Thermal Inactivation of Inoculum of Two *Phytophthora* Species by Intermittent Versus Constant Heat

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ABSTRACT

Research on solarization efficacy has examined the critical temperature and minimum exposure time to inactivate soilborne pathogens. Most mathematical models focus on survival of inoculum subjected to a constant heat regime rather than an intermittent heat regime that better simulates field conditions. To develop a more accurate predictive model, we conducted controlled lab experiments with rhododendron leaf disks infested with *Phytophthora ramorum* and *P. pini*. Focused in vitro experiments with *P. ramorum* showed significantly longer survival of inoculum exposed to intermittent versus constant heat, indicating that intermittent heat is less

damaging. A similar trend was observed in soil. Damage was evaluated by comparing the reduction in subsequent survival time of inoculum subjected to different intensities of sublethal heat treatments. Inoculum exposure to continuous heat reflected an increasing rate of damage accumulation. Multiple sublethal heat events resulted in a constant rate of damage accumulation which allowed us to calculate total damage as the sum of damage from each heat event. A model including a correction for an intermittent heat regime significantly improved the prediction of thermal inactivation under a temperature regime that simulated field conditions.

Solarization is a potential management tool for soilborne pathogens. It has been recently shown to be effective for *Phytophthora ramorum* in container nursery beds (Funahashi and Parke 2016). *P. ramorum* is a quarantine pathogen and causes sudden oak death. The pathogen is spread by movement of nursery plants (Goss et al. 2009), and there were 561 nursery detections in the United States between 2001 and 2014 (USDA-APHIS 2015). Once nursery beds become infested by the pathogen, they are extremely challenging to disinfect, and many nurseries are found to be recurrently positive in the test for the pathogen (USDA-APHIS, unpublished). Although soil solarization can be effective for disinfecting soil, an accurate predictive model is required to determine the feasibility and the appropriate duration of solarization treatment at different locations and soil depths.

Thermal inactivation (death) curves have been established to predict pathogen mortality in response to elevated temperature (Gamliel and Katan 2012). One of the most popular approaches is the degree-hour concept, in which mortality is related to the accumulated time of exposure above a certain threshold temperature (Shlevin et al. 2003). The degree-hour model has been proposed in various studies as a tool for evaluating the effectiveness of thermal inactivation of pathogens (Chellemi et al. 1994; Lopez-Herrera et al. 1994, 1997; Walkey and Freeman 1977). However, this model is not always successful because the degree-hour concept is empirical and it gives equal weight to all temperatures above a threshold temperature (Shlevin et al. 2003). Another popular approach is a first-order kinetics concept (e.g., Z-value model or Arrhenius model) (Corradini and Peleg 2006; Fujikawa and Itoh 1998; Sasanelli and Greco 2000) and Weibull model (Peleg and Cole 1998; Peleg and Pechina 2000; van Boekel 2002; Weibull 1951), in

which the rate of decline in a pathogen population is related to a first-order temperature equation. It is also common to build a model using the concept of lethal dose (LD) to indicate the lethality of a given temperature to a proportion of a pathogen population; for example, LD₅₀ (Pullman et al. 1981; Tamietti and Valentino 2001).

With any model, the strategy is to find a simple correlation between important physiological factors (e.g., temperature and water potential) and inactivation (death) of the pathogen. It is fundamental to include temperature in a predictive model as the first factor. However, some noticeable discrepancies have been found and other factors need to be included (Shlevin et al. 2003). It has been shown that many microbes are more heat resistant in drier conditions than in moist conditions (Gamliel and Katan 2012; Russell 2003).

Another important factor is the soil temperature regime. Pathogen propagules are exposed to a diurnal, fluctuating temperature regime during solarization, with highest temperatures achieved in the late afternoon and lowest temperatures by early morning. Models have been commonly constructed based on a cumulative heat effect assumption from lab experiments with constant heat treatments; for example, with *Escherichia coli* (Fujikawa and Itoh 1998) and a variety of organisms, including *Clostridium botulinum*, *Bacillus stearothermophilus*, *Salmonella typhimurium*, *Listeria monocytogenes*, *E. coli*, and *Vibrio vulnificus* (Peleg and Cole 1998), *Bacillus stearothermophilus* (Sapru et al. 1993), *Aphanomyces cochlioides* (Dyer et al. 2007), and theoretical simulation (Corradini and Peleg 2006; Peleg and Cole 1998). However, there is evidence that exposure to fluctuating temperature has significant effects on the pathogen response to heat. Porter (1991) evaluated the effect of intermittent heating of soil (6 h/day over 14 days to temperatures of 30 to 45°C) on the inactivation of *Sclerotinia sclerotiorum* sclerotia. Constant heat was more effective than intermittent heat for reducing the number of viable sclerotia. Although Coelho et al. (2000) did not conduct a direct comparison between constant versus intermittent heat on the reduction of a *P. nicotianae* population, they reported that 48 h of constant exposure to 44°C was required to reduce the population to below the detection level (1 propagule/g of soil) but, with intermittent heat (5 h/day for 15 days), a total of 75 h of exposure to the same temperature was required. Likewise, only 4 h of constant heat at 47°C was required, as compared with 9 h of intermittent heat (3 days of 3 h/day). In contrast, there was no

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significant effect of temperature fluctuation on *A. cochlidioides* survival in water at 45°C (4 h/day) (Dyer et al. 2007). Intermittent heat did not appear to be less effective than continuous heat in a soil experiment with *Sclerotium oryzae* sclerotia but, rather, appeared to be a little more effective (Usmani and Ghaffar 1986). In our previous field trials, extended survival was observed for *P. ramorum* at the 5-cm depth in nonsolarized plots even though the cumulative hours exposed to high soil temperature should have been adequate to inactivate the pathogen (Funahashi and Parke 2016). Because this is likely due to the combined effects of soil moisture and intermittent heat, it is relevant to assess each effect separately under controlled lab conditions.

The objectives of this study were to (i) compare the effects of constant versus intermittent heat on survival of *P. ramorum* inoculum, (ii) determine whether exposure to sublethal temperatures affected subsequent inoculum survival time, and (iii) determine whether a model predicting pathogen recovery of *P. ramorum* and *P. pini* inoculum would be improved by taking intermittent versus constant heat into account. Although the main focus of this work was *P. ramorum*, we also included *P. pini* Leonian in this study. *P. pini*, previously known as *P. citricola* I (Hong et al. 2011), is a common plant-pathogenic *Phytophthora* sp. in nurseries (Hong et al. 2008; Olson et al. 2013; Parke et al. 2014). We wanted to know whether *P. pini* and *P. ramorum* respond similarly to a fluctuating temperature regime; if so, *P. pini* could serve as a surrogate for *P. ramorum* in future field trials outside quarantine facilities to validate our model. *P. pini* has been reported to tolerate higher temperatures than does *P. ramorum* (Jung and Burgess 2009; Werres et al. 2001).

MATERIALS AND METHODS

***Phytophthora* spp. inoculum.** *P. ramorum* Werres, de Cock & Man in't Veld isolate Pr-1418886 and *P. pini* Leonian isolate Pc98-517 were used to produce infested leaf disk inoculum. *P. ramorum* zoospores were produced according to an established method (Parke and Lewis 2007) with dilute (one-third strength) V8 agar substituted for V8-corn meal agar. *P. pini* zoospores were produced according to Ochiai et al. (2011). Similar-aged rhododendron cultivar Catawbiense Boursault leaves were collected from plants maintained in a greenhouse at Oregon State University. Leaves were dipped into a zoospore suspension (5×10^4 zoospores/ml) and incubated in a moist chamber at 20°C (*P. ramorum*) or 24°C (*P. pini*). After 2 weeks, 6-mm-diameter disks were removed from leaf lesions using a hole punch. The batch of leaf disks was thoroughly mixed. The presence of chlamydospores (*P. ramorum*) or oospores (*P. pini*) within leaf tissue was confirmed by microscopic observation of cleared leaves prior to use as inoculum (Phillips and Hayman 1970).

Experimental description. Five types of experiments were conducted. *P. ramorum* was tested in all experiments and *P. pini* was included in two experiments. Infested leaf disk inoculum containing chlamydospores (*P. ramorum*) or oospores (*P. pini*) was used because they closely resembled naturally occurring inoculum sources in nursery soil and they were the most heat-resistant propagules. Mesh bags (2 by 2 cm), each containing five leaf disks, were made of plastic window screen material. Five replicated mesh bags per sampling period for each treatment were prepared. Temperature was recorded every 30 min with button temperature dataloggers (model DS1921G; Maxim Integrated Products Inc., San Jose, CA).

To assay recovery of *Phytophthora* spp., infested leaf disks were removed from the mesh bags and plated on *Phytophthora*-selective medium (PARPH) (Jeffers and Martin 1986) with a modified concentration of antibiotics (ampicillin at 200 mg/liter, rifamycin at 10 mg/liter, pentachloronitrobenzene at 66.7 mg/liter, hymexazol at 25 mg/liter, and Delvocid at 20 mg/liter) (DSM, Delft, The Netherlands). Plates were incubated at 20.5°C and examined after 20 days for outgrowth of colonies of *P. ramorum* or *P. pini*.

Recovery was quantified as the frequency of leaf disks (out of five) with outgrowth into the medium, expressed as a percentage.

Inoculum survival at constant high temperature (first experiment). Survival of *P. ramorum* and *P. pini* inoculum was tested after exposure to three different temperatures (35.5, 38, or 50°C) in distilled water. Mesh bags with leaf disk inoculum were kept in distilled water at room temperature (20.5°C) for 12 h to equilibrate the water potential, then transferred to water-filled beakers in growth chambers maintained at each temperature. All beakers were kept in moist chambers to minimize evaporation. Mesh bags were removed every 12 h for 35.5 and 38°C and every 30 min for 50°C, and immediately placed in new water-filled beakers at room temperature to quickly cool. Recovery of the inoculum was determined as described below. The experiment was planned to continue until the inoculum was inactivated. The experiment was conducted twice.

Comparison of survival in intermittent versus constant heat regimes (second experiment). Recovery of *P. ramorum* inoculum was compared after exposure of the inoculum to constant heat or an intermittent heat regime consisting of an alternating cycle of high and low temperature. The total duration of exposure to high temperature required to inactivate the inoculum was compared between the constant and intermittent regime. Two trials were conducted (trial 1 and trial 2).

In trial 1, conducted in distilled water, survival of *P. ramorum* inoculum after exposure to a constant heat regime (36.5°C) was compared with survival in the intermittent heat regime (24 h of exposure to high temperature followed by 12 h at 20.5°C per cycle). For every cycle, mesh bags were placed into distilled water previously equilibrated to each temperature to allow for rapid temperature adjustment. In the constant heat regime, inoculum mesh bags were removed from the growth chamber every 12 h and placed in water at room temperature to quickly cool. In the intermittent heat regime, inoculum bags were removed after each heat event and immediately placed in room temperature water as well. Recovery of the inoculum was determined as previously described. The trial was conducted twice. In trial 2, conducted in moistened soil to better simulate field conditions, the survival of *P. ramorum* was tested after exposure to a constant heat regime (36.5°C) and an intermittent heat regime (8 h at 36.5°C and 16 h at 20.5°C per cycle). Soil was collected from the Oregon State University Botany Farm and Field Lab, Corvallis. The soil was air dried, sieved through 2-mm mesh, and moistened with distilled water to achieve a soil moisture condition typical of field soil during solarization (Funahashi and Parke 2016). Soil was packed in small, lidded sample containers (11.6 cm³; Aqua Lab, Decagon Devices, Pullman, WA) with a mesh bag containing inoculum of *P. ramorum*. Bulk density of the soil was 1.46 g/cm³ and the water potential was -72 kPa, calculated with a water retention curve of the soil. Volumetric soil water content was 20.0%. Samples were left at room temperature for 12 h, then moved into a growth chamber. In the intermittent heat regime, samples were removed from the growth chamber and immediately replaced to quickly change the temperature to which the inoculum was exposed. Samples were taken out of the growth chamber every 8 h in the constant heat regime or after each heat event in the intermittent heat treatment, and then placed in a cold room (4°C) to quickly cool until processing within a few days. Survival was determined after rinsing leaf disks free of soil, then plating them on PARPH, as described above. The trial was conducted once.

Inoculum damage from different intensities of sublethal heat (third experiment). Sublethal heat is defined here as heat that is of shorter duration or of lower temperature than lethal heat after which outgrowth from the inoculum does not occur. The intensity of sublethal heat was quantified as a percentage of the intensity of lethal heat. In a heat treatment with a single temperature, its heat intensity is defined as the percentage of the treatment duration out of the lethal duration. Thus, 100% heat intensity is the condition where inoculum loses its ability to grow out, and 50% heat intensity is half

of the lethal heat duration. Inoculum damaged by a sublethal heat treatment was expected to survive less time in the subsequent survival assay at a certain temperature than nondamaged inoculum. Inoculum damage was quantified as the percent reduction in survival time in the subsequent survival assay compared with the survival time of nondamaged inoculum.

This experiment was designed to evaluate *P. ramorum* inoculum damage resulting from different durations of constant sublethal heat (Fig. 1A); therefore, it was conducted in distilled water instead of soil to avoid any potential effects of antagonistic soil microorganisms. Two trials (trial 1 and trial 2) were conducted at 35°C, at which nondamaged inoculum was inactivated in approximately 84 to 96 h according to the results of our first and second experiment. In trial 1, inoculum was exposed to heat treatment of 0, 24, 36, or 48 h at 35°C, cooled to 20.5°C for 12 h, and then returned to 35°C. Samples were then taken every 12 h and recovery was assessed as previously described. In trial 2, inoculum was exposed to 0, 24, 48, or 72 h at 35°C but was otherwise the same as the first trial. Each trial was conducted once. Mesh bags were transferred between beakers of distilled water equilibrated to each temperature.

Damage from multiple sublethal heat events (fourth experiment). This experiment was designed to test whether the damage from a given intensity of sublethal heat was consistent at all stages of inactivation (Fig. 1B). Two trials (trial 1 and trial 2) were conducted, in which the inoculum was exposed to zero, one, two, or three cycles of sublethal heat treatment of 24 h followed by 12 h at 20.5°C, then returned to the heat to determine the survival length. In the first trial, the sublethal heat treatment was 36.5°C but, in the second trial, it was lowered to 35°C. Samples were taken every 12 h (five replications) and survival was assessed as described previously. Each trial was conducted once.

Field temperature regime simulation (fifth experiment). The last experiment was designed to test survival of *P. ramorum* and *P. pini* at a temperature regime that simulated field conditions during a 2013 solarization trial in San Rafael, CA (15-cm depth), where the inoculum survived longer than predicted. Mesh bags enclosing leaf disk inoculum were kept in distilled water in a growth chamber set to mimic the daily temperature regime in the field (Supplementary Fig. S1). Inoculum was removed every 24 h for 12 days and survival was assessed as described previously. Inoculum survival from this experiment was compared with the output from predictive models established below. The experiment was conducted twice.

Recovery curve fitting, model establishment, and the calculation of damage. All model fitting and statistical tests were conducted using R statistics software, version 3.1.2 (R Development Core Team 2014) at a $P < 0.05$ level of significance. Probit models were fitted to recovery data of each treatment by using equations 1 and 2:

$$\Phi(x) = \int_{-\infty}^x \frac{1}{\sqrt{2\pi}} \exp(-u^2/2) du \quad (1)$$

$$\Phi^{-1}(y) = \beta_1 x + \beta_2 \quad (2)$$

where $\Phi(x)$ is error function, $\Phi^{-1}(y)$ is its inverse function, β_1 and β_2 are coefficients, u is an integral real-variable, and x and y are “day” and “frequency of recovery”, respectively. The probit model was selected because it has been used to describe thermal inactivation in many previous studies (Thomas and Mangan 1997). The heat duration to achieve 0.1% recovery ($LD_{99.9}$) was calculated for the data from the first, second, and fifth experiments to establish a conservative predictive model of inoculum survival and to evaluate the other results by comparing them to the model output. $LD_{99.9}$ is likely to reflect model fitting errors with tail effects because $LD_{99.9}$ changes significantly with a small change in β_1 or β_2 in the probit model. This leads to relatively large standard errors in $LD_{99.9}$, masking the treatment effects. Therefore, regarding our

minimum experimental resolution of 1/25 (4%), the duration of exposure to heat to achieve 5% recovery (LD_{95}) was used for the remaining experiments to compare treatment effects more clearly without being interrupted by the relatively large standard errors.

The measured $LD_{99.9}$ for each inoculum type from the different temperature treatments was used to determine parameters in the thermal inactivation (death) curve (log time versus temperature) model below to estimate $LD_{99.9}$ in various temperatures for both inocula:

$$\ln(LD_{99.9}) = a + b \times \text{Inoculum} + c \times \text{Temp} + d \times \text{Inoculum} \times \text{Temp} \quad (3)$$

where $\ln$ indicates natural logarithm, a , b , c , and d are coefficients, Inoculum = 0 for *P. ramorum* and 1 for *P. pini*, and Temp is temperature (°C). $LD_{99.9}$ was used to establish a conservative predictive model of inoculum survival. The thermal inactivation

Fig. 1. Schematic diagram of the treatments in A, the sublethal heat length experiment and B, the multiple sublethal heat experiment to which *Phytophthora ramorum* inoculum was exposed. A, Inoculum was exposed to heat treatment of 0 (control), 24, 36, 48, or 72 h at 35°C, kept at 20.5°C for 12 h, and then tested for survival at 35°C to compare the effects of the different sublethal heat length treatments. The experiment was conducted twice, except for the 36- and 72-h heat treatment. B, Inoculum was exposed to 24-h heat treatment zero (control), one, two, or three times at 36.5°C, with 12 h at 20.5°C, and then tested for survival at 36.5°C to compare the effects of the different number of sublethal heat length treatments. The experiment was conducted twice, except that the temperature of the heat treatment and the measurement of survival in the second trial was 35°C. Inoculum recovery was measured every 12 h after each treatment (five replications) to determine survival in both experiments.

curve has been used in many previous studies (Pullman et al. 1981), and the full model was selected by model comparisons using analysis of variance tests in a general linear regression and evaluation through application of the Akaike information criterion (AIC) (Akaike 1973). AIC is a suitable model selection criterion for addressing which model will best predict the next sample (Aho et al. 2014). The AIC was calculated by:

$$AIC = n\ln RMSE + 2p \tag{4}$$

where n is the number samples, $\ln$ indicates natural logarithm, RMSE is root-mean-square error, and p the number of parameters used in the prediction. The model with the smallest AIC is best. Heat intensity rate (r_T) at temperature T was defined as:

$$r_T(\text{day}^{-1}) = 1/LD_{99.9} \tag{5}$$

A rate summation model was applied to calculate heat intensity for various temperature regimes:

$$\text{Heat intensity}(\%) = 100 \times \int r_T dt \tag{6}$$

where dt is a unit time.

For the data from the third and fourth experiments of sublethal heat, LD_{95} for each treatment was calculated by fitting a probit model. LD_{95} of the control treatment in each experiment ($LD_{95\text{-control}}$) was normalized to 100% of heat intensity, and LD_{95} of each treatment ($LD_{95\text{-treatment}}$) was compared with each $LD_{95\text{-control}}$ to calculate the damage as the percent reduction in LD_{95} by each treatment:

Fig. 2. Relationship between 99.9% lethal dose ($LD_{99.9}$) values and survival of *Phytophthora ramorum* (closed symbol) and *P. pini* (open symbol) after exposure to different temperatures (35.5, 38, and 50°C). Regression lines derived from the general linear model are shown for *P. ramorum* (dotted line) and *P. pini* (solid line). Bars represent standard deviation.

TABLE 1. Summary of the output in the general linear regression model, 99.9% lethal dose = Intercept + Inoculum + Temperature + Temperature × Inoculum^a

Parameter	Estimate	SE	<i>t</i> value	<i>P</i> value
Intercept	14.43	0.12	118.6	<0.001
Inoculum	−1.20	0.28	−4.3	0.051
Temperature	−0.37	0.00	−153.9	<0.001
Temperature × Inoculum	0.05	0.01	8.9	0.012

^a Data from two trials were pooled. The residual standard error (SE) was 0.191 with 2 degrees of freedom, and R^2 (coefficient of determination) was > 0.999. The significant *P* values are shown in bold.

$$\text{Damage}(\%) = (1 - LD_{95\text{-treatment}}/LD_{95\text{-control}}) \times 100 \tag{7}$$

Heat intensity (%) of each sublethal heat treatment was calculated by the duration of the treatment (L) divided by $LD_{95\text{-control}}$:

$$\text{Heat intensity}(\%) = (L/LD_{95\text{-treatment}}) \times 100 \tag{8}$$

A power function regression model was used to fit the relationship between the sublethal heat intensity and damage:

$$\text{Damage}(\%) = [\text{Heat intensity}(\%)]^a / 100^{a-1} \tag{9}$$

The coefficient a was statistically tested if it was significantly different from 1 by using nonlinear regression analysis. If coefficient a is 1, damage is equal to heat intensity. Thus, $a > 1$ shows a concave relationship and $a < 1$ shows a convex relationship between damage and heat intensity.

The damage from multiple sublethal heat events was calculated with equation 7, and linearity of the relationship between damage and repeated sublethal heat events was tested by regression model fitting.

RESULTS

Inoculum survival at constant high temperature (first experiment). Survival of *P. ramorum* and *P. pini* inoculum was tested after exposure to three different temperatures (35.5, 38, or 50°C) in distilled water. There was a significant negative relationship between increasing temperature and $LD_{99.9}$ values for both *P. ramorum* and *P. pini* (Fig. 2). Temperature coefficients in the model were −0.37 for *P. ramorum* and −0.32 for *P. pini*. *P. pini* survived significantly longer at any temperature treatment than did *P. ramorum* (Fig. 2). The general linear regression model was applied and the summary of the output is shown (Table 1). In the full model, all parameters were highly significant, except for the Inoculum parameter ($P = 0.051$). Because the *P* value is still relatively small and AIC of the full model (−29.3) was smaller than the reduced model without the Inoculum parameter (−17.4), the full model was selected as:

$$\ln(LD_{99.9}) = 14.43 + 1.20 \times \text{Inoculum} - 0.37 \times \text{Temp} + 0.05 \times \text{Inoculum} \times \text{Temp} \tag{10}$$

This model was used to calculate heat regimes in all other experiments.

Comparison of survival in intermittent versus constant heat regimes (second experiment). Recovery of *P. ramorum* inoculum was compared after exposure of the inoculum to constant heat or an intermittent heat. The regression coefficients with both intercept and slope in the fitted probit models were all significant in the experiment ($P < 0.05$). *P. ramorum* survived significantly longer in the intermittent heat regime ($LD_{99.9} = 113 \pm 19$ confidence interval [CI] h) than in the constant heat regime ($LD_{99.9} = 48 \pm 7$ CI h) in the trial with distilled water (Fig. 3). In the constant heat regime, calculated $LD_{99.9}$ was within the range of values predicted by equation 10 ($LD_{99.9} = 50$ h). Similarly, in the trial with moistened soil, *P. ramorum* survived longer in the intermittent heat regime ($LD_{99.9} = 96 \pm 10$ CI h) than in the constant heat regime ($LD_{99.9} = 58 \pm 7$ CI h) (Supplementary Fig. S2). The calculated $LD_{99.9}$ for the constant heat regime in soil did not significantly differ from the predicted value in equation 10 ($LD_{99.9} = 50$ h) based on the initial experiments conducted in distilled water.

Inoculum damage from different intensities of sublethal heat (third experiment). This experiment evaluated *P. ramorum* inoculum damage resulting from different durations of constant sublethal heat. The longer sublethal heat treatments (48 and 72 h of exposure) significantly shortened the subsequent *P. ramorum* inoculum survival but there was no difference in subsequent survival length between 24 h of sublethal heat treatment and the control

treatment (Supplementary Fig. S3). Only the result of the second trial was shown but the first trial resulted in a similar pattern. The inoculum damage and the intensity of each sublethal heat treatment were calculated in each trial separately. The relationship between inoculum damage and the intensity of sublethal heat was fitted with a power function nonlinear regression model:

$$\text{Damage (\%)} = [\text{Heat intensity (\%)}]^{2.37} / 100^{1.37} \quad (11)$$

The coefficient $a = 2.4$ was significantly larger than 1 ($P < 0.001$), showing that the regression line was significantly concave from the 1:1 line. The concave shape of the relationship showed that the inoculum damage in the beginning of a single heat event was relatively small but increased rapidly by prolonged exposure to heat (Fig. 4A).

Damage from multiple sublethal heat events (fourth experiment). This experiment tested whether the damage from a given intensity of sublethal heat was consistent at all stages of inactivation. Every sublethal heat event significantly shortened the survival of *P. ramorum* inoculum (Supplementary Fig. S4). Only the result of the first trial was shown but the second trial resulted in a similar pattern. The inoculum damage by each treatment was calculated for each trial separately. The heat intensity of a single sublethal heat event was 56.0% in the first trial and 26.8% in the second trial. For both trials, a linear relationship between inoculum damage and repeated sublethal heat events were observed with significant first-order regression models (Fig. 4B, trial 1, $P = 0.005$; trial 2, $P = 0.005$). Relationships were considered to be linear because the second-order regression models did not significantly differ from the first-order regression model for each trial (trial 1, $P = 0.179$; trial 2, $P = 0.391$). The linear relationship assumes that the damage caused by a given intensity of sublethal heat is consistent at any stage of inactivation. The damage caused by a single sublethal heat event approximated the mathematical prediction calculated by the damage equation (equation 11). In trial 1, 17.2 ± 4.6 CI% damage was caused by 56.0% of heat intensity as opposed to the mathematical prediction of 25.3% (approximately 20.7 to 30.9%), and 9.3 ± 2.4 CI% damage in trial 2 was caused by 26.8% of heat intensity as opposed to the mathematical prediction of 4.4% (approximately 2.8 to 7.0%). Therefore, the inoculum damage from each sublethal heat event was calculated by:

$$\text{Damage}_i (\%) = [\text{Heat intensity}_i (\%)]^{2.37} / 100^{1.37} \quad (12)$$

where Damage_i and Heat intensity_i are the damage and the heat intensity, respectively, of i th sublethal heat event. Total damage was calculated as cumulative damage of all sublethal heat events by:

$$\text{Total Damage (\%)} = \sum \text{Damage}_i \quad (13)$$

Field temperature regime simulation (fifth experiment).

The last experiment tested survival of *P. ramorum* and *P. pini* at a simulated field temperature regime to evaluate the model, including a correction for an intermittent heat regime. The recovery of both *P. ramorum* and *P. pini* subjected to the simulated field temperature regime indicated that *P. pini* survived significantly longer than *P. ramorum*, as expected (*P. ramorum*: $\text{LD}_{99.9} = 138 \pm 12$ CI h and *P. pini*: $\text{LD}_{99.9} = 292 \pm 18$ CI h) (Fig. 5A). The measured daily

Fig. 3. Frequency of recovery (percent) of *Phytophthora ramorum* inoculum in distilled water during exposure to 36.5°C of constant heat treatment (filled circle) compared with recovery in intermittent heat treatment (24 h of 36.5°C and 12 h of 20.5°C) (open circle). Bars represent standard error and regression lines by probit model are shown.

Fig. 4. Relationships between **A**, different sublethal heat intensity (percent) or **B**, the number of sublethal heat events and *Phytophthora ramorum* inoculum damage (percent). **A**, The power function nonlinear regression line is shown as solid line. Dotted line shows a 1:1 line. Residual standard error was 0.169, with 7 degrees of freedom. **B**, Results are shown for trial 1, with 56.0% heat intensity of a single sublethal heat event (black bar), and trial 2, with 26.8% heat intensity of a single sublethal heat event (white bar). The first-order regression lines are shown (trial 1 = solid line and trial 2 = dotted line). Heat intensity is defined as the percentage of the treatment duration out of the lethal duration. Bars represent standard deviation.

temperature regime was imported into the model using equations 10, 5, and 6 without a correction for the intermittent regime, and output of heat intensities were plotted with inoculum recovery for each sampling period (Fig. 5B). The heat intensity at LD_{99.9} was calculated to be significantly greater than 100% (*P. ramorum*: LD_{99.9} = 265.0 ± 25.3 CI% and *P. pini*: LD_{99.9} = 281.7 ± 18.0 CI%).

Fig. 5. A, Observed frequency of recovery of *Phytophthora ramorum* (closed circle) and *P. pini* (open circle) inoculum during a simulated field temperature regime. B, Relationship between observed frequency of recovery of each inoculum and heat intensity calculated from the model without the intermittent heat regime correction. C, Relationship between observed recovery of each inoculum and calculated total inoculum damage from the model with intermittent heat regime correction. Bars represent standard error and regression lines with probit model are shown.

To take the intermittent heat regime into account, the heat intensity of each day was recalculated with the damage equation (equation 12) to reflect the actual inoculum damage, and the total inoculum damage was plotted for each sampling period (Fig. 5C). The same equations were used for both *P. ramorum* and *P. pini*. The model prediction was significantly improved and calculated damage at LD_{99.9} was less than 100% for both *P. ramorum* (LD_{99.9} = 92.5 ± 9.6 CI%) and *P. pini* (LD_{99.9} = 38.2 ± 2.5 CI%), although it was overcorrected for *P. pini*.

DISCUSSION

Our experiments demonstrated significantly longer survival of infested leaf inoculum of *P. ramorum* subjected to the same total number of hours of high temperature in intermittent heat than in constant heat (second experiment). Similar trends were observed in soil and in water. Intermittent heat apparently causes less physiological damage to the inoculum than exposure to constant heat. Nonlinear damage accumulation was observed in continuous heat and multiple sublethal heat events, resulting in development of a more accurate mathematical model to predict thermal inactivation under field conditions for both *P. ramorum* and *P. pini* inoculum (third, fourth, and fifth experiments).

Probit models were successfully fitted to all recovery data in assays using infested leaf disk inoculum, with significant coefficients of slopes and intercepts. This shows that the survival period of leaf disk inoculum follows a normal distribution. Leaf disk inoculum contains both hyphae and survival structures such as chlamydospores (*P. ramorum*) and oospores (*P. pini*). A mechanism at the molecular level behind the death of the inoculum (such as the inactivation of certain vital enzymes or denaturing of DNA) may differ among cell types (Peleg and Cole 1998; van Boekel 2002). However, measured LD are likely to reflect the response of the most heat-resistant spores. The effect of temperature on inoculum survival was successfully expressed with the semilogarithm regression model (Table 1). This is consistent with many previous studies that described a linear relationship between logarithms of LD₉₀ (D value) and temperatures for *Verticillium dahliae*, *Pythium ultimum*, *Thielaviopsis basicola*, *Rhizoctonia solani* (Pullman et al. 1981), and *E. coli* (Fujikawa and Itoh 1998). The longer survival of *Phytophthora pini* as compared with *P. ramorum* is also consistent with previous studies (Jung and Burgess 2009; Werres et al. 2001).

Other researchers have reported that intermittent heat was less effective in killing plant pathogens than was constant heat. For example, sclerotia of *Sclerotinia sclerotiorum* (Porter 1991), and chlamydospores of *P. nicotianae* (Coelho et al. 2000) required longer exposure to intermittent heat than to constant heat to become inactivated. A similar difference in inoculum survival between intermittent and constant heat regimes was observed in soil for *P. ramorum* (second experiment). The water potential of the soil used in the experiment was typical of field conditions under solarization after initial irrigation (−72 kPa). Therefore, the observed difference between intermittent and constant heat regimes in laboratory conditions is also likely to be significant under field conditions. The nonlinear, concave increase of inoculum damage during continuous heat treatment (third experiment) indicates an increasing rate of inoculum damage accumulation. It is unknown whether the curves would be the same shape under all temperature and water potential conditions. Damage caused by multiple heat events appeared to be constant (fourth experiment), although it is difficult to conclude definitively because only three heat cycles were tested. However, this allowed stepwise calculation of damage accumulation through multiple sublethal heat events to better predict inoculum survival. The damage concept explains the extended inoculum survival because less damage actually occurs in each sublethal heat event than would be expected by heat intensity alone. A model including a correction for intermittent heat regime utilizing the damage equation significantly improved the prediction

of thermal inactivation under a simulated field temperature condition (fifth experiment).

The low temperature threshold that separates a heat regime into multiple sublethal heat regimes is unknown. Russell (2003) reviewed lethal effects of heat on the physiology and structure of bacterial vegetative cells and spores; proteins and enzymes are considered to be the major targets of heat inactivation for both types of cells. There is a numerical correlation between the thermodynamic parameters of protein denaturation and the observed death rates of various organisms. The low temperature threshold and the increasing inoculum damage rate during continuous heat treatment probably reflect the kinetics of these inactivation mechanisms. It is also unknown how different low-temperature regimes cause different intermittent effects on pathogen survival. Longer periods of low temperature might trigger chlamydospore or oospore germination (Tooley and Carras 2011) and may affect thermotolerance through a different mechanism. Recovery under simulated field conditions confirmed that an intermittent heat regime correction with the damage equation is important (fifth experiment). The model without the intermittent heat regime correction predicted that 52 h of the simulated regime was enough to inactivate *P. ramorum* inoculum even though it actually required 138 h. Likewise, the model without an intermittent heat regime correction predicted that 104 h of the simulated regime was enough to inactivate *P. pini* inoculum even though it actually required 292 h. The model including the intermittent heat regime correction significantly improved the prediction for *P. ramorum* but somewhat overcorrected the damage to *P. pini*, possibly indicating a different response to intermittent heat than for *P. ramorum*. It is unclear whether different amounts of damage affect pathogenicity of surviving inoculum. Because delayed mortality (Assaraf et al. 2002) and decreased germination and pathogenicity (Arora et al. 1996) were reported for *Fusarium oxysporum* induced by the sublethal heat treatment, further study is necessary.

It is beneficial to use intermittent heat in controlled studies of thermal inactivation to better estimate conditions necessary to control plant pathogens by soil solarization (Coelho et al. 2000). However, it is difficult to employ cycling temperature treatments due to all the various possible temperature regimes, with different combinations of high and low temperature and different durations of each temperature. If the same structure of the damage equation (equation 9) could be used for different organisms, it would be easier to establish an accurate predictive model for each organism. Because the damage equation has only one coefficient to determine, technically, one test regime would be enough for this purpose.

In conclusion, this study demonstrated that intermittent heat is less damaging to inoculum of two *Phytophthora* spp. than is constant heat. The damage rate during continuous heat is not constant but rapidly increases. Therefore, corrections to the model are required for every sublethal heat event to accurately predict pathogen inactivation in fluctuating regimes. The same damage equation can be applied to each sublethal heat event because the achieved damage rate appeared to be similar at all stages of inactivation. However, more detailed studies are necessary to conclusively demonstrate that the rate of damage accumulation is constant over multiple heat events.

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