Association of the Phytoalexin Kievitone with Single-Gene Resistance of Cowpeas to Phytophthora vignae

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ABSTRACT

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Cowpeas monogenically resistant to *Phytophthora vignae* accumulated the isoflavonone phytoalexin kievitone after inoculation, but near-isogenic susceptible cowpea plants produced it at much lower rates. Kievitone was present in the resistant plants at 10-fold or higher concentrations than were required to inhibit mycelial growth of *P. vignae* in culture by 50%. Inoculated plants also produced the isoflavonoids daidzein and coumestrol, but no differences were seen in their rates of accumulation between inoculated resistant and susceptible plants. The data support the hypothesis that the basis for the expression of monogenic resistance of cowpea plants to *P. vignae* is a specific derepression of kievitone biosynthesis.

We isolated a fungitoxic compound from hypocotyls of cowpea (*Vigna sinensis* L. Endl. ex Hassk.) plants inoculated with the phytopathogenic fungus *Phytophthora vignae* Purss, and from germinating seeds of the same plant (5). The same chemical was also obtained from lima beans (*Phaseolus lunatus* L.). Spectral and chemical data disclosed that the antifungal compound was identical to kievitone, formulated as I (Fig. 1) by Burden et al. (3). Smith et al. (12) later confirmed the structure of kievitone from green beans and Bailey (1) reported its accumulation in virus-infected cowpea plants.

This study is concerned with the possible role of kievitone and related isoflavonoid compounds in the resistance of cowpea plants to *P. vignae,* the causal organism of stem rot of cowpea (9).

MATERIALS AND METHODS

Inoculation methods.—The cowpea cultivars Poona (susceptible to *P. vignae)* and Caloona (a near-isogenic resistant line) (10, 11) were generously supplied by G. S. Purss, Director of the Plant Pathology Branch, Department of Primary Industries, Queensland, Australia. Blackeye-5 cowpea seed was also used for production of kievitone by the seed method (5). Cowpea plants were grown in U.C. mix (2) in a growth chamber on a 12-hour photoperiod at 26,900 lx intensity and 27 C day temperature and 22 C night. The hypocotyls were inoculated with *P. vignae* race 2 (P606, G. S. Purss' isolate K901). Plants were inoculated by piercing the hypocotyls with a No. 22 hypodermic needle and introducing young mycelium of the fungus grown on pea broth medium (7). The hypocotyls were harvested at intervals for extraction of isoflavonoids.

Isolation of kievitone.—Cowpea hypocotyls or incubated germinating seeds (5) were ground in a Sorvall Omni-mixer with 2 ml of 95% ethanol per gram (fresh weight) of tissue. The ethanol extracts were concentrated to approximately one half the original volume and extracted with hexanes. The ethanol-water layer was saved, further concentrated, and then extracted three times with ethyl acetate. The ethyl acetate layers were dried with $MgSO_4$ and evaporated. After dissolution in a small volume of ethyl acetate, the extract was streaked onto 0.5-mm silica gel GF_{254} (E. Merck, Darmstadt, Germany) thin-layer chromatography (TLC) plates and developed with solvent I (Table 1). A single antifungal factor was detected by the TLC bioassay (7), and found to correspond to an ultraviolet (UV) light absorbing band. Thereafter, it was visualized by absorbance at 254 nm, and the appropriate band was scraped from the plates and eluted with acetone. The material was again streaked onto a TLC plate and developed with solvent VII (Table 1). After again recovering the material from the silica gel with acetone, the antifungal substance chromatographed as a single UV-absorbing spot in several solvent systems (Table 1). Only one spot was seen on the TLC plates when H_2SO_4 charring, fast blue salt B, or anisaldehyde-acetic acid (13) was used as a detection reagent. This purified material was identified as kievitone (3) by comparison with an authentic sample generously supplied by D. A. Smith: $[\lambda_{max}^{ethanol}$ (log E)] = 206 (4.38), 225 (s) (4.28), 294 (4.09) and 335 (s) (3.86); ethanolic NaOH = 337 nm (4.16);

TABLE 1. Solvent effects on chromatographic R_f values for kievitone on silica gel GF_{254} thin-layer chromatography plates.

	Solvent[a]	R_f
I.	Hexane:ethyl acetate:methanol (60:40:1, v/v)	0.15
II.	Hexane:ethyl acetate:methanol (50:50:1, v/v)	0.32
III.	Ethyl acetate:benzene:methanol:water (100:80:20:20, v/v)	0.69
IV.	Chloroform:acetone:20% NH_3 (65:35:1, v/v)	0.07
V.	Chloroform:acetone:acetic acid (90:10:1, v/v)	0.32
VI.	Chloroform:isopropanol (90:10, v/v)	0.25
VII.	Toluene:chloroform:acetone (40:25:35, v/v)	0.13

[a]Solvents were prepared on a volume basis.

$[\alpha_{20}^{MeOH}] = +6.50$; $M^+ = 356$ ($C_{20}H_{20}O_6$); other major peaks at m/e 338, 221, 205, 192, 177, 165, 136; NMR (d_6 acetone) (δppm); 6H singlet (1.40), 4H doublet (1.66), 2H doublet (4.80), 1H singlet (6.03), 1H doublet (6.20), 1H doublet (6.43), and 1H doublet (6.60). An authentic sample of kievitone gave identical mass spectral data with the cowpea and lima bean compounds and identical empirical formulae for the major fragments (12).

Quantitation of isoflavonoid compounds.—Ethanol extracts prepared as above from weighed cowpea hypocotyls were evaporated to dryness, transferred to small vials with peroxide-free ether, and the ether was expelled with N_2. The residue was redissolved in ethyl acetate [1 ml/g fresh weight (fr. wt)] and 80-μl aliquots spotted onto 0.375 mm silica gel GF_{254} plates. The plates were developed with solvent VI (Table 1) and areas corresponding to kievitone, daidzein, and coumestrol under 254 and 360 nm light were scraped from the plate and transferred to centrifuge tubes. One ml of 95% ethanol was added, stirred, and (after centrifugation) the supernatant fluids were analyzed for isoflavonoids.

Kievitone was estimated by absorption at 294 nm (E = 12447). Daidzein (II, Fig. 1) and coumestrol (III, Fig. 1) were analyzed by fluorimetry as described elsewhere (8).

Bioassays.—Kievitone was initially detected when crude extracts were run on TLC plates by the TLC bioassay method using *Cladosporium cucumerinum* (7). Activity of kievitone against *Phytophthora vignae* was determined by adding the purified material in 95% ethanol to petri dishes, followed by V-8 juice agar, so that the final ethanol concentration was 3% (7). Only ethanol was added to control plates. The molten agar was swirled in the plates before it solidified and agar plugs of *P. vignae* mycelium were placed at the center of the plates. Plates were incubated at 25 C, colony areas were determined at 24-hour intervals, and a standard dosage response curve was drawn.

RESULTS

Identification and antifungal activity of kievitone.

—Kievitone was not detected in healthy cowpea plants, but it was readily obtained from inoculated plants. Kievitone was active in the TLC bioassay at a minimum of 5 μg spotted. Preparations that had been stored for some time showed a higher R_f spot that also possessed weak antifungal activity. The identity of this presumed decomposition product was not determined. In agar bioassays against *P. vignae* (Fig. 2), kievitone had an ED_{50} concentration of 2.8×10^{-5} M (about 10 μg/ml) for inhibition of mycelial growth.

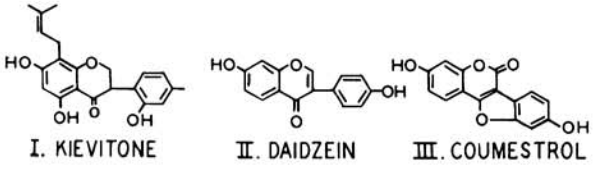


Fig. 1. Structures of cowpea isoflavonoid compounds: I, kievitone; II, daidzein; and III, coumestrol.

Isoflavonoid levels in cowpea hypocotyls.—The inoculation site of resistant Caloona hypocotyls became a purple-red color at 18 hours after inoculation and colonization by the fungus apparently had ceased by 24 hours. Inoculated susceptible plants acquired a similar color, but colonization by the fungus continued and the plants were about 90% rotted within 62 hours. Longitudinal cryostat sections through the inoculated areas of resistant and susceptible hypocotyls showed that the fungus had not ramified appreciably through resistant tissues after 18 hours, but the fungus rapidly colonized susceptible hypocotyls within that time and oospores of the fungus were seen at 48 hours.

Resistant inoculated Caloona hypocotyls contained detectable amounts of kievitone as early as 4 hours after inoculation (Fig. 3-A), and the compound accumulated until 24 hours and then leveled off. Kievitone was first detected in susceptible hypocotyls at 12 hours after inoculation and continued to increase until 62 hours after inoculation. The concentration attained, however, never

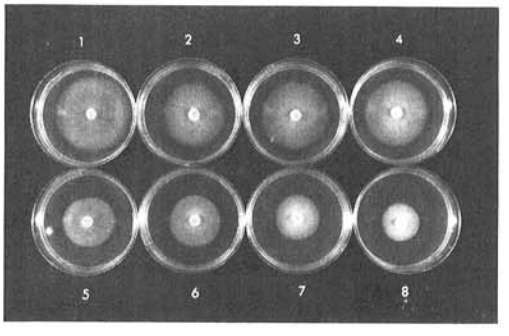
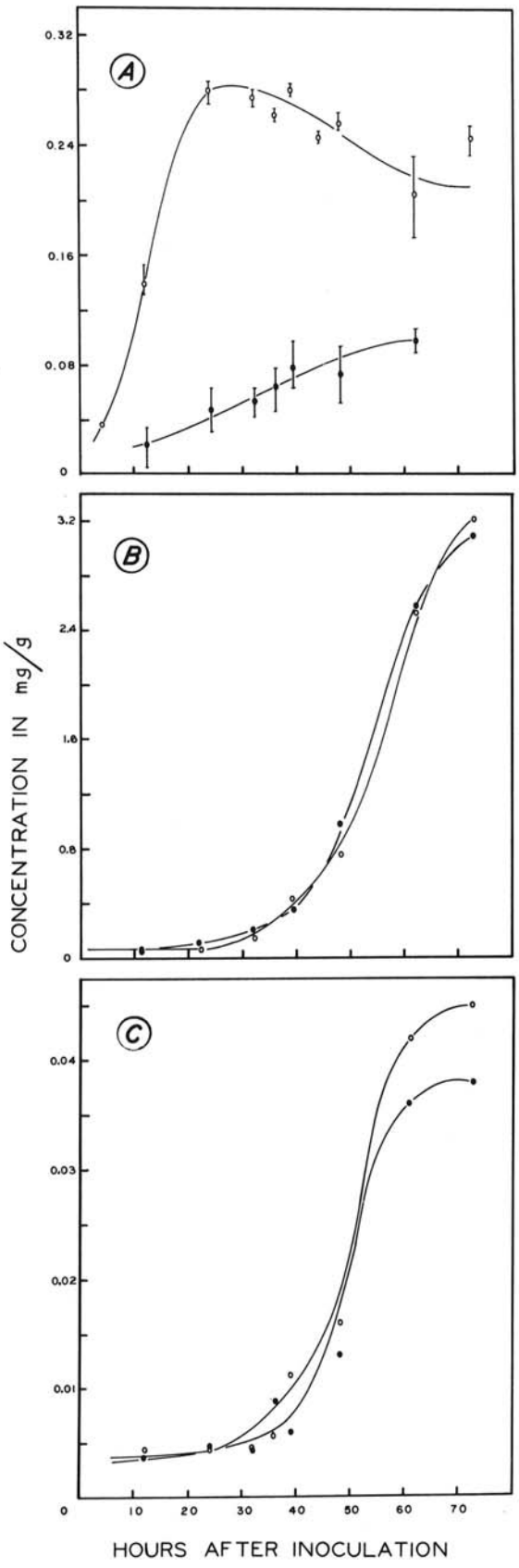


Fig. 2. Growth of *Phytophthora vignae* on V-8 juice agar supplemented with various concentrations of purified kievitone; numbers 1 through 8 denote concentrations of 0, 2.8×10^{-6} M, 5.3×10^{-6} M, 9.2×10^{-6} M, 2.8×10^{-5} M, 5.3×10^{-5} M, 9.2×10^{-5} M, and 1.4×10^{-4} M, respectively.



reached that in the resistant hypocotyls, and the production of kievitone in susceptible plants was at least five-fold slower than in the resistant Caloona. Kievitone was not detected in plants that were not wounded and inoculated. The compound was never detected in wounded (noninoculated) Poona plants, but occasionally was detected in wounded (noninoculated) Caloona plants.

Daidzein was present as a constituent of normal cowpea hypocotyls at 50 μg/g dry wt. This isoflavone accumulated in both resistant and susceptible inoculated hypocotyls, reaching 3,200 μg/g after 72 hours (Fig. 3-B), but no differences were observed in the concentration present in resistant or susceptible plants.

Coumestrol was detected in nonwounded cowpeas at 5 μg/g. It also accumulated in resistant and susceptible inoculated plants, reaching about 45 μg/g after 72 hours (Fig. 3-C). As with daidzein, no differences were seen in the curves for resistant and susceptible inoculated plants.

DISCUSSION

The production of kievitone in cowpeas inoculated with *P. vignae* meets several criteria for a phytoalexin: (i) it accumulated faster in response to fungus inoculation than to wounding and was not detected in uninjured plants; (ii) it accumulated more rapidly in monogenically disease-resistant than in near-isogenic susceptible hypocotyls; and (iii) accumulation in resistant plants occurred at or before the time (12-24 hours) when cessation of fungus growth occurred.

The fact that kievitone was produced more rapidly in resistant Caloona cowpeas than in near-isogenic susceptible Poona plants suggests that differential production of the compound is the basis for resistance to *P. vignae*. The concentration of the compound attained in resistant plants when fungal development ceased at approximately 24 hours (approximately 280 μg/g tissue) is also sufficiently in excess of the ED_{50} concentration for inhibition of *P. vignae* (approximately 10 μg/ml) to account for the inhibited growth of the fungus in the resistant plants. These data do not prove a cause-effect relationship, but they suggest that derepressed kievitone production is associated with the resistance of Caloona cowpeas to *P. vignae*.

If, as hypothesized for other legume host-parasite systems (4, 6, 8), the *Phytophthora*-resistance gene in cowpeas is a regulator gene for isoflavonoid biosynthesis, resistance should result in a coordinate derepression of isoflavonoid production. However, this was not observed. Daidzein and coumestrol, two isoflavonoids that have not been shown to have high antifungal activity, both accumulated after inoculation, but no differences were observed in resistant and susceptible reacting plants. Furthermore, unlike kievitone, which increased in the resistant plants at about 10 hours after inoculation, coumestrol and daidzein did not accumulate until about

Fig. 3. Levels of isoflavonoid compounds produced by susceptible Poona (closed points) and resistant Caloona (open points) cowpeas after inoculation with *Phytophthora vignae*. Brackets represent standard errors of three replicate analyses. A = kievitone; B = daidzein; C = coumestrol.

50 hours, a time when kievitone levels were declining. Therefore, it appears that, unlike plants such as soybeans (4, 6, 8), the expression of resistance in cowpeas to *P. vignae* involves a specific derepressed biosynthesis of only one isoflavonoid compound, that being kievitone.

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