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Induced antimicrobial activity in heat-treated woodchips inhibits the activity of the invasive plant pathogen *Phytophthora ramorum*

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Running title: Woodbased compounds inhibit *P. ramorum*

Abstract

We investigated the antimicrobial activity of heat-treated woodchips of three woody host species against the invasive oomycete plant pathogen *Phytophthora ramorum* to assess the potential of heated woodchips for disease suppression. Results demonstrated that heat-treated pine (*Pinus sylvestris*), Japanese larch (*Larix kaempferi*) and rhododendron (*Rhododendron ponticum*) woodchips inhibited the recovery of *P. ramorum* spores and mycelium compared with similar material that had only been air-dried. Effects were most evident with pine and larch; inhibition was maintained even when larch woodchips were diluted with soil. *In vitro* assays using methanol crude extracts from woodchips of the three species showed they all had an inhibitory effect on *P. ramorum* zoospores and reduced chlamydospore germination compared with air-dried wood extracts. Chemical analysis of the extracts revealed several induced compounds were present but in different concentrations for each species.

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Coniferaldehyde was the most active inhibitory against spores and mycelium, whilst the dominant resin acids, dehydroabietic and abietic acid, decreased the minimum inhibitory concentration of phenolic compounds tested against *P. ramorum* but were ineffective when used alone. An array of compounds, including dehydroabietic acid, methyl abietate, α -pinene and 3-carene, occurred at elevated levels in the living tissue of Japanese larch bark attacked by *P. ramorum*. These compounds may be part of the induced resistance response of larch to *P. ramorum*. Results of a field trial using heat-treated and air-dried woodchips were consistent with the crude extract bioassay results, suggesting that heat-treated woody materials have potential to reduce the survival of *P. ramorum* under natural conditions.

Introduction

The oomycete *Phytophthora ramorum* Werres, De Cock & Man in 't Veld is the causal agent of 'sudden oak death' in the western United States where the disease has resulted in extensive mortality of oaks and tanoaks since the early 1990s (Rizzo et al. 2002; Frankel 2008). After its formal description in 2001 (Werres et al. 2001) *P. ramorum* was quickly reported from a number of countries in Europe. In the UK, the first finding was made in a nursery in 2002 with further discoveries in the wider environment made soon after (Brasier et al. 2004a).

'Ramorum dieback' as it was first known in the UK, initially caused damage to a large number of ornamental and woody shrubs with only occasional impacts on broadleaf tree species such as beech (*Fagus sylvatica* L.) and some non-native oak species (Brasier et al. 2004b; Tracey 2009). In 2009, *P. ramorum* was unexpectedly found infecting Japanese larch (*Larix kaempferi* Lamb. Carr.) (Webber et al. 2010). Since then it has caused widespread mortality in plantation grown larch in many parts of the UK and Ireland (Brasier and Webber 2010, O'Hanlan et al. 2017). As a quarantine organism, *P. ramorum* control is mandatory in the UK and involves the early detection and removal of certain hosts, known as sporulating hosts, which generate huge numbers of spores (mainly sporangia and zoospores) when leaf and shoot tissue are infected. Initially the main sporulating host was found to be

rhododendron (Denman et al. 2005; Turner et al. 2005) and a programme of rhododendron clearance was launched by Defra in 2002 to control ramorum dieback of ornamentals in the UK (Defra 2008). However, the discovery that infected foliage of Japanese larch produced huge numbers of *P. ramorum* spores (Webber et al. 2010; Harris & Webber 2017) led to a major redirection of control efforts. As a consequence, millions of infected larch trees have been felled under Statutory Plant Health Notices, with initially 2,400 hectares felled or awaiting felling in 2009 rising to a total of more 20,000 hectares by 2017 (Webber and Brasier 2018).

Although removal of sporulating hosts such as infected larch and rhododendron is the accepted strategy for disease control (Sansford et al. 2009), it introduces significant biosecurity and economic impacts for the forest industry and nurseries. The same applies for gardens and parks that are open to the public and specialise in ornamental plants such as rhododendron, *Camellia* and *Pieris* which can be hard-hit by *P. ramorum* (Defra 2008). Moreover, leaves and needles of infected shrubs and trees can remain a source of inoculum even after removal of the plants, as *P. ramorum* chlamydospores that form in such tissue appear to be able to survive and persist in soil and leaf litter for some years after clearance (Turner et al. 2005; Harris 2015). Disease spread via contaminated soil or leaf litter on the shoes of walkers, or tools and vehicles that operate on *P. ramorum* infested sites can also occur (Cushman et al. 2008; Webber and Rose 2008). However, as complete clearance of infested material and soil from the wider environment is usually not practical or economically viable, other approaches are needed to limit inoculum levels and reduce the potential for movement of contaminated soil and litter.

One alternative strategy to reduce the spread of infected leaf litter and contaminated soil could involve covering the soil surface with a layer of woodchips that act as a physical barrier to reduce the mobility of *P. ramorum* inoculum. This approach has been studied in the USA for *P. ramorum* infested forest paths (Goheen 2013) and recognises that if the woodchips have antimicrobial activity the benefit may extend beyond just being a physical barrier. Chemicals associated with wood derived from certain tree species have been found to reduce the survival of *P. ramorum*, and in particular some conifer species

contain compounds that have high levels of antimicrobial activity (Manter et al. 2006). Heat treatment of wood is also known to be an efficient way of improving its resistance to attack from wood degrading fungi. The effectiveness of the process is not only due to the reduced moisture content but also because of enhanced bioactivity following the formation of new compounds in wood materials after heating (Esteves et al. 2008; Kamdem et al. 2002; Sundqvist 2004).

In an earlier study by Kalantarzadeh (2014), chemical analysis of heat-treated wood of Scots pine (*Pinus sylvestris* L.) showed the existence of several potentially antimicrobial compounds when tested against a range of bacterial pathogens of animals. Using a comparable approach, the aim of this study was to evaluate whether heat-treated wood including that sourced from host plants of *P. ramorum* showed antimicrobial activity against the pathogen and therefore might have a role in the disease management. The first objective was to assess the extent of any inhibitory activity in woodchips of Scots pine, both air-dried and heat-treated, when placed in direct contact with *P. ramorum* propagules, and then extend the assays to test rhododendron (*Rhododendron ponticum* L.) and Japanese larch, two major hosts of *P. ramorum* in Britain. The second objective was to undertake chemical analysis of the wood of all three species and individually test the different compounds isolated to determine if they inhibited the growth of *P. ramorum*. A similar analysis was also applied to Japanese larch bark in the process of being invaded by *P. ramorum* to explore the expression of any antimicrobial activity. Finally, to evaluate the strength and magnitude of any enhanced antimicrobial activity in heat-treated woodchips over an extended period, a field trial was set up with the objective of measuring the recovery of *P. ramorum* from pine, larch and rhododendron woodchips exposed to *P. ramorum* inoculum from naturally sporulating hosts over a twelve-month period.

Materials and Methods

Woodchip and inoculum preparation

Logs up to 80 m in length were obtained from Alice Holt Forest, Farnham, where ramorum dieback was absent. Woodchips (average dimensions of woodchips 1.0 by 1.5 cm) were prepared from

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debarked logs of rhododendron (*R. ponticum*), Japanese larch (*L. kaempferi*) and pine (*P. sylvestris*), using a commercial chipper and then air-dried or heat-treated. Woodchips were dried by spreading on trays whilst incubated at 20-25°C for 3 days or when the moisture content was $\leq 28\%$. Heat treatment involved loosely wrapping 100 g quantities in aluminium foil which were heated for 72 hours at 120°C in an electric oven.

Two isolates of *P. ramorum* were selected from the Phytophthora Culture Collection held at Forest Research, Farnham, Surrey, UK, with isolates P1578 and BRC01 cultured from infected rhododendron and Japanese larch respectively. Both were the most common genetic lineage of *P. ramorum* in Europe (EU1) and genotypes common to the British population (Grunwald et al. 2012; Harris et al. 2018). Both isolates were routinely cultured on Carrot Agar (CA) plates maintained at 20°C as described by Brasier and Kirk (2002). When isolations were undertaken from non-sterile material, a *Phytophthora* selective medium was used consisting of modified Synthetic Mucor Agar (SMA) (see Brasier and Kirk 2002).

Zoospore inoculum was prepared by growing each isolate of *P. ramorum* on 25-50 CA plates incubated under continuous artificial daylight at 18-20°C to encourage sporangial production. After 2 weeks, 5 ml of sterile distilled water was added to each plate, the sporangia gently dislodged using a plastic spreader and the spore-containing liquid tipped into a sterile beaker. The sporangial suspension was refrigerated at 4°C for 20 minutes and returned to room temperature for 45 minutes to induce zoospore release. The zoospore concentration in each suspension was counted using a haemocytometer. The average number of zoospores was estimated from four counts and adjusted to the required concentration prior to use.

Chlamydospore inoculum was prepared using the method of Tooley and Browning (2008). A 10^4 zoospore ml^{-1} suspension (300 ml) was prepared for each isolate and transferred to a zip-lock plastic bag. Leaf disks were cut from freshly harvested and water rinsed *R. ponticum* leaves using a 6 mm diameter cork-borer. Two hundred leaf disks were added to each spore suspension and incubated at

18-20°C for 5 days to allow colonisation. Under sterile conditions, the contents of each bag were sieved and then the leaf disks rinsed with sterile water and drained with the process repeated three times. The disks were transferred onto moistened tissue in plastic containers and incubated in the dark at 20°C. After 4 weeks, the disks contained abundant chlamydospores and their presence was confirmed by direct examination under a compound microscope.

Mycelium was prepared by adding 10 µl of zoospore suspension (10^4 zoospores ml⁻¹) to each well of a 96-well micro-titre plate pre-loaded with 100 µl sterile distilled water per well. The plates were incubated at 20°C in the dark and after 3 days mycelium formation in the wells was checked using a binocular microscope. The mycelium was then transferred to 96-well plates pre-loaded with known concentrations of the crude extracts derived from heated or non-heated woodchips of pine, larch or rhododendron or the purified compounds (see below).

Microcosm experiments using only woodchips

In these assays, treatments consisted of pine, larch and rhododendron woodchips, either air-dried or heat-treated, each challenged with a zoospore suspension of *P. ramorum* isolate BRC01, *P. ramorum* isolate P1578, or a water control. The woodchip samples were placed in sterile McCartney bottles (1g of woodchips per bottle) and each bottle was inoculated with 1 ml of a 10^4 zoospore ml⁻¹ suspension. Bottles were incubated at 20°C for 3 weeks, with ten replicate bottles for each isolate of *P. ramorum* /woodchip treatment set up. Controls were prepared in the same way, but 1 ml of sterile water was substituted for 1 ml of zoospore suspension. After 3 weeks, 1g of woodchips was removed from each bottle, placed onto an SMA plate, incubated for 7 days at 20°C and then checked for any outgrowth of *P. ramorum* as an indicator of survival. The number of plates with *P. ramorum* outgrowth from the woodchips was scored. In all, four separate trials of the assay were repeated.

Microcosm experiments using woodchip and soil mixtures

In these assays only larch woodchips were used in the soil mixtures, with each treatment challenged using two types of *P. ramorum* inoculum consisting of either a zoospore suspension or leaf disks permeated with chlamydospores. As before, isolates BRC01 and P1578 were used.

In the first assay, air-dried or heat-treated larch woodchips were ground into fine particles ≤ 2 mm in diameter and mixed thoroughly with non-sterile, but *P. ramorum*-free soil obtained from Alice Holt Forest, Farnham, Surrey. The ground wood was mixed with the soil in plastic bags in a 1:3 or 3:1 ratio (w:w) to make a total dry weight of 30 g. After mixing, sterile water was added to water holding capacity. The bags were closed and incubated at 25°C for 7 days but stirred with a sterile spatula every two days. Treatments consisting of just soil or ground woodchips were also set up. Additionally, perlite was substituted for woodchips and mixed with soil in the same proportions to act as controls. A 10^3 zoospores ml⁻¹ suspension was added to each bag (10 ml per 30g), thoroughly mixed and the bags incubated at room temperature (20°C) for 4 or 8 weeks. After incubation, four replicate 1 g samples of the mixture were removed from each bag, each diluted with 10 ml sterile water and 1 ml of the dilution spread onto SMA plates. The plates were incubated at 20°C for 7 days, counts of *P. ramorum* colonies were made and expressed as colony forming units (cfu) g⁻¹.

In the second assay, rhododendron leaf disks permeated with *P. ramorum* chlamydospores were incorporated into a 3:1 and 1:3 ratio of heat-treated larch woodchip/soil mix as already described, again with a perlite-soil mixture acting as the control. Each mixture was set up in a plastic pot and 12 leaf disks previously colonised with *P. ramorum* were added to each pot, with three replicate pots for each treatment. The pots were maintained at room temperature (20°C) for either 4 weeks or 12 weeks, after which the disks were recovered from the pots, rinsed with sterile water, placed onto SMA plates and incubated for 3 weeks at 20°C. As before, each disk was assessed for any outgrowth of *P. ramorum*.

Bioassays of crude wood extracts

Crude extracts were made from heat-treated and air-dried woodchips of pine, larch and rhododendron and tested against two types of *P. ramorum* inoculum consisting of either a zoospore suspension or leaf disks permeated with chlamydospores. As before, isolates BCR01 and P1578 were used.

Before extraction, the wood from each species was processed into fine shavings (*ca.* 500 g for each wood type) and then either air dried or oven dried. Subsequently, crude extracts were prepared by adding laboratory grade methanol to woodchips in a 5:1 ratio. Extractions were set up in triplicate with woodchips left to soak for 24 hours at room temperature on a roller shaker (SPIRAMAX 5, Denley Instrument Ltd.). The woodchips were then removed, and crude extracts were obtained by evaporating off the methanol. The crude extracts were finally decanted into sterile vials and stored at 4°C until used. For assays, each crude extract was dissolved in acetone at a concentration of 50 mg ml⁻¹ to create a standard stock solution.

The first assay tested the effect of diluted crude extracts on zoospores. Ninety-six-well micro-titre plates were pre-loaded with 9.6 ml carrot broth (250g carrots L⁻¹ of distilled water, boiled for an hour and filtered through cheese cloth) and 0.4 ml of a dilution series of the crude extract stock solutions (equating to 25, 12.5, 6.25 mg extract ml⁻¹) added into the broth. The micro-titre plates were left in a laminar flow cabinet for 5 hours to allow the acetone to evaporate. Three replicates were prepared for each extract concentration. Control wells received only acetone and carrot broth. Each well was inoculated with 5 µl of a 10⁴ ml⁻¹ zoospore suspension before the plates were incubated at 20°C for 24 hours. To determine if the extracts were inhibitory, 20 µl from each well was spotted onto CA plates and incubated for 24-48 hours. Inoculum spots with no growth were rated as inhibitory.

The second assay tested the effect of the crude extracts on chlamydospore impregnated rhododendron leaf disks using six-well plates. Each individual well in the six-well plates was loaded with six leaf disks and 1 ml of sterile water which contained 10 mg of crude extract. Each crude extract that was tested was replicated in two wells. To ensure good contact between the chlamydospores and the

extract, the six well plates were incubated for 48 hours on a platform shaker at room temperature. After incubation, the disks were removed with a pair of sterilised forceps, rinsed in sterile water for 1h and placed onto SMA plates. The plates were incubated for 5 days and the presence/absence of outgrowth of *P. ramorum* recorded for each disk. The entire assay was undertaken twice, and overall means calculated from the combined data.

Bioassays using specific antimicrobial compounds

Individual compounds originally derived from heat-treated pine wood were tested by preparing a dilution series of each compound (range 20 to 0.009 mmol) in dimethyl sulfoxide (DMSO). The compounds previously identified as coniferaldehyde, acetovanillon and vanillin (Kalantarzadeh 2014) were compared against the crude extract from pine.

Each diluted compound was added to a well, in 96-well plates, each well containing zoospores, chlamydospores or mycelium. All treatments were replicated three times. Plates were incubated at 20°C for 24 h for zoospores, and 48 h for chlamydospores and mycelia, then the contents of each well was placed onto a SMA plate and incubated for 5 days at 20°C to assess for any outgrowth of *P. ramorum*. The concentration at which no growth occurred was recorded as the minimum inhibitory concentration (MIC). To test whether the addition of resin acids lowered the MIC of the three compounds, the assay was repeated in combination with 20 mmol of the resin acids dehydroabietic acid (DHAA), abietic acid (AA) and pimaric acid (PA). Activity of resin acids was tested only against zoospores of *P. ramorum* isolates BRC01 and P1578.

Isolation, purification and identification of bioactive compounds

Gas chromatography mass spectrometry (GCMS), carried out with Agilent Technologies 7890A, GC system fitted with an Agilent Technologies 5975 inert XL EI/CI MSD triple axis detector, was used to analyse the methanol crude extracts of heat-treated wood samples. Samples were dissolved in chloroform or methanol (depending on solubility), 2µl of each sample injected and volatilised at 250°C

for 25 minutes using the HP-5-MS column (30 m x 250 μ m x 0.25 μ m) which was heated initially to 50°C for 3 minutes and then to 250°C for 1 minute, and then the temperature held for 30 minutes.

To isolate and purify different bioactive compounds from woodchips, a methanol extract from pine woodchips was reduced to 30 g of crude, dissolved in 100% methanol and mixed with 10 g silica gel (Merck 9385), followed by a drying step in a fume hood for 2 days. A 50 cm cylindrical gravity column with a 4 cm internal diameter was packed using a slurry of silica gel and 100% hexane. The dried crude extract/silica gel was added to the packed column, eluted using 100% dichloromethane (CH₂Cl₂) and collected in 75 ml portions giving around 260 fractions. Thin layer chromatography (TLC) was used to analyse the purity of each fraction. Fractions that had similar TLC bands were subsequently combined. A solution of *p*-anisaldehyde, sulphuric acid and cold methanol (1.5:2.5:96) was used to visualise the TLC spots. Combined fractions were further purified using small gravity columns (1 cm internal diameter) or preparative TLC plates and each purified product was identified using nuclear magnetic resonance (NMR) spectroscopic techniques. The NMR spectra were recorded at room temperature using either a 300 or 500 MHz Bruker AVANCE NMR spectrometer.

To identify potentially antimicrobial compounds from the bark of Japanese larch, material was collected from a naturally infected Japanese larch tree showing red and brown discolouration of the phloem typical of a *P. ramorum* colonised lesion and a GCMS analysis of different regions in and around the lesions was performed, following the preparation of refined dichloromethane (CH₂Cl₂) extracts of these regions as described above. The sample bark regions processed as CH₂Cl₂ extracts were: white tissue (not yet invaded), red tissue (indicating a response to *P. ramorum* but where the pathogen could not be detected either by isolation or PCR-based detection), dark brown tissue behind the red tissue margin (which usually yielded the pathogen when culturing or was PCR-positive for *P. ramorum*). Apart from these samples, a CH₂Cl₂ crude extract of the white phloem tissue from a healthy Japanese tree was also included for comparison.

Field assessments

A trial was set up to compare the potential of air-dried and heat-treated woodchips derived from the natural hosts of *P. ramorum* (rhododendron and Japanese larch) to inhibit the growth of *P. ramorum* under field conditions. This involved exposure to variable inoculum loads of sporangia and zoospores.

In November 2011, a location at Forestry Commission Largin Wood, Cornwall (OS Grid Reference: SX167647), was chosen as the field trial site because of its history of natural *P. ramorum* infection. Prior to the trial setup, *R. ponticum* foliage and soil samples were collected from three different locations at the site and checked for the presence of *P. ramorum* by plating onto SMA. As both foliage and soil samples at all three locations were positive for *P. ramorum*, fine mesh bags filled with either heat-treated and air-dried woodchips of pine, larch and rhododendron were placed on the litter layer at each location and sampled after 4, 8, 12, 20 and 52 weeks. Each bag contained 150 g of woodchips. Six bags of each woodchip type were placed at each location allowing the removal of one bag at each sampling time. To assess whether *P. ramorum* spores had contaminated the bagged woodchips and were viable, after each sampling time the contents of each bag was transferred to a plastic container in the laboratory, flooded with sterile water and five rhododendron leaf disks were placed floating on the water surface to bait for any viable *P. ramorum* associated with the woodchips as originally described by Tsao (1983). After 5 days, the leaf disks were removed and placed onto SMA plates, incubated at 20°C for 7 days, and the number of leaf disks with any outgrowth of *P. ramorum* recorded.

Statistical analysis

All experiments were designed with sufficient replication and balance to allow robust statistical analysis to be undertaken. A simple 1-way ANOVA which assumes a normally distributed error could not be adopted as a suitable model for analysis of the data recorded from this study.

The microcosm experiments using woodchip and soil mixtures which generated cfu count data as an indicator of zoospore survival (summary data shown in **Table 2**) and the field trial which assessed the inhibitory activity of larch, rhododendron and pine woodchips exposed over a period of 52 weeks to natural inoculum of *P. ramorum* (see **Table 7**) were analysed by fitting a series of Generalised Linear Mixed Models (GLMM) (see Schall 1991). For the microcosm experiments, woodchip type (different host species: air-dried or heat-treated), isolates and incubation time were taken as fixed effects, and the number of trials and replicates as random effects. The Poisson error distributions were defined with a logit function for cfu count data. For the field trial, just as before the woodchip type, sampling time and sample location were taken as fixed effects; replicates were random effects. The Poisson error distributions were defined with a logit function for the proportion of *P. ramorum* positive baits.

For all other assays fitting a series of GLMM was severely compromised by the large number of zero or 100% values that were recorded across the various combinations of woodchip treatment, isolate and time in each dataset. To counteract this, exact statistics for binomial proportions were used to obtain confidence intervals (Brown et al. 2001) for each individual set of trials (ie for each combination of woodchip type and isolation) and these tested for differences between woodchip treatments, isolates and time. Thus, each assay trial was considered to have two possible outcomes (growth of *P. ramorum* or no growth) and each trial was considered statistically independent.

Results

Microcosm experiments

Heat-treated pine, larch and rhododendron woodchips completely inhibited the recovery of viable *P. ramorum*, regardless of isolate, compared to the air-dried woodchips ($p<0.001$) (**Table 1**). All the inoculated air-dried wood treatments yielded colonies of *P. ramorum* after plating and the only notable difference was the slightly lower level of survival of P1578 zoospores compared to BRC01 which was found to be significant ($p<0.05$). In contrast, neither isolate of *P. ramorum* was recovered from any of the heat-treated woodchips or from any of the water only amended controls.

When similar numbers of zoospores were added to heat-treated larch woodchips mixed with non-sterile soil, there was no recovery of *P. ramorum* from the mixtures after 4 weeks of incubation even when the ground larch wood comprised only a quarter of the mix. After 8 weeks incubation, there was some recovery of *P. ramorum* from the same larch:soil mix (1:3), but the quantity was low (**Table 2**). More viable *P. ramorum* propagules (cfu) were always recovered from the soil only or soil-perlite mixtures compared with the larch-soil mixtures ($p<0.001$). Significantly more colonies were isolated from pure soil compared to the 1:3 ratio of perlite to soil mix, which itself produced significantly more colonies than the 3:1 perlite:soil mix ($p<0.001$). Isolate BRC01 also yielded significantly more cfu than P1578 from the soil-woodchip mixtures ($p<0.001$) (**Table 2**).

In further tests using leaf disks enriched with resilient *P. ramorum* chlamydospores placed in soil-larch woodchip mixtures, the heat-treated larch woodchips completely inhibited the growth of *P. ramorum* (**Table 3**). Even when larch woodchips were mixed with soil (3 parts larch to 1 part soil) *P. ramorum* was recovered from <6% of the leaf disks (2.7% and 5.7% of the leaf disks for isolates P1578 and BRC01 respectively) after 4 weeks of incubation. With the same treatment, no growth of *P. ramorum* was recorded after 12 weeks incubation (**Table 3**). In contrast, between 86 and 100% of the infected leaf disks placed in either soil alone or a soil-perlite mixture yielded *P. ramorum* (**Table 3**). Overall, differences between treatments that contained heat-treated larch woodchips and those without larch woodchips were highly significant ($p<0.001$) (**Table 3**). However, there were no significant differences between isolate or incubation time.

Bioassays of crude wood extracts

When the activity of methanol crude extracts made from woodchips of air-dried pine, larch and rhododendron was compared with extracts of heat-treated wood, strong differences were apparent (**Table 4**). None of the extracts from air-dried wood had an inhibitory effect on isolate BRC01 at any of the concentrations tested although isolate P1578 was inhibited by air-dried wood extracts from both pine and larch at a concentration of 25 mg ml⁻¹. In contrast, the methanol crude extracts from heat-treated pine, larch and rhododendron all inhibited *P. ramorum* growth ($p<0.001$) and although there

were slight differences depending on the isolate and wood species these were not statistically significant. The lowest minimum inhibitory concentration (MIC) when using extracts from heat-treated pine and larch was 6.25 mg ml⁻¹ against isolate P1578, and 12.5 mg ml⁻¹ and 6.25 mg ml⁻¹ against isolate BRC01 (pine and larch respectively). Methanol extracts derived from heat-treated rhododendron wood had a MIC of 25 mg ml⁻¹ against P1578 and 50 mg ml⁻¹ against BRC01 (**Table 4**).

Methanol crude extracts of heat-treated larch, pine and rhododendron at a concentration of 10 mg ml⁻¹ also significantly reduced the outgrowth of *P. ramorum* from chlamydospore-rich leaf disks by more than 90% compared to similar extracts from air-dried wood ($p < 0.001$, calculated at 95% confidence limits). Overall, the mean number of leaf disks yielding *P. ramorum* was much higher (>80%) when the crude extract was derived from air-dried wood, regardless of whether it came from pine, larch or rhododendron, compared to the mean number of leaf disks (<20%) yielding *P. ramorum* when the crude extract came from heat-treated woodchips (**Fig 1**).

Compound assessments and chemical analyses

Initial tests concentrated on using heat-treated pine wood crude extract and the pure wood-derived compounds acetovanillone, coniferaldehyde and vanillin which had already been shown to have antimicrobial activity against bacterial pathogens (Kalantarzadeh 2014). These compounds had activities 2-10 times greater than the crude extract when tested against zoospores, chlamydospores and mycelia of *P. ramorum* (**Table 5**). Coniferaldehyde proved the most inhibitory (MIC of 0.15 mmolar against zoospores; 1.7 mmolar against mycelium and chlamydospores) whilst acetovanillone and vanillin had similar antimicrobial properties (MICs of 1.5-1.6 mmolar against zoospores, 3.4-6.6 mmolar against mycelia and chlamydospores). All compounds (including the crude extract) had similar activities against the two *P. ramorum* isolates used in the assays.

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Addition of 20 mmol of the resin acid dehydroabietic acid to coniferaldehyde and acetovanillone reduced the MIC 8-fold, whilst in combination with vanillin the MIC was reduced 4-fold. The effects were similar with both isolates of *P. ramorum* (Table 6). Likewise, in a subsequent assay which combined acetovanillone, coniferaldehyde and vanillin with either dehydroabietic acid or abietic acid, the MIC decreased 4 to 16-fold. However, the addition of another resin acid, pimaric acid, had no significant effect on the MIC (Table 6).

GCMS analysis of the methanol crude extracts of heat-treated pine, larch and rhododendron (1 mg ml^{-1} methanol) revealed a number of chemical constituents (Fig 2, a-c). Spectra of all three crude extracts had peaks for vanillin at 13.7 minutes, vanillic acid at 15.8 minutes, and syringaldehyde at 16.9 minutes. The peak for vanillin was considerably higher in the crude extract of pine and larch compared to rhododendron. One peak at 17.7 minutes, identified as coniferaldehyde (NIST library), was only present in larch and pine extracts. In contrast, benzoic acid (18.67 minutes), syringic acid (18.73 minutes) and sinapaldehyde (20.34 minutes) were only present in the crude extract of rhododendron (Fig 2, c).

Analysis of dichloromethane (CH_2Cl_2) crude extracts of naturally infected Japanese larch bark revealed that prior to detection of the pathogen in invaded bark, the white phloem tissue at the edge of the lesion (i.e. apparently uninvaded tissue) showed an abundance of resin acid 2 (24.8 minutes) and much lower amounts of resin acid 1 (24 minutes) compared with the similarly coloured phloem tissue from an uninfected tree (control) (Fig 3 a, b). However, in the crude extract of the red-discoloured bark, resin acids 1 and 2 were present in considerable quantities, but in the brown tissue, where recovery of *P. ramorum* was usually the easiest, resin acid 2 was at levels similar to the control (Fig 3 c, d). Due to the trace quantity of resin acids, they could only be identified from a chromatogram of a spiked sample of CH_2Cl_2 crude extract with 10 mg of synthetic dehydroabietic acid and abietic acid. Resin acid 2 was identified as dehydroabietic acid (Fig 3 a) and resin acid 1 was identified as methyl abietate (Fig. 3 b), a derivative of abietic acid. The red discoloured tissue had raised levels of many other compounds compared with the control and white coloured phloem tissue. Monoterpenes, α -

pinene (6.14 minutes) and 3-carene (7.58 minutes) were only present in the extracts of red and dark brown phloem tissue and were apparently formed as a host response during pathogen invasion (Fig 3 c, d).

Field tests

No viable *P. ramorum* was detected from either the air-dried or heat-treated wood chips of pine, larch or rhododendron after 4, 8 and 12 weeks field exposure on the trial site in Cornwall (Table 7) but monitoring of infected plants on site during that twelve-week period indicated very little *P. ramorum* inoculum was produced between November to February. After 20 weeks, *P. ramorum* was isolated from the air-dried rhododendron woodchips, demonstrating that natural inoculum was being generated from infected host plants and coming into contact with the woodchips. Despite this, *P. ramorum* was not isolated from either the pine or larch woodchips although the difference in the amount of *P. ramorum* recovery between the three treatments was not statistically significant. However, after 52 weeks on the site (up to November 2012), there was more than a 2-fold increase in the recovery of *P. ramorum* from the air-dried larch and pine wood chips compared to the heat-treated wood chips ($p<0.001$). In contrast, although recovery from the heat-treated rhododendron woodchips was slightly lower than the air-dried material, levels of *P. ramorum* were not significantly different between the two (Table 7).

Discussion

Heat-treating chipped wood from rhododendron, pine and larch at 120°C for 72 hours enhanced the anti-microbial activity when compared to similar woodchips that had only been air-dried and not exposed to elevated temperatures. In particular, the wood from pine and larch appeared to become strongly inhibitory to spores and mycelium of *P. ramorum* after heat treatment and significantly reduced the recovery of the pathogen. Rhododendron woodchips, in contrast, never caused the same degree of inhibition. Furthermore, when heat-treated larch was mixed with soil in varying proportions and then exposed to zoospores and chlamydospores in the laboratory, the inhibitory effect continued

for up to 12 weeks although the two isolates of EU1 lineage *P. ramorum* used in the tests showed small but significant differences in sensitivity.

Earlier work by Manter and co-workers (2006, 2007) which evaluated the antimicrobial activity of yellow cedar against *P. ramorum*, demonstrated a correlation between bioassay results from the chemical components and the effectiveness of woodchips used in field treatments. Likewise, our study showed that both in the field and under controlled laboratory conditions heat-treated woodchips of larch and pine were strongly inhibitory to the pathogen. In the field trial the inhibitory effect of heat-treated pine and larch woodchips was evident for up to a year. Activity was also retained in soil/woodchip mixtures under moist conditions even though high moisture has been shown to favour survival and recovery of *P. ramorum* from infested soils and leaf litter (Fichtner et al. 2007).

Our bioassay tests showed the responses of the two *P. ramorum* isolates to crude extracts of heat-treated were dose dependent and the extracts appeared to contain a range of compounds, some of which were highly inhibitory. When pure compounds vanillin, acetovanillin and coniferaldehyde were tested they were found to be 2-10 times more active than the crude extracts. Since all three tested wood species showed some inhibitory activity, the potential inhibitors are likely to be produced through heat mediated chemical modifications of the wood. Chemical analysis of the methanol extracts showed some of the induced compounds were present in all three wood extracts but those with the most activity against *P. ramorum* were either absent or only present at lower concentrations in rhododendron wood as compared to larch or pine. With gas chromatography mass spectrometry analysis, the peak for vanillin, a phenolic aldehyde, was considerably higher in the extracts of pine and larch than in the rhododendron extract. In addition, coniferaldehyde which was only present in larch and pine extracts, proved to be the most active compound in limiting growth of mycelium, chlamydospores and zoospores of *P. ramorum*. The absence of coniferaldehyde in the crude extract of heat-treated rhododendron appears to be the reason why pine and larch extracts had a much greater inhibitory effect on *P. ramorum* than heat-treated rhododendron woodchips. A previous study by

Garbber et al. (1998) found that cell wall degradation of maize by fungal enzymes was severely limited by the presence of coniferaldehyde, highlighting the antimicrobial activity of this compound.

The results of chemical analysis and the bioassays of identified compounds in our study also suggest that the magnitude of antimicrobial activity may not just be due to some individual compounds, such as aldehydes and phenolic aldehydes, but result from the synergistic activity between some of the compounds. Dehydroabietic and abietic acid are diterpenoid components of heat-treated pine and larch and both these resin acids enhanced the inhibitory effect of aldehydes such as coniferaldehyde and vanillin. The same resin acids are known to be present in oleoresin produced by conifers (Savluchinske-Feio et al. 1999) and have low toxicity toward fungal pathogens but are of importance in sealing wounds and trapping insect invaders (Bollschweiler et al. 2007; Cleary 2010). In addition, higher concentrations of resin acids in decay resistant species are associated with the resistance of heartwood to brown-rot fungi, with abietic acid being the most abundant.

Interestingly, chemical analysis of the red discoloured region of Japanese larch bark in the process of being invaded by *P. ramorum*, not only revealed some of the same compounds found in the heat-treated larch wood but also provided evidence of resin acid production induced by *P. ramorum*. The increased resin acid content of infected phloem tissue suggests these compounds could play an important role in inhibiting the growth of *P. ramorum* as it attacks larch bark, but the effectiveness of the inhibition may depend on concentrations in the bark and potentially be overcome by the multiple infections that occur under natural conditions (Webber and Brasier 2018). The pattern of chemical changes observed in the phloem tissue of infected larch prior to, during and post invasion, also showed raised levels of many other compounds in addition to the resin acids. In particular, peaks for α -pinene and 3-carene in the extracts of red and dark brown phloem tissue were markedly higher. Both are found in the essential oils of conifers that exhibit toxic activity against many fungi and insects (Napierała Filipiak et al. 2002; Phillips and Croteau 1999).

Phenolics such as ellagic acid and a tyrosol derivative have already been found in the phloem of *Quercus agrifolia* Née (coast live oak), another species frequently infected by *P. ramorum* in the mixed-evergreen forests of California. Surviving coast live oaks appear to have constitutively higher levels of these phenolics than more *P. ramorum* susceptible individuals suggesting the compounds would be good candidates for examination as potential biomarkers for resistance to *P. ramorum* (Nagle et al. 2011; Conrad et al. 2014). For the same reasons, a greater understanding of what compounds are induced in Japanese larch bark during the early stages of pathogen attack could provide insights into what resistance mechanisms against *P. ramorum* are already operating. They could also be important traits to select for in any larch breeding programme aimed at finding genetic resistance to *P. ramorum*. Additionally, the active antimicrobial chemicals found in the heat-treated larch and pine wood could be novel candidates in the development of chemical treatments against *P. ramorum* and possibly other pathogens.

Conclusions

Extensive research efforts aimed at finding effective methods to eliminate or control *P. ramorum* have included chemical control to counter host infection (e.g. Garbelotto and Schmidt 2009), composting of infected residues (e.g. Swain et al. 2006) and searching for resistance (e.g. Conrad et al. 2014). Many of these disease-focussed studies have been summarised by Kliejunas (2010) but he also concluded that no single strategy is likely to be effective in eradicating *P. ramorum*, especially once it has established in the wider environment. The use of woodchips with enhanced antimicrobial activity against *P. ramorum* could offer another tool for reducing pathogen survival in wider environment outbreaks.

When taking action against *P. ramorum* infestations in natural and semi-natural environments, best management practices tend to concentrate on slowing disease spread as eradication efforts are difficult to implement successfully in these complex environments compared with the relatively controlled setting of nurseries. Safe disposal or containment of infested material from the wider environment outbreaks is also frequently challenging and costly. Spores, including persistent chlamydospores of *P.*

ramorum readily survive in soil and fallen foliage (Fichtner et al. 2007) and this inoculum reservoir is implicated in the infection of susceptible hosts such as rhododendron as it re-sprouts or reinvades following the clearance of infected hosts (Elliot et al. 2013; Harris 2015). Thus using a layer of woodchips to act as a barrier between infested soil/litter and the shrub or brush layer above could potentially prevent reinfection and inoculum multiplication.

Although our data indicated that the inhibitory activity of larch and pine woodchips generated after heat-treatment has the potential to operate in ecologically complex environments over several weeks and even for up to a year, the effect is likely to be modulated by external influences. Rainfall will probably leach the antimicrobials from the woodchips over time whilst soil microbes may break down the compounds *in situ*. Consequently, if woodchip treatments are to be used for long-term suppression of *P. ramorum* in natural environments further investigation and trialling is recommended, as the tests have only focussed on the EU1 lineage of the pathogen. For deployment, the method might be most useful in parks and gardens to treat limited areas following removal of infected plants to suppress any remaining inoculum and prevent reinfection of nearby susceptible hosts. Goheen (2013) also highlights the use of woodchip treatments for places where human activities likely to disperse *P. ramorum* are concentrated, such as roads, walk-ways and paths in infested forests and woodlands. However, there would be extra cost implications if woodchips are used that have to be heat-treated.

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Figure legends

Figure 1 Effect of methanol crude extracts of air-dried (AD) and heat-treated (H) wood of pine, larch and rhododendron on the outgrowth of *Phytophthora ramorum* from pre-colonised chlamydospores-rich leaf disks of rhododendron after plating onto selective medium. The mean number of disks with *P. ramorum* growth was calculated as a percentage from two trials, each with a total of 12 disks for each treatment.

Figure 2 Comparison of gas chromatography spectra of methanol crude extracts of (a) heated pine (b) larch and (c) rhododendron. Peak 1 is vanillin, 2 is vanillic acid, 3 is syringaldehyde, 4 is coniferaldehyde, 5 is benzoic acid, 6 is syringic acid, and 7 sinapaldehyde.

Figure 3 Comparison of gas chromatography spectra of crude CH₂Cl₂ extracts from Japanese larch bark. (a) phloem tissue of a healthy tree, (b) white phloem prior to invasion (c) red discoloration following invasion and (d) dark brown phloem formed as a response to invasion by *P. ramorum*. Peaks 1 and 2 represent two resin acids at 24.00 minutes and 24.7 minutes respectively.

Table 1 Recovery of *Phytophthora ramorum* three weeks after inoculation of air-dried and heat-treated woodchips of pine, larch and rhododendron. Woodchips were incubated with zoospore inoculum for three weeks and then placed onto selective medium to assess the growth of *P. ramorum* from surviving inoculum. *P* levels expressed for a 95% confidence limit

Treatment	No of trials	n	Recovery of <i>P. ramorum</i> from inoculated woodchips		Significance for isolate BRC01 vs P1578 across all woodchip types
			Isolate BRC01	Isolate P1578	
Air-dried woodchips					
Scots pine (<i>Pinus sylvestris</i>)	4	10	10.0*	9.0*	<i>p</i> <0.05
Japanese larch (<i>Larix kaempferi</i>)	4	10	9.8	8.1	
Rhododendron (<i>Rhododendron ponticum</i>)	4	10	10.0	10.0	
Heat-treated woodchips					
Scots pine (<i>P. sylvestris</i>)	4	10	0.0	0.0	
Japanese larch (<i>L. kaempferi</i>)	4	10	0.0	0.0	
Rhododendron (<i>R. ponticum</i>)	4	10	0.0	0.0	
Significance for air-dried vs heat-treated			<i>p</i> <0.001	<i>p</i> <0.001	

* Means calculated from four trials and 10 replicates for each treatment within a trial. Except for the larch woodchips *P. ramorum* was recovered from all replicates/trials of air dried woodchips and none of the heat-treated woodchips.

Table 2 Effect of diluting heat-treated larch (*Larix kaempferi*) woodchips with non-sterile soil on the survival of two isolates of *Phytophthora ramorum*, 4 and 8 weeks after inoculation with zoospores. Successful recovery of viable *P. ramorum* was demonstrated by the number of colony forming units (cfu) produced after plating a gram of the mixture onto *Phytophthora* selective medium.

Treatment	n	Mean zoospore recovery $\pm$ SE (cfu g ⁻¹)				<i>p value</i>
		Isolate BRC01		Isolate P1578		
		4 weeks	8 weeks	4 weeks	8 weeks	
All larch	4	0.0	0.0	0.0	0.0	
3 parts larch : 1 part soil	4	0.0	0.0	0.0	0.0	
1 part larch : 3 parts soil	4	0.0	0.6 $\pm$ 0.1	0.0	0.5 $\pm$ 0.1	
3 parts perlite : 1 part soil	4	3.8 $\pm$ 0.7	1.0 $\pm$ 0.0	2.1 $\pm$ 0.3	1.2 $\pm$ 0.1	
1 part perlite : 3 parts soil	4	5.6 $\pm$ 0.8	2.1 $\pm$ 0.2	4.4 $\pm$ 0.5	2.0 $\pm$ 0.0	
All soil	4	6.7 $\pm$ 0.2	4.2 $\pm$ 0.4	5.0 $\pm$ 0.2	3.5 $\pm$ 0.6	
Significance for larch + soil mixtures vs perlite + soil mixtures						<i>p</i> <0.001
Significance for 4 week vs 8 week incubation (only for 1:3 larch:soil mixture)						<i>p</i> <0.01
Significance for isolate BRC01 vs isolate P1578 across all mixtures						<i>p</i> <0.001

Table 3 Effect of diluting heat-treated larch (*Larix kaempferi*) woodchips with non-sterile soil on the germination of *Phytophthora ramorum* chlamydospores present in rhododendron leaf disks after 4 and 12 weeks of incubation. Successful recovery of viable *P. ramorum* was demonstrated by outgrowth from the leaf disks plated onto *Phytophthora* selective medium. *P* levels expressed for a 95% confidence limit.

Treatment	No. of trials (pots)	n	% leaf disks with <i>P. ramorum</i> outgrowth				<i>p</i> value
			Isolate BRC01		Isolate P1578		
			4 weeks	12 weeks	4 weeks	12 weeks	
All larch	3	12	0.0%*	0.0%*	0.0%*	0.0%*	
3 parts larch : 1 part soil	3	12	5.5%	0.0	2.7%	0.0	
3 parts perlite : 1 part soil	3	12	86.1%	91.7%	91.7%	94.5%	
All soil	3	12	100%	100%	100%	100%	
Significance for larch + soil mixtures vs perlite + soil mixtures							<i>p</i> <0.001
Significance for 4 weeks vs 12 weeks and isolate BRC01 vs isolate P1578							<i>p</i> >0.1

*Means calculated from three trials, with 12 leaf disks per trial

Table 4 Minimum inhibitory concentration (MIC) of the methanol crude extract (mg ml⁻¹) obtained from air-dried and heat-treated wood of pine, larch and rhododendron and tested against zoospores of *Phytophthora ramorum* derived from two isolates (BRC01 and P1578).

Crude wood extract	n	Minimum inhibitory concentration (mg ml ⁻¹)				<i>p</i> value
		Heat-treated wood		Air-dried wood		
		BRC01	P1578	BRC01	P1578	
Scots pine (<i>Pinus sylvestris</i>)	3	≥12.5	≥6.25	0.0	≥25	
Japanese larch (<i>Larix kaempferi</i>)	3	≥6.25	≥6.25	0.0	≥25	
Rhododendron (<i>Rhododendron ponticum</i>)	3	≥50	≥25	0.0	0.0	
Significance for air-dried vs heat-treated						<i>p</i> <0.01
Significance between isolates and between wood species						<i>p</i> >0.1

* MIC was the same for each replicate

Table 5 Minimum inhibitory concentration (MIC) of the compounds derived from heat-treated wood of Scots pine (*Pinus sylvestris*) tested against mycelia, zoospores and chlamydospores derived from two isolates of *Phytophthora ramorum* (BRC01 and P1578).

Compounds	n	Minimum inhibitory concentration (mmolar)					
		Mycelia		Zoospores		Chlamydospores	
		BRC01	P1578	BRC01	P1578	BRC01	P1578
Crude pine wood extract*	3	25	25	12.5	6.25	10	10
Acetovanillone	3	6.6	3.3	1.6	1.6	6.6	3.3
Coniferaldehyde	3	1.7	1.7	0.15	0.15	1.7	1.7
Vanillin	3	3.4	3.4	1.52	1.52	3.4	3.4

* MIC for crude extract expressed as mg ml⁻¹

Table 6 Minimum inhibitory concentration (MIC) of acetovanillone, coniferaldehyde and vanillin, with and without 20 mmol dehydroabietic acid (DHAA), 20 mmol abietic acid and 20 mmol pimaric acid, tested against zoospores of two isolates of *Phytophthora ramorum*.

Compounds	n	MIC* (mmol) against <i>P. ramorum</i>		
		Assay 1		Assay 2
		BRC01	P1578	BRC01
Acetovanillone + water	3	1.6	1.6	1.6
Acetovanillone + DHAA	3	0.2	0.2	0.2
Acetovanillone + AA	3	-	-	0.1
Acetovanillone + PA	3	-	-	1.6
Coniferaldehyde + water	3	0.15	0.15	0.1
Coniferaldehyde + DHAA	3	0.02	0.02	0.02
Coniferaldehyde + AA	3	-	-	0.02
Coniferaldehyde + PA	3	-	-	0.11
Vanillin + water	3	1.52	1.52	1.5
Vanillin + DHAA	3	0.38	0.38	0.38
Vanillin + AA	3	-	-	0.38
Vanillin + PA	3	-	-	1.5

* MIC was the same for each replicate

Table 7 Effect of heat-treated woodchips of larch, rhododendron and pine tested in the field on the survival of natural inoculum of *Phytophthora ramorum*. After harvesting each treatment replicate was baited with 30 rhododendron leaf disks per sample to test for the presence of viable *P. ramorum*.

Treatments	n	Sampling intervals						Significance air-dried vs heat-treated
		4, 8, 12 weeks		20 weeks		52 weeks		
		Air-dried	Heat-treated	Air-dried	Heat-treated	Air-dried	Heat-treated	
Japanese larch (<i>Larix kaempferi</i>)	18	0.0	0.0	0.0	0.0	13.5 ± 5.0	3.1 ± 2.1	<i>p</i> < 0.001
Scots pine (<i>Pinus sylvestris</i>)	18	0.0	0.0	0.0	0.0	16.1 ± 2.9	3.8 ± 2.5	<i>p</i> < 0.001
Rhododendron (<i>Rhododendron ponticum</i>)	18	0.0	0.0	2.8 ± 1.8	0.0	16.1 ± 5.0	8.5 ± 3.3	<i>p</i> > 0.05