth of competing mycoflora, which would have made interpretation of the data difficult as nearly all grain is contaminated with an indigenous population of fungi (unpublished observations) that would compete with added fungus. In this study only four time points (0, 7, 15, and 30 days) were used to establish the type of growth curve. The inclusion of additional time points, particularly in the early stages of growth, may have yielded more precise growth and toxin production curves.

An overall summary of results is provided in Table I. It may be concluded that peanut and soybean were much better substrates than rapeseed, wheat, and corn for OA and OB production by *A. alutaceus*, while corn and wheat were much better substrates for the production of OA and citrinin by *P. verrucosum* than the oilseed crops. Interestingly, citrinin was only produced by *P. verrucosum* on wheat and corn but not on oilseeds, while OB was only produced by *A. alutaceus* on oilseed crops but not on cereal crops. Substrate in addition to type of fungi clearly influences the nature and amount of toxic metabolites produced.

ACKNOWLEDGMENT

We thank K. Carrette for technical assistance. The support of this research by the grants of CIDA/NSERC, ERDA, and Western Grains Research Foundation is gratefully acknowledged.

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Received for review July 17, 1989. Revised manuscript received March 16, 1990. Accepted March 26, 1990.