The Isolation of Phytoalexins from Germinating Seeds of Cicer arietinum, Vigna sinensis, Arachis hypogaea, and Other Plants

N. T. Keen

Associate Professor, Department of Plant Pathology, University of California, Riverside, California 92502.
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ABSTRACT

Germinating seeds of several plant species challenged with the native microflora produced the same phytoalexins as other plant tissues. Processing moderate amounts of incubated seeds allowed the isolation of 30- to 100-mg amounts of the phytoalexins.

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Additional key words: demethylhomopterocarpin, maackiain, kievitone.

The isolation of highly purified phytoalexins from pathogen-infected plants in amounts sufficient for chemical characterization is often a tedious and time-consuming task. Although this difficulty can be circumvented by challenging fleshy plant tissues such as fruits, storage tissues and cotyledons with microorganisms or chemical elicitors (4), many plants do not form relatively large masses of such parenchymatous tissues. I report here that germinating seeds of several plant species challenged by the native microflora produce relatively large amounts of phytoalexins which can be readily isolated by conventional means.

Commercially purchased seeds that were not treated with fungicides were soaked in water for approximately 24 hours, sliced into 1- to 3-mm-thick pieces with a razor blade and placed in petri dish or cake-pan moist chambers with 0.01 M potassium phosphate, pH 7.5 for 3-5 days at 25 C. No microorganisms were added to the germinating seeds, but microorganisms present on the seeds multiplied greatly during incubation. The incubated seeds frequently became yellow-brown or dark-colored. Control seeds that were killed by freezing and thawing before the plates were incubated did not become pigmented. The incubated seeds were ground in a Sorvall Omnimixer for 20 seconds with two volumes of 95% ethanol, and the paper filtered extracts were concentrated at 40 C and then extracted with ethyl acetate as previously described (5). These crude extracts were chromatographed on thin-layer chromatography (TLC) plates and examined for ultraviolet-absorbing or fluorescing spots. The plates were then bioassayed for antifungal compounds as described (5). Antifungal substances were isolated from the crude extracts by preparative TLC and characterized by chemical and spectral methods.

To test the seed technique, several species of plants producing known phytoalexins were initially examined. Soybeans [*Glycine max* (L.) Merr.] seeds produced a single antifungal compound, which was identical to hydroxyphaseollin (5, 7), previously isolated from hypocotyls and other tissues. Control seeds that were killed by freezing and thawing before incubation contained no detectable antifungal activity in the TLC bioassay. With the seed technique green bean *(Phaseolus vulgaris* L.) produced phaseollin, peas *(Pisum sativum* L.) produced pisatin, and jackbeans *(Canavalia ensiformis* L.) produced demethylhomopterocarpin.

We previously observed that cowpea *(Vigna sinensis* L.) hypocotyls produced an antifungal compound upon inoculation with an incompatible race of *Phytophthora vignae* Purss. (Partridge and Keen, *unpublished),* but it was difficult to obtain large amounts of the phytoalexin due to the small size of the hypocotyls of the cowpea cultivars used. The seed technique, however, routinely gave 30- to 50-mg amounts of the same chemical as obtained from hypocotyls when approximately 100 g of dry seeds were used. The phytoalexin has been identified as kievitone (2, 8) (Partridge and Keen, *unpublished).*

Incubated peanut *(Arachis hypogaea* L.) seeds produced two antifungal chemicals as determined by the TLC bioassay. Seeds that were killed by freezing did not produce any detectable antifungal activity although they were heavily colonized by microorganisms. The same two antifungal chemicals were also detected in extracts of young stems of peanut plants inoculated with *Phytophthora megasperma* Drechs. var. *sojae* A. A. Hildb. or *Pyrenochaeta terrestris* (Hans.) Gorenz et al., both nonpathogens. The possible role of these inducibly formed chemicals as phytoalexins will be investigated further.

Application of the seed technique to *Cicer arietinum* L. showed that incubated living, but not dead, seeds produced an antifungal compound(s) which on TLC moved at the same R_f as demethylhomopterocarpin. Elution of the active TLC zones and re-chromatography with various solvents suggested a mixture of at least two antifungal compounds, but complete separation of the chemicals could not be achieved using TLC. Accordingly, the TLC eluates were silylated (5) and subjected to combined gas chromatography-mass spectrometry (GC-MS) using a 2 mm × 170 cm column of OV-1 on Gas-Chrom Q [131- to 149-μm particle size (100- to 120-mesh)] (Applied Science Laboratories, State College, Pennsylvania). The chromatograph was operated isothermally at 220 C, with helium as the carrier gas, and mass spectra were obtained using a Finnigan 1015C mass spectrometer with Systems Industries 150 computerized data handling equipment. The mass spectrometer was operated at 300 amperes and 70 eV. The GC-MS runs disclosed the presence of two chromatographic peaks, with retention times and mass spectra indistinguishable from silylated demethylhomopterocarpin and maackiain [authentic samples of demethylhomopterocarpin were isolated from jack beans (3, 6) and were also generously supplied by V. Higgins, University of Toronto, Canada; samples of maackiain were supplied by I. A. M. Cruickshank, CSIRO, Canberra, Australia, and by V. Higgins]. Whether these compounds function as phytoalexins against potential disease-causing organisms in garbanzo beans has not yet been determined.

Seeds of several plant species did not produce antifungal compounds as determined by the above techniques. These plants were *Cucumis sativa* L., *Wisteria* sp., *Oryza sativa* L., *Lens* sp., *Phaseolus aureus* L., *Avena sativa* L., and sunflower. Although inducibly-

formed antifungal compounds from these plants may have escaped detection here, it is noteworthy that phytoalexins with known structures have not been isolated from any of them.

The physiological relevance of compounds isolated by the seed technique in natural disease resistance would require assessment via experiments dealing with naturally susceptible tissues. No relationship of these compounds to natural disease resistance could be concluded in the absence of such data. However, the seed technique proved to be very advantageous in several cases for readily isolating sufficiently large amounts of the various inducibly formed antifungal chemicals for spectral characterization. The major advantages are that large quantities of seed may be processed in relatively small amounts of space and without the necessity of growing large numbers of intact plants. Due to the virtual absence of pigments and nonspecific phenolic substances in extracts from germinating seeds, extraction and subsequent purification of the phytoalexins was simplified. The technique could also be applied to screening various plants for production of possible phytoalexins.

A large literature has been concerned with the production of aflatoxins and mycotoxins in microorganism-infested seeds of higher plants. The data presented here indicate that such infested seeds could themselves produce antibiotic compounds in response to the presence of microorganisms. In view of the toxicity of several known phytoalexins to animal tissues (1, 9, 10), this possibility would appear worthy of further investigation.

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