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Original article

Thermal inactivation of *Phytophthora capsici* oospores

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ABSTRACT

Background: *Phytophthora capsici* is a major fungal plant pathogen that causes root and crown rot of pepper crops and its oospores are the most resistant propagules.
Objectives: To evaluate the effect of different temperature regimes and exposure times on the survival of *P. capsici* oospores.
Methods: Thermal inactivation treatments simulated field conditions, through the use of different constant and cycling temperature regimes, in moistened sterilized soil (15-53 °C) and sterilized water (45-53 °C). The plasmolysis method evaluated oospore viability. Relationships between oospores viability and exposure time were statistically determined by linear regression. Interpolation was used to calculate the estimated times required to kill a determined percentage of the population.
Results: The required time to reduce *P. capsici* oospores viability decreased with increasing temperatures. Times required to kill 100% of oospores were 199-22-6.6-4.7-1.0 hours at 40-45-47.5-50-53 °C respectively in moistened soil and 31-1.0-0.2 hours at 45-50-53 °C in water. Oospores were scarcely affected at temperatures ≤ 35 °C. With 1,680 hours at 15-35 °C, oospores survival in soil ranged from 88 to 36%. The 4 hours-40 °C regime killed 100% of oospores after 28 days, while the 5 hours-35 °C regime after 70 days killed only 75%. Time required to achieve total oospores death was remarkably shortened in water when compared with moistened soil.
Conclusions: The developed models can be used to predict survival values at any exposure time with constant temperatures ranging from 40 to 53 °C in moistened soil and from 45 to 53 °C in water. The weakening of *P. capsici* oospores under sublethal heating, is a useful observation that can be applied for pathogen control with solarization.

Inactivación térmica de las oosporas de *Phytophthora capsici*

RESUMEN

Antecedentes: *Phytophthora capsici*, importante fitopatógeno fúngico causante de la podredumbre radicular del pimiento, posee oosporas (los propágulos más resistentes).
Objetivos: Evaluar diferentes regímenes térmicos y tiempos en la supervivencia de las oosporas.
Métodos: La inactivación térmica simuló condiciones de campo, empleando diferentes regímenes constantes y cíclicos en suelo húmedo (15-53 °C) y en agua (45-53 °C) estériles. La viabilidad de las oosporas se evaluó mediante plasmólisis correlacionándola por regresión lineal con el tiempo de exposición. Los tiempos estimados necesarios para matar un determinado porcentaje de oosporas fueron calculados por interpolación.
Resultados: El tiempo requerido para reducir la viabilidad de las oosporas disminuyó al aumentar la temperatura. Los tiempos requeridos para matar al 100% de las oosporas fueron 199-22-6,6-4,7-1,0 horas a 40-45-47,5-50-53 °C respectivamente en suelo húmedo y 31-1,0-0,2 horas a 45-50-53 °C en agua. Las oosporas fueron escasamente afectadas por temperaturas ≤ 35 °C. Durante 1.680 horas a 15-35 °C, la supervivencia de oosporas en suelo varió entre 88 y 36%. El régimen de 4 horas-40 °C mató al 100% después de 28 días, mientras el régimen de 5 horas-35 °C después de 70 días sólo mató al 75%. El tiempo requerido para matar al 100% de las oosporas fue acortado significativamente en agua respecto al suelo húmedo.

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Conclusiones: Los modelos desarrollados pueden utilizarse para predecir valores de supervivencia para cualquier tiempo de exposición a temperaturas constantes de 40-53 °C en suelo y de 45-53 °C en agua. El debilitamiento de las oosporas de *P. capsici* bajo calentamiento subletal constituye una observación útil que puede aplicarse en el control del patógeno mediante solarización.

Phytophthora spp. are important soilborne pathogenic fungi for different crops; *Phytophthora capsici* is a major pathogen that causes root and crown rot of pepper crops and consequently, limits its production. Depending on environmental conditions, it also can damage other plant organs such as leaves, branches, stem and fruits. *P. capsici* is formed namely by vegetative propagules (mycelium, sporangia and zoospores, but rarely chlamydospores), and sexual resistant spores (oospores). Vegetative propagules cause rapid development of disease under favourable conditions while oospores are the residual inoculum in soil, which under non-favourable conditions, permit the disease persistence[11].

Postplant strategies used by growers for the control of root and crown rot of pepper are mainly based on soil-water management and application of fungicides. These latter methods do not always provide successful results and therefore, fungal inactivation treatments prior to the planting are widely used, such as chemical fumigation. A drawback of chemical fumigants is that their effectiveness may be limited due to reinfestation of soil after the treatment. Environmental pollution and their danger to humans are other inconveniences that have also made alternatives necessary[6].

One of these alternatives is soil solarization. Solarization is an hydrothermal process in which soil is moistened, covered with transparent plastic and exposed to sunlight to reach high temperatures capable of destroying plant pathogens. Soil solarization practices have shown satisfactory control of different types of diseases in many areas[4–6,13,18–20,22–30].

The effectiveness of solarization depends on different variables, such as the soil colour and structure, soil moisture, air temperature, length of day, and intensity of sunlight[6]. Thus, as solarization conditions can vary greatly from site to site, in vitro laboratory thermal inactivation studies of plant pathogens are mandatory effective tools to predict the utility of solarization as a disease management practice in the particular conditions of each site.

In addition to soil solarization, other heat treatments can be used to justify the interest of in vitro thermal inactivation assays: soil steaming disinfestation, thermotherapy of infected seedlings by pathogens before planting, and residue composting of infected crops[2,3].

Furthermore, little information is available concerning the exposure times necessary to kill soilborne plant pathogens at temperatures below 45 °C. These temperatures are most frequently found in solarized soils and are often considered to be "sublethal". Spare data with bacteria and fungi have also shown that temperatures below 45 °C can be lethal if maintained for long periods[7].

The aim of this in vitro study is to evaluate the effect of different temperature regimes and exposure times on the survival of *P. capsici* oospores.

Material and methods

In vitro production of oospores

P. capsici 02/155 (pepper isolate from Villanueva de Gállego, Zaragoza, Spain), of the A_1 compatibility type, was crossed with isolate *P. capsici* 59 (from the INRA collection, Antibes, France) of the A_2 compatibility type. A_1 and A_2 isolates were paired in Petri dishes (90-mm diameter) containing 15 ml of V8-juice broth (without agar). The plates were sealed with parafilm and incubated in darkness at 25 °C until they were used (ranging from 28 to 35 days).

Oospore fixation in nylon mesh

Oospores were extracted by blending the V8 liquid culture in 100 ml of deionized water. This suspension was filtered through a 100 μm nylon mesh and washed with deionized water. Sporangia and mycelium propagules remained on the filter and oospores were recovered from the filtrate. In the filtered suspension through the 100 μm nylon mesh, the oospores concentration was determined by a Fuchs-Rosenthal haemocitometer. Afterwards, the filtered suspension was collected and refiltered by vacuum through 25 μm nylon meshes (1 cm^2, CISA, Barcelona) mounted on the top of an analytical filter holder (Millipore®) connected to a membrane diaphragm vacuum pump (maximum flow: 1.9 m^3/h, maximum vacuum: 80 mbar, 230 V, 50/60 Hz, 1.1 A; Model ME-2, Vacuubrand®, Wertheim, Germany). A droplet of oospores suspension, containing at least 1,000 oospores, was drawn into each nylon mesh using vacuum (approximately 100 mbar). Consequently, oospores with a diameter size nearly the size of the mesh pores were embedded in the interstices and smaller ones passed through the mesh[21]. The nylon meshes containing embedded oospores were maintained at 5 °C (usually no more than three days) until they were used in the corresponding experiments.

Thermal treatments

Thermal inactivation treatments were carried out by simulating field conditions. Mesh squares containing oospores were introduced into glass test tubes (25 x 150 mm) filled with 30 grams of moistened sterilized soil. Soil water content was adjusted to field capacity (percent volumetric water content of 22.5% v/v). Tubes were loosely capped with plastic tops to retard water loss, but allowed for gas exchange. Tubes were left to equilibrate overnight and the next day, they were maintained at nine constant (15, 25, 30, 35, 40, 45, 47.5, 50 or 53 °C) and two daily cycling (4 hours at 40 °C, 20 hours at 25 °C; 5 hours at 35 °C, 19 hours at 30 °C) temperature regimes. The tubes in the lowest range of constant temperatures (15 °C) were placed in a programmable refrigerated precision temperature incubator (stability: ± 0.5 °C; Model Hotcold-UB-2101505, Selecta®, Abrera, Barcelona, Spain). Those tubes in the range of constant temperatures above 15 °C were placed in a water bath (stainless steel tank of 15 cm depth x 62 cm length x 50 cm width) with an immersion circulation heater (stability: ± 0.2 °C; Model Termotronic-3000389, Selecta®). The two daily cycling temperature regimes were maintained in water baths with immersion circulation heaters connected to the mains through a programmable timer. The two cycling temperature regimes were chosen to simulate daily temperature fluctuations at two soil depths (15 and 30 cm) during summer solarization (6 August-22 September 2009) in a closed greenhouse located at Derio (Northern Spain), a zone characterized by a humid mild climate. The temperatures recorded during the latter period were used to build the cycling regimes and represented the optimum conditions for evaluation of solarization in that area (fig. 1). Heat assays at constant temperatures (45°, 50° and 53 °C) were also carried out in a water
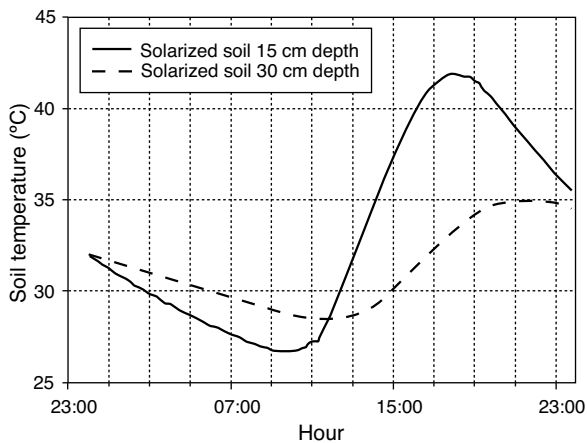
Figure 1. Hourly temperatures continuously recorded in solarized (moisten) soil for depths of 15 and 30 cm on 6 September 2009 in a greenhouse field experiment located at Derio (Northern Spain). Soil was tarped with 50-μm-thick (2 mil) transparent low density polyethylene plastic film from 6 August to 22 September 2009.

medium instead of in moistened soil in order to compare heat effectiveness in soil and in water, and with the aim to evaluate the use of hot water as a possible thermotherapy tool.

Exposure times were different at each temperature regime: at high temperatures rates, exposure times were less than at low levels. At each combination of temperature and exposure time, three mesh squares were removed and oospore viability percentages were measured.

Plasmolysis method

The plasmolysis method[17] was used to evaluate oospore viability. Mesh squares were introduced in 4 mol l⁻¹ sodium chloride solution for about 45 min and microscopically (Olympus CH40, x200) observed. The cytoplasm of the viable oospores was contracted to form a central ball-like structure and non-viable ones did not plasmolize as they had lost the differential permeability of their cellular membrane. Oospores were classified as viable (plasmolized) and unviable (non-plasmolized).

Quantification of oospores

In each different combination of temperature regime and exposure time, three glass test tubes (each containing one mesh square with embedded oospores) were used and 100 oospores were quantified in each mesh square. The exposed data, expressed as a percentage, were the average of three counts of 100 oospores. Each experiment was repeated once.

Statistical analysis

Oospore survival data points from constant temperature regimes in moistened soil were pooled and drawn in 3 dimensions against each exposure time for each temperature with Sigma Plot (version 10.0 for Windows) software (fig. 2). Relationships between the oospores viability percentage and the exposure time at each temperature regime were statistically determined by linear regression analysis, using the general-purpose procedure for regression (proc REG) of SAS (Statistical Analysis Software, SAS Institute, Cary, NC; release 9.1). The slopes of the regression lines for each temperature were compared with the use of an analysis of covariance, using the General linear models procedure (proc GLM). Viability percentage values were used as the dependent variable, temperature as class variable and the exposure time as covariate in a model
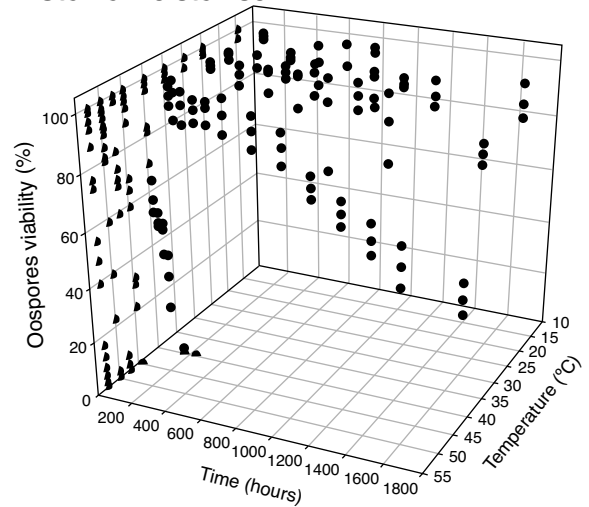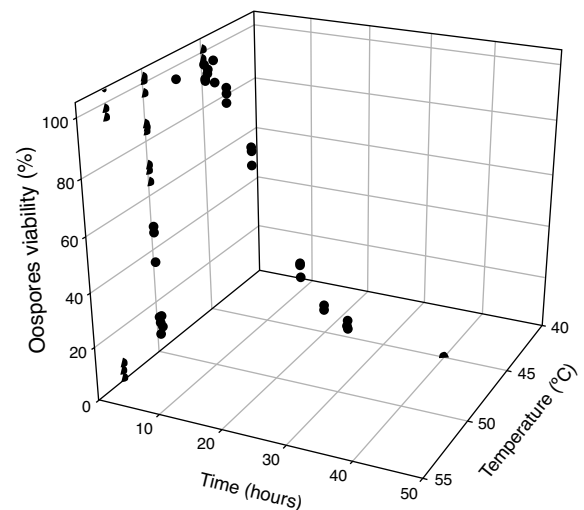

Figure 2. Thermal inactivation of *Phytophthora capsici* oospores at constant temperatures in different media. A. Sterile moistened soil (22.5% volumetric water content) (temperatures between 15 and 53 °C). B. Sterile water (temperatures between 45 and 53 °C).

that included temperature, exposure time and their interaction. At each temperature regime (constant or cycling), the lethal dose (LD) was defined as the exposure time required to reduce the oospores population by 25, 50, 75 or 100% (LD_{25}, LD_{50}, LD_{75} and LD_{100}) and was determined by the intersection of regression lines and horizontal lines corresponding to oospores viability values of 75, 50, 25 and 0% respectively (fig. 3). Exposure time at which 75% of the population of oospores was killed (LD_{75}) was plotted on a logarithmic scale against temperature. A new linear regression analysis of the data points was made according to Pullman et al[27] and repeated for LD_{25}, LD_{50} and LD_{100}. This provided a different regression line for each of the four LD percentages (fig. 4).

Results

Effect of constant temperature on the inactivation of oospores

Sigmoidal curves were obtained when oospores viability percentages were plotted against exposure times for each temperature in both assays (moistened soil and water) (fig. 2). Oospores were
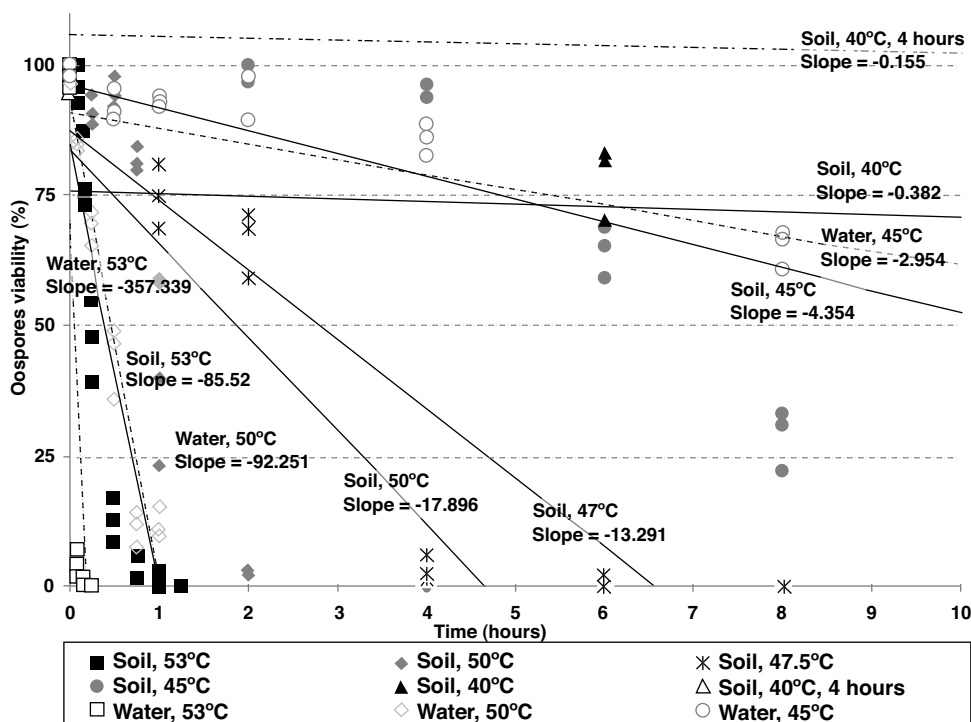
Figure 3. Regression lines corresponding to *Phytophthora capsici* oospores viability versus exposure time (first 10 hours) at different temperature regimes and media (constant temperatures in sterile moistened soil [continous lines]: 53, 50, 47.5, 45 and 40 °C; cycling temperature in sterile moistened soil [two dotted single dashed lines]: 40 °C for 4 hours and 25 °C for the remainder of each day; constant temperatures in water [dashed lines]: 53, 50 and 45 °C).

Table 1
Survival[U] of *Phytophthora capsici* oospores at each exposure time and each constant temperature regimen in different media[V].

Medium[V]-temperature (°C)	Exposure time (hours)												
	0.00	0.08	0.17	0.25	0.50	0.75	1.00	1.25	2	4	6	8	24
Soil[V]-53	100[X]	97.7[Y]	78.9	47.4	12.7	2.8	1.4	0.0					
Soil-50	100	-[Z]	-	91.5	94.8	81.9	40.7	-	2.6	0.4	0.0		
Soil-47,5	100	-	-	-	-	-	75.0	-	66.5	3.2	0.8	0.0	
Soil-45	100	-	-	-	-	-	-	-	100	93.0	64.6	28.8	0.0
Soil-40	100	-	-	-	-	-	-	-	-	-	78.5	-	58.2
Soil-35	100	-	-	-	-	-	-	-	-	-	-	-	90.2
Soil-30	100	-	-	-	-	-	-	-	-	-	-	-	-
Soil-25	100	-	-	-	-	-	-	-	-	-	-	-	-
Soil-15	100	-	-	-	-	-	-	-	-	-	-	-	-

Medium[V]- temperature (°C)	Exposure time (hours)												
	48	72	96	168	240	336	504	672	840	1,008	1,176	1,344	1,680
Soil-40	50.0	44.5	28.1	0.8	0.0								
Soil-35	89.8	-	87.1	86.3	86.7	86.3	81.0	73.5	65.9	58.3	50.7	43.2	35.6
Soil-30	-	-	-	100	-	98.0	97.1	99.6	88.2	95.5	86.5	-	82.0
Soil-25	-	-	-	100	-	100	95.7	97.6	-	98.0	-	95.7	
Soil-15	-	-	-	100	-	93.1	91.0	98.4	92.7	88.6	-	-	87.8

Medium[V]-temperature (°C)	Exposure time (hours)												
	0	0.08	0.17	0.25	0.5	0.75	1	2	4	8	16	20	24	40
Water[V]-53	100	4.2	0.5	0.0										
Water-50	100	84.8	-	68.8	43.8	11.2	12.0	0.0						
Water-45	100	-	-	-	92.0	-	93.1	95.0	85.8	65.1	24.1	10.0	5.0	0.0

[U] Survival assessments of oospores of *P. capsici* were determined by the plasmolysis method (Jiang and Erwin, 1990).
[V] Media: soil = sterile soil with moisture at field capacity (22.5% volumetric water content). Water = sterile water.
[X] At each temperature regimen, the values of oospores viability throughout exposure time are expressed as a percentage of the initial viability value.
[Y] Percentage values are the average of three counts of 100 oospores.
[Z] No data.

not affected by heat at temperatures equal or below 35 °C. The survival of oospores in moistened soil ranged from 88% at 15 °C to 36% at 35 °C (table 1; fig. 2) for an exposure time of 70 days (1,680 hours). However, heat sensitivity of oospores at temperatures above 40 °C was remarkable: treatments in moistened soil from 168 to 240 hours at 40 °C, from 8 to 24 hours at 45 °C, from 6 to 8 hours at 47.5 °C, from 4 to 6 hours at 50 °C and from 1 to 1.25 hours at 53 °C all proved to be lethal for oospores (table 1). Of note, time
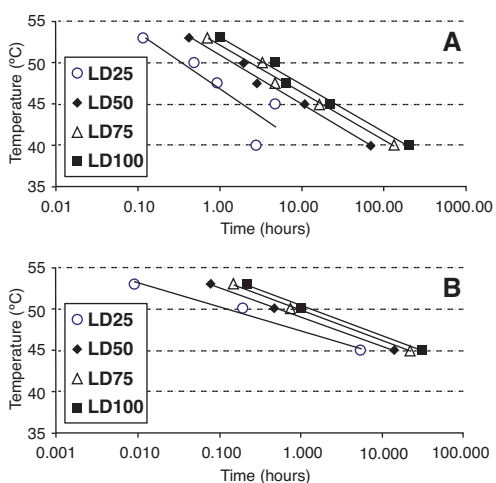
Figure 4. Times and temperatures exposures required to kill 25, 50, 75 and 100% (LD$_{25}$, LD$_{50}$, LD$_{75}$ and LD$_{100}$) of *Phytophthora capsici* oospores in different media. A. Sterile moistened soil (22.5% volumetric water content) (constant temperatures between 40 and 53 °C). B. Sterile water (constant temperatures between 45 and 53 °C).

required to achieve total lethality was significantly shorter in water, with values ranging from 24 to 40 hours at 45 °C, from 1 to 2 hours at 50 °C and from 0.17 to 0.25 hours at 53 °C (table 1).

This reduction in survival of *P. capsici* oospores was modeled using linear regression. The model selected for the comparisons among temperature regimes was $Y = a + b \cdot X$, in which Y is the oospores viability percentage and X is the number of hours of exposure to the temperature regime. A lack of fit of the model to the data was observed at temperatures below 35 °C with values of the coefficient of determination (R^2) equal to or lower than 0.35 (table 2).

As the temperature increased, the slope of the regression lines also showed an increase, implying that higher temperatures enhanced the rhythm of lethality. For constant temperatures in moistened soil, significant differences between the slopes of the regression lines were determined by analysis of covariance and pairwise comparisons between 53 and 50 °C ($P < 0.0001$), between 47.5 and 45 °C ($P < 0.0001$) and between 45 and 40 °C

($P < 0.0001$). Between 50 and 47.5 °C, there was no significant difference ($P = 0.1225$). The difference between the slopes of the constant and cycling exposures to 40 °C in moistened soil was also significant ($P < 0.0001$) (fig. 3; table 2).

For constant temperatures in water (53, 50 and 45 °C), the regression lines also showed significantly different slopes based on the analysis of covariance between 53 and 50 °C ($P = 0.0001$) and between 50 and 45 °C ($P < 0.0001$) (fig. 3; table 2).

Heat treatments in water inactivated oospores more rapidly than treatments in moistened soil at all the tested temperatures (53, 50 and 45 °C). These results are in agreement with the significant differences found in the slopes of the regression lines at 53 °C ($P = 0.0004$), at 50 °C ($P < 0.0001$), and at 45 °C ($P = 0.0107$). Notably, the oospores inactivation in water at 50 °C and in moistened soil at 53 °C showed no significant differences between the slopes ($P = 0.5939$) (fig. 3; table 2).

The analysis of the previous data is in accordance with the estimated exposure times required to kill a determined percentage of the oospore population: LD$_{100}$ values in water were only 0.22 hours at 53 °C and 1.01 hours at 50 °C, whereas in moistened soil these values were higher (0.99 and 4.68 hours respectively). However, LD$_{100}$ values were closer when comparing moistened soil and water at 45 °C (22.01 and 30.82 hours respectively), moistened soil at 50 and 47.5 °C (4.68 and 6.57 hours respectively), and moistened soil at 53 °C and water at 50 °C (0.99 and 1.01 hours respectively) (table 2).

Values obtained from the intersection points between regression lines and LD$_{25}$, LD$_{50}$, LD$_{75}$ and LD$_{100}$ percentages (fig. 3) were plotted on a logarithmic exposure time scale against temperature and new regression lines were obtained (fig. 4, A and B). These new regression lines showed highly significant determination coefficients in moistened soil and water, except the LD$_{25}$ parameter which was not significant (P values > 0.05) (table 3). The former models can be used to predict survival values at any exposure time with constant temperatures, ranging from 40 to 53 °C in moistened soil and from 45 to 53 °C in water.

Effect of cycling temperatures on the inactivation of oospores

The daily cycling temperature regime, consistent of 4 hours at 40 °C and 20 hours at 25 °C, killed 100% of oospores after 28 days, as opposed to the regime consistent of 5 hours at 35 °C and 19 hours

Table 2
Linear regression analysis[S] of survival[T] of oospores of *Phytophthora capsici* versus exposure time at each temperature regime (constant or cycling[U]) in different media[V]. Regression coefficients and estimated exposure times required to kill a determined population percentage.

Medium[V]	Temp (°C)	Slope	Intercept	R^2	P value[X]	LD$_{25}$[Z]	LD$_{50}$[Z]	LD$_{75}$[Z]	LD$_{100}$[Z]
Soil[V]	53	-85.517 B[Y]	84.95	0.80	< 0.0001	0.12	0.41	0.70	0.99
Soil	50	-17.896 C	83.78	0.69	< 0.0001	0.49	1.89	3.28	4.68
Soil	47.5	-13.291 C	87.33	0.84	< 0.0001	0.93	2.81	4.69	6.57
Soil	45	-4.354 D	95.85	0.82	< 0.0001	4.79	10.53	16.27	22.01
Soil	40	-0.382 F	76.04	0.85	< 0.0001	2.72	68.13	133.54	198.95
Soil	35	-0.045 H	96.17	0.75	< 0.0001	469.76	1,024.58	1,579.39	2,134.21
Soil	30	-0.010	100.39	0.35	0.0011				
Soil	25	-0.003	98.56	0.14	0.0952				
Soil	15	-0.007	97.81	0.29	0.0071				
Water[V]	53	-357.339 A	78.28	0.63	0.0020	0.01	0.08	0.15	0.22
Water	50	-92.251 B	92.98	0.94	< 0.0001	0.19	0.47	0.74	1.01
Water	45	-2.954 E	91.06	0.87	< 0.0001	5.44	13.90	22.36	30.82
Soil	40 (4)[U]	-0.155 G	105.63	0.94	< 0.0001	198.22	360.03	521.84	683.66
Soil	35 (5)[U]	-0.039 H	109.49	0.76	< 0.0001	896.29	1,548.98	2,195.67	2,845.36

[S]The model used for the analysis was: Viability = $a + b$ x (hours).
[T]Survival assessments of oospores were determined by the plasmolysis method (Jiang and Erwin, 1990).
[U]Cycling temperature regimes that simulated solarization consisted of temperatures increased daily to 40 or 35 °C for 4 and 5 hours respectively (length of time in parenthesis in hours); the remainder of each day was maintained at 25 or 30 °C respectively.
[V]Medium: Soil = sterile soil with moisture at field capacity (22.5% volumetric water content). Water = sterile water.
[X]Significance associated to the coefficient of determination (R^2).
[Y]Values of slopes followed by the same capital letter do not differ statistically ($P < 0.05$) according to the analysis of covariance.
[Z]Estimated exposure times (hours) at each temperature regime required to kill 25, 50, 75 and 100% of the oospores population respectively.

Table 3
Linear regression analysis[V] of constant temperatures versus exposure times required to kill a 25, 50, 75 or 100% population percentage (LD$_{25}$, LD$_{50}$, LD$_{75}$ and LD$_{100}$) of *Phytophthora capsici* oospores in moistened soil or in water.

Medium[X] (temperature range[Y])	Slope	Intercept	R^2	P value[Z]
Moistened Soil (40-53 °C)				
LD$_{25}$	-2.94	46.88	0.754	0.0562
LD$_{50}$	-2.56	50.86	0.988	0.0005
LD$_{75}$	-2.51	52.15	0.986	0.0007
LD$_{100}$	-2.49	52.97	0.985	0.0008
Water (45-53 °C)				
LD$_{25}$	-1.26	47.39	0.986	0.0755
LD$_{50}$	-1.54	48.99	0.999	0.0229
LD$_{75}$	-1.58	49.81	0.996	0.0400
LD$_{100}$	-1.59	50.35	0.995	0.0473

[V] The model used for the analysis was: Temperature = $a + b$ x ln (hours).
[X] Medium: Moistened soil = sterile soil with moisture at field capacity (22.5% volumetric water content). Water = sterile water.
[Y] Constant temperatures: 40, 45, 47.5, 50 and 53 °C in moistened soil; 45, 50 and 53 °C in water.
[Z] Significance associated to the coefficient of determination (R^2).

Table 4
Survival[V] of *Phytophthora capsici* oospores exposed to daily cycling temperatures regimes in moistened soil[X].

Temperature cycling regime	Exposure time (days)								
	0	7	14	21	28	35	42	49	70
40 °C 4 hours, 25 °C 20 hours	100[Y]	83.6[Z]	66.0	20.7	0.0				
35 °C 5 hours, 30 °C 19 hours	100	100	93.1	98.0	90.2	80.4	78.0	78.4	25.3

[V] Survival assessments of oospores were determined by the plasmolysis method (Jiang and Erwin, 1990).
[X] Sterile soil with moisture at field capacity (22.5% volumetric water content).
[Y] At each temperature regimen, the values of oospores viability throughout exposure time are expressed as a percentage of the initial viability value.
[Z] Percentage values are the average of three counts of 100 oospores.

at 30 °C, with which, after 70 days of exposure, 25% of oospores survived (table 4).

Discussion

Almost one century ago, heat started to be used to inactivate spores of different microorganisms, together with the implementation of the logarithmic transformation, and the application of simple linear regressions for the analysis of the results obtained [2,7,27].

Our results are in line with those reported in an early study demonstrating that the logarithmic relationship between time and temperature on the survival of four phytopathogenic soilborne fungi (microsclerotia of *Verticillium dahliae*, oospores of *Pythium ultimum*, chlamydospores of *Thielaviopsis basicola* and moniloid cells and runner hyphae of *Rhizoctonia solani*) was maintained at temperatures ranging from 37 to 50 °C[27]. Futhermore, in the latter study, it was observed that the exposure time required to kill 90% of *P. ultimum* oospores increased as the temperature decreased below 50 °C.

Seventy years ago, an early report described the results on the exposure to intermittent heat for the control of plant pathogens[14]. Since then, only a limited number of assays have been published [7,9,26,31]. An important fact is that the tolerance to heat exposure varies among different fungal species and consequently, further studies are needed to define the effect of heat in relationship to the different fungal plant pathogens[26].

Several authors have assessed laboratory thermal inactivation of different pathogens including pythiaceous fungi [2,3,7,18,27,32]. However, odd data available for heat inactivation of oospores only defines lethal treatment doses, with the exception of one current report in which survival models of thermal inactivation have been established with *Aphanomyces cochlioides* oospores[9].

In an early report on thermal laboratory inactivation[3], it was observed that 5-8 week old *P. capsici* oospores inoculated in moistened autoclaved soil and subjected to 50 °C for 30 minutes were still capable of producing disease in cucumber test plants. However, if survival of oospores was assessed by the plating technique on PDA medium instead of using test plants, 50 °C for 30 minutes was lethal. It has been shown that *Phytophthora cactorum* and *Phytophthora megasperma* oospores are less heat resistant: 45 °C for 30 minutes was lethal for oospores of *P. cactorum* and 45 °C for 20 minutes was sufficient to kill *P. megasperma* oospores (low temperature isolate). However, a high temperature isolate of *P. megasperma* survived (3-6%) a heat treatment of 45 °C for 30 minutes[18]. In other heat inactivation reports involving *P. ultimum*, *Pythium sylvaticum* and *P. aphanidermatum* oospores, the latter species was shown to be the most resistant one, and this fact is not surprising since it is considered a hot weather pathogen[3,27,32]. *Pythium aphanidermatum* oospores after 30 minutes exposure to 52.5 °C in moisten autoclaved soil were still capable of producing disease in cucumber test plants. Yet if the survival of oospores was assessed by the plating technique on PDA medium instead of using test plants, 52.5 °C for 30 minutes was lethal[3]. In the same way, *P. sylvaticum* oospores subjected to 50 °C for 30 minutes were not capable of producing disease, but when the survival of oospores was assessed by the plating technique, 45 °C for 30 minutes was lethal[3]. At 37 °C, the exposure time for LD$_{90}$ was 25.8 days for *P. ultimum* oospores and at 50 °C the LD$_{90}$ value was 27 minutes in moistened natural soil[27]. It also has been reported that *P. ultimum* thin-walled oospores were killed by heat at 50 °C for 30 minutes, but that thick-walled oospores needed higher temperatures (70 °C for 30 minutes)[32].

Our estimated LD$_{50}$ values in moistened soil were almost similar to those reported by Dyer et al[9] in water at 40, 45, 50 °C with *A. cochlioides* oospores. Considering that heat transmission in water is greater than in moistened soil for short periods of time[6], we can conclude that *P. capsici* oospores appear to be less heat tolerant than *A. cochlioides* oospores, even though the comparison between different published reports is hampered due to the different methodologies applied. Our estimated LD$_{50}$ values in water at 45 and 50 °C were lower than those estimated by Dyer at al[9]

and thus, it was reconfirmed that *A. cochlioides* oospores are more resistant than *P. capsici* oospores.

In conclusion, when comparing our results with those of other oospores of different species, such as *P. cactorum*, *P. megasperma*, *P. ultimum*, *P. sylvaticum* and *P. aphanidermatum* (and discussed above), *P. capsici* and *A. cochlioides* appear to need higher temperatures and/or longer exposure times to achieve eradication, and thus, their control is theoretically more difficult and complex.

In our work, using the plasmolysis method as viability test, oospores buried in moistened soil and heated at 50 °C during 30 minutes showed a survival rate of 95%, whereas between 4 and 6 hours of exposure time was needed to kill all the oospores (table 1). At the same temperature, through the use of linear regression, 4.68 hours was estimated as the necessary exposure time to achieve total lethality (table 2). Assessing oospore survival by plating techniques in selective agar media produces the problem in which germinability percentages for oospores of *P. capsici* are very low, ranging from 0 to 40%[15], and therefore, the survival of oospores is underestimated. In a solarization trial for the control of oospores of *P. capsici* and *Phytophthora nicotianae* buried at 10 and 25 cm soil depth[6], no consistent results were obtained because the survival evaluation was hindered by the poor germination rates. Only approximately 1% of the oospores were germinated in the *Phytophthora* selective agar medium PARPH[16].

In the present study, an exhaustive evaluation of temperature and time effect on oospore survival has been carried out with the aid of the plasmolysis viability test, leading to the construction of kinetic models for the study of thermal inactivation of *P. capsici* oospores. As in other previous reports[2,27], our models were calculated (by linear regression) relating the oospores viability with exposure time at each temperature and thus achieving satisfactory fitness. Nevertheless, within this model, constant temperatures of 30, 25 and 15 °C in moistened soil did not accurately describe inactivation. Instead, low determination coefficients were displayed (table 2). This could be explained by the fact that temperatures below 35 °C poorly affected oospore survival. A lack of fit of the model to the data was also observed at these temperatures in agreement with Coelho et al[7]. At constant temperatures of 40 °C or above, the required exposure times were low and the estimated models by linear regression fitted well with the data in moistened soil (R^2 values between 0.69 and 0.85). Our model was also well-fitted at a constant temperature of 35 °C ($R^2 = 0.75$) as well as at the two daily cycling temperature regimes of 4 hours at 40 °C ($R^2 = 0.94$) and 5 hours at 35 °C ($R^2 = 0.76$), despite the fact that the exposure times required to kill all the oospores were very high (2,134, 684 and 2,845 hours, respectively) (table 2). This is in keeping with the findings of the above mentioned study[7], that reported that *P. nicotianae* chlamydospores were hardly affected by temperatures of 35 °C. Maximum temperature for in vitro mycelium growth of *P. capsici* has been established between 35 and 36 °C[10], and consequently, it is not surprising that temperatures equal or below 35 °C do not affect the survival of oospores, since these propagules are widely considered more resistant than mycelium.

For this reason, solarization treatments in field conditions for the control of *P. capsici* will be most successful if application time and site conditions achieve temperatures above 35 °C. Our results obtained in the assay of daily cycling temperature regime (5 hours at 35 °C) proved that solarization would fail even if extended up to 10 weeks, since 25% of the oospores were still viable. As opposed to the above regime, the daily cycling temperature regime (4 hours at 40 °C) stamped out disease after 4 weeks (table 4).

Time and temperature data obtained in thermal inactivation studies with cycling temperatures under controlled environmental conditions may be used to develop reliable treatment guidelines for solarization under field conditions. Additionally, the cumulative number of hours at which soil temperatures exceed the threshold can be calculated to determine the duration of treatment necessary for the control of soilborne plant pathogens[8].

Since each pathogen requires a specific exposure time at each temperature for its control[8], thermal inactivation with cycling temperatures in controlled environmental conditions carried out in our study with *P. capsici* oospores can be useful in indicating the solarization effectiveness for the control of this pathogen under field conditions.

A method to relate cycling and constant temperature regimes can be very practical, because it offers the possibility to predict solarization effectiveness in each particular case based on the kinetic model of inactivation at constant temperatures.

The cumulative number of hours above 40 °C in the cycling regime (4 hours at 40 °C and 20 hours at 25 °C) required to kill 25, 50, 75 and 100% of the *P. capsici* oospores population in moistened soil were respectively: $LD_{25} = 33$, $LD_{50} = 60$, $LD_{75} = 87$ and $LD_{100} = 114$ hours. These values were obtained by dividing by six (= 24 hours day/4 hours at 40 °C) the total number of hours of incubation time in the cycling regime of 40 °C (table 2). When compared with the lethal doses estimated in the constant regime of 40 °C ($LD_{25} = 3$, $LD_{50} = 68$, $LD_{75} = 134$ and $LD_{100} = 199$ hours), the exposure times required in the constant regime were higher than in the cycling regime except for the LD_{25} value which was lower. This fact appears to indicate that once a minimum cumulative number of hours above 40 °C is achieved (which in our study has been estimated close to 60 hours), *P. capsici* oospores start to die faster in the cycling regime (when measured in the effective cumulative time). The latter observation could be caused by the weakening of oospores due to sublethal heating, which is a widespread phenomenon that has been demostrated with a variety of pathogens[1,12]. These authors speculate that an irreversible deterioration in viability of conidia of *Fusarium oxysporun* f. sp. *niveum* is inflicted by the heat shock, thus reaching a "point of no return" from which they can no longer recover. These authors also suggest that pathogen propagules might be affected under lower temperatures, possibly facilitating pathogen control by heating.

The results reported herein are also in agreement with Dyer et al[9] who determined that survival of *A. cochlioides* oospores exposed to alternating high and low temperatures in water (four 24 hours cycles at 45 °C for 4 hours and 21 °C for 20 hours) fitted closely to a cumulative effects model. They also confirmed that dormant oospores that survived a high temperature period remained dormant, accumulating heat-related damages.

The estimated cumulating hours above 35 °C were compared between the constant and the cycling regimes at 35 °C. The estimated exposure times required to kill the same percentage of oospore population were always lower in the cycling regime ($LD_{25} = 187$, $LD_{50} = 322$, $LD_{75} = 457$ and $LD_{100} = 593$ hours) than in the constant regime ($LD_{25} = 470$, $LD_{50} = 1,025$, $LD_{75} = 1,579$ and $LD_{100} = 2,134$ hours) as opposed to the constant and the cycling regimes at 40 °C. Of note, the LD values of the cycling regime at 35 °C were calculated by dividing by 4.8 (= 24 hours day/5 hours at 35 °C) the corresponding values shown in table 2. Our results at 35 °C are in agreement with Assaraf et al[1] who indicated that pathogen mortality can be achieved at temperatures that are lower than those needed for immediate full control, as long as sufficient time is allowed for the delayed heat mortality. The significance of this phenomenon can be applied to solarization, where soil heating is relatively mild.

Our study closely reproduces common temperature profiles observed in soil solarization assays by using soil moisture adjusted to field capacity. These conditions are equivalent to those found in real field conditions, except for the use of sterilized soil with the aim of the exclusive evaluation of thermal effects. Sublethal effects of high temperature, such as increased susceptibility of microbial infection, can also contribute to plant pathogens con-

trol under solarization[35]. Time and temperature requirements developed under sterile laboratory conditions will not reflect the possible contribution of antagonistic soil organisms or the release of chemical toxicants in the heated field soils to plant pathogens mortality[33,34]. The models constructed under these conditions are expected to overestimate the time necessary for pathogen death[8].

Consequently, the model developed in our study with sterile soils is also expected to overestimate the time needed for *P. capsici* oospores eradication.

Since constant and intermittent temperatures below $40\,^{\circ}\text{C}$ were not effective to inactivate *P. capsici* oospores, other alternatives that can improve these results have to be explored in the future. One possibility could be the combined use of sublethal temperatures and organic amendments for the improvement of *P. capsici* control by soil solarization. This can be especially useful in temperate regions with climatic conditions and a crop season unsuitable for the application of soil solarization. Under a particular climate and a compatible season with the crop production cycle, data presented in this study may provide a helpful tool for evaluating the effectiveness of solarization in reducing the soil oospores population of *P. capsici*, an economically important soilborne plant pathogen worldwide.

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Conflict of interests

Authors have no conflict of interests.

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