Stimulation of Phenylalanine Ammonia-Lyase Activity and Phytoalexin Production

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ABSTRACT

Phenylalanine ammonia-lyase (PAL) activity increases in excised pea and bean pod tissue within 8 hr after inoculation with pathogenic and nonpathogenic organisms. Phytoalexin production is correlated with increases in PAL activity. In general, facultative parasites are more potent than obligate parasites in stimulating PAL activity in these tissues. Fungal spore suspensions and chemical compounds that are effective inducers of PAL in pea and bean pod tissue do not significantly stimulate PAL in wheat, corn, and flax seedlings. Phytopathology 60:332-336.

In nature, "resistance is the rule rather than the exception" (20). This principle implies that every plant has some mechanisms(s) for resisting most of the microorganisms in its environment, some of which are pathogenic to other species. A given host may possess multiple features for resisting the array of microorganisms in its environment; however, this paper is concerned with only one host response, namely the activation of phenylalanine ammonia-lyase (PAL). Changes in PAL activity are frequently observed in host-pathogen interactions (6, 12, 14), and in response to light (21), chemical induction (6), wounding (13), and other stress conditions. PAL is also an intermediate enzyme in the production of pisatin (5), phaseolin (8), and possibly other structurally related phytoalexins (11), and in this study, phytoalexin production has been correlated with changes in PAL activity in bean and pea pod tissue.

MATERIALS AND METHODS.—*Culture of test organisms.*—Single-celled cultures of *Fusarium solani* (Mart.) Appel & Wr. f. sp. *pisi* (F. R. Jones) Snyd. & Hans., and *F. solani* f. sp. *phaseoli* (Burk.) Snyd. & Hans. were transferred from water agar to potato-dextrose agar, or potato-dextrose agar with 100 g/liter strained canned beans. After 3 weeks, macroconidia were harvested in sterile distilled water and adjusted to concentrations of approximately 1.2×10^6 spores/ml, and 1.5 ml of this suspension was the standard treatment.

Melampsori lini (Ehr.) Lév. Race 1 was harvested from Bison flax (*Linum usitatissimum* L.) and stored at 2 C. Ten mg of the uredospores were applied to pods by atomizing spores suspended in argon or by dusting spores onto tissue with a camel's-hair brush prior to the addition of 1.5 ml water. *Puccinia striiformis* West. and *Uromyces phaseoli* (Reb.) Wint. were applied in a comparable manner.

Helminthosporium carbonum Ullstrup, strains A and C (15), were grown on potato-dextrose agar. After 3 weeks, 10 ml sterile water was added to the surface of a culture in a 250-ml flask. The surface was scraped, and the scrapings were blended 15 sec in a Virtis homogenizer. The standard treatment on pea tissue consisted of 1.5 ml of this spores-mycelial suspension. The experiments with bean tissue utilized *H. carbonum* strain A grown on 2% V-8 juice agar, and the treatments were exclusively conidial suspensions prepared as for *F. solani.*

Induction treatments.—Immature Alaska pea (*Pisum sativum* L.) pods (less than 2 cm long) were harvested while still enclosed in the blossom to minimize contamination. The pods (1 g/treatment) were immediately split and placed in covered sterile petri dishes (60-mm diam). Treatments (1.5 ml of the desired spore suspension or inducer compound) were applied to the exposed endocarp (16), and the pods were incubated in the dark for the appropriate period before PAL extraction. L-phenylalanine-U-^{14}C (5.5×10^5 dpm, specific activity 365 mc/mmole) was added to endocarps in experiments in which pisatin production was being quantitated, as described previously (16).

Excised pea pods were utilized to take advantage of tissue that is uniform and nearly sterile. In pod tissue, the onset of senescence is very slow, excision and splitting results in minimal cell damage, and there is a maximal absorption area for uptake of isotope and inducer solutions. The obvious disadvantage in using pod tissue is that conditions were atypical for infection by the organisms used. Host resistance appears to be unaltered in pod tissue, although the symptom expression and the response intensity is somewhat different from that observed in seedling tissue.

Immature Tender Crop bean (*Phaseolus vulgaris* L.) pods (3-5 cm long) were harvested, and 1 g of pods (cut into 1-mm slices) was distributed in a sterile petri dish (60 mm diam). The treatments were applied as described above for pea pods. However, bean pods could not be harvested under the semisterile conditions attained with pea pod tissue.

Seven-day-old Nugaines wheat (*Triticum aestivum* L.) (5 g seedlings/treatment) and hybrid corn (*Zea mays* L.) seedlings (3 g with seed removed/treatment) were placed in tall petri dishes (100-mm diam), treated with 3.0 ml of the appropriate spore and inducer treatments, and incubated in the dark for 48 hr prior to the extraction of PAL. Seedlings were derived from surface-sterilized seeds.

Flax seedlings were grown in 3-inch pots in the

greenhouse for 14 days. The seedlings (8 plants/treatment) were repeatedly treated with actinomycin D (10 μg/ml), and with spore suspensions of *M. lini* (Race 1), *P. striiformis,* and *F. solani* f. sp. *pisi.* After a 24-hr incubation period, PAL was extracted from the respective treatments.

Phenylalanine ammonia-lyase extraction and assay.—One g tissue was homogenized in a mortar with 3 ml of 0.05 M borate buffer at pH 8.8, 1 g glass beads, and 0.1 g Polyclar. This and subsequent extractive operations were carried out at 2 C. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 20,000 *g* for 10 min. The supernatant was assayed immediately according to the procedure of Koukol & Conn (9), revised as follows: the reaction mixture contained 1.5 ml enzyme homogenate, 20 μmoles of L-phenylalanine, 2.8×10^5 dpm L-phenylalanine-U-^{14}C, and 200 μmoles of borate buffer pH 8.8 in a final volume of 2.7 ml. The mixture was incubated for 2 hr at 37 C.

Extraction and quantitation of pisatin and phaseolin.—Phaseolin and pisatin were isolated as described previously (8, 16). An ethanol extract of pod tissue was taken to dryness and further extracted with hexane or petroleum ether. The residue of the hexane extract is separated on silica gel thin-layer plates. Pisatin was detected on a silica gel thin-layer plate by converting it to anhydropisatin in HCl fumes. Anhydropisatin fluoresces under long-wave ultraviolet light. Phaseolin was detected with 3% ferric chloride in ethanol which produces a phaseolin-specific red color. Pisatin was quantitated on the basis of its absorbance at 309 nm and phaseolin by its absorbance at 280 nm. Radioactivity was measured in a liquid scintillation spectrometer.

RESULTS.—One constant measurement in this study was phenylalanine ammonia-lyase (PAL) activity. The variables were the hosts and the inducers. The relative abilities of various fungi to stimulate PAL activity in pea pod tissue is presented in Fig. 1, left. The three facultative parasites are more effective in inducing this response. PAL activity is increased more than twofold by all four fungi within 6 hr. Stimulation by *M. lini* peaks at this time interval. By that time, 20% or more of uredospores had developed spindly germ tubes (determined by examination of washings from treated pods at this time); the remaining spores had either not germinated or their germ tubes had lysed or broken. *H. carbonum* is reasonably effective in increasing PAL activity, which reaches a peak at 28 hr. At this time,

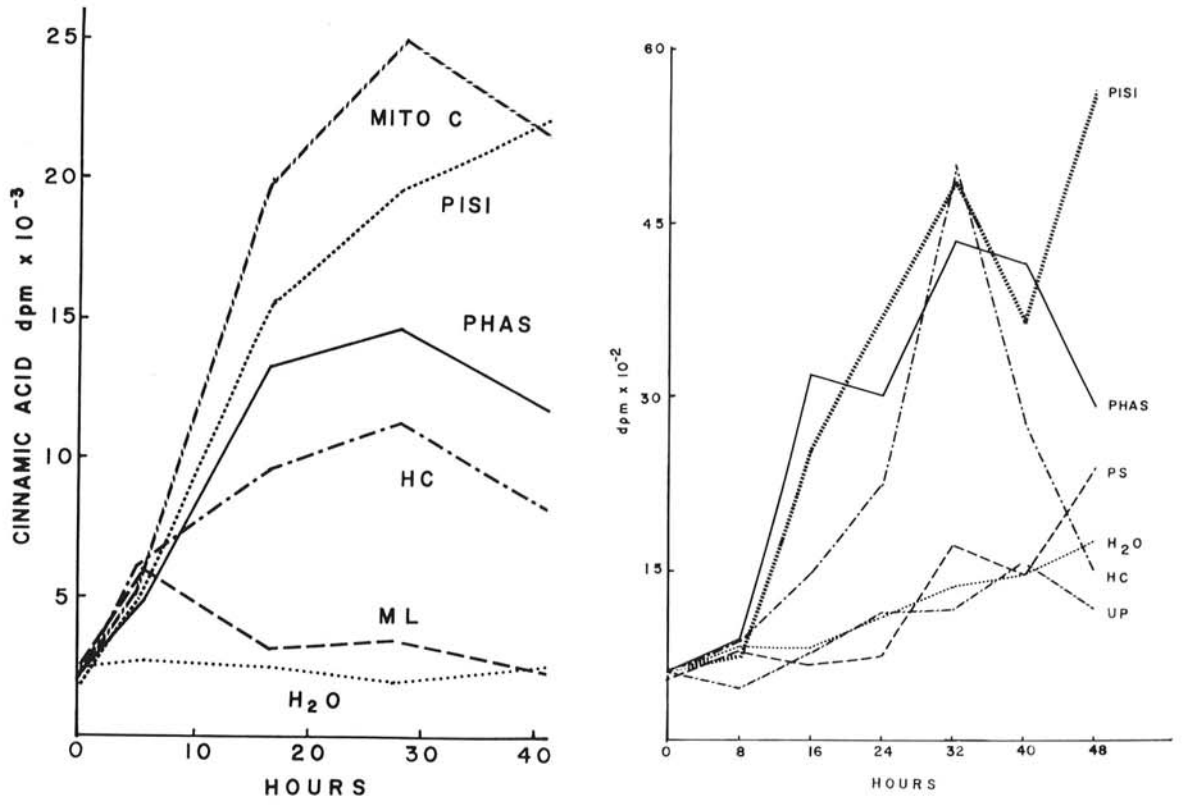

Fig. 1. (Left) Influence of various fungi on phenylalanine ammonia-lyase activity (PAL) in excised pea pods. Enzyme activity is expressed as dpm cinnamic acid-^{14}C (1,000 dpm = 0.036 mmole cinnamic acid) produced in 1 hr per g pea pod tissue. Abbreviations: PISI = *F. solani* f. *pisi;* MITO C = mitomycin C (100 μg/ml); PHAS = *F. solani* f. *phaseoli;* HC = *H. carbonum* strain A; ML = *M. lini.* (Right) Influence of various fungi on the stimulation of PAL activity in excised bean pods. Enzyme activity is expressed as dpm cinnamic acid-^{14}C (1,000 dpm = 0.036 mmole cinnamic acid) produced in 1 hr per g bean pod tissue. Abbreviations are defined in Fig. 1, except PS = *Puccinia striiformis;* and UP = *Uromyces phaseoli.*

most of the propagules have produced significant linear growth. *F. solani* f. sp. *pisi* (pathogen) and f. sp. *phaseoli* (nonpathogen) are both effective in increasing PAL activity more than sixfold in the first 18 hr. At 6 hr, the macroconidia of both *Fusarium* species have germinated (the germ tubes of the nonpathogen are actually twice as long as those of the pathogen).

At 42 hr, the *F. solani* f. sp. *pisi*-induced pods contain twice as much PAL activity as those induced with f. sp. *phaseoli*. *F. solani* f. sp. *pisi* is as good as the best chemically defined inducers of pisatin production in pea pods.

By 42 hr, *F. solani* f. sp. *pisi* has grown more in the vicinity of pod tissue than has f. sp. *phaseoli*. Mycelia of *F. solani* f. sp. *pisi* but not of f. sp. *phaseoli* have penetrated and parasitized the pollen grains associated with immature pods. Symptoms also indicate the relative virulence of the two fungi to peas. The endocarps exposed to *F. solani* f. sp. *pisi* are darkened and slightly mascerated, and the epidermis of the pod has pinhead-sized, dark brown lesions. The endocarps of pods incubated with f. sp. *phaseoli* are slightly discolored only around the edge of the pod and in areas damaged during excision; furthermore, while the epidermis of these pods has many more dark brown lesions, these are of but pinpoint size.

The ability of the two *Fusarium* forms to induce production of pisatin is compared in Table 1. *F. solani* f. sp. *pisi* induces more phytoalexin production within 40 hr than does f. sp. *phaseoli*. Pisatin degradation in the presence of f. sp. *pisi*, however, becomes an increasingly significant factor after 40 hr at this spore concentration. The data indicate that pisatin degradation is accelerated when pisatin is added immediately prior to the spore suspension of f. sp. *pisi*. Less pisatin was degraded when the pods are in the presence of the f. sp. *phaseoli* spore suspension.

The level of pisatin degradation in the control experiment is partly attributable to the slightly acidic solution associated with the pod which is capable of chemically dehydroxylating pisatin to anhydropisatin. Also, extraction efficiency is less than 100%.

The stimulation of PAL activity (Fig. 1, right) in excised bean pods by the various fungi was comparable to that in pea tissue. Again, the three facultative parasites were more effective in stimulating PAL activity than the obligate parasite. The activity of PAL stimulated by *H. carbonum* was comparable to that of *F. solani* f. sp. *pisi* and f. sp. *phaseoli* during incubation periods up to 30 hr. But during longer incubations, activity induced by *H. carbonum* was markedly reduced. Reduction in activity may implicate the lyase-inactivating system reported by Zucker (22). At 48 hr, activity induced by *F. solani* f. sp. *pisi* is greater than with the bean pathogen f. sp. *phaseoli*.

The increased production of phaseolin (Table 2) is evident up to 8 hr after increases in PAL activity. *F. solani* f. sp. *pisi* also stimulates greater increases in PAL activity than does the f. sp. *phaseoli*.

PAL activity (Table 3) in wheat and corn seedlings is not notably stimulated either by the various fungi or by the antibiotic, actinomycin D.

The activity of PAL in Bison (a variety of flax susceptible to *M. lini*, Race 1) is not significantly altered when incubated with *M. lini*, *F. solani* f. sp. *pisi*, or *P. striiformis*.

PAL activity was not significantly stimulated when Cass-M3 (a resistant line of flax, isogenic to the variety Bison except for the single M3 gene for resistance to Race 1) was inoculated with race 1 of *Melampsori lini* (Table 4).

DISCUSSION.—Host resistance responses may be induced by the pathogen via metabolites which activate genes for resistance in the host (7). Some genes dominant for avirulence in the pathogen may control the production of metabolites which function by activating host genes for resistance (7). There are numerous reports indicating that disease resistance depends on the

TABLE 1. Pisatin biosynthesis in pea pods in the presence of actinomycin D, *Fusarium solani* f. sp. *pisi*, and f. sp. *phaseoli*

Treatment[a]	Pisatin			
	Actual recovery	Expected recovery[b]	% Incorp.[c]	Isotope dilution
	μg/g *pod*	μg/g *pod*		
Water	[d]			
Water + pisatin	206		0.1	269,600
F. solani f. sp. *pisi*	381		1.9	22,340
F. solani f. sp. *pisi* + pisatin	368	587	1.1	36,220
F solani f. sp. *phaseoli*	237		1.6	17,090
F. solani f. sp. *phaseoli* + pisatin	473	443	1.3	40,680
Actinomycin D (10 μg/ml)	288		2.4	13,700

[a] L-phenylalanine-U-^{14}C (0.5 μc, specific activity 365 mc/mmole) was administered to split, immature pods, followed in certain experiments by the addition of 500 μg cold pisatin (in 20 μliters of ethanol). After 10 min, 1.5 ml of the inducer (actinomycin D [0.01 mg/ml] or spore suspension [1×10^6 spores/ml]) were applied, and the pods were incubated 40 hr in the dark at 22 C prior to the extraction of pisatin.

[b] Computed by adding the recovery in water control to the value obtained in the absence of added pisatin.

[c] Percent of L-phenylalanine-U-^{14}C incorporated into pisatin-^{14}C.

[d] Not detectable.

TABLE 2. Phaseolin production at indicated hr after treatment in excised bean pods in response to applications of actinomycin D (0.005 mg/ml), $CuCl_2$, H_2O, and spore suspensions of *Fusarium solani* f. sp. *pisi* and f. sp. *phaseoli*

Treatment[a]	μg phaseolin/g pods		
	16 hr	24 hr	48 hr
Water	tr[b]	30	62
$CuCl_2$	tr	79	195
Actinomycin D	tr	36	110
F. solani f. sp. *pisi*	40	128	167
F. solani f. sp. *phaseoli*	tr	80	72

[a] Treatments were applied as indicated in Methods. L-phenylalanine-U-^{14}C (0.5 μc, specific activity 365 mc/mmole) were applied prior to each treatment. Pods were incubated in the dark at 22 C.

[b] tr = Trace quantities which could not be accurately quantitated.

TABLE 3. Phenylalanine ammonia-lyase activity (PAL) in wheat and corn seedlings 43 hr after incubation with actinomycin D and various fungi

Treatment[a]	PAL activity		
	Corn hybrid Pr1 × K61	Corn hybrid Pr × K61	Nugaines wheat
Water	0.062[b]	0.075	0.051
Helminthosporium carbonum A	0.090	0.093	0.086
H. carbonum C	0.068	0.112	0.080
Fusarium solani f. sp. *pisi*	0.077	0.081	0.054
F. solani f. *phaseoli*	0.065	0.080	0.052
Puccinia striiformis	0.059	0.069	0.050
Actinomycin D	0.055	0.065	0.041

[a] Treatments were applied as indicated in METHODS. Five g of 7-day-old Nugaines wheat seedlings and 3 g hybrid corn seedlings (Pr1 × K61 and Pr × K61), resistant and susceptible, respectively, to *H. carbonum* (15).

[b] Enzyme activity is expressed as mmole cinnamic acid produced per hr per treatment at 37 C.

ability of the host to synthesize proteins (10, 12, 18, 19, 23)—which associates resistance responses to gene activation.

The increased activity of PAL is a host response associated with the synthesis of phytoalexins (6, 14). Also, PAL is involved in the production of lignin (1) and various phenolic compounds implicated in defense mechanisms in plants. Pisatin production in pea pod tissue involves a process of gene activation (16, 17), and depends on the synthesis of RNA, protein, and the enzyme PAL (6). We have used PAL activity as one indicator of early responses of various host species to obligate and facultative parasites, and to chemical induction. Our data relate the potential of various pathogens to activate a given host response (i.e., increase in PAL activity), and also demonstrate variability in the potential of different plant hosts to respond.

In pea tissue the pea pathogen (*F. solani* f. sp. *pisi*) and bean pathogen (f. sp. *phaseoli*) were comparable in their abilities to stimulate the activity of PAL. The inoculum of both organisms provided sufficient "inducer" to sharply increase the level of PAL in the initial 6 hr. The increased induction potential of *F. solani* f. sp. *pisi* as compared to that of f. sp. *phaseoli* after 18 hr was related to the increased growth of the fungus pathogenic on peas. However, that *F. solani* f. sp. *phaseoli* excels in inducing the PAL-inactivating system (22) in peas cannot be ruled out. When one compares the production of pisatin in response to these fungi, the picture is similar. Both *Fusarium* species induce high levels of pisatin; however, more pisatin can be extracted from the *F. solani* f. sp. *pisi*-treated pods up to 40 hr after inoculation. *F. solani* f. sp. *pisi* degrades the compound more rapidly (Table 1) (2, 3) and this degradation is important in evaluating pisatin accumulations after 40 hr.

TABLE 4. Phenylalanine ammonia-lyase (PAL) activity in near-isogenic lines of flax inoculated with *Melampsora lini* Race 1

Period following inoculation, hr	PAL activity[a]			
	Bison (susceptible)		Cass-M3 (resistant)	
	Healthy	Inoculated	Healthy	Inoculated
0	0.102	0.074	0.068	0.076
12	0.078	0.086	0.078	0.082
48	0.077	0.093	0.101	0.140

[a] PAL activity is expressed as mmole cinnamic acid produced/treatment per hr at 37 C. Enzyme was assayed as described under METHODS.

All of the plant pathogens tested more than double the level of PAL in pea pod tissue within 6 hr. After 6 hr, induction is somewhat correlated with the ability of the organism to grow on pea pod tissue. However, the induction potential of the obligate parasite *M. lini* is extremely low on pea pod tissue, considering the mass of uredospores which germinated.

The pattern of PAL induction in bean pods, in response to some of the same pathogenic organisms tested in pea pod tissue, is remarkably similar to that in pea pods. Induction of PAL by *F. solani* f. sp. *pisi* exceeds that by the true bean pathogen, *F. solani* f. sp. *phaseoli*. There was no obvious difference in the growth of the two *Fusarium* species on bean pods. Phaseolin production is greater in *F. solani* f. sp. *pisi*-treated bean pods than in pods treated with *F. solani* f. sp. *phaseoli* in the first 40 hr of incubation. The relative abilities of the two organisms to degrade phaseolin is not known.

All of the fungi tested stimulated the level of PAL activity in bean pod tissue in the first 8 hr except *U. phaseoli*, an obligate parasite of beans. This lack of response to *U. phaseoli* may be related to the suscepts inability to resist this rust at a critical period in the infection cycle.

PAL activity in wheat and corn seedling tissue inoculated with the various fungi was very low and variable in comparison to legume pod tissue. Since a moderate level of PAL is required for lignin production, the absence of dramatic changes in activity levels in the presence of nonpathogens and chemical inducers suggests that the gene for PAL synthesis is not activated by actinomycin D or metabolites of the fungal organisms.

The various plant pathogens were unable to stimulate increases in PAL activity when applied to flax seedlings. Further, there was no significant difference in PAL activity related to the single gene factor (for resistance to Race 1, *M. lini*) present in the flax line Cass M3 and absent in Bison.

Compounds derived from the C-6, C-3 structure of phenylalanine may not function as phytoalexin-type compounds in wheat, corn, and flax, especially in the systems tested. Fuchs et al. (4), in comparing the accumulations of L-phenylalanine-^{14}C metabolites in susceptible and resistant wheat leaves infected with stem rust have not discovered quantities which can readily be correlated with disease resistance. We have been unable to demonstrate significant differences between the accumulations of ethanol-soluble L-phenylalanine-^{14}C metabolites in resistant and susceptible

flax lines inoculated with *M. lini,* race 1 (L. Hadwiger, *unpublished data*). Such results suggest that responses other than increased PAL activity may be regulated by these genes for disease resistance.

Studies of host responses are complex even when comparisons are restricted to a single response such as PAL activity changes. However, our data indicate that (i) plant pathogens vary in their ability to induce a given host response, and (ii) pathogens possessing a potential to induce a given host response require a specific host to optimally realize this potential.

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