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Effects of Heat Shock on Disease Resistance
and Related Metabolism in Cucumber

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Bruce Allen Stermer

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EFFECTS OF HEAT SHOCK ON DISEASE RESISTANCE AND RELATED METABOLISM IN CUCUMBER

By

Bruce Allen Stermer

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ABSTRACT

EFFECTS OF HEAT SHOCK ON DISEASE RESISTANCE
AND RELATED METABOLISM IN CUCUMBER

By

Bruce Allen Stermer

A brief heat shock induced resistance to the scab pathogen, Cladosporium cucumerinum, in cucumber plants normally susceptible to the fungus. Immersion of seedlings in a 50 C water bath for 40 or 50 seconds was found to be the optimal treatment for the induction of resistance. Plants inoculated with C. cucumerinum as soon as 3 h after the heat shock exhibited increased resistance to the fungus; a 12 h interval from heat shock to inoculation allowed for development of maximum resistance. The resistance was still fully effective when plants were inoculated 48 h after heat shock. All scab susceptible cultivars that were tested became more resistant to C. cucumerinum after heat shock. There was a direct correlation between the activity of soluble peroxidase induced by heat shock and the resistance induced by the same treatment. Heat shocked cucumbers had an increase in activity of the same isoperoxidases seen to increase in cucumbers with systemic resistance induced by prior Colletotrichum lagenarium inoculation. The relationship of heat shock induced resistance to other stress responses and the role of peroxidases in induced resistance is discussed.

Within 6 h after the heat shock there were increases in the production ethylene and in its precursor 1-aminocyclopropane-1-carboxylic acid. Heat shock also enhanced the accumulation of extensin, a hydroxyproline-rich glycoprotein found in the cell walls of the seedlings.

Inoculations of heat shocked seedlings with C. cucumerinum 24 h after the shock resulted in further enhancement of extensin. Cell walls from heat shocked seedlings were more resistant to degradation by enzymes from C. cucumerinum than were cell walls from unshocked seedlings, but increased lignin deposition did not appear responsible. The accumulation of extensin after heat shock and its crosslinking by peroxidase is discussed as a possible mechanisms of resistance to C. cucumerinum.

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GENERAL INTRODUCTION

Heat shock has been widely used by plant pathologists. Kunkel reported in 1936 that dormant trees could be cured of yellows diseases by immersing tissues in a 50 C water bath for three to four minutes (9). In the following years heat treatments proved to be one of the most successful methods to eliminate viruses and yellows agents from infected plants (5). A summary of the therapeutic use of heat treatments on plant viruses has been published in a review by Hollings (5). Heat shock also has been used in studies of disease resistance mechanisms, e.g., the heat treatment of uninfected plants to increase their susceptibility to viruses and fungi. Yarwood found that immersion of bean leaves in hot water for a few seconds before inoculation increases their susceptibility to various viruses and fungi (18). Later work demonstrated that a brief heat shock could reduce disease resistance in many types of plants (2,17). Researchers attributed the decrease in resistance of heat shocked plants to a blocking of defense mechanisms, such as phytoalexin production (6). Section I of this thesis describes the use of heat shock to suppress pathogen-induced lignin deposition in cucumber cell walls and simultaneously increase susceptibility to fungi.

Wider interest in heat shock began with Ritossa's paper in 1962 (12). This paper showed that transient chromosome modifications, indicative of active gene loci, were dramatically induced in Drosophila by a brief heat shock. However, very little progress was made towards

understanding the phenomenon until 1974 when it was discovered that heat shock induced the synthesis of a small number of proteins and reduced normal protein synthesis (15). Since this time, heat shock has received considerable attention in model studies of gene expression in Drosophila (1). Analogous responses to heat shock were reported for cultured avian cells, bacteria, protozoans, yeast, and plants in 1978 (see ref. 13 for an excellent review). Thus, the heat shock response appears to be ubiquitous. In addition to heat shock other stress agents also induce heat shock proteins in various organisms, including amino acid analogs, metal ions, anoxia, viral infection, certain ionophores, and various antibiotics (13). The common occurrence of the heat shock response has been strengthened by the demonstration that antibodies to chicken heat shock protein cross-react with similar proteins of Drosophila, yeast, man, mouse, and frog (7).

Previous studies have shown that stress of cucurbits caused by a prior infection can render susceptible plants resistant to subsequent attack from many different pathogens. This induced resistance has many similarities with the resistance described in Section II where cucumbers develop the ability to resist C. cucumerinum infection approximately 24 h after a heat shock. Disease resistance induced in plants by prior infection has received considerable attention and is the subject of many reviews (8,11,14). Changes in epidermal cell walls appear to be involved in the mechanism of induced resistance in cucurbits against fungi. Correlated with the induction of disease resistance are an enhancement of cell-wall-associated peroxidase activity and lignin deposition (8). Increases in extensin, a hydroxyproline-rich glycoprotein

of plant cell walls, is also associated with disease resistance in cucurbits (3,4). The involvement of cell wall modifications in the resistance induced in cucumber by heat shock is examined in Section III. Recent reviews discuss extensin and its role in plants and also summarize knowledge about the role of lignification disease resistance (10,16).

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SECTION I

EFFECTS OF HEAT SHOCK ON CULTIVAR AND NONHOST RESISTANCE IN CUCUMBER SEEDLINGS

SECTION I

EFFECTS OF HEAT SHOCK ON CULTIVAR AND NONHOST RESISTANCE IN CUCUMBER SEEDLINGS

ABSTRACT

A brief heat treatment of cucumber seedlings immediately prior to inoculation with either Helminthosporium carbonum or Cladosporium cucumerinum temporarily induced susceptibility to these fungi; inoculations 24 h after heat shock demonstrated that resistance had returned. The heat shock did not appear to produce any permanent damage to seedlings. The ability of seedlings to deposit lignin at points of infection was associated with both cultivar and nonhost resistance. Heat treatments which induced susceptibility prevented the epidermal cell walls of seedlings from lignifying after inoculation. The data suggest that resistance, but not susceptibility, requires active host metabolism.

INTRODUCTION

Heat shock has been used by many researchers to manipulate the expression of resistance to fungi. Generally, heat shock applied prior to inoculation has prevented or delayed disease resistance in plants. Such heat shock can inhibit cell wall alterations, such as papilla formation (1), and also reduce phytoalexin production (8) and hypersensitive cell death (6,12). However, heat shock can also prevent or delay the susceptible response to the fungi that produce host-selective toxins; the shock reduces plant sensitivity to the toxin (2,3,11). The common denominator in all these effects of heat shock on disease resistance appears to be the temporary halt of many active processes. Thus, depending on which process requires active metabolism, heat shock may block resistance or susceptibility. Heat shock also has effects at the molecular level including the de novo synthesis of "heat shock proteins" and the reduction of normal protein synthesis (10).

Rapid lignification of epidermal cell walls is linked to disease resistance against some fungi (14). In cucumbers, lignin deposition is associated with resistance to the fungal pathogen Cladosporium cucumerinum (5,7). This study uses heat shock as a tool to 1) examine the association of lignin deposition with cultivar and nonhost resistance in cucumber seedlings, and 2) to investigate whether the seedlings require active metabolism for resistance or susceptibility.

MATERIALS AND METHODS

Plant and fungal material

Cucumber seedlings (Cucumis sativus L.) resistant (cv SMR-58) or susceptible (cv Marketer) to the fungus Cladosporium cucumerinum Ell. and Arth. were used. Seeds were germinated and grown for 5 days in darkness at 22 C in rolled-up germination paper (Anchor Paper Co., St. Paul, MN) before treatment (4). Helminthosporium carbonum Ull. race 1 and C. cucumerinum were grown on V-8 agar and potato dextrose agar, respectively, at 18 C (13).

Heat shock and inoculation of seedlings

The seedlings were given a heat shock by immersing their apexes and hypocotyls in a 50 C water bath for 40 seconds while holding on to the roots. The shocked seedlings were then placed in a covered 10 cm glass petri dish that contained one piece of moistened filter paper (9 cm diameter). Fungal cultures were gently rubbed with a bent glass rod in the presence of some water to dislodge spores. The spore suspension was filtered through 2 layers of cheesecloth, and the concentration of spores was adjusted to 10^6 spores per ml for C. cucumerinum and 10^5 spores per ml for H. carbonum. Seedlings were inoculated immediately or 24 h after heat shock by placing a line of 3 to 5 ul drops of spore suspension along the entire length of the hypocotyl.

Histochemical staining

The epidermis peeled from seedling hypocotyls was stained for lignin with phloroglucinol-HCl (9). A red chromogen is formed when phloroglucinol in HCl comes in contact with the cinnamyl aldehyde subunits of lignin. Separate epidermal tissues were stained with cotton

blue in lactophenol to visualize fungal structures (13). The stained epidermal peels were examined with a light microscope for evaluation of cucumber cell wall lignification and fungal development.

RESULTS

Effects of prior heat shock on resistance to Helminthosporium carbonum

The corn pathogen H. carbonum germinated a few hours after inoculation and formed appressoria within 6 to 10 h on untreated cucumber seedlings. By 18 h after inoculation, phloroglucinol-HCl staining produced a strong red color reaction in the cucumber cell walls around appressoria. Growth of the fungus into the epidermis stopped at about 24 h, and hyphal development in the tissues was restricted to the stained (lignified) areas. Both cultivars gave the same result.

In contrast, the production of lignin was totally suppressed in plants that were inoculated with H. carbonum immediately following a heat shock. The fungus readily penetrated the cucumber epidermis, and intracellular hyphae grew well and often entered adjacent cells by 24 h after inoculation. Within forty-eight hours after inoculation, the fungus had ramified through tissues; aerial mycelium was produced at inoculation sites by 72 h. Later, H. carbonum produced conidia on the cucumber hypocotyls. Although the cucumber tissues regained their ability to lignify by 48 h after the heat shock, and much lignin was present in the infected tissues after this time, the response by the seedlings apparently was too late to stop the fungus.

Seedlings that were inoculated 24 h after heat shock gave a typical lignification response and were resistant to the fungus. The

lignification of cell walls and the growth of H. carbonum in seedlings inoculated 24 h after heat shock were identical to the response of unshocked control seedlings. These delayed inoculations showed that normal resistance returned within 24 h after heat shock. The heat shock treatment did not appear to produce any permanent damage to seedlings.

The effects of heat shock were not only temporary, but the effects were also localized. This was demonstrated by heat shocking only half of the hypocotyl. Irregardless of whether the apical or basal half was used, only the portion given a heat shock lost the ability to lignify and lost resistance; the unshocked portion gave a normal resistant response (Fig. 1).

Effect of prior heat shock on resistance to Cladosporium cucumerinum

The fungus germinated several hours following inoculation on either cultivar; the response of SMR-58 (resistant) was lignification around sites of attempted penetration by 18 h, but the cell walls of Marketer (susceptible) did not contain phloroglucinol positive material at this time. The response of SMR-58 to C. cucumerinum was very similar to the response of either cultivar to H. carbonum. Development of C. cucumerinum was stopped in the resistant cultivar by 24 h after inoculations. However, C. cucumerinum continued growth and later ramified through the tissues of the susceptible cultivar.

When the normally resistant cultivar SMR-58 was inoculated with C. cucumerinum immediately after heat shock, no lignin deposition was observed at 24 h after inoculation. In addition, growth of the fungus into the tissues was similar to that seen for the susceptible cultivar. Delayed inoculations showed that the effects of heat shock on cucumber

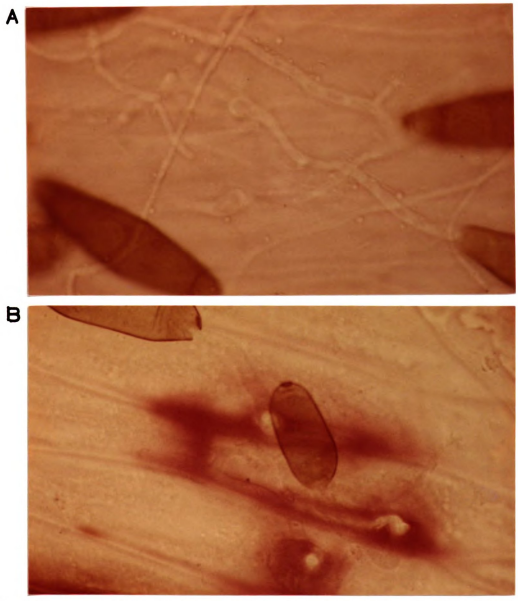

Fig. 1. Lignin deposition in epidermal peels from the heat shocked and unshocked halves of a cucumber hypocotyl inoculated with Helminthosporium carbonum. The apical half of a cucumber seedling (SMR-58) was heat shocked (40 seconds at 50 C), and entire length of the hypocotyl was then immediately inoculated. Twenty-four hours after inoculation epidermal peels were stained with phloroglucinol-HCl and photographed at 400X magnification. A, peel from a heat shocked portion of hypocotyl; B, peel from an unshocked portion of hypocotyl.

resistance to C. cucumerinum was also temporary; however, full resistance to C. cucumerinum returned slower than did resistance to H. carbonum (Table 1).

Additional observations of effects of heat shock on disease resistance

Two further observations are noteworthy. First, although normal resistance returned approximately 24 h after heat shock, both H. carbonum and C. cucumerinum continued to grow through tissues with recovered resistance when inoculated immediately after the shock. Apparently the fungi can overcome the host's resistance once an initial barrier is breached or a certain stage of fungal development is reached. Secondly, the cultivar susceptible to C. cucumerinum demonstrated an unexpected increase in resistance when inoculated 24 h after heat shock. The resistance induced by heat shock had many similarities to systemic induced resistance in cucumbers. Studies on the disease resistance induced by heat shock are presented in the following sections.

DISCUSSION

Heat shock delayed the expression of both cultivar and non-host resistance in cucumber seedlings. This effect was temporary, lasting less than 24 h, and was localized to the tissues actually shocked. A heat shock immediately before inoculation, however, did not provide any protection for the C. cucumerinum-susceptible cultivar against the pathogen. The major effect of heat shock on plant-pathogen interactions can be explained by the temporary halt of most active plant metabolism (10). In interactions where host susceptibility appears to be an active process, such as diseases involving host-selective toxins, a heat shock

Table 1

The effect of a heat shock prior to inoculation on lignin deposition and disease development in cucumber seedlings

Cultivar	Inoculated without prior heat shock		Inoculated immediately after heat shock[a]		Inoculated 24 h after heat shock[a]	
	Inoculum		Inoculum		Inoculum	
	C. carbonum	C. cucumerinum	H. carbonum	C. cucumerinum	H. carbonum	C. cucumerinum
Marketer						
lignin deposition[b]	+++[c]	-	-	-	+++	+/-
disease development[d]	none	extensive	extensive	extensive	none	limited
SMR-58						
lignin deposition	+++	++	-	-	+++	+
disease development	none	none	extensive	extensive	none	limited

[a]40 seconds at 50 C.

[b]Rated 24 h after inoculation using phloroglucinol-HCl.

[c]Arbitrary rating scale: (-) no staining, (+/-) sporadic staining, (+) light staining, (++) moderate staining, (+++) heavy staining.

[d]Evaluated 96 h after inoculation.

can prevent or delay the normal susceptibility of plants (2,3,11). Alternatively, if host defense is an active process, then heat shock will prevent or delay the normal resistance of plants (6,8,12). Because heat shock could block cultivar and nonhost resistance but not susceptibility to C. cucumerinum, it suggests that resistance to H. carbonum and C. cucumerinum in cucumber seedlings requires active host metabolism.

Heat shock also prevented the deposition of fungal-induced lignin in cucumber cell walls. There was a strong association between lignification and disease resistance. The prevention and later recovery of host resistance after heat shock was always correlated with the prevention and later recovery of the host's ability to deposit lignin. This is consistent with earlier work that indicated cell wall lignification by cucumbers is an important active defense against fungi (5,7).

Heat shock has several uses as a tool to study plant-pathogen interactions. Treatment of plants with a heat shock prior to inoculation can affect the success of the pathogen; this technique has been used with increasing frequency in studies of host plant responses to pathogen development in tissues (1,6,8). Chemical inhibitors of plant metabolism may have the same effect, but confusion can arise in inoculated tissues as to whether the effects seen are due to the action of the inhibitor on the host or on the pathogen. The study presented here shows unequivocally that one or more heat-sensitive structures or processes within cucumber seedlings are necessary for disease resistance. Heat shock does not simply kill treated tissues either because seedlings recover resistance and continue to grow.

Another use of heat shock that requires further study is the possible determination of active susceptibility in the plant. In the limited work reported, heat treatments have reduced the susceptibility of plants to fungi producing host-selective toxins (2,3,11), but have increased susceptibility to fungi not known to produce a host-selective toxin (6,8,12). Heat shock could provide a simple test of whether a toxin or a nontoxic compatibility factor (suppressor) is involved in a disease. More plant-pathogen systems need to be examined to see if this observation holds up.

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SECTION II

HEAT SHOCK INDUCES RESISTANCE TO *CLADOSPORIUM CUCUMERINUM* AND ENHANCES PEROXIDASE ACTIVITY IN CUCUMBERS

ABSTRACT

A brief heat shock induced resistance to the scab pathogen, Cladosporium cucumerinum, in cucumber plants normally susceptible to the fungus. Immersion of seedlings in a 50 C water bath for 40 or 50 seconds was found to be the optimal treatment for the induction of resistance. Plants inoculated with C. cucumerinum as soon as 3 h after the heat shock exhibited increased resistance to the fungus; a 12 h interval from heat shock to inoculation allowed for development of maximum resistance. The resistance was still fully effective when plants were inoculated 48 h after heat shock. All scab susceptible cultivars that were tested became more resistant to C. cucumerinum after heat shock. There was a direct correlation between the activity of soluble peroxidase induced by heat shock and the resistance induced by the same treatment. Heat shocked cucumbers had an increase in activity of the same isoperoxidases seen to increase in cucumbers with systemic resistance induced by prior Colletotrichum lagenarium inoculation. The relationship of heat shock induced resistance to other stress responses and the role of peroxidases in induced resistance is discussed.

INTRODUCTION

Certain environmental and biological stresses of plants applied prior to challenge by a microorganism can often alter the outcome of subsequent host-parasite interactions. For example, preinoculation heat shock stress of plant tissue has been demonstrated to induce a state of susceptibility to fungi normally non-pathogenic on the shocked plant (6,14). On the other hand, stress caused by prior, limited infection of one leaf of cucumber plants with bacteria, fungi or viruses has been shown to induce systemic resistance against bacteria, fungi and viruses (17). In cucumber, the systemic induced resistance was associated with a systemic enhancement of peroxidase activity, enzymes which often exhibit increased activity after certain types of stress.

When heat shocked cucumber plants were inoculated immediately after the shock with a nonpathogen the plants were found to be temporarily susceptible to the nonpathogen (26, section 1 this thesis). Inoculation of other cucumber plants at 24 h after heat treatment demonstrated that resistance to the nonpathogen had returned (26, section 1 this thesis). Further studies found that when cucumbers susceptible to scab, incited by C. cucumerinum, were inoculated with this fungus 24 h after heat shock the plants were also much more resistant to the pathogen.

This paper reports the induction of resistance to C. cucumerinum by heat shock and the association of enhanced peroxidase activity with the induced resistance. A preliminary report has been published (27).

MATERIALS AND METHODS

Culture of plants and pathogens

Cultures of *Cladosporium cucumerinum* Ell. and Arth. and *Colletotrichum lagenarium* race 1 (Pass.) Ell. and Halst. were maintained on potato dextrose agar and V-8 agar, respectively, at 18 C in the dark. Conidial spore suspensions were prepared from 7 to 10-day-old cultures. Suspensions were filtered through 2 layers of cheesecloth and the spore concentration determined with a hemocytometer. For most experiments, cucumber (*Cucumis sativus* L.) plants were grown in the dark in 2 layers of rolled germination paper (10). Five days after sowing, the seedcoats were removed, and the seedlings were heat treated and replaced in the germination paper. In other experiments, cucumber plants were grown in vermiculite in a growth chamber (18 hr photoperiod, 20 C). The scab-susceptible cultivar "Marketer" was used unless stated otherwise.

Heat shock treatments

Seedlings were treated by dipping their cotyledons and hypocotyls in a water bath.

Inoculations and disease ratings

Seedlings were inoculated by spraying with a spore suspension of *C. cucumerinum* ($3X10^5$ spores ml^{-1}) 24 h after the heat shock treatment unless stated otherwise. The etiolated seedlings were rolled up again in the germination paper and incubated at 22 C. Light grown seedlings were inoculated and incubated as described (11). Individual plants were rated for disease by a method modified from Hammerschmidt et al. (10) 4 days after inoculation; 0 to 10, >10 to 30, >30 to 60 and >60% of the hypocotyl area covered by lesions was rated a 0, 1, 2 and 3,

respectively. Averages were based on 18 to 22 plants per treatment for each experiment. All experiments were replicated at least twice. Resistance-inducing inoculations were performed by infiltrating the first true leaf of green plants with a suspension of C. lagenarium spores ($1X10^5$ spores ml^{-1}) as previously described (13).

Extraction and assay of soluble peroxidases

Tissue extracts for peroxidase assays were prepared from the apical 2 cm of 20 hypocotyls (minus the cotyledons). The hypocotyl segments (which were frozen at -20 C until used) were homogenized in 2.0 ml of ice-cold 0.5M sucrose-0.01 M phosphate buffer (pH 6.0) and then centrifuged (10,000 xg) for 20 minutes at 4 C (13). The clear supernatant was decanted and used for peroxidase determinations. Peroxidases were extracted from green plants as previously described (13). Protein content was estimated by the method of Bradford (3).

Peroxidase activity was assayed using guaiacol as the hydrogen donor. The reaction mixture, consisting of 1.5 ml of guaiacol solution (0.56% in 0.1M phosphate buffer, pH 6.0) and 1.5 ml of peroxide solution (0.6% in distilled water), was added to a cuvette immediately prior to the addition of the enzyme extract (0.1 ml) (21). The reaction was followed colorimetrically at 470 nm. Enzyme preparations were diluted to give changes in absorbance of 0.1 to 0.2 absorbance units ml^{-1}. Activity was expressed as the increase in absorbance at 470 nm $min^{-1}mg^{-1}$ protein.

Electrophoretic separation of peroxidase isozymes

Non-denaturing vertical slab gel electrophoresis was carried out using 10% polyacrylamide resolving gel (pH 8·8) and a 4% polyacrylamide

stacking gel of 1 mm thickness (16). Samples to be analyzed were in sucrose-phosphate buffer and contained a trace of bromophenol blue dye. Electrophoresis was performed at 10 mA per slab gel. Peroxidase isozymes were detected by soaking the gels in o-dianisidine (1 mM in 0.1 M acetate buffer, pH 4.5) for 30 to 60 minutes. The gels then were rinsed in distilled water and placed in 0.60% peroxide to visualize the peroxidases.

RESULTS

Effect of the temperature and duration of heat shock on inducing resistance

In general, the higher the temperature of the shock and the longer its duration at a given temperature the greater was the resistance induced against C. cucumerinum. However, when the heat treatments were increased to the point where they irreparably damaged the plant, as determined by watersoaking of tissues (60 seconds at 50 C and 30 seconds or longer at 52.5 C) resistance was reduced or not induced (Fig. 1). A 50 C heat shock for 40 or 50 seconds was the optimal treatment of those tested for the induction of scab resistance (Fig. 2).

Effect of inoculum level on heat shock induced resistance

Protection against C. cucumerinum induced by a 50 C shock for 40 seconds was evident at all spore concentrations used. Although inoculation at $3X10^6$ spores ml^{-1} produced the most consistent observations of reduction in disease symptoms, the induced resistance was greater at lower inoculum levels (Fig. 3).

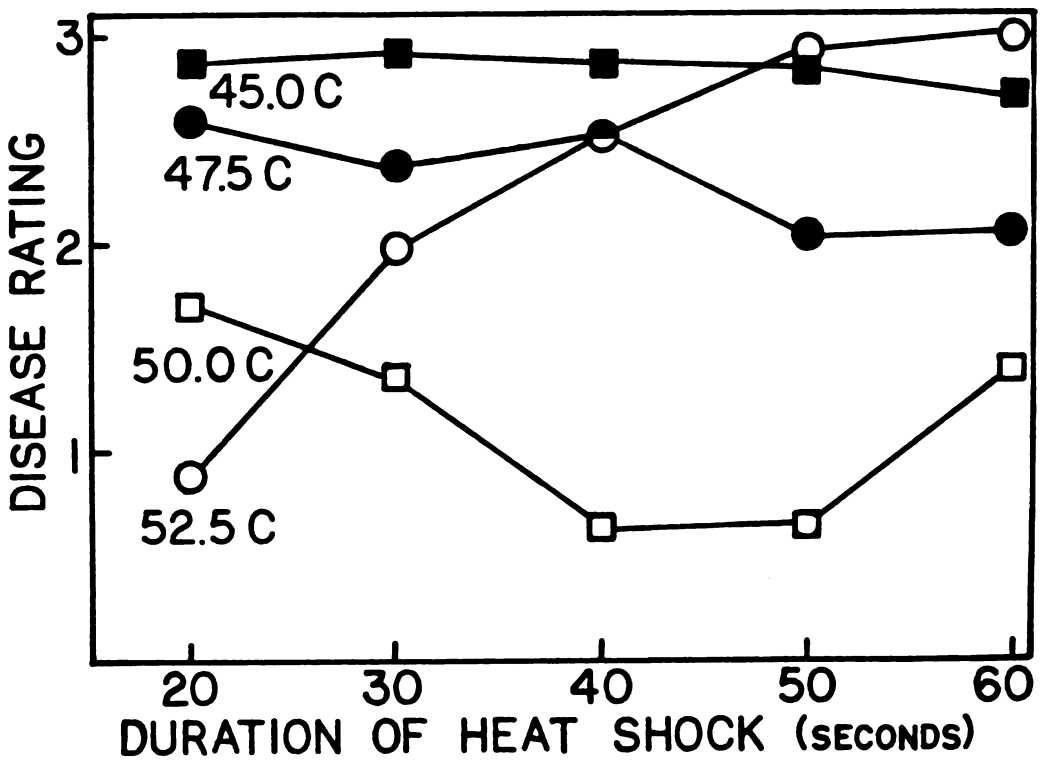

Fig. 1. The effectiveness of different heat treatments in protecting cucumber seedlings against C. cucumerinum. Five day old etiolated seedlings were heat shocked in a water bath at different temperatures for various durations. The plants were challenged 24 h after heat shock with C. cucumerinum ($3X10^5$ spores ml^{-1}) and incubated in germination paper in darkness. The seedlings were rated for disease 4 days after inoculation; 0 to 10, >10 to 30, >30 to 60 and >60% of the hypocotyl area covered by lesions was rated as 0, 1, 2 and 3, respectively.

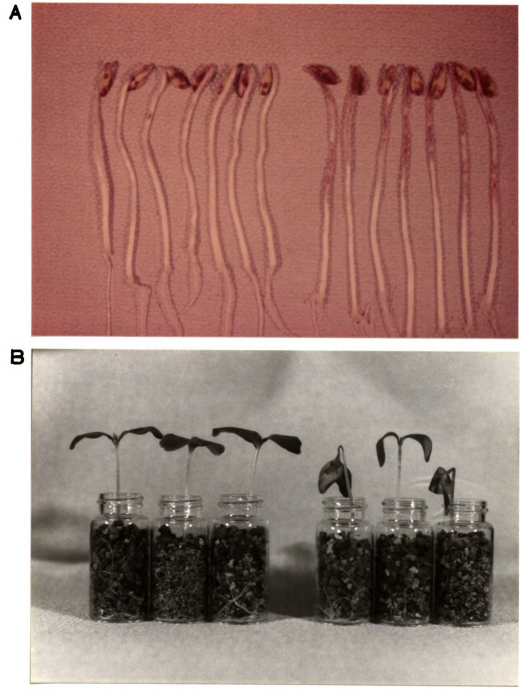

Fig. 2. Protection of cucumber plants against C. cucumerinum by heat shock. The plants were shocked for 40 seconds at 50 C and challenged with C. cucumerinum ($3X10^5$ spores ml^{-1}) 24 h later. A, dark grown seedlings 4 days after inoculation; B, plants grown in a lighted growth chamber at high relative humidity at 4 days after inoculation.

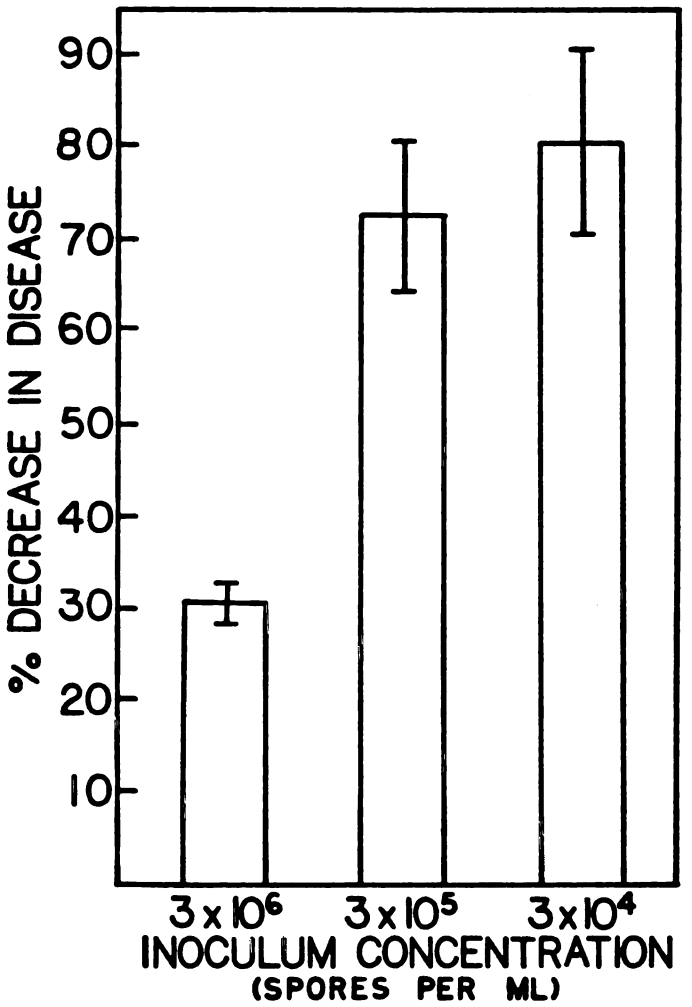

Fig. 3. The effect of challenge inoculum concentration on resistance observed after heat shock. Resistance was induced by a 40 second shock at 50 C, and the C. cucumerinum challenge inoculation was 24 h after heat treatment. Disease ratings were made 4 days after the challenge as described in Fig. 1.

Time course for heat shock induced resistance

By inoculation at various times after heat shock, resistance against C. cucumerinum was found to develop rapidly (Fig. 4). Plants inoculated as soon as 3 h after heat shock treatment demonstrated increased resistance to the fungus. Because there was a 12 to 18 h delay after inoculation before C. cucumerinum begins penetration of the plant, the actual onset of protection was probably 15 to 21 h after heat shock. A 12 h interval from heat shock treatment to inoculation allowed for development of maximum resistance. This resistance was still fully effective when plants were inoculated 48 h after heat shock. When inoculated after 48 h the unshocked control plants showed a gradual increase in resistance to the fungus. This resulted in a reduction in the disease rating when calculated as percent decrease compared to control.

Heat shock induced resistance in different cucumber cultivars

All of the scab susceptible cultivars that were tested became more resistant to C. cucumerinum after heat shock (Table 1).

Effect of temperature and duration of heat shock on enhancement of peroxidase activity

There was a direct correlation between peroxidase activity induced by heat shock and scab resistance induced by the same treatment (Fig. 5). The higher the temperature of the shock and the longer its duration the greater was peroxidase activity enhancement. As seen with induced scab resistance, temperature treatments which damaged the plants reduced the magnitude of the response.

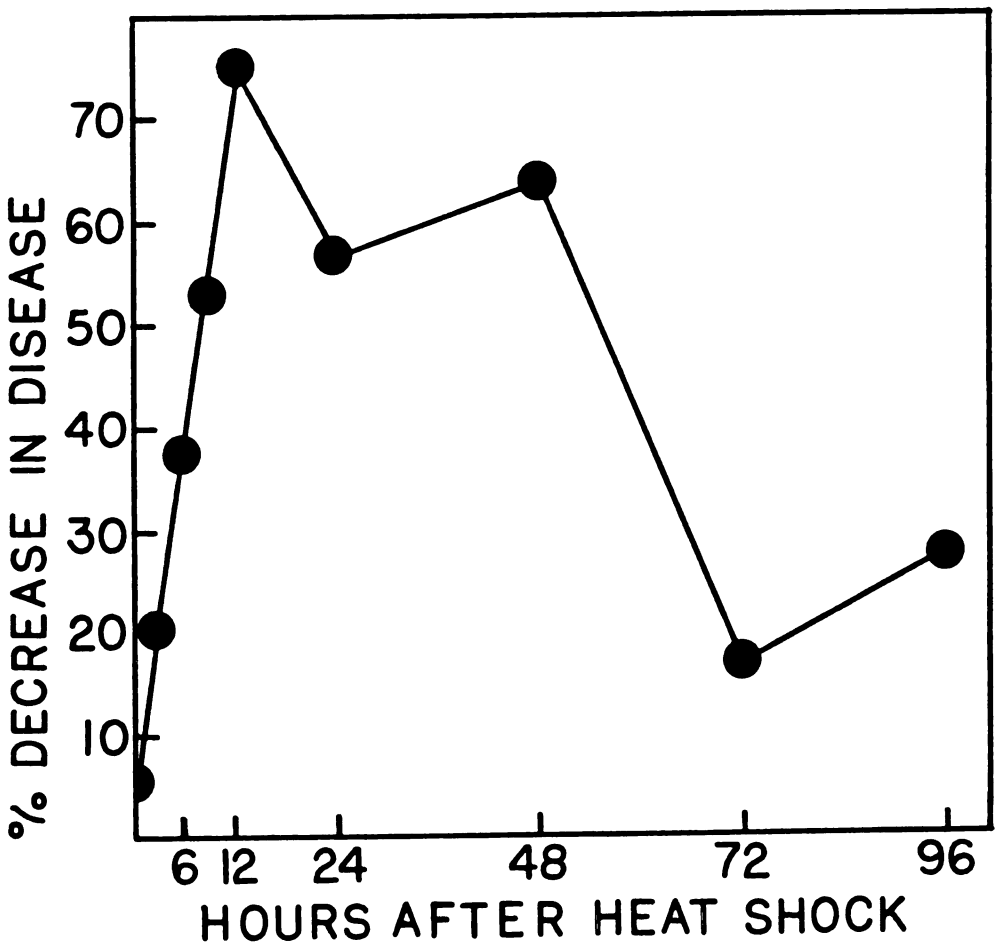

Fig. 4. The effect of the time of challenge on resistance observed after heat shock. Resistance was induced by a 40 second shock at 50 C. Disease ratings were made 4 days after the C. cucumerinum challenge inoculation ($3X10^5$ spores ml^{-1}). The disease ratings (explained in Fig. 1) were expressed as the percent decrease in disease rating of heat shocked plants when compared to unshocked control plants calculated by $(1 - \frac{hs}{c}) \times 100$.

Table 1

Resistance to Cladosporium cucumerinum induced in different cultivars of cucumber by preinoculation heat shock

Treatmentof seedlings	Disease rating of cultivars[a]			
	Marketer	Shamrock	Gemini	Straight-8
Heat shock[b]	0.80±.23	0.51±.29	0.61±.34	0.84±.35
No shock	2.32±.36	2.12±.42	2.29±.42	2.17±.28
Disease reduction by heat shock	65.5%	75.9%	73.4%	61.3%

[a]Disease ratings were made 4 days after challenge inoculation ($3X10^5$ spores ml^{-1}) as described in Fig. 1. Values are means and standard errors.

[b]Etiolated seedlings were heat shocked in a 50 C water bath for 40 seconds.

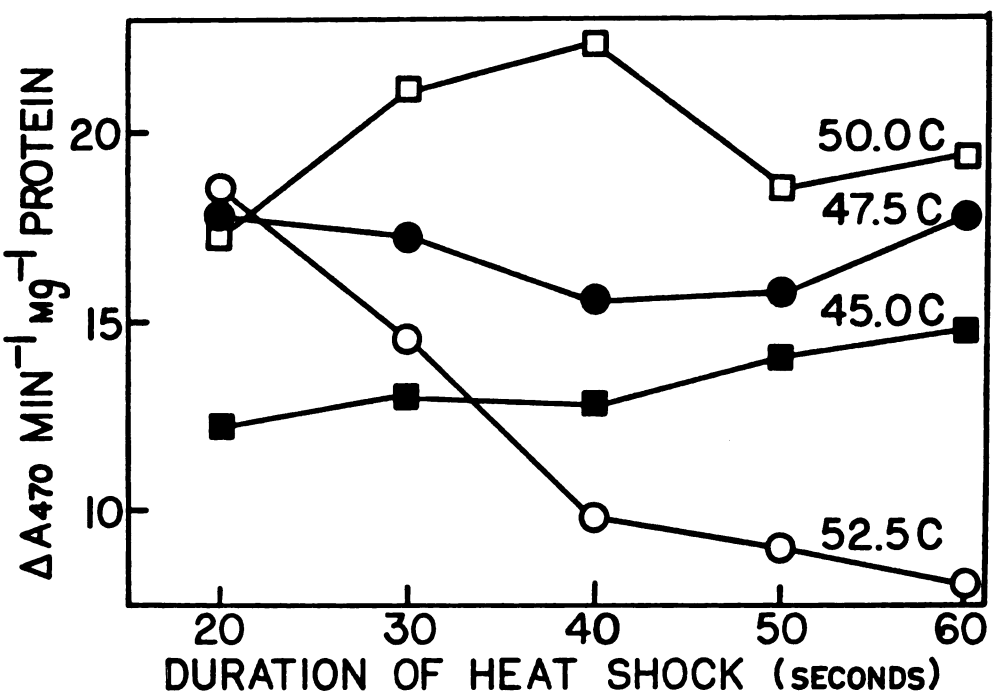

Fig. 5. The enhancement of peroxidase activity after heat treatments at various temperatures and durations. Five day old dark grown cucumber seedlings were heat shocked and hypocotyl samples were taken 24 h after the shock. Peroxidase activity is expressed as change in absorbance min $^{-1}$mg^{-1} protein using guaiacol as the hydrogen donor.

Time course for heat shock induced peroxidase activity

Peroxidase activity increased rapidly by 24 h after heat shock (Fig. 6). The rise in enzyme activity peaked at 2 days after the shock and then decreased. The peroxidase activity in the unshocked control seedlings remained relatively constant over this same period.

Separation of peroxidase isozymes by electrophoresis

Heat shocked cucumbers had an increase in activity of the fastest moving anodic isozymes when compared to control plants. The same isoperoxidases exhibited enhanced activity in cucumber plants with systemic resistance induced by prior C. lagenarium inoculation (Fig. 7) (13).

DISCUSSION

Preinoculation heat treatments of plants are known to alter the outcome of some host-parasite interactions. For example, susceptibility to fungi has been modified in many plants by a sudden rise in incubation temperature (heat shock) prior to inoculation. Heat shock pretreatment has increased the susceptibility of plants to fungal pathogens (32) and to nonpathogens (6,14). Heat shock inhibits plant defense responses to fungi (1,15,28). However, plants incubated at elevated temperatures can also become resistant to fungi that produce host-selective toxins. Loss of susceptibility to the toxin producing fungus has been associated with a loss of sensitivity to the toxin after heat treatment (4,5,19). The specific action of heat shock on disease resistance is unknown, although recent work has shown that heat shock causes the temporary inhibition of normal protein synthesis while increasing synthesis of a few "heat shock proteins" (2,7,23).

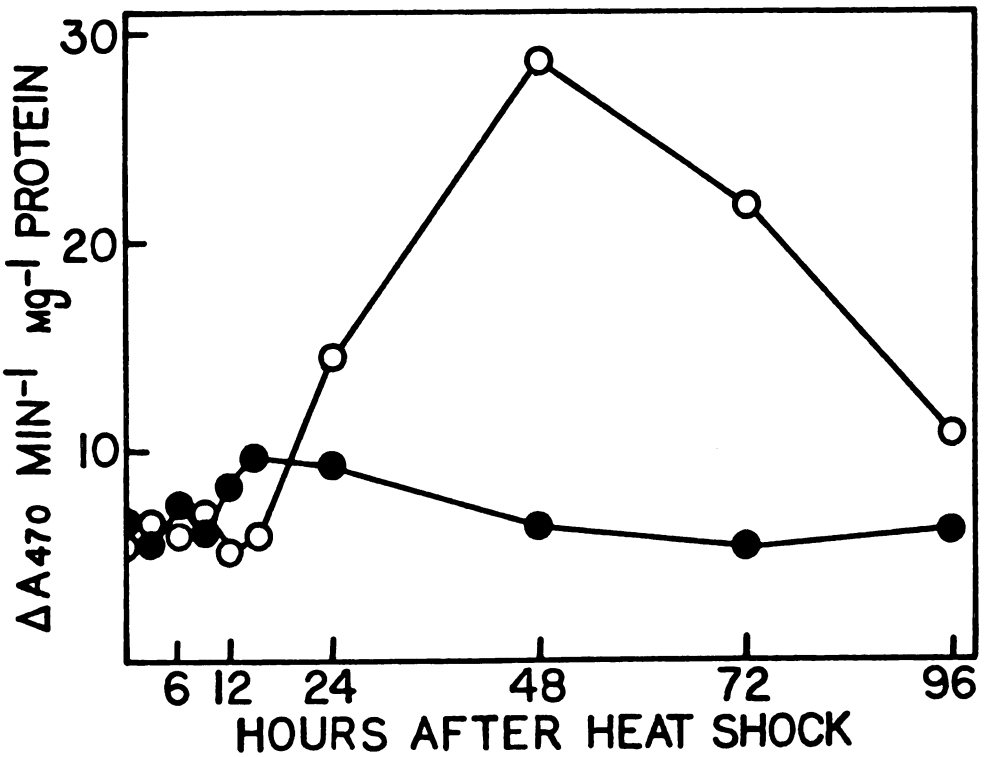

Fig. 6. Time course of peroxidase activity enhancement after heat shock. Dark grown cucumber seedlings were heat shocked 5 days after sowing and hypocotyl samples were taken 1 to 96 h after the shock. Peroxidase activity is expressed as change in absorbance $min^{-1}mg^{-1}$ protein using guaiacol as the hydrogen donor. Open circles represent values for heat shocked seedlings; solid circles represent values for unshocked seedlings.

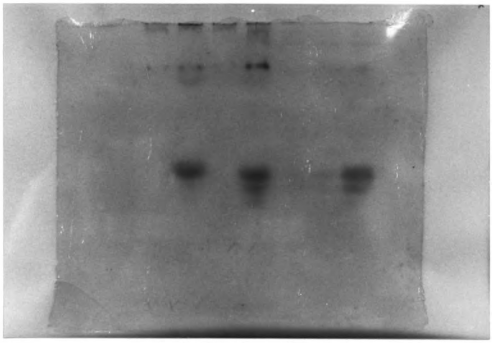

Fig. 7. Electrophoretic separation of anodic peroxidase isozymes from cucumber tissue with or without induced resistance to C. cucumerinum. For each sample 100 μg of protein was applied and electrophoresis was carried out with a 10% polyacrylamide slab gel (pH 8·8). Peroxidase isozymes were visualized with o-dianisidine as the hydrogen donor. Lane A, unshocked etiolated seedlings 6 days after sowing; B, etiolated seedlings 24 h after heat shock (40 sec at 50 C) and 6 days after sowing; C, unshocked etiolated seedlings 7 days after sowing; D, etiolated seedlings 48 h after heat shock (40 sec at 50 C) and 7 days after sowing; E, leaf 2 of green plants; F, leaf 2 of green plants that had leaf 1 infiltrated with H_2O 7 days earlier; G leaf 2 of green plants that had resistance induced by infiltration of leaf 1 with Colletotrichum lagenarium 7 days earlier.

A brief heat shock 24 h prior to inoculation induced a high level of resistance to C. cucumerinum in normally susceptible cucumber seedlings. Associated with the heat shock induced resistance was enhanced peroxidase activity in the seedlings. The optimal heat treatment for induction of peroxidase activity was identical to the treatment that resulted in the greatest protection against C. cucumerinum. Moreover, for all of the different heat shock treatments tested there was close correlation between the soluble peroxidase activity extracted and the amount of resistance observed against C. cucumerinum.

The level of heat shock induced resistance seen depended on the inoculum concentration used in the challenge. Resistance resulting from heat shock was more effective at the lower inoculum levels. Earlier work showed a similar effect of inoculum concentration in cucumbers with resistance induced by C. lagenarium (21).

Electrophoretic analysis of the peroxidase isozymes demonstrated that the same anodic isozymes are associated with resistance whether induced by heat shock or by prior C. lagenarium inoculations. Previous evidence indicates that these fast moving acidic isozymes are associated with the plant's cell wall (13,25). Thus, similar mechanisms may be implicated in resistance induced by either method, and the mechanisms may involve changes in the cell wall.

From the experiments described here it is not clear if the enhanced peroxidase activity is a cause or a consequence of heat shock induced resistance. Enhanced peroxidase activity has been associated with induced resistance in tobacco to tobacco mosaic virus (24,30). However, one study suggested that increases in peroxidase are not directly

involved in induced resistance against Pseudomonas solanacearum in tobacco (18). Recent work with cucumbers has shown that induced resistance and increases in peroxidase activity are at least causally related (13). Associated with the acquired resistance of cucumber was increased levels of lignification after challenge with C. cucumerinum (11). In addition, increased levels of extensin, a plant cell wall hydroxyproline-rich glycoprotein, has been implicated in resistance to C. cucumerinum (12). Peroxidase may be important in cucumber resistance because it is necessary for the crosslinking of extensin molecules (9) and also for the oxidative polymerization of hydroxycinnamyl alcohols to form lignin (29). Furthermore, peroxidase may contribute to induced resistance by increasing the concentration of fungitoxic free radicals formed from hydroxycinnamyl alcohols and other phenols.

The mechanism by which heat shock enhances peroxidase activity and induces scab resistance is unknown. The reduction of normal protein synthesis and the de novo synthesis of heat shock proteins caused by nonlethal heat stress appears to protect the plant against the deleterious effects of high temperatures (2). Recent research indicates that other types of stress also induce this response (8,22,31,32), suggesting that the heat shock response may be part of a more general response to stress that protects against cell damage. Perhaps disease resistance and peroxidase activity induced by heat shock are involved with this response.

Disease resistance induced in cucumbers by heat shock has many similarities with resistance induced by C. lagenarium and other biological agents. Heat shock may provide a good model to study the

mechanism of induced resistance as this system does not have the confounding effects of an inducing pathogen and can use very uniform plant tissue. Also, the heat shock induction of peroxidase activity may provide a good system to study enzyme regulation in plants.

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SECTION III

HEAT SHOCK INCREASES THE SYNTHESIS OF ETHYLENE AND ENHANCES THE ACCUMULATION OF HYDROXYPROLINE-RICH GLYCOPROTEIN IN CUCUMBER SEEDLINGS

ABSTRACT

Cucumber seedlings heat shocked for 40 seconds at 50 C developed increased resistance against the fungal pathogen Cladosporium cucumerinum. By 6 h after the heat shock there were increases in the production ethylene and in its precursor 1-aminocyclopropane-1-carboxylic acid. Heat shock also enhanced the accumulation of extensin, a hydroxyproline-rich glycoprotein found in the cell walls of the seedlings. Inoculations of heat shocked seedlings with C. cucumerinum 24 h after the shock resulted in further enhancement of extensin. Cell walls from heat shocked seedlings were more resistant to degradation by enzymes from C. cucumerinum than were cell walls from unshocked seedlings, but increased lignin deposition did not appear responsible. The accumulation of extensin after heat shock and its crosslinking by peroxidase is discussed as a possible mechanisms of resistance to C. cucumerinum.

INTRODUCTION

The response of plants to heat shock has recently received much attention. Many studies have examined the cytological, biochemical and molecular events in plants occurring within an hour after the heat shock. These events include the halt of cytoplasmic streaming (8), suppression of plant defenses against disease (9), and the rapid production of "heat shock proteins" (1). However, little is known about the long term response of plants to heat shock. One exception is the induction of disease resistance; 15 to 24 h after heat shock cucumber seedlings will develop resistance to the fungal pathogen Cladosporium cucumerinum (20). Concomitant with the induction of disease resistance in cucumbers is a marked enhancement by heat shock in the activities of peroxidase isozymes located in cell walls (section II this thesis). Although the mechanism by which heat shock enhances peroxidase activity and also induces disease resistance is unknown, recent research indicates that peroxidase may play an important role in the induced resistance (section II this thesis).

Cell wall modifications appear to be involved in the mechanism of resistance in cucumbers against C. cucumerinum. Hammerschmidt and Kúc found that the epidermal cell walls of plants with systemic induced resistance were lignified more rapidly and to a greater extent than were controls in response to attack by C. cucumerinum (5). Also, a recent study has shown that, in addition to enhanced lignification, an enhanced accumulation of bound extensin in cucumber cell walls was associated with cultivar resistance to C. cucumerinum (6). Thus, increased lignin

deposition and extensin accumulation may be involved in the resistance induced by heat shock.

Ethylene is often produced by plants undergoing various types of stress, such as mechanical or radiation injury, infection by microorganisms, or temperature deviations (21). Heat shock may also induce the production of ethylene, but this has not been examined. Interestingly, among the many plant processes reported to be stimulated by ethylene are the enhancement of extensin accumulation and peroxidase activity (2,10,18). The purpose of this study is to examine some prolonged effects of heat shock on cucumber seedlings, and to investigate the possible basis of disease resistance induced by heat shock.

MATERIALS AND METHODS

Cucumber seedlings Cucumis sativus L. cv Marketer) were used in all experiments unless stated otherwise. Seeds were germinated and grown for 5 days in darkness at 22 C in rolled germination paper (4).

Heat shock and inoculation of seedlings

The seedlings were given a heat shock by immersing their apexes and hypocotyls in a 50 C water bath for 40 seconds while holding on to the roots. Inoculation with the fungus Cladosporium cucumerinum Ell. and Arth. was by spraying seedlings with a spore suspension ($3X10^6$ spores per ml) 24 h after heat shock. C. cucumerinum is a pathogen of cv Marketer. After treatment, the etiolated seedlings were rolled up again in the germination paper and incubated until harvest.

Measurement of ethylene production

Immediately after the heat shock four seedlings were placed in a 50 ml flask containing 2 ml of water; the flask was then sealed with a serum bottle cap and incubated in darkness at 22 C. Four replicate flasks were used for each treatment. At various time intervals 1.0 ml gas samples were removed from each flask with a tuberculin syringe, and the concentration of ethylene in the sample was determined by gas chromatography (12). The accumulation of ethylene over time was measured and adjustments were made in calculations for ethylene removed during previous samplings.

Estimation of 1-aminocyclopropane-1-carboxylic acid (ACC) levels

The apical 2 cm of seedling hypocotyls (minus cotyledons) were excised and homogenized in 2.0 ml of buffer containing 100 mM K-Hepes (pH 8.0), 4 mM dithiothreitol and 0.4 mM pyrodoxal phosphate. The sample was then centrifuged at 12,000 RPM in a SS-34 rotor for 15 minutes at 4 C. The ACC levels in the supernatant were then estimated by determining the amount of ethylene produced from ACC after *in vitro* conversion of 0.1 ml samples in the presence of alkaline sodium hypochlorite in a sealed test tube (14). The ethylene was determined by gas chromatography as described above.

Estimation of bound extensin levels

The levels of cell-wall-bound extensin, a hydroxyproline-rich glycoprotein, were estimated by determining the amount of hydroxyproline remaining in extracted cell walls. The apical 2 cm of hypocotyl tissues were excised and ground to a powder in liquid N_2, then extracted with ca. 10 ml per gram fresh wt of the following: .015M Na-phosphate pH 6.0

(once), 0.5M NaCl (twice), 1.0 M NaCl (twice), and distilled deionized water (five times). The extracted cell walls were freeze-dried and 5 mg samples were hydrolyzed in 0.4 ml of 5.5N HCl at 110 C for 18 h. The hydrolysate was then reduced to dryness under nitrogen, resuspended in 0.5 ml of distilled deionized water, and 0.2 ml aliquots were removed for two determinations of hydroxyproline content by a spectrophotometric assay previously described (13).

Histochemical staining for lignin

The epidermis peeled from the apical 2 cm of a seedling's hypocotyl was stained with phloroglucinol-HCl (1% phloroglucinol in methanol/ concentrated HCl, 1:1) to visualize lignin deposition (11). Peels from at least four seedlings were examined with a light microscope for each observation.

Enzymic degradation of cucumber cell walls

Cell walls were prepared from the apical 2 cm of seedling hypocotyls (minus cotyledons) at 48 h after heat shock (40 seconds at 50 C). The excised tissues were ground with liquid N_2 to a powder, extracted three times with ca. 10 ml per gram fresh wt of 50 mM phosphate (pH 6.0), and then freeze-dried.

Cell wall degrading enzymes were produced by *C. cucumerinum* in liquid nutrient media containing 0.5% pectin and 0.5% polypectate or 1.0% cucumber cell walls as the carbon source (19). After six days on reciprocal shaker at 22 C the culture fluids were filtered through 4 layers of cheesecloth and centrifuged (12,000 g) for 15 minutes at 4 C. The filtrates were then dialyzed against water overnight. The partially purified filtrates were stored in scintillation vials at -20 C. The

Macerozyme solution was prepared fresh before each experiment. A one mg per ml solution of the Macerozyme powder (Kinki Yakult Mfg. Co., Japan) was passed through a Sephadex G-50 desalting column to remove contaminating reducing sugars.

The enzymic degradation was carried out in a 12 ml conical centrifuge tube containing 5 mg of cucumber cell walls, 0.25 ml of phosphate buffer (0.2M, pH 6.0) with .004% merthiolate, and 0.75 ml of enzyme preparation (16). The reaction mixture was incubated at 25 C for 16 h; then 0.1 ml aliquots were removed for estimation of the reducing sugars released (15). Values for control tubes containing only cell walls or only enzyme preparation were subtracted from the total amount of reducing sugars found.

RESULTS

Ethylene production after heat shock

A brief heat shock markedly increased the production of ethylene by cucumber seedlings. Ethylene accumulation was approximately two-fold higher by 6 h after heat shock with the shocked seedlings when compared to unshocked controls (Fig. 1). The increased production of ethylene was evident for at least 15 h in heat shocked seedlings.

ACC levels after heat shock

ACC levels were also higher in heat shocked seedlings. ACC levels were approximately two-fold higher in heat shocked seedlings than in unshocked seedlings by 6 h after the shock (Table I). Assays for ACC synthase must be considered inconclusive due to the low amounts of

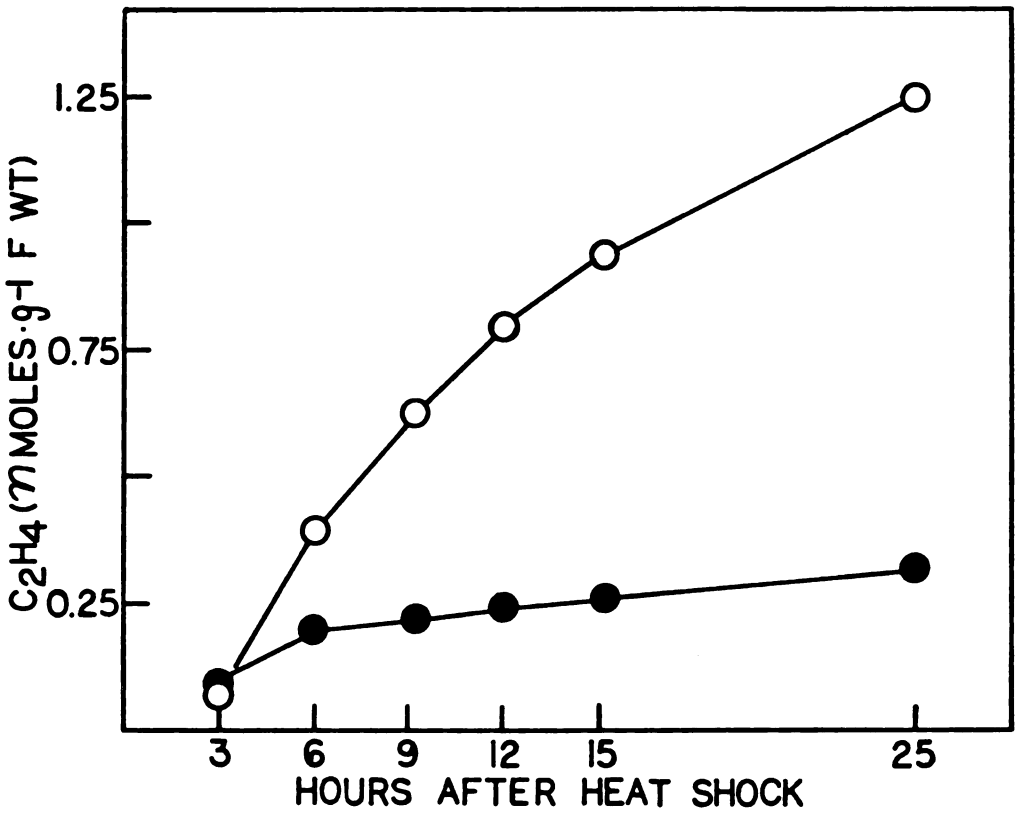

Fig. 1. Effect of heat shock on ethylene production by cucumber seedlings. Seedlings were heat shocked (40 seconds at 50 C) and four seedlings were placed in each 50 ml flask and sealed. One ml gas samples were removed at intervals and the ethylene concentration was determined by gas chromatography. Adjustments were made in subsequent measurements for the ethylene removed. Data represent the mean for two experiments each with four replicates per treatment. Open circles represent values for heat shocked seedlings; solid circles represent values for unshocked seedlings.

Table 1

Effect of heat shock on ACC levels in cucumber seedlings

	ACC (nmoles·g^{-1} fresh wt)[a]	
	Hours after heat shock	
Treatment	3	6
Heat shock[b]	1.44 ± .19	2.58 ± .20
Unshocked control	1.26 ± .11	1.20 ± .22

[a]Apical hypocotyl samples were excised at three or six hours after heat shock, homogenized, and centrifuged. The supernatant was used for determination of levels of 1-aminocyclopropane-1-carboxylic acid (data means ± SE for two experiments).

[b]40 seconds at 50 C.

activity extracted from tissues. However, preliminary results indicate heat shock increased levels of activity (data not shown).

Accumulation of bound extensin after heat shock

There was a marked increase in hydroxyproline content of extracted cell walls from heat shocked seedlings. Virtually all the hydroxyproline in extracted cell walls is found in extensin. Thus, there was an increased accumulation of bound extensin in the seedling cell walls after heat shock. The amount of hydroxyproline increased in treated cell walls relative to control seedling cell walls during a 72 h period after the heat shock (Fig. 2).

When heat shocked and unshocked seedlings were inoculated with the pathogen C. cucumerinum the pattern of hydroxyproline accumulation was changed. The fungus caused a slight decrease in the rate of hydroxyproline accumulation in both shocked and unshocked seedlings for up to 48 h after inoculation. However, between 72 and 96 h after inoculation there was a rapid rise in the hydroxyproline content of cell walls from both treatments. By 96 h after inoculation, the heat shocked seedlings, whether inoculated or not, had strikingly higher hydroxyproline levels than control seedlings (Fig. 2). The hydroxyproline content of the epidermis of seedlings also increased after heat shock. Assays of epidermal peels and of the underlying tissues indicated a comparable enhancement in both.

Lignin deposition in heat shocked and unshocked seedlings

Normally susceptible cucumber seedlings with heat-shock-induced resistance to C. cucumerinum showed only a slight increase in lignin deposition. In contrast, cucumber seedlings of the resistant cultivar

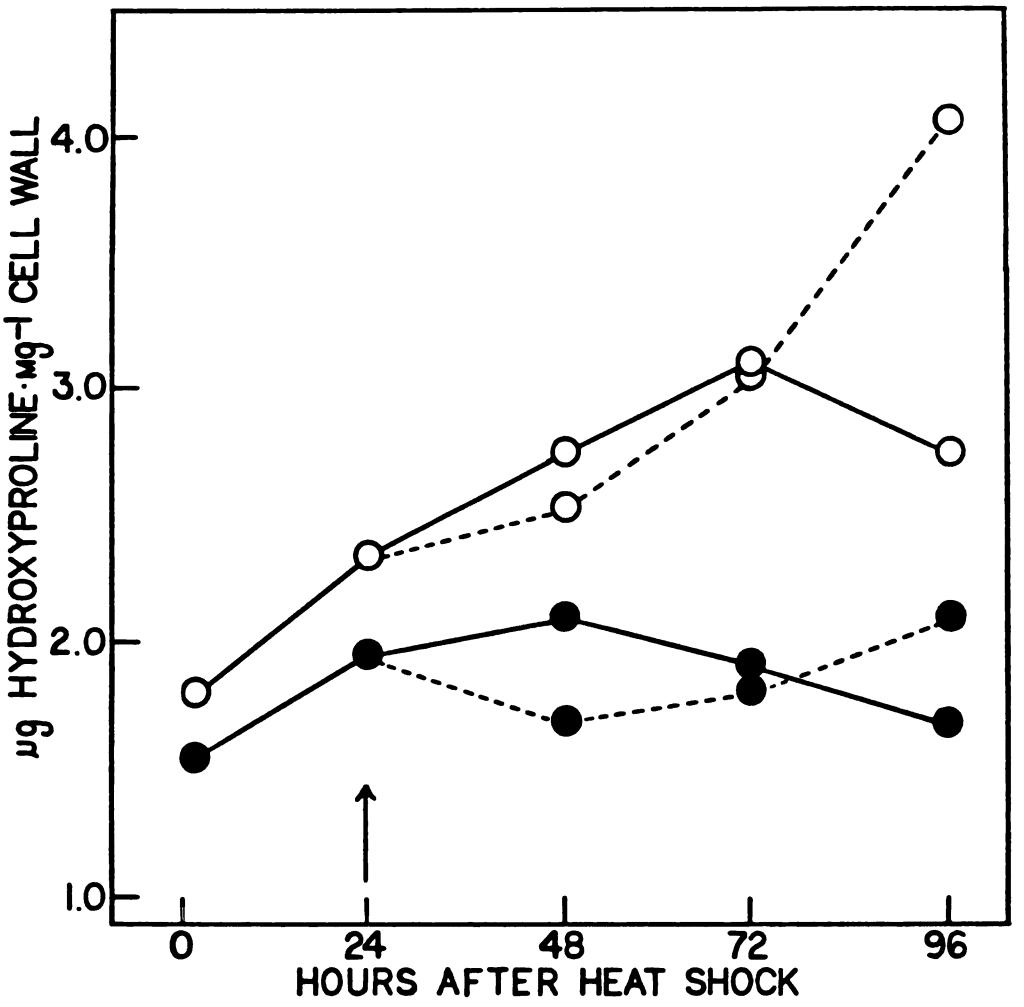

Fig. 2. Time course for the accumulation of cell wall hydroxyproline after heat shock. Cucumber seedlings were heat shocked (40 seconds at 50 C) and some were inoculated (ARROW) 24 h later with Cladosporium cucumerinum (10^6 spores per ml). At one day intervals the apical 2 cm of hypocotyls were excised for analysis. The hydroxyproline content of cell walls was estimated colorimetrically after acid hydrolysis. The data represents the mean for three experiments. (●—●) unshocked, uninoculated; (●···●) unshocked, inoculated; (○—○) heat shocked, uninoculated; (○···○) heat shocked, inoculated.

showed considerable lignification after the challenge inoculation (Table 2). The heat shocked seedlings produced sporadic flecks of phloroglucinol-HCl positive material without a challenge inoculation, probably due to the formation of "wound lignin" after the shock. However, increases in the amount of lignification seen in the normally susceptible seedlings after the inoculation were similar in heat shocked and unshocked seedlings.

The presence of lignin in epidermal peels was confirmed by CuO oxidation. Analysis of the epidermal degradation products by thin layer chromatography showed a marked increase in p-hydroxybenzaldehyde from inoculated seedling of the resistant cultivar (SMR-58) compared to uninoculated seedlings at two days after inoculation. Oxidation of epidermal peels of the susceptible cultivar (Marketer) after inoculation with C. cucumerinum yielded very low levels of p-hydroxybenzaldehyde for either the heat shocked or the unshocked seedlings. This indicates there was no significant increase in fungal-induced epidermal lignification due to a heat shock 24 h prior to inoculation. Vanillin and syringaldehyde were not detected in the oxidation products during these experiments.

Enzymic degradation of cell walls from heat shocked and unshocked seedlings

Cell walls from heat shocked seedlings were more resistant to degradation by C. cucumerinum culture filtrates than were cell walls from unshocked seedlings (Fig. 3). A 55% reduction in degradation of cell walls from heat shocked seedlings was seen when the cell walls were treated with culture filtrates produced by C. cucumerinum on the

Table 2

Effect of a heat shock 24 h prior to inoculation on lignin deposition in cucumber epidermal cell walls

Cultivar	Treatment	Phloroglucinol-HCl staining of epidermal peels Days after inoculation with C. cucumerinum[a] 0	1	2	3	4
Marketer[b]	heat shocked[c], uninoc.	+/-[d]	+/-	+/-	+/-	+/-
Marketer	no shock, uninoc.	-	-	+/-	+/-	+/-
Marketer	heat shocked, inoculated		+/-	+	+	+
Marketer	no shock, inoculated		-	+/-	+/-	+
SMR-58[b]	no shock, inoculated		++	++++	++++	++++
SMR-58	no shock, uninoc.	-	-	+/-	+/-	+/-

[a]Seedlings were sprayed with a spore suspension (10^6 spores per ml) and incubated in rolled germination paper.

[b]Marketer is normally susceptible to C. cucumerinum; SMR-58 is a cultivar resistant to C. Cucumerinum.

[c]40 seconds at 50 C, with inoculation following 24 h later.

[d]Arbitrary rating scale: (-) no staining, (+/-) sporatic staining, (+) light staining, (++) moderate staining, (++++) extensive staining.

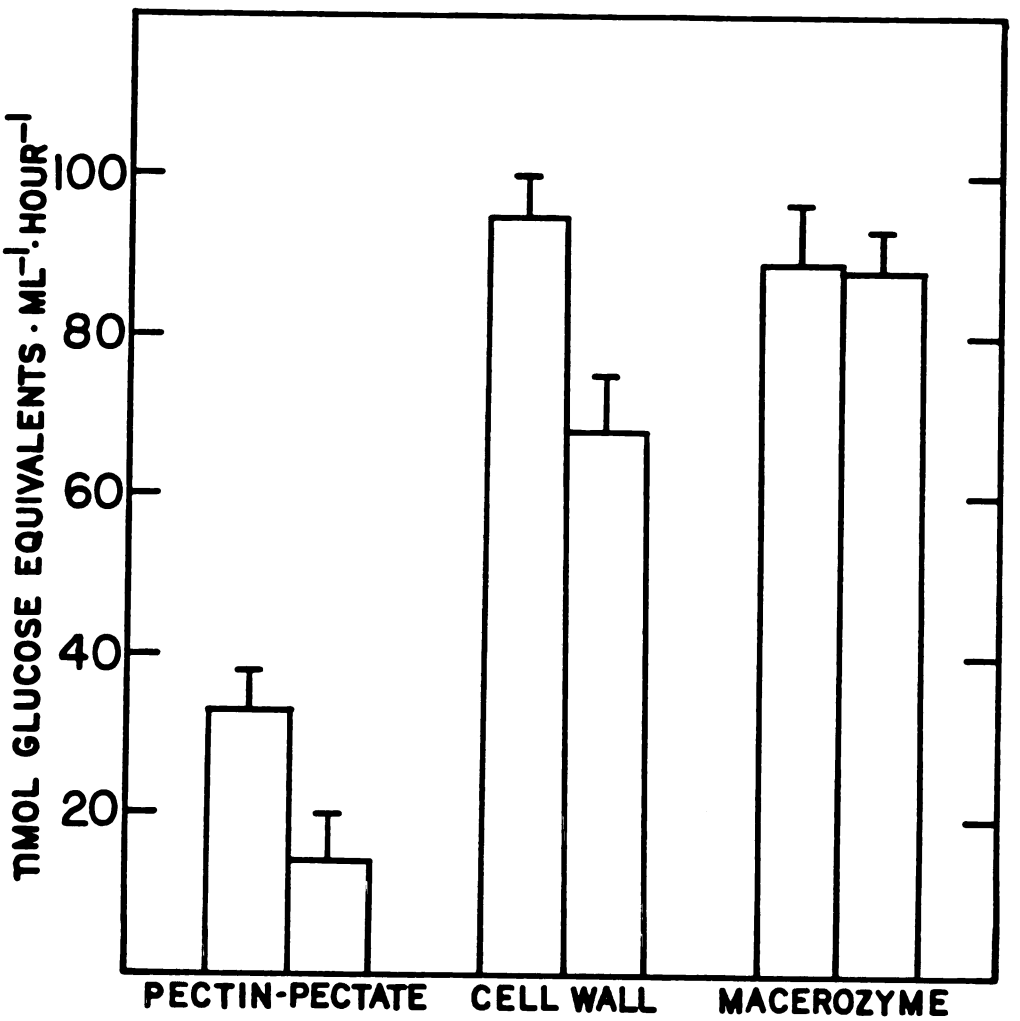

Fig. 3. Release of reducing sugars from cell walls of heat shocked and unshocked seedlings by wall degrading enzymes. Two days after the seedlings were heat shocked (40 seconds at 50 C) the apical 2 cm. of seedling hypocotyls (minus cotyledons) were ground to a powder with liquid N_2. Five mg cell wall samples were incubated with one ml of buffered enzyme preparation for 16 h at 25 C. Aliquots (0.1 ml) were then assayed for reducing sugars. Bars on the left of a pair represent cell walls from unshocked controls; bars on the right represent cell walls from heat shocked seedlings (mean ± SE).

pectin-polypectate medium. A 28% reduction in degradation of cell walls from heat shocked seedlings was seen using culture filtrates from the cell wall medium. The Macerozyme preparation, however, degraded cell walls from heat shocked and unshocked seedlings at the same rate.

DISCUSSION

A brief heat shock stimulated the synthesis of ethylene in cucumber seedlings and increased the accumulation of bound extensin in their cell walls. Estimates of the amounts of ACC in cucumber tissues indicated that increases in this ethylene precursor was responsible for the increase in ethylene production. The rise in ethylene production and extensin levels was not limited to the few hours immediately following heat shock; instead, the effect of heat shock on ethylene and extensin lasted for at least 24 h. This is the first report of the long term effects of heat shock on plant metabolism.

The enhanced accumulation of extensin in the cell walls of heat shocked seedlings supports the importance of this glycoprotein in cucumber resistance to C. cucumerinum. Accumulation of extensin in cucumber seedling cell walls is associated with resistance to C. cucumerinum. Resistant cucumber cultivars exhibited an average 61% increase in cell-wall-bound extensin content at 48 h after inoculation with C. Cucumerinum, while susceptible cultivars showed only an average 8% increase in bound extensin content (6). In another study the enrichment of bound extensin in the cell wall was associated with resistance in melon plants against Colletotrichum lagenarium (2). Moreover, in cucumber plants with systemic disease resistance induced by a localized

C. lagenarium infection, there is a systemic increase in bound extensin in the epidermis (Stermer and Hammerschmidt, unpublished). The apparent lack of lignin deposition in seedlings with heat-shock-induced resistance focuses more attention on the possible role for extensin in this resistance.

Cell wall alterations can contribute to disease resistance by forming a barrier that is more resistant to attack by the pathogen's cell wall degrading enzymes (17). The cell walls of heat shocked seedlings were tested to see if resistance to enzymic degradation could be involved in heat-shock-induced resistance. Results demonstrated that the cell walls from heat shocked seedlings were markedly more resistant than cell walls from unshocked controls to degradation by crude enzyme preparations from C. cucumerinum. This data further strengthens the hypothesis that cell wall alterations are important in cucumber resistance to C. cucumerinum. The commercial enzyme preparation degraded cell walls from heat shocked and unshocked seedlings at the same rate. Apparently, Macerozyme contains enzymes that can cleave cell wall components which C. cucumerinum filtrates can not.

A possible mechanism for the disease resistance induced by heat shock that is consistent with available data can now be proposed. This mechanism may also be important in the systemic resistance induced by localized infections. Increases in peroxidase activity is closely correlated with the onset of induced resistance whether brought on by prior infection or by prior heat treatment (7, section II this thesis). Furthermore, the peroxidase activity induced by C. lagenarium infection or by heat shock is in the same cell-wall-associated isozymes (section

II this thesis). Crosslinking of extensin molecules in the cell wall could result from peroxidase activity, and might be a factor in resistance induced by heat shock. Recent work has shown that extensin is crosslinked in plant cell walls by the coupling of tyrosine residues, probably by peroxidase (3). Because the extensin content of cucumber cell walls increases before the onset of resistance in heat shocked seedlings, it may be the crosslinking of extensin by the later increase in peroxidase activity that is crucial for disease resistance. The greater crosslinking could be responsible for the resistance of cell walls from heat shocked seedlings to digestion by C. cucumerinum enzymes.

The data suggest that perhaps a heat-shock-induced increase in ethylene production could stimulate the accumulation of extensin and peroxidase in the cell wall. The subsequent crosslinking of extensin in the cell wall by peroxidase may confer resistance to attack by pathogen.

Heat shock could be a useful took in the study of induced resistance and other plant responses linked to stress. Further studies with heat shock should increase our knowledge of the regulation of ethylene, peroxidase and extensin, and illuminate their roles in disease resistance.

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RECOMMENDATIONS

1. Explore the potential use of heat shock as a simple method to deduce if a host-selective toxin is involved in a plant-pathogen interaction.
2. Determine why seedlings inoculated immediately after heat shock do not stop the fungus later when the seedling's resistance has apparently returned.
3. Examine the levels of isodityrosine, the cross-linking amino acid of extensin, to see if levels are higher in cucumbers with heat-shock-induced or systemic induced resistance.
4. Determine if the peroxidase isozymes associated with induced resistance are capable of cross-linking the tyrosine residues of extensin molecules *in muro*.
5. Pursue other possible roles for peroxidase in induced resistance, such as the cross-linking of cell-wall-esterified ferulic acids.
6. Look for increases in chitinase activity in tissues with induced resistance.
7. Resolve if exogenous ethylene or ACC treatments can induce resistance in cucumber seedlings.
8. Look for changes in extensin, peroxidase, and isodityrosine levels after ethylene treatments of cucumber seedlings.
9. Examine the systemic effects of heat shock in larger plants.