

Inability of Storage Fungi to Invade Pea Embryos: Evidence Against Phytoalexin Involvement

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ABSTRACT

Storage fungi seldom invade embryos of pea seeds. Dry seeds (9 or 20% moisture content) noninfected or infected with *Aspergillus ruber* contained no phytoalexins or other detectable fungal growth inhibitors. Pisatin, however, was isolated from imbibed cotyledons challenged or unchallenged with *Alternaria alternata*. Thus, while imbibed pea seeds can produce measurable quantities of pisatin, the observed resistance of dry pea embryos to *Aspergillus ruber* invasion cannot be explained by the phytoalexin hypothesis.

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In an earlier paper (2), we demonstrated that pea and squash embryos were seldom invaded by pathogenic storage fungi even though dead parenchymatous layers between the testae and embryos were heavily infected. These fungi can readily invade the embryos of other seeds (e.g., wheat). We postulated that some material inhibitory to fungi may be present in the embryos of pea and squash seeds. Lindsey and Turner (3) demonstrated that seeds of peanuts contain materials inhibitory to fungal growth. In addition, phytoalexins (i.e., pisatin and inermine) (1, 4, 6) are produced by peas in response to fungal invasion, and if these metabolites were present, they should prevent storage fungi from invading seeds.

The present study was undertaken to search for preformed materials in dry pea seeds that are inhibitory to fungi, and to find whether phytoalexins are produced in dry infected pea seeds. All experiments were replicated, and most were repeated three or more times with similar results.

MATERIALS AND METHODS.—Untreated pea seeds (*Pisum sativum* L. 'Alaska') were used throughout this study. They were supplied by Asgrow Seed Company and were free of internal infection. We examined dry whole seeds (approximately 9% moisture content); seeds that were stored aseptically at 30 C and 92% relative humidity for 8 weeks (which resulted in a moisture content of 20%), and seeds also stored at 92% relative humidity for 8 weeks, but infected with *Aspergillus ruber* (Konig, Spieckermann, and Bremer) Thom and Church (2). We also examined pea cotyledons excised after 16 hours of soaking. These cotyledons were surface-sterilized in NaOCl (0.85%) for 1 minute and then plated

on potato-dextrose agar (PDA) in a ring 3 cm in diameter. Some of the cotyledons were challenged by placing a 5-mm diameter disk from the edge of an actively growing colony of *Alternaria alternata* (Fries) Keissler in the center of the ring of cotyledons. All of the cotyledons were incubated on PDA for 3 days, and then removed from the medium. Any cotyledons which exhibited contaminating microorganisms were discarded.

Extracts for bioassay or isolation of pisatin were obtained by grinding pea seeds or cotyledons in 80% ethanol (1:4, w/v) in a Waring Blendor. The resulting mixtures were clarified by centrifugation at 10,000 g for 10 minutes at 5 C. The supernatant solutions were concentrated at 40 C to 5-15% of their original volumes under reduced pressure, using a rotary evaporator to remove the ethanol.

Bioassays were accomplished by mixing 2 ml of double-strength PDA at 55 C with 2 ml of filter-sterilized pea extracts in petri dishes 5 cm in diameter. Extracts were sterilized by filtration through a 0.45 μ m pore-size Millipore filter. These were inoculated with a 5-mm diameter disk from the edge of an *A. alternata* colony and incubated 3 days at 25 C. Each bioassay contained extracts from approximately 50 whole peas or six to seven imbibed cotyledons.

Van Etten's (7) procedure was used to isolate and purify pisatin from the concentrated pea seed or cotyledon extracts. Identification of pisatin was accomplished by co-chromatography of purified materials with authentic pisatin (supplied by H. D. Van Etten) and by the characteristic ultraviolet absorption spectrum (4). Chromatography was done on silica gel thin-layer plates (250 μ m thick, E. Merck) on chloroform or in hexane: ethyl acetate:methanol (60:40:1, v/v) (7). Pisatin was quantitated by its molar extinction coefficient in ethanol [$\log \epsilon = 3.86$ at 309 nm (4)].

RESULTS AND DISCUSSION.—All extracts from pea seeds inhibited sporulation of *A. alternata* grown on PDA, an effect previously noted for extracts from dry seeds (5). However, linear vegetative growth of *A. alternata* was not inhibited on extracts from whole seeds at 9 or 20% moisture content, regardless of whether or not they were infected. In contrast, linear growth of *A. alternata* on PDA containing extracts from challenged or unchallenged cotyledons was only 39% of the control. Pisatin was isolated from challenged or unchallenged cotyledons (10 and 7 μ g per cotyledon, respectively) but could not be obtained from any dry whole seeds (9 or 20% moisture content).

Results of a previous study (2) indicated that some substance in pea embryos inhibited invasion by storage fungi, even though these fungi killed pea seeds. In the present study, we could not detect pisatin or any other substance which inhibited fungal growth in dry infected or uninfected pea seeds. Pisatin, was, however, easily obtained from imbibed cotyledons, indicating that our isolation technique was adequate, and that pea seeds can produce phytoalexins. Since phytoalexins or other growth inhibitions were not detected in dry pea seeds they cannot account for the observed resistance of pea embryos to storage fungi. Thus, the pea-*A. ruber* system represents a pea-pathogen relationship where the phytoalexin hypothesis cannot explain resistance or susceptibility.

Pea seeds apparently have the ability to impede the penetration of the embryo by storage fungi in the absence of fungitoxic host metabolites. It seems probable that resistance mechanisms exist within these seeds that are not detectable by *in vitro* assays of fungal growth on extracts of infected tissues. These mechanisms should be identified; their role, if any, in resistance to other seed-attacking microorganisms defined, and their relationship to other resistance mechanism (e.g., phytoalexins) elucidated.

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