

Observations on the Fungitoxicity of the Phytoalexin, Kievitone

David A. Smith

Lecturer, Plant Biology Department, The University, Hull, HU6 7RX, United Kingdom.

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ABSTRACT

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Kievitone, kievitone hydrate, and three other kievitone derivatives were bioassayed against *Aphanomyces euteiches* and *Rhizoctonia solani* in solid and/or liquid media. These five compounds also were assayed on thin-layer chromatography plates versus *Fulvia fulva* and *Cladosporium herbarum*. Results from these various bioassays suggested that the fungitoxicity of the phytoalexin, kievitone, depended upon its free phenolic hydroxyl groups and lipophilic side-chain. Kievitone inhibited the growth of *A. euteiches* and *R. solani* in solid and liquid aqueous media that were prepared without any organic solvent amendment. Although

kievitone had little effect on the radial mycelial growth of *Fusarium solani* f. sp. *phaseoli* on agar, it considerably reduced the increase in mycelial dry weight in liquid culture. Kievitone appeared to be as effective a fungitoxicant when assayed in solely aqueous media as it was when incorporated in similar media in the presence of small amounts of organic solvent. The radial mycelial growth of *A. euteiches* and *R. solani* was steadily repressed by kievitone; neither fungus appeared to adapt to the phytoalexin. *Fusarium solani* f. sp. *phaseoli*, on the other hand, was insensitive to kievitone in agar over a period of 6 days.

Additional key words: isoflavonoids, antifungal compounds.

Phytoalexins primarily are considered to be antifungal compounds (7), although some exhibit toxicity towards bacteria (9, 16), plants (24, 33), and animals (19, 33). Since certain phytoalexins may help to limit fungal colonization of plant tissues (7, 12, 20, 28), factors influencing their fungitoxicity merit close examination. It is important to establish how they exert antifungal activity as well as what features of their chemistry allow this rôle. Furthermore, as others have stated (3, 6), appropriate bioassay systems must be employed so that valid conclusions may be drawn concerning the function of phytoalexins in disease resistance. This paper attempts to answer some of these points as they affect the isoflavanone, kievitone (Fig. 1-A₁), a phytoalexin produced by members of the leguminous genera *Phaseolus* (4, 27), *Vigna* (1, 18, 20) and, apparently, *Dolichos* (T. K. Kandaswamy, *personal communication*).

The author previously reported several effects of kievitone on the vegetative growth of three pathogenic fungi (25, 26). These observations led to the suggestion that kievitone's fungitoxicity might be a function of its free phenolic groups. No direct evidence, however, was presented pertaining to this. Harborne et al. (10) proposed that the antifungal activity of the isoflavone luteone, which like kievitone possesses four phenolic hydroxyl groups, was enhanced, relative to that of other isoflavones, by its five-carbon side chain; kievitone has an identical substituent (4, 29). In an attempt to evaluate which structural feature(s) of kievitone primarily deter-

mine its antifungal activity, the fungitoxicities of a number of kievitone derivatives were compared; this paper, in part, presents these findings.

MATERIALS AND METHODS

Cultures.—The isolates of *Aphanomyces euteiches* Drechs., *Rhizoctonia solani* Kühn and *Fusarium solani* (Mart.) Sacc. f. sp. *phaseoli* (Burk.) Snyder & Hans. were those used previously (25). *Fulvia fulva* (Cooke) Ciferri (*Cladosporium fulvum* Cooke) was from the departmental culture collection; *C. herbarum* (Pers.) Link ex. S. F. Gray was obtained from the Commonwealth Mycological Institute, Kew, U. K. These two fungi routinely were grown on potato-dextrose agar at 25 C but were transferred to malt extract agar shortly before experimental use.

Sources and syntheses of kievitone and its derivatives.—Kievitone was purified as outlined earlier (25). Kievitone hydrate [KH (Fig. 1-C)] was provided by P. Kuhn (Plant Biology Department, The University, Hull); it had been purified as described elsewhere (15). Quantitation of these compounds was based on extinction coefficients of $\log E_{294nm} = 4.17$ (kievitone, 29) and $\log E_{293nm} = 4.22$ (KH, 15).

Following the general procedure outlined by Burden et al. (4) and J. Bailey (*personal communication*), treatment of kievitone with acid yielded the 2,2-dimethylchroman isomer. Since this derivative has the five-carbon side chain of kievitone cyclized with the C-7 hydroxyl (Fig. 1-A₁ and B₁), it will be given the trivial name "cyclized kievitone" (CK) hereafter. One to 2 mg of kievitone were

dissolved in 0.25 ml glacial acetic acid. An additional 0.75 ml of acetic acid, containing two drops of concentrated H_2SO_4 , was then added; the mixture was shaken and left to stand at room temperature for about 4 hr. At this time, the solution was transferred to a separatory funnel and approximately 30 ml of diethyl ether and 10 ml of water were added. After shaking and partitioning, the ether phase was collected. A second partitioning with diethyl ether was carried out and the combined ether fractions were taken to dryness under reduced pressure at about 40°C. The residue was dissolved in a small volume of ethanol prior to thin-layer chromatography (TLC) on pre-washed silica gel (sg). The CK and residual kievitone were separated by developing the TLC plate with toluene/ethyl formate/formic acid (7:2:1, v/v) (2) under unsaturated conditions; CK and kievitone had R_f values of 0.23 and 0.16, respectively. Both compounds were detected by their orange-brown reaction products upon spraying with diazotized *p*-nitroaniline (DPN) (30). The CK was eluted in ethanol from a nonsprayed portion of the plate.

Methylation of kievitone was accomplished by methyl iodide treatment (8). About 3 mg of kievitone was dissolved in 4 ml of dry acetone; 0.4 g of anhydrous K_2CO_3 and 0.2 ml of methyl iodide were added to the solution. The mixture was refluxed for 90 min at 60–80°C. After being refluxed, the contents of the flask were washed out with ethanol and filtered through glass fiber. The filtrate was dried, and chromatographed as outlined above for CK. The tetramethylated derivative of kievitone (Fig. 1-A₂), referred to hereafter as TMK, reacted slowly with DPN to yield a peach-colored product, R_f 0.89.

A sample of CK was methylated and the trimethylated product subsequently was purified by the procedure described above for TMK. This derivative, "cyclized, trimethylated kievitone" (CMK) (Fig. 1-B₂) also reacted slowly with DPN to give an apricot-colored reaction product, R_f 0.85.

The ultraviolet (UV) extinction coefficients of CK, TMK, and CMK were determined by weighing samples with a Cahn Model M-10 Electro-balance (Cahn Instrument Co., Paramount, CA 90723) and measuring the absorbance of aliquots of the same samples using a Pye Unicam SPI800 recording double-beam spectrophotometer. Mass spectrometric analyses of these three kievitone derivatives were carried out by A. Roberts, Chemistry Department, The University, Hull. Quantitation of CK, TMK, and CMK for bioassays was carried out by UV spectroscopy; the extinction coefficients are given in the Results section.

Kievitone derivative bioassays.—Incorporation of 0.01% Triton X-100 (BDH Chemicals Ltd., Poole, England) in conjunction with 2% ethanol or 2% dimethylsulfoxide (DMSO) maintained TMK and CMK in solution at the concentrations assayed. Although kievitone, CK, and KH were readily soluble in media containing only 0.5% ethanol or 0.5% DMSO, these three compounds also were assayed in media containing 2% ethanol + 0.01% Triton X-100 or 2% DMSO + 0.01% Triton X-100 to allow valid comparisons of relative fungitoxicities.

Radial mycelial growth bioassays were conducted by slight modification of the technique reported elsewhere

(25, 27). The "M-2" medium (17, 28) was used throughout, and the compounds were incorporated in the organic solvents described above. Glass petri plates (51 × 18 mm) were employed; 2.0 ml agar were dispensed into each plate. The fungi were incubated for 72 hr, at which time measurements of the extent of radial mycelial growth were recorded. Assay for *A. euteiches* was conducted in the presence of ethanol and that for *R. solani* in the presence of DMSO; *A. euteiches* grew very poorly when 2% DMSO was present. Each compound was assayed against *A. euteiches* at 7×10^{-5} M (25 μg kievitone/ml) and against *R. solani* at 1.4×10^{-4} M (50 μg kievitone/ml).

A liquid culture bioassay with *R. solani* as the test fungus was carried out as described previously (25), except that the organic solvents employed were 2% DMSO + 0.01% Triton X-100. The fungus was exposed to 1.4×10^{-4} M solutions of each of the five compounds.

Kievitone and its four derivatives were bioassayed in a third manner, by modifications of the *Cladosporium*-TLC plate procedure (2). Five separate spots of kievitone, CK, TMK, CMK, and KH were applied in ethanol to the central portion of a 20 × 20 cm TLC plate. The spots were about 3.5 cm apart; each received 5.6×10^{-8} mole (20 μg of kievitone) of the particular compound. When the ethanol had evaporated, the plate was sprayed with a spore suspension of *F. fulva* made up in liquid medium (25) containing about five drops of Tween-80 (Sigma Chemical Co. Ltd., Kingston-upon-Thames, England). The plate was placed in a moist chamber at 25 ± 2 °C in the dark for 5 days and then examined for zones of inhibition. A similar bioassay was conducted with *C. herbarum*. In this case, however, spores suspended in sterile water plus about five drops of Tween-80 were sprayed on the plate, which subsequently was oversprayed with molten potato dextrose agar.

Kievitone-in-water bioassays.—Dried kievitone was redissolved in sterile water and quantified by removing aliquots and measuring their absorbance in water acidified to pH 2.0 with HCl. Kievitone's ethanolic extinction coefficient was used since identical samples of kievitone dissolved in ethanol or water (pH 2.0) gave nearly superimposable UV absorption spectra. At pH values approaching or greater than neutrality, dissociation of kievitone's hydroxyl functions occurs, altering the UV absorbance (29). Acidified water was used only for kievitone quantitation; bioassays were conducted near neutrality.

For solid medium bioassays, a known amount of kievitone in 1.5 ml water was mixed with 1.5 ml double-strength (ds) M-2 agar in 51 × 18 mm glass petri plates. Controls comprised 1.5 ml water mixed with 1.5 ml ds agar. When the agar had cooled, inoculum disks were applied in the usual manner (25). *Aphanomyces euteiches*, *R. solani*, and *F. solani* f. sp. *phaseoli* were exposed to 2.8×10^{-5} M, 1.1×10^{-4} M, and 2.8×10^{-4} M kievitone (10, 40, and 100 μg/ml), respectively. The plates were incubated and mycelial growth subsequently was measured as described elsewhere (25).

For liquid culture bioassays, the medium and general procedure were as outlined earlier (25). However, each flask contained only 3.0 ml of culture medium for the first 24-hr incubation period. At this time, kievitone was added in 1.0 ml water; control flasks received only water. The fungi were incubated for a further day and harvested

in the normal manner. *Aphanomyces euteiches*, *R. solani*, and *F. solani* f. sp. *phaseoli* were bioassayed at 4.2×10^{-5} M, 1.4×10^{-4} M, and 2.8×10^{-4} M kievitone (15, 50, and 100 $\mu\text{g/ml}$), respectively.

Growth rate bioassays.—These bioassays followed the procedure outlined previously (25). Larger glass petri plates (51 \times 18 mm), however, were employed and 2.5–5.0 ml agar were dispensed into each plate. *Aphanomyces euteiches*, *R. solani*, and *F. solani*, f. sp. *phaseoli* were exposed to 2.8×10^{-5} M, 1.1×10^{-4} M, and 2.8×10^{-4} M kievitone, respectively. Increases in colony diameters were recorded for a maximum of 7 days.

RESULTS

Characterization of kievitone derivatives.—The mass spectrometry data indicated that CK, TMK, and CMK had molecular ions at 356, 412, and 398 m/e, respectively. All three compounds exhibited UV absorbance similar to that of kievitone with maxima in ethanol at 293 nm (log $E = 4.27$, CK) 289 nm (log $E = 4.16$, TMK) and 293 nm (log $E = 4.22$, CMK). The absorbance maximum of CK remained at 293 nm upon the addition of alkali as reported by Burden et al. (4); TMK and CMK also maintained their original absorbance maxima in alcoholic NaOH. All of the data obtained for CK, TMK, and CMK were consistent with the structures proposed (Fig. 1 - B₁, A₂, and B₂).

Kievitone derivative bioassays.—All bioassays in this series, whether in solid (Table 1) or liquid (Table 2) media, versus *A. euteiches* or *R. solani* or of *F. fulva* or *C. herbarum* on sg produced similar findings — only kievitone and CK exerted consistent antifungal activity. Any inhibition caused by TMK and CMK generally was negligible. On the other hand, KH exhibited some considerable toxicity in two of the five separate bioassays. This compound had no effect on *F. fulva* or *C. herbarum*

TABLE 1. The effects of kievitone and four kievitone derivatives on the radial mycelial growth of *Aphanomyces euteiches* and *Rhizoctonia solani*

Compound ^a	<i>A. euteiches</i>		<i>R. solani</i>	
	Growth (mm) ^b	(%) ^c	Growth (mm) ^b	(%) ^c
None	26.83 \pm 1.58	100	30.92 \pm 1.08	100
Kievitone	9.67 \pm 1.83	36	15.50 \pm 1.50	50
CK	8.25 \pm 0.25	31	15.67 \pm 1.08	51
TMK	25.92 \pm 1.92	97	33.42 \pm 2.58	108
CMK	26.75 \pm 0.25	100	29.33 \pm 0.92	95
KH	15.33 \pm 2.67	57	27.92 \pm 0.17	90

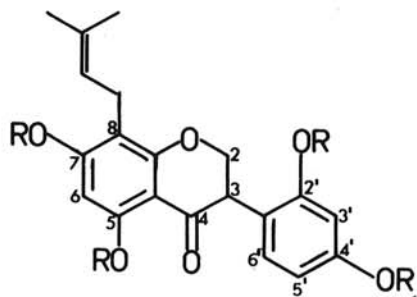
^aEach compound was incorporated in agar as described in the text. *Aphanomyces euteiches* was exposed to 7×10^{-5} M of each compound, *R. solani* to 1.4×10^{-4} M. Abbreviations: CK = cyclized kievitone, TMK = tetramethylated kievitone, CMK = cyclized, trimethylated kievitone, and KH = kievitone hydrate.

^bRadial mycelial growth was determined by measuring two diameters of three replicate colonies and subtracting the diameter of each inoculum plug after incubation at 25 C for 72 hr; the maximum variation of individual replicates from the mean of the three replicates is also shown.

^cThese values represent radial growth relative to that of the control, taken as 100%; each value is calculated from the mean growth (mm) of the three replicates.

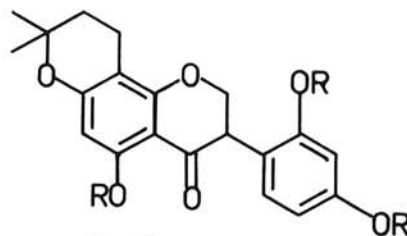
on sg, or on the radial mycelial growth of *R. solani* (Table 1). However, the growth of *A. euteiches* on agar (Table 1) and of *R. solani* in liquid culture (Table 2) was retarded by KH, though in neither case was the inhibitory effect so marked as that caused by kievitone and CK.

Kievitone-in-water bioassays.—When present in M-2 agar in the absence of any organic solvent, kievitone exerted considerable antifungal activity against *A. euteiches* and *R. solani*, but not against *F. solani* f. sp. *phaseoli* (Table 3). In aqueous liquid culture, kievitone



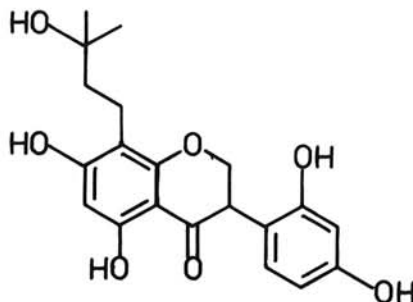
A₁, A₂

A₁: R = H; KIEVITONE
A₂: R = CH₃; TETRAMETHYLATED KIEVITONE



B₁, B₂

B₁: R = H; CYCLIZED KIEVITONE
B₂: R = CH₃; CYCLIZED, TRIMETHYLATED KIEVITONE



C

KIEVITONE HYDRATE

Fig. 1. The structures of kievitone and four kievitone derivatives.

induced mycelial dry weight losses in *A. euteiches* and *R. solani* over a 24-hr period (Table 4). Although kievitone caused no net loss of dry weight in *F. solani* f. sp. *phaseoli* during the same incubation time; nonetheless, growth of this fungus was retarded considerably (Table 4).

Growth-rate bioassays. — Radial mycelial growth of *F. solani* f. sp. *phaseoli* was repressed only marginally by kievitone (Fig. 2-C); inhibition of this organism never

TABLE 2. The effects of kievitone and four kievitone derivatives on the mycelial dry weight of *Rhizoctonia solani* attained in liquid culture

Compound ^a	Mycelium dry weight ^b (mg)	Change ^c (%)
None, 24 hr ^d	3.97 ± 0.57	—
None, 48 hr	8.50 ± 0.30	+114
Kievitone, 48 hr	2.43 ± 0.57	-39
CK, 48 hr	2.67 ± 0.17	-33
TMK, 48 hr	7.87 ± 0.27	+98
CMK, 48 hr	6.70 ± 1.50	+69
KH, 48 hr	4.63 ± 1.27	+17

^aEach compound was incorporated in liquid medium as described in the text. *Rhizoctonia solani* was exposed to 1.4×10^{-4} M of each compound. Abbreviations: CK = cyclized kievitone, TMK = tetramethylated kievitone; CMK = cyclized, trimethylated kievitone, and KH = kievitone hydrate.

^bEach dry weight value is the mean of three replicates; the maximum variation from that value of individual replicates also is shown.

^cThe dry weight attained after 24 hr (3.97 mg) was used to compare the changes in dry weight occurring in control and treatment flasks during the subsequent 24-hr incubation. Each value is the percentage change over the second 24-hr incubation period and is the mean of the three replicates.

^dAge of culture at time of addition of DMSO, Triton, and/or the appropriate compound.

TABLE 3. The effect of kievitone on the radial mycelial growth of *Aphanomyces euteiches*, *Rhizoctonia solani*, and *Fusarium solani* f. sp. *phaseoli*

Organism	Colony diameter (mm) ^a		Growth ^b (%)
	Control	Treatment	
<i>A. euteiches</i>	16.75 ± 1.00	7.58 ± 1.83	45
<i>R. solani</i>	29.42 ± 1.67	10.33 ± 0.42	35
<i>F. solani</i> f. sp. <i>phaseoli</i>	18.58 ± 2.58	17.50 ± 1.00	94

^aRadial mycelial growth was determined by measuring two diameters of three replicate colonies and subtracting the diameter of each inoculum plug after incubation at 25 C on M-2 agar. Each value is the mean of the three replicates; the maximum variation from the mean of individual replicates is also shown. *Aphanomyces euteiches*, *R. solani*, and *F. solani* f. sp. *phaseoli* were exposed to 2.8×10^{-5} M, 1.1×10^{-4} M, and 2.8×10^{-4} M kievitone, respectively. No organic solvent was used in the medium.

^bThese values represent radial growth in the treatment plates relative to that in the respective control plates, taken as 100%; each value is calculated from the mean growth (mm) of the three replicates.

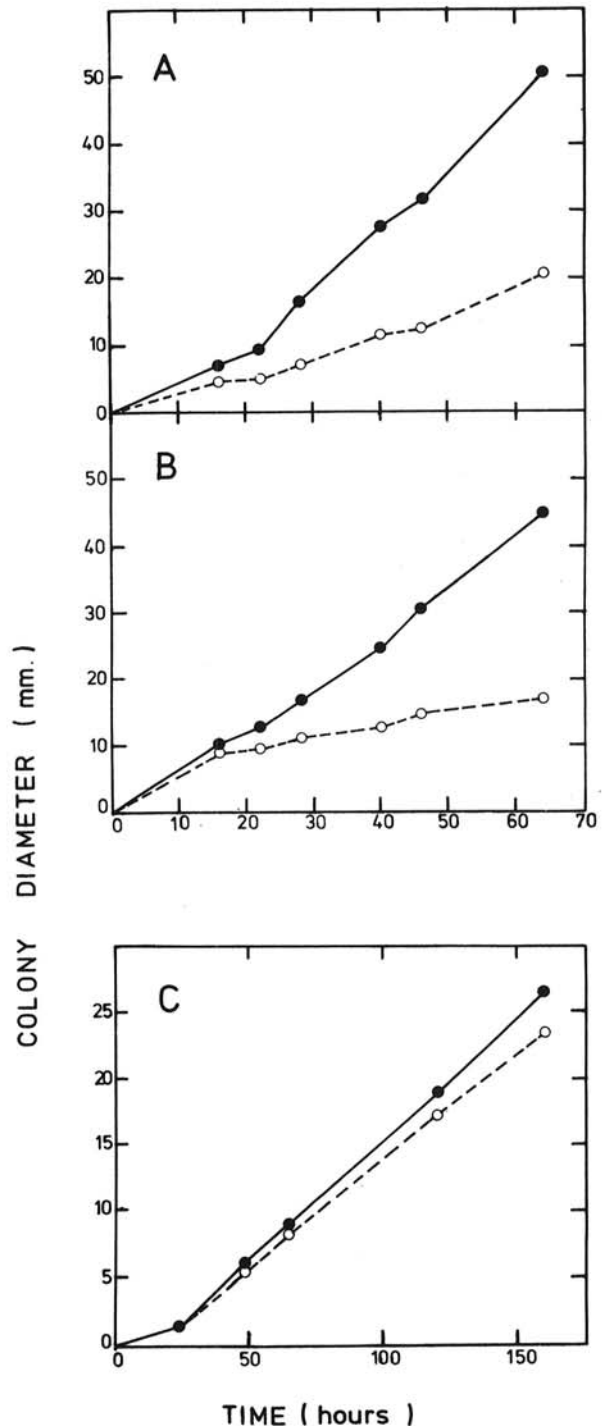


Fig. 2. The effect of kievitone on the increase in colony diameter of A) *Aphanomyces euteiches*, B) *Rhizoctonia solani*, and C) *Fusarium solani* f. sp. *phaseoli*. Kievitone was incorporated in M-2 agar in 2% ethanol (o---o); control plates received only 2% ethanol (●—●). *Aphanomyces euteiches*, *R. solani*, and *F. solani* f. sp. *phaseoli* were exposed to 2.8×10^{-5} M, 1.1×10^{-4} M, and 2.8×10^{-4} M kievitone, respectively. The plates were incubated at 25 C and two measurements of each colony diameter, subtracting the diameter of each inoculum plug, were made at intervals over several days. Each point represents the mean of four replicates.

exceeded 12% at any time during the 1-wk incubation. *Aphanomyces euteiches* and *R. solani* control cultures grew at similar rates, but both were much faster than *F. solani*, f. sp. *phaseoli* (Fig. 2). The growth rate of *A. euteiches* (Fig. 2-A) was consistently inhibited by kievitone throughout the assay period. A generally similar picture was evident for *R. solani* (Fig. 2-B). Here, however, the rates of growth in control and treatment plates were similar for the first 16 hr. Thereafter, the growth rates of *R. solani* in the presence and absence of kievitone diverged, the phytoalexin repressing radial mycelial growth for the following 48 hr.

DISCUSSION

Some years ago, Perrin and Cruickshank (21) proposed that the antifungal activity of pterocarpan isoflavonoids might be critically dependent upon a particular molecular stereochemistry. In a more recent investigation, which encompassed 17 isoflavonoids, VanEtten (32) could not confirm Perrin and Cruickshank's proposal and observed, generally, that the activity of antifungal isoflavonoids did not seem to require a common three-dimensional shape. Johnson et al. (12) pointed out the need to investigate the structure-antifungal relationships of isoflavones.

The possession of free phenolic hydroxyl groups may explain part of the antifungal activity of phenolic pterocarpan (31). Comparison of the fungitoxicities of kievitone, CK, TMK, and CMK (Tables 1 and 2) revealed that masking the phenolic hydroxyls by methylation annulled antifungal activity; bioassays of *F. fulva* and *C. herbarum* on sg confirmed this finding. In this connection, it should be noted that Ravise and Kirkiacharian (23) reported that, of the four isoflavonoids they tested, the least inhibitory to *Phytophthora parasitica* was a trimethoxy isoflavone. Although KH retains all four free phenolic functional groups of kievitone, it has less antifungal activity (Table 1). This compound did, however, cause considerable inhibition of *A. euteiches* on agar (Table 1) and of *R. solani* in liquid culture (Table 2) although, in neither instance, was the fungitoxicity so marked as that induced by kievitone and CK. The presence of Triton X-100 in both instances and of 2% DMSO, rather than 0.5% ethanol in the *R. solani* liquid culture, resulted in poorer-than-normal growth of controls (25) and this may, therefore, explain why KH

proved fungitoxic in these two assays. Certainly, the ineffective antifungal nature of KH has been established by more extensive bioassays presented elsewhere (13, 14).

Harborne et al. (10), working with the isoflavone luteone, which possesses a five-carbon substituent identical to that of kievitone, stated that luteone was more inhibitory to *Helminthosporium carbonum* Ullstrup than other isoflavones because of the side-chain. They suggested that this unsaturated five-carbon substituent might enhance lipid solubility and, consequently, fungitoxicity. This would be consistent with the results in this paper.

The data gained from this investigation of the fungitoxicities of various kievitone derivatives suggested that the antifungal nature of this phytoalexin was dependent upon two separate molecular features, the phenolic hydroxyl groups and the lipophilic dimethylallyl substituent. This proposal requires further investigation. Individual phenolic hydroxyl groups may contribute differentially to toxicity; bioassays of a series of partially-methylated kievitone derivatives might provide an answer.

Both VanEtten (32) and Perrin and Cruickshank (21) in their earlier studies reported the problem of insolubility of certain compounds in their assay media. Indeed, VanEtten (32) felt that the lack of antifungal activity exerted by 6 α , 11 α -dehydropisatin and 4*O*-methylcoumestrol might have reflected only insolubility of these molecules. With the occasional exception of KH, the five compounds exhibited consistent activity irrespective of the particular bioassay environment. Four fungi were employed in the current investigation, whereas Cruickshank and Perrin (21) used only one and VanEtten (32) and Johnson et al. (12), two. These four organisms responded in a generally similar fashion to the five compounds tested. It appeared that the relative fungitoxicities of the compounds tested were real and not unreasonably influenced by the particular procedure involved.

Daly (6) felt that actively-induced antifungal compounds were unlikely to contribute to resistance in situ unless they exerted antifungal activity in aqueous media lacking any organic solvent amendment. Since previous kievitone bioassays, used to assess the rôle of this phytoalexin in containing the invasion of bean hypocotyls by *R. solani*, were conducted in the presence of small amounts of ethanol (27, 28), the toxicity of

TABLE 4. The effect of kievitone on the mycelial dry weights of *Aphanomyces euteiches*, *Rhizoctonia solani*, and *Fusarium solani* f. sp. *phaseoli* attained in liquid culture

Time	<i>A. euteiches</i>		<i>R. solani</i>		<i>F. solani</i> f. sp. <i>phaseoli</i>	
	Mycelial dry weight (mg) ^a	Change ^b (%)	Mycelial dry weight (mg) ^a	Change ^b (%)	Mycelial dry weight (mg) ^a	Change ^b (%)
24 hr	3.9 ± 0.9	—	3.7 ± 0.2	—	6.1 ± 0.2	—
24+24 hr, C	9.2 ± 0.9	+136	10.2 ± 0.3	+176	19.3 ± 1.4	+216
24+24 hr, K	3.1 ± 0.1	-20	3.3 ± 0.4	-11	8.6 ± 0.6	+41

^aEach dry weight value is the mean of three replicates; the maximum variation from that value of individual replicates is also shown. The three organisms had been allowed to grow in liquid medium at 25 C for 24 hr prior to the addition of kievitone and/or water; no organic solvent was present in the culture medium. *Aphanomyces euteiches*, *R. solani* and *F. solani* f. sp. *phaseoli* were exposed to 4.2×10^{-5} M, 1.4×10^{-4} M, and 2.8×10^{-4} M kievitone, respectively, and their mycelial dry weights determined in control (C) and kievitone-containing (K) flasks after a subsequent 24 hr.

^bThese values represent the relative changes in dry weights in control and treatment flasks during the second 24-hr incubation.

kievitone was measured in media containing no organic solvent. The results presented in Tables 3 and 4 indicated that kievitone exerted considerable antifungal activity in solid and liquid aqueous media. The toxicities expressed towards *R. solani*, *A. euteiches*, and *F. solani* f. sp. *phaseoli* were similar to those reported previously and determined in the presence of ethanol (25, 27, 28). These bioassays emphasized that the use of small amounts of ethanol in assay media did not give invalid estimates of kievitone's fungitoxicity. In this connection, it should be noted that Pueppke and VanEtten (22) stated that pisatin remained antifungal in assays that did not involve an organic solvent.

Bailey and his colleagues concluded that a single evaluation of colony size could not give a reliable indication of the toxicity of phaseollin to *C. lindemuthianum* (3), which can metabolize this compound (5, 11). Initially, phaseollin caused complete inhibition of the radial growth of *C. lindemuthianum*; thereafter, growth occurred at equivalent rates in the presence and absence of phytoalexin. No such phenomenon was found in the present bioassays of *A. euteiches*, *R. solani*, and *F. solani* f. sp. *phaseoli* (Fig. 2). Growth rates of *A. euteiches* (Fig. 2-A) and *R. solani* (Fig. 2-B) generally and consistently were repressed by kievitone; no indication of adaptation to the phytoalexin was evident. Even *F. solani* f. sp. *phaseoli* (Fig. 2-C), which can metabolize kievitone (13, 15), was never strongly inhibited by kievitone; rather, cultures on control and treatment agars grew at similar rates throughout the duration of the experiment.

Kievitone accumulated earliest and most rapidly of the four phytoalexins found in *R. solani*-infected bean hypocotyls and this accumulation was localized at and immediately around lesions, which quickly became delimited (28). It now has been shown that kievitone effectively inhibited *R. solani* in aqueous media in the absence of an organic solvent at concentrations below those found in diseased tissues. Furthermore, there was no indication that *R. solani* adapted to the presence of kievitone, but it was, rather, steadily repressed.

This paper has therefore substantiated and amplified earlier findings with kievitone. However, much remains to be determined, particularly with regard to the precise mode of action of this phytoalexin.

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