

Tolerance of Mycelium of Different Genotypes of *Phytophthora infestans* (Mont.) De Bary to Exposure to Temperature below 0°C for Extended Durations.

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INTRODUCTION

Late blight of potato, caused by *Phytophthora infestans* (Mont.) de Bary, is a devastating disease that affects all potato growing regions in the world and has become a major factor adversely affecting potato production in the Northern United States (9). The source of annual epidemics of late blight of potato caused by *P. infestans* has led to debate as to the relative importance of over wintering sources of inoculum (23). Late blight over winters in potato tubers that are intended for replanting as seed (5,11,16,27), but the infection may also be harbored in waste or cull potato tubers (3,29) or within late blight infected volunteer potatoes returned to the soil during harvest the previous season, although it is generally agreed this source is of minor importance (8,29).

In North America, the probability that infected potato stems or foliage will emerge from an infected tuber is difficult to estimate as several factors can influence the fate of the infected tuber (20,24), temperature being one of the most important (18). The survival of viable host tissue from infection through dormancy to re-emergence the following spring is vital for survival of *P. infestans* (29).

Many investigators have used *in vitro* and soil assays to study the optimal and lethal upper temperatures for growth of *Phytophthora* spp. (2,4,15,28). The survival of *P. nicotianae* chlamydospores in soil was reduced considerably when temperatures were raised above 45°C (4). No studies have been found that examine the ability for *P. infestans* mycelium to survive at temperatures below zero. *P. infestans* can survive within infected tubers at 3°C as stored seed (18), however the fate of mycelium of *P. infestans* within potato tubers exposed to temperatures below 0°C has not been monitored. The objectives of this study were to investigate the ability of different genotypes of *P. infestans* to survive *in vitro* at temperatures below 0°C and to determine the duration of exposure at a range of temperatures below 0°C that was lethal to mycelium.

MATERIALS AND METHODS

Isolate descriptions. Isolates of four genotypes of *P. infestans* were obtained from foliage of potatoes with symptoms of late blight. The genotypes were determined by isozyme analysis (10), mefenoxam sensitivity (6) and mating type and were obtained from Kirk, MI (US1, mefenoxam sensitive, A1 mating type, MI95-6), Kirk, MI (US8, mefenoxam insensitive, A2 mating type, MI95-7, ATCC MYA-1769), Gudmestad, ND (US11, mefenoxam insensitive, A1 mating type, ND207-4) and Kirk, MI (US14, mefenoxam insensitive, A2 mating type, MI96-1). The isolates were maintained on sterile rye seed stock cultures and re-propagated from the long-term storage stock for each experiment on rye agar.

Substrate optimization. Prior to the experiment, several calibrations were performed. Determination of the optimal volume of clarified rye agar added to the 60 mm petri dishes that could be used to optimize the reflectance characteristics for the digital image analysis procedure (described below) was conducted. Clarified rye agar was prepared by

washing 100g organically produced rye seed which was boiled for 1 h in dH₂O, strained through cheese cloth to which was added 15g agar, 7.5g sucrose and dH₂O to bring the solution to 1L final volume. Volumes of 5, 10, 15 and 20 ml rye agar were pored into Petri dishes (60 mm diameter) and the reflectance intensity determined by digital image analysis. The scanned images were the rye petri plates without cover lids. The petri plates were placed open-surface down on a glass plate, 40 x 30 cm and 2 mm thick. The glass plate was used to prevent surface contamination of the scanner glass and permitted multiple samples to be prepared and moved to the scanner for image production. The glass plate was transferred to a flatbed scanner (HP ScanJet 4c, Hewlett-Packard Co., Houston, TX) controlled by an IBM-compatible PC. A 486DX2-80 CPU and a RAM capacity of 32 MB were adequate for the image processing. Scanner control software (DeskScan II ver. 2.4, Hewlett-Packard, Co., Houston, TX) generated an image of the petri plate agar surfaces against a black background. The image was formed from light reflected from the agar surfaces.

The brightness value of the image controlled the light intensity of every pixel in the image. The contrast value controlled the differences between light and dark regions of the image. While the scanner control software was able to automatically adjust the brightness and contrast of the image by comparing the relative area of the pale rye agar surfaces against the black background, the settings were manually set to 180 units (brightness) and 200 units (contrast) to ensure consistent readings. A photograph-quality image was taken and stored for analysis. A typical image in Tagged Image Format (*.tif) occupies about 1 megabyte. The image files created with the scanner software were loaded into the image analysis software (SigmaScan Pro ver. 5.0.0 build number 3981, **SPSS Science**, 233 S. Wacker Drive, 11th floor, Chicago, IL 60606-6307). The black background has 0 light intensity units (LIU), while pure white has 255 LIU. The clarified rye was pale gray. The image of the clarified rye surface was selected for analysis, and isolated from the adjacent regions of the image. The image was unedited. The area was selected with the “fill” tool, which encompassed all pixels within a given area brighter than the cut-off threshold. The area selection cut-off threshold was set to 10 LIU, effectively allowing the software to exclude all parts of the image darker than 10 LIU, e.g. the black background. The average reflective intensity (ARI) of all the pixels within the image gave a measurement of the plate without any growth of the sample. The calibrations resulted in optimal agar volumes of 10 ml substrate/60 mm diameter petri plates with mean ARI = 90.

Determination of developmental stage of sporangia on mycelial plugs after transfer from parent cultures. As the objective of the experiment was to determine the influence of duration of exposure of temperatures less than 0°C on mycelial survival, it was necessary to determine the length of time for all sporangia to germinate after transfer of mycelial plugs from parent cultures. *P. infestans* cultures of each isolate were grown on sterol-free rye agar plates for 14 days in the dark at 12°C. Ten 5 mm diameter cores were removed from the growing edge of each of 10 plates. The cores were placed into 10 ml distilled H₂O in test tubes and stirred on a magnetic stirrer for 1 hour to dislodge sporangia. The suspension was strained through four layers of cheesecloth and the concentration of sporangia was calculated using a haemocytometer. The number of non-germinated and germinated sporangia was counted at the time of transfer and again two days after incubation at 4°C. After two days only empty sporangia were observed in any

of the isolates tested and a pre-treatment of two days incubation at 4°C in the dark was imposed on all freshly transferred cores prior to exposure to temperature treatments.

Temperature exposure studies. The first set of temperature exposure experiments were conducted over a 24 h period. Fifty plates of each isolate were prepared 48 h prior to introduction to the temperature treatment. The plates were labeled with culture ID numbers and exposure times and bound together with parafilm and placed together on a fitted plastic loading tray transferred to a PTC-1 Peltier-effect temperature cabinet controlled by a PELT-3 Peltier-effect temperature controller (Sable Systems International, 2887 Green Valley Parkway #299, Henderson, NV 89014). The PTC-1 chambers were positioned in temperature-controlled environment chambers, 1.8 m³ volume (Environmental Growth Chambers, Chagrin Falls Ohio, USA) at 5°C. The PELT-3 Peltier-effect temperature controller was set for the exposure temperature 2 h prior to the start of the experiment. The tray with the culture plates was placed into the PTC-1 Peltier-effect temperature cabinet quickly to minimize temperature increase. Temperature equilibration was measured after the door of the chamber was opened, and at 0°C set temperature, temperature rose to 5°C after the door was opened and dropped to -0.3°C in 1.5 hr. At -3, -5, -10 and -20°C set temperatures, temperature rose to -1.2, -1.5, -3.5 and -4.8°C and recovered in 1.1, 1.3, 1.5 and 1.4 h respectively. Exposure times were measured from when the set temperature was reached.

Plates were removed after exposures of 1, 4, 8, 12 and 24 h. Temperature treatments were 0, -3, -5, -10 and -20°C. After plates were removed from the PTC-1 Peltier-effect temperature cabinet they were stored in the light at 12°C. After 14 days a sample (replication 1 of the experiment only) of 5 plates was scanned to determine amount of growth of mycelium. The second set of plates (n = 5) was retained for 28 days prior to evaluation. The lids were removed from the plates and the plates were placed face down and images generated (as described above). The experiment was repeated twice over the period from December 2000 to February 2001. The second and third replications of the experiment were evaluated 28 days after the temperature treatments.

The second set of temperature exposure experiments were conducted over a 7 day period. Temperature exposure treatments were selected after analysis of the first experiments (exposure over a 24 h period). The experiment was set up as described above except plates were removed from the PTC-1 Peltier-effect temperature cabinet after exposures of 1, 2, 3, 4 and 5 days. Temperature treatments were 0, -3 and -5°C. The experiment was repeated twice over the period from March to April 2001. Plates were scanned 28 days after removal from the temperature exposure treatment.

Data analysis. The relation between the ARI and weight of individual cultures was determined by linear regression (SigmaStat ver. 2.03, Jandel Scientific, San Rafael, CA). Interactions between temperature and duration of exposure were determined by three-way ANOVA for each replication of both experiments (24 h and 120 h maximum exposure) and if the replications were not significantly different at $p = 0.05$ the data were combined into a single analysis and an LSD generated for comparison of all treatments. To determine if mycelium survived exposure to the thermal treatment, the ARI of treated mycelium were compared to the ARI of a non-inoculated control (negative control) which was added to each exposure treatment using Bonferroni analysis (SigmaStat ver. 2.03, Jandel Scientific, San Rafael, CA).

RESULTS

Temperature exposure studies. Survival of isolates of different genotypes of *P. infestans* exposed to temperatures from 0 to -20°C for different durations up to 24 hours measured as ARI (LIU) of images of cultures incubated for 4 weeks after exposure at 12°C is shown in Table 1. All isolates survived exposure to 0 and -3°C for up to 24 h exposure except isolate 207-4 (US 11, A1) which did not survive exposure to -3°C for 12 or 24 h. Exposure to -5°C for up to 24 h was not lethal for the US8 genotype but 24 h exposure was lethal to the US14 genotype which survived exposure up to 12 h. The A1 genotype US1 was not able to survive exposure of greater than 1 h at -5°C but the genotype US11 survived after exposure of up to 8 h. All genotypes except US11 survived 1 h exposure to -10°C and both A2 genotypes survived exposure to -10°C up to 4 h. No genotypes survived exposure to -20°C.

All isolates of the genotypes of *P. infestans* survived *in vitro* exposure up to 120 h at 0°C (Table 2). Both A1 genotypes survived exposure of up to 48 h at -3°C and the US8 genotype survived exposure to 72 h but the US14 genotype survived after 120 h exposure. A1 genotypes did not survive exposure of 24 h at -5°C but both A2 genotypes showed some potential for recovery up 24 h after exposure to -5°C and US14 sometimes recovered even after 96 h exposure (Table 2).

DISCUSSION

The digital method of assessment of survival of mycelium of *Phytophthora infestans in vitro* relies on light reflectance from developing mycelium and therefore an increased average reflective intensity (ARI). Other studies have made use of light reflectance from biological materials that differ in the darkness of the sample e.g. Niemira et al., (1999) compared the differences between a common non-destructive visual rating system with the destructive digital method showed the potential for the use of computerized image generation and analysis for estimation of the amount and rate of tuber tissue infection caused by *P. infestans*. Image analysis is quantitative and objective and scanned images can be stored for future comparisons. Consistency of sample preparation was an essential element in the scanning method.

The relation between the ARI and mycelium weight was direct and established that ARI is a good estimation of survival of the temperature exposed samples. Radial growth of mycelium on plates may overestimate survival potential especially if the mycelium is growing sparsely. In this analysis, only the difference from non-inoculated plates was determined as an indicator of survival potential after exposure of mycelium to a range of temperatures for different durations. Although it is not possible to determine beyond doubt that some sporangia survived the pre-treatments prior to temperature exposure, all steps to ensure minimal production of sporangia in the parent cultures was attempted i.e. incubation in the dark at 12°C on sterol-free growth medium and then acclimatization at 4°C prior to exposure to the temperature treatment. As only empty sporangia were detected after this pre-treatment it was concluded that the plugs exposed to the temperature treatments consisted only of mycelium. No encysted zoospores were observed with the microscopic investigations.

The apparent increased tolerance of the two A2 genotypes of *P. infestans* to lower temperatures is cause for concern. Reports of increased average temperature in the Great Lakes region of the US has resulted in greater potential for survival of volunteer potatoes in fields and culled potatoes (1) which can potentially harbor inoculum of *P. infestans*.

As the environment in which the mycelium of *P. infestans* survives (potato tubers) is less frequently exposed to temperatures which normally cause substrate breakdown (about -3°C) the risk of survival of blighted tubers surviving winter also increases. Tolerance to temperature in the range of 0 to -3°C and the continued use of foliar applications of mefenoxam in potatoes for control of tuber disease such as pink rot caused by *Phytophthora erythroseptica* (25) may have resulted in the predominance of the US8 (A2, mefenoxam resistant) genotype in the Midwestern potato production areas of the US. Different genotypes of *P. infestans* vary in aggressiveness and virulence in foliar infections (19,21). This study supports the view that the US8 genotype is more virulent than those biotypes isolated prior to 1994 (17) and may partially contribute to a mechanism by which this increased virulence can be explained. As few commercial cultivars have substantive field resistance to foliar infection caused by US8 biotypes of *P. infestans* (7,12) the potential for survival of mycelium in tubers and production of initial inoculum in succeeding years is ominous for potato production.

The estimated base temperature for the development of *P. infestans* infection of tuber tissue has been reported to be about 3°C when tuber tissue was used as substrate (18). Base temperatures refer to the temperature at which development ceases (22) but are not good indicators of survival potential as mechanisms for tolerance to temperatures below the base temperature for development are known to exist in other fungi (26). Tubers are thought to be at greatest risk from infection by *P. infestans*, and perhaps other primary and secondary pathogens, immediately after harvest when tuber pulp temperatures are highest. Tuber vulnerability may persist into the first few weeks of storage prior to adjustment of the tuber tissue to the ambient temperature in store (11). A similar mechanism may occur for volunteer tubers left in fields after harvest.

Further work on the tolerance of mycelium from a wider range of isolates of *P. infestans* representative of genotypes not tested in this study and also of genotypes already tested is underway to determine if this tolerance is typical of the genotypes that have largely replaced the clonal lineage (US1) found prior to 1990. Attempts so far to determine survival of mycelium of *P. infestans* within tubers has failed due to degradation of tuber tissue at -3°C . In addition, as potato growers have commented on the apparent tolerance to ambient temperature in excess of 30°C and recovery potential of lesions after exposure to arid conditions (13,14) the tolerance of mycelium to temperatures in excess of 30°C is also being determined.

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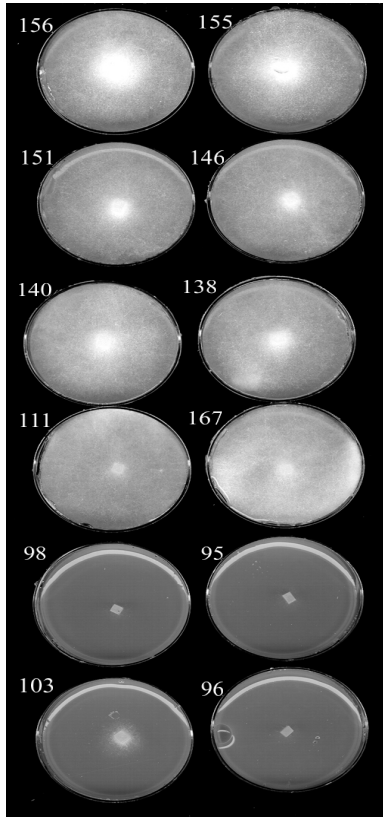


Figure 1. Example of images of *Phytophthora infestans* incubated on plates for 28 days after exposure to different durations of temperature. These are images of Pi95-7 (US8) exposed to -3°C for different durations then incubated at 12°C for 28 days. The values are the average reflective intensities (ARI) in light intensity units (LIU) as measured with Sigma Scan. Plates with no detectable growth of mycelium had ARI values of less than 100 LIU. Note the plate at the bottom left of the figure with minimal growth of mycelium and an ARI > 100 .

Table 1. Survival of isolates of different genotypes of *P. infestans* exposed to temperatures from 0 to -20°C for different durations up to 24 hours measured as average reflective intensity of images of cultures incubated for 4 weeks after exposure at 12°C.

Isolate identification	Exposure time ^a	Average Reflective Intensity (LIU ^b) of Images							
		Exposure temperature (°C)							
		0	-3	-5	-10	-20			
95-6 (US 1, A1)	neg con ^c	89		90		89		89	
	pos con ^d	147	* ^e	146	*	147	*	142	*
	1	147	*	138	*	139	*	109	*
	4	149	*	136	*	123	*	87	
	8	149	*	134	*	108	*	90	
	12	146	*	129	*	100		91	
	24	142	*	124	*	89		93	
LSD _{0.05}		8.6		13.0		12.4		9.2	
95-7 (US 8, A2)	neg con	91		91		93		89	
	pos con	154	*	149	*	143	*	141	*
	1	151	*	131	*	133	*	127	*
	4	152	*	136	*	128	*	107	*
	8	150	*	133	*	135	*	96	
	12	155	*	123	*	134	*	93	
	24	150	*	118	*	104	*	94	
LSD _{0.05}		8.4		12.1		8.4		9.4	
207-4 (US 11, A1)	neg con	88		90		90		91	
	pos con	136	*	126	*	141	*	133	*
	1	132	*	119	*	122	*	100	
	4	137	*	113	*	95		86	
	8	131	*	102	*	95		90	
	12	125	*	100		95		93	
	24	130	*	100		90		92	
LSD _{0.05}		12.5		11.7		14.9		10.7	
671 (US 14, A2)	neg con	90		90		90		90	
	pos con	158	*	145	*	129	*	127	*
	1	156	*	132	*	128	*	120	*
	4	156	*	131	*	122	*	105	*
	8	147	*	128	*	123	*	97	
	12	150	*	119	*	124	*	94	
	24	149	*	120	*	97		95	
LSD _{0.05}		7.3		9.7		7.4		8.0	

^a Exposure time (hours) of cultures to treatment temperatures.

^b Light intensity units of image, 0 = black and 255 = white.

^c neg con = negative control (non-inoculated agar plug, incubated at 12°C for 24 h).

^d pos con = positive control (inoculated agar plug, incubated at 12°C for 24 h).

^e * = Significantly different from negative control calculated for each genotype, temperature and exposure interaction.

Table 2. Survival of isolates of different genotypes of *P. infestans* exposed to temperatures from 0 to -5°C for different durations up to 120 hours measured as average reflective intensity of images of cultures incubated for 4 weeks at 12°C after exposure.

Isolate identification	Exposure time ^a	Average Reflective Intensity (LIU ^b) of Images					
		Exposure temperature (°C)					
		0		-3		-5	
95-6 (US 1, A1)	neg con ^c	92		89		94	
	pos con ^d	140	* ^e	152	*	149	*
	24	138	*	145	*	95	
	48	138	*	133	*	98	
	72	136	*	113	*	99	
	96	134	*	97		98	
	120	133	*	97		99	
LSD _{0.05}		14.9		11.5		6.9	
95-7 (US 8, A2)	neg con	91		93		94	
	pos con	142	*	164	*	140	*
	24	147	*	150	*	112	*
	48	145	*	144	*	95	
	72	143	*	128	*	96	
	96	142	*	99		99	
	120	142	*	100		98	
LSD _{0.05}		8.3		12.2		7.6	
207-4 (US 11, A1)	neg con	93		91		93	
	pos con	150	*	149	*	144	*
	24	146	*	133	*	98	
	48	149	*	125	*	98	
	72	154	*	103		98	
	96	150	*	103		99	
	120	150	*	105	*	99	
LSD _{0.05}		8.2		10.6		5.8	
671 (US 14, A2)	neg con	84		90		91	
	pos con	144	*	149	*	139	*
	24	142	*	140	*	106	*
	48	142	*	135	*	98	
	72	140	*	132	*	99	
	96	137	*	110	*	103	*
	120	140	*	109	*	98	
LSD _{0.05}		8.0		8.2		5.5	

^a Exposure time (hours) of cultures to treatment temperatures.

^b Light intensity units of image, 0 = black and 255 = white.

^c neg con = negative control (non-inoculated agar plug, incubated at 12°C for 24 h).

^d pos con = positive control (inoculated agar plug, incubated at 12°C for 24 h).

^e * = Significantly different from negative control calculated for each genotype, temperature and exposure interaction.

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