# Response of Monilinia fructicola Conidia to Individual and Combined Treatments of Anoxia and Heat

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ABSTRACT

Conidia of *Monilinia fructicola* were synergistically inactivated by treatment with heat followed by anoxia. By contrast, anoxia increased the resistance of spores to subsequent heating. A 45-min exposure to air after anoxia, however, caused the spores to revert to their original sensitivity. Sodium azide and sodium arsenite resembled anoxia in their effect on survival when conidia were treated with the respiratory inhibitors before or after heating. Phytopathology 61:61-64.

*Monilinia fructicola* (Wint.) Honey poses a constant threat to peaches and nectarines after harvest. Different postharvest chemical and physical treatments for reducing decay have been investigated (4). Heat treatments generally tolerated by fruit are capable of inactivating fungus lesions (10). Controlled atmospheres during storage and transit of fruit are becoming increasingly feasible (11). Benefits to fruit quality from postharvest anoxia have been claimed (9), but the effects of anoxia, alone or with other treatments, on postharvest fruit fungi have evidently not been extensively studied.

It was previously reported that heating sensitized *M. fructicola* to inactivation by anoxia (1). The purpose of this study was to further characterize the conditions leading to a synergistic interaction of heat and anoxia in the inactivation of conidia.

MATERIALS AND METHODS.—*Source of spores.*— Conidia of *M. fructicola* were obtained by growing fungus cultures for 7-9 days on V-8 juice agar in 500-ml flasks (12). Conidia were harvested by adding 200 ml of sterile Tween 80 (polyoxyethylene sorbitan monooleate) solution (1 drop/100 ml of distilled water), a few glass beads, and agitating the flask. Mycelial fragments were removed from the suspension of spores by filtering through eight layers of sterile cheesecloth. We washed conidia 3 times with fresh sterile Tween solution, centrifuging and decanting between washes to remove supernatant liquid from the pelleted spores. The concn was determined with a hemocytometer and adjusted to $3 \times 10^7$ spores/ml.

*Medium.*—Experiments were done using potato-dextrose broth (PDB) containing 8 g Difco potato extract, 20 g dextrose, 0.00005% resazurin (2) as a redox potential indicator, and deionized glass-distilled water to make 1 liter. The pH of the PDB was adjusted to 7.0 with KOH before autoclaving for 12 min at 121 C.

*Heat treatments.*—The conidia were heat-treated in a Viscosimeter constant-temperature ($\pm 0.02$ C) water bath. Seventy ml of PDB in 100-ml volumetric flasks were equilibrated with the temp of the water bath. One ml of suspension ($3 \times 10^7$ spores/ml) was added to the flask, followed by 10 sec of swirling for dispersal. The heat treatment was terminated by removal of the flasks from the heat-treatment bath to an ice bath and simultaneously adding 25 ml of chilled (0 C) PDB (potato-dextrose broth) followed by 15 sec swirling. The flasks were left in the ice bath for 5 min. After the volume of the suspension was adjusted to 100 ml by adding PDB, aliquots of 25 ml each were withdrawn for further treatments by exposure to periods of cold and anoxia. An aliquot was plated immediately, after appropriate dilutions, on potato-dextrose agar (PDA) in petri dishes to determine the percentage of spores surviving the heat treatments. Control spores were cooled identically, although they had not been heated.

*Exposure to anoxia.*—Anaerobic conditions were achieved by passing purified nitrogen through 125-ml Erlenmeyer flasks containing 25 ml of the spore suspension. To eliminate traces of oxygen, the effluent from liquid nitrogen was twice bubbled through cysteine hydrochloride-resazurin solutions (2). The nitrogen was delivered to each flask at a rate of 150 ml/min by means of capillary flow meters. Suspensions were shaken constantly (170 cycles/min) to hasten the elimination of dissolved oxygen initially present and to remove respiratory gases. The apparatus was installed in a constant temp room maintained at 23-25 C.

*Exposure to cold.*—Erlenmeyer flasks (125-ml) containing 25 ml of spore suspension ($3 \times 10^5$ spores/ml) were closed with 2 layers of Whatman No. 1 filter paper and placed for 24 hr on a rotary shaker (160 cycles/min) in a controlled-temp room at $0 \pm 1$ C.

*Metabolic inhibitors.*—In tests that involved the metabolic inhibitors, sodium azide and sodium arsenite, 1 ml of the inhibitor was added from stock solution to the cooled spore suspension after heat treatment. At the concn used, $1 \times 10^{-3}$ M, neither inhibitor affected the pH of the medium. A 25-ml aliquot was transferred to a 125-ml Erlenmeyer flask which was closed with filter paper as before and placed on a rotary shaker (170 cycles/min) for various periods at room temp (22-25 C). A second aliquot, diluted and plated immediately, served as a control. No effect on spore viability was detected when the inhibitor was added immediately before plating.

*Viability determination.*—Spore survival after each test was determined by appropriately diluting spore

suspensions and plating on potato-dextrose agar in 10-plate replicates followed by incubation at room temp. Details of methods have been described (2). Data were obtained from experiments performed at least twice.

RESULTS.—To determine the effect of anoxia on survival, freshly harvested, aerobically incubated spores (23-25 C/1.5 hr) were exposed to 24, 48, and 72 hr of purified $N_2$ gas. Neither the number (35%) nor length of germ tubes changed during anoxia, demonstrating that germination had been halted. When spores were plated, it was determined that 24- or 48-hr periods in anoxia caused no reduction in viability. After 72 hr in anoxia, however, only 50% of the incubated conidia survived as compared to 85% of the nonincubated conidia.

The kinetics of inactivation by heat of freshly harvested *M. fructicola* conidia are shown in Fig. 1. Little or no inactivation resulted from heating at 40 C for 16 min. By contrast, only 0.1% of the conidia survived heating at 50 C for 1 min. Thereafter, added increments of time were progressively less effective, and approx 0.02% survived a 16-min treatment. The reduced slope of the survival curve clearly revealed the presence of a resistant fraction in the spore population. At 45 C, a longer heating period was required to achieve equivalent inactivation, and a larger resistant fraction survived than at 50 C.

The effect of germination on heat sensitivity was tested. Survival of freshly harvested and incubated conidia (1.5 hr) was compared after heating at 40 C for 4, 8, 12, and 16 min. An increased sensitivity to heat accompanied germination, as shown by a slight reduction in survival of germinated spores after the 12-min treatment. After 16 min at 40 C, only 74% of the incubated conidia survived as compared to 96% of the nonincubated spores.

The possibility that treatments with heat and anoxia might interact synergistically was tested with heat applied immediately before or after a 24-hr period of anoxia. With a temp of 40 C for 16 or 32 min, only a slight synergism was detected. At higher temp, a striking synergism was seen when the heat treatment preceded anoxia. As shown in Fig. 2, heating at 45 C for 4 min reduced survival to about 30%, and anoxia reduced it to 93%. A survival of 27.9% should have been expected if the combined treatments were additive (30% × 93). Actually, only 7% survived. Furthermore, exposure to 50 C for 0.5 min before anoxia resulted in 1.5% survival instead of the 19.8% (20% × 99) expected if the effects were only additive.

By contrast, when anoxia preceded heat, the survival was much greater than would have been expected if the effects of heating and anoxia were additive (Fig. 2-A, B). The heat treatment of 45 C for 4 min after a 24-hr period of anoxia resulted in 80% survival, and 50 C for 0.5 min resulted in 70% survival instead of the 27.9 and 19.8%, respectively, expected had the treatments been additive. Clearly, the anoxia had rendered the spores less susceptible to the effects of subsequent heating.

The relationship between length of exposure to anoxia and the resulting increased heat resistance of conidia was established by exposing freshly harvested conidia to oxygen-free $N_2$ gas for various periods, followed by heating (45 C/4 min). Without exposure to anoxia, 9% of the conidia survived the heat treatment. When the spores were subjected to 3 hr of anoxia before heating, 42% survived. Lengthening the exposure to anoxia, up to 18 hr and particularly during the first 6 hr, increased the resistance to heat (Fig. 3-A). That the anaerobically-induced heat resistance was not permanent was demonstrated by exposing spores to air for various periods after 24-hr anoxia and before heating (Fig. 3-B). When heated immediately after 24-hr anoxia, about 80% of the conidia survived heat at 45 C for 4 min. After the first 15 min, conidia increasingly regained sensitivity to heat, with added time in air before heating. With a 60-min exposure to air before heating, only 4% survived, indicating that the anaerobically-induced heat resistance had completely disappeared.

As the altered response to heat might be associated with treatments, other than anoxia, that forestall germination, low-temp periods were provided before and after heating.

Preliminary experiments verified that conidia neither germinated nor lost viability during 24 hr at 0 C. Sequential treatments were then applied, consisting of a low-temp period (0 C/24 hr) and heating (45 C/4 min), or heating followed by the low-temp period. The results were compared with the inactivation following treatments of heat and anoxia (Fig. 4-A, B). Conidium survival after single treatments of heat, anoxia, and low temp were 12, 97, and 103%, respectively. Low temp, unlike anoxia, did not cause the spores to become more resistant to heat. Furthermore, the period of low temp after heat resulted in an increase in survival from 12% (heat only) to 44%. Many conidia appeared to recover from the effects of heating during the subsequent low-temp period.

Although 24-hr periods of either cold or anoxia forestall germination, anoxia reduced the sensitivity of conidia to heat; cold did not. Hence, the altered response to heat might be specifically related to anaerobic conditions rather than to the prevention of germination. To test this possibility, respiratory inhibitors, sodium azide, and sodium arsenite were used to prevent germination.

Sodium azide, an electron- and energy-transfer inhibitor, prevented germ-tube protrusion during a 6-hr incubation period at 25 C using a concn of $10^{-3}$ M in PDB. On transfer to fresh PDB, even after 24-hr contact with the inhibitor, spores remained capable of germination. The effect of the inhibitor on heat sensitivity was determined by heating (45 C/4 min) spores before or after 6 hr in the presence of sodium azide (Fig. 4-C).

Without exposure to the inhibitor, 17% of the conidia survived heating. After heating, exposing the spores to the inhibitor reduced survivors by about one-half. By contrast, 70% survived if spores were in-
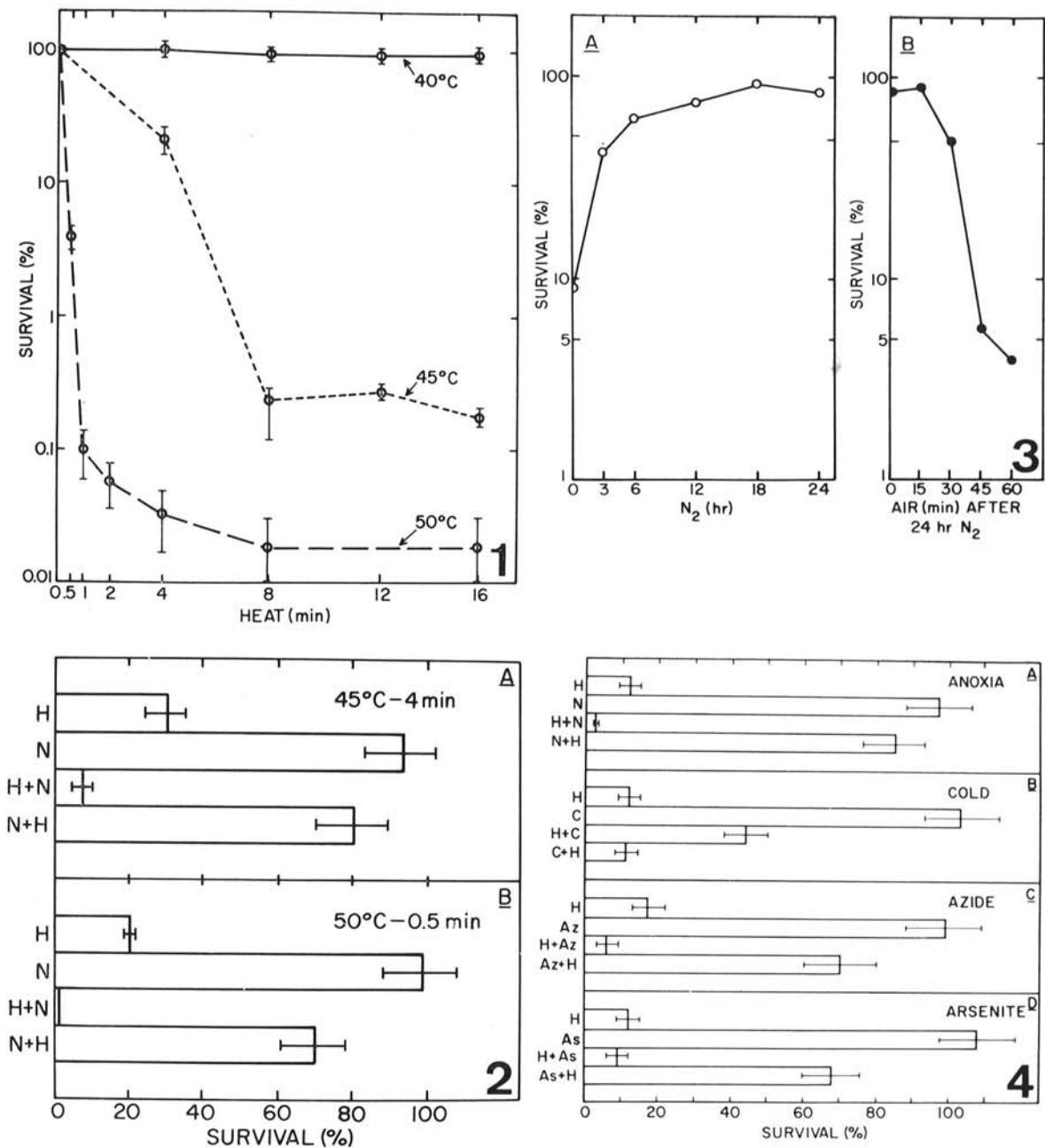
Fig. 1-4. **1)** Survival of nongerminated conidia after heat treatment at the indicated temp. Vertical lines through plotted points represent the standard deviation of the means. **2)** Survival of nongerminated conidia after single and sequentially combined treatments of heat and anoxia. H = heat only; N = 24-hr anoxia only; H + N = heat followed by 24-hr anoxia; N + H = 24-hr anoxia followed by heat. **A)** Heating at 45 C for 4 min. **B)** Heating at 50 C for 0.5 min. Horizontal lines represent the standard deviation of the means. **3) A)** Effect of anoxia and subsequent heat treatment (45 C for 4 min) on survival of nongerminated conidia. **B)** Effect of aerobic conditions after 24 hr of anoxia and before heat treatment (45 C for 4 min) on survival of nongerminated conidia. **4)** Survival of nongerminated conidia after single and sequentially combined treatments of heat (H = 45 C for 4 min) and **A)** ANOXIA:N = 24 hr of anoxia only; H + N = heat followed by anoxia; N + H = anoxia followed by heat. **B)** COLD:C = 24 hr at 0 C only; H + C = heat followed by cold; C + H = cold followed by heat. **C)** AZIDE:Az = incubation of conidia with $1 \times 10^{-3}$ M sodium azide for 6 hr; H + Az = heat followed by incubation with sodium azide; Az + H = incubation with sodium azide followed by heat. **D)** ARSENITE:As = incubation of conidia with $1 \times 10^{-3}$ M sodium arsenite for 1 hr; H + As = heat followed by incubation with sodium arsenite; As + H = incubation with sodium arsenite followed by heat. Horizontal lines represent the standard deviation of the means.

cubated in the inhibitor before heating. Clearly, sodium azide had increased the resistance of the spores to heat.

Sodium arsenite, an inhibitor of dithiol reactions and oxidative phosphorylation, similarly reduced the sensitivity of spores to heat (Fig. 4-D). At a concn of $10^{-3}$ M in PDB, sodium arsenite did not completely inhibit germination, as indicated by the appearance of a few germ tubes after 2-hr incubation. A 1-hr period in the inhibitor solution, however, was sufficient to increase the resistance of spores to heat. Without sodium arsenite, 12% survived heating (45 C/4 min). Sixty-eight per cent survived if spores were heated after treatment with the inhibitor.

DISCUSSION.—Complete elimination of oxygen is difficult. Commercial sources of $N_2$ gas commonly contained $O_2$ as a contaminant. Even after purification, care must be rigorously exercised to prevent recontamination. Permeation through rubber tubing, for example, is a source of oxygen contamination sometimes overlooked. The use of resazurin (2) permits visual verification of anaerobic conditions.

A previous report (6) that conidia of M. fructicola germinate under anaerobic conditions in the presence of dextrose but not in the presence of ethyl alcohol is not in accord with our results, in which no germination was observed in anoxia. We suspect that oxygen had not been completely eliminated during the earlier study. On the other hand, the possibility exists that different isolates of the organism may differ in requirements for oxygen during germination.

The germinated conidia (incubated for 1.5 hr) used in a few experiments had become only slightly more susceptible to anoxia than nongerminated conidia. Using longer incubation periods to increase susceptibility to anoxia was not possible because of clumping in the liquid medium, which prevented enumeration by plating. Nongerminated conidia used in most tests, however, were satisfactory for studying treatment interactions.

The heat-induced sensitization of M. fructicola conidia to inactivation by anoxia parallels their sensitization by heat to gamma irradiation (12). Presumably, sublethal heat injuries render many spores incapable of withstanding the subsequent stress of anoxia or irradiation. When irradiation preceded heat, the combined treatments were about additive. Different results, however, were obtained here when anoxia preceeded heating. During this period, the conidia had obviously undergone changes imparting resistance to heat.

A striking change in many cells subjected to anoxia is a reduction in size and number of mitochondria. Similar changes have been reported after treatment with certain respiratory inhibitors. For example, yeast cells grown anaerobically become devoid of mitochondria but regain them when aerated (7, 8, 13, 14). Mitochondria degenerated and migrated to the periphery when spores of Rhizopus stolonifer were subjected to anoxia. Upon introduction of air, mitochondria elon-gated, redeveloped extensive cristae, and dispersed from the periphery of the cell (3). Ekundayo (5) reported that degenerated mitochondria resulted from anoxia or treatment with sodium azide. Mitochondria in root tip cells of Sinapsis alba and Allium cepa were deformed when held in anoxia or treated with the respiratory inhibitors azide or cyanide (15).

Judging from observations cited above, the resistance to heat observed in this study was probably induced while mitochondria of the M. fructicola conidia were degenerating. The loss of resistance upon exposure to oxygen would presumably be associated with the return of mitochondria to normal structure and function.

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