Deterioration of Stored Pea Seed by Aspergillus ruber: Evidence for Involvement of a Toxin

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ABSTRACT

Invasion of pea seeds by *Aspergillus ruber* causes a rapid loss in seed viability, as measured by the ability of the seed to germinate normally. Infection of the intact seed precedes loss of viability and infection of the cotyledons or embryonic axes. However, death of the embryonic axes occurs before invasion of at least some of the axes. This suggests that a toxin is produced by *A. ruber* which kills the embryonic axes in advance of actual infection by the fungus.

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A previous paper (6) described a toxin produced by *Aspergillus ruber* (Konig, Spieckermann, & Bremer) Thom & Church that caused necrosis and inhibited the growth of healthy pea embryonic axes. There is no other direct evidence for involvement of a toxin produced by *Aspergillus* spp. in any plant disease, at least so far as the authors are aware. However, Anderson et al. (1) suggested that a fungal toxin might be involved in the deterioration of wheat seed.

In peas infected with *A. ruber*, we noted that even in advanced stages of infection, sporulation of *A. ruber* was confined largely to the hilum of the seed, and frequently could not be observed elsewhere. Since grain embryos are reported as favored sites of infection by storage fungi (3, 5), we wondered whether the interior portions of the pea seed were infected, and, if so, whether infection was correlated with deterioration of the seed. This communication describes an attempt to correlate fungus infection of various organs of the seed with germination and growth of the embryonic axes. If infection of the seed or seed parts is not correlated with loss of viability of the seed, then a role for toxins in loss of viability would be indicated.

MATERIALS AND METHODS.–Peas (*Pisum sativum* L. 'Alaska') from a single lot of seed were sterilized, inoculated or not inoculated, and incubated in a sealed desiccator over a saturated solution of $NH_4H_2PO_4$ (10) as previously described (6). After various periods of incubation, samples were removed from the desiccator, and levels of germinability and infection determined (6). Germination was considered normal when both the hypocotyl and epicotyl emerged and were not necrotic. Seeds were considered to germinate abnormally if the hypocotyl or epicotyl did not emerge, or if the emerging portions were curled and necrotic (Fig. 2). Dead seeds were those from which neither the hypocotyl nor the epicotyl emerged from the seed coat.

Seeds were dissected, and the infection of the seed parts and the ability of the excised embryonic axes to grow was determined. Seeds were initially softened by soaking in distilled water 120 to 150 min. The seed coat was then removed, surface-sterilized for 20 sec in 1.75% NaOCl, rinsed thoroughly with sterile distilled water, and plated on malt-salt agar (2, 6).The cotyledons were separated, and the embryonic axis was then gently removed. One cotyledon from each seed and the embryonic axis were surface-sterilized by the same procedure and plated on malt-salt agar.

Other samples of embryonic axes from the same batches of seed were removed from the seed by the same procedure and plated on a sucrose-mineral salts agar medium (6). With this procedure, healthy embryonic axes grew rapidly and were usually free of contaminating bacteria or fungi. Embryonic axes were grown for 3 days on sucrose-mineral salts agar, and the numbers of normal axes, abnormal axes, and dead axes were counted. The total weight of the axes in a given sample was also determined. Growth was considered normal when the radicle and hypocotyl grew fairly straight, with some bending of the hypocotyl. Abnormally growing axes were markedly curled, with the radicle and hypocotyl often forming a complete spiral. Dead axes were those which did not elongate (Fig. 3).

Several histological procedures were attempted to identify the site of infection of *A. ruber*. The most successful procedure was crushes of the tissues in 0.05% aniline blue in lactophenol (8) examined under the microscope at a magnification of $\times$ 400. Tissues from seeds softened in water, stored in a mixture of 95% ethanol:acetic acid:formaldehyde:water (10:1:2:7) (9), or untreated were equally satisfactory, and all three techniques were used in the results reported here. Cryostat sections as thin as 8 μ with various staining schedules were inferior for determining the presence or absence of fungal mycelium. The fungal growth was sparse and it was

difficult to determine if a short piece of material was of host or fungal origin.

RESULTS AND DISCUSSION.–Incubation of pea seed at 92% relative humidity and 30 C did not affect germination of sterile peas (Fig. 1-B). However, if the peas were inoculated with *A. ruber* prior to incubation, there was a steady decrease in the number of seeds germinating normally from 8 to 14 weeks. After 14 weeks' incubation, less than 10% of the peas were capable of normal germination and about 70% germinated abnormally (Fig. 1-B). The abnormally germinating seeds were so badly damaged that they would probably not have produced a seedling (Fig. 2). Abnormally germinating seeds and high percentages of dead seeds were apparently a direct result of fungal infection, since they were not observed in uninfected batches of seed (Fig. 1-B, 2).

Infection of intact seeds, as determined by growth of *A. ruber* from the seed on malt-salt agar, was well correlated with loss of normal germination. At all sampling times, the proportion of seeds infected was higher than the proportion which had lost the ability to germinate normally (Fig. 1-A, B), confirming the observations of Fields & King (4).

Infection of the embryonic axes was not correlated with ability of the embryonic axis to grow normally. The percentage of infected intact seeds was greater than the percentage of infected cotyledons, which in turn was greater than the percentage of infected embryonic axes (Fig. 1-A). Abnormal or no growth of the embryonic axes was evidently the result of infection by *A. ruber*, since these phenomena were never observed with healthy axes (Fig. 1-C, 3). The number of embryonic axes growing normally decreased more rapidly than the level of infection increased. After 14 weeks of infection, nearly 90% of the embryonic axes were dead (Fig. 1-C), but *A. ruber* grew from only 60% of the axes when they were plated on malt-salt agar (Fig. 1-A). These levels of infection varied considerably, but infection of all the embryonic axes of any sample was never observed, even after 14 weeks' incubation. In one batch sampled at 14 weeks, all the embryonic axes were dead, but only one axis of 10 sampled was infected.

These results were substantiated by histological examinations. Tissue from cotyledons and embryonic axes contained fungal hyphae in only two of six samples examined (at least five seeds/sample were examined). The two samples in which the tissues of the cotyledonary and embryonic axes contained hyphae were from heavily infected seed lots incubated 15 and 20 weeks. This period of incubation is longer than is required for death of the axes (Fig. 1-C). Even in these samples, hyphae could not be found in all the embryonic axes examined. Instead of growing in the embryo portions of the seeds, *A. ruber* appears to be confined primarily to the inner layers of the seed coats. The seed coats of peas are composed of several layers. The outer layer is a hard layer of palisaded epidermis, underlain by a layer of thick-walled subepidermal cells, and finally by a layer of thin-walled, largely empty parenchyma cells (7). In

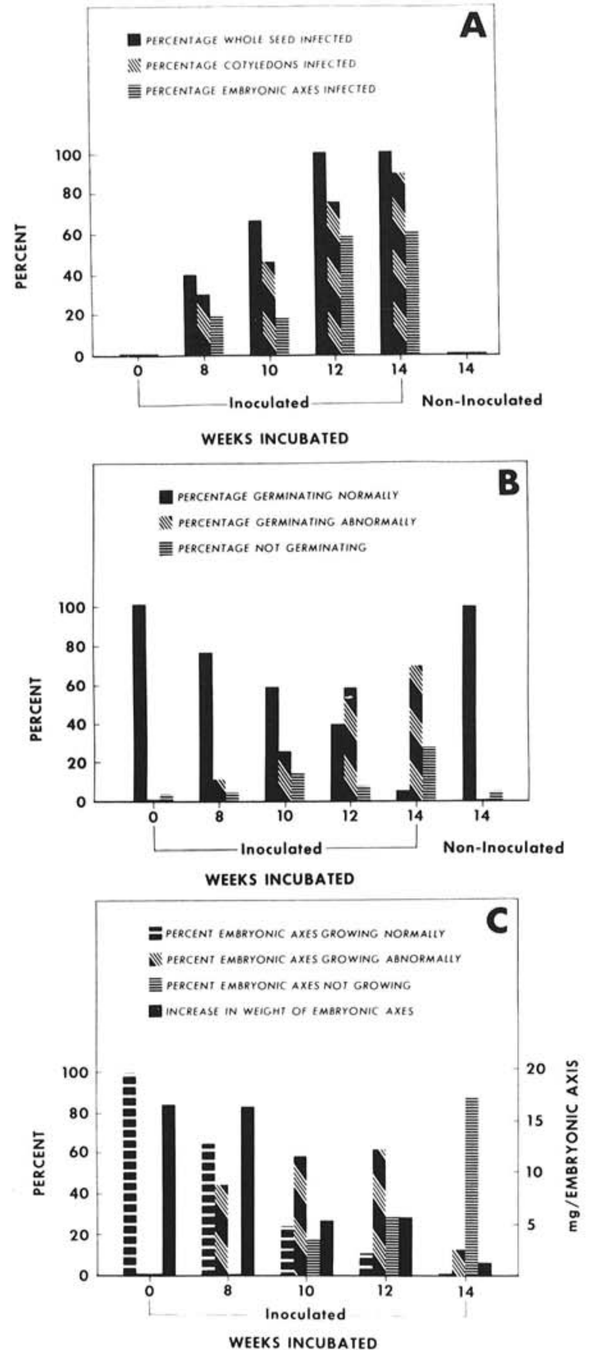
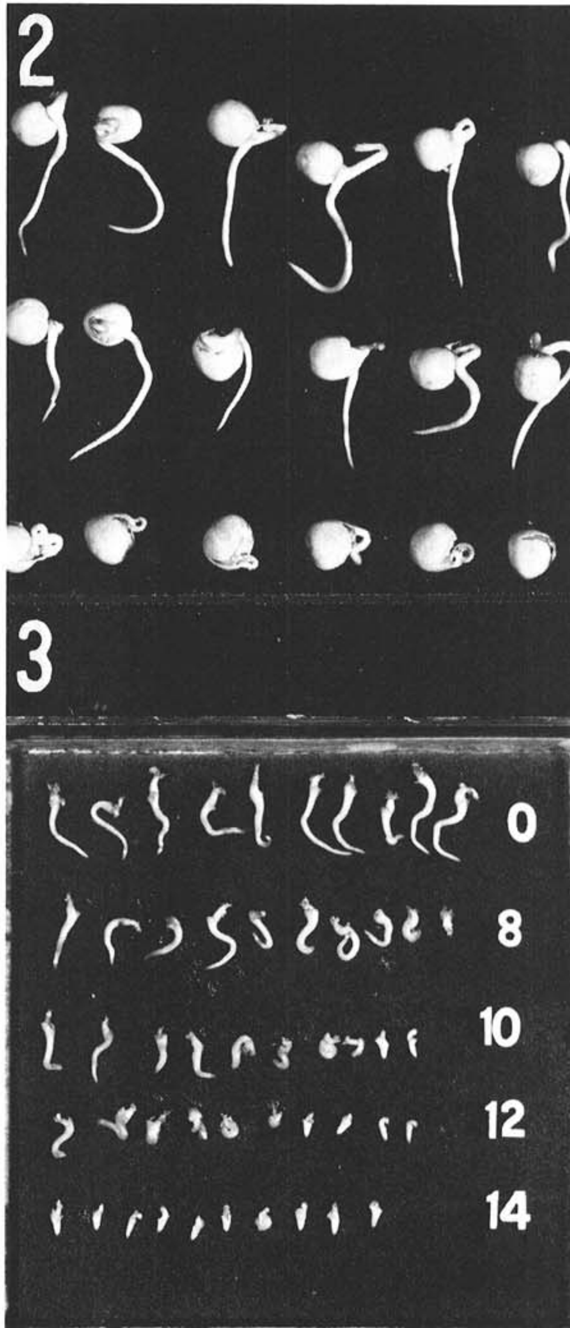


Fig. 1. All data presented represent the mean of at least 30 observations from at least two separate experiments. **A)** Percentage of infection by *Aspergillus ruber* of intact pea seeds, cotyledons, and embryonic axes after various periods of incubation. **B)** Percentage of intact seeds inoculated with *A. ruber* or not inoculated germinating normally, abnormally, or not germinating after various periods of incubation. **C)** Percentage of pea embryonic axes growing normally, abnormally, or not growing, and their mean increase in weight over the average weight (7 mg) of a single freshly isolated embryonic axis after storage for various lengths of time after inoculation with *A. ruber*.



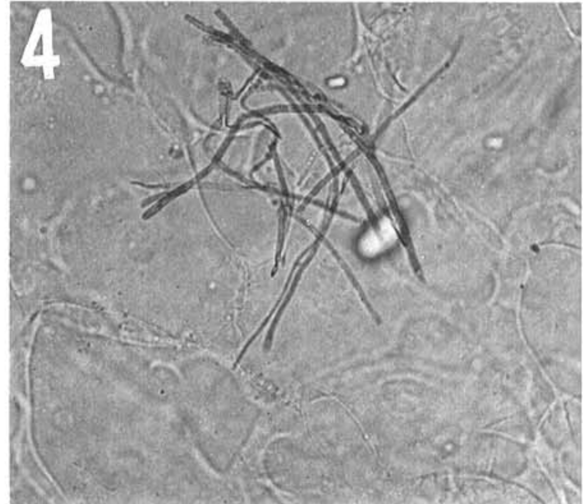


Fig. 2-4. 2) Normally germinating peas from a noninoculated, nonincubated sample (upper row) and from a sample of noninoculated peas incubated at 30 C over saturated $NH_4H_2PO_4$ for 14 weeks (middle row). Abnormally germinating peas inoculated with *Aspergillus ruber* incubated 14 weeks at 30 C over saturated $NH_4H_2PO_4$ (bottom row). **3)** Excised embryonic axes after 3 days' growth on sucrose-mineral salts agar. The numbers along the right side of the figure show the number of weeks the particular sample was incubated at 30 C over $NH_4H_2PO_4$ after inoculation with *A. ruber*. In the row of axes from peas incubated 12 weeks, the growth of the axis on the far left was considered normal, the next 5 abnormal, and the last 4 dead. **4)** A strip of the inner parenchymous layer of the pea seed coat and hyphae of *A. ruber* associated with it. Note that the hypha strands are not firmly attached to the parenchyma, and that the hyphae are of different widths (X 150).

the inner, largely dead parenchymous layer of the seed coat which forms an envelope completely surrounding the hypocotyl portions of the embryonic axis, most of the hyphae are found. The hyphae vary in size from 0.5 to 2.5 μ in width. They are not firmly attached to the parenchymous layer of the seed coat (Fig. 4), and the larger hyphae frequently float free in the mounting solution. Every infected seed examined contained hyphae in association with this layer, including the area enveloping the hypocotyl.

The numbers of embryonic axes found to be infected by plating on malt-salt agar often differed with histological observations. In lots where *A. ruber* grew from some of the embryonic axes, no hyphae were observed in crushed axes of the same lot upon microscopic examination. This may result either from the difficulty of seeing very small hyphae (0.5 μ wide) on examination of crushed axes, or from removing portions of the infected enveloping parenchymous layer along with the embryonic axes when transferring the axes to malt-salt agar.

The direct result of the fungal penetration itself would seem of minor importance in the death of the axes, regardless of whether a portion or all of the embryonic axes is infected when the axes are killed. The fungus is either present in very small quantities or still absent when the axes are killed. This fact, coupled with the fact that extracts from peas infected 14 weeks or from infected autoclaved peas are highly toxic to healthy embryonic axes whereas comparable

extracts from noninfected peas are not toxic (6), strongly indicates that deterioration of *A. ruber* infected pea seed is induced by a toxin produced by the fungus.

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