

Survival of Isolates of the US-22, US-23, and US-24 Clonal Lineages of *Phytophthora infestans* by Asexual Means in Tomato Seed at Cold Temperatures

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Abstract

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Survival of *Phytophthora infestans*, causal agent of potato and tomato late blight, is thought to be negligible when exposed to freezing conditions typical of a Wisconsin winter. However, the persistence of relatively new *P. infestans* clonal lineages US-22, US-23, and US-24 within a production region during 2010 to 2014 warranted further investigation. We used tomato seed as a culture medium to determine the survival of *P. infestans* isolates representing the three lineages under temperatures of 18, 4, 0, -3, and -5°C for 11 time points (1 to 112 days postincubation). Survival varied interactively with temperature, duration of time at a temperature, and clonal lineage of the *P. infestans* isolate. US-22, -23, and -24 isolates survived for 112 days at 18 and 4°C, 84 days at 0°C, and 14 days at -3°C. US-23 survived longer at -3 and -5°C than did US-22 or US-24. The vigor of US-22 and US-24 isolates decreased with increasing exposure to cold temperatures, a trend that was not observed for the US-23 isolate. By calculating the length of time needed

to kill the lineage isolates on infested tomato seed at five temperatures, we predicted that *P. infestans* would survive in 5% of tomato seed for 99, 25, and 16 days at 0, -3, and -5°C, respectively. We further applied a degree-day model to our empirical data to describe *P. infestans* survival as a function of cooling degree-day accumulations using archived soil temperatures at 5- and 10-cm depths at four Wisconsin locations over 27 years. The model indicated that survival of *P. infestans* in 5% of infested tomato seed would occur at 35 and 39% of the location-year combinations at 5- and 10-cm soil depths, respectively. Together, these data suggested that *P. infestans* has the potential to survive over the winter season by asexual means in infested tomato seed in Wisconsin and other Northern latitudes. Our cooling degree-day model for late blight in the tomato production system offers a tool for anticipating and mitigating disease based on integrated pest management concepts previously utilized for insects.

Phytophthora infestans (Mont.) de Bary, the causal agent of late blight, is one of the most destructive pathogens of potato (*Solanum tuberosum* L.) and tomato (*S. lycopersicum* L.) worldwide. The financial cost of field and storage losses to late blight in potato alone has been significant despite decades of enhanced pathogen understanding and management progress (Fry 2007; Guenther et al. 2001; Wustman 2007). Survival of *P. infestans* at freezing temperatures is believed to be limited because *P. infestans* is, functionally, an obligate parasite in its asexual state (Fry 2008; Stevenson et al. 2001); when infected plant tissues are killed with a winter freeze, the pathogen cannot survive because sexual reproduction and development of resilient oospores are not known to occur in the northern United States (Gavino et al. 2000; Goodwin et al. 1998; Kalischuk et al. 2012; Ristaino 2010). Although this pathogen is thought to be eliminated from northern regions each winter, when cold temperatures destroy living plant material in open field settings, any plant tissues that may be protected from extreme cold conditions can serve as potential pathogen reservoirs.

Overwinter survival of asexually reproducing *P. infestans* has been investigated in some regions and, in potato production systems, overwinter survival in cull piles has been demonstrated (Bonde and Schultz 1943; Kirk 2003a,b). Even in regions where winter air temperatures fall below 0°C, tubers located in the center of a cull pile can be insulated and remain above freezing (Kirk 2003a). If those tubers are infected with *P. infestans*, they have the potential to produce infected sprouts the following season (Bonde and Schultz 1943; Gigot et al. 2009; Johnson and Cummings 2013; Lambert et al. 1998; Zwankhuizen et al. 1998). Infected tubers can also remain in production fields postharvest. If deeply buried in soil or under heavy snow cover,

infected potato can be insulated from ambient air temperatures below 0°C, enabling the survival of the pathogen through the winter (Kirk 2003a,b; Kirk et al. 2001). In tomato production systems located in northern regions of the United States, *P. infestans* survival in fruit and seed is assumed to be reduced due to freezing winter temperatures but surviving over the winter in compost piles is thought to occur occasionally (Gevens et al. 2010). In California, where temperatures do not regularly drop below 0°C, infected seedlings have been observed emerging from buried tomato fruit a few weeks after the crop has been harvested (Vartanian and Endo 1985a). Other research has shown that wet or freshly extracted tomato seed infested with *P. infestans* and exposed to temperatures as low as 10°C can give rise to infected seedlings (Vartanian and Endo 1985b) but the survival of *P. infestans* in tomato seed below 10°C was not investigated.

A few studies have explored the ability of *P. infestans*, in an asexual life stage, to survive cold or below-freezing temperatures. In 1926, deBruyn (1926) tested the survival of *P. infestans* in various substrata and concluded that the pathogen could survive in manure for 5 days with temperatures as low as -25°C; however, the possibility that oospores formed in the cultures could not be eliminated. More recently, Kirk (2003b) tested survival of mycelia of four clonal lineages (CL) of *P. infestans* in culture media and found survival for up to 3 days at -3°C; however, the pathogen did not survive for more than 24 h at -5°C. Dandurand et al. (2006) demonstrated survival of *P. infestans* at low temperatures on natural substrata by noting that *P. infestans* remained viable in berries of nightshade weeds that were frozen for 48 h at -15°C. However, the potential for *P. infestans* to survive over the winter in production systems in northern latitudes still is not well understood because these experiments were short in duration when compared with the length of winters (or cold temperatures) typical of northern latitudes.

The US-22 CL of *P. infestans* was newly identified in the United States in 2009 when it caused widespread late blight, primarily on tomato, across the Eastern and Midwestern United States (Danies et al. 2013; Hu et al. 2012; Ristaino 2010; Seidl and Gevens 2013). In Wisconsin, tomato late blight caused by the US-22 lineage was widespread

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in 2009 and occurred again in 2010 (Seidl and Gevens 2013), raising questions about its ability to survive over the winter in production systems. Also in 2010, two new CL, US-23 and US-24, were identified in Wisconsin and became widespread in the state and across the United States (Gevens and Seidl 2013a,b). The objective of this study was to investigate the potential for surviving over the winter of asexually reproducing US-22, US-23, and US-24 CL of *P. infestans*. To do this, the survival of these CL was quantified by subjecting *P. infestans*-infected tomato seed to long-term freezing conditions. Additionally, vigor of the surviving “individuals” subjected to different durations of freezing conditions was assessed. A cooling degree-day (CDD) model was developed to predict *P. infestans* survival on tomato seed, and soil temperature data then were used to estimate the potential for *P. infestans* to survive over the winter in the Wisconsin environment.

Materials and Methods

***P. infestans* isolates.** All *P. infestans* isolates were collected from symptomatic plant tissues from Wisconsin in 2009 or 2010, and have been previously described (Seidl and Gevens 2013). A single isolate from each CL was used for the survival assays. Although of interest, we did not include comparison of one of the older lineages because isolates that have been maintained in long-term storage can lose virulence over time (Goth 1981; Hodgson and Grainger 1964; Thurston 1957). The US-22 isolate, Pi7-09a, is an A2 mating type and was collected from infected tomato fruit tissue. The US-23 isolate, Pi16-10a, is an A1 mating type and was isolated from infected tomato leaf tissue. The US-24 isolate, Pi21-10a, is an A2 mating type and was isolated from infected potato leaf tissue. All *P. infestans* cultures were derived from single zoospore isolations, and oospore production in single-isolate cultures was never observed. Isolates used for the survival experiment were transferred minimally in culture after isolation to maintain the original characteristics of the isolates (Goth 1981; Hodgson and Grainger 1964; Thurston 1957). For long-term storage, isolates were maintained in hemp seed (Lamour and Hausbeck 2001) in the Potato and Vegetable Pathology Laboratory at the University of Wisconsin-Madison. Single representative isolates of each *P. infestans* CL used in previous studies indicated that recently identified lineages showed low variation in biological characteristics (Danies et al. 2013; Mizubuti and Fry 1998). For the purpose of simplifying the results and discussion of our data pertaining to each isolate of US-22, US-23, and US-24, the representative isolate is often referred to as “the CL.”

***P. infestans* survival and vigor assays.** Commercially available nonfungicide-treated tomato seed (‘Brandywine Red’; Reimers Seeds, Saint Leonard, MD) were used for the survival assays. Three tomato seeds were added to each of 110 1.5-ml microcentrifuge tubes and sterilized in an autoclave. Sterile distilled water (0.75 ml) and one 7-mm-diameter plug of mycelia or sporangia from a 3-week-old *P. infestans* isolate actively growing on rye A media (Caten and Jinks 1968) were added to each tube. All tubes were sealed and incubated at 18°C for 4 weeks to allow for colonization of the tomato seed. Autoclaved tomato seed ($n = 3$) receiving sterile distilled water and no pathogen inoculation were included as an experimental control. The agar plug was removed from each tube after incubation. Tubes were resealed and randomly placed in each of five 80-well plastic microcentrifuge tube racks. The entire procedure was repeated for each of the three selected *P. infestans* isolates. Each microcentrifuge tube rack contained 66 tubes, 22 tubes per isolate, and one rack was placed at each temperature: -5, -3, 0, 4, and 18°C (Environmental Chamber and Refrigerator/Freezer, Hotpack Corp., Philadelphia). Incubator temperatures fluctuated no more than 0.5°C from the desired temperatures throughout the duration of experiments, as determined by temperature loggers (Spectrum Technologies, Aurora, IL; data not shown). Relative humidity was ambient at approximately 55% in the incubators (data not shown).

Survival of each isolate of *P. infestans* was tested at 1, 2, 3, 5, 7, 14, 21, 28, 56, 84, and 112 days after exposure to each temperature treatment. For the time points at or before 28 days, the sampling was performed at exactly the time specified, ± 5 min. Later time points were exact ± 2 h. For each of the 11 time points, two tubes of each

isolate at each temperature were randomly selected and removed from the incubators. Within 2 h of removal, the six seeds from the two tubes were placed on rye A medium in 9-cm petri plates and incubated at 20°C with a 12-h photoperiod. Individual seeds plated on media were evaluated using a dissecting microscope at 7, 14, and 21 days after plating. Survival of *P. infestans* was assessed as 0 (no growth) or 1 (visible growth). The pathogen was identified based on morphological features of *P. infestans* (Waterhouse 1963).

The amount of *P. infestans* growth from each seed was then quantified using a vigor index of 0 to 4, where 0 = no *P. infestans* growth, 1 = less than 10 short (<0.50 mm) mycelial strands, 2 = more than 10 longer (>0.50 mm) mycelial strands but not a wide-spreading colony, 3 = a wide-spreading colony, and 4 = a wide-spreading colony with a thick, profuse layer of mycelia or sporangia. The experiment was replicated twice in its entirety, and a third experimental replication was ended after the day-56 sampling.

Data analysis. The data from these experiments were examined in three different ways. First, a generalized linear modeling approach based on binomial regression (logit-link) was used to model *P. infestans* survival (i.e., alive or dead) as a function of CL and duration of time (Time) that a seed remained at an experimental temperature (Temp) prior to sampling (Venables and Ripley 2002). Initially, a full model, with Temp and CL as categorical covariates, Time as a continuous covariate, and all interaction terms among covariates (i.e., Time-CL, Time-Temp, CL-Temp, and Time-CL-Temp) was fit to the data. Experimental replication was included in the analysis as a blocking factor and allowed to interact with all model terms. Analysis of deviance was used to adjudicate the significance of the model terms. Because there were several significant interaction terms in the initial regression model, the full model was further subdivided to examine the importance of isolate and time within a temperature treatment and submodels then were evaluated separately. The submodels were used to calculate the length of time in days (ED), averaged over experimental replicates, needed to kill 50, 75, and 95% of US-22, US-23, and US-24 CL isolates of *P. infestans* on infested tomato seed at five temperatures.

The second analysis of these data modeled *P. infestans* survival as a function of simple CDD, calculated using a base temperature of 1°C (T_b) for each temperature and exposure time treatment (Campbell and Norman 1998). CDD below the base temperature were calculated as $CDD = T_b - T_x$, where T_x was the growth chamber temperature. Once CDD were obtained for each day, they were summed cumulatively for the duration of time that each treatment was maintained prior to sampling. The data from each experimental replication were aggregated for a CL and CDD estimate, resulting in the number of surviving and number of dead *P. infestans* samples for each CL-CDD combination. Logistic regression was used to model the survival of *P. infestans* as a function of log-transformed CDD calculated using a base temperature of 1°C. The logistic regression model can be represented as $\text{Log}_e(Y_i/1 - Y_i) = B_0 + B_1 X_i$, where the odds of survival are represented by $Y_i/1 - Y_i$, X_i is the log-transformed CDD (Log_e), and B_0 and B_1 are regression coefficients (the intercept and slope, respectively) (Kutner et al. 2004). Again, experimental replication was considered a blocking factor and allowed to interact with all model terms. Analysis of deviance was used to adjudicate the significance of the model terms. The average CDD necessary to have the probability of survival (Y) be 0.50, 0.75 and 0.95 was estimated by substituting 0.50, 0.75, or 0.95 for Y in the fitted regression equation, and solving for X, the CDD (log-transformed) (Kutner et al. 2004). These estimates were used to calculate the average number of days at 0, -3, and -6°C necessary to kill 50, 75, and 95% of *P. infestans* in the infested tomato seed. All logistic regression models were fit in R 3.0.2 (R Core Team 2014) using the *glm* function (binomial, logit-link). Parameter estimates, χ^2 test statistics, and ED estimates (\pm standard error [SE]) were extracted using the *summary*, *anova*, and *dose.p* functions in the MASS package (Venables and Ripley 2002).

Finally, for the third analysis, the three vigor ratings (taken at 7, 14, and 21 days after placing the seed on media) were summed for each of the exposure times and each CL-Temp combination for each

“individual” that survived. The resulting value was divided by 12, yielding a vigor index that was scaled to fall between 0 (no growth) and 1 (maximal growth) for each treatment combination. CDD ($T_b = 19^\circ\text{C}$) were calculated for each exposure Time–Temp combination and experimental replication (three total), as described above (Campbell and Norman 1998). The vigor index was then modeled as a linear function of CDD (log), CL, and the interaction term CDD (log)–CL, with experimental replication as a blocking factor and allowed to interact with all model terms. Because the interaction term CDD (log)–CL was significant, the regression model was subdivided to examine the relationship between vigor and CDD (log) for each CL separately. All linear regression models were fit in R (version 3.0.2) using the *lm* function and parameter estimates, and test statistics were extracted using the *summary and anova* functions (R Core Team 2014).

Environmental variability of *P. infestans* survival. To examine the potential for *P. infestans* to survive overwinter in Wisconsin, soil temperature data at two depths, 5 and 10 cm, were assembled from four locations. In total, 10, 27, 27, and 22 years of soil temperature data were obtained for Antigo (1988 to 1997), Arlington (1987 to 2013), Hancock (1987 to 2013), and Spring Green (1989 to 1997 and 2001 to 2011), respectively. These soil temperature data were downloaded from the University of Wisconsin Soil Science website (http://agwx.soils.wisc.edu/uwex_agwx/awon). CDD ($T_b = 1^\circ\text{C}$) were calculated using soil temperature data at 5 and 10 cm below the soil surface and daily values were summed for the winter (15 October through 1 May) at each location. The degree-day model (described above) was then used to determine the probability that an individual from a *P. infestans* CL would have survived the cold temperatures of each location’s winter. Data were then summarized to reflect the percentage of years in which at least 5, 25, and 50% of *P. infestans* individuals could have survived over the winter in a population of *P. infestans*-infected tomato seed at each location and soil depth.

Results

***P. infestans* survival.** No *P. infestans* or other microorganisms grew from the noninoculated, autoclaved control tomato seed. Survival of *P. infestans* was modeled as a function of Temp, Time, and the CL of the assayed isolate. When a full model including all of the interaction terms among the covariates was fit to the data, an analysis of deviance identified several significant interaction terms,

including the Time–CL–Temp term (Table 1). This finding suggested that survival varied interactively with Temp, Time, and CL of the *P. infestans* isolate.

To better understand these survival data, the full model was subdivided by Temp and individual models of survival as a function of Time, CL, and the Time–CL interaction term were fit (analysis of deviance tables for separate model fits, not shown). Survival of isolates of the three CL at 18°C was high, with 97% of samples surviving at day 112 of the experiment. In fact, the binomial model predicted it would take 304 and 268 days to kill 95% of each *P. infestans* isolate representing US-22 and US-23, respectively, in the infested tomato seed (Table 2). The high survival rate of *P. infestans* at this temperature caused difficulty in estimating some parameters for the binomial regression model, and survival times for the isolate representing US-24 could not be estimated. At 4°C , survival of *P. infestans* also was high,

Table 2. Average length of time in days (standard error) and proportion of *Phytophthora infestans* infested seed expected to contain live *P. infestans* for the US-22, US-23, and US-24 isolates at five temperatures (Temp) based on binomial regression modeling

Temp ($^\circ\text{C}$)	Pr (alive) ^b	Isolate ^a		
		US-22	US-23	US-24
18	0.50	212 (62)	176 (44)	NE
	0.25	246 (73)	210 (56)	NE
	0.05	304 (94)	268 (78)	NE
4	0.50	90 (6)	197 (89)	112 (14)
	0.25	105 (7)	228 (111)	143 (19)
	0.05	130 (11)	279 (149)	196 (29)
0	0.50	44 (4)	34 (3)	35 (4)
	0.25	59 (5)	49 (4)	51 (4)
	0.05	85 (7)	75 (6)	77 (6)
–3	0.50	4 (1)	4 (1)	4 (0)
	0.25	6 (1)	9 (1)	5 (1)
	0.05	10 (1)	17 (3)	8 (1)
–5	0.50	3 (0)	1 (0)	2 (0)
	0.25	4 (0)	3 (0)	3 (0)
	0.05	6 (1)	6 (1)	4 (1)

^a NE = not estimated.

^b Proportion (0.50, 0.25, or 0.05) of *P. infestans* infested tomato seed expected to contain living *P. infestans*.

Table 1. Analysis of deviance table for binomial generalized linear model describing the effect of temperature and duration of exposure to a temperature on the survival of *Phytophthora infestans* in infested tomato seed

Model terms ^a	DF residual ^b	Residual deviance ^c	DF ^d	Deviance ^e	Pr ($>\chi$) ^f
Time	448	2683	1	248	<0.0001
CL	446	2682	2	1.0	0.5944
Temp	442	748	4	1934	<0.0001
Exp	440	736	2	12	0.0028
Time \times CL	438	714	2	22	<0.0001
Time \times Temp	434	459	4	256	<0.0001
CL \times Temp	426	434	8	24	0.0019
Time \times Exp	424	423	2	11	0.0034
CL \times Exp	420	408	4	15	0.0044
Temp \times Exp	412	383	8	25	0.0017
Time \times CL \times Temp	404	360	8	23	0.0028
Time \times CL \times Exp	400	352	4	8	0.0919
Time \times Temp \times Exp	392	329	8	23	0.0038
CL \times Temp \times Exp	376	300	16	30	0.0206
Time \times CL \times Temp \times Exp	360	290	16	9	0.9108

^a Time refers to the number of days the treatment was exposed to a temperature, CL refers to clonal lineage of *P. infestans* isolates, Temp refers to temperature ($^\circ\text{C}$), and Exp refers to experimental replication.

^b Residual degrees of freedom.

^c Residual deviance of the model; residual deviance of the null model was 2,932 on 449 degrees of freedom; Akaike Information Criterion of full model was 675.7.

^d Degrees of freedom for model terms.

^e Reduction in deviance by the sequential inclusion of terms in the model.

^f *P* value, based on a χ^2 test, associated with the reduction in deviance by inclusion of terms in the model.

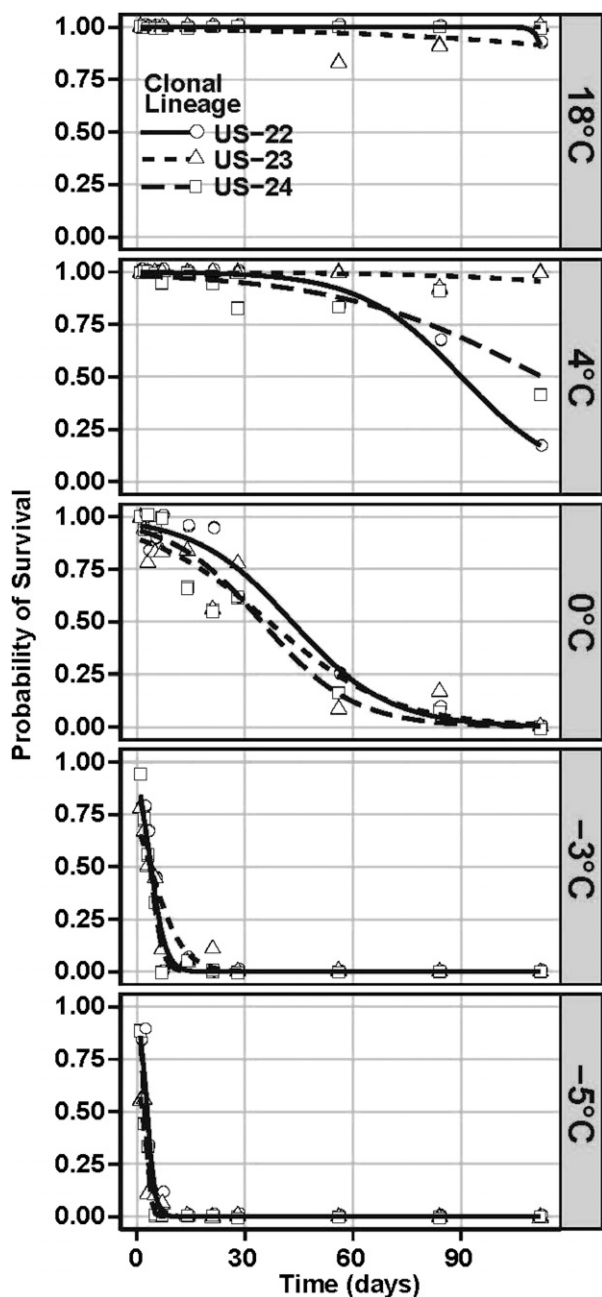


Fig. 1. Survival of the US-22, US-23, and US-24 isolates of *Phytophthora infestans* in tomato seed estimated at five temperatures for 112 days. Each symbol represents the mean survival of 18 seeds (6 seeds per replication, three experimental replications).

with 53% of samples surviving after 112 days. However, the survival of *P. infestans* varied interactively depending on the CL of the isolate and the Time; the regression slopes varied by CL (Fig. 1, χ^2 : Dev. = 7.6, DF = 2, $P = 0.02225$). For example, all samples of the isolate representing US-23 survived 112 days at 4°C (Fig. 1). However, for the two isolates representing the US-22 and US-24 CL, survival at 4°C started to decrease after 45 days, with only 17 and 42% of samples, respectively, surviving after 112 days. The binomial model for 4°C predicted it would take 130, 279, and 196 days to kill 95% of the US-22, US-23, and US-24 CL isolates, respectively, in the infested tomato seed (Table 2).

At 0°C, survival of *P. infestans* was documented until day 84 of the experiment but no survival of *P. infestans* was documented at day 112 of the experiment (Fig. 1). Additionally, at 0°C, the regression slopes did not vary among the three isolates. To kill 95% of the US-22, US-23, and US-24 isolates infecting the tomato seed at 0°C would take 85, 75, and 77 days, respectively (Table 2).

Survival of *P. infestans* at -3°C was documented until day 21 of the experiment but no survival was documented after day 21. However, again, the survival of *P. infestans* varied interactively depending on the CL of the isolate and the Time (Fig. 1, χ^2 : Dev. = 13.9, DF = 2, $P = 0.0009$). Only 5% of each US-22, US-23, and US-24 CL isolate in infested tomato seed would survive for 10, 17, and 8 days, respectively, at -3°C (Table 2).

At -5°C, survival was low, and documented until day 7 of the experiment. The isolate representing US-24 survived the shortest time, less than 5 days, and survival of the US-22 and US-23 isolates was documented at 7 days but not at 14 days. The binomial regression model predicted that *P. infestans* would not survive in 95% of the infested tomato seed for longer than 6 days at -5°C.

Degree-day model of *P. infestans* survival. The survival of *P. infestans* decreased as a function of CDD which, when incorporated into the model, accounted for 82% of the residual deviance. Other model terms accounted for an additional 3% of the residual deviance, and these model terms were primarily associated with the experimental replication. Similarly, the slopes of the regression model varied among CL but the variation was always associated with experimental replication (Table 3; Fig. 2). The variation associated with experiment was considered to be random variation and the regression model was reduced to provide an average slope and intercept that describes all isolates (Fig. 2). This model had an intercept of 4.20 and a slope of -1.53 on the scale of the linear predictor (Fig. 2; average B_0 : estimate = 4.20, SE = 0.17, $z = 24.7$, $P < 0.001$; average B_1 : estimate = -1.53, SE = 0.06, $z = -26.2$, $P < 0.001$). The CDD (Log_e) that would allow survival of 50, 25, or 5% of *P. infestans* individuals in a population of *P. infestans*-infested tomato seed was estimated to be 2.75 (± 0.04), 3.47 (± 0.05), and 4.68 (± 0.09), respectively (Fig. 2). Thus, 5% of *P. infestans* individuals in a population of *P. infestans*-infested tomato seed would be expected to survive for approximately 108, 27, and 18 days at 0, -3, and -5°C, respectively (Table 4). These estimates were consistent with the previous estimates of survival time at those temperatures (Tables 2 and 4).

Table 3. Analysis of deviance table for binomial generalized linear model describing the effect of the duration of cold temperature exposure, expressed as cooling degree-days (CDD), on the survival of *Phytophthora infestans* in infested tomato seed

Model terms ^a	DF residual ^b	Residual deviance ^c	DF ^d	Deviance ^e	Pr (> χ) ^f
CL	225	2,633	2	1	0.6239
Log (CDD + 1)	224	473	1	2,161	<0.0001
Exp	222	447	2	26	<0.0001
CL × Log (CDD + 1)	220	444	2	3	0.2684
CL × Exp	216	433	4	11	0.0230
Log (CDD + 1) × Exp	214	426	2	7	0.0373
CL × Log (CDD + 1) × Exp	210	399	4	27	<0.0001

^a CL refers to isolates of *P. infestans* clonal lineage and Exp refers to experimental replication.

^b Residual degrees of freedom.

^c Residual deviance of the model; residual deviance of the null model was 2634 on 227 degrees of freedom; Akaike Information Criterion of full model was 675.7.

^d Degrees of freedom for model terms.

^e Reduction in deviance by the sequential inclusion of terms in the model.

^f P value, based on a χ^2 test, associated with the reduction in deviance by inclusion of terms in the model.

***P. infestans* vigor index.** The vigor of the surviving *P. infestans* individuals was modeled as a function of cold-exposure quantified as CDD (log) calculated using a base temperature of 19°C. The slope of this relationship was dependent on the CL of the *P. infestans* isolate as determined by analysis of variance (Table 5). For this reason, individual simple linear regressions were performed on the data parsed by clonal lineage (Fig. 3). The vigor of isolates belonging to the US-22 and US-24 CL decreased with increasing exposure to cold temperatures. For the isolate representing the US-23 CL, a significant response to increasing cold exposure was not observed.

Soil survival of *P. infestans*. In Wisconsin, the magnitude of CDD calculated for soil temperatures measured at 5- and 10-cm soil depths was variable among years and locations (Table 6). Over all years for which data were acquired, the average values of winter-accumulated CDD were variable among locations ($F = 3.18$, $DF = 3, 164$, $P = 0.0256$) but did not vary significantly due to soil depth although, numerically, CDD values at 10-cm depths were lower when compared with those at 5-cm soil depths (Table 7). Based on the CDD model

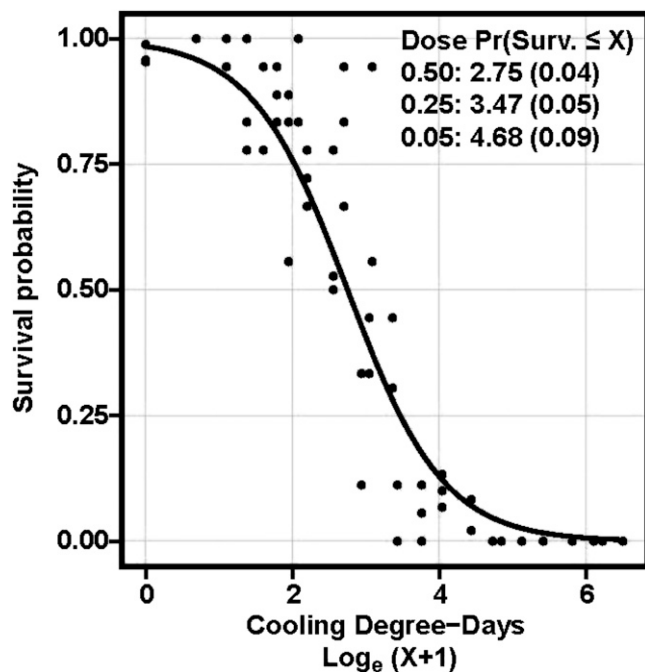


Fig. 2. Survival of the US-22, US-23, and US-24 isolates of *Phytophthora infestans* in infested tomato seed as a function of cooling degree-days (log). A base temperature of 1°C was used to calculate simple degree-days and all data for a clonal lineage were aggregated to degree-day values. The solid black line represents the average best fit line for all isolates, ignoring variation due to experimental replication.

(described above) and soil temperatures at a depth of 5 cm, 5, 25, and 50% of *P. infestans* individuals in the population of *P. infestans*-infected seed would have survived at, on average, 34, 14, and 14% of the location-year combinations, respectively. Using soil temperatures at a depth of 10 cm, the survival of *P. infestans* at 5, 25, and 50% *P. infestans* individuals in the population of *P. infestans*-infected seed would increase slightly and occur at 39, 18, and 14%, respectively, of the location-year combinations examined.

Discussion

Survival of *P. infestans* by asexual means at low temperatures has been studied previously in culture media assays outside of host tissues (Kirk 2003b; Mizubuti and Fry 1998), and on nightshade berries (Dandurand et al. 2006) for relatively short experimental durations, but has not been extensively explored in seed for longer durations. In this study, isolates representing the US-22, US-23, and US-24 CL of *P. infestans* recently identified and associated with tomato late blight epidemics were documented to survive on tomato seed for 79 days at 0°C, 12 days at -3°C, and 5 days at -5°C. When these data were described as a function of CDD, our model predicted that an accumulation of 108 CDD ($T_b = 1^\circ\text{C}$) would result in 5% survival of *P. infestans*. Additionally, CDD accumulations calculated from soil temperatures at a depth of 5 cm from 15 October through 15 May were less than 108 CDD for 20% of location-year combinations examined. These findings suggested that soil temperatures conducive for the survival of asexual propagules of *P. infestans* in infested seed may occur in Wisconsin.

Table 5. Analysis of variance table for the linear model describing the effect of cold exposure and clonal lineage on vigor of the surviving *Phytophthora infestans* individuals isolated from tomato seed

Model terms ^a	DF ^b	SS ^c	MS ^d	F value ^e	Pr(>F) ^f
CL	2	1.14	0.19	31.3	<0.0001
Log (CDD)	1	0.23	0.23	12.6	0.0004
Exp	2	0.11	0.06	3.01	0.0506
CL × Log (CDD)	2	0.16	0.08	4.3	0.01404
CL × Exp	4	0.12	0.03	1.7	0.1516
Log (CDD) × Exp	2	0.04	0.02	1.1	0.3230
CL × Log (CDD) × Exp	4	0.04	0.01	0.59	0.6715
Residuals	307	5.61	0.02

^a CL refers to isolates of a *P. infestans* clonal lineage, CDD refers to cooling degree-days base 19°C, and Exp refers to experimental replication. R^2 (adjusted) = 0.21, Overall F statistic = 5.96, $DF = 17,307$, and $P < 0.0001$.

^b Degrees of freedom associated with each model term.

^c Sum of squares explained by each model term.

^d Mean square (i.e., $MS = SS/DF$).

^e F value = $MS_{\text{model}}/MS_{\text{residual}}$.

^f $Pr(>F)$ is the probability of obtaining an F value more extreme than the F value.

Table 4. Average length of time in days (standard error) and proportion of *Phytophthora infestans*-infested seed expected to contain living *P. infestans* for the US-22, US-23, and US-24 isolates at three temperatures (Temp), as predicted by the cooling degree-day (CDD) model

Temp (°C)	Pr (alive) ^a	Dose	
		Log _e (CDD) ± SE ^b	CDD (°C)
0	0.50	2.75 (0.04)	14.0–15.3
	0.25	3.47 (0.05)	29.6–32.8
	0.05	4.68 (0.09)	98.5–116.9
-3	0.50	2.75 (0.04)	14.0–15.3
	0.25	3.47 (0.05)	29.6–32.8
	0.05	4.68 (0.09)	98.5–116.9
-5	0.50	2.75 (0.04)	14.0–15.3
	0.25	3.47 (0.05)	29.6–32.8
	0.05	4.68 (0.09)	98.5–116.9

^a Cold exposure that would result in survival of 50, 25, and 5% of the population of *P. infestans* in infested tomato seed (or mortality of 50, 75, and 95% of the population).

^b SE refers to standard error.

New CL of *P. infestans* have been identified in the United States since the 1980s, despite a lack of evidence for sexual recombination or spontaneous mutation. US-1 is believed to have been the first and predominant lineage in the United States up until the late 1980s, when the US-8 CL effectively displaced it in just a few years (Goodwin et al. 1998; Marshall-Farrar et al. 1998). Newer lineages have been identified and successively named but their occurrence generally has been ephemeral and their presence often limited to a particular geographic region (Ristaino 2010; Seidl and Gevens 2013; Seidl Johnson and Gevens 2014). In 2009, the US-22 CL quickly spread across much of the United States (Danies et al. 2013; Gevens et al. 2010, 2013a,b; Hu et al. 2012). In the following 4 years, US-23 and US-24 also were identified, with US-23 gaining predominance in most national potato and tomato late blight epidemics (Danies et al. 2013; Gevens et al. 2010, 2013a,b). The

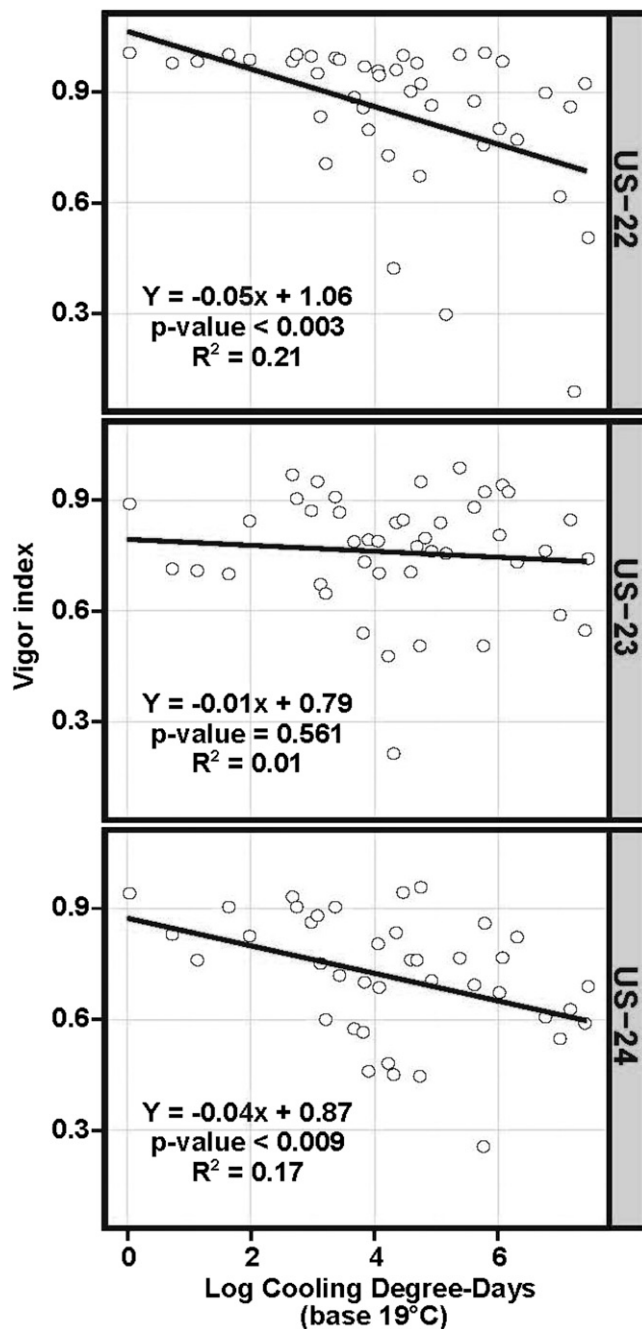


Fig. 3. Vigor of surviving *Phytophthora infestans* isolated from infested tomato seed following exposure to cold temperatures. Isolates of the A, US-22; B, US-23; and C, US-24 clonal lineages are represented.

displacement of CL since the 1980s suggests that the displacing lineages may have enhanced fitness over the displaced. Changes that can increase the probability that a CL will successfully survive, reproduce, and cause disease (Antonovics and Alexander 1989; deBruyn 1926) may include the ability to produce more sporangia (Hu et al. 2012; Van den Bosch and Kiessel 2007), infect a wider range of hosts (Golas et al. 2010; Grönberg et al. 2012; Vartanian and Endo 1985a; Wisconsin Department of Agriculture, Trade and Consumer Protection 2012), have greater rates of direct or indirect germination (Hu et al. 2012), or survive at broader temperature ranges (Goodwin et al.

Table 6. Calculated cooling degree-day accumulations using a base temperature of 1°C for temperatures measured at soil depths of 5 and 10 cm and in Antigo, Arlington, Hancock, and Spring Green, WI

Year	Antigo		Arlington		Hancock		Spring Green	
	5 cm	10 cm	5 cm	10 cm	5 cm	10 cm	5 cm	10 cm
1987	58	83	0	0
1988	15	7	266	268	40	60	324	284
1989	264	191	153	169	490	568	...	284
1990	93	18	4	13	35	90	156	157
1991	49	10	96	103	264	432	180	177
1992	9	0	44	158	310	614	366	379
1993	6	124	5	94	355	605	222	235
1994	43	137	109	262	391	287	229	289
1995	452	387	2	94	235	125	350	436
1996	239	201	232	192	56	0	326	440
1997	222	181	100	61	209	188	128	221
1998	168	142	121	107
1999	71	60	226	197
2000	170	135	49	4
2001	128	99	76	72	143	295
2002	510	419	479	444	395	632
2003	272	212	222	209	243	512
2004	251	224	333	322	120	570
2005	92	94	199	186	94	82
2006	224	211	236	209	127	84
2007	161	80	109	85	74	23
2008	317	192	216	196	120	45
2009	127	20	149	130	108	50
2010	203	165	207	186	231	121
2011	83	81	206	182	113	46
2012	147	98	207	183
2013	133	84	108	98

Table 7. Percentage of years in which 5, 25, and 50% of *Phytophthora infestans* in infested tomato seed would likely survive overwinter based on cooling degree-day (CDD) accumulations using soil temperatures at depths of 5 and 10 cm at four Wisconsin locations

Soil depth	Location ^b	CDD (SD) ^c	Pr (alive) ^a		
			0.05	0.25	0.50
5 cm	A	139 (149)	60	30	30
	B	152 (110)	37	11	11
	C	204 (130)	22	4	4
	D	184 (113)	18	9	9
	Average	...	34	14	14
10 cm	A	126 (123)	40	40	30
	B	141 (88)	51	7	4
	C	214 (177)	30	11	11
	D	231 (194)	36	13	9
	Average	...	39	18	14

^a Pr (alive) is the proportion (0.50, 0.25, or 0.05) of *P. infestans* individuals in a population of *P. infestans*-infested tomato seed expected to survive.

^b A = Antigo ($N_{\text{years}} = 10$), B = Arlington ($N_{\text{years}} = 27$), C = Hancock ($N_{\text{years}} = 27$), and D = Spring Green ($N_{\text{years}} = 22$).

^c Average CDD 1°C. SD refers to standard deviation.

1998; Montarry et al. 2007; Smart and Fry 2001; Van den Bosch and Kiessel 2007).

In this work, an isolate representing each of the three lineages survived on tomato seed at low temperatures for longer periods of time than has been previously reported (Dandurand et al. 2006; deBruyn 1926; Kirk 2003b). This difference could be due to the medium on which the survival assays were conducted. Autoclaved tomato seed were used in this study but previous studies have not used seed as a medium for a survival assay. The results may also indicate that new lineages are better able to survive temperatures at or below freezing than previously common lineages. In general, the *P. infestans* isolates representing the US-22, US-23, and US-24 CL performed similarly in the survival assays. However, small differences in survival at low temperatures were observed among the three CL (Danies et al. 2013; Hu et al. 2012; Kalischuk et al. 2012; Seidl and Gevens 2013). Specifically, US-24 was predicted to survive for the shortest duration (8 days) at -3°C while US-23 was predicted to survive over twice as long (17 days) at that temperature. It is not known whether the differences in survival among these isolates representing the different CL would be biologically important (or important in the field).

The ability of *P. infestans* to cause late blight on tomato seedlings from infected or infested seed has been demonstrated previously. For example, Vartanian and Endo (1985b) showed a 34% rate of disease on tomato seedlings arising from infected seed on sterile soil. Even on nonsterile field soil, 18% of seedlings arising from late-blight-infected seed showed signs and symptoms of late blight. Additionally, in a field setting, clusters of tomato seedlings infected with *P. infestans* at their crown have been observed (Vartanian and Endo 1985a). This finding suggested that the seedlings arose from an infected tomato fruit or seed that survived over the winter from the previous growing season; *P. infestans* infection from aerial inoculum is more likely to occur in the plant canopy and less likely to occur at the crown of the plant (Vartanian and Endo 1985a). Given that infected seedlings can arise from infested or infected seed containing viable *P. infestans*, it is reasonable to consider that volunteer tomato seedlings could provide a primary inoculum source for initiating late blight epidemics, even in regions with freezing winters.

The possibility that the asexual life stage of the new CL of *P. infestans* may be able to survive over the winter in the northern United States on tomato seed has important implications for tomato and potato late blight management in these areas. In Wisconsin, potato cull piles are carefully managed and even regulated because of their documented ability to be a source of inoculum for late blight, even in northern regions with freezing winters (Bonde and Schultz 1943; Kirk 2003a,b; Kirk et al. 2001; Wisconsin Department of Agriculture, Trade and Consumer Protection 2012). Tomato plants are known to freeze overwinter, destroying all of the living plant material needed for *P. infestans* to survive. However, tomato seed is known to survive over the winter and, unlike potato cull piles, volunteer tomato seedlings are not currently regulated in Wisconsin, even though the plants can produce sporangia that may be a source of inoculum for both tomato and potato production fields. Tomato growers should be aware of the potential for *P. infestans* to survive over the winter on or in tomato seed of infected tomato fruit that have been covered by soil or snow, and be prepared to manage volunteer plants in the subsequent spring.

Exposure to cold also affected the vigor of some of the surviving *P. infestans* isolates. For example, the vigor of the US-22 and US-24 isolates decreased as a function of cold exposure. This observed decrease of vigor was primarily due to a delayed initiation of *P. infestans* growth from the tomato seed after the cold treatment. Occasionally, *P. infestans* would not grow out of a tomato seed until the 3-week evaluation point of the experiment. In part, this delay may explain the phenology of late blight and why it is considered to be a mid- to late-season disease. If the observed growth rates of sterile media were extrapolated to tomato seedlings in the field, it would likely be a month or longer before late blight lesions would develop enough to become noticeable on a field scale. For the isolate representing the US-23 CL, vigor of the surviving individuals was not affected by exposure to cold. The US-23 isolate effectively had a head start in growth compared with the US-22 and US-24 isolates, which might also explain, in part, why

the US-23 CL has become predominant in the Wisconsin environment (Seidl and Gevens 2013).

Our initial data analysis considered calendar time as the correct scale for describing survival as a function of cold exposure; survival varied depending on the time spent at a given temperature. However, our second analysis described the survival of *P. infestans* as a function of CDD and considered the existence of a thermal time scale that relates to the survival of *P. infestans*. The calculation of CDD is an integration of temperature with respect to exposure time. It combines temperature and time into a single metric that represents exposure to cold temperatures. CDD can be calculated for time periods with varying temperatures and, assuming survival only depends on temperature, is a useful concept for extending constant temperature laboratory studies to the field. The CDD model was used to predict whether soil temperature conditions existed in Wisconsin that would be conducive for overwinter survival of *P. infestans*. In total, from CDD calculated from 15 October through 1 May, the model predicted 5% survival of *P. infestans* at 35 and 39% of the 86 location-year combinations at 5- and 10-cm soil depths, respectively. In roughly 14% of the winters examined, likely due to warmer winter air temperatures or deeper snow cover, the degree-day model predicted that 50% of *P. infestans* individuals in a population of infected seed would survive at both soil depths. These data suggest that, despite significant annual and site-specific variation of factors that influence soil temperature, (temperature) conditions that would allow *P. infestans* to survive over the winter in wet tomato seed likely occur at northern U.S. latitudes. However, survival of *P. infestans* over the winter is not typically observed in Wisconsin, suggesting that temperature is not the only factor determining survival; freeze-thaw cycles, desiccation, or antagonistic interactions with other soil microbes may greatly influence the survival of *P. infestans* over the winter.

Many factors can influence soil temperature and affect the overwintering ability of *P. infestans* in its asexual life stage in northern latitudes. For example, soil temperatures at different locations may fluctuate due to differences in air temperature and soil type, and moisture content can further influence soil temperature (Al-Kayssi et al. 1990). Soil microclimates can also exist at relatively small geographic scales. Backyard garden plots in cities often are more sheltered and warmer than garden plots in rural areas, temperatures in compost piles may be warmer due to microbial activity (Martin et al. 1992), and abundant snowfall in certain years or localized snow drifting may insulate the ground, preventing lower soil temperatures. Therefore, generalizing results from one or a few locations may lead to inappropriate conclusions about the ability of *P. infestans* to survive over the winter at all locations. However, soil temperature data that have been collected over multiple years and locations can help determine the long-term frequency in which *P. infestans* survival over the winter season is likely to occur. Long-term monitoring of soil temperature in northern climates will continue to be important as regions that may have normally had soil temperatures cold enough to eliminate *P. infestans* may become warmer due to climate change (Anderegg et al. 2010; Widrlechner et al. 2012). Further in situ investigations are necessary to determine the viability and transmissibility from seed to seedling of *P. infestans* after its survival over the winter season, given that soil temperatures can fluctuate greatly in the northern latitudes.

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