Heat Shock Protein Synthesis in Propagules of *Fusarium oxysporum* f. sp. *niveum*

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ABSTRACT

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Germlings of *Fusarium oxysporum* f. sp. *niveum* respond to elevated temperatures by synthesizing a set of heat shock proteins (HSPs) with apparent molecular masses of 95, 83, 80, 74, 70, 35, and 18 kD. Vital fluorescent staining in germlings decreases in intensity after exposure to elevated temperature levels. Reduction in percentage of intensely stained germlings at 35 and 36 C corresponds to appearance of HSPs. HSP synthesis is observed in germlings as early as 10 min after exposure to heat stress and declines gradually after transfer of the germlings to 25 C. Thermotolerance to high temperatures of 43 or 44 C is acquired by preheating germlings at sublethal levels. This was determined by measurement of survival upon dilution plating, by vital fluorescent staining and incorporation of L-[^{35}S]methionine into protein. The protection effect is abolished in the presence of cycloheximide, indicating dependence on protein synthesis.

Additional keywords: fluorescein diacetate, weakening.

Artificial heat treatments of soil and plant material and soil solarization are methods used for the control of plant pathogens (2,6). Heat treatment at sublethal levels may cause weakening of pathogens resulting in reduced survival capacity and pathogenicity at later stages (3,6,9,12). The weakening effect has been expressed as delayed spore germination, reduction in vital fluorescent staining, enhanced decline in viability, and reduction in disease incidence by propagules of the pathogen *Fusarium oxysporum* f. sp. *niveum* (3). This phenomenon depends on temperature level, exposure time, and the environment into which preheated propagules are introduced (3,6,16,19).

A wide variety of organisms respond to elevated temperatures by synthesizing a set of new proteins, namely heat shock proteins (HSPs), while normal protein synthesis is suppressed (10,15,17). Thermotolerance to lethal temperatures might be acquired after preexposing cells of different organisms to mild sublethal heating that causes HSP synthesis (10,11,14). In this work we characterized the response of germlings of *F. o. niveum* to elevated temperatures and studied HSP and normal protein synthesis, weakening, and thermotolerance.

MATERIALS AND METHODS

Pathogens. Isolates 1A and RC of the pathogen *Fusarium oxysporum* Schlecht f. sp. *niveum* (E.F. Smith) Snyd. & Hans. were used. Isolate 1A was used throughout this study, while RC was used only where specified. The pathogens were originally isolated from diseased watermelon plants grown in naturally infested fields.

Inoculum production. Mycelial disks from 5-day-old cultures of each isolate were placed aseptically into test tubes (30 ml), each containing 5 ml of liquid medium containing 0.5% yeast extract, 0.5% peptone, and 2% glucose in distilled water. After 24 hr of incubation at 25 C in a still liquid culture, 0.2 ml of the resulting conidial suspension was plated on the same medium solidified with 2% agar and incubated for 4 days at 25 C. Thereafter, conidia were suspended in sterile water for 18 hr at 25 C for conidial germination. The germling suspensions were then centrifuged at 8,000 *g* for 10 min at room temperature and adjusted to the indicated germling concentrations.

Radioisotope-labeling and heating of inocula. Quantities of 1 ml of aqueous suspensions of germinating conidia (5×10^7 germlings per milliliter) were placed in Eppendorf tubes, centrifuged at 8,000 *g* for 10 min at room temperature, and resuspended in 200 μl of sterile water. Cellular proteins in each tube were pulse-labeled periodically, as indicated, with 50μCi of L-[^{35}S]methionine (specific activity, >1,000 Ci/mmol; Amersham Laboratories, England). Pulse-labeling of proteins in germlings was done immediately upon transfer to heated shaker baths (0.1 C accuracy) at temperatures of 25, 33, 35, 36, 38, 40, 42, and 44 C for the duration of 1 hr. Similarly, the kinetics of protein synthesis was determined at 40 C for 10, 20, 40, and 60 min by pulse-labeling samples during last 10 min of exposure. Protein patterns were compared to those of identical samples, which were incubated at 25 C for 60 min.

Recovery from a heat shock of 40 C for 60 min was examined in germlings after transferring them to 25 C. Proteins were pulse-labeled at various periods after initial heat shock and compared to those synthesized at 25 and 40 C for 60 min each. Label was applied 30 min before extraction of protein, at the end of each period of recovery. In parallel, fluorescent staining and viability of heated and unheated propagules were determined, as described below.

Protein extraction and determination of radiolabel incorporation. After each treatment, the germlings were centrifuged (8,000 *g* at freezing temperature) and the precipitate ground with 100 μg of chemically pure sand in Eppendorf tubes. The homogenate was resuspended in 200 μl of a modified Laemmli buffer (7) containing 62.5 mM Tris-HCl (pH 7), 2% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM EDTA, 10% glycerol, and 5% mercaptoethanol. The homogenate was centrifuged (8,000 *g* at freezing temperature) for 10 min, and the supernatant solution was collected. Acid-precipitable radioactivity of 10-μl samples of this solution was counted by liquid scintillation spectrometry (Beckman LS 1701 analyzer) to determine the level of incorporated label. Proteins were precipitated overnight at −20 C with 800 μl of acetone per 200 μl of homogenate. The precipitate was collected by centrifugation in 40 μl of Laemmli buffer solution containing a few grains of bromophenol blue and then boiled for 3 min at 100 C.

Gel electrophoresis of protein extracts. Protein extracts were

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separated by one-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) (8% acrylamide), essentially as described by Laemmli (7). For each extract, aliquots (5–30 μl) containing equal amounts of radioactivity were applied per well. Gels were 1.5 mm thick and 14 cm long. After electrophoresis for 15–16 hr at 60 V, in a buffer composed of 25 mM Tris, 192 mM glycine, and 0.1% SDS (pH 8.3), the gels were stained with 0.2% Coomassie Brilliant Blue R-250 in 50% methanol, 45% acetic acid, and 5% water, then destained by several changes in a solution containing 25% methanol and 7% acetic acid in water and dried under vacuum. Autoradiographs were prepared with Agfa X-ray film and exposed for periods of 8–24 hr at −70 C. Apparent molecular weights were determined by reference to Coomassie blue-stained low weight protein standards of Pharmacia (Sweden) and prestained SDS markers of Sigma (St. Louis, MO).

Fluorescent staining of inocula. Vital fluorescent staining with fluorescein diacetate, to determine the recovery of germlings from heat shock, was performed as previously described (3,18). Viable nonheated propagules (control) are intensely stained and fluoresce brightly under microscopic examination. The percentage of germlings that are intensely stained out of total examined was determined in 20 germlings in each of four microscope fields per treatment. Results were expressed as the percent of untreated control (25 C incubation) at zero time.

Viability of inocula. Viability of heated and unheated germlings was determined by dilution plating on a peptone-PCNB medium selective to *Fusarium* spp. (13), acidified with lactic acid (l ml/L) and supplemented with chloramphenicol at 250 $\mu g\ ml^{-1}$. Each treatment was carried out with three replicates of 10 dishes each. Percent survival was calculated after 5 days of incubation at 27 C by comparing with the unheated control.

Acquired thermotolerance of inocula. Thermotolerance to high temperatures (43 or 44 C) acquired by preheating germlings at sublethal temperatures (38 or 40 C) for the indicated periods, was determined by dilution plating, vital fluorescent staining, and incorporation of radiolabel in proteins, as compared to unheated controls at 25 C. Germlings were radioisotope-labeled with 5 μCi of L-[^{35}S]methionine per 200 μl of germling suspension (5×10^7 germlings per milliliter) in Eppendorf tubes to determine the rate of protein synthesis. In a parallel experiment, thermotolerance of germlings in soil was determined by mixing propagules in Rehovot sandy soil (0.6% organic matter; 3.8% clay; 12% field capacity; pH = 7.6) to a population of 10^5 colony-forming units per gram. The soil was brought to a moisture of −0.26 bar. The germlings and soil then were heated or maintained at 25 C, as indicated, and viability was assessed by dilution plating.

All experiments were performed twice or more with similar results. Results show data of one of these experiments. Statistical analyses of the data were done by Duncan's multiple range test ($P = 0.05$) or by calculation of standard error.

RESULTS

The effect of elevated temperatures on the patterns of protein synthesis and fluorescent staining. Germlings of *F. o. niveum* were radioisotope-labeled with L-[^{35}S]methionine during exposure to different heat regimes for a period of 60 min. Protein extracts of heat-treated cells, separated on one-dimensional SDS-PAGE and visualized by autoradiography, revealed either newly induced or enhanced levels of heat shock proteins when compared to that of untreated germlings at 25 C (Fig. 1). The predominant HSPs correspond to molecular masses of 95, 83, 80, 74, 70, and 35 kD. A minor HSP of 18 kD also was observed. The protein band of 70 kD was prominent in the 38 C treatment, but absent in the 40 and 42 C treatments. Many of the normal proteins synthesized at 25 C were less prominent, especially at 40 and 42 C. No incorporation of label into proteins was detected after heating at 44 C. Five major HSPs (95, 83, 80, 74, and 35 kD) were clearly visible at 35 C, while only HSP 74 was readily observed at 33 C (Fig. 2). Another isolate (RC) of *F. o. niveum* exhibited identical HSP patterns (not shown) as in 1A, the isolate used extensively in this work.

The percentage of intensely stained germlings with fluorescein diacetate, measured immediately after the termination of heating, was negatively correlated with temperature level (Fig. 3). Fluorescence in germlings was reduced by 27 and 54%, respectively, in the treatments of 35 and 36 C for 60 min, as compared to the control at 25 C. Reduction in fluorescence at 35 and 36 C is associated with appearance of five major HSPs (95, 83, 80, 74, and 35 kD) upon heating (Fig. 2).

Kinetics of HSP synthesis at 40 C. Germinating conidia were heat-treated at 40 C for the duration of 10, 20, 40, and 60 min with L-[^{35}S]methionine being present during the last 10 min in each case. The increase in synthesis of HSP 74 was detected as early as 10 min after exposure to the heat treatment, while the synthesis of four other HSPs (95, 83, 80, and 35 kD) was observed 20 min after heat shock (Fig. 4). The protein bands increase in intensity with the extension of the heat treatment over a 60-min period.

Changes in protein synthesis and fluorescent staining during recovery from heat shock. Return of germlings to 25 C after a 60-min heat shock at 40 C caused a gradual decline in levels of the major HSPs after 4 hr (Fig. 5). Eight hours after heat shock, a further decline of visible HSPs was seen. The return of germlings to 25 C after heating at various temperatures for 60 min was expressed in increasing fluorescence during incubation (Fig. 3). Thus, percentage of intensely stained germlings increased from 46% immediately after heating at 36 C for 60 min to 74% after 6 hr of recovery at 25 C.

Effect of preheating on thermotolerance. Thermotolerance was acquired by preheating germlings at sublethal temperatures (which do not reduce viability) before subsequent exposure to higher temperatures. This was determined by viability assessment upon

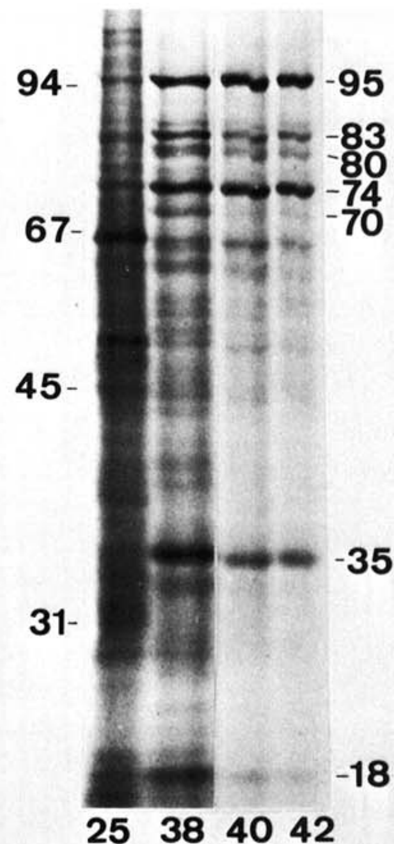

Fig. 1. Effect of heating on the pattern of protein synthesis in germlings of *Fusarium oxysporum* f. sp. *niveum*. The autoradiogram shows L-[^{35}S]methionine-labeled proteins separated by SDS-PAGE. Germlings were labeled during exposure to 25, 38, 40, and 42 C (as indicated) for the period of 60 min. Double the amount of radioisotope-labeled protein was applied to lane 25. Molecular weight markers in kilodaltons are indicated on the left, and the apparent molecular weights of the major heat shock proteins appear on the right.

dilution plating, by fluorescent staining, and by incorporation of L-[^{35}S]methionine into proteins after heat treatments.

Preheating germlings at a sublethal temperature of 38 C before exposing them to drastic heating of 44 C improved their survival from 22 to 94% (Fig. 6). All heat treatments caused a sharp decline in the percentage of intensely stained germlings immediately after heating (Fig. 6). Similar to the survival results, the combined 38–44 C treatment was less detrimental than the 44 C treatment alone. Thus, in the combined treatment, the percentage of intensely stained germlings, during the 6-hr incubation period, increased from 16 to 69%. In contrast, only a slight increase in fluorescence was measured during the same period of time in germlings exposed to 44 C alone. Similar results were obtained with the isolate RC when germlings were subjected to the same treatments. Thus, survival percentages for the 25, 38, 44, and combined 38–44 C treatments were 100, 100, 11, and 81, respectively.

Thermotolerance was also evident when germlings were preheated at 38 C for 15 or 60 min and 40 C for 60 min before exposure to various high temperatures of 43 C for 90 or 120 min and 44 for 120 min. The same phenomenon was observed in nongerminating conidia.

The protective effect of preheating was diminished if the preheated germlings were not exposed immediately thereafter to high temperatures, but incubated at 25 C for a recovery period of 6 hr, prior to exposure. This was evident since survival of germlings exposed to sublethal (40 C for 30 min) followed by high (43 C for 90 min) temperatures, immediately or after a 6-hr interval at 25 C, was 69 and 29%, respectively. This 6-hr "escape" period corresponds with the reduction of HSPs in recovery experiments at 25 C (Fig. 5).

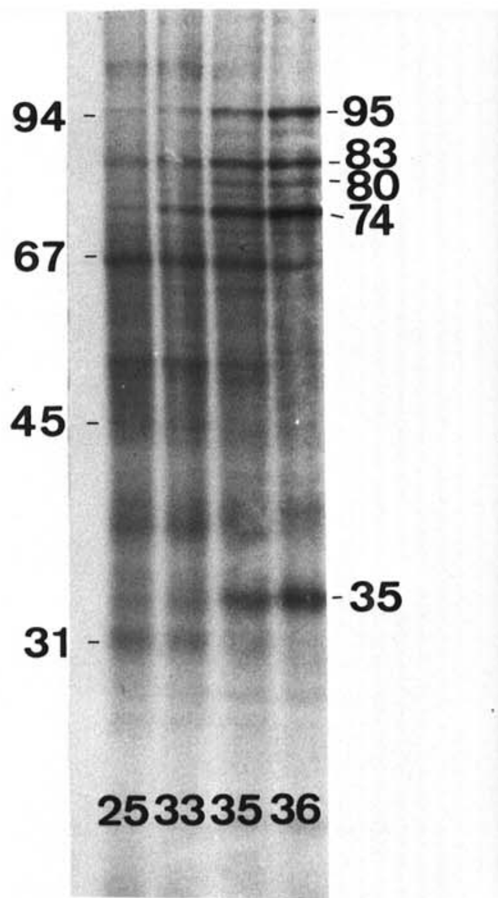

Fig. 2. Effect of heating at mild temperature levels on the pattern of protein synthesis in germlings of *Fusarium oxysporum* f. sp. *niveum*. The autoradiogram shows L-[^{35}S]methionine-labeled proteins separated by SDS-PAGE. Germlings were labeled during exposure to 25, 33, 35, and 36 C (as indicated) for the period of 60 min. Equal counts of radioisotope-labeled protein were applied to each lane. Molecular weight markers in kilodaltons are indicated on the left, and the apparent molecular weights of the major heat shock proteins appear on the right.

Thermotolerance or hardening in germlings subjected to sublethal temperatures followed by exposure to high temperatures was also determined in soil (10^5 germlings per gram of soil). Survival of 100 and 33% was observed in germlings subjected to sublethal (38 C for 30 min) or high (44 C for 60 min) temperatures, respectively. Preheating at 38 C for 30 min before subsequent exposure to 44 C for 60 min increased survival in germling population to 92%, thus essentially confirming thermotolerance, as observed in aqueous suspensions of germlings (Fig. 6).

Cycloheximide, an inhibitor of protein synthesis, was used to determine whether acquisition of thermotolerance depends on protein synthesis. Percentage of survival in germlings, subjected to the above combination of sublethal (38 C) and subsequent high (44 C) temperatures, with or without cycloheximide, was 7 and 62%, respectively, as compared to 4% survival at 44 C, without cycloheximide. Cycloheximide at the concentration used (100 μg per milliliter of inoculum suspension) had no toxic effect on an unheated population of germlings when incubated with the chemical for 3 hr, as determined by dilution plating. Toxic effects (10 and 78% reduction in population survival) were evident only at concentrations of 500 and 1,000 μg per milliliter of inoculum suspensions, respectively. The inhibition of protein synthesis by cycloheximide was demonstrated in a parallel experiment. Thus, in the presence of cycloheximide, a reduction of 83 and 78% in incorporation of radiolabel into protein in germlings was observed after incubation for 30 min at 25 and 38 C, respectively.

Incorporation of radiolabel into proteins demonstrates that cells preheated at 38 C for 30 min acquired thermotolerance to the subsequent drastic treatment as expressed in increased percentages of survival and incorporation (Table 1). However, incorporation of radiolabel was still much lower in these cells as compared to those heated at 38 C only.

DISCUSSION

Germlings of *F. o. niveum* respond to elevated temperatures by synthesizing HSPs having apparent molecular masses of 95, 83, 80, 74, 70, 35, and 18 kD. These are similar to the major HSPs of *Neurospora crassa* (5,14) and the yeast, *Saccharomyces cerevisiae* (11), as observed on one-dimensional SDS-PAGE gels.

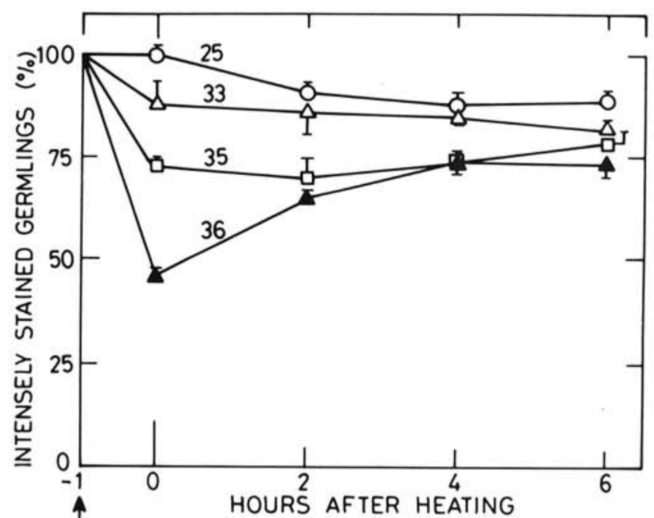

Fig. 3. Effect of heating on vital fluorescent staining in germlings of *Fusarium oxysporum* f. sp. *niveum*. Germling fluorescence was assessed by determining percent of intensely stained germlings of total observed. Numbers denote temperature. The vertical arrow indicates beginning of heat treatment, which lasted for 60 min (from −1 to 0 hr). The vertical lines denote standard error.

The high molecular weight HSPs of *F. o. niveum* appear to correspond to the HSP 80 and 70 families, which are highly conserved in eukaryotic organisms (10). The smaller molecular mass HSPs of about 20 kD are common in plants and fungi (4,5,14); however, the 35 kD HSP of *F. o. niveum* and other fungi (14), is not commonly found in other groups of organisms. An HSP 70 gene was identified in *Fusarium* using a *Ustilago maydis* HSP gene as a probe (8) indicating homology of this gene within different species of fungi. In *F. o. niveum* as in other organisms, HSP 83 is expressed at regular growth temperatures, and its synthesis is enhanced at an elevated temperature of 35 C

TABLE 1. Effect of sublethal preheating on thermotolerance in germlings of *Fusarium oxysporum* f. sp. *niveum* as determined by percentages of survival and incorporation of L-[^{35}S] methionine

Treatment (temperature, C)	Exposure time (min)	Survival[w] (% of control)	cpm[x] (% of control)
25 (control)	60	100 a[y]	100.0 a
38	30	100 a	86.0 a
44	60	15 c	6.3 c
38–44[z]	30–60[z]	81 b	17.0 b

[w]Determined by dilution plating.
[x]L-[^{35}S]methionine radiolabel was applied for 30 min to germlings, 6 hr after each treatment and incorporation (cpm, counts per minute) into protein extracts was measured.
[y]In each column, figures with a common letter are not significantly different ($P = 0.05$), according to Duncan's multiple range test.
[z]Germlings were preheated at 38 C for 30 min and thereafter exposed to 44 C for 60 min.

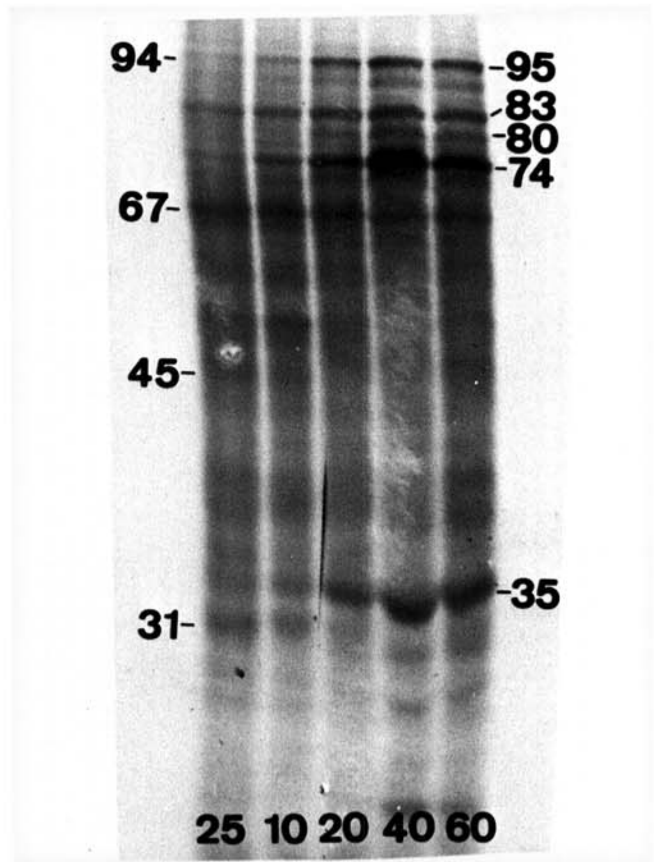

Fig. 4. Kinetics of protein synthesis at 40 C in germlings of *Fusarium oxysporum* f. sp. *niveum*. The autoradiogram shows L-[^{35}S]methionine-labeled proteins separated by SDS-PAGE. Germlings were labeled during the last 10 min of exposure to heat and proteins extracted after 10, 20, 40, and 60 min (as indicated). Lane 25 shows the protein profile of germlings labeled at 25 C for 60 min. Equal counts of radiolabeled protein were applied to each lane. Molecular weight markers in kilodaltons are indicated on the left, and the apparent molecular weights of the major heat shock proteins appear on the right.

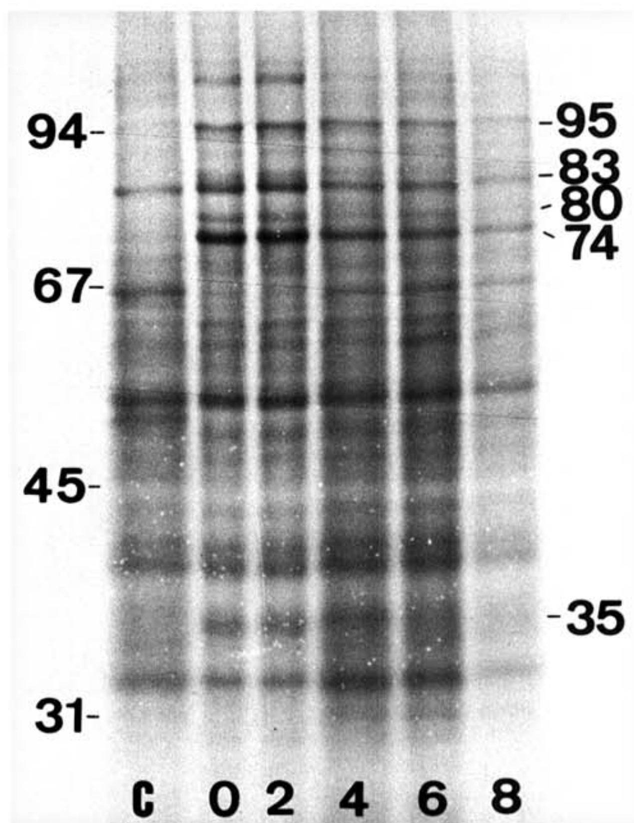

Fig. 5. Pattern of protein synthesis in germlings of *Fusarium oxysporum* f. sp. *niveum* during recovery at 25 C after heat shock treatment at 40 C for 60 min. The autoradiogram shows L-[^{35}S]methionine-labeled proteins separated by SDS-PAGE. Label was applied to germlings 30 min before protein extraction at the end of each period of recovery (2, 4, 6, and 8 hr as indicated in the appropriate lanes). Lanes C and 0 show protein profiles of germlings labeled for 60 min at 25 and 40 C, respectively. Equal counts of radioisotope-labeled protein were applied to each lane. Molecular weight markers in kilodaltons are indicated on the left, and the apparent molecular weights of the major heat shock proteins appear on the right.

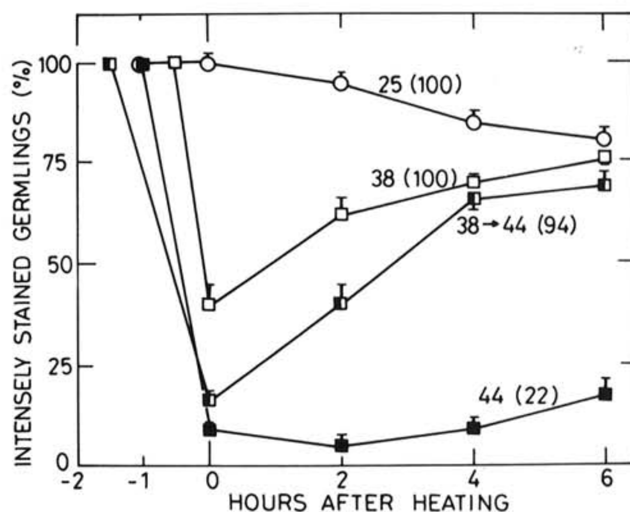

Fig. 6. Effect of sublethal preheating at 38 C for 30 min on thermotolerance to subsequent heating at 44 C for 60 min (indicated as 38→44) in germlings of *Fusarium oxysporum* f. sp. *niveum*. Numbers not in parentheses denote temperature in C. Survival percentage (numbers in parentheses for each treatment) was assessed by using dilution plating. Vital fluorescent staining was measured over a 6-hr period by determining percentage of intensely stained germlings out of total observed, and expressed as percent of control (25 C) at zero time. Germlings were heat-treated for the desired period until 0 hr before incubation at 25 C. The vertical lines denote standard error.

and higher. A gradual increase in temperature above 33 C, causes an enhanced synthesis of all HSPs, with different levels of HSPs at different temperatures. The synthesis of HSPs 80, 70, 35, and 18 declines at 40 C, whereas that of HSPs 95 and 74 continues even at near lethal temperatures.

A decrease in vital fluorescent staining with fluorescein diacetate in fungi is well correlated with a decline in growth and respiration rate (18). This has also been shown in a previous work, where sublethal heating at 40 C of conidia of *F. o. niveum* resulted in reduced vital staining (3). Heating results in enhanced synthesis of HSPs and a gradual reduction in frequency of intensely stained germlings of *F. o. niveum* (Figs. 2 and 3). Furthermore, recovery from heat shock in *F. o. niveum* is accompanied by a decline in HSPs, as well as increased fluorescent staining. Therefore, reduced vital fluorescent staining and HSP production seem to be reliable indicators of heat stress.

Thermotolerance is acquired by pretreating cells at sublethal temperatures before transfer to lethal ones (10,11,15; Table 1 and Fig. 6). This was expressed in reduced heat mortality, increased fluorescence, and increased incorporation of radiolabel into protein. In this study, cycloheximide inhibited protein synthesis and eliminated the development of acquired thermotolerance. Similarly, thermotolerance in *F. o. niveum* was nullified after an elapsed period of 6 hr between sublethal preheating and exposure to lethal temperatures. The requirement of protein synthesis for acquired thermotolerance in *F. o. niveum* is in accordance with other reports (10,11,14,15).

Sublethal heating of conidia or chlamydospores of *F. o. niveum* caused only slight initial reduction in propagule populations but resulted in a weakening effect in the surviving propagules, which was expressed in enhanced decline of the population density of propagules (3). The weakening effect caused by sublethal heating was also demonstrated with *Sclerotium rolfsii* (9), *Rosellinia necatrix* (19), and *Armillaria mellea* (12) as well as with other weakening agents, e.g., the mild fumigant carbon disulfide (1). Similarly, other stress agents, e.g., arsenite, ethanol, heavy metals, and certain antibiotics, may induce synthesis of HSPs (5,10). The initial synthesis of HSPs might signal the first events of weakening in an organism upon heat stress. HSP production takes place at the sublethal heating range, which corresponds to the temperature range associated with the weakening phenomenon.

Soil solarization for the control of soilborne pathogens, at increasing soil depths (20–60 cm), relies on relatively low temperatures (within the sublethal range of 40–35 C), for the eradication of inocula (6,16,19). A diurnal cycle of a gradual build up in temperature followed by a gradual decrease takes place during solarization (6). These daily cyclic changes in temperature are repeated 30–40 times during a typical solarization process. A time factor is required for the recovery of normal protein synthesis and decline in HSP synthesis during extended periods of heat stress in fungi (15 and Fig. 5). The hardening effect of acquired thermotolerance might also depend on the environment into which propagules are introduced, as was demonstrated with the weakening of propagules caused by exposure to sublethal temperatures (3). Therefore, it is difficult at the present state of knowledge to predict whether the heating regime during solarization will induce thermotolerance in pathogens or cause a cumulative weakening effect (in an environment hostile to the pathogen) by inflicting irreversible damage beyond the threshold of hardening. Such knowledge may enable improved control of the fate of weakened or possibly hardened propagules exposed to sublethal heating during solarization or during heat treatment of plant propagation material.

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