Weakening and Delayed Mortality of *Fusarium oxysporum* by Heat Treatment: Flow Cytometry and Growth Studies

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ABSTRACT

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Survival of *Fusarium oxysporum* f. sp. *niveum* following heat treatments was studied using flow cytometric, physiological, and microscopic assays. We exposed germinating conidia to sublethal temperatures from 36 to 42°C for 60 min, followed by rhodamine 123 staining and flow cytometry, and found increasing levels of fluorescence that reflect a change in mitochondrial membrane potential, indicating a weakening induced by stress. Viability of conidia or germinating conidia of the fungus exposed to heat decreased with increasing temperature, as assessed by fluorescent staining. However, viability was higher than that assessed with the 5-day-long plate count method and was further reduced 13 and 24 h after treatment, suggesting delayed mortality of the heat-treated ger-

minating conidia. Delayed mortality was substantiated by observing these conidia with light and fluorescent scanning electron microscopy and by subculturing single germinating conidia that had been previously heated. Programmed cell death was not observed in heat-treated conidia or germinating conidia of *F. oxysporum* based on the detection of plasma membrane phosphatidylserine translocation, cell-cycle measurements, detection of DNA fragmentation, or microscopic observation of apoptotic bodies. We hypothesize that propagules, which survived the heating and apparently are alive, may undergo further irreversible detrimental processes, eventually leading to their death by yet unidentified mechanisms. These findings suggest that pathogen propagules also might be affected under lower temperatures, possibly facilitating pathogen control by heating.

Additional keywords: apoptosis, sublethal heating, vital staining.

Heat is a common stress agent in nature and also can be used to control soilborne plant pathogens (16). When a pathogen is exposed to a sublethal dose of a killing agent, only part of the population dies. The individuals that survive the treatment may be stressed, weakened, and more vulnerable to additional abiotic or biotic stresses (11,17). Damage to the weakened propagules of a fungal pathogen can be expressed in various ways (e.g., delayed germination, reduced vital staining, or reduced disease incidence) (8,11,18). However, data on irreversible damage and eventual mortality without exposure to additional stress have not been reported with soil fungi.

A wide range of fluorescent dyes and markers for different structural and functional cellular properties and cell viability are available (13,35). Freeman and Katan (11) showed that the fluorescence level of heated, surviving but weakened conidia of *Fusarium oxysporum* f. sp. *niveum* stained with fluorescein diacetate (FDA) is lower than that of nonheated conidia. When fluorochromes are combined with flow cytometry (4,35), they provide a powerful analytical tool for studies of microbial physiology (40) and can discriminate between cells that differ physiologically or phenotypically (6). As compared with the plate count method, flow cytometry can detect cells that are still metabolically active but cannot be cultivated, and possibly even weakened propagules (30). This approach has not been used with phytopathogenic forms of *Fusarium*.

Apoptosis, also known as programmed cell death (PCD), is an important physiological process by which cells are eliminated during development and morphogenesis. It is a common feature in

multicellular organisms (26,37). In apoptosis, the cell is an active participant in its own demise. Cells undergoing apoptosis show typical biochemical and morphological events. These include membrane blebbing without the loss of its integrity; aggregation of chromatin at the nuclear membrane; formation of membrane bound vesicles (known as apoptotic bodies), and mitochondria becoming leaky due to pore formation involving proteins of the bcl-2 family. Apoptosis is a tightly regulated process, involving activation and enzymatic steps. It is an energy (ATP)-dependent process and includes nonrandom mono- and oligonucleosomal length fragmentation of DNA (ladder pattern after agarose gel electrophoresis), prelytic DNA fragmentation, release of various factors (Cytochrome C, apoptosis-inducing factor [AIF]) into cytoplasm by mitochondria, activation of caspase cascade, and alterations in membrane asymmetry.

Soil heating for pathogen control (e.g., by steaming or solarization) is a common method for disease control. In the past, emphasis was placed on achieving lethal levels of heating in an attempt to obtain maximum pathogen killing. These elevated temperatures also may be detrimental to beneficial soil microflora (16-18). Therefore, there is increasing interest in the effect of sublethal heating on pathogen viability and in the possibility of reducing pathogen populations using less drastic heating. The effect of heat at sublethal temperatures is especially important to soil solarization, which involves mild heating (16). We hypothesize that sublethal heating weakens and reduces the viability of the surviving propagules; some of these propagules may undergo irreversible detrimental processes.

The objectives of this study were to (i) assess and quantify reduced viability and weakening of *F. oxysporum* f. sp. *niveum* propagules following heat treatment by using a variety of parameters, and (ii) characterize and quantify the delayed mortality of the heat-treated propagules.

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MATERIALS AND METHODS

Organism and growth conditions. Biotype OR1 of *F. oxysporum* Schlechtend.:Fr. f. sp. *niveum* (E. F. Sm.) W. C. Snyder & H. N. Hans. (a derivative of FON-AA-O, ATCC 66054), pathogenic to watermelon plants, was grown on yeast extract (YE) agar medium as described previously (10). Thereafter, conidia were suspended in sterile 10 mM sodium phosphate buffer (pH 7.2). The conidial suspension was filtered and washed with buffer by centrifugation at $6,400 \times g$ for 8 min at room temperature (11). The conidial pellet was resuspended in 10 mM sodium phosphate buffer (pH 7.2), and the suspension adjusted to a concentration of 5×10^7 conidia ml^{-1} . The suspension (1 ml) was added to a 250-ml Erlenmeyer flask containing 49 ml of YE broth medium (pH 5.5). Conidia were synchronized by placing the flasks at 4°C for 10 h in the dark. Conidia were pelleted by centrifugation at $6,400 \times g$ for 8 min at room temperature. The conidia were washed once by resuspending the pellet in 10 mM sodium phosphate buffer (pH 7.2). Conidial concentration was adjusted to 5×10^6 conidia ml^{-1} . Conidia were heated at the indicated temperatures by placing 2-ml sterile screw-cap test tubes (Sarstedt, Numbrecht, Germany) containing 1 ml of 5×10^6 conidia ml^{-1} in a heated water bath (Fried Electric, Haifa, Israel) at an accuracy of $\pm 0.1^\circ\text{C}$ for 60 min. Incubation at 25°C served as the nonheated control.

Dilution plate count. We used this procedure to determine the size of the *F. oxysporum* population using a selective agar medium, based on Nash-Snyder medium (36). The modifications were: chloramphenicol (0.25 g liter^{-1}) was added and the pH was adjusted to 4.5 with lactic acid. Conidia were counted in a hemacytometer. Three 1-ml aliquots of conidial suspension per treatment were each separately heated and diluted in sterile distilled water. An aliquot of 0.2 ml of suspension containing approximately 100 conidia was spread on each of five plates containing the selective medium and incubated at 28°C, in the dark. Colonies were counted after 5 days or after 13 days when indicated. Mortality was calculated by comparing these plates with the plate counts of nonheated (25°C) control conidia.

Fluorescence studies: flow cytometry. For viability assessment, FDA (Sigma-Aldrich, St. Louis), acridine orange (AO, Sigma-Aldrich), and propidium iodide (PI, Sigma-Aldrich) were used according to a previously described staining procedure (30), with some modifications, as described below. FDA, a nonfluorescent compound, enters the cell and is hydrolyzed to fluorescein and acetate by nonspecific esterases. Fluorescein is retained by the cell if the plasma membrane is intact (28). Heat-treated conidia were centrifuged at $6,400 \times g$ for 5 min at room temperature and then washed in 0.1 M phosphate-buffered saline (PBS, pH 7.0). FDA working solution was kept on ice in the dark for up to 30 min. A 7.5- μl (7.5 ng) aliquot of FDA working solution was added to 300 μl of conidial suspension at 5×10^6 ml^{-1} , and incubated at 4°C for 6 min in the dark. Conidial fluorescence was analyzed in a flow cytometer (FACScan; Becton Dickinson, San Jose, CA). Excitation of FDA was monitored at 488 nm and emission was detected at 530 ± 15 nm as FL1 fluorescence. The dye AO does not freely cross intact plasma membranes and, as such, serves as an indicator of cellular membrane integrity (12). The phenanthridinium dye, PI, intercalates into double-stranded nucleic acids to produce red fluorescence, proportional to the nucleic acid content, when excited by blue light (13). This dye cannot pass through intact cell membranes and is used to stain nucleic acids stoichiometrically in dead or dying cells with injured membranes. Conidial staining with AO and PI was carried out as described for FDA staining. Excitation of AO or PI was obtained at 488 nm and emission was detected at 585 ± 21 nm as FL2 fluorescence. Rhodamine 123 (Rh123) is a cell-permeant, cationic, lipophilic fluorescent dye that is sequestered by active mitochondria without inducing cytotoxic effects (14). Rh123 stains only mito-

chondria. Before staining with Rh123 (Molecular Probes, Eugene, OR), heat-treated germinating conidia were washed by centrifugation at $6,400 \times g$ for 5 min at room temperature. Conidial suspensions at 5×10^6 ml^{-1} were resuspended in 0.1 M PBS (pH 7.0) containing from 0.5 to 50 $\mu\text{g ml}^{-1}$ Rh123. Rh123 staining followed a procedure similar to that of Diaper et al. (7) for bacterial cells, except that the washing steps were omitted. After incubation for 30 min at 28°C on a linear shaker (150 rpm min^{-1}), samples were kept on ice and analyses were completed within 30 min (approximately 60 min after the termination of the heat treatments). Excitation of Rh123 was obtained at 507 ± 3 nm and emission was detected at 529 ± 4 nm as FL1 fluorescence.

When indicated, fluorescence staining and germination studies also were carried out after 13, 24, or 26 h of incubation to check the delayed mortality. This incubation was carried out at 4°C, in the dark, to prevent new growth of the germlings, which could plug the cytometer tube. Preliminary experiments showed that incubation at 4°C did not affect the results compared with incubation at room temperature. For example, FDA fluorescence for conidia stained 13 h after heating at 45°C were 88 and 86.9% when incubated at 4°C or at room temperature after treatment, respectively. A similar trend also was obtained with AO staining.

All flow cytometry results were obtained after collecting 2×10^4 conidia from each treatment, in two replicates, using a high flow rate. List mode data acquired from the FACScan were converted from Hewlett Packard to Apple (Macintosh) computer format using the computer program Facs-Convert (Becton Dickinson, San Jose, CA). Data then were analyzed using CellQuest software (version 3.3; Becton Dickinson). The percentage of viable (FDA-positive) conidia was determined by comparing the fluorescence of the 47°C-heated conidia to that of the control (25°C). The percentage of nonviable (AO-positive and PI-positive) conidia was determined by counting the number of stained heated conidia and comparing them to the number of stained nonheated control conidia.

FUN-1 staining. We determined the activity of specific vacuolar enzymes by using FUN-1 (2-chloro-4(2,3-dihydro-3-methyl-1,3-thiazol-2-yl)-methylidene)-1-phenylquinolinium iodide) and a Live/Dead Yeast Viability Kit (Molecular Probes). FUN-1 differentially stains living and dead cells (13,32,33). Viable cells (i.e., those that are metabolically capable and have an intact plasma membrane) stained with FUN-1 process and concentrate it within the vacuoles, generating red cylindrical intravacuolar structures (CIVSs) (13,27). In nonviable cells, FUN-1 stays in the cytosol and fluoresces a diffuse orange-green, with no discernable red structures. Conidia at zero time, or 13 h earlier, were heat treated at 42 to 47°C for 60 min or nonheated (25°C). A portion of the heat-treated conidia was stained immediately with FUN-1. The remainder was suspended in YE broth medium and placed on a linear shaker (75 rpm min^{-1}) at 28°C for 13 h, after which they also were stained with FUN-1. The staining procedure was: 50 μl of 5×10^6 ml^{-1} suspension of conidia, or germinating conidia, was suspended in 895 μl of 10 mM Na-HEPES (Sigma-Aldrich), pH 7.2, and 105 μl of 20% D-glucose and washed by centrifugation ($6,400 \times g$ for 5 min). The pellet was resuspended in 160 μl of 10 mM Na-HEPES, pH 7.2, and 20 μl of 20% D-glucose. This suspension was stained with 18 μM FUN-1 (final concentration) by adding 40 μl of 100 μM FUN-1 working solution. The suspensions were incubated at 28°C for 45 min in the dark on a linear shaker (150 rpm min^{-1}). Conidia or germinating conidia were viewed in an Axioscope epifluorescence microscope (Carl Zeiss, Oberkochen, Germany), equipped with a fixed filter of BP 450 to 490 nm and LP 520 nm. Photographs were taken using an Axiovert 100 M confocal laser scanning microscope (Carl Zeiss) equipped with an argon laser. Excitation was obtained at 488 nm and emission was detected using BP at 505 to 550 nm and LP at 650 nm. Photographs were viewed using the 2.02 version LSM 510 Imager software (Carl Zeiss).

Germination studies. Conidial suspensions were diluted after heat treatment and 150,000 conidia in 200 µl were spread on YE plates and incubated at 28°C for 22 h in the dark. Germination percentage was determined by counting approximately 200 conidia per treatment. A conidium was considered germinating when its germ tube was at least as long (6 to 10 µm) as the conidium from which it originated. For FUN-1 staining of germinating conidia, a 50-µl aliquot of 5×10^6 ml⁻¹ conidial suspension was suspended in a 250-ml Erlenmeyer flask containing 50 ml of YE broth. These conidia were germinated at 28°C for 8 h in the dark on a linear shaker (75 rpm min⁻¹), then stained with FUN-1.

Germinating conidia studies. The ability of germinating conidia to form colonies was determined. For each treatment, 200 germinating conidia were individually transferred to YE agar medium under an inverted microscope, using a sterile stainless insect pin (No. 1; Bioquip Products, Gardena, CA).

Germinating conidia were stained with 0.05% (wt/vol) aniline blue solution in 85% lactic acid for light microscopic observations. They were prepared for scanning electron microscopy (SEM) as described previously (19). Samples were viewed under a Jeol JSM 35 microscope.

Apoptosis studies. *Translocation of plasma membrane phosphatidylserine.* The Annexin V-FITC apoptosis detection probe (BioVision, Palo Alto, CA) was used according to manufacturer's instructions. It is based on the translocation of membrane phosphatidylserine (PS) from the cytoplasmic to the extracellular side of the cell membrane (20,24). A 100-µl conidial suspension of 5×10^6 ml⁻¹ was centrifuged at $6,400 \times g$ for 5 min at room temperature and then resuspended in 200 µl of binding buffer. Annexin V-FITC (5 µl) was added to the conidial suspension, which then was incubated at room temperature for 5 min in the dark. Detection was carried out by using a fluorescent microscope and a flow cytometer (set as for FDA viability assessments).

Cell-cycle measurements using the AO and PI DNA denaturation assays by flow cytometer. Staining procedures of AO and PI were carried out as described (2,5), with slight modifications. Conidia were heated and then placed at 4°C; samples were taken for the analysis at different time points within 32 h from the termination of the heat treatments. During the last 9 h of the above period, conidia were transferred to YE broth medium for their germination at 28°C, on a linear shaker (75 rpm min⁻¹). Conidia or germinating conidia (1 ml at approximately 1.5×10^6 ml⁻¹) were centrifuged at $6,400 \times g$ for 5 min at room temperature and pellets were fixed for 12 h to 3 days, as described (2,5). For PI staining, the washed and RNase (Sigma-Aldrich)-treated conidial pellet were resuspended directly in PI working solution to a final concentration of 50 µg ml⁻¹, followed by incubation for 15 min at room temperature in the dark. For AO staining, conidial or germinating conidial suspensions were added to 0.5 ml of 0.1 M HCl. After a 40-s incubation at room temperature, 2 ml of AO staining solution (pH 2.6) containing AO at 6 µg ml⁻¹ was added (4). Conidia or germinating conidia were then analyzed by flow cytometer using the FL3 and FL2 channels (FL2 channel for the PI protocol).

DNA fragmentation. Conidia were heated, placed at 4°C, and sampled during the following 24 h. Genomic DNA was isolated

from conidial samples as described (41). DNA samples were electrophoresed and blotted (34). Blots were probed with [α^{32} P]dCTP hexamer-labeled DNA probe (Prime-a-Gene; Promega Corp., Madison, WI) using either complete or *HindIII*-digested total genomic DNA of the above isolate as a template. In parallel, gels were stained with ethidium bromide (Sigma-Aldrich) and visualized with ultraviolet light for the detection of DNA fragmentation (3).

Appearance of apoptotic bodies. Heated conidia were placed at 4°C, sampled during 32 h after heating, and stained with PI as described for FDA staining. During the last 9 h of the above period, conidia were transferred for germination, as described earlier. Conidia and germinating conidia (240 conidia from each heat treatment) were visualized under a light microscope for morphological changes (e.g. cell shrinkage, cytoplasm condensation, and vacuolization) (33). All the apoptosis experiments were carried out two or three times with similar results.

Experimental design. The dilution plate counts and germination studies were carried out in three replicates. These studies and FDA, AO, PI, Rh123, FUN-1 staining, single germinating conidia, light and scanning electron microscopy, and apoptosis studies were carried out three to five times with similar results. Results from one representative experiment are presented for each study. Comparison of temperatures and times was performed by two-way analysis of variance and specific means comparisons were performed by least significant difference ($P = 0.05$).

RESULTS

Survival of conidia following heat treatment. The viability of conidia following exposure to elevated temperatures was assessed immediately after termination of heat treatment by dilution plate count (Table 1). Heating the conidia at 45 and 47°C for 60 min resulted in pronounced mortality in the population, reaching 81 and 94%, respectively, compared with the nonheated (25°C) control (Table 1).

Alteration in Rh123 fluorescence of heat-treated conidia and germinating conidia. Germinating conidia were exposed to temperatures between 36 and 45°C for 60 min and stained with Rh123 at dye concentrations between 0.5 and 50 µg ml⁻¹. Changes in Rh123 fluorescence of heated germinating conidia were detected only at low dye concentrations (0.5 to 8 µg ml⁻¹). Survival of germinating conidia was not affected by heating to temperatures between 36 and 42°C, as assessed by dilution plate count (data not shown). However, their fluorescence values increased by 42 to 50% above the nonheated propagules (Fig. 1), indicating a weakening of the heat-stressed propagules. At 45°C, the fluorescence level increased by 50%. In contrast to germinating conidia, no changes in fluorescence were detected in heat-treated conidia at temperatures between 33 and 49°C (data not shown).

Flow cytometry of conidia following heat treatment. Conidia were exposed to heating at two temperatures and their response was assessed, using several fluorescent dyes and flow cytometer, at various times after heating. Exposure of the conidia to 45 and 47°C for 60 min, immediately followed by staining with FDA, revealed a decrease in their vital fluorescence by 3.1 and 22%, re-

TABLE 1. Fluorescence (%) of conidia of *Fusarium oxysporum* f. sp. *niveum* stained with fluorescein diacetate (FDA) and acridine orange (AO) at various times after heating for 60 min, as determined by flow cytometer, in comparison to the percent survival determined using the plate dilution method (plate)^y

Time (h) ^z	25°C			45°C			47°C		
	Plate	FDA	AO	Plate	FDA	AO	Plate	FDA	AO
0	100	94 a ± 0.5	2.7 a ± 0.8	19 a ± 0.8	91 a ± 0.2	25 b ± 5.6	6.1 a ± 0.4	72 a ± 0.0	55 b ± 2.4
13	100	89 b ± 0.6	2.1 a ± 0.2	18 a ± 0.7	88 a ± 0.0	21 b ± 0.1	5.5 a ± 0.4	60 b ± 5.0	63 a ± 0.0
24	100	90 b ± 1.8	2.3 a ± 0.3	12 b ± 0.6	82 b ± 0.2	34 a ± 3.1	5.6 a ± 0.3	42 c ± 0.3	67 a ± 1.1

^y Values in each column denoted by different letters differ significantly by the least significant difference test ($P = 0.05$), ± standard error.

^z Time after heating.

spectively, relative to the nonheated conidia (Table 1). In parallel, heat-treated conidia showed an increase in AO fluorescence, indicating reduced membrane integrity (Table 1, Fig. 2). The cytosolic enzymatic activity of the heat-treated conidia, as expressed in fluorescence, was higher than that assessed by the dilution plate method (Table 1). Compared with time zero, a further decrease in FDA fluorescence and increase in AO fluorescence were observed in the heated conidia 13 h after heat treatment, indicating a further reduction in cytosolic enzymatic activity. This trend was more marked and significant after 24 h. The further changes in fluorescence with time were not attributed to the incubation of conidia at 4°C, because similar changes also were obtained when incubation was carried out at room temperature. Heating conidia at 45°C for 60 min and staining with PI (which also indicates mortality), and assessing with flow cytometer, resulted in 29% nonviable conidia, compared with 2.9% in the nonheated control.

Staining with FUN-1. We used FUN-1 staining to distinguish between living and dead propagules. Nonheated conidia stained with FUN-1 appeared diffuse green. Within 40 min of incubation, bright red, round intravacuolar structures (RIVSs), resembling CIVSs (13), became visible (data not shown) and appeared to be dynamic structures that moved vigorously within the vacuoles. However, when conidia were killed following their exposure to a lethal heat treatment (e.g., 50°C for 60 min), they contained no inclusions and fluoresced orange-green. FUN-1 staining of conidia heated at 47°C showed a reduction in viability by 17% (Table 2). The percentage of viable heated conidia decreased further 13 h after heat treatment, similar to the trend observed with FDA and AO staining. A more pronounced decrease in viability was evident when germinating conidia originating from the heat-treated conidia were used, especially at the higher temperatures (Table 2).

Effect of heating on conidial germination. Exposure of conidia to heating at 45 and 47°C for 60 min resulted in a decrease in germination percentage (Table 3). Similar to the reduction in FDA fluorescence of heated conidia assessed by fluorescence (Tables 1 and 2), the percentage of germination in the heated conidia decreased further 13 and 24 h after heating.

Colony formation by single conidia. Dilution plate counts of heated conidia showed greater decreases in viability (Table 1) than did fluorescence staining (Tables 1 and 2) or conidial germination (Table 3). Results with the latter two methods are assessed shortly after the termination of heating, whereas dilution plate counts require 5 days for colony formation. Fluorescence or germination percentage decreased further after incubation for 13 or 24 h

Fig. 1. Changes in rhodamine 123 fluorescence of heated (36 to 45°C) and nonheated (25°C) germinating conidia of *Fusarium oxysporum* f. sp. *niveum*, as detected using flow cytometer. Data represents fluorescence values above nonheated control (25°C).

(Tables 1 to 3). One hypothesis to explain the lower survival observed with the plate counts is that viability of the previously heated propagules further declined with time, resulting in delayed mortality. This hypothesis was directly tested in the following experiments.

Single germinating nonheated or previously heated conidia were transferred, separately, to YE agar medium. The plates were incubated at 28°C, and monitored for 13 days for colony formation. Of the nonheated germinating conidia, 84 to 95% formed colonies, indicating that most of them were indeed initially viable. In contrast, only 10 to 32% of the germinating conidia that had been previously heated eventually formed colonies (Table 3). Apparently, many of the heat-treated conidia lost their viability during or after their germination. Colony counts of the transferred germinating conidia were made for 13 days, 8 days longer than the usual 5-day incubation, to detect any possible late appearance of fungal colonies. No additional colonies appeared on the plates after the fourth day's count, nor was any more growth of the germinating conidia evident microscopically. Therefore, germinating conidia that did not form colonies were considered to have eventually died. Preliminary experiments showed that similar results were obtained by using either YE or the selective medium for the incubation of the germinating conidia.

Germinating conidia originating from the nonheated conidia were firmly attached to the solid agar medium; when separated from that medium, a piece of the surrounding agar came with, indicating that the germ-tubes actively penetrated the agar medium. In contrast, it was easy to remove most of the germinating, heat-

Fig. 2. Single-parameter histograms, as detected by flow cytometer, of acridine orange-stained conidia of *Fusarium oxysporum* f. sp. *niveum* heated at **B and E**, 45°C and **C and F**, 47°C for 60 min, compared with **A and D**, nonheated control conidia (25°C), immediately (left column) or 24 h (right column) after heat treatment. Note that higher counts (indicating dead conidia) were observed after 24 h.

treated conidia from the solid agar medium, indicating that these germinating conidia were not established in the medium and had not penetrated it.

The possibility of a further decline in viability with time also was assessed by dilution plate counts. The conidial suspension was heated and then kept at 4°C for 13 and 24 h. The suspension then was diluted and plated on a selective medium to assess conidial survival. In contrast to the other parameters assessed immediately after heating (Tables 1 to 3), survival values using the plate count method at 13 and 24 h after heat treatment were, in most cases, similar to those obtained immediately after heat treatment (Table 3).

Light and scanning electron microscopy. We studied the morphology of heated and nonheated germinating conidia. In the nonheated (25°C) or sublethally heated (40°C, data not shown) germinating conidia, the resultant germ tubes and conidia showed no distinct morphological changes. In contrast, many of the 45 to 49°C-heated conidia and their germ tubes were devoid of cytoplasmic material, as detected by aniline blue staining of the cytoplasm (Fig. 3). Germ tubes appeared thickened, collapsed, withered, and distorted. More prominent changes were observed using SEM. Nonheated (25°C) as well as sublethally heated (40°C) germinating conidia were intact, with no distinct morphological changes. Conidia and germ tubes of the heated (45 to 47°C) germinating conidia were withered and distorted (Fig. 4). Sinking and collapse of the cell surface was observed, while cross-walls remained intact. Perforation of the conidia and germ tube also was visible.

Apoptosis study in conidia after heat treatment. The following four approaches (which include five methods) were used to determine whether the delayed death of heated conidia is a manifestation of PCD.

Detection of translocation of plasma membrane PS in heated conidia using the Annexin V-FITC assay. Conidia were nonheated (25°C) or heated at 45 or 47°C for 60 min, and were monitored by flow cytometer at various time points for 24 h after heat treatment for possible initiation of PCD. There was no evidence of Annexin V-FITC binding to PS (as would be indicated by an increase in FL1 fluorescence) in the heated conidia, relative to the nonheated controls, at any time during the examination period (data not shown).

Cell-cycle measurements by the AO and PI DNA denaturability assays via flow cytometer. Staining with AO revealed no difference in fluorescence between heated and nonheated conidia, before or after germination (data not shown). However, with the PI staining procedure, we detected a difference in fluorescence within 22 h after the heat treatment, which was higher in sub-G1 phase of the 47°C-heat-treated conidia compared with the G1 phase of the nonheated conidia by 10% in one experiment (not statistically different) and 20% in a second experiment (statistically different, *t* test). This difference decreased with time after heat treatment, and decreased further following germination of these conidia. No differences were detected when conidia were heated at 40 or 45°C.

Measurement of DNA fragmentation. Southern blot analysis of the total genomic DNA of conidia that previously had been heated at temperatures between 40 and 49°C, and probed with either complete or *HindIII*-digested total genomic DNA of the above isolate, did not reveal any fragmentation of the DNA samples at any time point after heating (data not shown). Similar results were obtained with agarose gels following staining with ethidium bromide (data not shown).

Tracking of the occurrence of cell blebbing and the appearance of apoptotic bodies. Microscopic visualization for the occurrence of cell blebbing and the consequent formation of membrane-bound vesicles (apoptotic bodies), typical of apoptosis, was carried out in heated conidia and germinating conidia, for 32 h after the termination of the 42 and 49°C heating for 60 min. No typical apoptotic morphological changes, including apoptotic bodies, were observed in the heated conidia or germinating conidia during this period.

DISCUSSION

A population of heated propagules consists of individuals with various levels of viability and capacities to recover. Part of that population is dead, as determined shortly after treatment, while the remainder is considered surviving, based on commonly used parameters (17). Some of the surviving propagules may recover, depending upon their genetic background, their heat tolerance, and the occurrence of additional stresses. Another segment of the stressed population, although showing residual enzymatic activities as reflected by vital staining, exhibits decreased viability with

TABLE 2. Viability of nonheated or heated conidia or germinating conidia (GC) of *Fusarium oxysporum* f. sp. *niveum*, immediately or 13 h after heating, as determined by FUN-1 staining, using a fluorescent microscope

Time after heating (h)	Type of propagules	Live conidia or GC (%), at temperatures (°C) ^z			
		25	42	45	47
0	Conidia	97 a ± 2.0	91 a ± 1.0	83 a ± 2.0	80 a ± 1.0
13	Conidia	96 a ± 1.0	79 b ± 5.0	70 b ± 5.0	67 b ± 1.0
13	GC	96 a ± 1.0	83 a ± 3.0	65 a ± 4.0	12 a ± 3.0
26	GC	92 a ± 2.0	83 a ± 2.0	16 b ± 3.0	8.3 a ± 1.0

^z Conidia or GC were considered live when their cytoplasm appeared diffuse green and their vacuoles contained bright-red, round inclusion bodies. Nonviable conidia or GC contained no inclusions and fluoresced bright orange-green. Values in each column, and for each type of propagule denoted by different letters, differ significantly by the least significant difference test (*P* = 0.05); ± standard error values are also given.

TABLE 3. Germination (GE) of and colony formation (CF) by nonheated or heated conidia of *Fusarium oxysporum* f. sp. *niveum*, at various times after heat treatment^a

Time after heating (h)	25°C		45°C		47°C	
	GE ^y	CF ^z	GE	CF	GE	CF
0	100	84 b ± 4	91 a ± 7	24 a ± 4	37 a ± 2	32 ± 5
13	100	95 a ± 2	61 b ± 0	10 b ± 3	30 a ± 2	ND
24	100	ND	61 b ± 6	ND	26 a ± 1	ND

^a Values in each column denoted by different letters differ significantly by the least significant difference test (*P* = 0.05); ± standard error values are also given. ND = not determined.

^y Germination at 25°C (control), at 0, 13, and 24 h after treatment, was 83.5, 80.0, and 85.0%, respectively, and these values were taken as 100%.

^z Germinating conidia were transferred to yeast extract agar medium and observed for 13 days for colony formation.

time (Tables 1 and 2) and eventually dies (Table 3). Apparently, a third segment of this population is at an intermediate stage in terms of levels of viability and ability to withstand additional stresses and recovery.

According to Jones (15), staining of yeasts can differentiate the cell population into four physiological classes, ranging from those in excellent condition to those that are dead. Various studies have demonstrated weakening caused by stress agents. Tjamos and Fravel (38) demonstrated the harmful effects of heating reflected in reduced germination and melanization of *Verticillium dahliae* microsclerotia that leads to impaired survival. Heat-stressed chlamydospores of *F. oxysporum* f. sp. *ciceri* and *Sclerotium rolfsii* sclerotia lose organic substances, are more readily colonized by soil microorganisms, and exhibit reduced germination (1,21). Exposure of sublethally heated sclerotia of *S. rolfsii* to soil microbial activity increased the mortality rate, indicating a weakening effect (8). Germinating conidia exposed to elevated temperatures followed immediately by Rh123 staining have a marked increase in fluorescence (Fig. 1), reflecting an increase in mitochondrial membrane potential (13). This increase may be due to an immediate demand for large amounts of energy to overcome the stress. Similar to our finding, heat-treated bacteria stained with Rh123 exhibit consistently higher fluorescence (25). However, nonspecific energy-independent binding should not be excluded. We found that this shift in Rh123 fluorescence (Fig. 1) also occurred at sublethal temperatures (i.e., lower than those causing immediate cell death), thus indicating a weakening effect in heat-stressed germinating conidia. This effect was not observed with ungerminated conidia, which are apparently less vulnerable to heating than those that are germinating conidia.

Using a variety of viability assays, the present study indicates a phenomenon not described before with weakening; namely, part of the propagule population, although having survived the heat,

further declined with time and eventually died. These propagules suffered from a detrimental effect of the heat that is manifested only at later stages. Therefore, we defined this phenomenon as delayed mortality because the mortality of these propagules could not be determined immediately after the termination of the heating process. This delayed reaction can be regarded as an extreme case of weakening involving irreversible damage and may occur even under axenic conditions. Various lines of evidence support this conclusion: (i) With all parameters tested, except for the plate count, viability of heat-treated propagules, expressed in fluorescence staining or germinability, decreased with time (Tables 1 to 3, Fig. 2). We assume that irreversible detrimental processes continue to take place after the removal of the stress agent (i.e., heat). (ii) Mortality rate of heat-treated propagules was higher in the 5-day dilution plate count assay (Table 1), than in the more immediate tests, such as vital staining or germination. Prudencio et al. (30) exposed yeasts to stress induced by acetic acid and found a similar gap between flow cytometry and plate count values. They suggested that this gap resulted from the stress imposed on some cells that still retain some metabolic activity as measured by flow cytometer but cannot grow to form colonies. (iii) Most of the conidia that were heated and later germinated ceased to grow, did not form colonies, and eventually died (Table 3), suggesting that irreversible damage resulted from the heat treatment. (iv) Germ tubes of heat-treated conidia became distorted and collapsed, as evidenced by light, fluorescent, and scanning electron microscopy (Figs. 3 and 4).

Changes with time after heat treatment in esterase activity, intracellular dye processing, and membrane integrity show that only a small portion of the conidial population can be declared dead when assessed by FDA or AO immediately after treatment. This portion of the population probably reflects necrotic or cytotoxic cell mortality, following lethal heat treatment. However, the fate of the propagules that appear viable when tested shortly after the termination of the treatment is not clear because their physiological status may change with time and only a certain portion eventually recover (23). The term "viable" might be questioned because, although these propagules have activity that can be followed by fluorescent stains or other physiological assays and may even germinate, they do not form colonies and do not grow much beyond germination. Therefore, these propagules cannot be con-

Fig. 3. Germination of **A**, nonheated (25°C; **A**) and heated (**B**, 45°C; **C**, 47°C; **D** to **F**, 49°C) conidia of *Fusarium oxysporum* f. sp. *niveum* stained with aniline blue. Although most of the heated conidia germinated, many of their germ tubes are withered and distorted. Bars = 5 µm.

Fig. 4. Ultrastructure of heated and nonheated germinating conidia of *Fusarium oxysporum* f. sp. *niveum*, observed by scanning electron microscope. **A**, Nonheated (25°C) germinating conidia were intact and showed no distinct morphological changes, whereas conidia and germ tubes of the heated (**B** and **C**, 45°C; and **D**, 47°C) germinating conidia were withered and distorted. Sinking and collapse of the cell surface can be observed, while cross-walls remain intact.

sidered ecologically viable. Growth and colony formation involve a wider spectrum of metabolic activities than those connected with fluorescence staining.

One of two processes occurs in propagules that survive the heat treatment. Some propagules recover and survive the stress, depending on the presence of additional stress agents but, for others, the damage is irreversible. Tsuchido et al. (39) observed that the permeability of the outer membrane of *Escherichia coli* cells to the dye 1-*N*-phenylnaphthylamine is damaged by heat stress (55°C) but is repaired during subsequent sublethal heating (37°C). Likewise, Fravel (9) suggested that the slower growth rate detected in *V. dahliae* microsclerotia exposed to sublethal doses of the fungicide metham sodium may reflect a period of repair in the damaged cells. The weakening of various types of propagules caused by sublethal heating was demonstrated with a variety of pathogens and appears to be a widespread phenomenon (1,8,9,11,18,21,38). In many studies, including those with *F. oxysporum* f. sp. *niveum* (10), heat-shock proteins are synthesized and play a crucial role in tolerance to heat or other stress agents (22,29). It appears that, at 45 to 47°C, such mechanisms cannot protect at least part of the *F. oxysporum* f. sp. *niveum* population from the irreversible deterioration in viability inflicted by the heat shock, thus reaching a "point of no return" from which they can no longer recover. It is not known what is the importance of delayed heat mortality in the rapid decline of population of heated conidia of *F. oxysporum* f. sp. *niveum* (11). In the present study, conidia rather than chlamydospores were used, since the latter could not be synchronized. In a previous study (11), sublethal heating of either conidia or chlamydospores resulted in reduction in length of germ tubes beyond reduction in viability, but higher temperatures were needed for suppressing chlamydospores. Populations of heated conidia declined faster in soil, were more vulnerable to soil microorganisms, and caused lower disease incidence compared with unheated conidia. However, further studies needed to establish the relevance of the weakening to disease control under more realistic conditions.

We used several approaches in attempt to connect delayed mortality of heated conidia and apoptosis. For 24 h after the termination of the heat treatment, no asymmetrical events in plasma membrane PS, typical to the initiation of apoptosis (20), were detected. However, the conidial cell wall may have interfered with the natural affinity of Annexin V protein to plasma membrane PS. No DNA fragmentation was detected for 24 h after heating. Tracking of the occurrence of cell blebbing and the appearance of apoptotic bodies also failed to reveal apoptosis. Based on the five assays we used, the only apoptotic process we detected was some increased proportion of heat-treated conidia with a lower DNA content with time after the treatment. Additional studies are needed before the involvement of apoptosis can be definitively excluded.

Our study indicates that pathogen mortality might be achieved at temperatures that are lower than those needed for immediate full control, if sufficient time is allowed for the delayed heat mortality. The significance of this phenomenon can be pertinent to solarization, where soil heating is relatively mild (16). Using a mild heating for pathogen control also will reduce the harmful effects of high temperatures on beneficial soil organisms and on soil biological balance (16), which were often observed when drastic measures were used. A determination of whether the delayed heat mortality phenomenon can also be triggered by other stress agents, such as starvation (which is frequently connected with biocontrol) or reduced dosage of pesticides is also worth pursuing. This information could then be useful when attempting to combine means of improving pathogen control and reducing environmental hazards (18). New effective methods of integrated control of these pathogens are especially important because methyl bromide, a major soil fumigant, has been banned for use (31).

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LITERATURE CITED

- Arora, D. K., Pandey, A. K., and Srivastva, A. K. 1996. Effects of heat stress on loss of C, germination and pathogenicity from chlamydospores of *Fusarium oxysporum* f. sp. *ciceri*. Soil Biol. Biochem. 28:399-407.
- Belloc, F., Dumain, P., Boisseau, M. R., Jallouste, C., Reiffers, J., Bernard, P., and Lacombe, F. 1994. A flow cytometric method using Hoechst 33342 and propidium iodide for simultaneous cell cycle analysis and apoptosis determination in unfixed cells. Cytometry 17:59-65.
- Burgoyne, L. A., Hewish, D. R., and Mobbs, J. 1974. Mammalian chromatin substructure studies with the calcium-magnesium endonuclease and two-dimensional polyacrylamide-gel electrophoresis. Biochem. J. 143:67-72.
- Darzynkiewicz, Z., Robinson, J. P., and Crissman, H. A. 1994. Flow Cytometry, Methods in Cell Biology, vol. 42, 2nd ed., parts A & B. Academic Press, Orlando, FL.
- Darzynkiewicz, Z., Traganos, F., Sharpless, T., and Melamed, M. R. 1975. Thermal denaturation of DNA *in situ* as studied by acridine orange staining and automated cytofluorometry. Exp. Cell Res. 90:411-428.
- Deere, D., Shen, J., Vesey, G., Bell, P., Bissinger, P., and Veal, D. 1998. Flow cytometry and cell sorting for yeast viability assessment and cell selection. Yeast 14:147-160.
- Diaper, J. P., Tither, K., and Edwards, C. 1992. Rapid assessment of bacterial viability by flow cytometry. Appl. Microbiol. Biotechnol. 38:268-272.
- Eshel, D., Gamliel, A., Grinstein, A., Di Primo, P., and Katan, J. 2000. Combined soil treatments and sequence of application in improving the control of soilborne pathogens. Phytopathology 90:751-757.
- Fravel, D. R. 1996. Interaction of biocontrol fungi with sublethal rates of metham sodium for control of *Verticillium dahliae*. Crop Prot. 15:115-119.
- Freeman, S., Ginzburg, C., and Katan, J. 1989. Heat shock protein synthesis in propagules of *Fusarium oxysporum* f. sp. *niveum*. Phytopathology 78:1656-1661.
- Freeman, S., and Katan, J. 1988. Weakening effect on propagules of *Fusarium* by sublethal heating. Phytopathology 78:1656-1661.
- Guasch, R. M., Guerri, C., and O'Connor, J. E. 1995. Study of surface carbohydrates on isolated golgi subfractions by fluorescent-lectin binding and flow cytometry. Cytometry 19:112-118.
- Haugland, R. P. 1996. Handbook of Fluorescent Probes. Molecular Probes Inc., Eugene, OR.
- Johnson, L. V., Walsh, M. L., and Chen, L. B. 1980. Localization of mitochondria in living cells with rhodamine 123. Proc. Natl. Acad. Sci. USA 77:990-994.
- Jones, R. P. 1987. Measures of yeast death and deactivation and their meaning: Part I. Process Biochem. 22:118-128.
- Katan, J. 1989. Soil temperature interactions with the biotic components of vascular wilt diseases. Pages 493-499 in: The Interaction of Genetic and Environmental Factors in Development of Vascular Diseases of Plants. C. Beckman and E. Tjamos, eds. Springer-Verlag, Berlin.
- Katan, J., Ginzburg, C., and Assaraf, M. 1996. Pathogen weakening as a component of integrated control. Pages 320-326 in: Advances in Biological Control of Plant Diseases. T. Wenhua, R. J. Cook, and A. Rovira, eds. China Agricultural University Press, Beijing.
- Katan, J., Ginzburg, C., and Freeman, S. 1992. The weakening effect as a trigger for biological control and criteria for its evaluation. Pages 55-61 in: Biological Control of Plant Pathogens. E. C. Tjamos, G. Papavizas, and R. Cook, eds. Plenum Press, New York.
- Kessel, R. G., and Shih, C. Y. 1974. Scanning Electron Microscopy in Biology. Springer-Verlag, New York.
- Koopman, G., Reutelingsperger, C. P. M., Kuijten, G. A. M., Keehnen, R. M. J., Pals, S. T., and Van Oers, M. H. J. 1994. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 84:1415-1420.
- Lifshitz, R., Tabachnik, M., Katan, J., and Chet, I. 1983. The effect of sublethal heating on sclerotia of *Sclerotium rolfsii*. Can. J. Microbiol. 29:1607-1610.
- Lindquist, S., and Craig, E. A. 1988. The heat-shock proteins. Annu.

- Rev. Genet. 22:631-677.
23. Lloyd, D., and Hayes, A. J. 1995. Vigour, vitality and viability of microorganisms. FEMS Microbiol. Lett. 133:1-7.
 24. Martin, S. J., Reutelingsperger, C. P. M., and Green, D. R. 1996. Annexin V, a specific probe for apoptotic cells. Pages 107-119 in: Techniques in Apoptosis: A User's Guide. T. G. Cotter and S. J. Martin, eds. Portland Press Inc., London.
 25. Mason, D. J., Lopez-Amoros, R., Allman, R., Stark, J. M., and Lloyd, D. 1995. The ability of membrane potential dyes and calcafluor white to distinguish between viable and non-viable bacteria. J. Appl. Bacteriol. 78:309-315.
 26. Metcalfe, A., and Streuli, C. 1997. Epithelial apoptosis. Bioessays 19:711-720.
 27. Millard, P. J., Roth, B. L., Truong Thi, H. P., Yue, S. T., and Haugland, R. P. 1997. Development of the FUN-1 family of fluorescent probes for vacuole labeling and viability testing of yeast. Appl. Environ. Microbiol. 63:2897-2905.
 28. Ormerod, M. G. 1990. Flow Cytometry: A Practical Approach. Oxford University Press, Oxford.
 29. Parsell, D. A., and Lindquist, S. 1993. The function of heat-shock proteins in stress tolerance: Degradation and reactivation of damaged proteins. Annu. Rev. Genet. 27:437-496.
 30. Prudencio, C., Sansonetty, F., and Corte-Real, M. 1998. Flow cytometric assessment of cell structural and functional changes induced by acetic acid in the yeasts *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae*. Cytometry 31:307-313.
 31. Ristaino, J. B., and Thomas, W. 1997. Agriculture, methyl bromide and the ozone hole: Can we fill the gaps? Plant Dis. 81:964-974.
 32. Roth, B. L., Millard, P. J., Yue, S. T., and Haugland, R. P. 1995. U.S. patent 5,445,946.
 33. Roze, L. V., and Linz, J. E. 1998. Lovastatin triggers an apoptotic-like cell death process in the fungus *Mucor racemosus*. Fungal Genet. Biol. 25:119-133.
 34. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
 35. Shapiro, H. M. 1995. Practical Flow Cytometry, 3rd ed. Wiley-Liss, New York.
 36. Singleton, L. L., Mihail, J. D., and Rush, C. M. 1992. Soilborne Phytopathogenic Fungi. The American Phytopathological Society, St. Paul, MN.
 37. Smith, S. C. 1997. Placental apoptosis in normal human pregnancy. Am. J. Obstet. Gynecol. 177:57-65.
 38. Tjamos, E. C., and Fravel, D. R. 1995. Detrimental effects of sublethal heating and *Talaromyces flavus* on microsclerotia of *Verticillium dahliae*. Phytopathology 85:388-392.
 39. Tsuchido, T., Aoki, I., and Takano, M. 1989. Interaction of the fluorescent dye 1-N-phenyl-naphthylamine with *Escherichia coli* cells during heat stress and recovery from heat stress. J. Gen. Microbiol. 135:1941-1948.
 40. Ueckert, J., Breeuwer, P., Abee, T., Stephens, P., Nebe Von Caron, G., and Ter Steeg, P. F. 1995. Flow cytometry applications in physiological study and detection of foodborne microorganisms. Int. J. Food Microbiol. 28:317-326.
 41. Yatzkan, E., Szoor, B., Feher, Z., Dombradi, V., and Yarden, O. 1998. Protein phosphatase 2A is involved in hyphal growth of *Neurospora crassa*. Mol. Gen. Genet. 259:523-531.