Survival of *Phytophthora ramorum* hyphae after exposure to temperature extremes and various humidities

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Abstract: We examined the effect of short-term exposure to high and low temperatures and a range of relative humidity (RH) on survival of *Phytophthora ramorum* hyphae. Spore-free hyphal colonies were grown on dialysis squares atop V8 medium. Colonies were transferred to water agar plates positioned at 27.5–50 °C on a thermal gradient plate and incubated 2.5–480 min. For low temperature trials colonies were transferred to vials of distilled water and incubated in a water bath at −5 to −25 °C for 1–24 h. In the relative humidity trials hyphal colonies were transferred to sealed humidity chambers containing various concentrations of glycerin for 1–8 h. Relative humidity was 41–93% at 20 °C and 43–86% at 28 °C. Survival in all trials was characterized by growth from dialysis squares into V8 medium. Temperatures of 37.5–40 °C were lethal to *P. ramorum* hyphae within several hours, and temperatures of 42.5–50 °C were lethal within minutes. Exposure to 32.5 and 35 °C resulted in reduced survival over 8 h, while 30 °C had no effect on three of four isolates. Hyphal colonies demonstrated considerable tolerance to cold, with all isolates surviving a 24 h exposure to −5 °C. Survival diminished over time at lower temperatures, however a few colonies survived 24 h exposure to −25 °C. Temperature also affected the ability of hyphal colonies to withstand reduced humidity. A RH of 41–43% was lethal in 2 h at 28 °C compared to 8 h at 20 °C. Three of four isolates were unaffected by an 8 h exposure to 81 and 95% RH at 20 °C, and 73 and 86% RH at 28 °C. Isolate differences were apparent in tolerance to freezing temperatures and reduced humidity. From these results it is apparent that the cold temperatures found in the northeastern USA are not likely to prevent the establishment of *P. ramorum*. There is also the potential for hyphae, and presumably spores, to survive periods of high humidity on the leaf surface in the absence of free water.

Key words: mycelium, sudden oak death, vegetative

INTRODUCTION

*Phytophthora ramorum* Werres, DeCock & M in’t Veld, causal agent of sudden oak death, poses a serious risk to hardwood forests on the west coast of the USA (Rizzo et al 2005). This pathogen has a broad host range, including many commonly grown nursery plants (USDA-APHIS 2007). New hosts frequently are identified and the full impact of this disease has yet to be realized. A climatological map, including host plant distribution, was constructed in an effort to predict the potential for establishment of *P. ramorum* throughout the USA (Venette and Cohen 2006, Magarey and Fowler 2007). Temperature limits for growth and sporulation have been determined (Werres et al 2001, Englander et al 2006), and efforts are under way to determine the ability of *P. ramorum* to survive the temperature and moisture extremes found in summer and winter in many areas of the country. Chlamydospores, thick-walled asexual spores, have been observed in/on leaves (Tooley et al 2004, Davidson et al 2005), twigs and stems (Pogoda and Werres 2004, Lewis and Parke 2006, Parke et al 2007) and fruit (Moralejo et al 2006). Chlamydospores in potting medium, sand and soil have proven to be long-lived at moderate temperatures (Shishkoff 2007, Colburn et al 2005, Fichtner et al 2007, Linderman and Davis 2006). At extreme temperatures free chlamydospores survived a 7 d exposure to 30 °C and 0 °C, with diminished survival at temperatures above and below, respectively. Enhanced survival of *P. ramorum* in plant tissue was observed with cold temperatures only (Tooley et al 2008). Chlamydospore production however can be variable depending on host species (Tooley et al 2004). There also have been reports of a reluctance of chlamydospores to germinate (Fichtner et al 2005, Smith and Hansen 2007), so their role in nature as survival structures is not clearly understood.

While survival of *Phytophthora* species during temperature and/or moisture stress usually relies on resistant spores, there are documented cases of long-term survival by hyphae, most notably with *P. infestans* (Zwankhuizen et al 1998). *P. fragariae* mycelia are
capable of surviving extended periods in soil, although oospores are likely the primary over-wintering structure (Duncan 1980). With some Phytophthora species, the production of resistant oospores is limited by geographical distribution due to the absence of a compatible mating type, as with P. infestans (Crosier 1934). This is presently the case with P. ramorum; one mating type is found in Europe (A1) and the other is found in North America (A2), although the A1 mating type was identified at an Oregon nursery (Hansen et al 2003). The likelihood of the production of oospores in nature should the opposite mating type be introduced is unknown, as is their potential role in survival. In the absence of resistant spores, the ability of hyphae to survive temperature and moisture extremes takes on greater significance. Moralejo et al (2006) describe the production of P. ramorum hyphal aggregates produced under the epidermis on several Mediterranean plants. While it is unknown whether the formation of stroma is a common occurrence in the disease cycle of P. ramorum, these hyphal aggregates have much greater potential for long-term survival than do individual hyphae in plant tissue.

In the short term the higher temperatures and drier conditions experienced on a diurnal cycle may limit the pathogen’s ability to remain active. Atmospheric humidity is usually at its lowest when temperatures are at their warmest. However a microclimate exists at the leaf surface where temperatures are often below ambient and relative humidity (RH) above atmospheric (Gates 1968). With evening comes cooler temperatures and saturation of the atmosphere resulting in dew formation on leaf surfaces. If hyphae and spores present on the leaf surface are not capable of surviving daytime temperatures and humidity, then successful disease establishment rests with an ability to sporulate, germinate and penetrate quickly in the presence of dew. Otherwise infection will occur only during periods of extended moisture at conducive temperatures. Formation of sporangia is dependent on 6–8 h of favorable conditions for P. infestans (Crosier 1934), 24 h for P. cactorum and 2–3 h for P. colocasiae (Trujillo 1965). Davidson et al (2005) reports the production of P. ramorum sporangia within 48 h of flooding infected leaves. P. infestans and P. citrophthora can penetrate plant tissues in 2 h (Pristou and Gallegly 1954, Gerlach et al 1976). Three hours of moisture is sufficient for infection by P. palmivora (Timmer et al 2000) and 3–7 h for P. cactorum (Grove et al 1985). Experiments in our lab with detached leaves and intact rhododendron plants have shown that 8 h of moisture may result in infection of 30% of leaves on intact plants and that some disease may occur with a moisture period of as little as 1 h for intact plants and 2 h for detached leaves (Tooley et al 2005). The ability of hyphae to survive daytime conditions on the leaf surface, more likely to occur deep within the plant canopy where humidity is highest, would mean that when the temperature cools and atmospheric humidity rises, sporangia production could recommence quickly for maximum efficiency. Yet, in spite of the potentially significant role hyphae may play in the epidemiology of the disease on a wide range of host species, the conditions under which P. ramorum hyphae may survive have not been explored.

Therefore our first objective was to identify the temperatures (high and low) and corresponding exposure periods limiting survival of spore-free hyphal colonies of P. ramorum. Our second objective was to evaluate survival of hyphae at various constant atmospheric humidities. In addition we evaluated survival of hyphae exposed to a 24 h diurnal cycle of fluctuating temperatures and humidity.

**MATERIALS AND METHODS**

Four North American isolates of P. ramorum were used in these studies: Pr5, Pr6, Pr52 and 73101, as described in Englander et al (2006). P. ramorum was cultured on 10% V8 medium (1.7% agar or broth) containing 0.3 g CaCO₃ and 0.02 g β-sitosterol per liter and incubated at 20–22 C.

The effect of high temperature on survival.—P. ramorum hyphal colonies, growing on dialysis membrane squares, were exposed briefly to high temperatures and survival was assessed. Two thermal gradient plates, consisting of long rectangular aluminum plates with a cold bath at one end and heating bath at the other, were used. Petri dishes (60 × 15 mm), containing 10 mL 1.5% water agar (WA), were arranged on gradient plates. Agar temperature was allowed to equilibrate for 1 h after which agar surface temperatures were measured with a microsurface thermistor sensor probe without lifting the lid of the Petri dish. Gradient plate temperatures corresponding to agar surface temperatures of 27.5–50 C, in increments of 2.5 C, were identified.

Spectra/Por cellulose dialysis membrane tubing (12 000–14 000 MWCO; Spectrum Labs, Rancho Dominguez, California) was moistened, cut into single-layer 1.5 cm squares and autoclaved in distilled water. Ten squares were arranged on 10% V8 agar in each 100 × 15 mm Petri dish and each square was inoculated with 20 µL of a spore-free hyphal suspension prepared by homogenizing mycelia from four prespore forming V8 broth cultures (ca. 7 d old) in 30 mL sterile distilled water for 30 s at one-third speed in a Waring blender. Dialysis square colonies were incubated 3–4 d before use. The absence of spores in broth cultures and dialysis square colonies was confirmed microscopically before use.

WA dishes were positioned on the thermal gradient plates at 27.5–50 C, in increments of 2.5 C and allowed to
equilibrate 1 h. Two cultures growing on dialysis squares were transferred to each of three dishes per isolate/temperature/exposure period. After 2.5, 5, 7.5, 10, 12.5, 15, 30, 45, 60, 120, 240, 360, and 480 min incubation, the dialysis square colonies were transferred to V8 agar. Petri dishes were examined periodically for 14 d for growth of hyphae from dialysis squares into the agar. Survival was characterized as growth or no growth. Two isolates were tested simultaneously and experiments were performed three times.

The effect of low temperature on survival.—A LAUDA Brinkman ECO-line RE106 water bath, filled with 50% ethylene glycol, was used to expose hyphal colonies to low temperatures. A wire basket, capable of holding 12 glass vials (27.25 × 57 mm), was constructed to fit into the bath. Hyphal colonies were produced on dialysis squares as described previously. Glass vials, each containing 20 mL sterile distilled water and nine dialysis square colonies, were incubated overnight at 4 C and transferred to the bath for 1, 2, and 4 h followed by another series of tests at 8, 12, and 24 h. Colonies of all four isolates were incubated simultaneously, 1 vial per time period. Temperatures of –5, –10, –15, and –20 C were tested consecutively. It was determined with a submersible thermometer that vial contents equilibrated with water bath temperatures within 30 min of immersion; vial contents thawed within 2 h of their removal from the water bath. After incubation vials were removed from the bath and held at room temperature to thaw. Dialysis square colonies then were transferred to V8 agar. Survival, characterized as growth from dialysis squares into the medium, was monitored 14 d. Experiments were performed three times.

The effect of relative humidity on survival.—Hyphal colonies grown on dialysis squares (as described previously) were exposed to relative humidity resulting from 0, 20, 60 and 80% glycerin in a closed system as follows. A sterile 100 mm Petri dish lid was inverted inside a 1500 mm dish and filled with 20 mL glycerin solution. A sterile 13 cm nylon mesh circle was placed atop the dish. Two dialysis square colonies from each of four isolates were arranged on the mesh circle above the glycerin solution. The lid of the enclosing dish was rimmed with stopcock grease and set in place. Colonies were incubated at 20 and 28 C for 1, 2, 4 and 8 h. Three sets of dishes were assembled for each concentration and exposure period for a total of 48 dishes/temperature. A fourth set of dishes also was assembled, and a hobo logger with thermal and hygrometric capabilities (Onset Corp., Pocasset, Massachusetts) was attached to the outside of each dish with grease, aligning the sensing vent with a corresponding hole which had been cut in the lid. Temperature and relative humidity within the sealed chamber were monitored continuously. After incubation, dishes were opened. Dialysis square colonies were dipped in sterile water to rehydrate and then transferred to V8 agar. Survival, characterized as growth from dialysis squares into agar medium, was monitored 14 d. Experiments were performed two times.

Hyphal colonies growing on dialysis squares also were exposed to fluctuating temperature and relative humidity for 24 h, designed to simulate cool, wet conditions found overnight and in the early morning followed by warmer, drier conditions in the afternoon. Incubator temperatures (14-28 C) were adjusted throughout the experiment. Target humidity was 60-90% and was achieved by transferring hyphal cultures among humidity chambers containing different concentrations of glycerin. Twenty humidity chambers were assembled with Petri dishes as described previously and filled with 20, 40 or 60% glycerin, or distilled water. Two colonies from each of four isolates were added to the first set of five humidity chambers to be employed. Hyphal colonies were exposed to varying concentrations of glycerin through transfer among humidity chambers six times during the 24 h experiment. Another set of humidity chambers was used to monitor changes in temperature and humidity with a hobo data logger (Onset Corp., Pocasset, Massachusetts) attached to the lid, as described previously. At the end of the experiment, dialysis squares containing colonies were removed from the humidity chambers, dipped in sterile distilled water to rehydrate and plated on V8 agar. Survival, characterized as growth from dialysis squares into the agar medium, was monitored 14 d. This experiment was performed twice.

Statistics.—Data were analyzed by logistic regression modeling the odds of growth of individual hyphal colonies. The analyses were conducted with PROC LOGISTIC of the SAS statistical package (SAS Institute 2004) with logit values as the dependent variable.

For high and low temperature studies, logistic regressions first used experimental runs and isolates as class variables, and temperature within isolate and exposure period (time) within isolate as covariates. Single-degree-of-freedom contrasts were written to compare intercepts and slopes (based on logit values) among isolates and generate Wald Chi-square values and tests of significance for each comparison. Data from the relative humidity studies were analyzed similarly. Intercepts presented for all regression analyses were beyond the range of the data (0 C and 0 h exposure) and are presented to allow for reconstruction of the complete regression lines.

RESULTS

The effect of high temperatures on survival.—Survival was markedly reduced at 37.5–50 C compared to 27.5–35 C (Fig. 1). A 240–480 min exposure to 35 C was required to observe diminished survival. Temperatures exhibiting the most variable response were 37.5 and 40 C with cooler temperatures having little effect during the period tested and warmer temperatures exhibiting a detrimental effect in a matter of minutes. Lethal exposure periods were 240 min at 37.5 C, 120 min at 40 C, 15–30 min at 42.5 C, and 2.5 min at temperatures of 47.5 and 50 C. High temperature (27.5–50 C) and time of exposure were highly significant (probability of a greater Chi-square ≤ 0.01) in terms of the probability of hyphal growth (logit values) for each of the four isolates of P.
BROWNING ET AL: HYphaE SURVIVAL

100
80
60
40
20
0

-120
120
240
360
480

Exposure period (Minutes)

Fig. 1. Survival of *P. ramorum* hyphae from four North American isolates after exposure to high temperatures on water agar plates positioned on a thermal gradient plate. Three experiments were performed using six colonies each.

*P. ramorum* tested (Table I). Slopes for temperature were all negative and significantly different than zero for all isolates, indicating that probability of hyphal growth, for all isolates, significantly decreased as temperatures increased from 27.5 to 50 C. Slopes for time of exposure were all positive and significantly different than zero for all isolates indicating that, despite the negative effect of increasing temperature, the probability of hyphal growth increased with increasing exposure time (i.e. hyphal growth may have adjusted to high temperatures over time). However the positive effect of increased exposure time was minimal in comparison to the negative effect of increasing temperature (see differences in magnitude of slope estimates for temperature and exposure time Table I). There were significant differences among isolates for the intercepts and slopes of both temperature and time of exposure (Table I). Although all isolates were affected in the same manner by increasing temperature or exposure time, they were not all affected to the same extent (as indicated by slope differences).

The effect of low temperatures on survival.—*P. ramorum* hyphae exhibited considerable resistance to short-term cold temperature injury. A 24 h exposure to −5 C had no impact, while survival was diminished at all other temperatures tested (Fig. 2). However growth of colonies was observed by two isolates (Pr52, Pr6) after a 24 h exposure to −25 C. Low temperature (−25 to 5 C) and length of exposure had significant effects on the probability of hyphal growth (logit values) for each of the four isolates of *P. ramorum* tested (Table II). Slopes for temperature were all positive and significantly different from zero for all isolates, indicating significantly increased probability of hyphal growth with increasing temperature (from −25 to −5 C). Slopes for exposure period were all negative and significantly different from zero for all isolates, indicating that despite positive response to increasing temperature longer exposure at low temperatures led to decreased probability of hyphal growth (i.e. hyphal growth did not seem to adjust to prolonged exposure to low temperature). However the negative effect of increased exposure time was minimal in comparison to the positive effect of increasing temperature (see differences in magnitude of slope estimates for temperature and exposure time in Table II). There were no significant differences among isolates for slopes attributable to exposure period. There were significant differences among isolates for the intercepts and slopes for temperature and for intercepts for time of exposure. Probabilities for hyphal growth

| Table I. Effects of temperatures 27.5–50 C and time on probability of hyphal growth (logit values) of four different isolates of *Phytophthora ramorum* |
|---|---|---|
| **Isolate** | **Temperature (C)** | **Time (min)** |
| | **Intercept** | **Slope** | **Intercept** | **Slope** |
| Pr5 | 27.568 a² | −0.7052 a² | −0.334 a² | 0.0026 ac² |
| Pr6 | 19.148 b | −0.4825 b | −0.194 a | 0.0019 c |
| Pr52 | 34.449 c | −0.9658 c | −0.677 b | 0.0039 b |
| 73101 | 31.815 ac | −0.8349 ac | −0.609 b | 0.0033 ab |

¹The intercept term represents the logit value at zero for the respective variable.

²Means followed by the same letter do not differ significantly (*P* ≤ 0.05) according to Wald Chi-square tests.
corresponding to logit values at 0°C (intercept), ranged from 0.97 for isolate Pr5 to 0.93 for isolate Pr52.

*The effect of relative humidity on survival.*—Humidities equilibrated within 20 min of sealing the chambers. Relative humidity resulting from distilled water with 0, 20, 60 and 80% glycerin were respectively 93, 81, 62 and 41% at 20°C, and 86, 73, 58 and 43%, respectively, at 28°C. Three of the four isolates tested were unaffected by an 8 h exposure to relative humidities of 81 and 93% at 20°C, and 78 and 86% at 28°C. Isolate Pr5 however exhibited reduced survival within 2–4 h (Fig. 3). A 2 h exposure to 43% humidity at 28°C was lethal to all isolates. At 20°C, an 8 h exposure to 41% RH was necessary to kill hyphae from all isolates. There were no significant effects due to experimental runs. The probability of hyphal growth at 20°C (0.60) was significantly greater than the probability of hyphal growth at 28°C (0.54), and thus data collected at the two temperatures were analyzed separately (TABLE III). Slopes for glycerin concentration and time of exposure were all negative and significantly different than zero for all isolates at both temperatures, indicating that probability of hyphal growth of all isolates significantly decreased with increasing glycerin concentration and time of exposure. There were significant differences in slopes (which represent the probability of hyphal growth) among isolates in response to increasing glycerin concentration and time of exposure at both temperatures (TABLE III).

Hyphal colonies also were exposed to a 24 h diurnal regimen of temperature/humidity. Recorded temperature and relative humidity values are provided (Fig. 4). The highest temperature achieved was 27.5°C with a corresponding RH of 63.8%. The lowest temperature recorded was 14.9°C with a corresponding RH of 92.2%. One hundred percent survival resulted from exposure to these temperature/RH regimens in both experiments.

**DISCUSSION**

We report here temperatures of 37.5–40°C to be lethal to *P. ramorum* hyphae within several hours and 42.5–50°C to be lethal within a matter of minutes. Diminished survival of hyphal colonies was observed after 4–8 h at 35°C. Exposure of hyphae to 30°C and 32.5°C for 8 h had no effect on survival of three of

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Temperature (C)</th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intercept¹</td>
<td>Slope</td>
</tr>
<tr>
<td>Pr5</td>
<td>3.838 a²</td>
<td>0.3210 a²</td>
</tr>
<tr>
<td>Pr6</td>
<td>3.097 b</td>
<td>0.2065 b</td>
</tr>
<tr>
<td>Pr52</td>
<td>2.640 b</td>
<td>0.1592 c</td>
</tr>
<tr>
<td>73101</td>
<td>2.664 b</td>
<td>0.1968 b</td>
</tr>
</tbody>
</table>

¹The intercept term represents the logit value at zero for the respective variable.
²Means followed by the same letter do not differ significantly ($P \leq 0.05$) according to Wald Chi-square tests.
Survival of *P. ramorum* hyphae from four North American isolates incubated inhumidity chambers (filled with 0, 20, 60, 80% glycerin) at 20 and 28 °C in two experiments using six colonies each.

Four isolates while an exposure of 8 h at 32.5 °C reduced the survival of isolate Pr6. In contrast, chlamydospores of *P. ramorum* exhibited declining survival rates at 35 °C in a week, requiring 7 d exposure to be lethal at 35 °C and 1–2 d at 40 °C (Tooley et al. 2008). Swain et al. (2006) report similar results with cultures of *P. ramorum* killed by 24 h exposure at 40 °C, 2 h at 45 °C and 1 h at 55 °C. Comparing these results it appears that chlamydospores are more resistant to the detrimental effects of high temperature than are hyphae.

Examining results with other *Phytophthora* species, *P. cinnamomi* hyphae were killed within 1–2 h at 38 °C and chlamydospores were killed within 1–2 h at 40 °C (Gallo 2007). Nesbitt et al. (1979) report lysis of *P. cinnamomi* hyphae in both sterile and nonsterile soil within several days at 36 °C. *P. capsici* hyphae were killed by 30 min exposure to 42.5–45 °C (Bollen 1985, Coelho et al. 2001), while 6 h exposure at 45 °C was necessary to kill hyphae and sporangia of *P. cambivora* (Wicks 1988). Extreme temperatures such as these will not be encountered in nature, but they do apply to composting, which has been explored as a procedure for inactivating *P. ramorum* propagules present in municipal greenwaste and soil (Garbelotto 2003, Swain et al. 2006).

Survival of extreme temperatures likely will occur in colonized plant tissues—attached leaves and stems and decomposing leaves in contact with soil. When *P. ramorum* was present in infected rhododendron leaves, in the form of chlamydospores and perhaps hyphae, survival at 35 °C declined within 2 d, with no

**TABLE III.** Effects of four relative humidity levels, two temperatures and time on probability of hyphal growth (logit values) of four different isolates of *Phytophthora ramorum*.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Glycerin concentration</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 °C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intercept¹</td>
<td>Slope</td>
</tr>
<tr>
<td>Pr5</td>
<td>2.286 a²</td>
<td>-0.047 a²</td>
</tr>
<tr>
<td>Pr6</td>
<td>5.814 b</td>
<td>-0.105 b</td>
</tr>
<tr>
<td>Pr52</td>
<td>9.229 b</td>
<td>-0.156 b</td>
</tr>
<tr>
<td>73101</td>
<td>7.201 b</td>
<td>-0.124 b</td>
</tr>
</tbody>
</table>

¹ The intercept term represents the logit value at zero for the respective variable.
² Means followed by the same letter do not differ significantly (*P* ≤ 0.05) according to Wald Chi-square tests.

**FIG. 4.** Recorded temperatures and relative humidity that *P. ramorum* hyphal colonies were exposed to for 24 h in a diurnal regimen of temperature/humidity. Two experiments were performed using 10 colonies from each of four North American isolates.

*P. cinnamomi* hyphae were killed within 1–2 h at 38 °C and chlamydospores were killed within 1–2 h at 40 °C (Gallo 2007). Nesbitt et al. (1979) report lysis of *P. cinnamomi* hyphae in both sterile and nonsterile soil within several days at 36 °C. *P. capsici* hyphae were killed by 30 min exposure to 42.5–45 °C (Bollen 1985, Coelho et al. 2001), while 6 h exposure at 45 °C was necessary to kill hyphae and sporangia of *P. cambivora* (Wicks 1988). Extreme temperatures such as these will not be encountered in nature, but they do apply to composting, which has been explored as a procedure for inactivating *P. ramorum* propagules present in municipal greenwaste and soil (Garbelotto 2003, Swain et al. 2006).

Survival of extreme temperatures likely will occur in colonized plant tissues—attached leaves and stems and decomposing leaves in contact with soil. When *P. ramorum* was present in infected rhododendron leaves, in the form of chlamydospores and perhaps hyphae, survival at 35 °C declined within 2 d, with no
survival observed by 4 d (Tooley et al 2008). Exposure to 40 C for 2 d also was found to be lethal. In this instance enclosure in plant tissue did not appear to provide protection from the detrimental effects of high temperature as might be expected (Garbelotto 2003). However recovery of P. ramorum from infected rhododendron leaf disks held in soil and exposed to diurnal temperatures reaching 33 C was near 100% for 12 wk (Tooley et al 2008). The temperature regime in that study was selected to represent summer temperatures in southeastern USA. The effect of reduced moisture in combination with high temperatures was not studied here. It is possible that exposure to 30–32.5 C, temperatures just outside the range supporting growth of P. ramorum (Werres et al 2001, Englander et al 2006), in conjunction with reduced moisture could prove to be injurious to hyphae. Recovery rates of P. ramorum from infected, attached California bay laurel leaves as well as those in leaf litter have been found to decline during the summer when conditions are warm and dry (Davidson et al 2002, Fichtner et al 2007). Linderman and Davis (2006) reported the sensitivity of P. ramorum to desiccation with no recovery from air-dried, colonized rhododendron leaves, a treatment that two other Phytophthora species tolerated.

In our studies P. ramorum hyphal colonies exhibited considerable tolerance to freezing temperatures of short duration. Exposure to −5 C for 24 h had no impact, but survival was diminished at −10 C and below over 24 h. However there was some survival by two of four isolates after 24 h exposure to −25 C. Tooley et al (2008) report similar results with chlamydospores, with little to no survival observed in 7 d at −10 and −20 C. When present in infected leaf disks however P. ramorum exhibited near 100% survival after 1 wk at −10 C. In this case it appears that enclosure in leaf tissue did afford the fungus some protection from extreme cold. Based on the similarity in cold tolerance of hyphae and chlamydospores, survival of P. ramorum in plant tissue in the cold may be as likely to occur in the form of hyphae as in chlamydospores. Survival of hyphae would be enhanced by the presence of hyphal aggregates as observed by Moralejo et al (2006). In addition, because the production of chlamydospores appears to be host-dependent (Tooley et al 2004), survival of hyphae would serve to increase the reservoir of hosts for over-wintering.

Much information is available on cold tolerance of other Phytophthora species due to concerns of over-wintering potential. Benson (1982) reported that chlamydospores of P. cinnamomi had no greater tolerance for cold than did hyphae. Spore-free colonies were killed by exposures of 2, 6 and 16 d at −6.7, −3.8 and −1.4 C, respectively, while chlamydospores in soil were killed in 2, 17 and 29 d at −6.4, −3.4 and −0.5 C, respectively. P. infestans is known to survive as hyphae in potato tubers. Temperatures of −20 and −10 C were lethal to P. infestans cultures within 1 h and −5 C within a day, with reduced survival over 5 d at −3 C (Kirk 2003). De Bruyn (1926) reported a much greater cold tolerance, with cultures of P. infestans surviving up to 10 d at −17 C and up to 5 d at −25 C. Gerlach et al (1976) suggested that P. citrophthora may over-winter as mycelia because regrowth in Pieris japonica leaves after exposure to cold most often originated with hyphae in vascular tissue. Cultures of P. citrophthora survived 2 d at −21 C. Preincubation at 4 C was found to enhance the survival of P. citrophthora at freezing temperatures (Gerlach et al 1976) but had no effect on the survival of P. cinnamomi (Benson 1982) and P. parastica (Kuske and Benson 1983).

We also examined the ability of P. ramorum hyphae to survive reduced humidity. At 20 C, an 8 h exposure at 43% RH was required to kill hyphal colonies of all isolates. At 28 C, the lethal exposure was reduced to 2 h. Also exposure to a diurnal cycle of temperature and humidity ranging from 27.5 C and 63.8% RH to 14.9 C and 92.2% RH had no deleterious effect on survival of hyphal colonies. Differences in isolate sensitivity to reduced humidity were apparent, with Pr5 showing diminished survival over 2–8 h exposure to atmospheres of 81 and 73% at 20 and 28 C, respectively. Hyphae from the other three isolates were unaffected by 8 h exposure. Davidson et al (2002) reported that P. ramorum zoospores and chlamydospores were killed by a 30 min exposure to 30% RH while both survived a month on moistened filter paper. This is an extremely low humidity that may be more representative of conditions found in an abscissed leaf. While it is difficult to predict the temperature and relative humidity present in the microclimate at the leaf surface, these results indicate that there is potential for survival of hyphae, and likely spores, during the daytime hours in the absence of free water. Growth and sporulation then can resume in the evening when temperatures are cooler and dew is present on leaf surfaces.

A microclimate exists at the leaf surface where temperatures are often below ambient and relative humidity above atmospheric (Gates 1968). Leaves moderate surface temperatures through size, shape and orientation. Transpiration and evaporation also
serve to cool the leaf and raise the relative humidity (Vogel 1970). During a dry, hot period the measured RH in a wheat field did not fall below 40%. Irrigated turf maintained a humidity of 70% while atmospheric humidity was up to 40% (Burrage 1971). Atmospheric humidity is usually lowest when temperatures are at their highest. As evening approaches temperatures fall and the atmosphere approaches saturation with dew condensing on leaf surfaces. So fungi present on a leaf surface must be able to withstand the declining relative humidity found on a sunny, summer day until they can resume growth and sporulation when more favorable conditions return in the evening.

Diem (1971) compared the resistance of germ tubes to relative humidity of 40–95% at 20°C. Hyaline fungi were more sensitive than were dematiaceous. For example Aspergillus species were killed by 4 h exposure to 85% RH, and Colletotrichum graminicola and Penicillium sp. suffered some detriment after 8 h. In contrast two species of Cladosporium, which dominate in the phylloplane, showed good resistance to reduced humidity, with minimal damage resulting from exposure to 65% RH. In some fungi survival was due to regermination by a multicelled spore or from a newly formed branch on the germ tube. In a study comparing phylloplane fungi and soil fungi, Park (1982) demonstrated experimentally that it is the ability to withstand dry atmospheres that lets phylloplane fungi survive on leaf surfaces. When soil fungi did survive exposure to low humidity, regrowth originated at the center of the colony or from a resting spore and was delayed. In contrast phylloplane fungi resumed growth from hyphal apices with no delay when favorable conditions returned. We should note that the uniform survival of P. ramorum hyphal colonies at 73% RH reported here does not imply that there was no injury to hyphae, especially at the periphery of colonies. It is possible that regrowth after treatment originated at the center of the colony where mycelium concentration was densest.

Alternating periods of wetting and drying may be more deleterious to survival than extended periods of drought. Pseudomonas destructor spores were able to survive sunny and warm conditions but not alternating wet and dry conditions, especially when dew deposition was low (Hildebrand and Sutton 1984). Diem (1971) has speculated that perhaps the fluctuating humidity found at the leaf surface might have a larger role than UV resistance in determining the composition of the phylloplane community. We report 100% survival of P. ramorum hyphal colonies exposed to a diurnal cycle of temperature/humidity values for 24 h, consisting of a high temperature of 27.5°C with 63.8% RH and a low of 14.9°C with a RH of 92.2%. While atmospheric humidity likely will be lower than 64% on a warm, sunny day, this value is not unreasonable to expect on the surface of the leaf, especially deep in the canopy or close to the soil surface where humidity is at its highest.

We have identified lethal temperatures and exposure periods for hyphae of P. ramorum. By necessity we used young, spore-free hyphal colonies. More mature hyphae may exhibit a higher degree of tolerance to high temperatures. Chlamydospores appear to be more resistant to high temperatures than are hyphae (Tooley et al 2008) and so will likely be the over-summering propagule. Based on our studies P. ramorum seems capable of tolerating summer temperatures encountered in southern USA, especially when enclosed in plant tissue. Free chlamydospores did not exhibit increased resistance to freezing temperatures so survival during cold periods may occur in the form of hyphae and/or chlamydospores. It does not appear that low temperatures are likely to limit the establishment of P. ramorum in the northeastern USA either. Because infected leaves are more likely to drop than are healthy leaves (Davidson et al 2002) desiccation likely will pose a greater challenge to survival than will exposure to temperature extremes. Therefore future studies investigating the affect of a combination of moisture and temperature extremes on recovery of P. ramorum are warranted.

**LITERATURE CITED**


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