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Effect of temperature on survival of *Phytophthora kernoviae* oospores, sporangia, and mycelium†

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Abstract

Phytophthora kernoviae Brasier, Beales & Kirk, recently found in the UK and New Zealand, is a pathogen of more than 30 host species. It is not known to produce chlamydospores, but is homothallic and produces abundant oospores and sporangia.

This study was conducted to examine long-term survival of oospores, sporangia, and mycelium buried in sand at different temperatures. Viability of oospores buried in sand kept at 4, 10, 20 or 30 °C was assessed by staining with tetrazolium bromide solution. After 1 year at these temperatures, 82, 81, 79, and 58% of oospores of a New Zealand isolate respectively had survived. Corresponding values for an English isolate were 86, 75, 82, and 78%. Necrosis was observed on *Rhododendron* L. leaf discs exposed to oospores that had been buried for 1 year at temperatures below 30 °C. Oospores exposed for 1 and 6 h at 50 °C and 24 h at 40 and 50 °C were less viable than controls and did not germinate.

Sporangia or mycelium of two New Zealand and two English isolates introduced to moist sand and kept at different temperatures showed a population decline within 1 week. Numbers of colony-forming units then remained at a low but steady level over time. Sporangia and oospores were formed at 4, 10 and 20 °C but not at 30 °C. The ability of *P. kernoviae* to persist in sand for long periods of time at different temperatures is likely to be one of the factors determining the rate of spread of this pathogen.

Keywords: mycelium, oospores, persistence, *Phytophthora kernoviae*, sporangia, temperature

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Introduction

The Oomycete *Phytophthora kernoviae* Brasier, Beales & Kirk was first described in England, where it causes cankers on *Fagus sylvatica* L. (Brasier et al., 2005). This pathogen was not known to exist elsewhere until Ramsfield et al. (2009), using molecular techniques, found that some previously-undescribed *Phytophthora* sp. isolates in New Zealand matched *P. kernoviae* isolates from Cornwall, England. Recently, *P. kernoviae* has been reported from Scotland (Anonymous, 2008). To date, the species has not been isolated in the United States of America (USA) but there is concern about the damage it could cause there if it became established, due to its broad host range. Since its first discovery in 2003 on *F. sylvatica* and *Rhododendron* spp. (Brasier et al., 2005), *P. kernoviae* is now known to affect 34 host species (Department for Environment, Food and Rural Affairs (DEFRA), 2009).

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Phytophthora kernoviae is not known to produce chlamydospores. It is homothallic and produces large numbers of oospores and caducous sporangia (Brasier et al., 2005). The potential role of these propagules in the epidemiology of diseases caused by *P. kernoviae* is currently unknown. This study was undertaken in order to gain specific knowledge about the survival of propagules at different temperatures. Information gained was likely to be useful for the development of management strategies and for prediction of the extent of disease impact in other regions.

Materials and Methods

Pathogen

Four isolates of *Phytophthora kernoviae,* derived from soil or plant material in England and New Zealand (Table 1), were studied. All isolates were maintained for long term storage on Rye A agar (Caten & Jinks, 1968) medium and transferred to clarified 20% V8 agar (Mitchell & Kannwischer-Mitchell, 1992) medium during experimental use.

Experiment 1. Effect of temperatures up to 30 °C on oospore survival from two isolates

Oospores of *Phytophthora kernoviae* isolates PK-1 and PK-4 were produced in clarified 20% V8 broth (Mitchell & Kannwischer-Mitchell, 1992) for 6 weeks and released from the mycelium by sonication as described by Widmer (2010a). After sonication to release the oospores, the suspension was filtered through four layers of cheesecloth to separate the oospores from any residual mycelial fragments. The filtrate containing oospores was collected and the concentration of oospores in the filtrate was determined by counting the number in a diluted sample on a microscope slide. For each isolate tested, the filtrate was pipetted onto nylon screens (1 cm by 1 cm; 20-µm-mesh) sitting on a Büchner funnel so that there were approximately 1000 oospores per screen. Washed masonry sand, obtained from a local source (Frederick, MD, USA), with particles ranging in size of 0.6 to 0.15 mm, which has virtually no organic matter or added nutritional value, was sterilized by autoclaving. After cooling, it was adjusted to 7% moisture content with sterile distilled water and used to fill sterile plastic Petri plates (90-mm-diameter; approximately 40 cm^3). Thirty-two screens containing oospores were buried in the sand in each plate and the plates were wrapped with plastic wrap. There were two replicated plates for each isolate per treatment. The plates were kept at 4, 10, 20, or 30 °C in the dark. Four screens were removed from each plate after 1, 2, 4, 8, 16, 26, 34, and 52 weeks. This experiment was conducted three times.

Experiment 2. Effect of temperatures higher than 30 °C on oospore survival from four isolates

The effect of high temperature on survival of oospores of all four isolates was determined by modification of a procedure described by Fay and Fry (1997). Oospores were produced in clarified 20% V8 broth over a period of 6 weeks and were then released from the mycelium by sonication (Widmer 2010a). The broth was then passed through four layers of cheesecloth and frozen at -20 °C for 24 h to kill any residual, viable mycelium. After determination of oospore concentration in the suspension, seven 1-mL aliquots for each isolate were transferred to 1.8-mL sterile microcentrifuge tubes. Six of the seven tubes were immersed in water baths either set at 40 or 50 °C (three each). A tube, not exposed to high temperatures, was kept at 20 °C (ambient temperature) for a control. Tubes were removed from the water baths after 1, 6, and 24 h. Subsamples were assessed for oospore viability, infection potential and germination potential, described below. The experiment was conducted twice. Data were subjected to analysis of variance using PROC GLM in SAS for Windows version 9.2 (Statistical Analysis Software, Cary, NC, USA) and the means were separated using Duncan's Multiple Range test and were considered significant if $P \leq 0.05$.

TABLE 1: Description of *Phytophthora kernoviae* isolates used in the present study.

Isolate	Original Designation[a]	Origin	Original isolation	Year[b]	Source[c]
PK-1	ICMP 15082	Trounson Kauri Park, New Zealand	Soil	2003	TR; MC
PK-2	ICMP 14761	Northland, New Zealand	*Annona cherimola* Mill.	2002	TR; MC
PK-4	CSL 2378	Cornwall, United Kingdom	*Rhododendron ponticum* L.	2004	KH
PK-5	CSL 2286	Cornwall, United Kingdom	*Rhododendron ponticum* L.	unknown	KH

[a] *Isolate name designated by source.*

[b] *Year isolated.*

[c] *Name of originator: MC, Mike Coffey, University of California, Riverside, CA, USA; KH, Kelvin Hughes, The Food and Environment Research Agency (Fera), York, UK; and TR, Tod Ramsfield, Scion, Rotorua, New Zealand.*

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Analytical Methods for Experiments 1 & 2

Oospore viability test (after Sutherland & Cohen, 1983)

One of the four nylon screens per isolate removed at each time point in Experiment 1 or a 0.25 mL sample from Experiment 2 was placed in a microcentrifuge tube containing 0.25 mL of 0.1% tetrazolium bromide (MTT) solution and 0.25 mL of water. The tubes were wrapped in aluminum foil to exclude light and placed in an incubator at 35 °C. After 2 days, either the screen was removed in Experiment 1 or a sample of the oospore suspension in Experiment 2 was removed after vortexing and transferred to a glass slide. One hundred randomly selected oospores embedded on the screen or in the suspension were examined under a microscope. The number of purple- or rose-coloured spores was noted. For Experiment 1 only, regression analysis was used to compare relationships between temperature and percentage of stained (viable) oospores for each isolate. After arcsine square root transformation of the data, the slopes and y-intercepts of the regression lines were compared using SAS for Windows Version 9.2.

Potential of oospores to infect rhododendron leaves

For Experiment 1, three of the four screens per isolate removed at each time point were each placed on the abaxial side of an 11-mm-diameter disc taken from a *Rhododendron* 'Cunningham's White' leaf. In addition, three screens that were not imbedded with oospores were placed on leaf discs for non-inoculated controls. All of the leaf discs were then transferred to a plastic mesh screen, sitting on a piece of moist paper towel in a 90-mm-diameter Petri plate. The plates were covered with the lid and placed in an incubator at 20 °C in the dark. After 7 days, the leaf discs were removed and each was examined visually for necrosis. The leaf discs were then surface sterilised in 70% ethanol for 15 seconds, rinsed three times in sterile water, and plated on a *Phytophthora* selective medium (Ferguson & Jeffers, 1999) amended with 20 mL per liter of clarified V8 broth (PARPH+V8). The plates were incubated at 20 °C in the dark. After 4 days, the plates were inspected visually to verify *P. kernoviae* infection.

In Experiment 2, infection potential of the oospores of the controls and those exposed to higher temperatures for different time periods was determined. *Rhododendron* 'Cunningham's White' leaf discs (11-mm-diameter) were placed on a plastic mesh screen sitting on a moist paper towel in a Petri plate (90-mm-diameter). Aliquots containing 1000 oospores from each sample were pipetted onto the leaf discs. If necessary, a volume of water was first pipetted onto the discs so that the final liquid volume after addition of the oospores on each disc would be 50 µL. There were five discs per plate per treatment. The plates were covered and placed in the dark at 20 °C. After 7 days, the leaf discs were examined for necrosis, surface sterilized in 70% ethanol for 15 seconds, rinsed three times in sterile water, and plated on PARPH+V8 medium. After incubation for 4 days in the dark at 20 °C, the disks were examined visually for resulting growth of *P. kernoviae* into the agar.

Oospore germination test (Experiment 2 only)

After each exposure time and the initial control, an aliquot (50 µL) of each sample was pipetted into a well of a micro-titer plate containing 1 mL of sterile water. A second aliquot (50 µL) of each sample was pipetted into 500 µL of sterile water previously pipetted onto the surface of a plate containing PARPH+V8 medium. This diluted sample was mixed, and then distributed evenly over the surface of the plate with a glass rod. All the samples were incubated at 20 °C. The micro-titer plates were placed under continuous light while the PARPH+V8 plates were kept in the dark. After 3 days, the percentage of oospore germination was determined for both treatments by observing 100 randomly chosen oospores and counting those that germinated. An oospore was considered germinated if an emerging germ tube was at least the diameter of the oospore or there was a resulting sporangium. The plates were re-examined after a further 3 days.

Experiment 3. Effect of temperature on survival of sporangia

Sporangium production was stimulated using a method described by Widmer (2010b). Five plugs (5-mm-diamter) of V8 agar containing mycelium taken from the edge of an actively growing culture of Isolates PK-1, PK-2, PK-4 or PK-5 were placed in a 50-mm-diameter Petri plate containing 6 mL of 10% clarified V8 broth. Plates were closed but not sealed and placed in an incubator at 20 °C under continuous cool-white fluorescent lighting (approximately 3000 lux). After 5 days, sporangia were abundant. No oospores were observed. Sporangia were released from the mycelium by shaking the plate and the broth was filtered through four layers of cheesecloth. The concentration of sporangia in the filtrate was determined and a portion of the suspension added to autoclaved sand (moisture content 7%) to achieve a density of approximately 200 sporangia/cm^3.

An estimate of the actual sporangium density in the sand was made at this stage. After thorough mixing, a 5 cm^3 sample of sand was added to 95 mL of 0.2% water agar slurry. Following further mixing, 1 mL aliquots were plated out on PARPH+V8 medium with five plates being prepared for each isolate. After a 4-day incubation period at 20 °C in the dark, the sand was gently washed away and the number of colony-forming units (CFU) recorded. Population density was

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determined by multiplying mean CFU by the dilution factor.

The main sand culture of each isolate prepared above was divided into four portions, each of which was placed in a plastic bag. Bags were closed, but not sealed, and placed in incubators in the dark at 4, 10, 20, or 30 °C. Samples were taken after 1, 2, 4, and 8 weeks, then at 4-week intervals up to 1 year and plated out on PARPH+V8 medium, as described above. The procedure was conducted three times for each isolate at each temperature.

Direct observation of the propagules in sand culture was done after 1, 4, and 8 weeks, when a 5 cm^3 portion was mixed with 10 mL of sterile water by vortexing for 10 s. After settling for 5 s, the supernatant liquid was passed through a 100-µm sieve on to a 20-µm sieve. A minimal amount of water was used to transfer collected material to a 50-mm diameter Petri plate. A stereoscope was used to identify *P. kernoviae* propagules.

Experiement 4. Effect of temperature on survival of mycelia

Mycelium of isolates PK-1, PK-2, PK-4 and PK-5 was generated by transferring five agar plugs (5-mm-diameter), taken from the edge of an actively growing colony, to 20 mL of clarified 20% V8 broth and incubating under continuous fluorescent lighting at 20 °C. The plugs were removed aseptically after 4 days and 2 mL of sterile-filtered 1 mM trifluoroperazine was added to each culture to inhibit production of *Phytophthora* propagules (Judelson & Roberts, 2002; Widmer, 2009). Cultures were examined after 7 days. Development of oospores in PK-1 and PK-2 precluded their inclusion in this part of the investigation.

Mycelium of isolates PK-4 and PK-5 was removed from the broth, placed in a blender cup with 20 mL sterile water and blended for 20 s. After settling for 1 min, the suspension was blended for a further 20 s and then poured into a plastic bag containing 1000 cm^3 sterile sand (moisture content 3%). The blender cup was rinsed with 20 mL of sterile water and the rinsings were added to the sand. The final moisture content was 7%.

The sand culture prepared for each isolate was mixed well. The initial density of *P. kernoviae* CFU was measured by plating on PARPH+V8 medium. A 5 cm^3 sample was removed then added to 95 mL of 0.2% water agar slurry. Aliquots (1 mL) of this suspension were spread on each of ten PARPH+V8 plates. After a 4-day incubation period at 20 °C in the dark, the sand was gently washed away and the CFU recorded. Population density was determined by multiplying the mean CFU by the dilution factor.

The remaining contents of the bags of sand were divided into four approximately equal portions. Each portion was transferred to a new plastic bag that was closed but not sealed and placed in an incubator at 4, 10, 20 or 30 °C. Samples taken after 1, 2, 4, 8, 12, 16, 20, and 24 weeks were plated out on PARPH+V8 medium as described above. The whole procedure was conducted three times for each isolate/temperature combination. Direct observation of propagules in the sand was done after 1, 4 and 24 weeks using the procedure described above for sporangia.

Results and Discussion

Oospores of isolates PK-1 and PK-4, embedded on nylon screens and stored at a temperature of 4, 10, or 20 °C, were subsequently associated with necrosis in at least one rhododendron leaf disc at all times tested. Colonisation by *P. kernoviae* was confirmed in each case. No necrosis was observed on the control discs exposed to screens without oospores. For oospores stored at 30 °C, necrosis and infection of rhododendron leaf discs was not observed beyond 2 weeks of exposure to PK-1 or 1 week of exposure to PK-4, even though oospore viability was relatively high (>70 and 60% for PK-1 and PK-4, respectively) for up to 52 weeks (Figure 1). After transformation to linearise the data (data not shown), slopes of the viability/time regression lines at different temperatures were not significantly different for either PK-1 (P = 0.355) or PK-4 (P = 0.942). Differences between the y-intercepts (P<0.0001) indicated a significant effect of temperature on viability.

One explanation for the discrepancy between lack of necrosis and apparent oospore viability could be that the oospores became dormant at 30 °C. *Phytophthora* oospore dormancy is known, but not well understood (Boutet et al., 2010; Lippman et al., 1974; Lutz & Menge, 1986). Exposure to lower temperatures may be needed to break dormancy on certain species. Blackwell (1943) recommended exposing *P. cactorum* oospores to 1°C to break dormancy, but Zentmeyer & Erwin (1970) did not see an increase in *P. megasperma* var. *sojae* oospores when exposed to this temperature. According to Sutherland and Cohen (1983), staining with MTT can differentiate dormant (rose-coloured) and active (purple-coloured) oospores. In this study, it was difficult to distinguish colour differences and no attempt was made to determine dormancy. Singh et al. (2004) reported a decrease in positively-stained oospores of *P. infestans* (Mont.) deBary after burial in sand in the sub-tropical plains of India for 150 days where temperatures reached up to 44 °C. They also reported that the time required for germination increased from 2 days in newly-formed oospores to 48 days in oospores buried for 150 days under the stated field conditions. If *P. kernoviae* oospores behave in the same manner, a period longer than 7 days may

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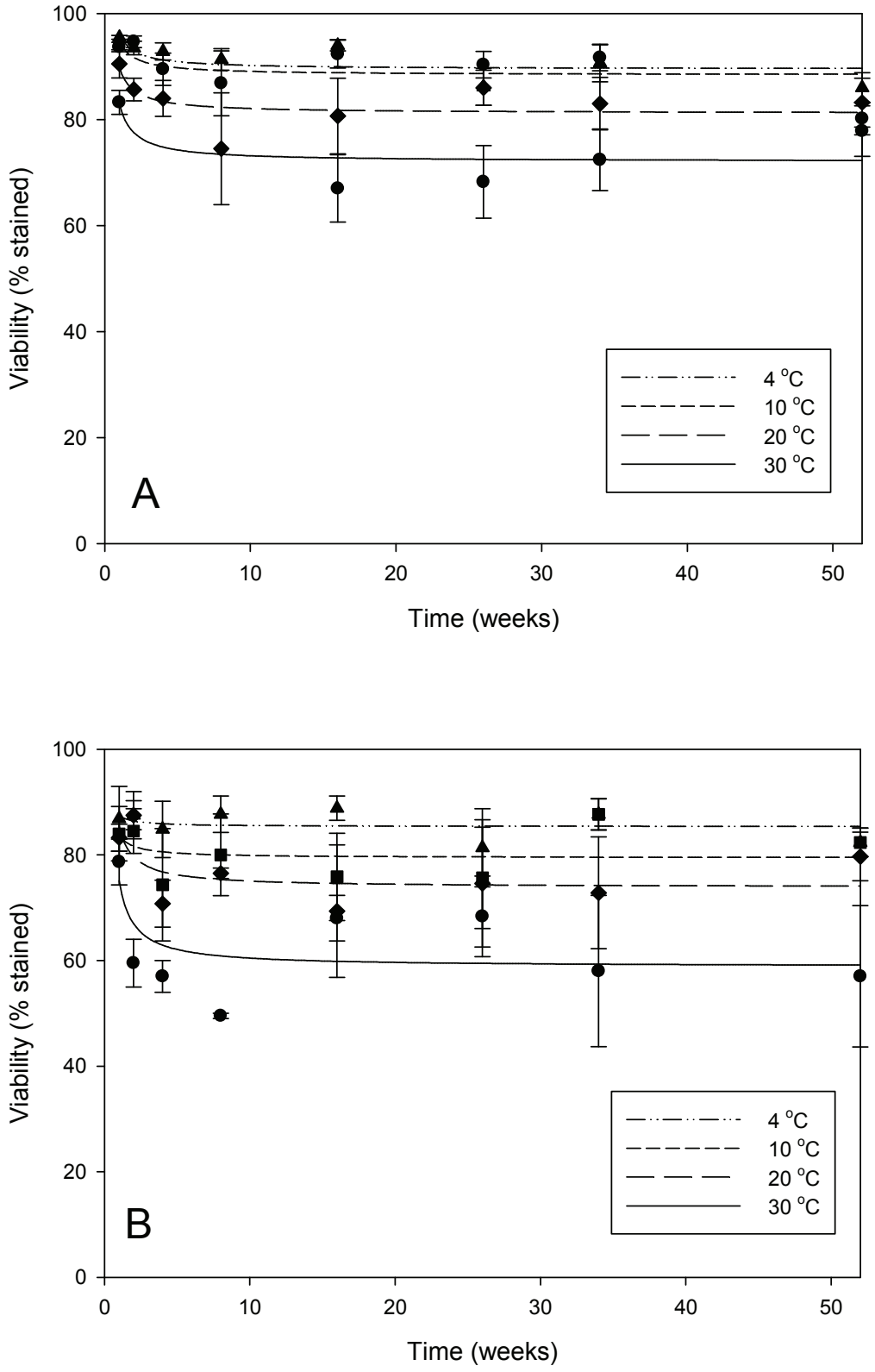

FIGURE 1: *Phytophthora kernoviae* oospore viability following burial in moist sand at 4, 10, 20, and 30 °C for increasing periods of time. A: Isolate PK-1 (New Zealand); B: Isolate PK-4 (England). Lines indicate relationships based on an inverse first order equation giving the best fit to actual data points for 4 (▲), 10 (■), 20 (♦), and 30 °C (●). Bars represent standard error of the means.

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have been required for viable spores to germinate and cause infection.

Exposure to high temperatures of 40 or 50 °C reduced viability and germination of oospores (Table 2). At 40 °C, germination rate was reduced after 1 h and completely inhibited after 6 h. At 50 °C, no germination was observed. However, no observations were made beyond 6 days, and the work of Singh et al. (2004) suggests that dormant spores might have germinated after a longer period.

In the present study, Experiment 2 showed that infection occurred only when leaf discs were inoculated with oospores that had not been exposed to high temperature or had been kept at 40 °C for only 1 h. Drenth et al. (1995) reported that *Phytophthora infestans* oospores exposed to temperatures of 40 °C or higher were not infectious. Our observations of the inhibition of oospore germination after exposure to 40 °C for periods exceeding 1 h or to 50 °C for 1 h are similar to those of Fay and Fry (1997) for *P. infestans* and Juarez-Palacios et al. (1991) for *P. cactorum* (Leb. & Cohn) Schröeter.

Differences between isolates that had not been exposed to high temperature (i.e. controls) were observed in terms of viability (P = 0.0091) and germination in water (P = 0.0203), but not germination on PARPH+V8 (P = 0.174). There was no difference of oospore germination observed after 3 and 6 days (data not shown). No isolate effect was apparent among spores kept at 40 or 50 °C (data not shown). The fact that *Phytophthora kernoviae* oospores do germinate on PARPH+V8 medium, albeit at a lower rate than in water (Table 2) indicates that an assay method for detection of viable oospores in soil could be based on use of a *Phytophthora*-selective medium. Oospore survival may be affected by the proximity of host material. Tooley et al. (2008) compared the germination rate of *P. ramorum* chlamydospores in infected rhododendron leaf discs with the rate in liquid suspension and found that a longer period of incubation at 40 °C was required for inhibition of germination in leaf discs. Oospore survival in plant material was not tested in the present study.

Data for survival of sporangia and mycelium in sand are not shown because the originating propagule type for colonies developing on PARPH+V8 could not be confirmed. The number of CFU observed after plating-out of sand treated with a suspension of sporangia declined to 5-10% of the original after exposure for 1 week at 4, 10, or 20 °C. After approximately 16 weeks, population size remained steady at approximately 1-2% of the original until observation ceased at 52 weeks. No CFU developed from sand samples exposed to temperatures of 30 °C. Linderman and Davis (2006) detected *Phytophthora ramorum* in soil that had been treated with a suspension of sporangia and kept at 20 °C for 6 months, but did not attempt a soil extraction to determine the propagule type. In the present study, empty sporangia were observed in sand extracts after 1 week of exposure to 4, 10, 20, or 30 °C. Sporangia containing cytoplasm were observed in sand kept at 10 °C for 1 week. After 4 weeks at temperatures of 4, 10, or 20 °C, only empty sporangia and some mycelium were seen. Oospores were observed after an 8 week exposure to temperatures of 4, 10 or 20 °C. This suggests that CFU observed on the PARPH+V8 medium after more than 1 week were likely to have arisen from cysts or oospores.

TABLE 2: Effect of high temperature on viability and germination of *Phytophthora kernoviae* oospores from four isolates (PK-1, PK-2, PK-4 and PK-5) at different exposure times.

Temp. (°C)	Time (h)	% viability [1]				% spores germinated in water [2]				% spores germinated PARPH+V8 [2]			
		PK-1	PK-2	PK-4	PK-5	PK-1	PK-2	PK-4	PK-5	PK-1	PK-2	PK-4	PK-5
20 [3]	0	91	94	89	72	53	74	46	18	34	46	31	8
40	1	88	84	81	59	18*	11*	10*	4*	6*	3*	7*	5
50	1	60	71	39*	34*	0*	0*	0*	0*	0*	0*	0*	0*
40	6	74	81	60*	46	0*	0*	0*	0*	0*	0*	0*	0*
50	6	10*	14*	4*	13*	0*	0*	0*	0*	0*	0*	0*	0*
40	24	17*	3*	4*	6*	0*	0*	0*	0*	0*	0*	0*	0*
50	24	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*

[1] Viable oospores were determined as those stained rose or purple after exposure to tetrazolium bromide solution.

[2] Germinated oospore were determined as those where the germ-tube diameter was equal or greater than oospore diameter after 3-days at 20 °C under continuous light.

[3] Ambient temperature

** Mean values in the same column differ from the Control value (20 °C) at P<0.05 (Duncan's Multiple RangeTest).*

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Numbers of CFU derived from sand treated with a suspension of mycelium decreased to about 50% of the original after 1 week when kept at temperatures of 4, 10, or 20 °C. A slight increase to approximately 60% was observed until the 12th week, after which there was a decline to approximately 20%. This level was maintained until observation ceased at 24 weeks. Some of the mycelium (representing less than 1% of the original) survived for up to 2 weeks at 30 °C. After 1 week, direct observation revealed only mycelium in the sand extract. After 4 weeks at 4, 10, or 20 °C, mycelium, sporangia and oospores were observed. Oospores were more abundant and observed to germinate, which terminated into a sporangium, after 24 weeks. The spike in CFU numbers after 2 weeks may have been related to the release of zoospores from recently-formed sporangia after plating out on the PARPH+V8 medium.

It is not possible to make definitive conclusions on the survival of sporangia or mycelium specifically in sand over time at various temperatures from this data. However, *Phytophthora kernoviae* is clearly able to persist in sand for long periods of time at temperatures up to 20 °C through formation of new propagules. This observation is similar to that made by Duncan (1980) for *P. fragariae* Hickman. In his study, mycelium was not detectable in soil stored for 63 days at either 15 or 30 °C, but naturally infested soil remained infective for 8 months at 15 °C. Duncan concluded that persistence of *P. fragariae* in field soils must depend on oospore formation. Shishkoff (2007) reported that *P. ramorum* mycelium and chlamydospores buried in mesh bags in sand survived at low levels (approximately 10%) for a year under greenhouse conditions.

This study has shown that *Phytophthora kernoviae* populations are unlikely to be eliminated from soil by leaving an infested soil fallow for up to 1 year. It is, however, acknowledged that survival may be affected by soil moisture, as shown by Matheron and Porchas (2009) for *P. nicotianae* Breda de Haan and by Roberts et al. (2005) for *P. capsici* Leonian. Only one moisture level was investigated in the present study. The saprophytic behavior of *P. kernoviae* was not included in this study, but *Phytophthora* spp. are considered to be poor saprophytes in soil (Malajczuk, 1983) and, therefore, must rely on propagule formation for long-term survival. It is not surprising that sporangia were observed over time in sand, which has no nutritive value that was originally infested with mycelium only. Some *Phytophthora* spp. require the nutrients to be washed away before significant sporangia production occur (Ribeiro, 1983). Although the exact nutritional status of the sand used in the present experiments was not determined, the vast amount of organic matter would have been washed away in the processing of the sand. The formation of oospores should also not be surprising since it has been hypothesised that oosporgenesis is induced by the exhaustion of a nitrogen source and the amount of sugar remaining in the medium (Elliott, 1989). In this study, propagules were observed at a temperature of 4 °C in sand, which is in contrast to what was reported earlier on artificial medium at the same temperature (Widmer, 2010a). It is unknown whether this is related to the nutritional status of the growing conditions.

Conclusion

Oospores of *Phytophthora kernoviae* can survive for long periods at temperatures of 30 °C and below. Their viability is reduced by exposure to higher temperatures (40 °C for 6 or 24 h; 50 °C for 1, 6 or 24 h). These results are similar to those reported for other *Phytophthora* species. Individual sporangia and mycelial fragments do not survive in sand at 30 °C for more than 1 or 2 weeks, respectively. Persistence of *P. kernoviae* in soil at lower temperatures is most likely related to the ability to produce new propagules (oospores and sporangia).

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