Metabolic Nature of the Infection-Limiting Effect of Heat on Bean Anthracnose

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ABSTRACT

Colletotrichum lindemuthianum, the causal agent of bean anthracnose, does not grow at temp above 32 C. The disease is also heat-sensitive. Thermal sensitivities of conidia, appressoria, and infection hyphae on etiolated bean hypocotyls, and conidia and mycelia on agar were investigated. The infection-limiting effect of heat treatment reflected deleterious effects of heat on conidia and appressoria, but mycelium on agar was not killed by five times the duration of heat treatment that prevented further development of infection hyphae in the host. Infection-limiting heat treatment of infected plants induced protection against subsequent infection by the same race of the fungus; but previously uninfected plants were more susceptible after heat treatment. The response of previously infected and uninfected plants to a varietal-nonpathogenic race of *C. lindemuthianum* was not affected by heat treatment. We conclude that heat treatment of the host-fungus complex prevents further development of the pathogen via initiation of a response that also protects the host against subsequent infection. Phytopathology 60:1005-1009.

Temperature exerts a profound influence on biological systems, and it is not surprising that temp associated alterations of disease resistance in plants are frequently observed (7, 14, 16). Both high and low temp alter disease resistance in *Phaseolus vulgaris* L. Schulz & Bateman (15) reported that treatment of bean seeds at 5 C during the first 24 hr of germination increased susceptibility to infection by *Rhizoctonia solani.* Yarwood (17) observed that susceptibility of primary leaves of young bean plants to anthracnose and certain viruses was increased by immersion of the leaves in hot water for a few sec prior to inoculation.

Colletotrichum lindemuthianum (Sacc. & Magn.) Scribner is also temp-sensitive. Edgerton (4, 5) reported that growth of the fungus on bean pod agar was near maximal at 18-26 C, and that no growth occurred at temp above 30-31 C (5). Leach (9) observed maximum growth of the fungus in culture at 22.5 C, and the highest percentage of spore germination at 27.5 C. Limiting temp for infection have been reported at 26.6 C (8), 30 C (18), and 32 C (10).

The histology of infection by *C. lindemuthianum* has been investigated by Leach (9), Dey (3), and Rahe et al. (13). Conidia, appressoria, and infection hyphae occur during infection. The relative thermal sensitivities of these structures and the effect of temp on the fungus in regard to the thermal sensitivity of the disease is reported here. A preliminary report has been published (11).

MATERIALS AND METHODS.—*Growth of bean seedlings and fungi.*—Bean seeds, cultivar Topcrop, were washed in tap water and kept for 5 days in the dark in vermiculite moistened with Hoagland-Snyder nutrient solution. The seedlings were washed in 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 and kept in a dark moist-chamber at 22-24 C. Inoculations were begun when the seedlings were 11-13 days old. The β and γ races of *C. lindemuthianum* were grown in the dark on bean juice agar. Inocula were prepared from 8-day-old cultures by flooding plates with tap water and rubbing the cultures with a glass rod. The suspensions of conidia and mycelia were filtered through cheesecloth and centrifuged for 10 min at 5,000 *g*. The pellets were suspended in deionized water, adjusted to 1-2 $\times$ 10^6 conidia/ml, and sprayed on the bean hypocotyls.

Heat treatment.—Heat treatment was accomplished by placing the rag dolls in an electrically heated incubator maintained at approximately 99% relative humidity. Temperature equilibration at the center of the rag dolls occurred within 1.5 hr. The temp in the incubator cycled through a range of 5 C during regular 1-hr intervals at any of the settings used. Variation within rag dolls, however, was less. Figures denoting duration and temp of heat treatment refer to the total time rag dolls were kept in the incubator and to the mid-point of the temp range.

Plants were observed for 10 days after heat treatment or final inoculation. Strips of epidermis were sectioned freehand from the bean hypocotyls at intervals after inoculation and/or heat treatment, and examined with a light microscope.

RESULTS.—*Effects of various temp on bean seedlings, fungus, and disease development.*—The variety Topcrop is susceptible to the β race and resistant to the γ race of *C. lindemuthianum.* Susceptibility was characterized by flecks or small areas of discolored tissue which developed into reddish-brown, necrotic lesions. Symptoms first appeared at 72-84 hr after inoculation and occurred sequentially from the cotyledons to just above the roots within 24-36 hr. Resistance was characterized by flecks which did not enlarge.

The number and size of lesions decreased with increasing temp of incubation within the range of 28-

32 C. Lesions did not develop on plants kept at temp above 32 C. Conidia of *C. lindemuthianum* germinated and formed short germ tubes on bean juice agar at 32 C, but further growth did not occur. Occasional colonies developed when the cultures were returned to 22-24 C. No growth occurred at 22-24 C after exposure of conidia to 37 C for 9 hr.

The general condition of etiolated seedlings kept at 32-34 C for up to 4 days remained similar to that of plants kept at 22-24 C. The rate of elongation of seedlings was reduced at 35-38 C, but the plants were not otherwise visibly affected and resumed growth when returned to 22-24 C. Extended exposure at 39-41 C caused injury to the epicotyls of seedlings.

Effects of heat treatment at various stages of infection on disease development.—Seedlings were kept at 22-24 C for 45 hr after inoculation, then heat-treated for various lengths of time before being returned to 22-24 C. Decreasing numbers of lesions and increasing time for the appearance of symptoms were associated with increasing durations of heat treatment and increasing temp of exposure. The number of lesions on plants held for 8 hr or more at any temp above 35 C was markedly reduced. Symptoms were not observed within 10 days after inoculation on plants treated for 40-48 hr at 35 C, 24-32 hr at 36 C, or 8-16 hr at 37 C; the plants were indistinguishable from noninoculated controls. The effect of these treatments on the intensity and time of appearance of symptoms is summarized in Table 1.

In subsequent experiments, treatments were at 37 C for various lengths of time and at various times after inoculation. The effect of these treatments on the intensity and time of appearance of symptoms is summarized in Table 2. When plants were treated for 10 hr at 39 C immediately after inoculation, symptoms appeared 12-24 hr later than on untreated plants. The intensity of infection was similar on both treated and untreated plants. The number of lesions was markedly reduced when the plants were exposed to 37 C for 14 hr, however, and the time of appearance was 3 days longer than on untreated plants. Symptoms did not develop within 10 days on plants which were heat-treated for 78 hr or longer.

When heat treatments were begun at or later than 64-72 hr after inoculation, symptom development on plants at 37 C was initially similar to and concurrent with that on plants kept at 22-24 C, but ceased within 7-9 hr at the elevated temp and did not resume when the plants were returned to 22-24 C. Flecking similar to that characteristic of resistance to *C. lindemuthianum* frequently occurred 24-36 hr after heat treatment. Flecking developed over the entire hypocotyl on plants treated at 78 hr after inoculation, but occurred primarily in the area of the cotyledonary node following treatment at 69 hr, and was not observed following treatment at 56 hr.

Induced resistance associated with heat treatment at 37 C of anthracnose-infected bean seedlings.—When infected and noninoculated heat-treated plants and noninoculated untreated plants were inoculated with the race of *C. lindemuthianum* 24 hr after heat treatment, symptoms were markedly reduced on plants in which the initial β race infection had been stopped by heat treatment. In contrast, heat treatment of the previously noninoculated plants resulted in increased susceptibility relative to that of noninoculated untreated plants (Fig. 1). Less protection was observed when heat treatment was begun 44 hr after inoculation than was observed following treatments begun 69-94 hr after inoculation. Resistance to the γ race of *C. lindemuthianum* was not visibly affected by prior heat

TABLE 1. Effect of heat treatments of etiolated bean seedlings 45 hr after inoculation with *Colletotrichum lindemuthianum* on the appearance of symptoms[a]

Time of treatment[b]	Temp of treatment, C	Time of observation[b] and response[a]				
		96	120	144	192	240
Untreated	22-24	++++	+++++	+++++	+++++	+++++
45-53	35	−	+++	++++	++++	++++
	36	−	+++	+++	+++	+++
	37	−	++	++	++	++
45-61	35	−	++	+++	+++	+++
	36	−	+	++	++	++
	37	−	−	−	−	−
45-69	35	−	−	+	++	++
	36	−	−	−	+	+
	37	−	−	−	−	−
45-77	35	−	−	+	++	++
	36	−	−	−	−	−
	37	−	−	−	−	−
45-85	35	−	−	−	−	+
	36	−	−	−	−	−
	37	−	−	−	−	−
45-93	35	−	−	−	−	−
	36	−	−	−	−	−
	37	−	−	−	−	−

[a] − = No symptoms; + = one-4 lesions/plant; ++ = Five-9 lesions/plant; +++ = Ten-29 lesions/plant; ++++ = 30 or more lesions/plant; +++++ = Coalescing lesions and general necrosis.

[b] All numbers refer to hr after inoculation of etiolated seedlings with the β race.

TABLE 2. Effect of exposure at 37 C of etiolated bean seedlings inoculated with *Colletotrichum lindemuthianum* on the appearance of symptoms[a]

Time of treatment[b]	Time of observation[b] and response[a]									
	72	84	96	108	120	144	168	192	216	240
Untreated	+	++	+++	++++	+++++	+++++	+++++	+++++	+++++	+++++
0-10	−	−	+	+++	+++++	+++++	+++++	+++++	+++++	+++++
0-14	−	−	−	−	−	−	(++++)	(++++)	(++++)	(++++)
0-18	−	−	−	−	−	−	−	−	−	−
45-52	−	−	−	−	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)
45-58	−	−	−	−	−	−	−	−	−	(++++)
45-69	−	−	−	−	−	−	−	−	−	−
56-64	−	−	−	−	−	−	−	(++++)	(++++)	(++++)
67-74		+	+	+	+	+	(++++)	(++++)	(++++)	(++++)
69-78		+	+	+	+	+	+	+	(++++)	(++++)
69-86			+	+	+	+	+	+	+	+
69-93			+	+	+	+	+	+	+	+
78-86	+		++	++	++	++	++	++	(++++)	(++++)

[a] − = No symptoms; + = flecking barely detectable in area around cotyledons; ++ = moderate discoloration in area around cotyledons; +++ = numerous lesions on upper half of hypocotyls; ++++ = numerous lesions over full length of hypocotyls; +++++ = coalescing lesions and general necrosis; ( ) = occasional lesions distributed randomly over hypocotyl.

[b] All numbers refer to hr after inoculation of etiolated Topcrop seedlings with β race.

treatment of noninoculated or β race-infected Topcrop seedlings.

Histological observations of infected, heat-treated plants.—Microscopic examination of hand-sectioned epidermal strips from plants kept at 22-24 C indicated germination of some conidia of the β race of *C. lindemuthianum* within 4 hr after inoculation. Hyaline appressoria were observed at 11 hr, and nearly all germinated conidia had formed pigmented appressoria at 24 hr. Penetration from appressoria was observed near the cotyledonary node at 40-48 hr, but not until 60 hr at mid-hypocotyl.

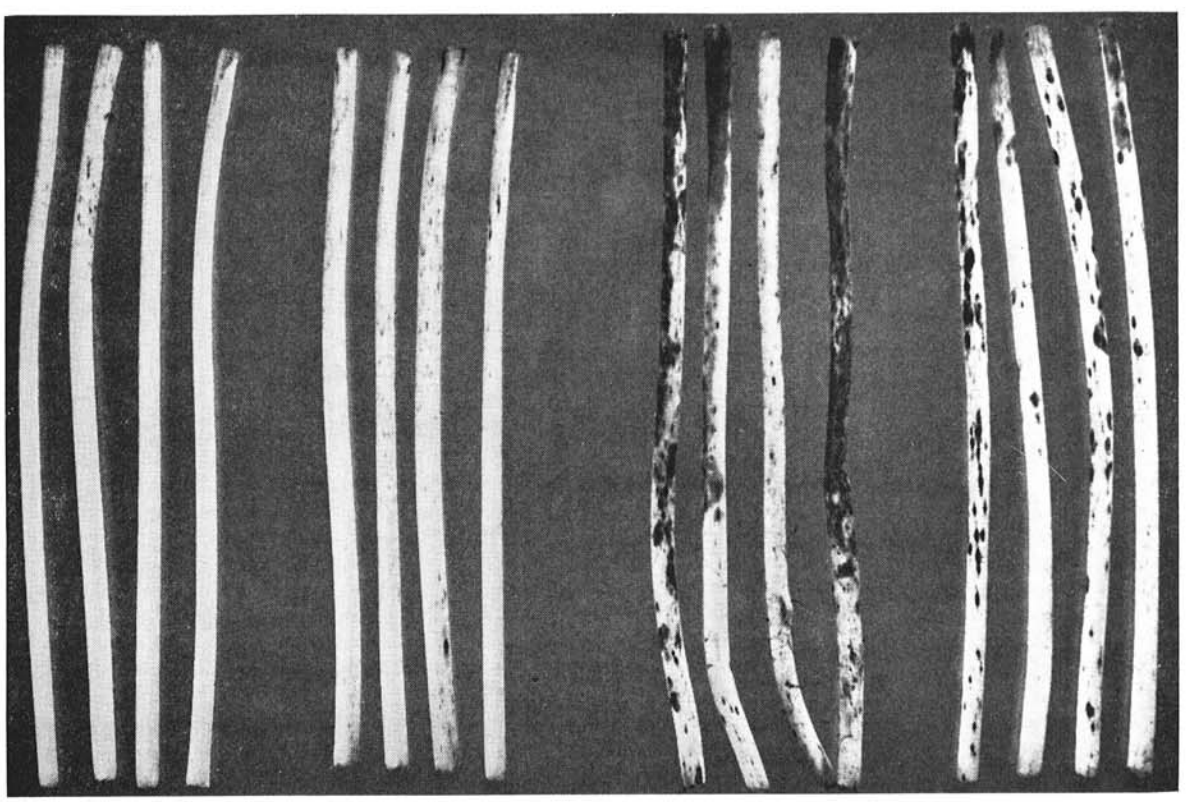

Fig. 1. Effect of heat treatment (at 37 C, 78-86 hr after inoculation) of β race infected and control Topcrop seedlings on their response to subsequent inoculation 24 hr after heat treatment with the β race of *Colletotrichum lindemuthianum*: groups of hypocotyl sections were (left to right) infected heat-treated-not reinoculated, infected heat-treated-reinoculated, control heat-treated-inoculated, and control untreated-inoculated.

When plants were placed at 37 C immediately after inoculation, conidia appeared undamaged after 10 hr, but had not germinated. Germination and appressoria formation occurred when the plants were returned to 22-24 C. As the length of exposure at 37 C increased, however, fewer conidia were detected, and of these, many appeared plasmolyzed or deformed. Germination of conidia at 22-24 C was not observed following treatments of more than 14 hr at 37 C.

Epidermal strips were removed from plants 5 days following heat treatment 74-82 hr after inoculation. Lesions restricted by heat treatment and flecks that appeared after the plants had been returned to 22-24 C were apparent on these plants. An appressorium was observed at nearly every fleck, and infection hyphae were frequently distinguishable within some of the host cells constituting a fleck. These cells were plasmolyzed and granular in appearance, and visibly similar to cells characteristic of hypersensitive or resistant response (9).

Effect of heat treatment on C. lindemuthianum growing on agar.—Enlargement of 3-day-old colonies of *C. lindemuthianum* growing on bean juice agar in 10-cm petri dishes ceased at 37 C, but resumed when the cultures were returned to 22-24 C after exposures of 15 hr or less. Heavy sporulation occurred within 3 days in the heat-treated portions of the colonies. Longer exposures at 37 C resulted in lag periods prior to resumption of growth at 22-24 C and visible evidence of damage to the fungus. The mycelium at the centers of colonies treated for 18 hr at 37 C collapsed soon after treatment, and collapse of entire colonies occurred during heat treatments lasting 24 hr or longer. Resumption of growth was observed within 2 and 5 days after 24-hr and 35-hr treatments, respectively.

To test whether the heat-treated fungus was self-inhibitory, colonies approximately 2 cm in diam were treated for various lengths of time at 37 C. Fresh conidia were introduced adjacent to the treated colonies 24 hr after heat treatment. The growth of colonies from these transfers was not inhibited by the treated or collapsed mycelium, and was similar to the growth of colonies on fresh agar in the absence of treated colonies.

DISCUSSION.—*Colletotrichum lindemuthianum* does not develop at temp above 31-32 C in culture, and infection does not occur in the field during hot weather (4, 5). The highest temp at which undiminished infection occurred on etiolated seedlings was 27 C; lesions decreased in size and number with increasing temp of incubation between 28-32 C. We observed penetration of epidermal cells of bean hypocotyls by *C. lindemuthianum* only from appressoria (13). Appressoria developed when conidia germinated on the plants at 27 C but not at 32 C. Ishida & Akai (6) reported that although conidia of *Colletotrichum lagenarium* germinated at 32 C, appressoria formation decreased at temp above 28 C, and did not occur at 32 C.

We observed, consistent with the reports of Dey (3) and Leach (9), that conidia began germination 4-6 hr after inoculation, and formed pigmented appressoria within 24 hr at 22-24 C. Penetration of host cells from the appressoria on the upper portions of seedlings began 40-48 hr after inoculation, but the average time at which penetration occurred on the mid-hypocotyl portions of plants was about 60 hr after inoculation. The sequential appearance of symptoms reflected the time at which penetration was observed on different portions of the plants, and indicated that the appearance of symptoms occurred 30-40 hr after penetration. We propose that heat treatments begun immediately after inoculation affect only the conidial stage of the fungus, and treatments begun at 45 hr primarily affect appressoria. Treatments later than 45 hr are considered to affect increasing numbers of infection hyphae and correspondingly fewer appressoria.

Growth of the fungus ceased at temp above 32 C. This effect was observed microscopically for both conidia and mycelium growing on bean juice agar, and for conidia and appressoria on etiolated bean hypocotyls. The cessation of symptom development at elevated temp suggested that mycelium within host tissues was similarly affected. Resumption of growth or development by the various structures of the fungus was dependent on the duration of heat treatment. The infection-limiting effect of heat treatment of conidia or appressoria may reflect direct physical or physiological damage to these structures. Similar damage apparently delayed the resumption of growth by mycelium on bean juice agar, although the fungus was not killed by up to 35 hr at 37 C. Presumably equivalent heat treatment would have similarly affected infection hyphae within the host. The fact that as little as 7-hr heat treatment at 37 C directed against infection hyphae irreversibly interrupted symptom development suggests that (i) infection hyphae within the host are more sensitive to heat than is mycelium growing on bean juice agar; or (ii) conditions within the host after treatment prevent further invasion by the infection hyphae.

Bell & Presley (1, 2) describe effects of temp on phytoalexin synthesis and resistance to *Verticillium* wilt in cotton. Varieties susceptible at 22 C were resistant at 32 C. Heat-killed conidia of a pathogenic strain of *Verticillium albo-atrum* induced phytoalexin synthesis in susceptible and resistant varieties, and protection was observed when the induced plants were inoculated 7 days later with live conidia of the same strain of the fungus. The authors state that the level of induced resistance was directly related to the amount of phytoalexin induced.

We have described protection against normally pathogenic races of *C. lindemuthianum* (12) induced by varietally nonpathogenic races, or by other fungi to which beans are hypersensitively resistant. The development of flecks after heat treatment suggested a resistant response by the host, possibly induced by a resumption of growth by the infection hyphae. The apparent similarity of the fleck response after heat treatment and the hypersensitive response characteristic of resistance suggested the possibility of induced protection following heat treatment. Subsequent challenge of the heat-treated plants with a pathogenic race

of *C. lindemuthianum* indicated that protection was induced when an earlier infection by the same race was arrested by heat treatment. Equivalent heat treatment of noninfected plants resulted in increased susceptibility to subsequent infection by the pathogenic race; heat treatment, per se, did not induce protection in the host. Heat treatment did not alter the response of seedlings to a varietal-nonpathogenic race of the fungus. Resistant response to the γ race of *C. lindemuthianum* was similarly expressed by infected and previously noninoculated heat-treated plants and previously noninoculated untreated controls. This indicated that previous infection by a varietal-pathogenic race of the fungus did not predispose the infected plants to susceptibility to varietal-nonpathogenic races of the fungus. Two distinct mechanisms of defense are apparent; one quantitative in nature and heat sensitive, and the other qualitative, and not altered by the heat treatments. Only the former mechanism is consistent with Yarwood's (17) observation of increased susceptibility of heat-treated bean leaves to anthracnose.

Heat-treated or heat-killed mycelium of *C. lindemuthianum* on bean juice agar did not inhibit the growth of subsequently introduced colonies of the same fungus, suggesting that the heat-treated infection hyphae, per se, were also not a source of protection in the host. The induced protection must reflect an active response of the host cells to the fungus which was triggered by heat treatment of the host-fungus complex.

Rahe et al. (12) stated that reciprocal cross protection indicated that each of the bean varieties tested was potentially capable of a resistant response, and that each of the races of the fungus used was potentially capable of inducing such a response. The interaction controlling the expression of these potentials determines the outcome of any host-fungus encounter. One explanation for the protection demonstrated in these experiments is that heat treatment selectively initiates the expression of the potential of the fungus for inducing a resistant response. Our observations suggest that a protective response occurs because heat treatment (i) causes an alteration of fungus structure or extracellular metabolites, and/or (ii) slows the growth of the fungus in such a way that the normal response-infectivity equilibrium (susceptibility) is irreversibly shifted in favor of the host (resistance). These alternatives imply that the potential of the fungus to induce a protective host response is self-repressed or host-repressed in susceptible varieties (but is de-repressed by heat treatment), or that the response mechanism of the host is activated by infection but repressed by the fungus in susceptible varieties. In the latter case, varietal response would be determined by the rate of synthesis and stability of the fungal repressor.

We conclude that, while the infection-limiting effect of elevated temp reflects direct physiological or structural damage to conidia and appressoria, the effect against infection hyphae may be indirect via activation of the varietal resistant response of the host against the normally pathogenic fungus. Such an effect would be particularly significant at marginally high temp or at the daily high temp of short duration commonly encountered in the field.

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