Weakening Effect on Propagules of *Fusarium* by Sublethal Heating

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ABSTRACT

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Sublethal heating of conidia and chlamydospores of *Fusarium oxysporum* f. sp. *niveum* at 38–42 C caused 0–33% reduction in propagule viability and resulted in a weakening effect in the surviving propagules. This weakening effect was expressed as a delay in germination, in reduction in growth of conidial and chlamydospore germ tubes, and in enhanced decline of the population density of viable conidia in soil. Viability of conidia that were heat-treated or exposed to solarized soil declined faster than unheated conidia in a soil suspension culture. Vital fluorescent staining with fluorescein diacetate showed that heated conidia were less brightly stained than unheated conidia even when apparent viability, as measured by dilution plating, remained 100%. Disease incidence in watermelon seedlings inoculated with heat-treated conidia of *F. o. niveum* was reduced by 35–82%. A. similar trend was observed with *F. oxysporum* f. sp. *melonis* in muskmelon seedlings. This study showed that heating at sublethal temperatures may adversely affect spore viability of *Fusarium*, resulting in pathogen control beyond the initial mortality rate caused by heating.

Additional keywords: Fusarium wilt.

Reduction of pathogen populations in soil or in propagation material is achieved principally by chemical or physical means, e.g., by fumigation, artificial heat treatments, and solarization (3,5,7,10). The death rate of a population depends on both the dosage and exposure time, causing various degrees of reduction in viability (10,15). However, sublethal doses (at near lethal range), which eliminate only a part of the population, also may affect the surviving and, possibly, weakened propagules.

Previous studies have shown that the weakening of propagules of various pathogens following sublethal treatments may result in reduced survivability and pathogenicity. Thus, exposing sclerotia of *Sclerotium rolfsii* to sublethal heating, drying, heat shock, or to metham sodium caused reduced viability resulting from microbial activity in the damaged sclerotia (6,9,12,16). In certain cases, disease incidence also was reduced. In other studies, sublethal doses of heat have rendered the pathogens *Armillaria mellea* and *Rosellinia necatrix* more vulnerable to antagonism by *Trichoderma* spp. (13,19).

The purpose of our research was to study the effect of sublethal heating on the possible weakening of propagules of *Fusarium* leading to an increased vulnerability to microorganisms or reduced pathogenicity.

MATERIALS AND METHODS

Pathogens. Pathogenic isolates of *Fusarium oxysporum* Schlecht. f. sp. *niveum* (E. F. Smith) Snyd. & Hans. (isolate 1A) and *F. oxysporum* Schlecht. f. sp. *melonis* Snyd. & Hans (race 0, isolate RM 1) were used. The two pathogens were isolated from diseased watermelon and muskmelon plants, respectively, grown in naturally infested fields.

Inocula production. Mycelial disks from 5-day-old cultures of the indicated pathogen were placed in Erlenmyer flasks (250 ml), each containing 100 ml of liquid medium of yeast extract (5 g/L), peptone (5 g/L), and glucose (20 g/L). Flasks were placed on a shaker at 100 strokes per min and maintained at 30 C. After 4 days, contents of the flasks were filtered through eight layers of sterile surgical gauze. The microconidial suspensions then were washed with distilled water by centrifugation at 6,200 *g* for 10 min at 0 C and adjusted to the indicated concentrations.

Water suspensions of 10^7 conidia ml^{-1} of *F. o. niveum* were mixed with sterile Rehovot sandy soil (0.6% organic matter; 3.8% clay; 12% field capacity; pH 7.6) to a population of 10^6 colony-forming units (cfu) g^{-1} soil, bringing soil moisture to −0.26 bar. Infested soil then was incubated at 30 C. After 60 days, chlamydospores were the only propagules detected in the soil, as determined by microscopic examination of soil samples stained with 0.1% aniline blue.

Plants. The following plants were used: watermelon (*Citrullus vulgaris* L. 'Sugar Baby'), susceptible to *F. o. niveum*; and muskmelon (*Cucumis melo* L. 'Ananas Yokneam'), susceptible to *F. o. melonis*.

Heat treatment of inocula of *Fusarium* sp. Experiments involving heating were carried out within a temperature range that caused a partial reduction (mortality rate of 0–33%) of conidial and chlamydospore viability. Conidial suspensions, or soil containing chlamydospores moistened to −0.26 bar, were placed aseptically in 30-ml test tubes and heat-treated in a water bath (Fried Electric, Haifa, Israel; 0.1 C accuracy) at the indicated temperatures and exposure times. In all experiments, viability of the inocula after heat treatments was determined by dilution plating on a peptone-PCNB medium selective to *Fusarium* spp. (20). Mortality was calculated by comparing with the untreated control inocula maintained at 25 C.

Germination of conidia and chlamydospores of *Fusarium*. Heat-treated and untreated conidial suspensions of the pathogens at 4×10^5 conidia ml^{-1} were plated on potato-dextrose agar (PDA; 0.1 ml per dish), incubated for 10 hr at 18 C, and further incubated at 27 C. Germination percentage and germ tube length were assessed microscopically at 250× after 12–21 hr of incubation at 27 C, as indicated in each experiment. Appearance of a germ tube was used to assess germination. The length of germ tubes of 20 conidia also were measured in each of four microscopic fields per treatment, and results were expressed as a percent of maximum in the unheated control.

Heat-treated and untreated samples of soil containing chlamydospores were diluted in 0.1% sterile water agar (1:1, w/v),

and 0.2-ml samples were spread over plates of PDA. Germ tube length of chlamydospores was assessed by the same method as described for conidia.

Decline in population density of conidia of *Fusarium* in soil. Heat-treated and untreated aqueous conidial suspensions of *F. o. niveum* were added to Rehovot soil to a concentration of 10^5 cfu g^{-1} soil, bringing the soil moisture to −0.26 bar. Samples (50 g) of these soils were placed in 100-ml flasks and incubated at 27 C. Survival of initial populations of *Fusarium* was determined by dilution plating of soil samples taken immediately and periodically over 7 wk. Percent survival in each treatment was calculated by comparing viable population density at the indicated period with that surviving in the treatment immediately after heating.

Decline in viability of conidia of *Fusarium* by soil bacteria. This experiment was performed essentially as previously described (8). Five ml of heat-treated or untreated suspensions of conidia (5×10^7 ml^{-1}) were transferred to 100-ml flasks containing 45-ml Czapek dox mineral solution (without sugar). These conidia, serving as a sole carbon source, were subjected to soil bacteria by adding 500 mg of untreated or solarized Rehovot soil (which served as a source for soil organisms) to the medium in each flask, which was incubated in a shaker bath at 30 C. The solarized soil was taken from a plot solarized by a standard method (10,11). Samples of the suspension were withdrawn from the flasks over a 6-day period to assess survival percentage of conidial populations by dilution plating, as described in the previous section.

Fluorescent staining of conidia of *Fusarium*. This vital staining technique essentially was that described by Söderström (17). Fluorescein diacetate (FDA) (Sigma Chemical Co., St. Louis, MO) was dissolved in acetone (2 mg ml^{-1}) and stored at −20 C. Dilutions were made to a final concentration of 10 μg ml^{-1} in phosphate buffer (10 mM, pH 7). A drop of heat-treated or untreated suspension at 2×10^6 conidia ml^{-1} of *F. o. niveum* and a drop of diluted FDA solution was pipetted onto a microscopic slide and covered with a cover slip. The preparation was studied after 2 min under a standard RA Zeiss fluorescent microscope (C. Zeiss, Oberkochen, W. Germany) with an excitation filter at 390- to 490-nm transmission and a 515-nm barrier filter. FDA staining was determined in 20 conidia in each of four microscope fields per treatment. Percentage conidial fluorescence was calculated by determining percent of brightly stained conidia of total observed. Results were expressed as the percent of untreated control at zero time. In parallel, viability was determined by dilution plating of the heat-treated and untreated conidia from the same stock on a selective medium.

Inoculation of seedlings with *Fusarium*. The methods of Cohen et al (2) were used. Watermelon and muskmelon seeds were planted in sandy uninfested Rehovot soil in the greenhouse. After 5–6 days, seedlings were removed, washed with running water, and roots of these seedlings were dipped for 30 sec in heat-treated or untreated suspensions containing 5×10^4 conidia ml^{-1} (or at other concentrations as indicated) of *F. o. niveum* or *F. o. melonis*. Seedlings were transplanted into Rehovot sandy soil, maintained in the greenhouse at 24–30 C, and examined daily to determine disease incidence. Inoculated seedlings began to exhibit wilt symptoms 6–7 days after inoculation. Disease incidence was expressed as the percentage of totally wilted plants in a population. Greenhouse experiments were performed with four replicates of 15 seedlings each. The experiments were carried out in a completely randomized design.

Inoculation of seedlings with *Fusarium* in a vermiculite rooting medium. Watermelon seedlings were germinated in a sterile vermiculite rooting medium in a growth chamber at 25 C and inoculated with *F. o. niveum* as described in the previous section. The inoculated seedlings were transplanted into pots of the same medium and incubated in the growth chamber. Disease percentage was determined as previously described. Experiments were carried out with six replicates of eight seedlings each and in a completely randomized design.

All experiments in this work were conducted at least twice with similar results. Results shown represent data from one of these experiments at random. Statistical analyses of the data was done by linear regression with transformations where indicated at a significance level of $P = 0.05$. Values for 50% survival or germ tube length were calculated from regression equations and compared by Duncan's multiple-range test ($P = 0.05$).

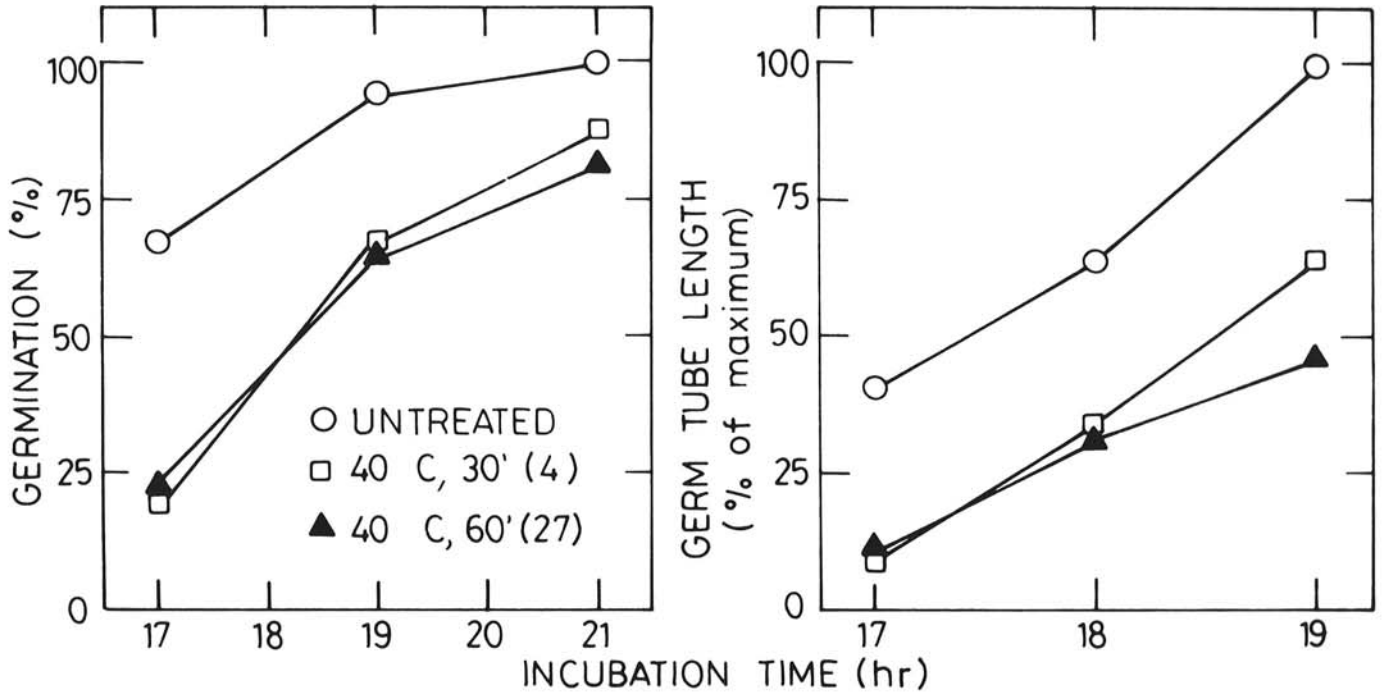

Fig. 1. Effect of sublethal heating at 40 C for 30 and 60 min on conidial germination and germ tube length of *Fusarium oxysporum* f. sp. *niveum*. Numbers in parentheses denote mortality of conidia following heating, expressed as a percentage of germination in the unheated control. After log transformation of incubation time, the linear regression coefficients ($0.83 < R^2 < 0.94$) are significant. The slope value for percent germination of the untreated control is significantly different from that of each of the heat treatments ($P = 0.05$). The slope value for germ tube length of the untreated control is significantly different from that of the heat treatment of 40 C for 60 min ($P = 0.05$).

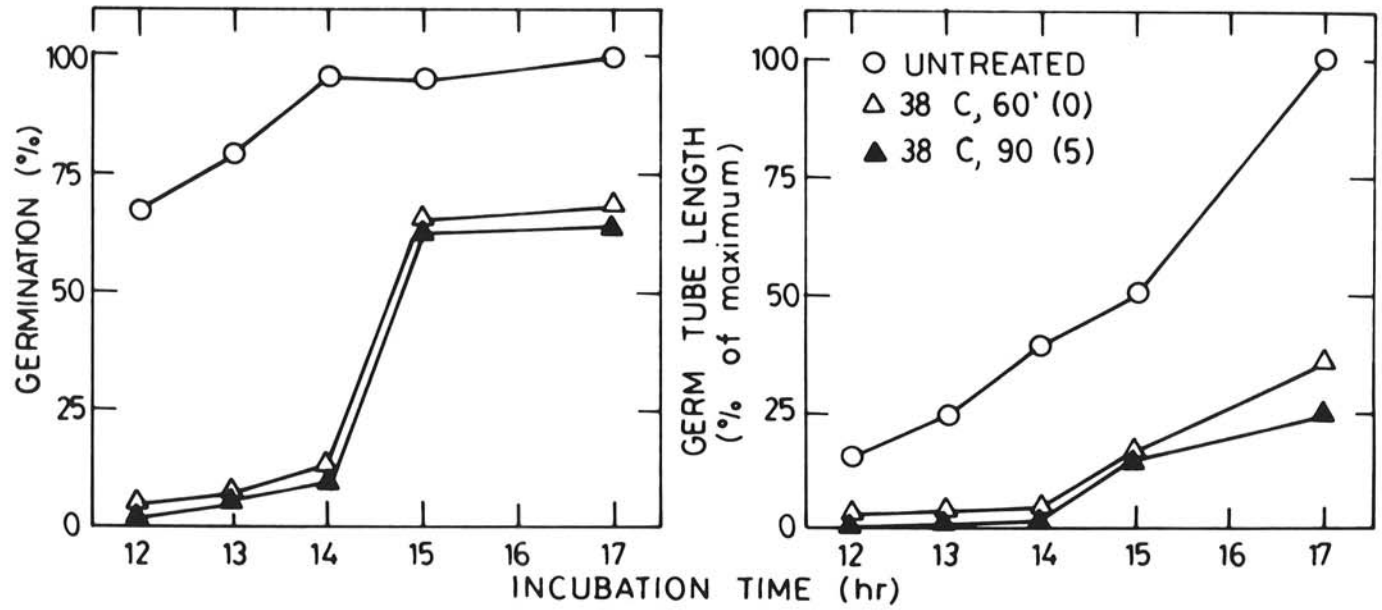

Fig. 2. Effect of sublethal heating at 38 C for 60 and 90 min on conidial germination and germ length of *Fusarium oxysporum* f. sp. *niveum*. Numbers in parentheses denote mortality of conidia following heating, expressed as a percentage of germination in the unheated control. After probit transformation of germination percent and log transformation of incubation time, the linear regression coefficients ($0.54 < R^2 < 0.85$) are significant. The slope values for the untreated controls are significantly different from those of each of the comparable heat treatments ($P = 0.05$).

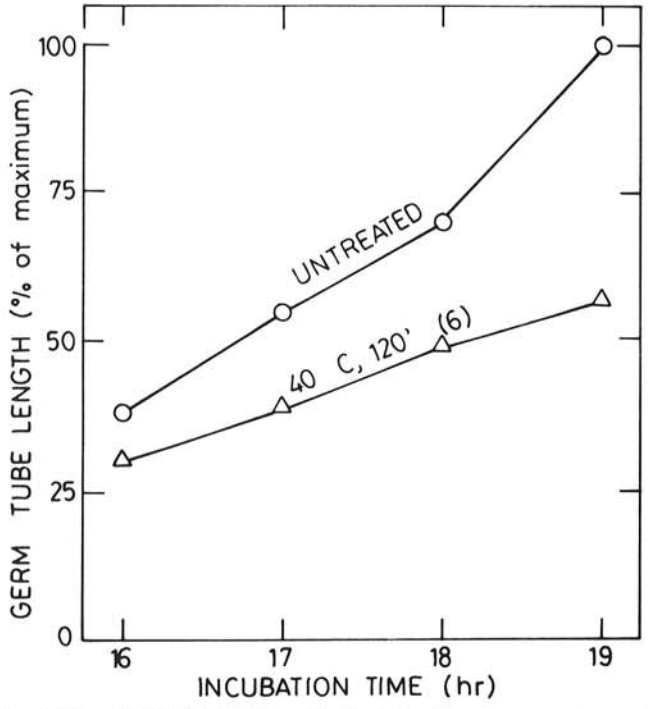

Fig. 3. Effect of sublethal heating at 40 C for 120 min on germ tube length of chlamydospores of *Fusarium oxysporum* f. sp. *niveum*. The number in parentheses denotes mortality of chlamydospores immediately following heating, expressed as a percentage of germination in the unheated control. After linear regression analysis, both coefficients ($0.9 < R^2 < 0.95$) are significant. The slope value of the untreated control is significantly different from that of the heat treatment ($P = 0.05$).

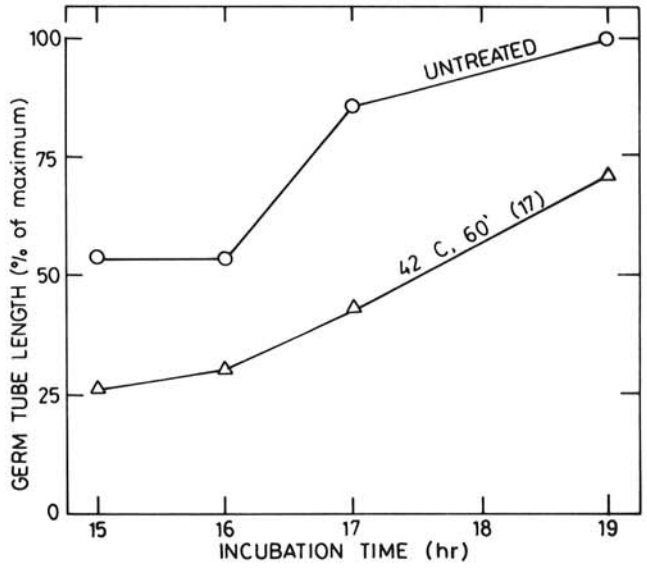

Fig. 4. Effect of sublethal heating at 42 C for 60 min on germ tube length of chlamydospores of *Fusarium oxysporum* f. sp. *niveum*. The number in parentheses denotes mortality of chlamydospores following heating, expressed as a percentage of germination in the unheated control. After linear regression analysis, both coefficients ($0.64 < R^2 < 0.74$) are significant. The slope values are not significantly different ($P = 0.05$). The time required to reach 50% germ tube length in the untreated control is significantly different from that of the heat treatment ($P = 0.05$).

RESULTS

Effect of sublethal heating on germination and mycelial growth of conidia and chlamydospores. Heat treatment of conidia of *F. o. niveum* at 38 and 40 C for 30–90 min caused a delay in germination and a reduction in length of germ tubes (Figs. 1 and 2). These effects were more pronounced than reduction in their viability (0–27%) at these temperatures. Similar results (not shown) in delay in germination and reduction in germ tube length were found with heat-treated (40 C for 30 and 60 min) conidia of *F. o. melonis*.

Preliminary experiments showed that chlamydospores of *F. o. niveum* were less affected by heat treatment than conidia; thus, higher temperatures or longer exposure times were required to achieve a similar effect of sublethal heating. Heat treatment of chlamydospores at 40 and 42 C for 120 and 60 min, respectively, caused a reduction in germ tube growth similar to that in conidia (Figs. 3 and 4). Heating at 40 and 42 C killed 6 and 17% of the chlamydospores, respectively.

Effect of sublethal heating on survival of conidia in soil. A sharp decline of 36% in conidial survival of *F. o. niveum* was observed 4 days after sublethal heat treatment (40 C for 60 min; Fig. 5). After

41 days of incubation, only 22% of the originally introduced heat-treated conidia (40 C for 60 min) survived, as compared with 45% survival in the unheated control. Heat-treated conidia at 40 C for 30 min had a similar survival rate to the unheated control.

Effect of soil bacteria on heat-treated conidia. After 2 days of incubation, viability of heat-treated (40 C for 60 min) conidia was reduced by 22%, whereas that of the unheated control remained unchanged (Fig. 6). The percentages of surviving heated conidia were lower than unheated conidia throughout the test period. Viability of untreated conidia in solarized soil declined more rapidly than heated conidia in untreated soil in the first 4 days. After 6 days, only 10–17% of heated conidia in untreated soil and of unheated conidia in solarized soil survived, as compared with 44% of unheated conidia in unheated soil. The combined treatment of heating and solarization was not more effective than solarization alone in reducing viability. Viability of conidia tested immediately after heating was not reduced.

Effect of sublethal heating of conidia on fluorescent staining and viability. Percentage of conidia accepting the vital fluorescent stain was lower in heat-treated conidia at 40 C for 1–4 hr than in the untreated control (Figs. 7 and 8). Although heat treatment for 3 hr reduced conidial viability by only 1%, fluorescent staining (in percentage of brightly stained conidia) was reduced by 41%. At all heating exposure times, level of fluorescence was lower than survival. Similarly, in an additional experiment, heating of conidia at 38 C for 90 and 120 min reduced fluorescent staining by 45 and 47%, respectively, whereas viability was reduced by only 7 and 9%, respectively (data not shown). An incubation period of 4 hr at 25 C did not affect the fluorescent staining capacity of untreated conidia. Conidia that were killed by exposure to 60 C for 60 min did not accept the stain.

Effect of sublethal heating of conidia on disease incidence. Heating conidia of *F. o. niveum* only partially reduced viability by 0–33%. However, the incidence of diseased seedlings at the end of the experiment was reduced by 35–82%, and disease progress was delayed following inoculation with the heat-treated conidia (Fig. 9). Similarly, heat treatment of conidia of *F. o. niveum* at 38 C for 240 min delayed disease progress and significantly reduced disease incidence by 70% (Fig. 10), although initial reduction in conidial viability was only 26%. Disease incidence in this treatment was even lower than that of plants treated with unheated conidia at half concentration. Reduction in disease incidence following inoculation with conidia heated at 38 C for 120 min was less pronounced and did not differ statistically from the control, whereas heating at 38 C for 60 min had no effect on either conidial viability or disease incidence. Similarly, final disease percentage in an additional experiment with inoculated watermelon seedlings at conidial concentrations of 5×10^4 conidia ml^{-1} was 64, 17, and 9 for unheated; heated at 40 C for 60 min; and 40 C for 90 min, respectively. Percentage of disease in seedlings inoculated with unheated conidia at half concentration was 48. Reduction in viability of conidia heat-treated at 40 C for 60 and 90 min was 0 and 33%, respectively.

In an experiment in a growth chamber with sterile vermiculite as rooting medium, watermelon seedlings were inoculated with conidia of *F. o. niveum* at a concentration of 2×10^4 conidia ml^{-1}. Treatments were: unheated, heated at 38 C for 60 min, and heated at 38 C for 90 min. Disease percentages among these treatments were similar throughout the duration of the experiment. Final disease percentages after 16 days were 94, 100, and 90, respectively. The reduction in conidial viability by heating for 60 and 90 min was 11 and 14%, respectively. A similar trend with the vermiculite system was observed when conidia were heated at 40 C for 30 and 60 min.

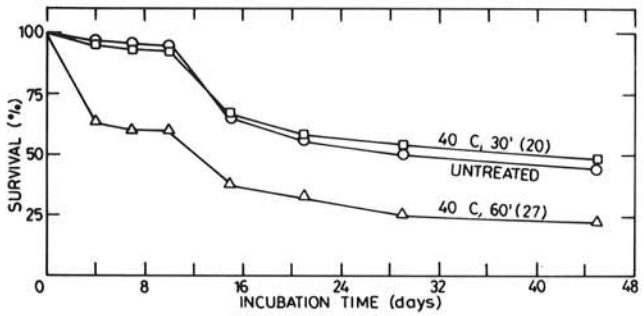

Fig. 5. Effect of sublethal heating at 40 C for 30 and 60 min on survival of conidia of *Fusarium oxysporum* f. sp. *niveum* in soil. The numbers in parentheses denote mortality of conidia following heating, expressed as a percentage of each treatment at zero time. With log transformation of incubation time, linear regression coefficients ($-0.94 < R^2 < -0.92$) are significant. The slope values are not significantly different ($P = 0.05$). The time required to reach 50% survival in the unheated control is significantly different from that of the heat treatment of 40 C for 60 min ($P = 0.05$).

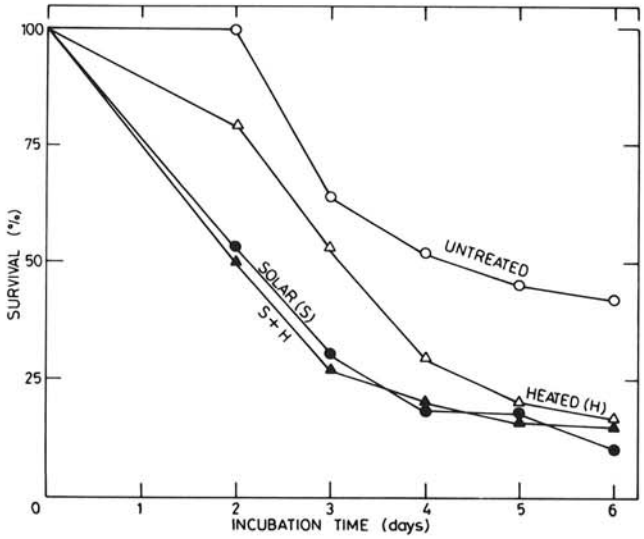

Fig. 6. Effect of heating at 40 C for 60 min, incubation with solarized soil, and combined treatments on survival of conidia of *Fusarium oxysporum* f. sp. *niveum* in a soil suspension culture. At zero time, no mortality of conidia was observed following heating. Results are expressed as a percent of each treatment at zero time. After probit transformation of survival percentage and log transformation of incubation time, linear regression coefficients ($-0.94 < R^2 < -0.77$) are significant. The slope value of the untreated control is significantly different from those of each of the treated ones ($P = 0.05$).

DISCUSSION

Sublethal temperatures, at near lethal range, weakened the propagules of *Fusarium* and reduced their survival beyond the

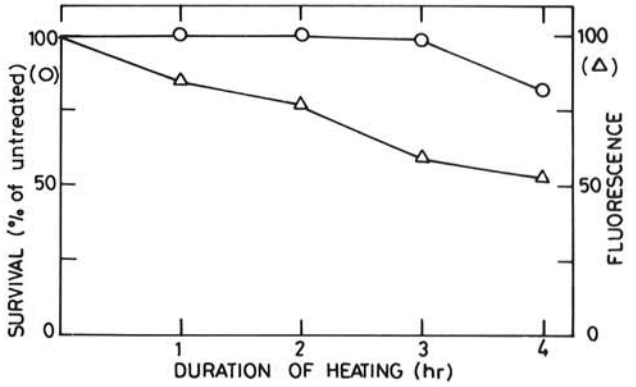

Fig. 7. Effect of sublethal heating at 40 C for 1–4 hr on percent fluorescent staining of conidia of *Fusarium oxysporum* f. sp. *niveum*. Ninety-four percent of untreated conidia were brightly stained after 4 hr of incubation at 25 C. After a linear regression, both coefficients ($-0.92 < R^2 < -0.4$) are significant. The slope value of survival percentage is significantly different from that of percentage of fluorescence ($P = 0.05$).

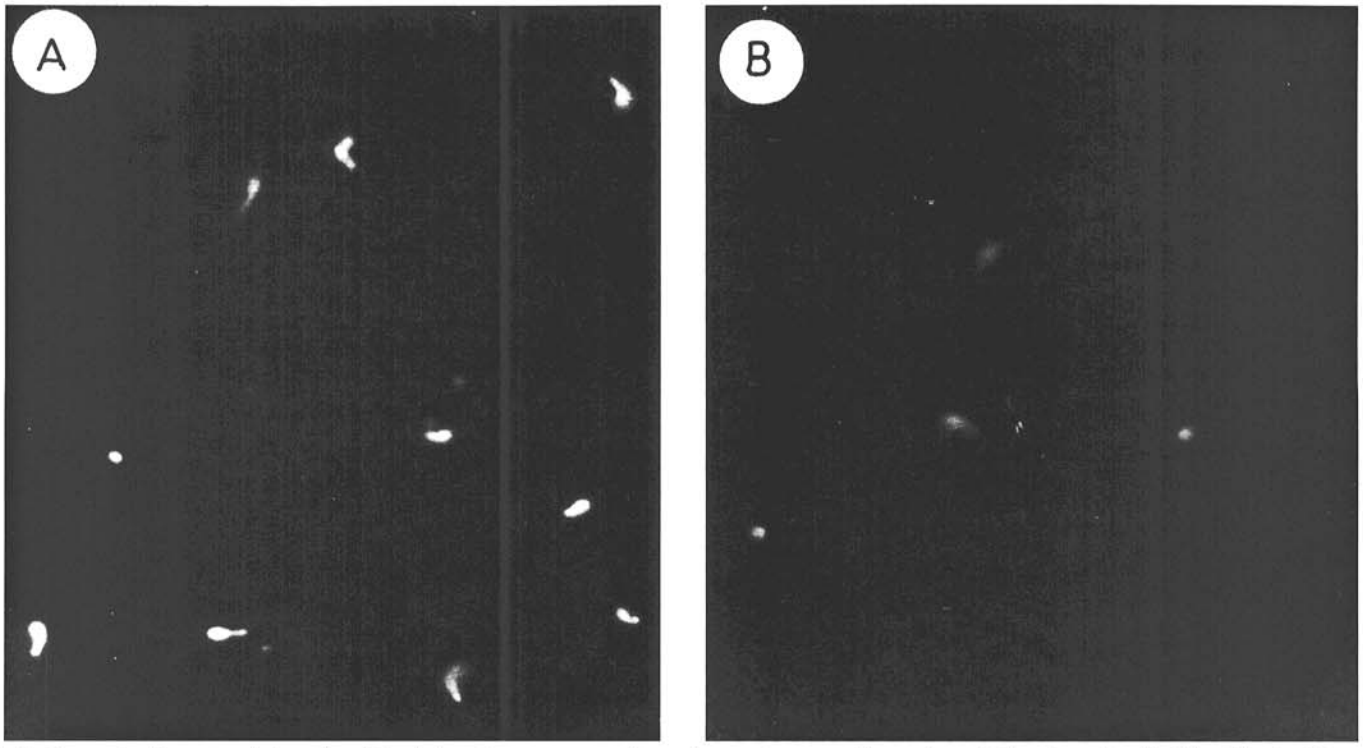

Fig. 8. Florescein diacetate staining of conidia of *Fusarium oxysporum* f. sp. *niveum* that were either unheated (**A**) or heated at 40 C for 240 min (**B**).

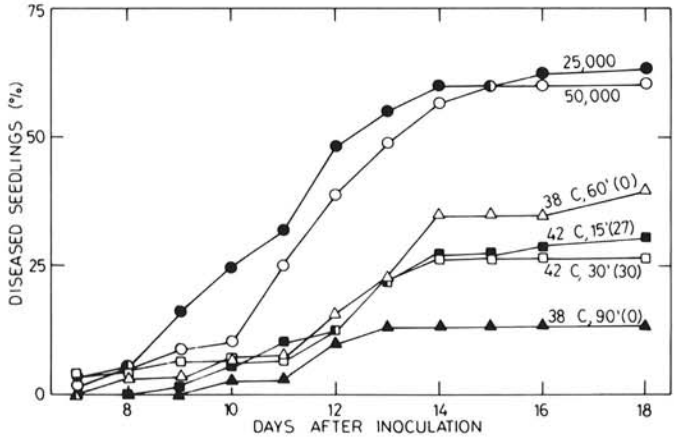

Fig. 9. Effect of sublethal heating at various temperatures on wilt incidence in watermelon seedlings inoculated with conidia of *Fusarium oxysporum* f. sp. *niveum*. Temperature in C and exposure time in min are shown, with numbers in parentheses denoting percent mortality of conidia following heating. Untreated controls are shown at conidia concentrations of 25,000 (2.5×10^4 conidia ml^{-1}) and 50,000 (5×10^4 conidia ml^{-1}). Heat-treated conidia were adjusted to a concentration of 5×10^4 conidia ml^{-1} before heating. After probit transformation of percentage of disease incidence and log transformation of days, the linear regression coefficients ($0.76 < R^2 < 0.98$) are significant. The slope values of the unheated controls are significantly different from those of the comparable heat treatments ($P = 0.05$).

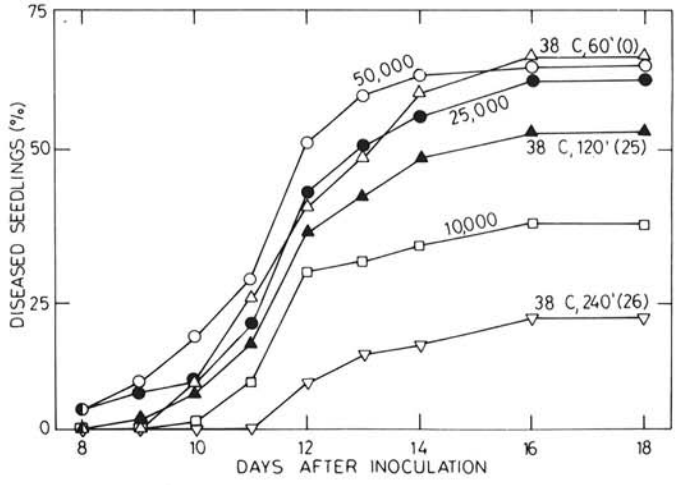

Fig. 10. Effect of sublethal heating at 38 C for 60–240 min on wilt incidence in muskmelon seedlings inoculated with conidia of *Fusarium oxysporum* f. sp. *melonis*. Temperature in C and exposure time in min are shown, with numbers in parentheses denoting percent mortality of conidia following heating. Untreated controls are shown at conidia concentrations of 10,000 (10^4 conidia ml^{-1}), 25,000 (2.5×10^4 conidia ml^{-1}), and 50,000 (5×10^4 conidia ml^{-1}). Heat-treated conidia were adjusted to a concentration of 5×10^4 conidia ml^{-1} before heating. After probit transformation of percentage of disease incidence and log transformation of days, the linear regression coefficients ($0.73 < R^2 < 0.91$) are significant. The slope value of the unheated control of 25,000 is significantly different from that of the heat treatment of 38 C for 240 min ($P = 0.05$).

initial 0–33% killing by heat. The weakening was expressed in delayed spore germination (as also shown with various pathogens) (15) and germ tube growth; reduction in vital fluorescent staining; and enhanced decline in viability of propagules of *Fusarium*, either in culture or in a natural soil, apparently due to microbial activity. Disease incidence also was reduced in spite of the severe root-dip technique that was used. In certain cases, the weakening effect was evident even when no initial heat mortality was observed (Figs. 2, 6, 7, and 9). Extended heating of conidia was associated with decreased fluorescent staining (Fig. 7). Similarly, decreased relative vital staining of fungi with FDA was associated with decline in respiration rate (17). The continuous decline in time of the population of *Fusarium* in culture, where conidia served as a sole carbon source for soil bacteria (Fig. 6), suggests that lysis possibly was involved in this decline, as previously demonstrated (8). Heat mortality curves usually describe the lethal effect (10,15,19), thus possibly underestimating the damage inflicted on surviving but weakened propagules by sublethal heating. The effective control of pathogens by solarization (18,19) also may be

related to biological control factors operating against the weakened propagules (10,11). The microbial effect on the sublethally treated propagules usually is expressed after termination of experiments, as in the present and other studies (1,13,18). Therefore, a certain delay in planting following treatment might be desirable.

Disease incidence was reduced in seedlings inoculated with heat-weakened conidia and transplanted in untreated soil, but not in sterile vermiculite. Thus, disease reduction may be attributed to reduction in inoculum density or potential due to enhanced microbial activity against the slow-germinating propagules, rather than to reduced pathogenic capacity of the heated propagules.

The weakening of pathogen propagules by sublethal treatments has been demonstrated in various studies. Carbon disulfide had no direct toxic effect on *A. mellea*, but the treated fungus was later killed in fumigated soil by the activity of *T. viride* (1). Garrett (7) suggested that the fumigant might weaken the resistance of the pathogen to attack by *T. viride*. Similarly, sublethal heating stressed *A. mellea*, which subsequently was controlled by *T. viride* (13). Exposure of sclerotia of *S. rolfsii* to metham sodium or to heat treatments predisposed them to attack and degradation by *T. harzianum* (9). Combining heating and *T. harzianum*, both at sublethal doses, resulted in improved control of *S. rolfsii* (6) and *R. necatrix* (19). Sublethal heating of sclerotia of *S. rolfsii* caused cracks in the rind, increased leakage of water-soluble organic compounds, increased colonization of the sclerotia by bacteria and streptomycetes, and, consequently, decreased disease incidence (12). Dried and remoistened sclerotia of *S. rolfsii* leaked large quantities of sugars and amino acids and, subsequently, rotted in soil (16). Similar to heating, solarized soil enhanced decline of the pathogen population in culture (Fig. 6), as previously demonstrated (8).

The weakening effect depends on temperature level, exposure time, and the environment into which the preheated propagules are introduced. For example, all heated conidia plated on an agar medium immediately after heating produced colonies, demonstrating complete recovery from the heat treatment (Fig. 6). However, mortality of these heated conidia reached 22% after 48 hr of incubation with a soil suspension. A similar trend was obtained when conidia were incubated in natural soil (Fig. 5). Apparently, the partially heat-damaged and slow-germinating propagules have a better chance of recovery in the presence of nutrients under axenic conditions in culture than in a hostile environment, unless the damage reached an irreversible stage. On the other hand, a certain threshold of heating has to be reached to obtain a detectable weakening effect (Figs. 5 and 10).

The weakening effect achieved at sublethal temperatures and the improved pathogen control obtained by combining it with other control agents (6,10,13,19) might be applicable to other methods of control, e.g., fumigation. Combining partial soil disinfestation with biocontrol agents (5) or fungicides at low dosage may further intensify the weakening effect and improve pathogen suppression. These approaches might be followed for integrated control of soilborne pathogens.

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