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Effect of Conditioning Treatments on the Survival of *Radopholus similis* at High Temperatures

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Abstract: Heat treatments are an environmentally safe method for eliminating quarantine pests from tropical foliage. Conditioning heat treatments can induce thermotolerance against subsequent and otherwise phytotoxic temperatures in tropical foliage, allowing heat treatments to be even more effective. However, if thermotolerance is also induced in nematodes of quarantine significance like *Radopholus similis,* heat treatments would be rendered ineffective. A lethal thermal death point ($LT_{99.9}$) was established for *R. similis* by recording mortality at 25 (control temperature), 43°C, 45°C, 47°C, or 49°C after a 0, 1-, 2-, 4-, 6-, 8-, 10-, 12-, or 15-minute exposure. In a second experiment, nematodes were conditioned at 35, 40, or 45°C for 0, 15, 30, 60, 120, and 180 minutes, allowed to rest for 3 hours, and then challenged at 47°C for 5 minutes. No nematodes survived the challenge heat treatment; rather, nematode mortality was hastened by the conditioning treatment itself. In a third experiment, *R. similis* inside anthurium roots were conditioned at 25°C or 40°C for 15 minutes and then treated at 45°C for up to 8 minutes. Mortality of conditioned and unconditioned nematodes was similar ($P > 0.1$). Conditioning treatments increase plant thermotolerance but do not induce thermotolerance in *R. similis.* Heat treatments have promise as disinfection protocols for quarantines.

Key words: Anthurium, burrowing nematode, conditioning, hot water, quarantine, *Radopholus similis,* survival, thermotolerance.

One pesticide-free approach to eliminate nematodes from high-value tropical foliage plants is heat treatment (Arcinas et al., 2004; Tsang et al., 2001; 2004). This approach holds promise for plants infected with *Radopholus similis* exported to areas such as Japan that quarantine the nematode. However, heat treatment is effective only if the foliage plant tolerates higher temperatures than the nematodes. Unfortunately, *Anthurium andraeanum,* a good host to *R. similis,* does not tolerate high temperatures (Hansen et al., 1992; Ishii et al., 1956).

Many plants can be conditioned to tolerate higher temperatures by the induction of heat shock proteins (Hsp) (Yarwood, 1967). The induction of Hsp is accomplished by stressing plants with temperatures 5°C to 10°C above ambient temperature (Paull and Chen, 1990). The cowpea (*Vigna sinensis*) exhibited maximum temperature adaptation when stressed or conditioned for 20 seconds in 50°C hot water followed by 8 hours at 40°C in an air incubator. Lag periods of approximately 3 hours are often required between stress/conditioning treatment and subsequent challenge heat to minimize plant damage (Yarwood, 1967).

The induction of Hsp and induced thermotolerance is not unique to plants. Many organisms, including nematodes, have a thermotolerance response to elevated temperatures. *Heterorhabditis bacteriophora* subjected to 35 °C for 3 hours followed by a 1- to 2-hour lag period remained infective at 35°C and 40°C, unlike unconditioned nematodes (Selvan et al., 1996). Thermotolerance was associated with the presence of 70-kDa Hsp in the conditioned nematodes (Selvan et al., 1996). A heat shock response has also been identified in *Caenorhabditis elegans* (De Pomerai et al., 2000), and several genes involved in the process have been identified (Heschl and Baillie, 1989; Nikolaidis and Nei, 2004). The induction of thermotolerance in plant-parasitic nematodes is an important quarantine issue. If the heat shock response found in *C. elegans* and *H. bacteriophora* is ubiquitous among nematodes, which is not an unreasonable expectation, heat treatments designed to disinfest plants of *R. similis* may fail. The objective of the experiments reported herein was to determine if *R. similis* developed thermotolerance.

Materials and Methods

A series of three experiments was conducted to determine if thermotolerance was induced in *R. similis* through conditioning treatments. Nematodes were reared on alfalfa root callus (Ko et al., 1996). Twenty-four hours prior to use, nematodes were extracted from the callus using Baermann funnels, counted, and suspended in water at a density of approximately 10,000 vermiform stages/L water.

The first experiment was designed to establish an in vitro thermal death point for unconditioned nematodes. As a subtropical-to-tropical nematode, *R. similis* tolerates temperatures up to 32°C (DuCharme, 1969). Consequently, nematodes were subjected to treatments of 25 (control temperature), 43, 45, 47, or 49°C for 0, 1, 2, 4, 6, 8, 10, 12, and 15 minutes. Treatments were arranged factorially (five temperatures × nine exposure times) and replicated five times. The experiment was repeated once. One-ml aliquots of the nematode suspension were pipeted into 1.5-ml Eppendorf tubes, placed in a dry bath incubator, and heated to treatment temperature. Digital thermometers were used to determine when nematode suspensions reached the target

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temperature, at which point treatment time measurements were begun. Immediately after exposure, nematode mortality was recorded as nonmotile nematodes that failed to respond to tactical stimulus after the treatment. Because the number of nematodes in each 1-ml aliquot varied, the initial nematode number exposed to the treatment was based on a maximum likelihood estimate from the number of nematodes recovered in the control treatment (Wadley, 1949). Data from the two experiments were tested for homogeneity of variance, found to be similar for all temperatures except 49°C, and combined for further analysis. Data from the 49°C treatments were analyzed using Pearson's χ^2 as the predictive value. Lethal temperature (LT) values were estimated by probit analysis (Finney, 1971). The in vitro thermal death point was determined as the exposure time at which 99.9% of the treated population ($LT_{99.9}$) with 95% confidence interval was killed. The in vitro thermal death point for *R. similis* was determined by plotting mortality against exposure time for the different treatment temperatures. The in vitro thermal death point was subsequently used as the challenge heat temperature in other experiments.

The goal of the second experiment was to determine the effect of in vitro heat conditioning on the $LT_{99.9}$ of *R. similis*. Nematodes were conditioned at 35°C, 40°C, and 45°C for 0, 15, 30, 60, 120, or 180 minutes in a dry bath incubator. The Eppendorf tubes containing the conditioned nematodes were then placed in an agitated ambient water bath for 3 hours to allow development of tolerance and then challenged at 47°C for 5 minutes. A challenge at ambient temperature for 5 minutes served as a control treatment. After the challenge treatment, surviving nematodes, as determined by movement or a tactile response, were counted. Treatments were arranged in a $3 \times 6 \times 2$ factorial with five replications. The experiment was repeated once. Data were analyzed for variance.

The third experiment sought to determine the effect of in planta heat conditioning on the $LT_{99.9}$ of *R. similis*. Fourteen *A. andraeanum* cv. Mickey Mouse growing in 15-cm square plastic pots filled with three parts 3-cm crushed volcanic cinder to two parts sphagnum peat moss (No. 4 Sunshine Mix, Sun Gro Horticulture, Bellevue, WA) were inoculated with 10,000 mixed life stages of *R. similis* delivered in 20-ml aliquots 17 weeks prior to treatment. Seven plants were conditioned by placement in a continuously recirculating 40°C hot water unit for 15 minutes (Tsang et al., 2001). The remaining seven plants were placed in an ambient temperature water bath. Three hours later, medium was removed from the roots. The roots from seven plants in each conditioning treatment were composited, cut into 3- to 5-cm pieces, mixed, and divided into twenty 20-g subsamples. These subsamples were placed into 5-cm^2 330-µm-pore mesh bags and randomly assigned to heat treatments of 45°C for 0, 2, 4, 6, or 8 minutes. Challenge heat was applied by immersion in a continually agitated hot water bath maintained at target treatment temperature. Each challenge treatment was replicated four times and the entire experiment repeated once. After the challenge heat treatment, the bags were placed in a mist chamber and nematodes collected over the next 24 hours (Barker, 1985). Living nematodes, as determined by movement or a tactile response, were counted. The data from the two experiments were arcsine transformed, tested for homogeneity of variance, found to be similar, and combined for analysis. Parameters of the probit regression estimates of mortality at 45 °C were compared between conditioned and unconditioned nematodes using analysis of variance.

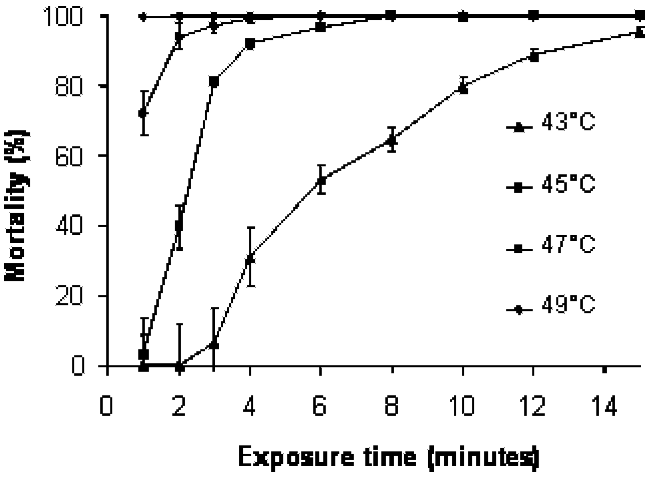

FIG. 1. Mortality curves for aqueous suspensions of *Radopholus similis* at different temperatures. Percentage mortality is calculated as 1-(Pf/Pi) × 100 where Pf is the number of survivors and Pi is the mean of survivors recovered from control treatment. Bars represent standard error of the mean.

RESULTS AND DISCUSSION

Radopholus similis survived longer at lower temperatures (Fig. 1). Nearly all nematodes died within 1 minute of exposure at 49°C. At 45°C, 100% mortality of *R. similis* occurred within 10 minutes. $LT_{99.9}$ values of 27, 7, and 5 minutes at 43, 45, and 47°C, respectively, were derived from probit analysis (Table 1). An $LT_{99.9}$

TABLE 1. Probit analysis of mortality rates of *Radopholus similis* at 43°C, 45°C, and 47°C.

Temperature (°C)	Probit regression[a]	$LT_{99.9}$[b] (minutes)	χ^2deviation from observed values[c]
43	$y = 7.1x - 5.7$	27	$P = 0.16$
45	$y = 9.3x - 3.2$	7	$P < 0.01$
47	$y = 6.0x - 0.9$	5	$P = 0.99$

[a] Percentage mortality between 0 to 15 minutes transformed by 1n (% ÷ (1 – %)) and time transformed by 1n (time). Regression equation units back-transformed to minutes.

[b] Maximum likelihood probit estimate of minutes required for 99.9% mortality of treated population.

[c] Pearson χ^2 goodness-of-fit test for deviation of regression from observed values. Values less than 0.05 indicate poor fit of probit regression to observed values.

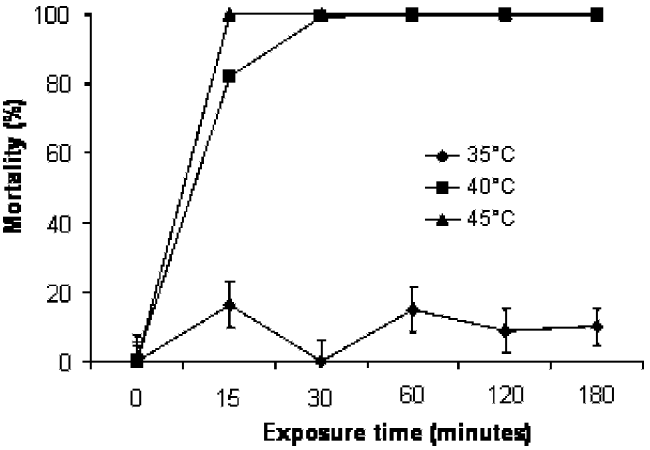

FIG. 2. Mortality of *Radopholus similis* conditioned at 35°C, 40°C or 45°C for 0, 15, 30, 60, 120, and 180 minutes. Each point represents a mean of eight replications. Bars represent standard error of the mean.

at 47°C for 5 minutes was selected as the challenge heat treatment because it was similar to the 49°C for 12-minute hot water treatments being tested commercially for *A. andraeanum* (Tsang et al., 2004).

Radopholus similis did not develop thermotolerance with in vitro conditioning. Few *R. similis* survived the 40 and 45°C conditioning temperatures (Fig. 2). At 40°C, conditioning was lethal at exposures greater than 30 minutes. At 45°C, all nematodes were dead after 15 minutes. Nematodes surviving the 35°C and 40°C conditioning treatments appeared debilitated at the end of the 3-hour acquisition period. Debilitated nematodes were inactive and had clear intestinal regions. Many conditioned *R. similis* in the 25°C control challenge treatment were slow moving or nonmotile upon the final observation. This debilitation suggests an increase in susceptibility to heat rather than a thermotolerance response at the tested temperatures. None of the nematodes conditioned at 35°C or 40°C survived the subsequent 47°C, 5-minute challenge treatment. *Radopholus similis* regularly encounters temperatures below 35°C and would therefore not be likely to act as conditioning treatments.

Nematode survival inside roots was not different from nematode survival in aqueous suspensions. Probit analysis regression estimates for survival at 45°C were linear for both conditioned and nonconditioned nematodes infecting anthurium roots. Survival regression equations were:

Nonconditioned: $y = 0.11 + 1.72x$
Conditioned: $y = 0.15 + 1.68x$

where y = percent nematode survival and x = time of exposure at 45°C. The slopes did not differ between conditioned and nonconditioned nematodes ($P = 0.85$). Relative potency, the ratio of equally effective doses, was 1.0005. The LT_{50} (lethal time for 50% mortality) was not different between the treatments, 56 seconds for nonconditioned nematodes and 55 seconds for conditioned nematodes (Fig. 3).

Radopholus similis did not develop thermotolerance after conditioning at 35°C or 40°C in aqueous solutions or in plant tissue. Sublethal temperatures resulted in

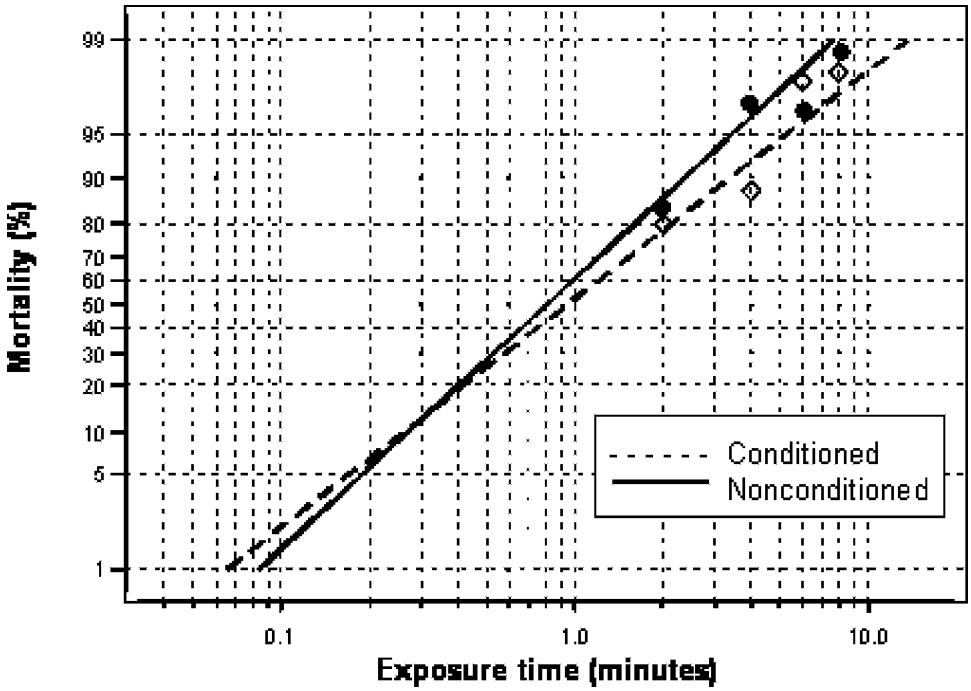

FIG. 3. Probit regression estimates of mortality of *Radopholus similis* conditioned at 40°C and exposed to 45°C for 15 minutes. Percent mortality and time were ln transformed for analysis. Unit shown are back-transformed values on log scale axes. Points represent mean of eight replicates combined from two runs of the experiment.

additive effects on mortality of *R. similis* instead of inducing thermotolerance.

Conditioning plants to increase thermotolerance remains a viable option in quarantine measures against *R. similis*. The conditioning treatment and acquisition period appropriate for engaging thermotolerance in *A. andraeanum* do not lead to induction of thermotolerance in *R. similis*. Consequently, conditioning treatments can reduce phytoxicity associated with elevated temperatures without comprising the efficacy of the nematode eradication treatment.

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