Pathogenicity and Infection Sites of Aspergillus Species in Stored Seeds

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ABSTRACT

Wheat, pea, squash, and tomato seeds were inoculated with seven different *Aspergillus* isolates representing common species of storage fungi. Wheat seeds were infected and the percent germination was reduced by all isolates. Pea seeds were infected, and the percent germination was reduced, by members of the *A. glaucus* and *A. restrictus* group species. Squash seeds were infected by six of the isolates, but germination was reduced by a single isolate of *A. flavus*. Tomato seeds were unaffected by any of the isolates. Embryos of wheat seeds were readily invaded. The infected embryos were dark, and they were jellylike when imbibed. Embryos of squash and embryonic axes of peas were seldom invaded by pathogenic isolates and the fungi in both seeds were confined to layers of dead cells between the seed coat and the embryo.

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Certain storage fungi, primarily *Aspergillus* spp., are serious pathogens of seeds (2, 3). Although it is known that different *Aspergillus* spp., or different isolates of certain *Aspergillus* spp., vary in pathogenicity to similar hosts (14, 15), there is little known about the pathogenicity of single isolates to a wide range of hosts.

Reports also indicate that embryos of stored seeds are primary and favored sites of infection for storage fungi, and that invasion causes death of host seeds (2, 3, 4, 13). However, colonization of peanut embryos by *A. flavus* is limited (11), and *A. ruber* sometimes kills pea embryonic axes before invasion (8).

This study was conducted to determine the pathogenicity of various *Aspergillus* isolates to various seeds and to determine which seed parts were invaded by pathogenic isolates.

MATERIALS AND METHODS.—Four kinds of seeds were chosen as representatives of a wide range of

TABLE 1. Infection of whole seeds and seed parts by various *Aspergillus* spp.[a]

Seeds[b]	Infecting spp.	Normal germination (%)[c,d]	Infection (%)	
			Whole seeds	Embryos (Wheat and squash or embryonic axes (peas)
Pea	None	87	0	0
Pea	*A. ruber*	46	99	0
Pea	*A. chevalieri*	43	87	7
Pea	*A. restrictus*	57	97	5
Wheat	None	97	0	0
Wheat	*A. ruber*	73	100	55
Wheat	*A. chevalieri*	77	99	47
Wheat	*A. restrictus*	73	95	45
Squash	None	80	0	0
Squash	*A. flavus*	50	95	14[e]

[a]Data represent averages of data from two to four separate experiments.

[b]Pea seeds were stored 10 to 12 wk, wheat seeds 8 wk, and squash seeds 12 wk at 30 C and 92% relative humidity.

[c]Germination of all seed lots was between 97 and 100% before storage under conditions of high temp and humidity.

[d]Seeds that had sprouted but that had dead or distorted plumules or radicles were considered abnormal and were not included in normal germination counts.

[e]While the embryos were not infected, the perisperm-endosperm layer enveloping the embryos were infected in all seeds examined.

seed types that could be obtained free of internal infection. All seed lots used in this study had at least 97% germination. Pea seeds (*Pisum sativum* L. 'Alaska') were obtained from the Asgrow Seed Company, whereas squash, tomato, and wheat seeds were produced near Geneva, N.Y. according to Christensen's suggestions for obtaining uninfected seeds (1). Wheat plants (*Triticum aestivum* L. 'Redbobs') were grown in a greenhouse when the surrounding landscape was usually snow-covered. Plants were surface-irrigated. Squash (*Cucurbita pepo* L. 'Table Queen') and tomato seeds (*Lycopersicon esculentum* Mill. 'Heinz 1350') were obtained from ripe, unblemished field-grown fruits. Seeds and pulp were removed from fruits, water was added to moisten the mixtures, and the mixtures were allowed to ferment overnight. Concentrated H_2SO_4 (0.5 ml/kg pulp) was added to tomato pulp, the mixture was stirred and allowed to stand for 15 min. The pulp was washed from tomato and squash seeds and the cleaned seeds were dried at 35 C for 2 days. After harvest, all four kinds of seeds were stored at 10 C and 20% relative humidity (RH) to minimize physiological aging.

Isolates of common storage fungi were obtained from the USDA Northern Marketing and Nutrition Research Division Collection; they bear NRRL numbers. Included were representatives of the *A. flavus* group (*A. flavus* Link NRRL 482, *A. flavus* NRRL 5523); the *A. glaucus* group [*A. ruber* (Konig, Spieckermann, and Bremer) Thom and Church, NRRL 52, *A. chevalieri* (Mangnin) Thom and Church NRRL 78, *A. amstelodami* (Mangnin) Thom and Church NRRL 113, *A. repens* de Bary NRRL 13], and the *A. restrictus* group (*A. restrictus* Smith NRRL 148). The fungi were maintained on autoclaved peas at 50% moisture content.

Before storage, seeds of wheat, tomato, pea, and squash were surface-sterilized with NaOCl (1.75%) for 1, 3, 5, and 10 min, respectively. The seeds were then rinsed thoroughly with distilled water, and dried asceptically at 45 C to less than 10% moisture content. Wheat seeds (6 g), pea seeds (30 g), and tomato seeds (1 g) were treated and dried in cotton-stoppered sterile containers suitable for storage. For adequate drying of tomato seeds it was necessary to place a sterile filter paper wick in the moist seeds. Squash seeds (20 g) were dried in sterile bags, then transferred to containers for storage. For studies on the site of infection, pea and squash seeds were stored in open beakers. This procedure insured uniform infection throughout the samples. Seed samples were inoculated with freely sporulating cultures of *Aspergillus* isolates growing on autoclaved peas. Inoculated seeds were then placed in loosely sealed autoclaved screw-capped jars containing a saturated solution of $NH_4H_2PO_4$ which resulted in a RH of 92% (19). Uninoculated controls were treated similarly. The jars were kept at 30 C.

After storage for different intervals of time, thirty seeds per sample were tested for germination and thirty were tested for infection. Seeds were germinated on moist blotters (squash, wheat, and tomatoes) or between sterile moist towels (peas) in sterile boxes at 25 C. Sprouted seeds with dead or distorted plumules or radicles were considered abnormal. Whole seeds were tested for infection by first washing them in a 1% (w/v) sodium dodecyl sulfate solution to remove spores, then by treating with 0.85% NaOCl for 2 min, and by plating on Czapek-Dox agar containing 30% sucrose (17). The fungi involved in this study were readily identified by their growth characteristics on this medium. Contaminated samples were discarded.

A histological procedure was substituted for plating methods commonly used for detecting fungi in dissected seed parts. This was required because spores were produced on the seed coats during storage and were mechanically distributed onto other seed parts during dissection. These spores were frequently not killed by NaOCl treatments as evidenced by the presence of viable spores in sterile water used to wash NaOCl from apparently uninfected seed parts, and by the observation of ascospores on tissues where no cleistothecia were present. To avoid this problem, we modified a procedure used to detect *Ustilago nuda* in barley embryos (18) and applied it to detect *Aspergillus* in the embryos or embryonic axes of the seeds in this study. Embryos of

wheat and squash and embryonic axes of peas were removed from seeds soaked in water under a partial vacuum (430 mm Hg) for 3 to 5 h. Embryos of wheat and embryonic axes of peas were then placed in 5 ml of a solution containing 25 g NaOH, 0.25 g trypan blue, and 1 drop Triton X-100 per 100 ml, then heated at 100 C 40 to 60 min. Squash embryos required 1 h of heating in a 25% (w/v) NaOH solution followed by replacing the NaOH solution with the trypan blue solution and 2 h of additional heating to be adequately stained. The trypan

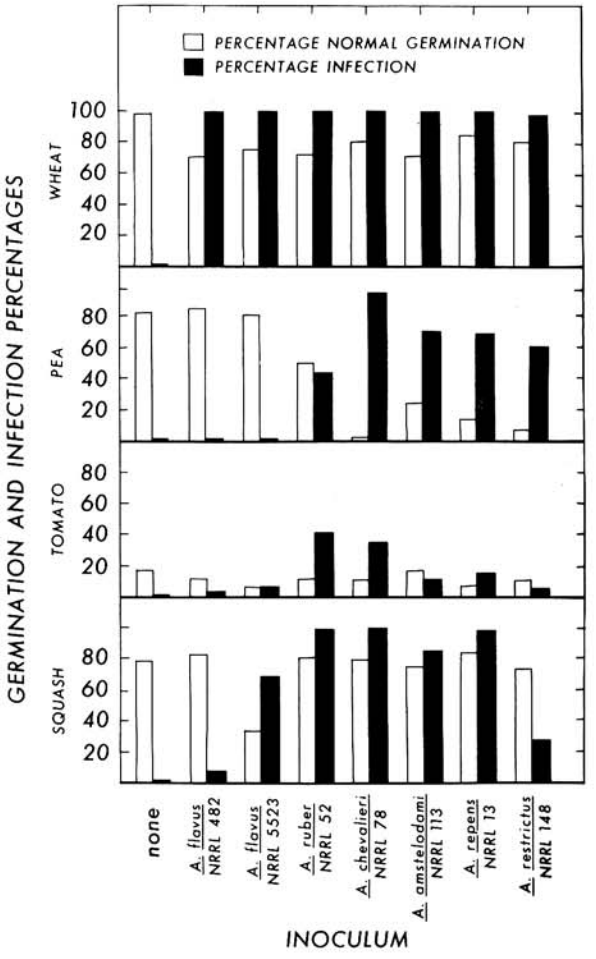

Fig. 1. Percentage normal germination and infection of wheat seeds stored 7 wk, pea seeds stored 14 wk, and tomato and squash seeds stored 16 wk after inoculation with various *Aspergillus* spp. Storage was at 30 C and 92% relative humidity (RH). Seeds that sprouted, but that had dead or distorted plumules or radicals were considered abnormal and were not included in normal germination counts; the normal germination of all seed lots was at least 97% before storage at high temp and RH. All data represent averages of two to four separate experiments.

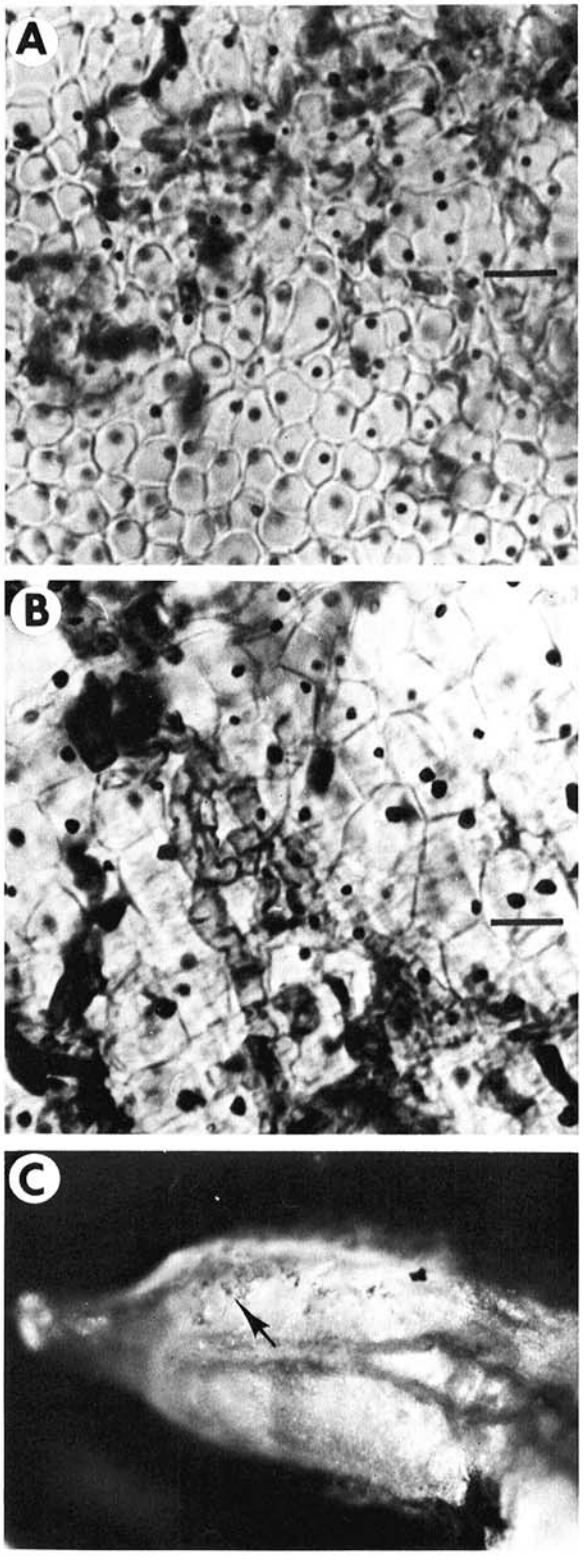

Fig. 2-(A to C). Hyphae of *Aspergillus* isolates in various host seed tissues after staining with trypan blue as described in the text. **A)** Hyphae of *A. chevalieri* in wheat embryo tissues (the bar represents 5.0 μm). **B)** Hyphae of *A. flavus* NRRL 5523 in the endosperm-perisperm layer of squash seeds (the bar represents 2.5 μ). **C)** Hyphae (arrow) of *A. flavus* NRRL 5523 on the outer layer of a whole dead or dying squash root tip (the portion of the root shown is ca. 3.0 mm long).

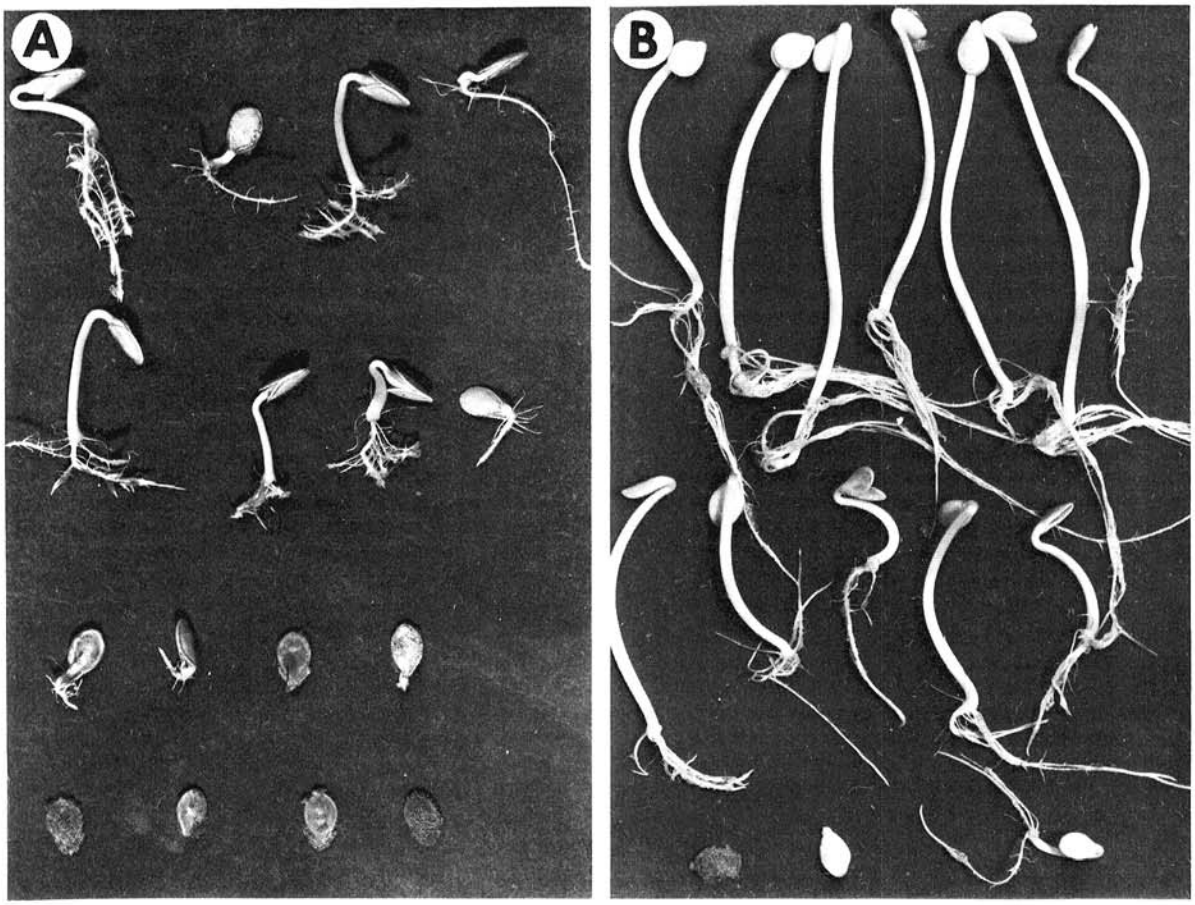

Fig. 3-(A, B). Germination and growth of squash seeds and embryos from a single sample of seeds infected with *Aspergillus flavus* NRRL 5523. **A)** Germination and growth of seeds on blotters after 7 days of imbibition. **B)** Growth of embryos separated from seed coats and endosperm-perisperm layer after 6 days of incubation on mineral salts agar. Both photographs were taken of seeds on blotters 13 × 23 cm.

blue solution was then replaced with lactophenol (1:1:1:5 v/v/v/v lactic acid: liquefied phenol: glycerine: water) and heated at 100 C for 60 min. The lactophenol was decanted and fresh lactophenol was added and heated. This process was repeated until the host tissues were nearly colorless while the hyphae remained bright blue (Fig. 1). The axes or embryos were then placed in fresh lactophenol, crushed on a microscope slide and thoroughly and independently examined by both authors. At least 10 axes or embryos per sample were examined.

Only certain representative, seed-pathogen combinations were used for histological studies. *A. chevalieri, A. ruber,* and *A. restrictus* were used for pea and wheat seeds because the three were pathogenic to both kinds of seeds (Table 1), because the pea-*A. ruber* combination has been studied extensively (e.g., 6, 7, 8), and because the three fungi represent two *Aspergillus* group species (17). For squash seeds, *A. flavus* NRRL 5523 was chosen because it was the only isolate pathogenic to squash (Table 1). Since none of the fungi used in this study was pathogenic to tomato seeds, these seeds were not investigated further.

RESULTS.—The *Aspergillus* isolates were differentially pathogenic to the four kinds of seeds. Wheat seeds were invaded by all isolates and the germination of all infected samples was reduced by approx. 25% (Fig. 1). Pea seeds were not infected by either isolate of *A. flavus,* but were infected and the germination was lowered by *A. ruber, A. chevalieri, A. amstelodami, A. repens,* and *A. restrictus* (Fig. 1). Squash seeds were invaded by all isolates except *A. flavus* NRRL 482, but only *A. flavus* 5523 lowered seed germination (Fig. 1). Tomato seeds were not seriously affected by any of the fungi although *A. ruber* and *A. chevalieri* invaded seeds after extended storage (Fig. 1).

The site of invasion also differed markedly among wheat, pea, and squash seeds (Table 1). Eight wk after inoculation, germination of wheat seeds was reduced by about 25%, and approx. 50% of wheat embryos were invaded by *A. chevalieri, A. ruber,* or *A. restrictus*. When the bran layers were removed from the embryos of heavily infected seeds, the embryos were found to be dark and frequently were covered by conidiospores and/or cleistothecia. Dark embryos were brittle when dry and jelleylike after imbibition. Stained hyphae in infected

embryos were clearly visible (Fig. 2-A). Embryos of squash or embryonic axes of peas were not usually invaded even when germination was markedly reduced (Table 1). However, when squash seeds infected with *A. flavus* NRRL 5523 were dissected, the perisperm-endosperm layers enveloping the embryo were all found to be infected (Fig. 2-B). Similarly, we reported earlier (8) that pea seeds contained a layer of dead tissue between the seed coat and embryos, and that most of the hyphae of *A. ruber* were found in this layer.

Unlike pea seeds, which were killed before imbibition (8), squash embryos were not killed until germination began. Most of the squash seed (84%) grew normally if seed coats and the perisperm-endosperm layers were removed and the resulting embryos were lightly surface-sterilized and plated on mineral salts agar (6), without sucrose. In contrast, only 50% of the whole undissected seeds germinated normally (Fig. 3). However, among seeds that did not germinate normally, only 25% of the embryos were heavily infected after being kept on blotters for 5 days. The remainder were infected superficially in small areas on the radicle (Fig. 2-C) while the cotyledons were uninfected. Although *Aspergillus* spp. were unable to invade sound pea or squash embryos, the fungi were able to do so if the seeds were mechanically damaged.

DISCUSSION.—The various *Aspergillus* isolates exhibited host specificity. Wheat and pea seeds were infected and their germination was reduced by all seven and by five isolates, respectively. Squash seeds were infected by all isolates except *A. flavus* NRRL 482. However, *A. flavus* NRRL 5523, which was originally isolated from cucumber seeds, was the only isolate that lowered germination of squash seeds. None of the isolates affected tomato seeds. Similarly, Kulik (10) reported that two *Aspergillus* spp. had little effect on nine kinds of vegetable seeds.

Embryos of some seeds, e.g. wheat and corn (5), were readily invaded, while embryos of other seeds, e.g. peas and squash, were seldom invaded. Thus, we have found exceptions to the generalization that embryos are favored sites of infection (2, 3, 4, 13).

The mechanism by which different seeds were killed by pathogenic isolates seemed to be dependent upon the kind of seed. Wheat embryos were heavily invaded and when imbibed they became a jellylike mass. This suggested that invasion and perhaps the production of cell wall-degrading enzymes may be important processes in wheat seed deterioration. In contrast, pea and squash embryos were killed without physical invasion, indicating involvement of diffusable toxins. This concept was previously suggested for the pea-*A. ruber* system (8), and a toxin specific to peas was isolated (6, 7). While diffusable toxins were apparently involved in deterioration of squash and pea seeds, damage to pea seeds occurred during storage, whereas damage to squash seeds occurred during germination.

Inability of the various fungi to invade tomato seeds or squash embryos or pea embryonic axes seemed especially significant to us. Pea cotyledons were not readily invaded by *A. ruber* (8). It suggested that some seeds contained substances capable of protecting them against invasion. Similar findings (11) led Lindsey to search for and find an antifungal substance in fresh peanut seeds (12). Extracts from dry whole pea seeds inhibited sporulation of *Alternaria alternata* (16). The tannin content of sorghum grain appears to be correlated with resistance to seed molds (9). The ability of some *Aspergillus* spp. to infect mechanically damaged squash or pea embryos and to invade wheat embryos indicate they are capable of infecting embryos of various seeds. Even though dead parenchymous layers between the seed coats and embryos of sound squash and pea seeds became heavily infected, the embryos themselves were seldom infected. These results indicate the existence of preformed chemicals which inhibit invasion, probably located in the outer layers of pea and squash embryos.

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