Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Composting is an effective treatment option for sanitization of *Phytophthora ramorum*-infected plant material

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Keywords
exotic pathogen, heat treatment, molecular detection, oomycete, plant substrates, quantitative PCR, sanitation, sudden oak death.

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2005/1148: received 30 September 2005, revised 8 February 2006 and accepted 9 February 2006

doi:10.1111/j.1365-2672.2006.03008.x

Abstract

Aims: To determine the effects of heat and composting treatments on the viability of the plant pathogen *Phytophthora ramorum* grown on both artificial and various natural substrates.
Methods and Results: *Phytophthora ramorum* was grown on V8 agar, inoculated on bay laurel leaves (*Umbellularia californica*) and on woody tissues of coast live oak *(Quercus agrifolia)*. Effects on growth, viability and survival were measured as a result of treatment in ovens and compost piles. Direct plating onto PARP medium and pear-baiting techniques were used to determine post-treatment viability. No *P. ramorum* was recovered at the end of the composting process, regardless of the isolation technique used. By using a PCR assay designed to detect the DNA of *P. ramorum*, we were able to conclude the pathogen was absent from mature compost and not merely suppressed or dormant.
Conclusions: Some heat and composting treatments eliminate *P. ramorum* to lower than detectable levels on all substrates tested.
Significance and Impact of the Study: Composting is an effective treatment option for sanitization of *P. ramorum*-infected plant material. Assaying for pathogen viability in compost requires a direct test capable of differentiating between pathogen suppression and pathogen elimination.

Introduction

Phytophthora ramorum, the causal agent of the disease commonly referred to as sudden oak death (SOD), is presumed to be an introduced pathogen (Rizzo and Garbelotto 2003; Ivors *et al.* 2004). This oomycete can infect not only several oak species (*Quercus* spp.) and the related tanoaks (*Lithocarpus densiflorus*), but also a large number of plant species native to the California central coast (Rizzo and Garbelotto 2003), and several plant species widely used in the nursery trade (Davidson *et al.* 2003). New plant species, displaying a variety of symptoms, continue to be added to the host list (Garbelotto 2003; Hüberli *et al.* 2003, 2004, 2005; Murphy and Rizzo 2003). Furthermore, the disease is spreading to new locations both through infected nursery stock and perhaps by other unknown means (Davidson *et al.* 2001; Werres *et al.* 2001; Orlikowski and Szkuta 2002). The number of infected plants growing in landscaped and natural areas of California is difficult to estimate, but is likely to be in excess of tens of thousands. Leaves of California bay laurel (*Umbellularia californica*) can remain infectious for several weeks, even when dry (D. Rizzo, personal communication), and viable cultures can be obtained from tanoak logs and from oak firewood (Davidson *et al.* 2005). Thus, sanitation of infected material remains a serious issue for extended periods of time.

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Composting has been shown to be an effective tool for sanitization of phytopathogen-infested green waste (Hoitink *et al*. 1976; Burge *et al.* 1981; Bollen 1985, 1993; Lopez-Real and Foster 1985; Christensen *et al.* 2002). At least three main processes are involved in pathogen suppression while composting plant material: (i) high temperatures (typically 40–70°C) reached during the thermophilic composting phase (Burge *et al.* 1981; Yuen and Raabe 1984; Bollen 1985); (ii) colonization of compost by a variety of different micro-organisms that either prey on or outcompete the target microbe(s); and (iii) the production of antibiotic compounds by organisms involved in the composting process (Hoitink *et al.* 1997; Hoitink and Boehm 1999; McKellar and Nelson 2003). Temperature is the most standardized variable to measure when assessing the quality of the composting process (Burge *et al.* 1981). It is also likely that heat may be the most important of the three processes mentioned above from a pathogen reduction standpoint (Hoitink *et al.* 1976; Bollen 1985).

Given the broad host range of *P. ramorum* and its ability to infect different plant parts on its numerous hosts, the pathogen may be carried into composting facilities via infected green waste. However, as *P. ramorum* has a limited geographical distribution and seems to be more aggressive than other common nursery *Phytophthora*s (Linderman *et al.* 2002), the USDA and the international community have imposed regulations designed to avoid its introduction into new areas (http://www.aphis.usda.gov/ppq/ispm/pramorum/regulations.html). Potential restriction of sale and transport of commercial compost depends on the ability of the composting process to eliminate all *P. ramorum* inoculum (Paswater 2003).

While several studies have focused on the suppressive effects of compost on plant pathogens (Hoitink *et al.* 1976; Spring *et al.* 1980; Burge *et al.* 1981; Bollen 1985, 1993; Lopez-Real and Foster 1985; Hoitink and Fahy 1986; Déportes *et al.* 1998; Labrie *et al.* 2001; Suárez-Estrella *et al.* 2003), few have actually addressed pathogen eradication in the compost substrate (Hoitink *et al.* 1976; Hardy and Sivasithamparam 1991). In studies on bacteria pathogenic to humans, eradication through composting of sewage sludge and municipal solid waste (EPA 1989; Hay 1996) was regarded as successful if pathogen levels were reduced below threshold levels (EPA 1989; Hay 1996).

In this study, we describe three experiments designed to test heat treatments and composting as sanitation tools for plant material infected by *P. ramorum*. The first experiment was designed to determine time × temperature combinations lethal to *P. ramorum in vitro*. The second experiment investigates the effects of heat treatments and composting on plant material infected by *P. ramorum*, by placing infected plant material in incubators or in compost piles. We finally present a case study in which a turned windrow compost pile containing large amounts of *P. ramorum*-infected plant material is used to address several questions, including:

i What is the contagion risk posed by heavily infected compost piles?

ii What is the variability of sanitization within a heavily infected compost pile?

iii Can composting actually eliminate the pathogen when the inoculum density is extremely high?

Materials and methods

Experiment 1: the effect of dry heat on colony growth and sporulation of *P. ramorum*

Three isolates of *P. ramorum* were used for this study: Pr 1 (CBS 110534), Pr 2 (UCD Pr2) and Pr 5 (ATCC MYA-2434). For each time and temperature treatment, three replicates of each isolate were subcultured on 5% V8 growth medium (Erwin and Ribeiro 1996) and incubated at 18–20°C until the colony diameter had reached approximately 1 cm. Colonies were then incubated in the dark at the following temperatures: 22 (control), 35, 45 or 55°C and for durations of 30 min, 1, 2, 4 and 24 h in a complete factorial design. Growth was recorded 5 and 8 days postexposure. Plates were then flooded with 5 ml of sterile water and 1 ml of the resulting suspension was plated on clean V8 agar to assess viability. Experiment 1b was performed by using an identical approach, but testing growth only at 22 and 40°C; results from both tests were combined. Average daily colony growth values were calculated as the means of nine growth measurements (three replicates for three isolates) taken on days 5 and 8, and the growth rate corrected for differences in colony size at day 5. Growth rates among isolates were not significantly different, thus they were pooled for subsequent analyses. A 99% confidence interval, based on the standard error of the mean, was calculated for each group to compute significant differences among treatments.

Experiment 2: the effect of composting and heat treatment on infected plant material

Wood chips

Wood chips were excised from natural and inoculated (Pr 102) cankers on living *Quercus agrifolia* trees in Marin County. Infected chips from each tree were bagged separately, placed in coolers and cultured for *P. ramorum* prior to use. Experiments were started within 48 h of collection.

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Stem sections
Quercus agrifolia saplings (2–4 cm diameter) were inoculated by placing a plug of inoculum (Pr 102) under the excised bark. Inoculation wounds were then sealed with grafting wax before being wrapped in foil. Stems were inoculated at 30-cm intervals with two inoculations on opposite sides of the stem on each interval. The resulting lesions will hereafter be referred to as cankers. After 12–15 weeks, individual segments were cut and kept with one open side submerged in water.

Laurel leaves
Umbellularia californica leaves were collected from 10 different trees on the University of California, Berkeley campus, to account for potential differential susceptibility among individuals (Hüberli *et al.* 2003). A slurry of water and V8 agar inoculated with a mixture of *P. ramorum* isolates (Pr 102 and Pr 52) was poured over the leaves. In experiments 2 and 3, leaves were layered with moist paper towels and high humidity was maintained by keeping them in a sealed plastic container. The leaves were incubated at 12°C until lesions were fully developed (10–14 days).

Oven trials
The infected plant material was placed in an oven and incubated at 55°C for 2 weeks. After treatment, the material was kept for a week at 20°C before attempting isolation of *P. ramorum* on PARP.

Experiment 2A: treatment for turned windrow compost
Two composting facilities in Marin County, California were selected, and a single compost pile with four replications was used in each location. Each replicate was made up of infected plant material including 10 infected *Q. agrifolia* wood chips, three inoculated stem sections of *Q. agrifolia* and 10 infected *U. californica* leaves. The infected plant material was contained in a nylon mesh bag, and a Hobo digital temperature recorder was added to each replicate (Onset Computer Corporation, Pocasset, MA, USA). Replicates were placed at 10 m from the end of the pile, at 50–100 cm beneath the surface, two on each side of the pile, each approximately two-third the way up each side. The compost piles were turned a minimum of five times during this experiment and the replicates were carefully removed and replaced every time the pile was turned. The compost pile at site 1 was approximately 18 m long, 4 m wide and 1·5 m tall, and was a mix of unground landscape debris amended with 25% horse stall tailings (manure mixed with wood chips), 28% cow manure and a very small percentage of olive waste. The pile at site 2 was approximately 20 m long, 8 m wide and 2·5 m tall, and was composed of a mix of ground green waste. To ensure pathogen viability at the start of the experiment, isolations were performed from all substrates before placing the infected material into the piles. Four additional replicates were used as a control and incubated at 20°C for the duration of the experiment. The experiment took place during April 2002 and was repeated in May of the same year.

Experiment 2B: treatment for forced-air static-pile compost
Two trials were carried out at a single location in Sonoma County, California. Two compost piles, each containing four replicates of artificially infected plant material (as described in Experiment 2A: treatment for turned windrow compost, above), were employed in the first trial in September 2002, while only a single compost pile was employed for the second trial in October 2002. Each compost pile was approximately 25 m long, 6 m wide and 2·5 m tall. The piles were a mix of ground landscape debris subsequently augmented with either manure or chemical fertilizers to achieve the correct nutrient and pH balance for composting. Air was mechanically blown into perforated pipes, which were placed beneath each compost pile as needed to maintain adequate aeration and temperature, initially for several hours and subsequently for about 2 h per day. Isolations were performed as described above and all plant material from each replicate was directly plated after 2 weeks as described in Direct plating, below. Untreated replicates (controls) were stored at 12°C rather than at 20°C as described above for trials on windrow piles.

Direct plating
Viability of the pathogen was determined by counting how many sample fragments (normally about 1–4 mm^2 in size) formed colonies, when plated on P_{10}ARP (PARP) selective media (Erwin and Ribeiro 1996, modified to 25 $\mu g\ ml^{-1}$ PCNB). Fragments were taken from the infection margin of each of 10 wood chips, 10 leaves or three stem sections before placement into the piles, and at the end of the composting process. Plates were incubated in the dark at 20°C and scored for the presence of *P. ramorum* 2 and 6 weeks after plating. Each sample was also pear baited as outlined in Pear baiting below. If the resulting colonies were not easily identifiable as *P. ramorum* by morphology alone, a polymerase chain reaction (PCR) test was performed using selective primers (Hayden *et al.* 2004) to confirm the species identification. As a positive control, one plate from every batch of PARP medium was infected with Pr52 and Pr102.

Pear baiting
Each bag of material or group of stems was also baited with green, unripe D'Anjou pears at the end of the

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experiment. The substrate to be tested was placed into one-gallon sealable plastic bags and flooded with de-ionized water at a 4 : 1 water to substrate ratio as described by Tsao (1983) or enough water to cover the bottom third of the bait pear, whichever was greater. A single pear was placed into each bag, and incubated for 4 days and then left to dry on paper towels for an additional 4–5 days, all at 12°C. All lesions developing on pears were then plated on PARP. Five isolations per pear were attempted, and recovery was determined after 2 and 8 weeks on PARP. As a positive control, one pear was placed into a bag with two 1-cm discs of V8 agar, one disc infested with Pr52 and one with Pr102.

Statistical analysis

Data were analysed in two ways:

i Overall effectiveness of composting and heat treatment (ovens), using the difference of means test. For this analysis, we treated each replicate as an experimental unit. The efficacy of each treatment, namely oven, turned windrow and forced air, was determined by comparing culturing data for all substrates combined, before and after treatment. Identical data for untreated controls was used as a term of comparison. In light of the lack of differences between time of the experiment and sites within each treatment type, data from replicated trials and from different sites were combined. Mean survival rates were calculated for each experimental unit and the differences of means between treatments and controls was then calculated. Such differences express the average change in the number of surviving subsamples per experimental unit, per treatment. Ninety-nine per cent confidence intervals were also calculated based on standard error values for each mean.

ii Effectiveness of either forced-air or turned-windrow composting on each of three plant substrates, using a difference of means test. For this analysis, each type of substrate in each replicate was used as the experimental unit. Besides isolation success, an analysis of baiting success was also included in this analysis. This was performed for both pretreatment and post-treatment data. These data were then analysed using a difference of means approach, as in (i), above.

Experiment 3: survival and spread of *P. ramorum* within a turned windrow compost pile characterized by high levels of inoculum

Inoculation substrate

More than 1 m^3 of *U. californica* leaves was collected from multiple trees on the University of California at Berkeley campus, and placed into plastic greenhouse planting flats. The flats were flooded with de-ionized water and inoculated with isolates Pr52 and Pr102. Based on a random sampling of 30 artificially infected leaves *vs* naturally infected leaves, the lesions were, on average, approximately three times larger on artificially infected leaves than those typically seen on naturally infected leaves collected from the most recent year's growth on *U. californica* trees (data not shown). Quantification of infected area was determined by scanning the infected leaves and analysing the images with Assess 1.01 (APS Press, St Paul, MN, USA).

Initial inoculum levels

Viability of the pathogen was determined by counting the number of plated leaf fragments (of 35 randomly selected leaves) giving rise to a viable colony on PARP after 2 weeks. Quantitative PCR was performed following the single round Taqman protocol by Hayden *et al.* (2006) on inoculated bay leaves to estimate the precomposting pathogen DNA level.

Turned windrow compost pile

Inoculated leaves were thoroughly mixed with 9 m^3 of ground green waste, resulting in a pile containing at least 10% highly infected material. Because the artificially inoculated leaves are more thoroughly colonized than naturally infected leaves, we estimate the amount of *P. ramorum* introduced was comparable to a pile comprised of approximately 30% naturally infected bay leaves. The uncovered pile was wetted heavily upon assembly on 3 April 2003 and irrigated the following day to produce enough runoff for sampling, as described in Runoff collection, below. Four days after inoculation, the pile was turned in an attempt to dry it, as moisture content was above the 50% moisture content optimum (Laisin 2002). On the 11th day of the trial (April 14), the pile was subject to heavy rains, from which we again took runoff samples. The cold weather and excess moisture caused pile temperatures to fall, and required turnings the next day, and again 1 week later, to dry. Thereafter, the nutrients in the pile appear to have been exhausted, as pile temperatures did not adequately recover by day 34, in spite of pile moisture contents in the vicinity of 50%. A small amount of fresh, high-nitrogen material was added on day 38, and the windrow was turned again on days 43 and 62. The compost pile was disassembled on day 71 (12 June 2003).

Temperature probes

Hobo digital temperature probes were placed in 24 predetermined locations within the pile, in three layers. The bottom layer consisted of 12 temperature probes in a 4×3 array, the middle layer consisted of eight probes in a 4×2 array and the top layer consisted of a 4×1 array

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of probes. The temperature probes were removed each time the pile was turned and then returned to their location as the pile was reassembled during turning.

Runoff collection

Three water traps measuring 30 cm × 30 cm × 5 cm were placed on pier blocks on a longitudinal line within the pile and connected by hoses to collection bins on the outside of the pile. Runoff collected was pear baited as outlined in Pear baiting for experiment 2, above. The wetted pile was irrigated thoroughly, and we collected the runoff the morning following its initial assembly. Ten days after the compost pile was assembled, enough rain fell to cause further runoff, and a subsample of this was collected and sampled. Zoospores from strains Pr 52 and Pr 102 were introduced to a 0·25 l subsample from each basin as positive controls, and each subsample was then pear baited as above. The pile was then allowed to dry to moisture levels more conducive to the composting process (approximately 50%).

Direct plating

Viability of the pathogen was determined by plating symptomatic leaf tissue fragments (normally about 4 mm^2 in size) onto PARP, as described in *Direct plating* for experiment 2.

Compost dilution plate method

For direct assessment of compost viability, PARP medium that was still only barely molten (less than 40°C) was poured at a 4 : 1 ratio onto 160 plates containing sieved (1 mm) mature compost and allowed to cool. Plates were checked for growth of *P. ramorum* after 2 and 8 weeks. As a positive control, 160 additional plates had two 5-mm diameter V8 agar plugs, one each infected with Pr52 and Pr102, placed onto the plate with the sieved compost sample. These plates were otherwise treated as above.

Pear baiting

At the end of the composting phase, two compost samples, of approximately one quarter-litre volume each, were taken within 10–15 cm of each digital temperature recorder location, for a total of 48 direct samples. These were then baited as described in *Pear baiting* in experiment 2. Twenty positive controls, each containing five 1-cm^2 discs each of Pr52 and Pr102 manually mixed into the compost, but otherwise prepared as above, were employed to insure that the compost media itself did not suppress sporulation.

Flood sampling

At the end of the composting phase, half of the compost pile was transferred into six plastic pools that had been raised up on palettes. Each pool was equipped with a filter made of wire mesh and cheese cloth enclosed in PVC pipe sections. Each filter ran through the bottom of the pool and connected to a hose on its outside, which was then connected to a 100 μm irrigation filter, and finally to one of 120 collection buckets (20 l cap.). The pools were filled with enough untreated well water to just cover the compost and allowed to sit for 2 days. The water was then drained through the filters into the buckets. Pilot studies had indicated that the wire mesh and cheese cloth filters would not trap *P. ramorum* hyphae or spores to a significant extent, but the fine mesh (100 μm) irrigation filters might potentially catch some hyphae and attached chlamydospores (data not shown). Once the pools were drained, the three irrigation filters (each filter was joined by a junction to two pools) were cut up and plated onto PARP, and all compost drainage was baited with three unripe D'Anjou pears per bucket at 12°C. The pears were removed from the buckets after 4 days and then treated as in Pear baiting, above. Positive controls consisted of twelve 20-l buckets filled halfway with compost leachate into which 10–15 × 10^4 zoospores were introduced, and nine 20-l buckets into which we emptied a single 84 mm Petri dish each of strains Pr102 and Pr52 grown on 10% V8 agar.

Sentinel plants

Thirty *Rhododendron* cv 'Cunningham's White' were used to test for the possibility of infection of plants nearby the composting facility during the composting process. A total of 30 plants were placed downwind from the compost pile, in groups of 10 at distances of 1, 5 and 10 m. Five plants were also placed approximately 50 m upwind from the compost pile to test for the presence of local inoculum unrelated to the composting process. Prior to use in the field, every plant was tested for susceptibility by inoculating two detached leaves using a zoospores suspension. Sentinel plants in the field were inspected for infection on a biweekly basis. Any leaves containing lesions were removed, and all lesions both directly plated and pear baited as described above.

At the conclusion of the composting phase of the experiment, these same *Rhododendron* plants were planted into the resulting compost, in a shade house. The plants received regular overhead watering using a combination of large and small droplet sizes, to facilitate splash dispersal onto the leaves of any spores that might be present. Splash dispersal is a potential transmission pathway for *P. ramorum* (Davidson *et al.* 2005). Splash dispersal has been demonstrated from the soil surface to 'Cunningham White' *Rhododendron* leaves (S.A. Tjosvold, personal communication). The plants were checked monthly and symptomatic leaves were tested as described above.

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Starting in December 2003, five compost samples were taken on a monthly basis from around the rhododendrons, and these were tested as described in *Pear baiting*.

Molecular detection of P. ramorum in compost

In February–April 2004, 430 0·3 g compost samples were taken from the compost pile and analysed for the presence of *P. ramorum* DNA. Bulk DNA was extracted using the Qiagen QIAmp DNA-stool Mini Kit (Valencia, CA, USA) and PCR amplified using the nested Taqman protocol of Hayden *et al.* (2006) for detection of *P. ramorum*. All sample extracts were then spiked with 80 pg of *P. ramorum* DNA and processed again through the PCR assay, to ensure detection was not limited by the presence of PCR inhibitors in the compost substrate. If detection was inhibited at the 80 pg level, then the samples were re-extracted and re-spiked with 0·8 ng of *P. ramorum* DNA and rerun.

A standard curve was generated for quantification of PCR products (Fig. 4) by amplifying known amounts of *P. ramorum* DNA (starting concentration 240 μg ml^{-1}) and plotting the number of PCR cycles required to cross the amplification threshold (C_t) (Hayden *et al.* 2004). To ensure the assay was sensitive enough to detect the pathogen in bay leaves infected for several months, we selected a set of naturally infected leaves with symptoms characteristic of older infections, and quantified the amount of *P. ramorum* DNA using the standard curve described above and a quantitative PCR protocol exclusively designed to detect and quantify *P. ramorum*. All samples selected were positively identified as infected by *P. ramorum* via the PCR assay (Hayden *et al.* 2006), but the set included both samples that had produced viable cultures when plated, and samples that produced no cultures. DNA quantification from these samples was then compared on the curve with DNA titres that were detectable by the PCR assay in presence of compost, a substrate known to greatly affect the efficiency of the PCR reaction.

Results

Experiment 1: the effect of dry heat on colony growth and sporulation of *P. ramorum*

Mild temperatures and low treatment times (e.g. 35 and 40°C for periods up to 2 h) had little effect on pathogen growth rates. Combinations of milder temperatures with long treatment times (e.g. 35°C for 24 h or 40°C for 4 h) or higher temperatures with shorter treatment times (e.g. 45°C for 1 h, 55°C for 0·5 h), appeared to suppress the pathogen temporarily, allowing for vigorous regrowth after treatment (Fig. 1). Longer treatment times combined with higher temperatures (e.g. 40°C for 24 h or more or 55°C for 1 h or more) killed the pathogen, resulting in no recovery. When fungal growth was completely arrested, so were the production of any type of reproductive structure. We identified seven lethal treatments: 1, 2, 4 and 24 h at 55°C; 4 and 24 h at 45°C; 24 h at 40°C. Flash heat treatments at 55°C were not effective and even prolonged exposure (4 h) at temperature as high as 40°C were not lethal to *P. ramorum* (Fig. 1).

Experiment 2: the effect of composting and heat treatment on infected plant material

Phytophthora ramorum was not recovered from any substrate both after oven exposure at 55°C for 2 weeks

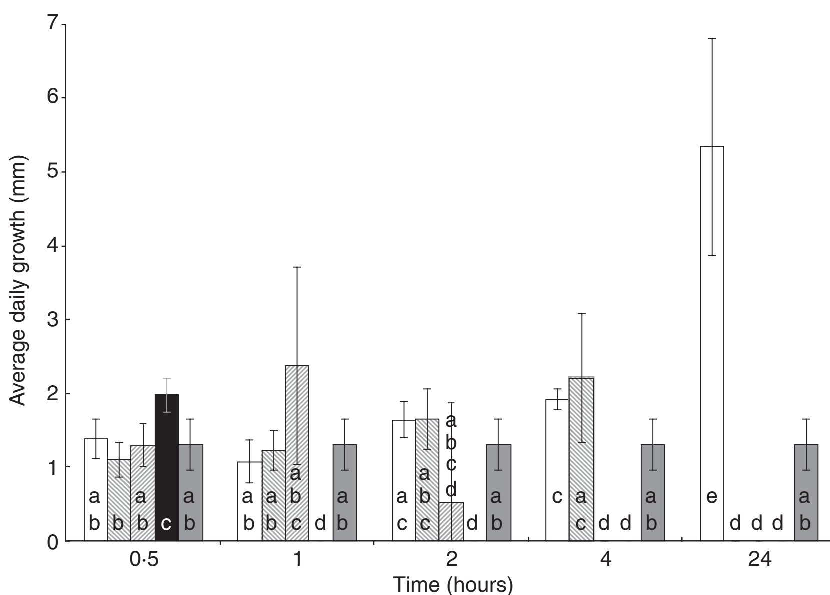

Figure 1 Average daily growth represents the mean growth rate of three isolates. Different shading patterns indicate different temperatures to which plates were exposed. Error bars represent the 99% confidence intervals for the means. Letter designations represent statistical significance groupings for $P < 0·01$. □, 35°C; ▧, 40°C; ▨, 45°C; ■, 55°C; ▩, 22°C.

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Table 1 Percentage isolation success of *Phytophthora ramorum* from infected plant substrates before and after heat and compost treatments

Treatment	%Pre (SE)	%Post (SE)
Turned window (TW)	36 (3·0)	0
Oven	31 (4·0)	0
Control – TW and oven	35 (3·0)	28 (1·4)
Forced air	68 (3·5)	0
Control – forced air	67 (2·4)	50 (7·8)

%Pre, percentage isolation success before treatment; %Post, percentage isolation success after treatment.

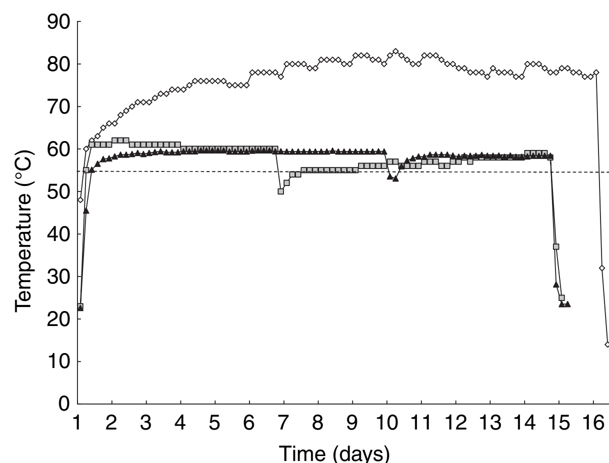

Figure 2 Average temperature profiles for composting trials in experiment 2. Dashed line represents the critical 55°C temperature value. ◇, Forced air; ■, turned windrow no. 1; ▲, turned windrow no. 2.

and at the end of each compost trial. In contrast, viability of the pathogen from control-infected plant material remained high and similar to viability at the beginning of each experiment (Tables 1 and 2). When analysing untreated controls by direct plating, cankers on artificially inoculated stems provided the highest percentage of successful isolations (87–88%), followed by isolations from artificially inoculated bay leaves (55–56%) and from wood chips (31%). Positive isolation from baiting was highest from bay leaves (38%), while very limited baiting success was obtained from stems (19%). Baiting from wood chips was always unsuccessful. Independently of ease or method of isolation, all substrates were always negative for the growth of *P. ramorum* after treatments. Viability of the pathogen by substrate did not change significantly in the controls during the length of our experiments (Tables 1 and 2).

Compost temperature profiles (Fig. 2) indicated the 55°C threshold required by EPA and CCR guidelines for commercial composting (i.e. 2 weeks with five turnings for turned windrow composting and three consecutive days for forced air static pile composting, as described by the California Code of Regulations 2005) had been reached in all trials.

Experiment 3: survival and spread of *P. ramorum* within a turned windrow compost pile characterized by high levels of inoculum

This experiment was conducted in the spring of 2003, a time when weather conditions should have been favourable for the survival and spread of *P. ramorum.* Average mean temperatures ranged from 10 to 12°C, rising slightly to 15°C at the end of May. The experiment received a total of approximately 22 cm of rainfall, over half of this falling in April. The temperature profile (Fig. 3) revealed that heat loss was greatest in the bottom corners, or 'toes', of the compost pile. Turning while the component material was still fresh appeared to increase temperature in the toe areas. Internal pile temperatures were consistently higher than toe temperatures (data not shown), as shown in compost heat variation studies such as Burge *et al.* (1981). In the course of experiment 3, composting was extended until 55°C was reached and

Table 2 Percentage success of isolations and baiting from three plant substrates subjected to forced-air or turned-windrow composting

	Plating					Baiting			
Substrates	%Pre	%Con	$P < 0{\cdot}01$	%Post TW	%Post FA	% Con	$P < 0{\cdot}05$	%Post TW	%Post FA
Leaves	55 (4·3)	56 (5·3)	a	0	0	38	a	0	0
Stems	87 (2·7)	88 (5·8)	b	0	0	19	a	0	0
Chips	31 (5·5)	36 (8·4)	c	0	0	0	b	0	0

Standard errors in parentheses. $P < 0{\cdot}01$, different letters indicate significant differences for %Con at the indicated confidence level.
%Pre, percentage success before treatment; %Post, percentage success after treatment; TW, turned windrow composting; FA, forced air composting.

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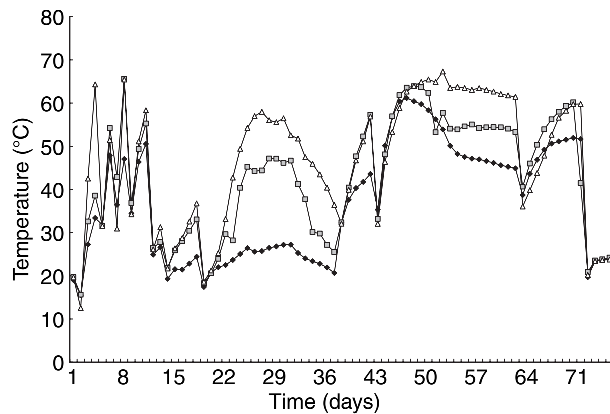

Figure 3 Temperature variation in the turned windrow compost pile in experiment 3. '1st Corners' represents the average temperature of all of the bottom level outside corners, or 'toe' of the pile; 'Mid Lvl' represents the average temperature halfway up the pile from the toe; 'Ridge' represents the temperatures along the top of the pile. Data points represent the mean of four values. ◆, Corners; ▩, mid pile; △, ridge.

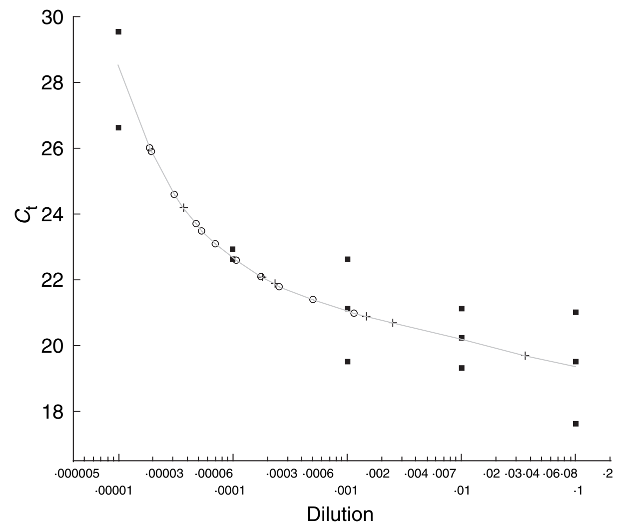

Figure 4 Standard curve for second-round PCR results. Block points represent known quantities of DNA and '+' or 'O' points on the curve represent cultures tested on the curve. Viable samples (culture +) always had amplification threshold (C_t) values ≤25 and non-viable samples (culture −) always had $C_t \geq 21$. Our detection limit is between C_t values of 37·5 and 40 or DNA concentrations 10 times below those expected for non-viable samples. ■, Curve standard; +, cultured positive; ○, cultured negative.

maintained for a sufficiently long period of time as to meet EPA and CCR guidelines for commercial composting in California.

We were unable to obtain any viable culture of *P. ramorum*, regardless of the method used, from solid compost samples taken during and after the composting process. Positive controls for both baiting ($n = 20$) and direct plating ($n = 160$) of compost samples failed as well, indicating the suppressive effect of compost on *P. ramorum* is so strong to invalidate the use of these approaches to test the survival of this pathogen in compost. All 430 compost samples collected in the 8 months following the composting process tested negative for *P. ramorum*, using the nested PCR procedure. Results obtained by spiking the compost extract with *P. ramorum* DNA indicated the PCR assay could detect 80 pg of *P. ramorum* DNA in 82% of the compost samples and 0·8 ng in the remaining 18%. Quantitative second round PCR analysis determined that all naturally infected leaves contained more than 0·8 ng of *P. ramorum* DNA even several months after infection, indicating the sensitivity of the assay would suffice to detect the pathogen even in aged, naturally infected *U. californica* leaves (Fig. 4). On average, compost infested with inoculated leaves contained 37·8 μg of *P. ramorum* DNA per sample prior to composting (data not shown). The assay is therefore sensitive enough to detect an estimated pathogen reduction of between five and six orders of magnitude.

Phytophthora ramorum was never baited from runoff water or from flooded compost. On the other hand, successful baiting occurred in all the runoff control samples ($n = 3$) and in 60% ($n = 7$) of the compost flood control samples when agar discs colonized by the pathogen were added to these two substrates. Additionally, bait pears were successfully infected in seven of 12 flooded compost control samples when zoospores of *P. ramorum* were added to the water during the baiting process.

All baiting ($n = 36$) and direct plating attempts ($n = 248$) to isolate *P. ramorum* from sentinel plants yielded negative results for the entire duration of the study (12 months). In contrast, leaves from 29 of the 30 sentinel plants could easily be infected when agar discs colonized by *P. ramorum* were placed in the same bags where the leaves were being floated.

Discussion

Experiment 1: the effect of dry heat on colony growth and sporulation of *P. ramorum*

Results from experiment 1 showed that temperatures above 35°C merely slowed down the growth rate of *P. ramorum*, and even exposure to 40°C for 4 h did not affect pathogen survival. The ability to withstand exposure to such high temperature suggests this pathogen may be able to become established and survive in warmer regions. Heat tolerance of *P. ramorum in planta* has been shown by Harnik *et al.* (2004). For sanitation purposes,

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three treatments were found to kill the pathogen: 24 h at 40°C, 2 h at 45°C and 1 h at 55°C; 55°C is the minimum required temperature that needs to be obtained (and maintained for at least five pile turns) in commercial turned windrow composting facilities (Hay 1996). Based on these observations, we suggest that prolonged exposure to 45°C may be as effective at eliminating *P. ramorum* as 55°C. As temperature fluctuations are hard to avoid in most field situations, it is preferable to choose the highest temperature for the shortest period of time. However, flash-heat treatments of 55°C for 30 min are not likely to successfully eliminate the pathogen.

It should be noted that these results were obtained on cultures. Survival of the pathogen in infected plant tissue may vary significantly depending on plant species, type of substrate (e.g. wood, cambium, leaves; see below), and propagules produced by the pathogen on each host or host part.

Experiment 2: the effect of composting and heat treatment on infected plant material

All tested treatments reduced recovery of the pathogen to zero. Furthermore, pathogen recovery levels were significantly different when compared to those of untreated samples ($P < 0{\cdot}01$). This was true regardless of type of treatment or substrate. At the end of the experiments, recovery of the pathogen in untreated controls was undistinguishable ($P < 0{\cdot}01$) from recovery recorded before treatment. Direct plating results were used for the analysis, but pear baiting provided identical results.

Wood chips represented the most variable and least favourable substrate for recovery of *P. ramorum*. Baiting always failed from wood chips, suggesting that the pathogen does not sporulate on this substrate, or that leachates from the substrate inhibit sporulation. Isolations from cankers caused by the artificial inoculation of coast live oak saplings yielded the largest percentage of successful isolations, but only limited baiting success, suggesting that the stem sections supported mycelial growth beneath the bark, but provided only limited opportunities for sporangial formation. We hypothesize sporangia production on these stems occurred solely at the inoculation site, perhaps on the inoculum plug. Isolations from bay leaves were moderately successful, but baiting was significantly more successful than with the two other substrates. This is not surprising as bay leaves are known to support prolific sporulation of *P. ramorum* (Davidson *et al.* 2005).

Both turned windrow systems maintained temperatures above 55°C for the time mandated by EPA guidelines for commercial composting in California, as did the forced air static pile system. However, it should be noted that in these studies the infected plant material and temperature recorders were placed in comparatively 'ideal' locations within each pile, in an experimental design referred to as direct process evaluation (Christensen *et al.* 2002). While results from these experiments clearly indicate that the composting process is capable of sanitizing infected green waste under the conditions tested, they do not necessarily prove that composting as a whole achieves identical results. As Christensen points out, deviations from the conditions tested may affect the outcome and direct process evaluation is unreliable by itself as an evaluation tool for monitoring the overall sanitary process. The frequency and intensity of potential deviations from the conditions tested here will define the reliability of composting as a tool to eliminate *P. ramorum* from infected plant material. Results from experiment 2 suggest that, if conditions met in our sampling points are met throughout the pile, composting will be successful in eliminating *P. ramorum* from infected plant material.

Experiment 3: survival and spread of *P. ramorum* within a turned windrow compost pile characterized by high levels of pathogenic inoculum

Our last experiment was designed to test the validity of the composting process by monitoring viability and presence of the pathogen in a compost pile largely composed of infected plant material. In this instance, rather than use direct process evaluation as in experiment 2, we used spot test analysis, a method whereby many samples are taken throughout the pile, and tested for presence of *P. ramorum*. This is a much more accurate method for the analysis of the sanitary process (Christensen *et al.* 2002). Sampling in this experiment was either complete or very intensive, to maximize our chances of detecting even limited pathogen survival. A complex sampling strategy was adopted including baiting, direct plating, PCR-based detection and the use of sentinel plants. Each diagnostic test had individual strengths and weaknesses. The use of baiting techniques allowed us to sample large volumes of substrate, but relies on active infection of the bait by motile zoospores. Therefore this method was used to determine active sporangia production on the tested substrate. Zoospores are generally regarded as the primary infection propagules for *Phytophthora* species, including *P. ramorum* (Werres *et al.* 2001) and *P. cinnamomi* (Zentmeyer 1980). When using baiting as a diagnostic detection assay, negative results have to be interpreted with caution, as it has been shown that pear baiting may be insensitive enough to miss *Phytophthora* spore densities that are high enough to cause disease (Yamak *et al.* 2002). Composts can suppress sporangial production below detectable limits without eradicating *P. cinnamomi* (Hardy and Sivasithamparam 1991), and suppressed iso-

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lates may recover sufficiently to become a threat under more favourable conditions (Hardy and Sivasithamparam 1991; Sidhu *et al.* 1999).

The positive controls used for our baiting trials failed to produce any lesions on the pear bait, indicating that sporulation of *P. ramorum* was suppressed. In contrast to our inability to bait positive controls from solid compost samples, the flood baiting controls were successful, suggesting that the leachate itself is not suppressing pathogen activity, but that suppression requires either intimate contact with the compost substrate or that suppression of *P. ramorum* in compost is severe enough that sporangia are not formed on *U. californica* leaves once they have been subjected to the biotic or chemical environment of a compost pile. Some evidence for the latter comes from another study in which we were unable to retrieve *P. ramorum* from 24 infected *U. californica* leaves 6 h after placing them into a cool (approx. 15°C) pile of ground and wetted green waste, while we were able to retrieve *P. ramorum* from 10 of 12 leaves placed on the surface of the pile.

While compost dilution plate methods do not require pathogen sporulation, they can realistically be used to sample only a significantly smaller subset of substrate. Results should be interpreted with caution, as negative results may be the result of pathogen suppression rather than eradication. No *P. ramorum* colonies were formed on 160 PARP plates of a mixture of sieved inoculated compost and PARP, but positive controls simultaneously inoculated with *P. ramorum* failed to grow out as well. This result indicated that compost contains chemical or biological factors capable of suppressing *P. ramorum* growth. Suppression of *Phytophthora* species has occurred in compost preparations in the absence of high temperatures (Hardy and Sivasithamparam 1991) or in compost-amended soil (Hoitink and Fahy 1986; Hoitink and Boehm 1999, McKellar and Nelson, 2003), and has repeatedly been attributed to biological or chemical factors (Hoitink *et al.* 1976; Yuen and Raabe 1984). We believe that the antimicrobial properties of compost suppressed *P. ramorum* in this test, including the positive controls.

The use of highly susceptible sentinel plants at various distances downwind from the pile is a good indicator that infected green waste is non-contagious once in a compost pile. The closest plants were 1 m from the infested pile and were routinely showered with debris as the pile was turned. This occurred under environmental conditions conducive to infection (Davidson *et al.* 2001), particularly the turning conducted in the cold, wet, windy weather of 12–25, April 2003 (days 10–23). It is possible that foliar application of compost caused a suppression of infection due to induced resistance (Elad and Shtienberg 1994) during this phase of the experiment. However, the rhododendrons were later planted in this compost, prewatered with a fine mist and then heavily watered with large droplets allowing splash dispersal of any propagules in the compost. After a year of this treatment, not a single infected leaf was found, while control leaves retained 97% infection. In light of the recent nursery infections that have occurred on *Rhododendron*, *Camellia* spp. and other nursery stock, it seems clear that *P. ramorum* will readily infect such susceptible species given adequate conditions to do so (http://www.aphis.usda.gov/ppq/ispm/pramorum/regulations.html). The lack of infection strongly suggests that *P. ramorum* is heavily suppressed or eliminated under composting conditions.

Detection by PCR enabled detection of *P. ramorum* regardless of dormancy or reproductive status. As our above results seem to corroborate Malajczuk's (1983) statement that *Phytophthora* species are easily suppressed by competing fungi and bacteria (or perhaps are suppressed by chemical compounds), PCR as a detection tool compliments the use of viable culture methods. Positive PCR results may therefore be problematic from a regulatory perspective, because the technique may detect dead cells. Although PCR is performed on small volumes of substrate, our sampling assay was intensive enough (430) and sensitive enough (at 0·8 ng of DNA per sample) to provide >99% confidence that if *P. ramorum* DNA were still present at only one part in 100 000 of the introduced amount, it should have been detected. Unlike the other tests, PCR-based assays can detect dormant chlamydospores or any other resting structure and vegetative mycelium. We found no positive samples.

In experiment 3, the temperatures in the compost were initially lower than those required by US EPA 40 CFR Part 503. Limited heating was due to the excessive moisture level in the pile, due initially to a watering regime designed to produce collectable leachate and subsequently to the heavy rains occurring in that period. Turning of the pile caused a significant reduction in moisture level, but heating did not occur after this point (especially in the toe of the pile), until further nitrogen-rich material was incorporated in the pile. It is interesting to note that the toes of the pile heated up as quickly as the rest of the pile, but cooled down faster than the rest of the pile. Maximum exposure to high temperatures can thus be obtained by shifting composting material in different locations in the pile through turnings (EPA 1989). In spite of the fact that only moderate temperatures were reached in the first part of the experiment, no isolates of *P. ramorum* were obtained from the leachate. This result further indicates that the pathogen is at least effectively suppressed during the composting process due to biologically or chemically mediated processes. It should be noted that the compost piles used in this study were both

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quite large and carefully monitored, as should be typical of commercially run facilities. Smaller composting piles or those not turned and/or monitored on a rigorous schedule as might be found in 'back yard' composting, frequently do not meet the temperature requirements for effective sanitization (Yuen and Raabe 1984; Ryckeboer *et al.* 2002), and therefore should not be construed to meet phytosanitary conditions outlined in this report.

The combined results from the three experiments conclusively show that some heat and composting treatments suppress and eliminate *P. ramorum* from a variety of infected plant substrates. Even when the pathogen was present in the leaves of *U. californica*, its most resilient wild plant host, the host material was effectively sanitized by following the guidelines described by US EPA 40 CFR Part 503 for composting (Hay 1996). Our experiments also indicate that, when analysing compost for the presence of a pathogen like *P. ramorum*, it is necessary to employ an assay capable of directly assessing for pathogen presence. We believe the approach we describe here is extremely appropriate for this type of analyses. The approach included a very sensitive and specific nested Taqman PCR assay in which the detection threshold for the DNA of the target organism was checked for each sample. The ability to quantify the detection threshold combined with quantification of the DNA of the target organism prior to treatment allows to determine not only presence/absence of the pathogen but also to quantify the magnitude of detectable pathogen reduction.

Whether compost may serve as a route of spread for this aggressive plant pathogen will depend on other factors such as the ability of the pathogen to survive or colonize mature compost. *Phytophthora ramorum* has been recovered from dust samples immediately surrounding tub grinders used to process green waste (Shelly *et al.* 2006), and preliminary data suggest that *P. ramorum* is capable of surviving in finished compost if introduced, so although compost appears to eliminate the pathogen when processed correctly, this should not be construed to mean that all compost originating from infected green waste is safe by simple virtue of the fact that it has been processed in accordance with US EPA guidelines.

Acknowledgements

This research was funded in part by the California Integrated Waste Management Board, by the USDA Forest Service Pacific Southwest Region and by the Gordon and Betty Moore Foundation. We would like to thank David Rizzo and Jenny Davidson, both of UC Davis, and Jennifer Parke, with the USDA in Corvallis, OR, for their input on specific issues in this text. We would also like to thank McEvoy Ranch, the Bolinas composting facility, Sonoma Compost, and Chip Sanborne and Don Liepold of Grab & Grow Soil Products for their invaluable help in conducting this research. We are grateful for the extremely insightful comments of two anonymous referees.

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