Relation Between Production of Extracellular Kievitone Hydratase by Isolates of *Fusarium* and Their Pathogenicity on *Phaseolus vulgaris*

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ABSTRACT

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Twenty-eight wild-type isolates of *Fusarium* and *Nectria* were surveyed for ability to produce kievitone hydrate from the phytoalexin kievitone and for their pathogenicity towards *Phaseolus vulgaris*. In culture, at least 14 of the isolates were able to form kievitone hydrate, but only three were highly virulent on bean. Similarly, only three of the 28 isolates yielded cell-free culture filtrates capable of producing kievitone hydrate; these same three isolates were the most aggressive pathogens. Kievitone hydratase appeared to be the enzyme involved in each instance of extracellular production of kievitone hydrate.

Additional key words: *Fusarium solani* f. sp. *phaseoli*, metabolism, *Nectria haematococca*, phytoalexin, phytoalexin detoxification.

Phytoalexin accumulation is a putative defensive reaction expressed by some plants against fungal pathogens (2,10). In the French bean, *Phaseolus vulgaris* L., several postinfectional compounds constitute the plant's phytoalexins (18). Kievitone has been cited as an important phytoalexin in *P. vulgaris* (11,12) and in at least one other legume, cowpea (6). This isoflavonoid is detoxified in vitro (3,4) and in vivo (3) by *Fusarium solani* (Mart.) Sacc. f. sp. *phaseoli*, an aggressive pathogen of *P. vulgaris* (19). Detoxification is catalyzed by an extracellular glycoenzyme, kievitone hydratase (KHase) (1,4, and T. E. Cleveland and D. A. Smith, *unpublished*), which generates kievitone hydrate (KH) from kievitone (4,5). Previous research on kievitone detoxification by *Fusarium* involved only one isolate of the fungus (1,3,4,5). The present survey was undertaken to assess the frequency of occurrence of KHase in wild-type isolates and to investigate any potential relationship between the ability to generate KH and pathogenicity on bean.

Comparable work (15,17) with the pea phytoalexin, pisatin, and numerous field isolates of *Nectria haematococca* Berk. and Br. revealed that all isolates highly virulent on pea were able to demethylate, and so detoxify, pisatin. A subsequent genetic analysis (14) indicated that pisatin tolerance and pisatin demethylating ability in *Nectria* were either required for virulence on peas, or genes for virulence were closely linked to genes for phytoalexin tolerance and demethylating ability.

MATERIALS AND METHODS

Fungal cultures. Seventeen isolates of *Fusarium* were used in the survey (Table 1). Isolate FB1-S was used in the earlier work on kievitone detoxification by *F. solani* f. sp. *phaseoli* (1,3,4,5,9). Eleven isolates of *N. haematococca* MP VI, the perfect stage of *F. solani* (17), were also investigated (Table 1). The codes assigned to the various isolates incorporate those used by the investigators who provided the fungi and should aid comparisons with previously published work (eg, 17). The fungi were routinely maintained on

potato-dextrose agar (PDA) at 25 ± 2 C and transferred to bacteria-proof filters (0.22-μm, type GS; Millipore Corporation, Bedford, MA 01730) resting on "M-2" agar (7,12) 4–7 days prior to inoculation of liquid culture medium.

Phytoalexin. Isolation of kievitone has been described elsewhere (7,12). Quantification of this phytoalexin by ultraviolet (UV) spectrophotometry has likewise been reported (8).

Kievitone hydrate formation by intact fungi. Each of the 28 fungal isolates was treated in a comparable manner. Two or three 4-mm-diameter mycelial disks, still attached to their supporting pieces of bacteria-proof filter, were removed from "M-2" agar and added to 4 ml of heat-sterilized liquid medium (9) in a 25-ml Erlenmeyer flask. All flasks were incubated on a reciprocal shaker at 27 ± 2 C for 24–40 hr prior to the addition of kievitone. Solutions of kievitone in ethanol were added to all treatment flasks to give an initial phytoalexin concentration of ~25 μg/ml and an ethanol concentration of 1%. Control flasks received only ethanol. Incubation was continued in the same conditions.

The formation of KH by the various isolates was assessed, by slightly different protocols, at two time periods:

After 24 hr. All flasks contained two mycelial disks per 4 ml of medium and were incubated on a shaker for 1 day. At this time, and irrespective of the amount of mycelial growth apparent, kievitone was added. The cultures were then incubated for a further 24-hr period prior to extraction of the media for isoflavonoids.

After 6 hr. The 24-hr survey, described above, indicated differential growth rates between the isolates. To minimize the possibility that lack of KH formation merely reflected insufficient growth, a modified survey was conducted. An additional mycelial disk was used as inoculum, where required, and preincubation was extended to a maximum of 40 hr for slow-growing cultures. Mycelium was then decanted from the flasks and collected on 5 μm Millipore filters. About 40 mg (fresh weight) of mycelium was returned to the residual culture filtrate; the volume was increased to ~4 ml with fresh medium. Kievitone was added and incubation continued for 6 hr prior to extraction of the isoflavonoids.

Residual kievitone and any KH formed were extracted from the culture filtrate after the mycelium had been removed on Whatman No. 1 paper (R. and W. Balston Ltd., England). A water wash (~1.5 ml) of the mycelium was collected with the filtrate. The isoflavonoids were extracted with diethyl ether and separated by thin-layer chromatography (tlc) on silica gel as described

previously (9). A maximum of three sequential tlc steps on separate plates were undertaken. The solvent systems employed were chloroform:methanol (50:3, v/v) (kievitone R_f = 0.27, KH R_f = 0.13), chloroform:methanol:acetic acid (8:1:1, v/v) (kievitone R_f = 0.73, KH R_f = 0.55), and toluene:ethyl formate:formic acid (7:2:1, v/v) (kievitone R_f = 0.23, KH R_f = 0.12). Quantification of isoflavonoids in ethanol eluates was by UV spectrophotometry (8,9). The extraction procedure, after one tlc step, was about 50% efficient (9). The data were not corrected for extraction efficiencies, unless subsequent tlc was required. If so, any additional loss was taken into account, such that the yields presented in Table 1 and Fig. 2 are, or are equivalent to, those achieved after the first tlc step.

In the 6-hr assays, only arbitrary scores (+ or −) of KH formation were recorded, based upon the characteristic color reaction (5) with diazotized p-nitroaniline (13) at the appropriate R_f value and the characteristic UV absorption spectrum (5). In the 24-hr assays, KH was precisely quantified.

Pathogenicity tests. *P. vulgaris* 'Dark Red Kidney' seeds were purchased locally. Fifty seeds (five rows of 10) were sown in "Premier Pro-Mix" (Premier Brands Inc., New Rochelle, NY 10801) in 51 × 26 × 5-cm flats and incubated under greenhouse conditions (~23–31 C under daylight supplemented with fluorescent light). When seedlings emerged, 5–7 days after seeds were sown, they were inoculated without wounding. Two types of inoculum were used. The first was mycelial plugs (1 cm in diameter) excised from cultures growing on PDA. One plug was set against each emerging hypocotyl, at or just below soil level. The mycelium was placed in direct contact with the plant. The second type of inoculum was mycelial pieces taken from 4- to 5-day-old liquid cultures growing in 30 ml of medium (9). Clumps of mycelium were again placed directly against the hypocotyls, at or just below the soil surface. The plants were maintained in the greenhouse. Symptoms were recorded 1 wk after inoculation, and assigned to the following categories: severe = consistent red-brown spreading lesions that girdled the stem; mild = occasional red-brown lesions, with some flecking but no stem girdling; slight = considerable

TABLE 1. Surveys of the ability of *Fusarium* and *Nectria* to form kievitone hydrate, and their pathogenicity to *Phaseolus vulgaris*

Isolate	Code[a]	Remarks from source[b]	Kievitone hydrate production[c]		Disease reaction on beans[f]
			6 hr[d]	24 hr[e]	
F. solani phaseoli	FB1-S	Bean pathogen	+	31	Severe
	S136-T	Bean pathogen	−	10	Mild
	W8-BK	Bean pathogen	+	32	Severe
	W9-BK	Bean pathogen	+	32	Severe
F. solani	S499-T	From bean; pathogenicity not tested	−	≤5	Slight
	S602-T	From bean; pathogenicity not tested	−	0	Insignificant
F. solani	S239-T	From birch	−	0	Insignificant
F. solani	S289-T	Soil saprophyte	−	14	Insignificant
	S333-T	Soil saprophyte	−	18	Slight
F. solani cucurbitae race 2	S198-T	Curcurbit pathogen	−	0	Insignificant
F. oxysporum cucumerinum	B1-GK	Cucumber pathogen	+	50	Slight
F. oxysporum lycopersici	M811-GK	Tomato pathogen	−	0	Insignificant
	M811c-GK	Tomato pathogen	−	0	Slight
F. oxysporum melonis	B6-GK	Melon pathogen	+	44	Insignificant
F. oxysporum pisi	F49-BK	Pea pathogen (root rot)	+	18	Insignificant
F. oxysporum pisi race 1	F80-BK	Pea pathogen (wilt)	−	0	Insignificant
F. oxysporum pisi race 2	F81-BK	Pea pathogen (wilt)	+	42	Slight
N. haematococca MP VI	T8-V	From pea	+	14	Slight
	T27-V	From chickpea	+	14	Insignificant
	T30-V	From pea	+	30	Mild
	T69-V	From tuliptree	−	0	Insignificant
	T78-V	From alfalfa	−	27	Slight
	T86-V	From potato	−	0	Slight
	T111-V	From cottonwood	−	0	Insignificant
	T200-V	From red clover	−	≤5	Slight
	T214-V	From cottonwood	−	≤5	Insignificant
	T224-V	From soil	−	≤5	Slight
	T231-V	From potato	−	0	Insignificant

[a] The letters and numbers preceding each hyphen designate the isolate codes provided by the various sources. The letters following each hyphen designate the source: S = Smith (author); T = Toussoun, Pennsylvania State University; BK = Burke and Kraft, USDA, Washington State; GK = Gessler and Kuć, University of Kentucky; and V = VanEtten, Cornell University.

[b] These remarks present condensed descriptive comments of each isolate as provided by the sources.

[c] One hundred micrograms of kievitone were added in ethanol (initial ethanol concentration in the medium, 1%) to liquid mycelial cultures of each fungal isolate. The cultures were incubated on a reciprocal shaker at 27 C. Kievitone hydrate (KH) and any residual kievitone were subsequently extracted from the medium by partitioning into diethyl ether and separated by thin-layer chromatography.

[d] For each isolate, two flasks were harvested after 6 hr, and the production of KH was scored. Two parameters were employed for a + score: the characteristic color reaction at the appropriate R_f value upon spraying with diazotized p-nitroaniline (13) and the characteristic ultraviolet absorbance spectrum (5). In a few instances, where only trace amounts of KH were apparent, replicates were bulked after initial tlc in order to have sufficient absorbance for spectral verification.

[e] For each isolate, two flasks were harvested after 24 hr, and the production of KH was quantitatively determined by ultraviolet spectrophotometry after thin-layer chromatography. Each value, in micrograms of KH per flask, is the mean of two replicates.

[f] Each isolate was assayed three times for its pathogenicity towards *Phaseolus vulgaris*. Mycelial inoculum was placed against and around the hypocotyls; the plants were not wounded. Symptoms were recorded 1 wk after inoculation. Four disease reaction categories were recognized (Fig. 1).

red-brown flecking with occasional small lesions but no stem girdling; insignificant = no reaction or, at most, minor pin-point flecks. Representative examples of these symptoms are shown in Fig. 1. Pathogenicity assessments were conducted three times, twice with a mycelial plug and once with mycelial pieces. On each occasion, one row of emerging plants was inoculated with a particular isolate.

Kievitone hydrate formation by cell-free culture filtrates. Cultures were prepared as described above with two 4-mm-diameter mycelial disks in 4 ml of liquid medium. The flasks were incubated on a shaker for ~41 hr. Each flask was then harvested, and the mycelium was removed by passage of the remaining medium through a 0.22-μm Millipore filter. The cell-free filtrate from each flask was collected and 2 ml were then dispensed into a fresh flask. Eight flasks for each isolate were prepared in this manner. Solutions of kievitone in ethanol were added to the cell-free filtrates to give an initial phytoalexin concentration of ~25 μg/ml and an ethanol concentration of 1%. The contents of two flasks were each immediately extracted with diethyl ether (9). The six remaining flasks were subjected to additional incubation; two flasks were harvested thereafter at 2, 4, and 6 hr. Isoflavonoids were subsequently partitioned into diethyl ether, resolved by tlc, and quantified spectrophotometrically.

Characterization of KHase from cell-free culture filtrates. Flasks with 250 ml of liquid medium (9) were inoculated with two 1-cm mycelial plugs from 1-wk-old PDA cultures of isolates W8-BK and W9-BK. Cultures were incubated at 27 C on a reciprocal shaker for 3 days. Separate cell-free filtrates were prepared from each isolate by first passing the appropriate liquid medium through four layers of cheesecloth and then through sterile 0.22 μm Millipore filters. The cell-free filtrates were lyophilized and redissolved in double-deionized water to give volumes approximately one-tenth those of the original filtrates. The preparations were placed in dialysis membrane bags (50,000 MW exclusion limit) and dialyzed for 24 hr against 6 L of double-deionized water and then for 24 hr against 50 mM potassium phosphate at pH 6.0 (dialysis buffer). Enzymic activities remaining within the dialysis bags were characterized as follows.

A portion (5 ml) of each retained solution was applied to a column (1.5 × 8 cm) of DEAE-A50 Sephadex anion exchanger (Pharmacia Fine Chemicals, Piscataway, NJ 08854). The column was eluted with 30 ml of dialysis buffer followed by 30 ml of dialysis buffer containing 0.35 M NaCl. The latter eluate was allowed to flow through a column (1.5 × 8 cm) of Concanavalin A-Sepharose (Pharmacia). This column was eluted with 30 ml of dialysis buffer containing 0.1 M NaCl prior to elution with 30 ml of 200 mM 1-O-methyl-α-D-glucopyranoside (MG) (Sigma Chemical Co., St. Louis, MO 63178) in the same buffer.

The KHase in the MG eluate was incubated at 27, 65, or 70 C for 50 min in a shaking water bath. These preparations were allowed to return to 27 C and then assayed for enzymic activity.

All KHase activity was determined in essentially the same manner. Generally, 30 μg of kievitone in 5 μl of ethanol were incubated in 1 ml of enzyme solution for 3 hr at 27 C. When necessary, dialysis buffer was used to dilute enzyme preparations. Incubation time was increased for preparations possessing low levels of enzyme activity. To terminate the reaction, kievitone and KH were partitioned into diethyl ether, resolved by tlc, and quantified spectrophotometrically. Enzymic activity was considered negligible in any incubation mixture if no KH was detected with diazotized p-nitroaniline and spectrophotometry.
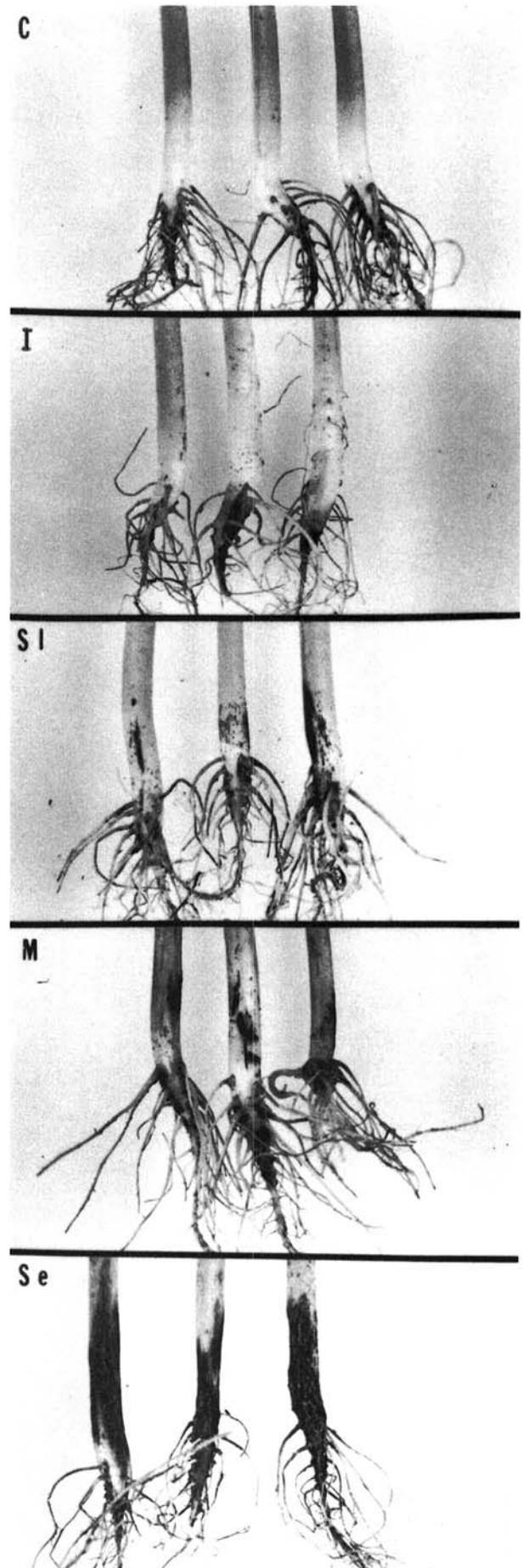

Fig. 1. Representative symptoms occurring on *Phaseolus vulgaris* hypocotyls one week after inoculation with isolates of *Fusarium* or *Nectria*. The disease reactions were placed in one of four categories, depending upon symptoms: C = control, I = insignificant (no reaction or at most, minor pin-point flecks), Sl = slight (considerable red-brown flecking with occasional small lesions but no stem girdling), M = mild (occasional red-brown lesions, with some flecking but no stem girdling), Se = severe (consistent red-brown spreading lesions that girdled the stem).

RESULTS

KH formation by intact fungi. Ten of the 28 isolates generated KH 6 hr after the addition of kievitone; another four isolates (S136-T, S289-T, S333-T, and T78-V) yielded KH only in the 24-hr assay (Table 1). The ability to form KH was not restricted to a particular forma specialis or to a species.

Pathogenicity tests. Because each bean seedling was directly exposed to a substantial piece of mycelial inoculum, it was not surprising that reactions occurred with several isolates in addition to those previously recorded as bean pathogens (Table 1). Severe symptoms (Fig. 1), however, developed only with three (FB1-S, W8-BK, W9-BK) of the four *F. solani* f. sp. *phaseoli* isolates. The fourth "bean pathogen" (S136-T) did not prove aggressive; only mild symptoms (Fig. 1) occurred in the interaction. The pea pathogen, T30-V (17), also caused a mild disease reaction. All other isolates were poor pathogens (Table 1, Fig. 1).

KH formation by cell-free culture filtrates. The formation of KH by isolates listed in Table 1 was assumed to reflect K Hase activity. This enzyme was previously reported to be primarily extracellular (4). Therefore, all isolates which yielded KH after 6 hr in the initial survey (Table 1) as well as S136-T, the poor "bean pathogen," and one wholly negative isolate (T231-V), to serve as a control, were assayed for KH formation in cell-free culture filtrates. The results are presented in Fig. 2. Cell-free filtrates from only three isolates (FB1-S, W8-BK, W9-BK) produced KH in the time periods considered.
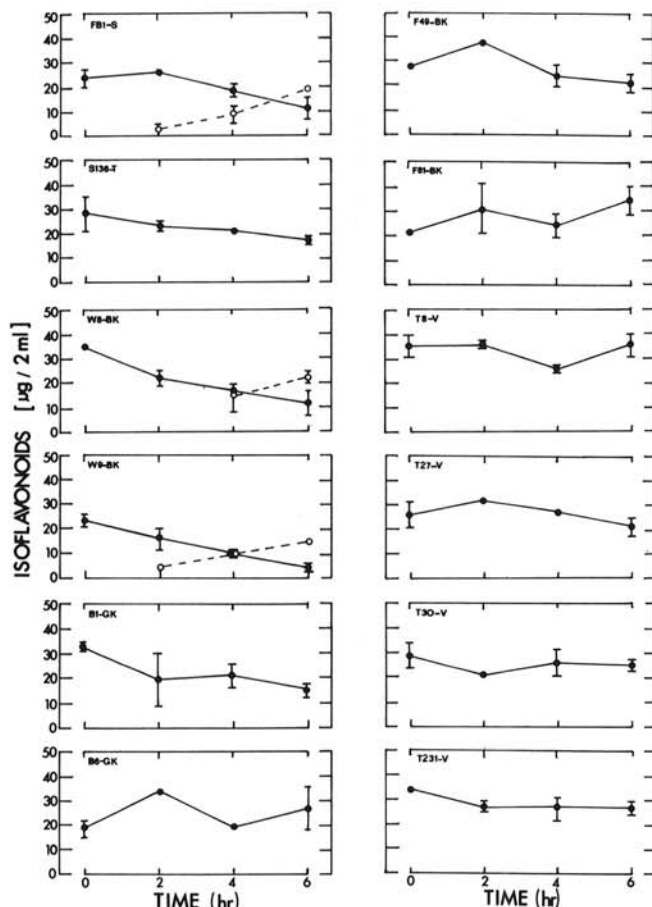
Characterization of KHase in cell-free culture filtrates. Purification and characterization of K Hase from cell-free culture filtrates of FB1-S has been described (1,4, and T. E. Cleveland and D. A. Smith, *unpublished*). To determine if the kievitone transformation found to occur in cell-free culture filtrates of isolates W8-BK and W9-BK also reflected the probable presence of K Hase, some preliminary characterization of the presumably enzymic activities of W8-BK and W9-BK were undertaken.

Cell-free K Hase from isolates W8-BK and W9-BK was shown to be the same as that from isolate FB1-S (1,4), within the limits of this characterization study. All three isolates produced K Hase with a molecular weight exceeding 50,000, which bound to DEAE-A50 Sephadex and Concanavalin A-Sepharose. The enzyme was eluted from these gels with 0.35 M NaCl and MG, respectively. The K Hase from all three sources was inactivated by more than 60% after 50 min of exposure to 70 C. All activity was destroyed when the enzyme was heated to temperatures approaching 100 C for 2 min.

DISCUSSION

The survey of KH formation by intact fungi (Table 1) indicated that the ability to convert kievitone to KH was not restricted to the isolate (FB1-S) originally investigated (3), but apparently occurs widely in *Fusarium* and *Nectria*. Pathogenicity tests (Table 1, Fig. 1) revealed three isolates (FB1-S, W8-BK, and W9-BK) that could be readily distinguished from the other 25 by their consistently aggressive development in *P. vulgaris* hypocotyls.

The composite results in Table 1 showed that some nonpathogens of bean (eg, isolate B6-GK) produced KH. Even if kievitone detoxification were an essential characteristic of a fungal pathogen of *P. vulgaris*, other traits would probably be required for pathogenicity (16). Furthermore, the diverse metabolic capabilities of fungi would anticipate that some nonpathogens of bean could structurally modify kievitone. An association between kievitone detoxification and the pathogenicity of *Fusarium* to *P. vulgaris* was nonetheless indicated, since all three virulent isolates (FB1-S, W8-BK, and W9-BK) produced KH efficiently, forming this metabolite after 6 hr and yielding >25 μg after 24 hr. The corollary makes this clearer; no aggressive bean pathogen was detected that could not generate KH. Similarly, all isolates of *Nectria* that were virulent pathogens of pea were tolerant of pisatin and able to demethylate this phytoalexin (17).

The results also allow speculation about whether there might be some biochemical parameters common to isolates of *Fusarium* and *Nectria* that are legume pathogens. VanEtten et al (17) reported that, of the 11 isolates of *Nectria* listed in Table 1, only three (T8-V, T27-V, and T30-V) consistently demethylated pisatin. These same isolates were the only isolates of *Nectria* to produce KH after 6 hr (Table 1). Even T78-V, which eventually did produce a substantial amount of KH, was also isolated from a legume.

The survey of cell-free production of KH (Fig. 2) revealed a distinct biological pattern—only those isolates of *Fusarium* that were aggressive pathogens of bean (Table 1, Fig. 1) possessed demonstrable K Hase activity. It is unclear why isolates such as B1-GK, which readily produced KH from kievitone when the intact fungus was exposed to the phytoalexin (Table 1), showed no cell-free enzymic activity (Fig. 2). Perhaps metabolism of kievitone by B1-GK occurs intracellularly or perhaps any extracellular enzyme(s) is inactivated during preparation of the cell-free filtrate. Either situation would imply an appreciable difference from traditional K Hase activity (1,4). That the cultural conditions were uniquely favorable to the secretion of extracellular K Hase only by virulent fungi seems unlikely, since similar conditions did not impede kievitone transformation by several intact fungi that were poor pathogens of bean. Nonetheless, further analysis of the influence of cultural conditions on enzyme production would help establish whether the extracellular occurrence of K Hase is an essential characteristic of virulent isolates.

Multiple chemicals may comprise a phytoalexin response. Furthermore, this response can be assumed to represent only one

Fig. 2. Kievitone hydrate formation by cell-free culture filtrates of *Fusarium* and *Nectria* isolates. Kievitone in ethanol (ethanol concentration in the medium, 1%) was added to cell-free culture filtrates of isolates of *Fusarium* or *Nectria* prepared as described in the text. Fifty micrograms of phytoalexin (25 μg/ml) were added to each filtrate. The filtrates were incubated in flasks on a reciprocal shaker at 27 C. At various time intervals, the concentrations of kievitone (●——●) and KH (o——o) were determined. The isoflavonoids were extracted in diethyl ether, separated by tlc, and quantified by UV spectrophotometry; each value is the mean of two replicates. Scatter bars indicate the ranges; where no bars are shown, the individual replicate values fall within the area of the symbol.

part of a continuum of mechanisms governing natural disease resistance (2). Consequently, demonstration of a causal association between detoxification of a single phytoalexin by a fungus and the microorganism's pathogenicity might prove difficult, because many other factors could mask any relationship. Nonetheless, this paper reveals an association between phytoalexin detoxification by *Fusarium* and its pathogenicity on bean. Whether the association is one of cause or effect remains to be clarified. Must *F. solani* f. sp. *phaseoli* detoxify phytoalexins to be a pathogen, or does it detoxify phytoalexins because it is a pathogen? These options are, of course, pertinent only to situations in which phytoalexins may be considered a significant part of the resistant response of a plant. This dilemma between cause and effect might be resolved by investigating the pathogenic capacity of a mutant of isolate FB1-S (or of isolates W8-BK and W9-BK) that differ from the wild-type only in an absence of KHase.

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