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Potential for eradication of the exotic plant pathogens *Phytophthora kernoviae* and *Phytophthora ramorum* during composting

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Temperature and exposure time effects on *Phytophthora kernoviae* and *Phytophthora ramorum* viability were examined in flasks of compost and in a large-scale composting system containing plant waste. Cellophane, rhododendron leaf and peat-based inoculum of *P. kernoviae* and *P. ramorum* isolates were used in flasks; naturally infected leaves were inserted into a large-scale system. Exposures of 5 and 10 days respectively at a mean temperature of 35°C in flask and large-scale composts reduced *P. kernoviae* and *P. ramorum* inocula to below detection limits using semi-selective culturing. Although *P. ramorum* was undetectable after a 1-day exposure of inoculum to compost at 40°C in flasks, it survived on leaves exposed to a mean temperature of 40·9°C for 5 days in a large-scale composting system. No survival of *P. ramorum* was detected after exposure of infected leaves for 5 days to a mean temperature of ≥41·9°C (32·8°C for *P. kernoviae*) or for 10 days at ≥31·8°C (25·9°C for *Phytophthora pseudosyringae* on infected bilberry stems) in large-scale systems. Fitted survival probabilities of *P. ramorum* on infected leaves exposed in a large-scale system for 5 days at 45°C or for 10 days at 35°C were <3%, for an average initial infection level of leaves of 59·2%. RNA quantification to measure viability was shown to be unreliable in environments that favour RNA preservation: high levels of ITS1 RNA were recovered from *P. kernoviae*- and *P. ramorum*-infected leaves exposed to composting plant wastes at >53°C, when all culture results were negative.

Keywords: *Phytophthora pseudosyringae*, *Rhododendron ponticum*, temperature, *Vaccinium myrtillus*

Introduction

The oomycetes *Phytophthora kernoviae* and *Phytophthora ramorum* are pathogens introduced to the United Kingdom (UK). *Phytophthora ramorum* has also been introduced to many other European countries, as well as the USA where it causes the widespread death of native oaks in California and Oregon known as sudden oak death (Harnik *et al.*, 2004; Jennings, 2008). More recently in the UK the widespread death of Japanese larch (*Larix kaempferi*), caused by *P. ramorum*, has been reported (Webber *et al.*, 2010). *Phytophthora kernoviae* is also present in New Zealand where it was first reported in 2006 (Sansford, 2008). Both pathogens have a wide plant host range, including members of the *Fagacaeae* such as oaks and beech, members of the *Lauraceae*, as well as plants in several other taxonomic families (Linderman & Davis, 2006). In the UK, *Rhododendron* has been particularly susceptible (Sansford & Woodhall, 2007; Sansford, 2008), and the introduced and invasive *Rhododendron ponticum* is an important intermediate host and source of multiplication and promotion of pathogen inoculum for susceptible native plants such as bilberry (*Vaccinium myrtillus*; Beales *et al.*, 2009) as well as for trees. Detached leaves and stems infected with *P. ramorum* can remain infectious for several weeks and *P. ramorum* and *P. kernoviae* can survive for at least 2 years in soil, so that management of plant wastes in infected areas is problematic and poses an ongoing phytosanitary risk (Swain *et al.*, 2006; Sansford & Woodhall, 2007; Sansford, 2008). Composting of contaminated plant waste is a potential management option. Temperatures achieved in well-managed composting systems are usually sufficient to 'eradicate' most plant pathogens including *Phytophthora* species, where eradication is defined as 'reducing the inoculum to below a detectable limit' (Noble & Roberts, 2004; Noble *et al.*, 2009). Exposing infected plant material for 2 weeks in compost reaching at least 55°C has been shown to be effective in eliminating *P. ramorum* (Swain

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et al., 2006), although the efficacy of shorter and cooler composting processes in achieving eradication has not been investigated. Previous research into the effect of exposure of *P. ramorum* inoculum to different time-temperature combinations *in vitro* and *in planta* has produced conflicting results (Noble *et al.*, 2009). This may, at least partly, be due to the inocula containing different structures with differing resiliency such as chlamydospores, sporangia, oospores and mycelial fragments. Jennings (2008) showed that *P. kernoviae* was eradicated *in vitro* and *in planta* after exposure to temperature treatments that were effective in killing *P. ramorum*. However, there are no previous studies on the efficacy of composting in eradicating *P. kernoviae*.

Semi-selective culturing followed by morphological identification of *Phytophthora* colonies is the usual method for detecting *Phytophthora* viability, but this does not provide quantitative measures of viability of inocula (Noble *et al.*, 2009). Swain *et al.* (2006) used a PCR assay to detect *P. ramorum* DNA post-composting, but could not distinguish live and dead material. RNA quantification by real-time polymerase chain reaction (RT-PCR) has been used as an alternative method to measure viability of *Phytophthora* inoculum (Chimento *et al.*, 2008). As part of this study, we have assessed this approach on composted samples with comparison to viability results obtained from semi-selective culture.

The aims of this work were to establish the minimum compost temperatures and exposure times required to eradicate *P. ramorum* and *P. kernoviae*. The influence of controlled temperatures on inocula containing different types of propagules of known isolates was examined in flasks of compost, followed by studying the influence of measured temperatures on naturally infected leaves in a large-scale composting system containing shredded plant wastes. The influences of compost temperatures and exposure times on *P. kernoviae* and *P. ramorum* were compared with the influences on another *Phytophthora* species that infects heathland bilberry, *Phytophthora pseudosyringae* (Beales *et al.*, 2010).

Materials and methods

Phytophthora species and inoculum

Phytophthora kernoviae isolate CC95 (Food and Environment Research Agency (Fera), York) and *P. ramorum* isolate CC47 (Fera) were used for the bench-scale flask experiments. Each isolate was grown on *Rhododendron cuneatum* cv. Cunningham's White leaves, cellophane discs by placing the discs over agar cultures, and in a peat-based medium (Noble *et al.*, 2011). The *P. ramorum* leaf and cellophane inocula contained hyphae, sporangia, and chlamydospores; the *P. kernoviae* leaf and cellophane inocula contained hyphae, sporangia, and oospores. Previous measurements conducted on the same types of leaf inocula produced under the same conditions have shown a mean of eight sporangia and four chlamydospores per 100 mm^2 for *P. ramorum* and five sporangia per 100 mm^2 for *P. kernoviae*. The cellophane disc inoculum contained hyphae and sporangia at concentrations of 180 colony forming units (CFU) mm^{-2} for *P. ramorum* and 170 CFU mm^{-2} for *P. kernoviae*. The initial peat-based inoculum contained hyphae and chlamydospores at a concentration of $2{\cdot}3 \times 10^6$ CFU g^{-1} for *P. ramorum* and hyphae and oospores at a concentration of $7{\cdot}2 \times 10^5$ CFU g^{-1} for *P. kernoviae*. Three squares (10×10 mm) of leaf and cellophane inocula were placed individually in polyester mesh bags with 3 g of the composted green waste used in the flasks. Sample bags were also filled with 3 g of peat inoculum.

For large-scale composting systems, naturally putative diseased leaves, predominantly rhododendron and *Viburnum* spp., with symptoms typical of *P. kernoviae* or *P. ramorum* and diseased bilberry stems with symptoms typical of *P. pseudosyringae* were used. The levels of infection with each *Phytophthora* species in the samples of putative diseased leaves or stems were determined as *post hoc* using semi-selective plate culturing.

RNA quantification

Inoculum samples from the composting tests described below were analysed by reverse transcriptase (RT) real-time PCR (RT-Taqman) assays, to quantify RNA and hence live *Phytophthora* cells, because RNA is very labile in exogenous conditions. RNA was extracted from 100 mg of inoculum using the RNeasy Plant mini kit (QIAGEN). DNA was removed according to the manufacturer's instructions by prolonged incubation with RNAse-free DNAseI. Two Taqman assays were designed to specifically detect *P. ramorum* and *P. kernoviae*, based on the assay of Hughes *et al.* (2006) which targeted the ITS1 region of nuclear ribosomal DNA. The *Cox*I region used previously (Chimento *et al.*, 2008) was found not to be sufficiently polymorphic to allow design of assays specific to *P. ramorum* and *P. kernoviae*. Oligonucleotides were as follows (5′–3′): *P. ramorum*, forward = CT GGCTTCGGCTGGCT, reverse = AAAAGTGGGCT ACTAGCTCAGAC, probe = 6′FAM-CTCTATCATGG CGAGCGCTTGAGCCT-TAMRA; *P. kernoviae*, forward = CTCGTTGGCAGTTTCGACTGT, reverse = GGTTTAAAAAAGAAACTCTCAATTCCGACT, probe= 6′FAM-AGTTCTATCAAACGATCGATTTGGGCT GCA-TAMRA. RT-Taqman reactions were run as follows in 25 μL volumes in an ABI7900 SDS machine: $1 \times$ Absolute QPCR ROX Mix (Thermo); 5 U Superscript III reverse transcriptase (Invitrogen), 5 mM dithiothreitol, 0·5 μM each oligonucleotide. Run parameters were: 42°C 30 min, 95°C 10 min; and 40 cycles of 95°C 15 s and 60°C 1 min. Cycle threshold (Ct) values were recorded and equivalent *Phytophthora* genomes per reaction were calculated using previous calibrations of known numbers of *P. ramorum* chlamydospores (using an average of 12 genomes each) and *P. kernoviae* oospores (data not shown). These calibrations were only intended to provide a relative scale between composting

conditions and not to indicate absolute numbers of spores or genomes.

Bench-scale flask composting

Composting was conducted in bench-scale equipment consisting of Quickfit multi-adapter flasks (Fisher Scientific) immersed in thermostatically controlled water baths, each holding up to four 2-L flasks (Noble *et al.*, 2011). Each flask contained 1200 g of compost and was connected to ancillary equipment to aerate the compost for 2 min in every 15 min at a flow rate of 150 mL min^{-1}. The temperature of the compost was monitored with Squirrel® multipoint temperature data loggers (Grant Instruments) and gaseous conditions in the compost using methods described in Noble *et al.* (2011). The green waste compost used in the flasks was prepared in turned windrows using wastes from parks and gardens and fruit and vegetables for 9 months (Organic Recycling Ltd). Water was added to the compost to obtain an initial moisture content of 41 (±1)% w/w (Table 1).

The following compost temperature × exposure time treatments were examined on three types of inoculum of *P. kernoviae* and *P. ramorum* in an incomplete factorial design experiment: 18 and 35°C for 1, 3, 5 and 10 days; 40 and 45°C for 1 and 3 days. There were between two and six replicate flasks of each temperature treatment (Table 2). Each flask contained sufficient sample bags of each *Phytophthora* species and inoculum type to enable a bag of each species and type to be retrieved at each of the exposure times. Sample bags containing *P. kernoviae* and *P. ramorum* leaf inoculum exposed to compost at 35 and 45°C for 1 or 3 days were also kept for a further 14 days at 20°C in the compost to detect viability after potential constitutive or heat-induced 'dormancy'. Two replicate flasks of each temperature treatment contained additional cellophane, leaf and peat inoculum of *P. ramorum*, which was tested for viability post-composting by RNA quantification. All the remaining *Phytophthora* inocula retrieved from sample bags post-composting were tested for viability by semi-selective plate culture on PDA + PARPH agar (Mitchell *et al.*, 1986). Plates from cellophane and leaf inocula were classed as positive if mycelia or spores characteristic of *Phytophthora* were present; plates from peat samples were classed as positive if germinating chlamydospores or oospores were detected by the emergence of hyphae. The detection limit in the peat-based inoculum was 33 CFU g^{-1} for both *P. kernoviae* and *P. ramorum*.

Large-scale composting

Composts were prepared at Trengwainton Gardens, Penzance, Cornwall in open bays measuring 2·9 × 2·2 × 2 (height) m internally, and at Cannock Chase, Staffordshire in an insulated cargo container measuring 3·7 × 2·4 × 2·6 (height) m internally. The bays were constructed of insulated wooden walls on three sides mounted on a concrete base. After filling with *c.* 2·9 t wetted plant wastes (mainly rhododendron at Trengwainton and bilberry at Cannock Chase) to a height of *c.* 1·5 m, the open ends of the bays or containers were closed by horizontal wooden boards fitted into vertical slots on the ends of the side walls, and the wastes were covered with an insulating waterproof layer. The temperature of the wastes was allowed to rise through the natural composting process for about 7 days. Samples of putative *P. kernoviae* and *P. ramorum* infected leaves or *P. pseudosyringae* infected bilberry stems were enclosed in 500 × 300 mm nylon mesh bags containing 2 kg of the same plant wastes that were in the composting system, together with a temperature probe connected to a data logger (Grant Instruments). The bags were inserted at different locations in the composting systems (centre, corners, sides), at the surface and at depths of 0·7 and 1·5 m (base), to obtain a range in exposure temperatures. Gaseous conditions in the composts were measured as described previously.

Four compost batches were prepared in bays at Trengwainton from shredded and wetted woody wastes, predominantly rhododendron (Table 1). In the first batch, six to eight putative *P. kernoviae* or *P. ramorum* infected leaves were placed in each bag and 30 leaves were used as day 0 controls. In the subsequent three batches, nine putative infected leaves were inserted in the bags and 81–100 leaves were used as day 0 controls. Depending on the batch, between 8 and 21 bags were inserted into the composting wastes, and between 4 and 17 bags were retrieved after 5 and 10 days. In the third and fourth replicate batches, bags of leaves were only positioned in the corners and sides of the bay because these were considered to be the most critical positions in terms of compost temperature and pathogen survival.

Table 1 Properties of green waste composts used in flasks and of wastes used in large scale composting systems, before wetting, and at the start and at the end of the temperature incubation experiments, and gaseous conditions in the materials during incubation. Each value is the mean of at least three replicate samples

	Moisture (% w/w)			pH		Ash (% DM)				
Compost or waste	Before	Start	End	Start	End	Start	End	O_2 (% v/v)	CO_2 (% v/v)	NH_3 (mg m^{-3})
Green waste compost	32	41	38	7·6	7·4	76·5	78·0	17·4	2·8	0·4
Woody plant wastes	48	63	56	6·1	6·5	2·6	3·7	13·1	4·2	0·2
Bilberry waste	45	64	61	5·1	5·9	3·8	5·2	12·7	4·6	0

Table 2 Viability of *Phytophthora kernoviae* and *Phytophthora ramorum* in different inoculum media following different temperatures and exposure times in flask composts, determined by semi-selective culturing. Each flask contained three samples of cellophane and leaf inoculum and a sample of peat-based inoculum

		Temperature (°C)							
		18		35		40		45	
Time (days)	Medium[a]	*P. kernoviae*	*P. ramorum*	*P. kernoviae*	*P. ramorum*	*P. kernoviae*	*P. ramorum*	*P. kernoviae*	*P. ramorum*
1	Cellophane[b]	67	100	0	72	0	0	0	0
	Leaf[b]	100	100	0	78	0	0	0	0
	Peat[c]	$1{\cdot}3 \times 10^4$	$1{\cdot}8 \times 10^4$	0	$1{\cdot}4 \times 10^4$	0	0	0	0
3	Cellophane	72	94	0	6	0	0	0	0
	Leaf	100	83	0	39	0	0	0	0
	Peat	$3{\cdot}1 \times 10^4$	$9{\cdot}3 \times 10^3$	0	$2{\cdot}4 \times 10^3$	0	0	0	0
5	Cellophane	0	100	0	17	–[d]	–	–	–
	Leaf	67	100	0	17	–	–	–	–
	Peat	$1{\cdot}0 \times 10^5$	$3{\cdot}7 \times 10^4$	0	$4{\cdot}2 \times 10^3$	–	–	–	–
10	Cellophane	0	100	0	0	–	–	–	–
	Leaf	83	100	0	0	–	–	–	–
	Peat	$7{\cdot}1 \times 10^3$	$1{\cdot}0 \times 10^5$	0	0	–	–	–	–

[a]Each value is the mean of six flasks, except for 5 and 10 day, and 40°C treatments ($n = 2$) and 3 days at 45°C treatments ($n = 4$).
[b]Mean values for cellophane and leaf samples are percentages of positive samples.
[c]Mean values for peat samples are colony forming units per gram.
[d]Dash indicates treatment not tested.

Three compost batches were prepared in the cargo container at Cannock Chase from chopped and wetted bilberry waste (Table 2). In each batch, five putative *P. pseudosyringae* infected bilberry stems were inserted in each of eight bags; 18–24 putative infected stems were used as day 0 controls.

After retrieval from the composts, putative *P. kernoviae* and *P. ramorum* infected leaves were divided in two, with one half used for semi-selective plate culturing and the other half used for RNA analysis as described earlier. Viability of *P. pseudosyringae* on retrieved stems was determined by semi-selective plate culturing.

Statistical analysis

Relationships between compost temperature and exposure time and *Phytophthora* survival in the large-scale systems were investigated using a logistic regression (a generalized linear model with a logit link function). This compared the proportion of the leaves or bilberry stems in each bag that were still positive for *P. kernoviae*, *P. ramorum* or *P. pseudosyringae* at the end of the experiment. However, in order to account for the fact that not all leaves or stems were positive at the start of the experiment and that this proportion differed between the compost batches, an offset variable (the logarithm of the proportion of positive leaves or stems at the start of each compost batch) was used. This allowed for the fact that bags from composts where larger proportions of *Phytophthora* positives were observed at day 0 could be expected to have a greater probability of containing *Phytophthora* positives at the end of the compost batch than bags from other batches. All bags from the fourth compost batch from Trengwainton were only used in statistical analysis for *P. ramorum* because none of the day 0 or post-composting leaves were positive for *P. kernoviae*. All analyses were conducted in GENSTAT 12·1 or 13·1.

Results

Bench-scale flask composting

During flask incubation, aerobic conditions were maintained in the composts, there were low but detectable levels of ammonia, and the moisture content of the compost declined slightly (Table 1).

The shift from positive to negative results was sudden and occurred in the same temperature × time treatment for almost all flask replicates, which prevented any mathematical model to be fitted to the data (Table 2). In peat-based inoculum bags where survival was detected post-composting, there were at least 889 CFU g^{-1} for *P. kernoviae* and 367 CFU g^{-1} for *P. ramorum* at the end of the composting process, although the mean values of the smallest numbers of detected propagules were slightly higher (Table 2). These values represent log reductions of 3·4 and 3·3 from the original inoculum concentrations. There was consistent survival of the inoculum of both species in compost at 18°C for up to 10 days; the exception was for *P. kernoviae* as cellophane inoculum, which lost some viability after 1 and 3 days and did not survive after 5 and 10 days (Table 2). *Phytophthora kernoviae* survived as peat-based inoculum exposed to compost for 1 day at 35°C but did not survive this treatment as cellophane or leaf inocula. There was no survival of *P. kernoviae* after exposure to compost for 3 days at 35°C or for 1 day at 40°C. No viability was detected in

the leaf inoculum samples that were exposed to compost to 35 or 45°C for 1 or 3 days, and then for a further 14 days at 20°C, i.e. all values were zero (dormancy testing).

Phytophthora ramorum, as cellophane, leaf and peat-based inocula, survived in compost at 35°C after 5 days but was undetectable after 10 days (Table 2). There was no survival of *P. ramorum* after exposure to compost at 40°C for 1 day. No viability was detected in the leaf inoculum samples that were exposed to compost at 35 or 45°C for 3 days or to 45°C for 1 day and then for a further 14 days at 20°C, i.e. all values were zero.

ITS1 RNA levels (Fig. 1) showed a clear decrease with increasing compost temperature (to 35°C) and exposure time (to 3 days) for *P. ramorum* leaf and cellophane inoculum, but not for peat inoculum. No ITS1 target was detected after 1 day of incubation at 45°C in any of the three types of inoculum. At 35°C, results between inoculum types were variable: cellophane and peat inoculum maintained a low level of RNA after 3 days (Fig. 1a,c), whereas none was detected on leaves after 3 days (Fig. 1b). Generally the level of ITS RNA showed the same trend as the culturing results (Table 2), except cultures from leaves were positive after exposure to 35°C for 3 days whereas no RNA was detected. Also, after a 3-day exposure to compost at 18°C, all inoculum types produced positive cultures but no RNA was detected in leaf and peat samples. In the flask experiments, RNA analysis was conducted on different samples to those used for culturing, so some incongruity between the test results could be expected. RNA results for inoculum samples exposed to compost for an additional 14 days at 20°C (dormancy testing) were all negative.

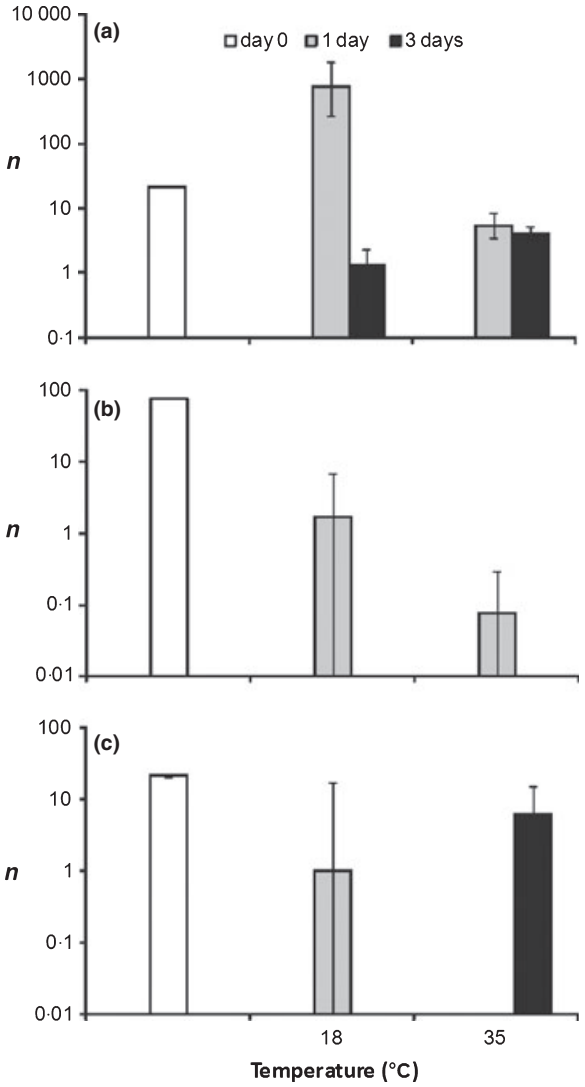

Figure 1 Mean relative amount of ITS1 RNA, as measured by RT-Taqman PCR, in (a) cellophane (b) leaf and (c) peat samples of *Phytophthora ramorum* inoculum following different temperatures and exposure times in flask composts. Error bars are the 95% confidence limits of the measurements. No RNA was detected in samples exposed to compost at 45°C. Each value is the mean of two replicate flasks.

Large-scale composting

Mean and maximum temperatures of 58–64°C and 62–70°C respectively were achieved in the centre of each of the four batches of shredded plant wastes at Trengwainton. The minimum mean temperatures in the composting plant wastes were recorded in the lower corners of the bay (15–44°C). Mean ambient air temperatures ranged from 6°C during the fourth composting batch to 18°C during the second composting batch. Mean and maximum temperatures in the centre of the three batches of composting bilberry waste at Cannock Chase were lower than those recorded at Trengwainton (49–53°C and 58–60°C respectively). The minimum mean temperatures in the composting waste were recorded in the lower corners of the container (23–29°C). Mean ambient air temperatures were 11–15°C. Standard deviations in temperature of the composting wastes were about 10% of the mean values in each recording location at both sites.

The plant wastes used in the large-scale systems had higher moisture contents and lower pH values and ash contents than the green waste composts used in the flasks. During the 10-day composting periods, compost moisture declined and pH increased slightly (Table 1). Mean O_2 and CO_2 concentrations in the composting plant wastes were lower and higher respectively than those recorded in the flasks (Table 1). Ammonia concentrations in woody plant waste composts at Trengwainton were only just detectable with gas detector tubes; no ammonia was detected in the composting bilberry wastes at Cannock Chase (Table 1).

Post-composting, leaves that tested positive for *P. kernoviae* or *P. ramorum*, or bilberry stems that tested positive for *P. pseudosyringae* were only retrieved from the corners of the composting systems. All leaves retrieved from bags positioned in the surface, base, sides, back, front and centre of the composting bay, where mean compost temperatures were ≥41·9°C over a 5-day

period, tested negative for both *P. kernoviae* and *P. ramorum*. All bilberry stems retrieved from the same relative positions in the composting container, where mean compost temperatures were ≥37·3°C over a 10-day period, tested negative for *P. pseudosyringae*. There were strong negative effects of mean compost temperature on the proportions of leaves that tested positive for *P. kernoviae* (P = 0·001, Fig. 2a) or *P. ramorum* (P = 0·001, Fig. 2b) or stems that tested positive for *P. pseudosyringae* (P = 0·001, Fig. 2c) post-composting. The fitted regressions in Figure 2 have the following equation:

$$\ln(P/(1-P)) = bT + a + \ln(I_0) \qquad (1)$$

where P is the proportion of positive *P. kernoviae* or *P. ramorum* leaves or *P. pseudosyringae* stems after composting (after exposure times of 5 or 10 days), T is the mean compost temperature, I_0 is the proportion of positive *P. kernoviae* or *P. ramorum* leaves or *P. pseudosyringae* stems in the day 0 samples, and a and b are the values of the model parameters (intercept and slope). The fitted values in Eqn (1) for a, b and the observed value for I_0 for exposures in composting plant wastes of 5 and 10 days of *P. kernoviae* and *P. ramorum* infected leaves and *P. pseudosyringae* infected stems are shown in Table 3.

The 10-day exposure in composting plant wastes resulted in a significantly (P = 0·003) smaller proportion of leaves that tested positive for *P. kernoviae* than the 5-day exposure. There was no evidence that the effect of mean compost temperature on the proportion of leaves that tested positive for *P. kernoviae* was different between the exposure times. However, only six observations (out of 46) showed some survival of *P. kernoviae* after composting (four after 5 days and only two after 10 days). After 5-days exposure to mean compost temperatures exceeding 32·8°C, there were no leaves that tested positive for *P. kernoviae* (Fig. 2a).

Unlike for *P. kernoviae*, there was no overall difference between the two compost exposure times, 5 and 10 days, in the proportion of leaves that tested positive for *P. ramorum* post-composting in the large-scale system. However, there was a significant interaction (P = 0·002) between the effects of compost temperature and exposure time, with a more rapidly decreasing proportion of *P. ramorum* survival with increasing temperature for leaves exposed for 10 days than for those exposed for 5 days. This is shown by the greater negative slope for 10 days than for 5 days exposure in Table 3. After exposure to compost for 5 days, there were no leaves that tested positive for *P. ramorum* at compost temperatures of ≥41·9°C; the corresponding temperature for a 10-day exposure was 31·8°C. The fitted survival probabilities of *P. kernoviae* and *P. ramorum* on leaves, or *P. pseudosyringae* on stems, based on all the test data, indicate that survival is still a small possibility, even at higher temperatures (Fig. 2). The probabilities of obtaining a positive result for *P. kernoviae* or *P. ramorum* on leaves or *P. pseudosyringae* on stems, exposed to compost for 5 days at 45°C or for 10 days at 35°C, for the initial mean proportions of

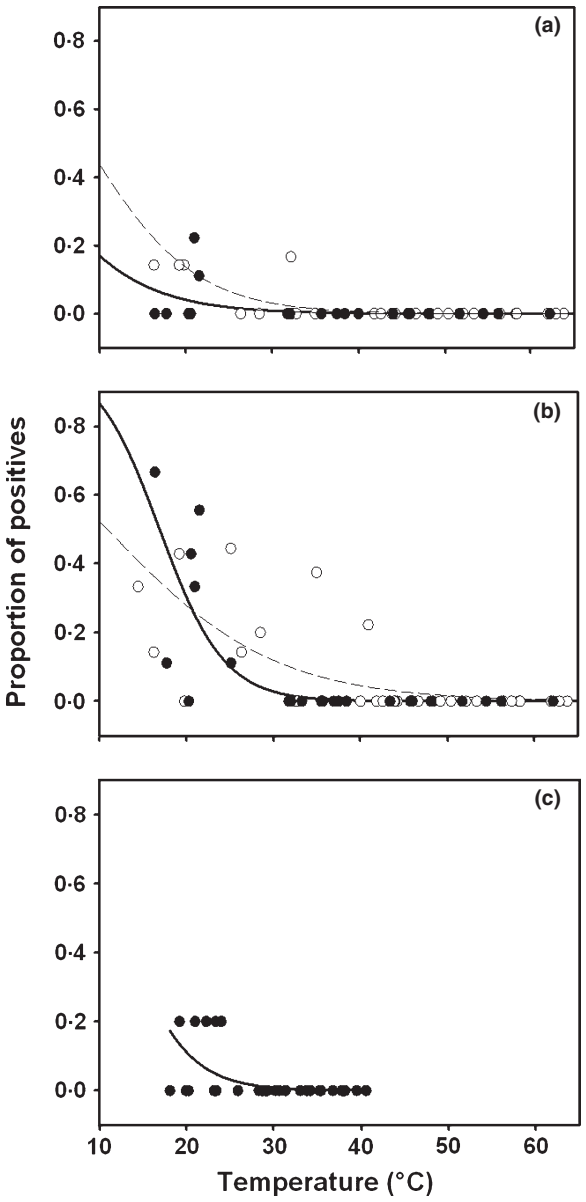

Figure 2 Fitted regressions for mean compost temperature and proportion of positive leaves of (a) *Phytophthora kernoviae*, (b) *Phytophthora ramorum* and (c) *Phytophthora pseudosyringae* after 5 days (○ and dashed line) and 10 days (● and solid line) in composting plant wastes in a large-scale system. The values in the regression equation (1) are shown in Table 3.

infected leaves or stems shown in Table 3, were <3% (Table 4).

RT-Taqman results from the large-scale composting experiment showed the same trend as the culture results, with a logarithmic decline in ITS1 RNA in both species correlated with increasing temperature (Fig. 3). However, in both *P. kernoviae* and *P. ramorum* there were two important differences between ITS1 RNA measurements and viability as assessed by culturing. First, in the temperature range 41–45°C no viable cultures were

Table 3 Fitted slope and intercept, on the logit scale, for the regressions between mean compost temperature and the proportion of positive leaves with *Phytophthora kernoviae* or *Phytophthora ramorum* or positive stems with *Phytophthora pseudosyringae* after exposure to composting plant wastes for 5 or 10 days in large-scale systems. I_0 is the mean proportion of positive leaves or stems at day 0

Phytophthora species	Exposure (days)	Slope (SE[a])	Intercept (SE)	I_0
P. kernoviae	5	−0·16 (0·04)	3·83 (0·84)	0·084
P. kernoviae	10	−0·16 (0·05)	2·46 (1·11)	0·084
P. ramorum	5	−0·10 (0·02)	1·67 (0·63)	0·592
P. ramorum	10	−0·27 (0·06)	5·10 (1·26)	0·592
P. pseudosyringae	10	−0·27 (0·08)	4·24 (1·27)	0·404

[a]Standard error.

Table 4 Fitted probability and 95% confidence intervals of obtaining *Phytophthora kernoviae* and *Phytophthora ramorum* positive leaves or *Phytophthora pseudosyringae* after exposure to composting plant wastes for 5 days at 45°C or 10 days at 35°C in large-scale systems, for the mean proportions of positive leaves at the start (I_0) in Table 3

	Fitted probability (and 95% confidence intervals) (%)	
Phytophthora species	5 days at 45°C	10 days at 35°C
P. kernoviae	0·29 (0·05, 1·59)	0·40 (0·09, 1·79)
P. ramorum	2·73 (1·14, 6·39)	0·76 (0·13, 4·27)
P. pseudosyringae	–	0·22 (0·02, 1·90)

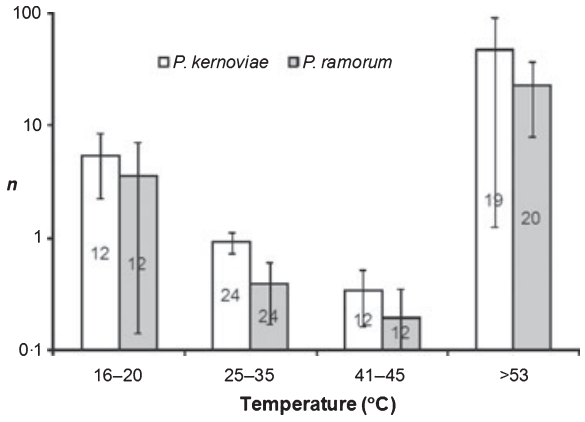

Figure 3 Mean relative amount of ITS1 RNA, as measured by RT-Taqman PCR, in leaves retrieved from four temperature ranges in a large-scale composting system. Error bars are the 95% confidence limits of the measurements at each temperature. The value in each bar is the number of samples in each temperature range.

found, but RNA was still detected at approximately 5% of the level observed at 16–20°C; second, at temperatures >53°C there was a higher level of ITS1 RNA than that recovered from leaf samples exposed to a temperature range of 41–45°C.

Discussion

The observation that *P. kernoviae* has a lower compost temperature tolerance than *P. ramorum* is in agreement with *in vitro* results of Turner *et al.* (2008) who showed that mycelium of *P. kernoviae* was killed after shorter exposure periods and at lower temperatures than mycelium of *P. ramorum*. Although *P. ramorum* was undetectable after a 1-day exposure to a constant flask compost temperature of 40°C, leaf inoculum survived a 5-day exposure to a mean temperature of 40·9°C in composting plant wastes in a large-scale system. These results are in broad agreement with those of Tooley *et al.* (2008) who reported that *P. ramorum*, as free chlamydospores and in rhododendron tissue, could survive a 4-day treatment at 35°C but not at 40°C. The pathogen survived a 2-day treatment at 40°C as chlamydospores but not in infected leaf tissue. Turner *et al.* (2008) found that wet heat treatment at 40°C was effective in killing *P. ramorum* mycelium after 15 min, at 42·5°C was effective in killing sporangia after 10 min, and at 45°C was effective after 20 min in eradicating *P. kernoviae* and *P. ramorum* from leaves inoculated with sporangia 24 h earlier. Swain *et al.* (2006) showed that *P. ramorum* could not be isolated from infested leaves, wood chips and cankered stems after 24 h exposure to dry heat at 40°C. Harnik *et al.* (2004) were able to reisolate *P. ramorum* from artificially inoculated California bay laurel (*Umbellularia californica*) leaves held at 55°C for up to 1 week, although such temperature tolerance has not been demonstrated by other workers. Linderman & Davis (2006) found that *P. ramorum* survived as chlamydospores for 12 months and as sporangia for 6 months in soil and various potting mix components but could not be detected in infected rhododendron pieces after 1 month.

The majority of tests on other *Phytophthora* species, including *P. cactorum*, *P. cinnamomi*, *P. infestans*, *P. megasperma*, and *P. nicotianae*, have shown that exposure to a temperature of ≤50°C for ≤7 days is sufficient to achieve eradication, as defined earlier (Noble & Roberts, 2004; Noble *et al.*, 2009). However, Noble *et al.* (2009) found survival of oospores of *P. nicotianae* after exposure to compost at 54°C for 7 days, and Downer *et al.* (2009) found survival of *P. cinnamomi* for up to 7 days in composting piles that reached 60°C. Results here indicated that compost temperatures that were effective in reducing inocula of *P. kernoviae* and *P. ramorum* to be below detectable limits were also effective for plant material infected with *P. pseudosyringae*.

Previous studies have shown RNA quantification as a reliable indicator of viability in *Phytophthora* (Chimento *et al.*, 2008). In this study, this was found to be true only within a certain temperature range, i.e. within which enzymatically driven RNA degradation (both intra- and extracellular) can operate. However, at elevated temperatures, RNA degradation no longer corresponded with

Phytophthora viability and ITS1 RNA levels increased above 53°C, compared to lower temperatures, even though no viable *Phytophthora* was found in the cultures of the same samples. The most likely explanation for this is that elevated temperatures prevented enzymatic degradation of RNA. Juvonen *et al.* (2010) also observed this effect after heat treatment of *Lactobacillus*, but it was not observed by Yaron & Matthews (2002) in *Escherichia coli* after heat treatment. Many RNAses, e.g. RNAseA, are known to be thermally stable, but it could be the case that the compost environment was not favourable for such thermostable enzymes or that they were not present. It is also possible that heat shock temporarily raised expression levels of ITS1 RNA prior to killing *Phytophthora* cells, therefore giving the observed increased RNA level compared to lower incubation temperatures since heat shock has been shown to raise rRNA expression in other organisms (de León & Mellado, 1997; Carlson *et al.*, 1999). A combination of chemical, physical and biological factors is likely to influence the persistence of RNA in different environments. Therefore without prior validation of non-cellular RNA in such conditions and their careful control during experiments, RNA cannot reliably be used as an indicator of viability. It should be noted that this may be true for any species, not just *Phytophthora*. This problem could be overcome by measuring RNA only after a further brief incubation of a sample at optimum temperature for the target organism, just long enough to allow degradation of exogenous RNA from any dead organisms; however, this would negate the quantitative and speed advantages of using RNA and RT-Taqman methods. Also, if the RNA preservation were due to chemical conditions the extra incubation would still not degrade RNA from dead cells. In complex environments such as the compost examined here, it cannot be determined beforehand whether RNA preservation may occur.

The higher temperature tolerance of *P. ramorum* observed in the large-scale composting system compared with that observed in the flasks may be due to differences in the natural and artificially produced inoculum and/or the substrates used, especially peat, such as water potential. Turner *et al.* (2008) and Jennings (2008) found that exposure to dry heat required longer and hotter treatments to achieve eradication of *P. ramorum* than exposure to wet heat. This is consistent with the effect of compost moisture on eradication temperatures of *P. nicotianae* and other pathogens (Noble *et al.*, 2009). Although the plant wastes used in the large-scale system had a higher gravimetric moisture content than the flask composts, much of this was contained within stems and leaves, rather than the freely available moisture applied to the mature compost used in the flasks. The mature flask compost had a higher pH value than the plant wastes used in the large-scale system (Table 1). Waste feedstocks have been found to exert an effect on the temperatures needed to eradicate *P. nicotianae*, although the effect of compost pH within the range 6–9·5 on pathogen eradication is usually minimal (Noble *et al.*, 2009). Even in the absence of elevated temperatures, composts, through biotic and abiotic antagonism, are known to reduce the viability of the inocula of *Phytophthora* species (Noble & Coventry, 2005) including *P. ramorum* (Fichtner *et al.*, 2009).

Mean temperature values were used in the fitted regressions for compost temperature and the proportion of *Phytophthora* positive leaves (Fig. 1). This was justified in that the standard deviations in compost temperatures in sampling locations were small due to the composting wastes first being allowed to rise in temperature before leaves were inserted and the composting system being insulated against subsequent heat loss. However, in situations where compost temperatures are more variable, other parameters such as peak temperature or day-degrees may be more appropriate for relating temperatures to pathogen survival characteristics (Noble & Roberts, 2004; Noble *et al.*, 2009).

Temperatures above those required to reduce levels of *P. kernoviae*, *P. ramorum* and *P. pseudosyringae* to below detectable limits were achieved in the majority of the composting systems filled with shredded and wetted woody and leaf wastes from rhododendron and other shrubs and trees, or chopped bilberry. Survival of these pathogens was only detected in infected leaves positioned in bags in the cooler upper and lower corners of the bay. Survival risk was minimized by refilling the composting wastes into a second bay where the separated corner material was positioned into the centre and then achieved sanitizing temperatures on reheating. By using the results of this work, the risk of survival of *P. kernoviae*, *P. ramorum* and *P. pseudosyringae* in a composting process can be estimated by monitoring time-temperature profiles in different locations, particularly in waste in the sides and corners of the system. This information can be used in decisions regarding the subsequent use, disposal or further treatment requirements of the composted waste. A well-managed and monitored composting system can therefore be considered to be an effective and phytosanitary treatment method for plant wastes infected with the *Phytophthora* species studied in this work.

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