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Technical Note

Production of Trichothecene Mycotoxins on Cereal Grains by *Myrothecium* spp.

ABSTRACT

Three small grain cereals—barley, oats and wheat—were inoculated with six isolates of Myrothecium *spp. and the amounts of roridin A and verrucarin A quantified. The highest levels of roridin A and verrucarin A were detected in oats and wheat, respectively, whilst the lowest level was reported in barley.*

INTRODUCTION

As well as unpleasant flavors, odors, or other undesirable changes, certain fungi produce secondary metabolites called mycotoxins, which are poisonous and cause harmful effects when food and feed containing mycotoxins are consumed by humans and animals. Among the mycotoxins, aflatoxins have been thoroughly investigated in various human and animal foods and feeds, since their discovery, as a result of the death of 100 000 turkey poults at 500 locations in England, in 1960 (Butler, 1973). The aflatoxins are produced by two species of *Aspergillus—Aspergillus flavus* (Link Ex Fries) and *A. parasiticus* (Speare)—and are commonly found in air and soil. Aflatoxin contamination has been extensively studied in agricultural products such as wheat, oats and sorghum (Shotwell *et al.*, 1969*a*), soybeans (Bean *et al.*, 1972), winged bean (Bean & Fernando, 1985), amaranth (Fernando & Bean, 1985), corn (Shotwell *et al.*, 1969*b*) and peanuts (Bampton, 1963).

Another group of mycotoxins, commonly called trichothecenes, has been extensively investigated, both chemically and biologically (Bamburg & Strong, 1971). Among the various genera of fungi that produce

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trichothecenes, *Fusarium* and *Stachybotrys* spp. have been well studied because of their growth on cereal grains, and hay and straw, respectively (Ueno, 1980). Their toxigenic effects on humans and farm animals are well documented. For example, red-mold disease in barley and wheat by *Fusarium graminearum* was responsible for severe intoxication in man and farm animals in Japan, and stachybotriotoxicosis caused by *Stachybotrys atra* was extremely important in the Soviet Union because of the death of many horses after the consumption of contaminated straw and hay (Ueno, 1983). Besides *Fusarium* and *Stachybotrys*; *Myrothecium* is another genus that produces trichothecenes and has been identified as a pathogen in various agricultural crops (Stevenson & McCollock, 1947; Cognee & Bird, 1964; Schieber & Zentmeyer, 1968; Bruton, 1982; Leath & Kendall, 1983) including small grain cereals, barley, oats and wheat (Crosier, 1962). The production of trichothecene mycotoxins on cereals and other agricultural crops by *Myrothecium* species has not been investigated, as has that by *Fusarium* and *Stachybotrys* species.

A comparison of several mycotoxins by biological assay has revealed that trichothecene mycotoxins, i.e. roridin A and verrucarin A, were the most virulent among thirty-two mycotoxins investigated. For example, roridin A and verrucarin A were cytotoxic at concentrations as low as 0·0001 ng and 0·1 ng, respectively, on HEp-2 human epithelial cells, whereas the lowest toxicity levels for the others were: aflatoxin B_1, 100 ng; aflatoxin B_2, 100 ng; aflatoxin G_2, 1000 ng; T-2 toxin, 10 ng; HT-2 toxin, 10 ng; Deoxynivalenol, 100 ng and Zearalenone, 100 ng. Aflatoxin G_1, Ocratoxin A, and Rubratoxin B were not cytotoxic even at 1000 ng (Robb & Norval, 1983).

Even though these mycotoxigenic fungi infect different agricultural commodities, the production of mycotoxins depends upon the substrate suitability. For example, several investigators have reported that the soybean is less favorable for the production of aflatoxin, although the *Aspergillus* infection has been identified in the soybean (Hesseltine *et al.*, 1966; Detroy *et al.*, 1971; Gupta & Venkitasubramanian, 1975). Also, the production of trichothecene mycotoxins in potato tubers was low, even the *Fusarium* growth being detected (El-Banna *et al.*, 1984). The present survey was carried out to determine in what capacity barley, oats and wheat provide a suitable substrate for *M. roridum* and *M. verrucaria*, the two species infecting grains (Crossier, 1962) with the production of the potent trichothecene mycotoxins, roridin A and verrucarin A. No previous attempt has been made to examine the production of roridin A and verrucarin A in these small grains.

MATERIALS AND METHODS

Seed samples (25 g) were weighed into 250 ml Erlenmeyer flasks, moistened to 20% of water content and sterilized for 15 min at 15 psi and 120 °C. The isolates of *M. roridum* and *M. verrucaria* were obtained from the American Type Culture Collection, Rockville, Maryland, USA, and maintained on potato dextrose agar slants at 25 °C. Seven-days-old culture slants bearing greenish-black conidia were used to prepare the spore suspension containing 3×10^6 conidia per millilitre. After inoculation, flasks were incubated at 25 °C for 14 days. The flasks were hand-shaken, once every 3 days, in order that the inoculum was properly mixed.

After 14 days' incubation, 100 ml of acetone were added to each flask and the contents were transferred and ground in a Waring blender for 3 min. The ground samples were passed through Whatman No. 1 filter paper and the residue was washed twice with 25 ml of acetone. The acetone extract was then partitioned with an equal amount of ethyl acetate in a separatory funnel. The acetone layer containing pigments was discarded and the ethyl acetate extract dried over anhydrous sodium sulphate (5·0 g) and flash-evaporated in a water bath at 40 °C. The residue was dissolved in 5 ml of methylene chloride and passed through a silica gel (10 μ) mini-column (1·0 × 15 cm). The column was eluted with 50 ml of methanol–methylene chloride (10:90, v/v) (Bean *et al.*, 1984). The eluent was evaporated to dryness under vacuum and the residue was saved for thin-layer chromatography (TLC) and high pressure liquid chromatography (HPLC).

TLC and HPLC Assays

Ten microliter samples were spotted on pre-coated silica gel (60 F-254) plates and developed in methanol and methylene chloride (5:95, v/v). On the same plate 10 microliters of roridin A and verrucarin A standards (1 mg/10 ml methylene chloride) were spotted. Developing time was *ca.* 10–15 min, for a solvent movement of 10 cm. Chromatographic plates were examined under short wave uv light (254 nm) for fluorescent compounds. The roridin A and verrucarin A were identified by comparing the R_f of the samples with the standards spotted on the same plate.

Quantification of roridin A and verrucarin A was carried out by HPLC. The HPLC system used in this study was a Varian Vista model 5000 liquid chromatograph (Varian Associates Inc., CA) equipped with a

model 280 variable wavelength ultraviolet absorbance detector and a Rheodyne model 7125 syringe loading sample injector loop. The operating parameters were Versapack silica column, 25 cm × 4·1 mm inside diameter (Alltech Associates, Inc., Deerfield, IL 60015, USA) and an absorbance detector with a fixed wavelength at 254 nm. The solvent system was methylene chloride–methanol, with a gradient of 100% to 90% methylene chloride, in 16·0 min, at a flow rate of 1·0 ml/min. Chromatographic peak areas were determined by a Varian Vista series CDS-401 integrator. The quantification of roridin A and verrucarin A was carried out by comparing the relative retention time and the estimation of the peak areas of the sample extracts relative to standards of roridin A and verrucarin A.

RESULTS AND DISCUSSION

The amounts of roridin A and verrucarin A produced in barley, oats and wheat are summarized in Table 1. The amounts of roridin A and verrucarin A produced varied, depending upon the isolate and the substrate used. For example, the highest levels of roridin A (350 μg) and verrucarin A (210 μg) were detected in oats and wheat, respectively. In

TABLE 1

Roridin A and Verrucarin A Production by *Myrothecium verrucaria* and *M. roridium* Isolates on Barley, Oats and Wheat

(Micrograms RA or VA per gram of seed. Values are means of three replicate samples)

Isolate	*Substrate*					
	Barley		*Oats*		*Wheat*	
	RA[a]	*VA*[b]	*RA*	*VA*	*RA*	*VA*
M. verrucaria ATCC 24571	160	60	350	120	301	198
M. roridum ATCC 26146	93	35	207	75	162	81
M. verrucaria ATCC 937	45	47	175	62	101	210
M. verrucaria ATCC 23470	61	28	152	92	114	78
M. roridum ATCC 36872	61	38	123	61	141	121
M. verrucaria ATCC 25621	18	21	97	62	41	23

[a] RA, Roridin A.

[b] VA, Verrucarin A.

barley, the amounts of roridin A and verrucarin A produced were much lower than in oats and wheat. On the other hand, the highest amount of roridin A (350 μg) was produced by the isolate *M. verrucaria* ATCC 24571 while the lowest (18 μg) was recorded by the isolate *M. verrucaria* ATCC 25621. The lowest (21 μg) and highest (210 μg) amounts of verrucarin A were produced by the isolates *M. verrucaria* ATCC 25621 and *M. verrucaria* ATCC 937, respectively.

Production of roridin A and verrucarin A by isolates of *M. roridum* and *M. verrucaria* is deemed to be important, since the infected cereal kernels could produce mycotoxins before harvest. The pre-harvest mycotoxin (aflatoxin) contamination and subsequent destruction of the entire crop has been reported for corn, in the southeastern United States (McMillan *et al.*, 1978). On the other hand, trichothecene mycotoxins are used in many laboratory experiments, but the chemical synthesis is more expensive and poor in yield, compared with other methods. It has been reported (Won Lee & Mirocha, 1984) that the use of cereal grains increased production several-fold.

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