Deterioration of Stored Pea Seed by Aspergillus ruber: Extraction and Properties of a Toxin

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ABSTRACT

Peas infected with *Aspergillus ruber* lost their ability to germinate normally in about 14 weeks, whereas sterile seeds under the same storage conditions were not affected. Aqueous extracts from infected peas induce necrosis and growth inhibition of healthy embryonic axes, but axes grow normally on extracts from noninfected seed. The toxic principle(s) is heat-stable, and a portion is dialyzable. It is partially soluble in methanol, ethanol, and 80% (v/v) aqueous acetone, but insoluble in absolute acetone, chloroform, or ethyl acetate. The properties of the toxic principle(s) from infected living peas or from *A. ruber* cultures on autoclaved peas are similar.

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Environmental conditions favoring infection of stored seed by fungi, especially *Aspergillus* and *Penicillium* spp., have been thoroughly studied (4, 7), but the biochemical and physiological events leading to loss of viability are poorly understood. It has recently been suggested that toxins may be involved in cellular membrane damage in infected seed (1), but in spite of the attention paid to mammalian and avian toxins produced by these fungi, effects of toxins produced by storage fungi on plant systems have been ignored. We have attempted to isolate toxins that might be involved in seed deterioration by *Aspergillus ruber* (Konig, Spieckermann, & Bremer) Thom & Church (8). This paper describes extraction, a rapid semiquantitative bioassay, and some of the properties of a toxin produced by *A. ruber*. To the best of our knowledge, this constitutes the first report of a toxin from an *Aspergillus* spp. which may play a role in plant disease.

MATERIALS AND METHODS.–Peas (*Pisum sativum* L. 'Alaska') were obtained from a lot of high-quality seed with a germination of about 98%. Alaska pea seeds were chosen for these investigations because they can be obtained in large quantities free of internal infection, and are resistant to deterioration when storage fungi are absent (6).

Aspergillus ruber Northern Utilization Research and Development Division Culture 52 (NRRL 52) was chosen as representative of storage fungi in general because preliminary experiments indicated that it was pathogenic to pea seed. It is only slightly toxic to avian or mammalian systems (11), and is a member of the *A. glaucus* group, which is frequently associated with spoilage of stored seed (4). *Aspergillus parasiticus* (NRRL 2999), *A. flavus* (NRRL 482), and *A. candidus* (NRRL 303) were not pathogenic in preliminary experiments after 8 weeks' incubation, while *A. restrictus* (NRRL 148) was less pathogenic than *A. ruber*, at least under the conditions described below.

About 200 seeds were surface-sterilized with 1.75% (w/v) aqueous NaOCl for 4 min, washed with sterile distilled water, and placed in 50-ml autoclaved polypropylene bottles stoppered with sterile cotton. They were then dried at 45 C for 24 hr, inoculated with a freely sporulating *A. ruber* colony growing on autoclaved peas (6), or not inoculated, and placed in a desiccator over a saturated $NH_4H_2PO_4$ solution (15). Seeds were incubated at 30 ± 1 C. The moisture content of the peas in equilibrium with the humidity in the desiccator (ca. 92%) was 21%, confirming the observations of Fields & King (6). In preliminary experiments, this moisture level was reached after incubation for not more than 8 weeks. *Aspergillus ruber* was also grown in culture on autoclaved peas (40 to 50% moisture content) at room temperature. On autoclaved peas, the fungus produces a very luxuriant growth in 7 days or less.

After incubation for various lengths of time, the living seeds were sampled to determine levels of fungus infection and their ability to germinate. To determine the infection of whole seeds, sample batches were surface-sterilized for 1 min in 1.75% NaOCl, thoroughly rinsed in sterile distilled water, and plated on a medium containing 2% (w/v) agar, 2% (w/v malt extract, and 10% (w/v) NaCl (malt-salt agar) (3). After 5 to 7 days' incubation at room temperature, the number of seeds from which *A. ruber* was growing was counted. Similar samples of the same batches of seed were also surface-sterilized for 1 min, then germinated at 20 C between moist autoclaved paper towels. After 5 to 6 days, counts were made of normally germinating seeds, abnormally germinating seeds, and dead seeds.

Extracts were made of infected, ungerminated peas, abnormally germinating infected peas, or from

cultures of *A. ruber* grown on autoclaved peas. As controls, extracts were made of similarly treated, noninoculated peas. The extracts were made by homogenizing peas in distilled water (2 ml/pea) in a Waring Blendor. The resulting slurries were centrifuged, the pellet was discarded, the supernatants were stored at 10 C overnight and recentrifuged, and the final supernatants sterilized by passage through a 0.45 μ pore size Millipore filter.

Extracts were tested for toxicity by the plating of eight healthy embryonic axes on a medium containing 2 ml of the extract to be tested, 2 ml of a sucrose-mineral salts agar, and 80 units of procaine penicillin G and 10 μg dihydrostreptomycin sulfate in 0.4 ml sterile distilled water. The sucrose-mineral salts agar contained 1.45 g $Ca(NO_3)_2 \cdot 4H_2O$, 0.25 g KNO_3, 0.51 g $MgSO_4 \cdot 7H_2O$, 0.25 g KH_2PO_4, 10 g sucrose, and 20 g agar/liter (13). The pH was adjusted to 6.5 before autoclaving because the pH of aqueous extracts of pea seed ranged from pH 6.3 to 7.2. The embryonic axes were removed from healthy seeds by removing a small piece of seed coat from a number of peas, imbibing the seeds by soaking 8 to 16 hr under reduced pressure, then removing the seed coats, separating the cotyledons, and carefully removing the intact axes. The axes were surface-sterilized in 1.75% NaOCl for 20 sec, washed thoroughly in sterile distilled water, and plated on the sucrose-mineral salts agar. The axes were incubated 3 days at 20 C. All experiments reported here were repeated at least twice.

RESULTS AND DISCUSSION.—After 14 weeks' incubation, the number of peas germinating abnormally or not germinating increased dramatically in samples inoculated with *A. ruber*, and the fungus had infected nearly 100% of the seeds. Germination of sterile seed incubated 14 weeks was not affected (9). Therefore, all extracts of infected, nonautoclaved peas were made after incubation for 13 to 15 weeks. Extracts from infected peas in water or methanol were brown, and the extracts from autoclaved pea cultures were very dark. Extracts from noninfected peas, either autoclaved or fresh, were a yellow-brown color.

Embryonic axes from healthy, cold-stored peas were placed on the sucrose-mineral salts medium containing extracts from infected peas or noninfected seed. Axes placed on a medium containing extracts from infected peas became necrotic, and root hair production and growth of the axes were inhibited (Fig. 1). These effects could be detected in 1:1,000 dilutions of extracts from autoclaved peas, from 1:100 dilutions of abnormally germinating peas, and from 1:10 to 1:50 dilutions of extracts from ungerminated peas. These differences probably reflect the differences in the quantity of fungus in peas following these treatments. Axes on extracts from noninfected peas grew well, without browning or growth inhibition.

Necrosis and inhibition of growth were evaluated as bioassays. Growth inhibition was not reproducible with crude extracts. There may be factors in these extracts counteracting the effects of the toxin on growth. In some instances, as toxic extracts were diluted, growth inhibition became more pronounced. Growth of axes on media containing extracts from noninfected peas was greater than on media containing distilled water and the sucrose-mineral salts agar only. Levels of necrosis permitted a semiquantitative assay. A scale utilizing the percentage of the axis which was necrotic (necrosis index, Table 1) was utilized, and gave a logarithmic response over a 100-fold range in concentration of the extract from diseased peas (Fig. 2). Such an assay is rapid, and although somewhat subjective, is valuable for screening large numbers of samples.

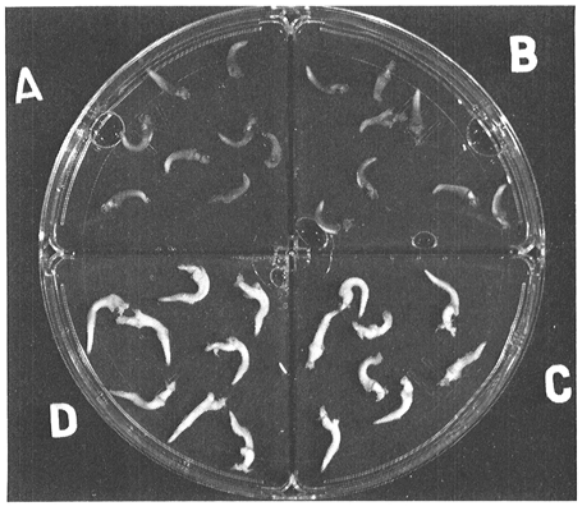

Fig. 1. Effects of extracts from autoclaved pea cultures on healthy embryonic pea axes after 3 days' incubation. **A)** Axes on an extract from peas inoculated with *Aspergillus ruber*. **B)** Axes on the same extract after autoclaving 20 min. **C)** Axes on autoclaved extract from noninoculated peas. **D)** Axes on an extract from noninoculated peas. The axes in A and B had a necrosis index of five; the extracts in C and D had a necrosis index of less than one.

Certain properties of the toxins extracted from the various sources were determined. All tests were conducted at pH 6.5 to 7.0. The activity in the pea embryonic axes assay was not affected by autoclaving at 120 C at 17 psi for 20 min. Pea extracts were

TABLE 1. Necrosis index used for semiquantitative estimation of toxin concentration in extracts of *Aspergillus ruber*-infected peas

Necrosis index	Description of index
0	No necrosis
1	Less than 10% of the embryonic axis necrotic; necrosis confined to the epicotyl and cotyledonary node
2	10-25% of the axis surface necrotic
3	25-50% of the surface of the axis strongly necrotic
4	50-75% of the surface of the axis strongly necrotic
5	More than 75% of the axis necrotic, growth of the axis slight

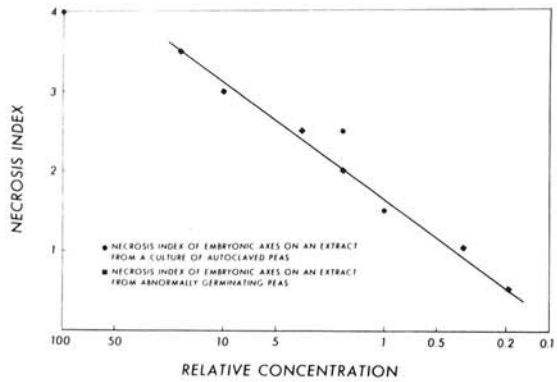

Fig. 2. Necrosis index of healthy embryonic pea axes on extracts from abnormally germinating peas infected with *Aspergillus ruber* or from an autoclaved pea culture of *A. ruber*. Concentrations were adjusted by dilution with sterile distilled water.

dialyzed against distilled water for 48 hr with two changes of water. About 50% of the activity, as determined by the necrosis index, passed through the dialysis tubing. Extracts from autoclaved peas or from abnormally germinating peas behaved similarly. This suggests that at least two compounds of differing molecular weight are present, although it is possible that some of the toxin is aggregated with large molecular constituents in the extracts.

Solubility of the toxin from *A. ruber* in various solvents was determined. Aliquots of the aqueous extracts were evaporated to dryness in a rotary evaporator at 35 C under reduced pressure, three to four volumes of solvent were introduced, and the residue was thoroughly washed. The solvent was removed and the residue redried in a stream of air, then redissolved in distilled water. The solvent was then evaporated, and the resulting residue dried and again taken up in distilled water. This procedure indicated that 40 to 70% of the toxic activity dissolved in methanol, whereas about 10% dissolved in ethanol and 80% (v/v) aqueous acetone. The solubility in each solvent was similar regardless of the source of the toxin. None of the extracts contained toxic materials soluble in acetone, chloroform, or ethyl acetate. These results, along with the similarities in biological activity, heat stability, and dialysis suggest that a similar toxin or mixture of toxins were present in all extracts regardless of whether they were derived from autoclaved fungus cultures on autoclaved peas or from living infected peas. The solubility characteristics demonstrate that the toxic principles from *A. ruber* are not any of a number of common mammalian or avian toxins or antibiotics produced by *Aspergillus* spp. which are soluble in acetone or chloroform (i.e., the aflatoxins, the sterigmatocystins, patulin, ascladiol, the aspergillic acids, and kojic acid) (2, 5, 10, 12, 14). The presence of the toxin in infected seed but not in healthy seed, and other evidence (9), strongly suggest a role for a toxin in deterioration of pea seed by *A. ruber*. Other seed infected with storage fungi should be examined to determine whether toxins may also be involved in their deterioration.

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