

Phytoalexin Nature of Heat-Induced Protection Against Bean Anthracnose

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ABSTRACT

Heat treatment of anthracnose-infected bean hypocotyls prevented the development of lesions and the large increases of phenylalanine ammonia-lyase (PAL) and total acetone-soluble phenolic substances associated with pathogenesis. Instead, smaller increases of PAL and total acetone-soluble phenolic substances occurred following heat treatment, as well as necrotic flecks within which infection hyphae were restricted.

Phaseollin synthesis was stimulated in infected heat-treated plants to levels far in excess of those occurring in the normal susceptible interaction. Additional phenolic and/or fluorescing substances were detected chromatographically in extracts from infected hypocotyl tissue, but not from healthy tissue. Distinctive

differences in the apparent quantities and/or times of appearance of these substances were observed in natural resistant and susceptible interactions. Comparisons of fluorescing and phenolic substances associated with natural resistant and susceptible interactions and with the heat-treated susceptible interaction suggested that the nature of host-parasite interaction is shifted from susceptibility to resistance by heat treatment. Differences in the levels of phaseollin which occurred in naturally resistant and heat-induced "resistant" responses are discussed in terms of differences in the numbers of cells occupied by infection hyphae at the time phaseollin synthesis was stimulated.

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Interruption of pathogenesis by elevated temperatures has been reported in cotton infected with *Verticillium albo-atrum* (3, 4) and French bean (*Phaseolus vulgaris* L.) infected with *Colletotrichum lindemuthianum* (Sacc. & Magn.) Scribner (11). Data suggesting that protection in cotton resulted from increased synthesis of gossypol and related phytoalexin-like substances were obtained in the former case. We describe here the occurrence of phaseollin in amounts toxic to *C. lindemuthianum* induced by heat treatment of anthracnose-infected bean plants.

MATERIALS AND METHODS.—Bean seeds, cultivar 'Topcrop', were kept for 6 to 7 days in the dark in vermiculite moistened with Arnon-Hoagland nutrient solution. Seedlings were rinsed with tap water after removal of seed coats, placed between two sheets of seed germination paper and rolled inside a waxed paper cover (rag doll). The rag dolls were moistened with nutrient solution and kept in the dark at 24 C. The β and γ races of *C. lindemuthianum*, pathogenic and nonpathogenic, respectively, on the cultivar Topcrop, were used for inoculations. Inocula were prepared as previously described (11), adjusted to 4 to 8×10^5 conidia/ml and sprayed evenly onto the seedlings. Heat treatment was accomplished by placing moist rag dolls in an unlighted Percival Model 1-36 incubator at 36 C.

Phenylalanine ammonia-lyase (EC 4.3.1.2.) (PAL) and acetone-soluble phenolic substances were determined essentially as described earlier (12). Acetone powders were prepared from hypocotyl sections cut 2 cm below the cotyledonary nodes and 2 cm above the root zones. Each acetone filtrate, representing seven or eight plants and 12 to 18 g fresh wt of tissue, was concentrated under vacuum at 40 C

to 3 to 5 ml of residual aqueous material which was acidified with 1 ml of 0.5 N HCl and made up to 10 ml with distilled water. This was extracted three times with 10 ml volumes of ethyl acetate. The ethyl acetate fractions were combined, concentrated to dryness under vacuum at 40 C and the residue dissolved in 95% ethanol (0.1 ml/g fresh wt of tissue).

Quantitative measurement of phaseollin in the ethanolic solutions described above was accomplished directly from thin-layer chromatograms by using a Zeiss PMQ-II Chromatogram Spectrophotometer. Relative peak areas were obtained by means of a Sargent Welch Model SRLG recorder equipped with Series 200 disc integrator and Model 610 automatic printer.

Ten- μ liter samples were applied at 2.5-cm intervals as narrow bands 0.8 cm long to layers (0.25 mm nominal thickness) of Silica Gel G (E. Merck, supplied by Brinkmann Instruments, Canada, Ltd.) previously activated for 2 hr at 110 C on 20 X 20 cm glass plates. The chromatograms were developed in a glass tank with ascending benzene-ethyl formate-formic acid 75:24:1 (v/v/v). Vapor saturation was provided by placing the plate being developed between thin layers on which the solvent had previously ascended. Developed chromatograms were placed in a fume hood for 2 hr prior to taking absorption measurements.

The plates were scanned in the coordinate of development at a speed of 50 mm/min with the instrument arranged in mode M-Pr (deuterium source \rightarrow monochromator \rightarrow sample \rightarrow photomultiplier). The wavelength used was 280 nm, with slit dimensions of 0.5 X 14.0 mm. Relative areas of at least three scans in both the forward and reverse directions over an area containing phaseollin were averaged for each analysis. Quantitative measurement of phaseollin in extracts

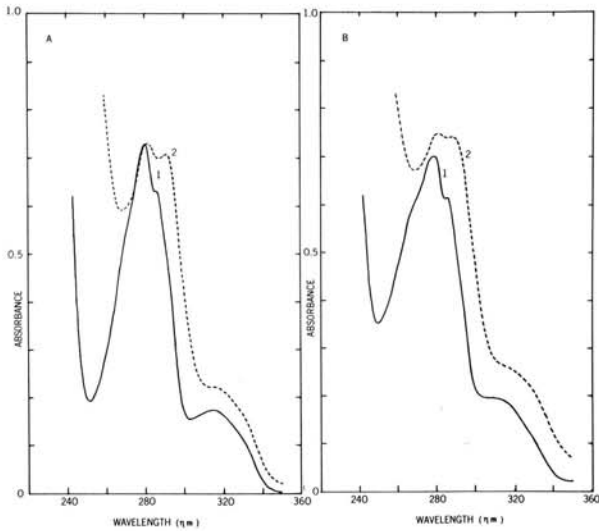


Fig. 1. Ultraviolet spectra in absolute ethanol (1) and 0.002 M sodium ethylate (2) of A) phaseollin and B) component with corresponding *R_F* eluted from thin-layer chromatographic separation of extract made 72 hr after heat treatment of 'Topcrop' bean hypocotyls infected with the β race of *Colletotrichum lindemuthianum*.

was accomplished by using a standard curve prepared from known amounts of phaseollin. In addition, three samples containing different known quantities of phaseollin were applied to each assay plate, and all values from any plate on which the peak areas of any of the known quantities varied by more than 10% from the corresponding values obtained from the standard curve were rejected. Purified phaseollin was obtained by the method of Van Etten & Bateman (14) from etiolated Topcrop hypocotyls 10 days after inoculation with *Thielaviopsis basicola*. Identification of the isolated material as phaseollin was based upon comparison of its ultraviolet spectra in absolute ethanol and 0.002 M sodium ethylate (Fig. 1-A) with published data, and quantitative measurements were based upon the reported extinction at 279 nm in absolute ethanol (5).

The ethanolic solutions were also examined for additional phenolic substances associated with infection. Developed chromatograms were inspected under ultraviolet light (254 nm and 366 nm) before and after being reacted with diazotized sulfanilic acid (DSA).

All experiments were replicated at least once, and values reported in Fig. 2 represent averages of duplicate determinations from each of the replicate experiments.

RESULTS.—Duration of heat treatment required to prevent pathogenesis.—Exposure at 36 C was begun 72 hr after spraying plants with water or conidia of the β race of *C. lindemuthianum*, at which time infection hyphae had proliferated within 1 to 10 or more host cells at individual penetration sites, depending upon location along the hypocotyl.

Heating was continued for periods of 11, 15 and 19 hr, after which the plants were returned to 24 C. The cytoplasm of host cells occupied by infection hyphae became granulated 6 to 36 hr after initiation of heat treatment. These cells appeared as minute tan flecks which did not enlarge, and further proliferation of infection hyphae was not observed. The precise time of appearance of flecks is a function of location on the hypocotyl, and occurs earliest near the cotyledonary node and progressively later at more distant locations. During the 12 days following heat treatment no lesions appeared on any of the plants heat-treated for 19 hr, and fewer than 25% of the

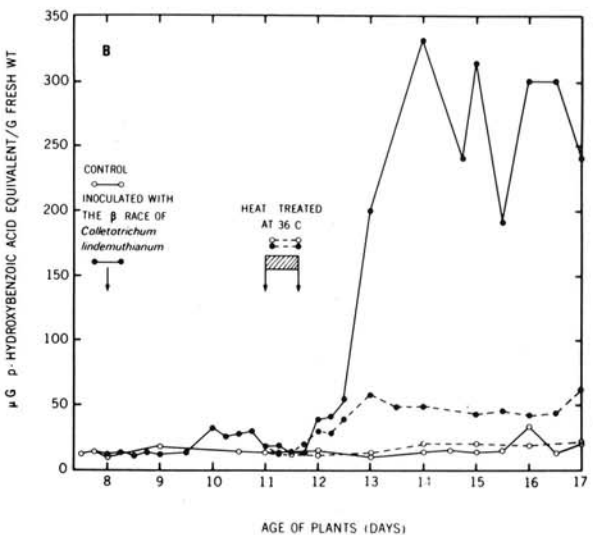
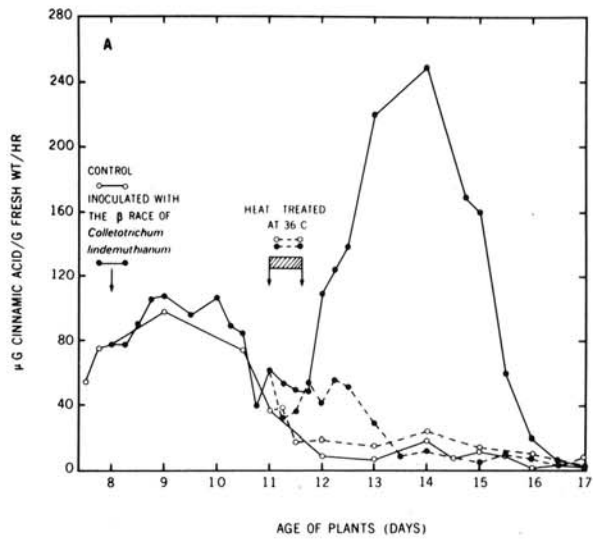


Fig. 2. Effect of heat treatment on A) phenylalanine ammonia-lyase activity and B) acetone-soluble phenolic substances from 'Topcrop' bean hypocotyls uninfected and infected with the β race of *Colletotrichum lindemuthianum*.

plants treated for 15 hr developed even one to two lesions. All plants heat-treated for 11 hr developed a limited number of lesions 3 to 4 days after heat treatment. Fifteen hr at 36 C was taken as the minimum heat treatment required to stop pathogenesis, and was used in subsequent experiments.

Apparent relation of PAL to maturation of hypocotyls.—The activity of PAL from hypocotyl sections of uninfected etiolated seedlings was correlated with hypocotyl development and was typically 5- to 6-fold higher when plants were 8 to 10 days old than after elongation ceased at 11 to 12 days (Fig. 2-A). The interdependence of PAL and development was further indicated by the fact that heat treatment of 9- to 10-day-old plants interrupted growth by elongation and depressed PAL to levels characteristic of non-elongating seedlings. Resumption of elongation and elevated PAL occurred simultaneously when the plants were returned to 24 C. In contrast, the level of acetone-soluble phenolic substances in infected plants was not affected by heat treatment, and remained virtually constant during the period of assay (Fig. 2-B).

Effect of heat treatment on host-parasite interaction, PAL and acetone-soluble phenolic substances. Lesions and coalescing necrosis which developed on plants infected with *C. lindemuthianum* and not heat treated occurred about 90 hr after inoculation, although the precise time of appearance ranged from 78 to 120 hr depending upon location on the hypocotyl. The activity of PAL increased dramatically and concurrently with the appearance of lesions, and increase of acetone-soluble phenolic substances occurred approximately 12 hr later (Fig. 2). The appearance of lesions on infected plants was prevented by heat treatment, as were the associated large increases of PAL and phenolic substances. Instead, minute flecks consisting of granulated host cells occupied by infection hyphae developed on these plants 6 to 36 hr after initiation of heat treatment. A corresponding smaller PAL response occurred concurrently with or, in some experiments, up to 21 hr before the appearance of most of the flecks which developed on infected heat-treated plants, and this was also associated with increased phenolic content. The activity of PAL returned to a level similar to that of uninfected heat-treated plants within 30 hr, but the increased content of acetone-soluble phenolic substances persisted during the following 4 days.

Qualitative analysis of extracts by thin-layer chromatography.—A number of fluorescing and phenolic substances were detected in extracts from infected plants and, in general, their appearance paralleled the development of visible symptoms. Of particular interest among these substances were three (R_F 0.19, 0.25 and 0.29) which fluoresced light blue under illumination of 366 nm, and a substance which corresponded exactly to marker spots of phaseollin with respect to mobility (R_F 0.50), fluorescence, and DSA response and which, when eluted, gave ultraviolet spectra in absolute ethanol and 0.002 M

sodium ethylate characteristic of phaseollin (Fig. 1-B). The former three substances gave a very weak reaction with DSA but precipitated with basic lead acetate; the latter substance was considered to be phaseollin on the basis of the criteria indicated.

In resistant interactions (γ race - Topcrop) the light blue-fluorescing substances appeared sequentially. The component at R_F 0.19 always occurred first, being detected initially in extracts made about 72 hr after inoculation. The components at R_F 0.25 and 0.29 appeared at 84 to 90 hr. Phaseollin was initially detected concurrently with the appearance of the latter two components. In susceptible interactions (β race - Topcrop) the component at R_F 0.19 was also detected initially about 72 hr after inoculation, and the components at R_F 0.25 and 0.29 at 84 to 90 hr, but only trace amounts of the latter two substances were detected. Visibly similar trace amounts of phaseollin occurred simultaneously.

The previous description is intended as a general guide. In individual experiments, variations were observed with respect to the exact time, but not sequence, of appearance of the substances described, as well as the apparent overall levels which occurred. In every experiment the substances occurred in apparently stable ratios in relation to each other for a given type of interaction, although the overall level of response varied greatly in individual experiments. The variations in overall levels of these substances which occurred in extracts from resistant interactions were in apparent proportion to the intensity of necrotic flecking associated with the interaction in each experiment.

Additional fluorescing and/or phenolic substances were detected in extracts but the ones described comprised patterns which clearly and reproducibly distinguished extracts from resistant and susceptible interactions (Fig. 3). Extracts from heat-treated susceptible interactions (β race - Topcrop: heat-treated) were virtually identical to those from naturally resistant interactions with regard to the sequence of appearance and apparent ratios of the four substances described. The component at R_F 0.19 was first detected in extracts made at the time heat treatment was begun (72 hr after inoculation). Phaseollin and the components at R_F 0.25 and 0.29 were first detected in extracts from heat-treated plants taken 6 hr after the beginning of heat treatment (78 hr after inoculation).

Quantitative analysis of phaseollin in extracts.—Phaseollin occurred in extracts from infected heat-treated and infected nonheat-treated plants, but was not detected in extracts from uninfected plants, irrespective of heat treatment (limit for detection by the procedures described was 1 to 2 $\mu\text{g/g}$ fresh wt of hypocotyl tissue). As described above, phaseollin was initially detected in infected heat-treated plants 6 hr after the beginning of heat treatment. In the duplicate experiment for which data are presented in Fig. 4 (extracts from plants in two rag dolls independently analyzed), phaseollin increased markedly during the next 66 hr

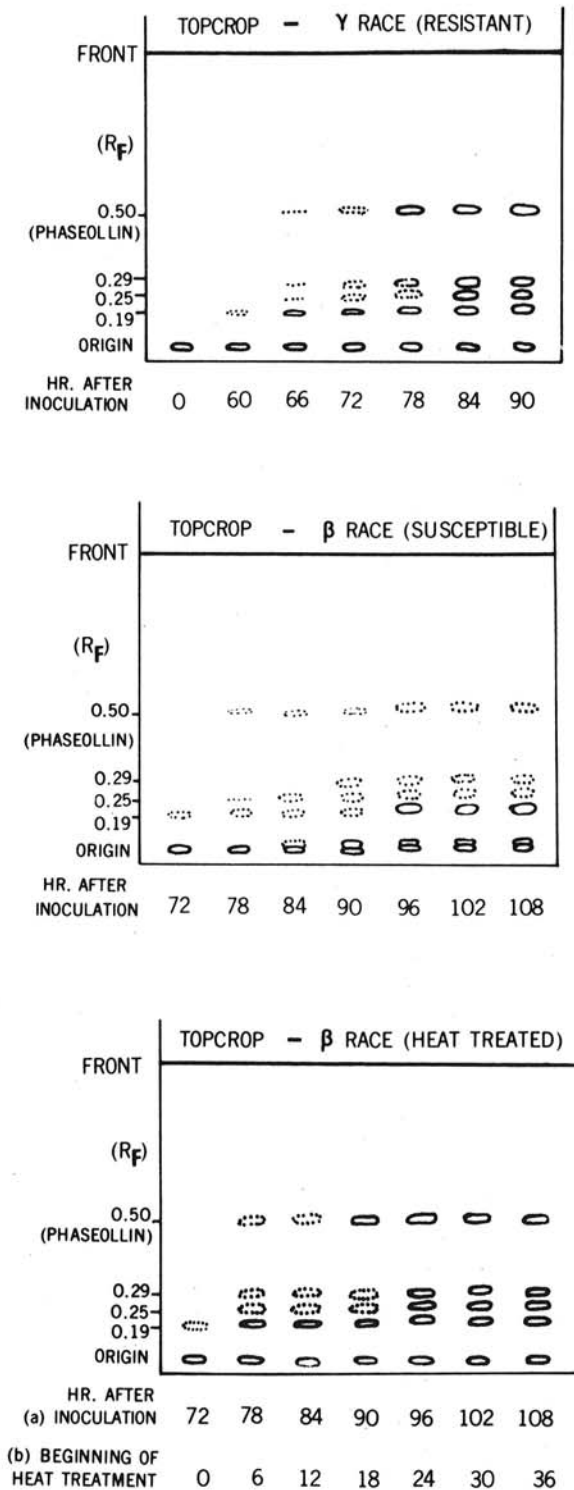


Fig. 3. Occurrence of phaseollin and blue-fluorescing substances characteristic of reaction type in resistant, susceptible, and heat-treated interactions between 'Topcrop' bean hypocotyls and *Colletotrichum lindemuthianum*. Visual estimates of substances: --- = barely detected, ⊖ = relatively small amount, ⊕ = relatively large amount.

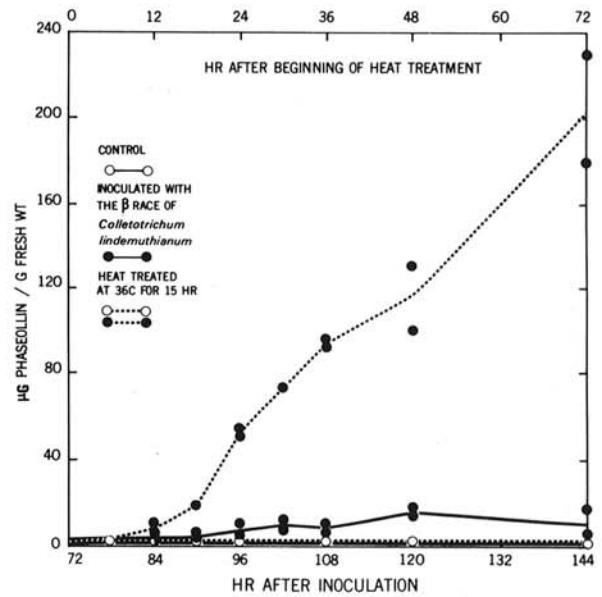


Fig. 4. Effect of heat treatment on levels of phaseollin occurring in 'Topcrop' bean hypocotyls uninfected and infected with the β race of *Colletotrichum lindemuthianum*.

to levels of more than 200 μg/g fresh wt of tissue at 3 days after the beginning of heat treatment. In a prior duplicated experiment somewhat lower levels of phaseollin occurred at 24 hr after the beginning of heat treatment (25 and 31 μg/g fresh wt), and relatively little increase occurred during the next 2 days (42 and 53 μg/g fresh wt maximum at 3 days after beginning of heat treatment).

Although precise counts were not recorded, numbers of conidia and numbers of penetration sites per unit area of hypocotyl tissue were similar in every experiment. The only difference observed among the experiments was the variation in intensity of necrotic flecking induced by heat treatment in the individual experiments. As in the case of the blue-fluorescing substances at R_F 0.25 and 0.29, the intensity of necrotic flecking was apparently in proportion to the observed levels of phaseollin. Microscopic examinations clearly suggested that the intensity of necrotic flecking varied in proportion to the average number of cells occupied by hyphae at penetration sites at the time of heat treatment.

The time of appearance of phaseollin is closely associated with, and possibly precedes, the appearance of visible flecking induced by heat treatment. In one experiment phaseollin was detected chromatographically before the appearance of flecks. The progressive appearance of flecks as a function of their location along the hypocotyl precludes a precise statement as to the temporal relationship between symptom appearance and occurrence of phaseollin when the latter is extracted from entire hypocotyl sections.

The occurrence of phaseollin in infected plants

which had not been heat treated was similarly associated with the appearance of necrotic lesions, but the levels attained remained low, not exceeding 20 $\mu\text{g/g}$ fresh wt during the period of assay in any of the experiments.

DISCUSSION.—Definitive conclusions regarding the role of phaseollin in natural defense of *P. vulgaris* are presently precluded by the limited data concerning rates of synthesis and levels reached at individual infection sites in various types of interactions. Bailey & Deverall (1) have recently presented data by which they compute levels of phaseollin in excess of 3,000 $\mu\text{g/ml}$ cell volume in hypersensitive cells. There can be little question as to the fungitoxicity of the contents of such cells. Fungitoxicity of phaseollin to *C. lindemuthianum* has been reported at 50 $\mu\text{g/ml}$ (ED_{50} for germ tube elongation in agar medium) (5), and 10 $\mu\text{g/ml}$ (spore germination), 3 $\mu\text{g/ml}$ (germ tube growth following direct application) and 0.9 to 12.6 $\mu\text{g/ml}$ (mycelial growth in liquid culture) (1). The data reported here also show unquestionably fungitoxic levels of phaseollin occurring after heat treatment of infected hypocotyl tissue, even if one assumes an even distribution of the phytoalexin throughout the hypocotyl.

The question as to when fungitoxic levels are reached relative to when growth of infection hyphae ceases remains unanswered, however. Our data clearly indicate a proportional relationship between levels of phaseollin and the intensity of necrotic flecking which occurs in both naturally resistant and heat-induced "resistant" interactions, as well as an apparent proportional relationship between the levels of phaseollin and the blue-fluorescing substances with R_F 0.25 and 0.29, which characterize these interactions. Although a close temporal relationship between necrotic flecking and the occurrence of these phenolics is also shown, our data do not prove a cause-effect relationship, nor do they reveal the relationship between cessation of hyphal growth at individual infection sites and the occurrence of necrosis and/or phaseollin.

If phaseollin and the blue-fluorescing substances at R_F 0.25 and 0.29 are linked proportionally with necrotic flecking in resistant interactions, it must be concluded that necrotic flecking is biochemically distinct from lesion necrosis. The latter reaction frequently coalesces over entire hypocotyls, and very low levels of phaseollin and the other two substances are found in extracts of these tissues. The magnitude of PAL and total acetone-soluble phenolic responses do appear proportional to necrosis, whether this be flecking or lesions. This is not surprising since a large number of phenolic substances are associated with both lesion necrosis and hypersensitivity (12). Measurements of PAL and total acetone-soluble phenolic substances, while sensitive indicators of phenolic response, cannot be used to distinguish the nature of response. The levels of phaseollin and the blue-fluorescing substances at R_F 0.25 and 0.29 in relation to the level of the blue-fluorescing substance

at R_F 0.19 clearly distinguished resistant and susceptible interactions in these experiments.

Whether phaseollin (and the blue-fluorescing substances at R_F 0.25 and 0.29) is cause or product of resistant interaction, the data and observations presented permit speculation concerning control of its synthesis. That enzymes catalyzing the various presumed reactions for synthesis of phaseollin are products of the host is indicated by the large number of abiotic inducers of phaseollin synthesis (6). The relative non-specificity of induction is further indicated by reported synthesis of phaseollin in response to a wide range of fungi (1, 2, 6, 9, 10, 11), bacteria and viruses (2, 6, 13). If phaseollin constitutes a non-specifically induced defense substance, pathogenicity could be accounted for by (i) tolerance, (ii) detoxification, (iii) non-induction or (iv) induction (of a phaseollin "operon") and simultaneous blockage at a particular step in the synthetic pathway. Tolerance is an observed characteristic of some pathogens of *P. vulgaris*, particularly among bacteria (6, 13). The author is not aware of any reports of complete non-induction associated with pathogenicity, nor of pathogenicity associated with detoxification. Phaseollin is reported to be metabolized by *C. lindemuthianum* in liquid culture, however (1), and breakdown of pisatin by a number of fungi has been shown (7).

The occurrence of phaseollin in both resistant and susceptible interactions between *P. vulgaris* and *C. lindemuthianum* indicates induction of a metabolic sequence for the synthesis of phaseollin by both pathogenic and non-pathogenic races of this fungus. That a sequence of reactions is involved is suggested by the studies on biosynthesis of phaseollin reported by Hess et al. (8). Development of large areas of fungus colonization without appreciable phaseollin in susceptible interactions could be accounted for by blockage of the biosynthetic sequence at some critical step in the pathway during colonization. If blockage of this step requires continuous metabolic action by the pathogen upon the induced machinery for phaseollin synthesis, then any factor which slows fungal metabolism would result in increased synthesis of phaseollin.

Rahe et al. (11) have reported that when cultures of *C. lindemuthianum* on bean juice agar medium are heated at 36 C for 15 hr, growth ceases but the fungus is not killed. If the synthetic machinery for phaseollin synthesis were induced only in those cells directly affected by infection hyphae and simultaneously blocked at a critical step by some factor required to be continuously synthesized by the pathogen, heat treatment which stops metabolism of the fungus in the host would permit phaseollin synthesis in those cells in which prior induction of the synthetic machinery had occurred. This would account for production of phaseollin following heat treatment of infected plants, as well as the observed proportional relationship between the levels of phaseollin occurring and the number of cells colonized at individual infection sites at the time of heat treatment. It also accounts for the fact that

phaseollin is not detected following heat treatment of uninfected plants. The hypothesis is amenable to test in that any factor which slows or prevents metabolism by pathogenic races of *C. lindemuthianum* in the host should result in synthesis of phaseollin and, if phaseollin is responsible for resistance, convert susceptibility to resistance.

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