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Epidemiology and microevolution of *Phytophthora ramorum* during a controlled disease outbreak in a simulated plant production facility

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Phytophthora ramorum outbreaks have been documented to occur in natural settings during favourable environmental conditions, and to be caused by the overdominance of highly infectious genotypes. However, little is known about the dynamics of outbreaks in nursery settings. Through the description and quantification of symptoms, as well as through systematic pathogen isolation and genotyping, this study examines the scale and dynamic of spread of four different genotypes of *P. ramorum* in soil, water and leaves among *Rhododendron* plants in a nursery setting. *Phytophthora ramorum* isolation success was highest from leaves and intermediate from soil, reaching peak values at the end of the wet-warm season. The observed disease outbreak was of moderate intensity, and abundance among the four genotypes used as inoculum varied, depending on substrate and isolation time. The spread mechanism of the disease was mostly through leaf-to-leaf contagion, followed by leaf-to-soil, and the scale of pathogen spread was less than 2 m in the 20 months of the experiment. Surprisingly, a large number of novel genotypes were detected during the experiment, and all were clearly derived from the four used as inoculum. The frequency of two such novel genotypes in the post-outbreak phase was comparable to the frequency of some of the original four genotypes, suggesting they may be competitive. The creation of new genotypes in a nursery setting poses a threat to the industry itself, as well as to wildlands, due to the increase in pathogen adaptability often associated with new genetic variation.

Keywords: genetic diversity, multilocus genotypes, nursery setting, spread pathways

Introduction

The knowledge on the biology and epidemiology of *Phytophthora ramorum* is rapidly expanding both for wildlands and for nursery settings (Ivors *et al.*, 2006; Tjosvold *et al.*, 2008; Garbelotto & Hayden, 2012; Croucher *et al.*, 2013; Eyre *et al.*, 2013; Grünwald *et al.*, 2019). Nonetheless, significant gaps in knowledge still exist. In particular, the spatial scale and rate of spread in nurseries, and comparative levels of plant infection between soil and water infestation by *P. ramorum* in nurseries are still unclear. Some of the limitations of research on *P. ramorum* in nurseries have been dictated by the difficulty of performing manipulative experimentation due to strict government regulations on the pathogen (USDA Animal Plant Health Inspection Service, 2002; EPPO, 2016), and by the need to eradicate outbreaks in nurseries once they are detected. This difficulty is additionally compounded by the fact that nurseries in North America and Europe are characterized by a different composition of evolutionary lineages of the pathogen, each with its own distinctive genetic and phenotypic traits (Ivors *et al.*, 2006; Elliott *et al.*, 2011; Eyre *et al.*, 2014), and by a different climate.

Water, soil, and even symptomless plants have all been broadly indicated as potential reservoirs of pathogen inoculum both in Europe and in North America (Werres *et al.*, 2007; Eyre *et al.*, 2013; Eyre & Garbelotto, 2015), but the relative importance of each is still unclear. Experimentation has shown water-mediated spread of infection in natural systems and production facilities (Davidson *et al.*, 2005; Tjosvold *et al.*, 2008) without fully quantifying such spread, while circumstantial but convincing evidence has indicated that intense rain events leading to partial flooding of nursery beds can cause severe outbreaks of the disease in production nurseries (Garbelotto & Rizzo, 2005; Chastagner *et al.*, 2010). Inert potting mix has been shown to harbour viable chlamydospores of the pathogen for years (Linderman & Davis, 2006; Shishkoff, 2007), and the presence of root infection in some ornamental plants, notably rhododendrons, points to a possible soil-to-root pathway of infection (Rizzo *et al.*, 2005). In summary, soil, water and plants represent substrates with potentially different epidemiological roles and with possible substrate-driven genotypic and ecological adaptation (Eyre *et al.*, 2013; Eyre & Garbelotto, 2015).

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Additionally, evidence from research performed in natural settings on all three substrates indicates that pathogen populations in the three substrates respond differently to varying environmental conditions (Eyre *et al.*, 2013, 2014). Ecological constraints have been shown to be the most important limiting factor in the spread of *P. ramorum* in natural settings (Eyre *et al.*, 2013; Garbelotto *et al.*, 2017; Kozanitas *et al.*, 2017), hence the differential response of populations of the pathogen in different substrates adds a significant and relevant complexity to the disease cycle. Finally, it has recently been shown that different hosts can substantially affect the pathogenicity of identical genotypes of *P. ramorum* through epigenetic regulation (Kasuga *et al.*, 2016), but there is no information on whether the same may be true for isolates originating from different substrates such as soil, water and plants.

Seasonal fluctuation of populations of the pathogen both in nurseries and in forests has been identified in Europe (Werres *et al.*, 2007; Junker *et al.*, 2016) and North America (Tjosvold *et al.*, 2008; Chastagner *et al.*, 2010; Eyre & Garbelotto, 2015). Eyre & Garbelotto (2015) have shown that both pathogen viability and DNA-based detection in water decrease when water temperature increases and when rainfall ceases, at least in central California. This pattern of decreasing viability and ability to detect the pathogen during the dry months, combined with the observation that water populations from one year are genotypically different from the year before, suggests water is reinoculated every year from infected leaves, at least in a natural setting (Eyre & Garbelotto, 2015). Whether these findings may apply to a nursery setting, where the environment is in part controlled, remains to be tested.

Finally, as expected for an infectious disease, forest outbreaks occur during favourable environmental conditions, such as abundant rainfall and warm temperatures in the case of sudden oak death, and are caused by the overdominance of highly infectious genotypes (Eyre *et al.*, 2013), but there is no knowledge of how pathogen populations may change genotypically during an outbreak in a nursery setting.

This study provides an opportunity to elucidate some aspects of the epidemiology of pathogen populations in three substrates in a nursery setting, in a way analogous to the study that has been recently completed for wildland settings in San Mateo County (Eyre & Garbelotto, 2015), but through a controlled experiment.

The main goals of the study are: (i) to understand how pathogen populations fluctuate seasonally in terms of overall abundance, genotypic richness and evenness in soil, water and plants in a nursery setting; (ii) to determine if such fluctuations are associated with changes in disease incidence and/or severity and if the seasonal pattern of these changes is the one expected, given the known biology of the pathogen; (iii) to establish at what spatial rate the pathogen spreads in simulated nursery beds regularly irrigated over a period of 20 months; (iv) to ascertain the preferential spread pathways such as leaf-to-leaf, leaf-to-soil, soil-to-leaf or soil-to soil; (v) to determine whether there are differences in prevalence among isolates coming from different substrates (soil, water, leaves) that may suggest either a differential fitness or an adaptation to inhabit different substrates; (vi) to explore the potential of new genotypes to emerge in a nursery setting, and to determine at what pace this microevolutionary process occurs, and if it is occurring at the same rate for each of the genotypes initially employed; and (vii) to determine if new genotypes are rare or abundant, and if they are competitive with the original genotypes used to inoculate plants.

Materials and methods

Isolates

Four isolates, each representing a different multilocus genotype (MLG), were used as inoculum: one had been found to be dominant in soil (S), one on leaves (L), one in water (W), and one was found in all three substrates (A) during the course of an intensive sampling study conducted in forest stands spanning two drainages in the San Francisco Peninsula (Eyre & Garbelotto, 2015). According to their colony macromorphology and microsatellite profiles, these isolates all belong to the NA1 lineage of the pathogen. Before inoculations and to ensure comparable viability of the four isolates, each was inoculated into a detached rhododendron leaf by placing an agar plug of mycelium onto a pinprick wound on the abaxial surface of each leaf (Eyre *et al.*, 2014). After 1 week of incubation at 18 °C in a moist chamber, small portions of leaf showing visible lesions were plated onto PARP medium (Jeffers & Martin, 1986) to reisolate the culture. All the isolates are stored at the Garbelotto laboratory culture collection, at the University of California, Berkeley (UCB).

Zoospore inoculum preparation

Phytophthora ramorum isolates were separately grown up on Petri dishes containing 10% clarified V8 agar (Vettraino *et al.*, 2010) for 2 weeks in the dark at 18 °C. In order to induce production of sporangia in the resulting mycelia, 1 cm^2 pieces of cultures were flooded with 20 mL of 1% soil tea and incubated in the dark at 18 °C for 2 days, according to the methodology described by Eyre *et al.* (2014). At that time, zoospore release was induced through cold shocking of culture plates by placing them on a bed of ice for 30 min, followed by inoculation at room temperature for 1 h. The production of zoospores was counted in a haemocytometer and inoculum was adjusted to 5×10^4 zoospores mL^{-1} for each isolate (Eyre *et al.*, 2014).

Experimental set-up and plant inoculation

Two experimental plots were set up at the National Ornamental Research Site of the Dominican University of California (NORSDUC) greenhouse facilities, each containing two square subplots, with a side of 3.6 m, and partitioned in 8 × 8 grids, each containing a 4.5 L (1-gallon) pot with a *Rhododendron* 'Cunningham's White' plant (Fig. 1a). The canopies of the plants were touching, and the distance between two main stems on a row was 0.45 m. In each subplot, four plants located in one corner were inoculated with zoospore suspensions from each of the

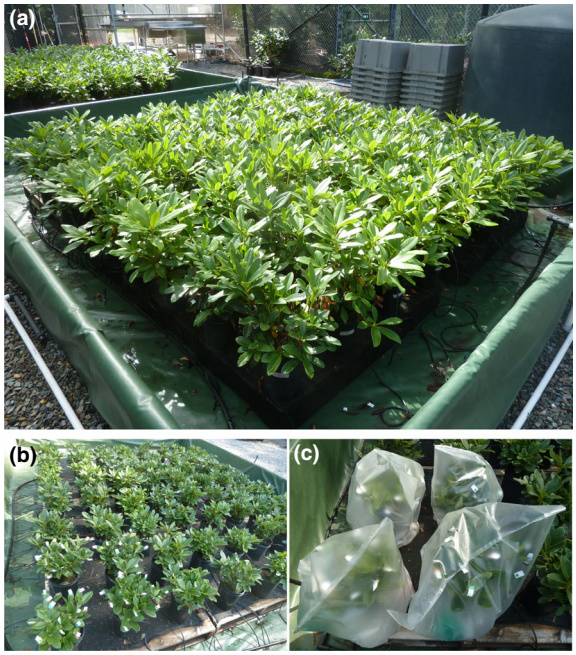

Figure 1 (a) One of the experimental plots showing the healthy condition of rhododendron plants before the inoculation; (b) for the four plants in the nearside corner, each leaf was labelled with a colour-coded tag to denote which of the four inoculum types (A, S, L or W genotype) it would be inoculated with; (c) 24 h after the inoculation, the four inoculated plants were covered with bags to maintain a moist environment and to buffer any extreme dips in temperature, in order to promote the establishment of the pathogen on each plant. [Colour figure can be viewed at wileyonlinelibrary.com].

four genotypes (Fig. 1b). That was accomplished by inoculating 16 leaves per plant, with each of the four genotypes inoculated on four leaves. Leaves were randomly assigned to a genotype and were labelled with a different colour tag, depending on the genotype. Each leaf was inoculated with 100 µL of zoospore suspension pipetted into a Parafilm cup previously made around the stem and base of a leaf, where a small wound had been previously generated using the sharp tip of a hairpin. Twenty-four hours after inoculation and in order to promote equal infection success, inoculated plants were covered with bags to maintain a moist environment and to buffer any extreme dips in temperature for 1 week (Fig. 1c). After that time, lesions were visible in inoculated leaves. Inoculations were performed in February 2013.

During the experiment, an overhead irrigation system was set up around each plot to simulate rain and facilitate the spread of the pathogen. Plants were irrigated twice a week in the absence of rain, or 3 days after any substantial rain event. After the third sampling, irrigation water for two of the four plots came from the tanks collecting run-off water from the experimental plant beds. This was done to test whether using recycled water would result in an increase in infection rate.

Experiment monitoring and sampling

Six weeks, and 3, 6 and 9 months after inoculation, *P. ramorum* disease symptoms (lesions on the stem and leaves, and wilting leaves) were assessed for each plant. Additionally, soil and leaf samples were collected from a number of plants and corresponding pots in the experiment. The number of sampled plants and pots increased at each sampling time, with the first sample limited to the four plants that had been originally inoculated, and later samplings including increasingly more pots around the original four (Fig. 2). Samples were taken from tissue with symptoms if available, or from a randomly selected leaf, if no symptoms were visible. Plants with obvious symptoms and the soil in their pots were sampled, even if not included in the set of plants to be tested at each sampling time.

Foliar sampling

At each sampling time (Fig. 2), a single hole punch (4 mm diameter) was taken from 10 leaves per plant. Punches were taken rather than whole leaves to maintain an inoculum on the plant if present. On the original four inoculated plants, only leaves that were not those originally inoculated were sampled to ascertain if infection had spread within the inoculated plants. Foliar lesions that could be potentially caused by *P. ramorum* were sampled by taking punches at the edges of each lesion. If there were no visible lesions, then symptomless leaves were punched.

All leaf discs were plated into PARP + H selective grown medium (Vettraino *et al.*, 2010) and incubated in the dark at 18 °C. *Phytophthora ramorum* colonies were selected based on microscopy morphology and subcultured onto clean PARP medium.

Soil sampling

A small soil sample (a tablespoon) was taken from the pot of each plant that was sampled (Fig. 2). Each sample was a composite of four collections made around the root collar. Soil was tested for the presence of the pathogen using 10 leaf discs of uninfected Cunningham's White rhododendron as bait for *P. ramorum* (Eyre *et al.*, 2014). After 7 days of incubation, leaf discs were plated on PARP + H medium and treated as leaf samples as described above.

R8	R7	R6	R5	R4	R3	R2	R1
R16	R15	R14	R13	R12	R11	R10	R9
R24	R23	R22	R21	R20	R19	R18	R17
R32	R31	R30	R29	R28	R27	R26	R25
R40	R39	R38	R37	R36	R35	R34	R33
R48	R47	R46	R45	R44	R43	R42	R41
R56	R55	R54	R53	R52	R51	R50	R49
R64	R63	R62	R61	R60	R59	R58	R57

Figure 2 Layout of each plot. Each square represents a plant. The yellow squares in the top right-hand corner indicate the location of the original four inoculated plants and the surrounding squares represent the new plants sampled at each time point: grey for the first and second sampling, orange for the third and green for the fourth. [Colour figure can be viewed at wileyonlinelibrary.com].

Sampling of water tanks

Run-off water from each of the two main plots was collected in two dedicated onsite tanks. Due to the availability of only one tank per plot, the water from the two subplots within each plot were mixed. Samples were taken from the top and bottom layers of the water within the tank by collecting water as it drained. Four 2 L samples were taken from the first water to drain out (i.e. bottom layer), then, after the majority of water had run through, four 2 L samples from the top layer were collected. Each sample was baited with whole uninfected Cunningham's White leaves. To have a number of water baits equivalent to the number of baits or isolations from soil and leaves, respectively, 90 leaves were used to bait the 'top water', and 90 leaves were used to bait the 'bottom water' layer for each tank, resulting in a total of 360 baits.

Leaves were floated in the collected water samples and the containers held within the sites for 7 days. Leaves were collected, assessed for the presence of symptoms, and 2 × 2 mm sections from each leaf, from the margins of lesions if present, were plated on to PARP + H selective medium.

DNA extraction from mycelium

Mycelial isolates were inoculated into 12% pea broth liquid (Eyre *et al.*, 2014) culture and grown for 1 week at room temperature, followed by vacuum harvesting on filter paper and lyophilization. Lyophilized mycelium was ground to powder using a glass bead per sample in a FastPrest amalgamator (Bio 101). DNA was extracted from lyophilized material using the NaOH extraction protocol described by Osmundson *et al.* (2013). DNA extracts were stored at −80 °C.

PCR detection of *P. ramorum* from bait leaves

Plants that were negative for *P. ramorum* isolation by plating were tested using a DNA-based assay. A single hole punch per leaf was taken and placed in 2 mL tubes for DNA extraction. Leaf discs were first frozen at −20 °C and then lyophilized for 48 h, before grinding them to powder in a bead amalgamator. Leaf DNA was extracted from the resulting leaf powder using the ROSE extraction protocol (Osmundson *et al.*, 2013), and tested for the presence of the pathogen using a *P. ramorum*-specific TaqMan real-time PCR diagnostic assay (Hayden *et al.*, 2006). DNA was extracted from punches of uninfected lettuce leaves and tested alongside the samples as control (Eyre & Garbelotto, 2015).

Microsatellite genotyping

A total of eight *P. ramorum*-specific microsatellite loci: PrMs39a, PrMs39b, PrMs45a, PrMs45b, PrMs43, PrMs18, PrMs64 and Ms145 (Croucher *et al.*, 2013) were selected for extracted DNA amplification. PCRs were performed in a final volume of 10 μL using 10 ng genomic DNA, 200 μM dNTPs, 5× reaction buffer, 1.5 mM $MgCl_2$, 0.1 μM of each primer and 0.025 U *Taq* DNA polymerase (Promega). Thermal cycling programmes varied for different primers sets and were taken from the literature (Ivors *et al.*, 2006). Fragment analyses of PCR products were run with a LIZ 500 size standard on a 3730 ABI Sequencer (Applied Biosystems). Fragment sizes were scored using PEAKSCANNER v. 2 (Applied Biosystems). Original soil, leaf, water and 'all substrates' MLGs were used for comparative analyses of genotypes among isolates (Eyre *et al.*, 2013).

Genetic diversity indices and minimum spanning networks

The following indices were calculated for the genotypes recovered from each sampling time and each substrate: (i) clonal genotype diversity, $R = (G - 1)/(N - 1)$, where G is the number of MLGs present in a sample and N is the sample size; (ii) Stoddart and Taylor's index of genotyping diversity, $G = 1/\sum p_i^2$, where p_i is the frequency of the ith MLG; (iii) $\hat{G}$, which is G/no. of individuals; and (iv) MLG evenness, using the index E_5: $E_5 = (1/\lambda) - 1/eH' - 1$, where λ = Simpson's index of diversity and H' = the Shannon–Wiener diversity index. If $E_5 = 1$ then all genotypes are equally represented, whereas as E_5 approaches 0 a few genotypes or a single genotype become dominant. Indices (Shannon & Weaver, 1949; Stoddart & Taylor, 1988; Grünwald *et al.*, 2003) were calculated for all the plots pooled together using the software POPPR (Jimenez *et al.*, 2017).

Genetic distances were calculated according to Bruvo *et al.* (2004) in GENODIVE v. 2.0b23 (Meirmans & Van Tienderen, 2004), using number of repeats corresponding to the allele size, to produce a minimum spanning network (MSN) to represent the relationship and interconnectedness of genotypes using SPLITSTREE4 v. 4.13.1 (Huson & Bryant, 2005, 2010).

Pearson's one-way chi-square tests were performed to compare the frequency of detection of the different MLGs found at each sampling time on all substrates together, on leaves and soil. Two-way chi-square tests were used to compare the frequency of detection of the different MLGs at the three different sampling times. The analysis was repeated for all substrate data, leaf data and soil data.

Results

Phytophthora ramorum isolation

Disease incidence can be described as the number of plants from which the pathogen was successfully isolated or detected via PCR out of all the plants sampled. Incidence measured by pooling culture and PCR positives was greatest at sampling 2, during the outbreak phase (Table 1). At the first sampling time, only the original four plants were found to have symptoms and be infected by the pathogen; however, all positive isolations came from leaves that were not originally inoculated, indicating the pathogen was able to spread within plants in a period of 6 weeks, in one to four plants depending on the plot.

In the three later samplings, isolations were attempted from the four plants originally inoculated and from neighbouring plants in an increasing concentric area centered around the original four. The outbreak sampling in the spring, at the end of April, had the highest percentage of positive plants, while the post-outbreak samplings provided progressively lower numbers. Two-way chi-square tests indicated that isolation success rate and incidence of the different MLGs were significantly different when comparing different sampling times (Figs 3 & 4). Using recycled water for irrigation did not result in any detectable increase in infection rate.

Isolation success was highest from leaves and intermediate from soil, reaching the highest percentage of

Table 1 Disease incidence: time of sampling, number of plants sampled and number where pathogen recovered.

Sampling			No. plants sampled	No. isolated from	Incidence (%)	
					Based on actual sample size[a]	Based on n = 80[b]
1	Establishment	Sep 2013	36	8	22.2	10.0
2	Outbreak	Apr 2014	36	21	58.3	26.0
3	Post-outbreak	Dec 2014	100	15	15.0	19.0
4	Fourth	Apr 2015	256	6	2.3	7.5

[a]Size varied among sampling times.
[b]Twenty was picked as the number of plants (a grid of 5 × 4 plants) that realistically could have been infected per plot by the pathogen given the specific conditions met in this experiment. The number was further corroborated by positive isolations, in other words 20 is the effective population size that could have been infected per plot, resulting in a total number of 80. This sample size allows for a better comparison of disease incidence among sampling times, without the confounding factor of variable sample size.

isolation from both substrates during spring, 3 months after plants were inoculated and at the end of the peak of the warm spring rainfall (Fig. S1), followed by a sharp decrease in isolation success as the experiment continued into the dry summer and autumn months. Changes between sampling times in pathogen incidence values in the soil, and in the percentage of pots that yielded positive baits, mirrored changes of disease incidence values measured between sampling times.

Severity of disease can be described by the percentage of positive isolations and PCR positives made at each sampling time from plants. Severity of infestation is instead calculated using results from soil sampling. This was calculated overall by combining the two severity indices (Table 2a) and for each substrate (Table 2b). Disease severity was greatest at outbreak sampling, and at all time points disease severity from leaves was greater than severity of soil infestation.

Genotyping of isolates and diversity indices

Data was obtained from all microsatellite loci for 149 isolates out of the 168 collected. All genotypes from the first sampling matched one of the four original genotypes inoculated, but surprisingly, eight and 13 new MLGs emerged in outbreak and post-outbreak samplings, respectively (Table 3). Unfortunately, isolation success was particularly low in the fourth sampling and it was not possible to fully genotype any of those isolates. Consequently, the diversity indices were calculated for the first three sampling points only, and for all the plots together.

Figure 5 shows that, in general, diversity increased over time as shown by increasing values of R, G and $\hat{G}$. This is in part expected, as the establishment sampling only recovered the original four genotypes that were used in the initial inoculations. The outbreak and post-outbreak samplings recovered not only the original

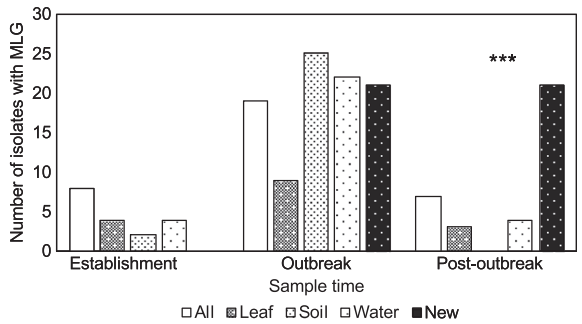

Figure 3 Distribution of the multilocus genotypes (MLGs) at each sampling time. Frequencies of MLGs varied significantly when comparing different sampling times (two-way chi-square 37.8, $P < 0.001$). Asterisks indicate significant difference in frequency of MLGs within one sampling time (***$P < 0.001$).

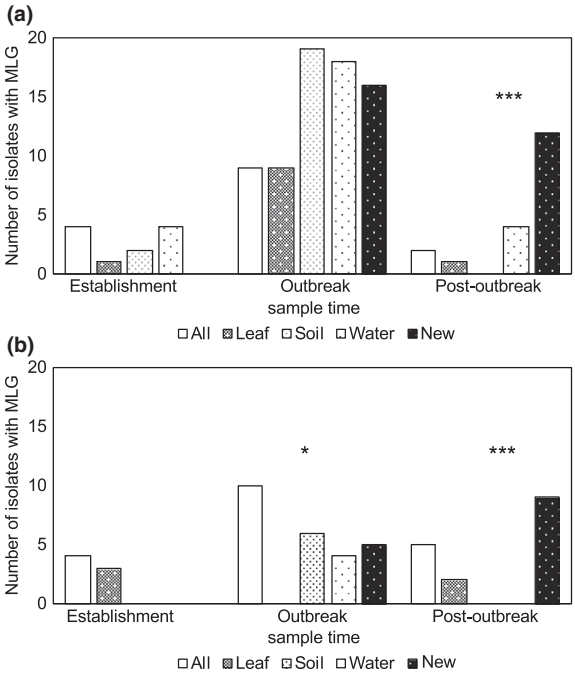

Figure 4 Distribution of the multilocus genotypes (MLGs) at each sampling time in leaf only (a) and in soil only (b). Frequencies of MLGs varied significantly when comparing different sampling times in leaf (two-way chi-square 22.8, $P = 0.035$) and soil (two-way chi-square 26.5, $P < 0.001$). Asterisks indicate significant difference in frequency of MLGs within one sampling time (*$P < 0.05$; ***$P < 0.001$).

genotypes, but also additional novel genotypes that were closely related to each of the original four (see 'minimum spanning network' section below), indicating a microevolutionary process had taken place during the experiment. Finally, the evenness index, E_5 (Fig. 5) decreased between establishment and outbreak samplings, and then slightly increased between outbreak and post-outbreak samplings.

Table 2 Disease severity. (a) Isolation attempts and successful isolation for each sampling time and substrate. (b) Successful isolation per infected plant for each sampling and substrate (samples 1 and 2 and samples 3 and 4 can be compared, but 1–2 and 3–4 cannot be compared due to different sampling intensity per plant).

(a)

			Successful isolation					
Sample	Total isolation attempts	Isolation attempts per substrate	Leaf	Soil	Both	Leaf success (%)	Soil success (%)	Total success (%)
1	720	360	16	11	27	4.4	3.7	3.8
2	720	360	79	29	108	21.9	9.7	15.0
3	600	300	25	18	43	8.3	6.0	8.0
4	2048	1024	6	0	6	0.6	0.0	0.3

(b)

			Total isolation attempts			Successful isolation					
Sample	No. infected plants	Isolation attempts per substrate per pot	Leaf	Soil	Both	Leaf	Soil	Both	Leaf success (%)	Soil success (%)	Total success (%)
1	8	10	80	80	160	16	11	27	20.0	14.0	17.0
2	21	10	210	210	420	79	29	108	38.0	14.0	26.0
3	15	3	45	45	90	25	18	43	55.0	40.0	53.0
4	6	4	24	24	48	6	0	6	25.0	0.0	12.5

Table 3 Genotypes originally inoculated, and new genotypes derived from them, allelic composition at six SSR loci and frequency (*n*).

			MS18		MS19				MS43		MS45		MS64		MS145	
		n	a	b	a	b	a	b	a	b	a	b	a	b	c	d
Original four genotypes[a]	S	27	220	275	131	248	374	486	167	188	343	381	168	179	200	243
	L	16	220	275	131	248	370	490	167	188	343	381	168	179	200	243
	W	30	220	275	131	248	358	474	167	188	343	381	168	179	200	238
	A	34	220	275	131	248	370	482	167	188	343	381	168	179	200	243
New MLGs mostly present in the 2nd sampling	1	2	220	273	131	248	358	474	167	188	343	381	168	179	200	237
	2	8	220	273	131	248	374	486	167	188	343	381	168	179	200	243
	3	1	220	275	131	248	355	474	167	188	343	381	168	179	200	237
	4	1	220	275	131	248	370	444	167	188	343	381	168	179	200	243
	5	1	220	275	131	248	370	494	167	188	343	381	168	179	200	243
	6	1	220	275	131	248	374	482	167	188	343	381	168	179	200	243
	7	8	220	275	131	248	374	490	167	188	343	381	168	179	200	243
	8	1	220	279	131	248	358	474	167	188	343	381	168	179	200	237
New MLGs present in the 3rd sampling only	9	2	220	273	131	248	358	474	167	188	343	378	168	179	200	237
	10	1	220	273	131	250	370	490	167	188	343	378	168	179	200	243
	11	1	220	273	131	250	370	444	167	188	343	378	168	179	200	243
	12	2	220	275	131	250	374	490	167	188	343	378	168	179	200	243
	13	2	220	273	131	248	370	482	167	188	343	378	168	179	200	243
	14	1	220	273	131	250	374	490	167	188	343	381	168	179	200	243
	15	3	220	275	131	250	358	474	167	188	343	381	168	179	200	237
	16	2	220	275	131	250	358	474	167	188	343	381	168	179	200	245
	17	1	220	275	131	250	370	482	167	188	343	381	168	179	200	245
	18	1	220	273	131	250	370	482	167	188	343	381	168	179	200	243
	19	1	220	275	131	250	374	486	167	188	343	381	168	179	200	248
	20	1	220	275	131	250	370	493	167	188	343	381	168	179	200	245
	21	1	220	275	131	250	370	490	167	188	343	381	168	179	200	245

[a]S, soil genotype; L, leaf genotype; W, water genotype; A, all substrates genotype.

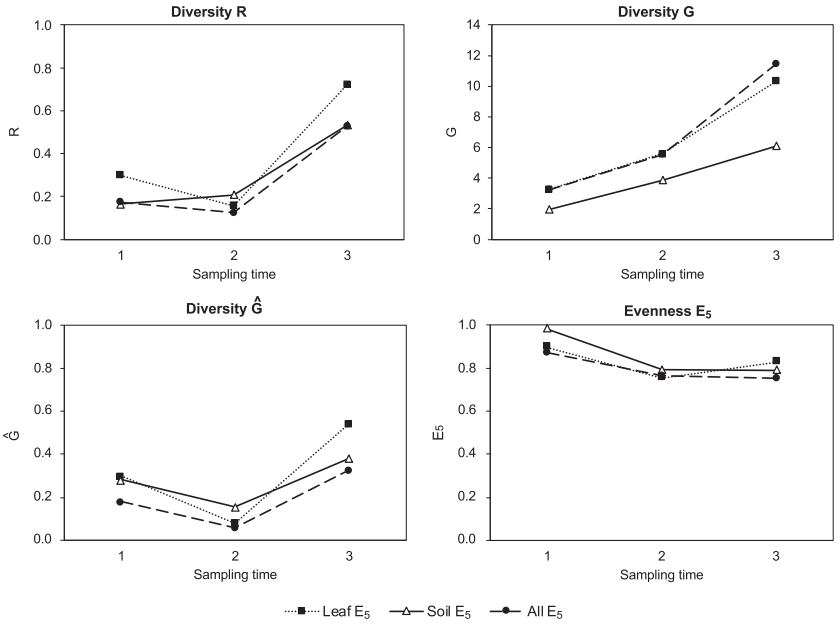

Figure 5 Diversity indices for each sampling time divided by the substrate from which the isolations were made. R = clonal genotype diversity, G = Stoddart and Taylor's index, $\hat{G} = G/N$ where N = sample size, and E_5 = evenness index.

Persistence of the original four genotypes

The frequency of isolation of the different MLGs over time provided information on their respective fitness. As expected, only the original four genotypes were found in the establishment sampling. Even though the 'A' MLG was the most frequent and the 'L' MLG was the least frequent in this sampling time, there were no statistical differences (chi-square $P = 0.23$) in incidence of MLGs in the first 'establishment' sampling (Fig. 3). In the second sampling time (outbreak), there were more isolates recovered than in the first sampling, and overall, the incidence of each of the four original genotypes was statistically different from that of the first sampling based on a two-way chi-square analysis ($P = 0.02$). Genotypes 'W' and 'S' were slightly more abundant than 'A', indicating a rapid ability to produce infectious propagules as environmental conditions change through time. In this sampling, a novel group of 'New' MLGs was also detected and, overall, this group included a variety of new genotypes that, pooled together, matched the abundance of the original genotypes. However, by the post-outbreak sampling, the genotype 'S' disappeared and the other original three genotypes appeared to have been in part replaced by the group of 'New' MLGs (Fig. 3). Based on a two-way chi-square analysis ($P = 0.0002$), the frequency of genotypes at this sampling time was significantly different from that at the previous sampling time. It should be noted though that, within this 'New' category, no single genotype dominated and none reached a frequency notably superior to that of other genotypes, and that genotype 'A' was still the most abundant, while one or two new genotypes had frequencies approaching those of genotypes 'W' and 'L'. This 'dominance' is thus obtained by pooling together several new genotypes, each of them characterized by a relatively low frequency. This particular detail is critical for a proper interpretation of the results concerning the appearance and establishment of these new genotypes.

When considering only the isolates that were recovered from leaves at the different sampling times (Fig. 4a), a pattern similar to the one described above was identified. The four original MLGs were roughly equal at the establishment sampling (chi-square $P = 0.48$) and in the outbreak sampling, but in the post-outbreak sampling, the frequency of MLGs obtained was statistically different (chi-square $P < 0.0001$) and the 'W' genotype was the one most frequently detected. Additionally, the frequency of MLGs in this sampling time was statistically different from the frequency of MLGs in the previous sampling time (two-way chi-square $P = 0.0075$).

Fewer isolations overall were made from soil than from leaves (Fig. 4b), but the distribution of MLGs was different from that found on leaves, and differences in frequency of detection among MLGs were almost significant at the first sampling time ($P = 0.06$) and were significant at the two later sampling times ($P = 0.03$ and $P = 0.001$). There were similar changes in relative abundance between time points, i.e. least at establishment sample, and most at outbreak sample. However, in the first sampling, only the 'A' and 'L' isolates were present, and in the second sampling, the 'L' MLG was completely absent. Unlike that observed in leaves, the 'A' MLG was most abundant at outbreak sampling, and was still relatively well represented in post-outbreak sampling. The 'New' MLG group again increased over time. Overall, it appears that the generalist 'A' MLG survived best in the soil.

Minimum spanning network

The MSN (Fig. 6) showed that the new MLGs detected during the course of the experiment were not

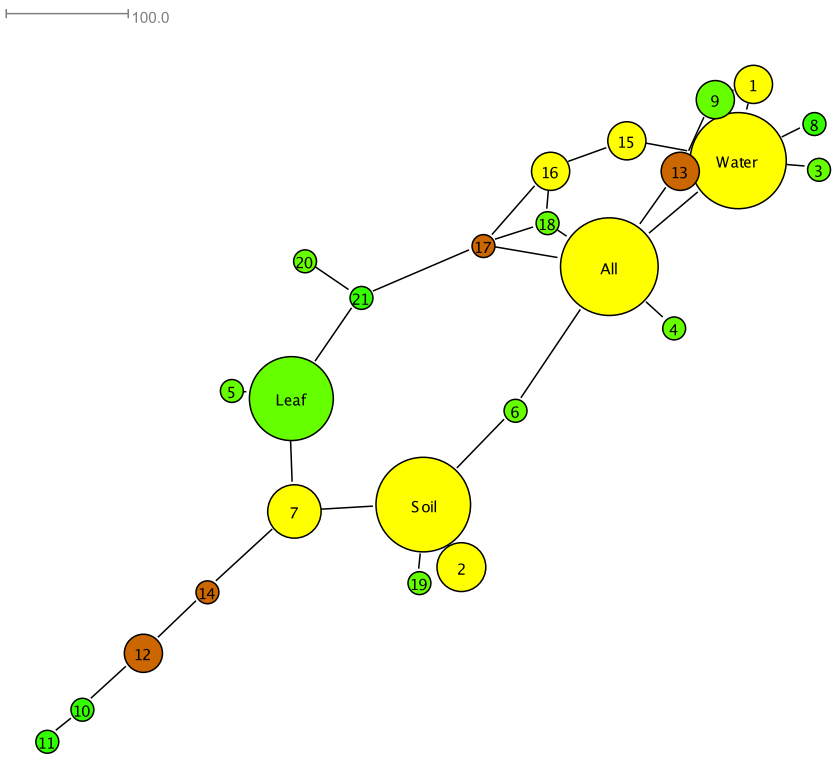

Figure 6 Minimum spanning network of multilocus genotypes (MLGs) detected from cultures isolated from second and third rounds of sampling of rhododendron. MLG codes: Water, Soil, Leaf and All represent the original genotypes used for inoculations, found to be dominant in one of these substrates, or all substrates; nodes 1–21 represent the new MLGs derived from the original MLGs. Nodes are proportional to the number of isolates with each MLG in eight sizes classes: 1 = 1 isolate, 2 = 2–5 isolates, 3 = 6–10, 4 = 11–15, 5 = 16–20, 6 = 21–25, 7 = 26–30, 8 = 31–35. Nodes are coloured according to the substrate in which they were found. Yellow, found in soil and leaf samples; green, found only in leaves; brown, found only in soil. Lines between nodes are proportional to Bruvo distance between MLGs. [Colour figure can be viewed at wile yonlinelibrary.com].

contaminants, but clearly all derived from the four original MLGs, at first directly from them and then later from novel genotypes originating from them. Based on the location of new genotypes on the MSN, and assigning those that were ambiguously placed in between two of the original genotypes to both of them, it appears that novel genotypes were generated in comparable numbers (three to five) by each one of the four original genotypes. However, it is evident that the substrate where the isolates were growing, and not the substrate of origin, differently affected the generation of new genotypes. In this experiment, the frequency of new genotypes found in leaves was significantly higher than the frequency of new genotypes found in other substrates (chi-square = 5; $P = 0.02$), suggesting microevolution of the pathogen is driven by aerial populations of the pathogen. To test the validity of this statement, the number of new MLGs that were present in different substrates was calculated: soil only = four new MLGs; leaf only = 12; leaf and soil = five; total leaf = 17; and total soil = nine.

Changes in frequency of the four original MLGs at different sampling times (Figs 3 & 5) suggest there may be variation in fitness among MLGs, and point to a reduced overall fitness of genotype 'S' and to a prolonged fitness of the isolate that was originally present in all substrates, genotype 'A'. Results also suggest an adaptation of the 'W' genotype to different substrates. The variation in abundance of different genotypes in the first three samplings (Fig. 7) shows that the 'A' genotype seems to be the most successful one in terms of original establishment (result in establishment sampling), spread during the outbreak, and survival after the outbreak. The 'L' genotype seems to have an overall lower establishment success, with a similar pattern during and after the outbreak. The 'S' genotype was less successful in the establishment phase, but became extremely abundant in the outbreak phase. However, it appeared to fare poorly after the outbreak. The 'W' genotype seemed to fare very well during the outbreak phase.

There were two new MLGs (MLGs 2 and 7) that increased their frequency during the outbreak phase, almost reaching 'L' genotype frequency. However, the 'A' genotype was found to be dominant in the post-outbreak phase, a phase otherwise characterized by a large number of poorly represented MLGs.

Spread dynamics

Figure 8 identifies plants that were positive for *P. ramorum* at each sampling time, and allows for the quantification of the extent of the spread of the pathogen during the course of the experiment. It should be highlighted that the experiment did not include flooding of beds, such as that caused by excessive watering or storms, but it did include rainfall (Fig. S1) and a generous overwatering regime.

Given the conditions of the experiment, the maximum spread was of three pots from previously infected pots, corroborating a relatively small scale (135 cm) spread of the pathogen in the nursery over 20 months. Although a rare event, plants and their pots at times were skipped. That means that, at least occasionally, plants adjacent to

infected plants escaped infection, but the plant next to them and in the direction opposite to the established infestation became infected. Taking into consideration results from all sampling times, it appears that this event occurred twice, and in both cases only a single plant was skipped (Fig. 8; outbreak and post-outbreak samplings).

Cumulatively a total of 46 pots were positive for *P. ramorum* in the leaves, versus 17 with positive results from the soil. With a single exception, newly positive pots (i.e. pots that were not positive the previous sampling period) were either positive for both leaves and soil or positive in the leaves only. This indicates the spread dynamic of *P. ramorum* in nurseries, and in the absence

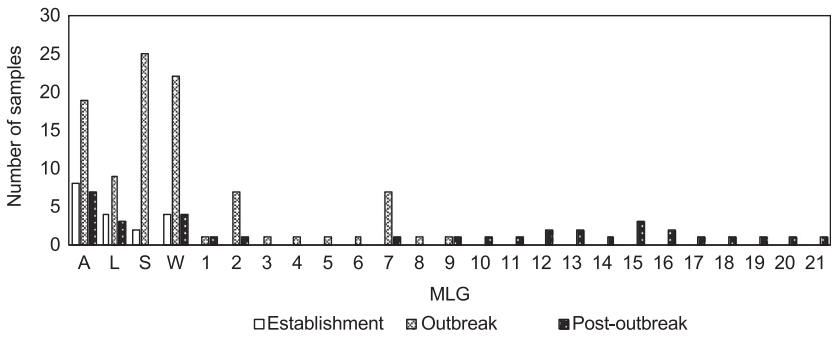

Figure 7 Abundance of different genotypes in the first three samplings (establishment, outbreak and post-outbreak phases).

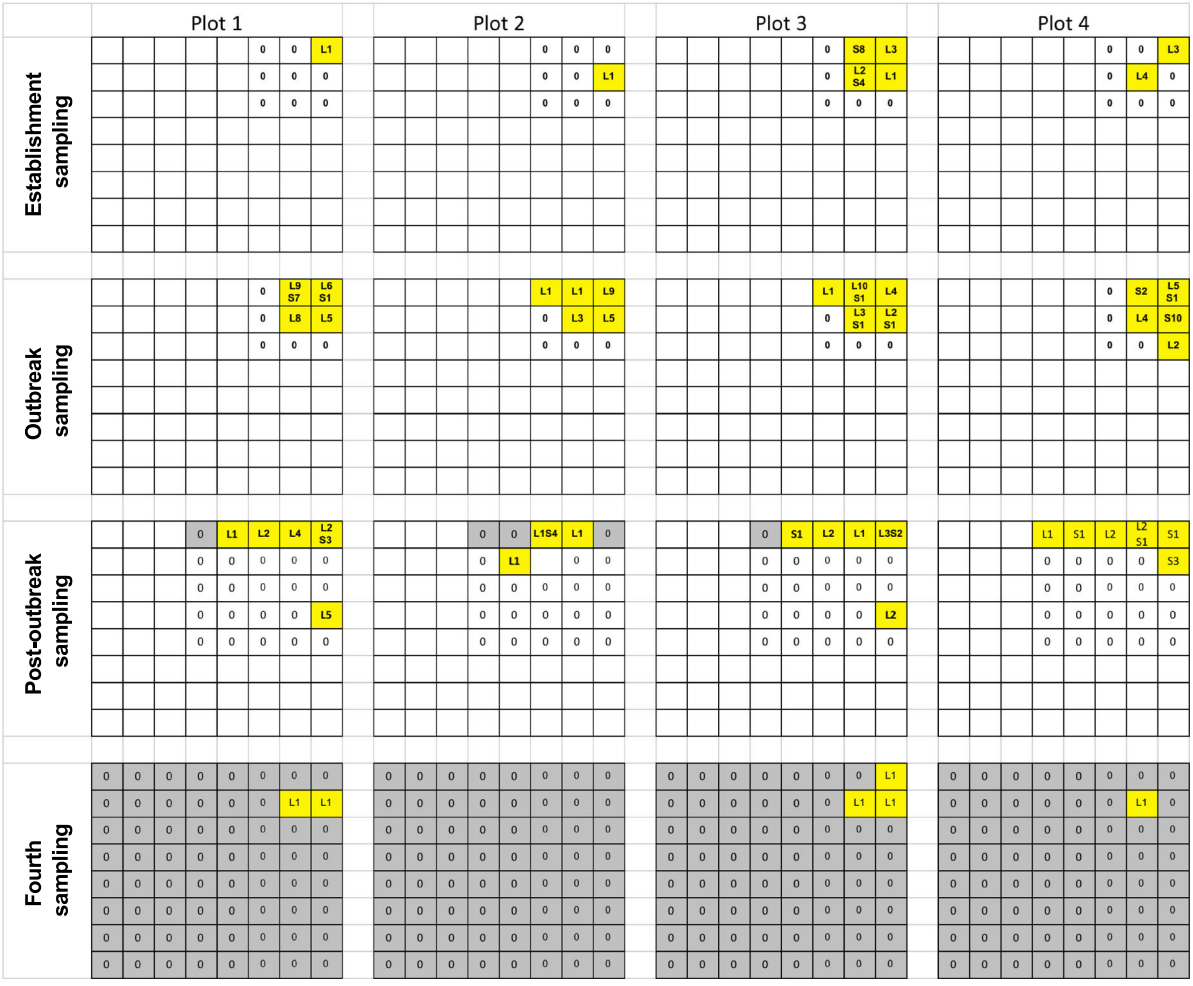

Figure 8 Plants positive for *Phytophthora ramorum* isolation at each sampling time. L identifies a positive isolation from leaves, and S from soil. [Colour figure can be viewed at wileyonlinelibrary.com].

of flooding, follows mostly a leaf-to-leaf pathway, followed by a leaf-to-soil one.

Isolations from water tanks

Although water infestation levels were tested by baiting the pathogen from the water in the tanks capturing irrigation water, results were inconsistent between the two tanks and among sampling times. *Phytophthora ramorum* recovery was very low from the water, and isolation success was restricted to the top section of one of the two tanks, where 15 cultures of *P. ramorum* were recovered (Table 4). There were a lot of symptoms on water baits from both sites and tank sections, but the majority of them were not caused by *P. ramorum*. Isolates from the last sample (May–June 2015) were identified by a comparison of their ITS4, ITS5 and *RPS10* sequences with sequences deposited in GenBank using the BLAST function (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and by morphological identification of cultures growing in agar medium at 28 °C to favour chlamydospore production. Terminal and lateral chlamydospores were found at 28 °C, and additionally, non-papillate and persistent sporangia were produced in water. Sexual reproduction was not observed. According to these morphological and DNA sequence results, all isolates were identified as *Phytophthora* taxon *pg chlamydo*, currently *Phytophthora chamydospora* sp. nov. (Hansen *et al.*, 2015).

Discussion

Rates of establishment and spread, both in wildlands and in nurseries, of plant diseases caused by *Phytophthora* species, are driven by pathogen attributes such as spread and infectivity proficiency or adaptability to new substrates, as well as by favourable environmental conditions and the susceptibility of available hosts (Rizzo *et al.*, 2005). This study examined in detail the spread dynamic of four different genotypes of *P. ramorum*, independently in soil, water and leaves, across pots containing susceptible *Rhododendron* plants (Werres *et al.*, 2001) under nursery conditions. Moreover, by using a genetic approach, the genotypes used as inoculum were tracked, and this information provided insights into variation in adaptability of four genotypes and on the evolution of novel genotypes derived from them.

Table 4 Percentage of *Phytophthora ramorum* recovery from water baiting of top and bottom of collection tanks in each site from all samples.

	Tank section	
	Top	Bottom
Site 1	0.0	0.0
Site 2	16.0	1.0
Total %	8.0	0.6

Individual percentages are from the 90 bait leaves used to bait the top/bottom of each tank separately. Total percentage is from all samples taken from top water across both sites/tanks (180 bait leaves total).

Although the experiment effectively only covered a single disease cycle without replication, the higher isolation success of the pathogen measured during the wet-warm season, a few weeks after the peak of rainfall, is in agreement with previous studies in forest settings (Eyre *et al.*, 2013; Garbelotto *et al.*, 2017). Nonetheless, the correlation between the onset of the disease outbreak and wet and warm weather in an outdoor nursery setting indicates that, at least in the Californian environment, outdoor climatic patterns may be important drivers of disease epidemiology, even in the presence of artificial shading and regular abundant watering typical of most outdoor plant production facilities.

Values of genetic diversity and genotypic indices recorded during the experiment (see below) all indicate the inoculation resulted in a moderate outbreak with the dominance of a few highly infectious genotypes (Frank, 1992; Eyre & Garbelotto, 2015), followed by a post-outbreak chronic disease state in which multiple genotypes were present at comparable and rather low frequency of isolation. Additionally, multiple lines of evidence support the notion that the new genotypes arose from the four used to inoculate plants, and were not the result of external contamination. First, bay laurels or other sources of infection were not present immediately nearby; secondly, the MSN places all of the novel genotypes in a cloud of genotypes surrounding the ones used initially; thirdly, all infected plants were near the ones originally inoculated in all four subplots, while infection from outer sources would be expected to be distributed more broadly across the experimental plots.

The experiment outbreak led to an increase in overall genotypic diversity G and to a reduction in evenness index, suggesting the larger the pathogen population size, the greater the number of genotypes generated (Eyre & Garbelotto, 2015). In fact, despite its exclusively clonal reproduction mode (Ivors *et al.*, 2006), there is ample evidence that new MLGs emerge both through mutation (Croucher *et al.*, 2013) and through genomic modification events (Kasuga *et al.*, 2016), and it is expected that the frequency of such events may be proportional to the size of the population (Eyre *et al.*, 2013). However, the appearance of new MLGs in such a short period is remarkable, and the reduction in evenness is dictated by the fact that some genotypes dominated during infectious outbreaks, as expected based on the theory of infectious diseases (Frank, 1992; Eyre & Garbelotto, 2015). In the present experiment, during the outbreak sampling, the four original and two new MLGs markedly dominated the genotype diversity. In contrast, after the first spring outbreak and when disease incidence and severity decreased, evenness stopped decreasing or slightly increased, due to the fact that a large proportion of the new MLGs generated during the third sampling were singletons (Frank, 1992). These singletons were not endemic, as plants were tested and free of pathogens at the beginning of the experiment, and no direct source of

inoculum was present in the immediate vicinity of the experimental plots.

The relatively low decrease in evenness and the large number of new genotypes detected both suggest environmental conditions at the experimental site were not particularly favourable to *P. ramorum* (Eyre & Garbelotto, 2015). Because the experimental design itself included regular and abundant watering and shading, the fluctuations in disease incidence were not limited by the absence of water, a key factor for pathogen sporulation and plant infection (Garbelotto *et al.*, 2017), but may have been driven by overall climatic conditions or by one limiting climatic factor at the experimental research facility. Given that temperatures recorded at the experimental site at the times of sampling were consistently close to 20 °C, a temperature regarded as optimal for *P. ramorum* sporulation and infection (Garbelotto *et al.*, 2017), it is believed that the most limiting factor may have been lower than optimal relative humidity (Garbelotto & Hayden, 2012). Several previous studies have corroborated the indispensable presence of abundant rainfall, as well as warm temperatures, to activate *P. ramorum* sporulation and infection (Davidson *et al.*, 2005; Garbelotto & Hayden, 2012; Eyre & Garbelotto, 2015), but additionally, it has been shown that infection of Cunningham's White requires moisture periods of 24–48 h (Tooley *et al.*, 2009).

Similar patterns of changes were observed for plant infection and soil infestation levels, but changes were markedly stronger in leaves. All but one newly infected plant were found to be positive in leaves or in both leaves and soil, suggesting the disease spread mechanism is mostly through leaf-to-leaf contagion, followed by leaf-to-soil pathogen spread. Sporulation of the pathogen occurred rapidly and abundantly in leaves at the onset of favourable climatic conditions, as previously described by Eyre *et al.* (2013) and Garbelotto *et al.* (2017). In fact, based on correlations found between leaf and soil populations, Eyre & Garbelotto (2015) suggested forest soil may be mostly colonized by propagules of *P. ramorum* genotypes formed on leaves. Patterns of infection and of pathogen population genetics indices corroborate that in this experiment, disease was driven by foliar populations of the pathogens. Chi-square analyses comparing frequency of MLGs showed that in leaves the original four genotypes maintained a comparable frequency all through the outbreak phase, but MLGs in soil populations changed their respective frequency quite rapidly, suggesting that soil is not a major substrate for sporulation but is inoculated from leaves and thus more easily subjected to fluctuations in MLG frequencies. It should be noted that the pattern observed in this study was generated in the absence of plot flooding, which happens in some facilities, and may result in substantial soil infestation.

The scale of spatial movement of the pathogen among plants was limited to one to three plants, or 45–135 cm away, from a source, e.g. an infected plant. The presence of pots/plants that were skipped by the pathogen further corroborates an aerial but splash-dispersed movement rather than a dispersal through the flow of water, resulting in a quite modest scale of spread during a year. These data indicate splash-dispersal of *P. ramorum* inoculum in nursery conditions is limited. Additionally, movement was more marked along plot boundaries than inside the plot, indicating a role of airflow in the dispersal of propagules, as expected for a putative splash-dispersed plant pathogen. Overall, these data support the current prescription of elimination of all plants within 2 m from a confirmed source in a nursery setting (Swiecki & Bernhardt, 2008). Longer dispersal and more extensive plant eradication, 5–20 m away from a transmissive hosts, is instead recommended to significantly reduce the risk of infection in forest settings (Davidson *et al.*, 2005; Garbelotto *et al.*, 2017).

With respect to the investigation on genotype microevolutionary processes, this is the first study to provide evidence of differences in fitness, dispersal potential and survival ability among different genotypes of the NA1 lineage of *P. ramorum* in a nursery setting. Previous studies had focused on differences in pathogenicity on ornamental plants, but when comparing different lineages (Elliott *et al.*, 2011; Eyre *et al.*, 2014). The present findings were particularly interesting in light of the fact that all four genotypes were selected because they were successful in different natural substrates (water, soil, leaves) or successful in all three natural substrates. No isolates from oak or tanoak stems were used because of the possibility of genomic alterations affecting their reproductive potential, life expectancy and overall vigour (Kasuga *et al.*, 2016). It is remarkable that the genotype selected because it was dominant in soil, increased its frequency dramatically between the samplings of the establishment and outbreak phases, surpassing all other genotypes, possibly indicating a rapid and substantial production of sporangia (i.e. infectious propagules), but disappeared in the post-outbreak sampling, possibly indicating a poor production of chlamydospores (i.e. survival or resting propagules). This result is in agreement with results from other studies, which have documented a turnover of genotypes in forest soils between one year and the next (Fichtner *et al.*, 2007; Eyre *et al.*, 2013), and may also suggest a trade-off between phenotypic traits in *P. ramorum*. Conversely, the three genotypes selected because they were dominant on either all substrates, leaves or water, displayed smaller changes in frequency among samplings, but persisted with significant frequency in the post-outbreak phase. The frequency ranking of 'A', 'S' and 'L' genotypes may have been in part facilitated by the frequent overhead watering prescribed during the course of the experiment. Although the long-term survival and fitness of these genotypes was not studied here, due to the end of the experiment after 20 months, the results may suggest that genotypes that display intermediate success in outbreak, as well as in pre- and post-outbreak phases, may be the most successful in the long term.

This is also one of the first experiments in which a high generation of novel genotypes was artificially induced, suggesting that the nursery environment facilitates the generation of these new individuals. Interestingly, many new genotypes were generated even as the disease level decreased, maybe because of the decreased presence of dominant genotypes lowering intergenotypic competition, allowing for the establishment of new individuals (Frank, 1992; Eyre & Garbelotto, 2015). Some genotypes were ecologically fitter than others (Eyre *et al.*, 2013). Based on the MSN, all new genotypes were shown to represent a tight cluster of closely related genotypes, one or a few mutational steps away from the four original genotypes, and thus it can be asserted that none of the new genotypes were contaminants. The majority of new genotypes were generated in the leaves (Eyre *et al.*, 2013), although soil may also have been a significant substrate for the generation of novel genotypes during the post-outbreak phase. The higher frequency of new genotypes found in leaves clearly supports the hypothesis that leaves are the substrate where the largest genotypic diversity was generated. It should be pointed out that each initial MLG was inoculated on leaves, hence it could be assumed that a longer period of time may be associated with a higher frequency of generation of new genotypes; however, 6 weeks after inoculations, no new genotypes were detected at all, suggesting that the effective time for this microevolutionary process to occur is actually comparable between leaves and soil. However, this conclusion regarding soil needs to be verified by another controlled experiment, due to the fact that soil has been previously shown to be inoculated by aerial propagules generated on leaves (Eyre *et al.*, 2013), and the genotypes only encountered in soil here may have also been present in leaves, but missed during the sampling.

This creation of new genotypes in a nursery setting poses a threat to the industry itself, as well as to wildlands, due to the increase in pathogen adaptability often associated with new genetic variation; this has been shown to be true for the plant pathogen *Seiridium cardinale* (Garbelotto *et al.*, 2015), and has been recently hypothesized to be one of the mechanisms responsible for the development of resistance to fungicides in *Phytophthora* (Hunter *et al.*, 2018). Unfortunately, the length of the experiment was too short to properly evaluate the fitness of newly generated genotypes. Nonetheless, at least two of nine new genotypes generated before the end of the spring, namely MLGs 2 and 7 derived from the soil genotype, were isolated at high frequency during the outbreak phase, even if their establishment must have necessarily occurred significantly later than the establishment of the original four genotypes used for the inoculation.

Finally, despite good and consistent isolation success from both leaves and soil, isolation success was lowest and unpredictable from irrigation water contained in tanks. It was significant that all positive isolations came from the top layer of one tank, indicating survival and proliferation of *P. ramorum* is best in the more aerobic top layers of water, and may be suppressed in the anaerobic bottom layers. It is unclear why isolation success was markedly low from the water tanks, but possible factors may be increased anaerobiosis in a tank with little water circulation compared to an open-air body of free-flowing water (Erwin & Ribeiro, 1996), and increased water temperatures in a closed container, considering that high water temperatures have been reported to be a limiting factor for the viability of *P. ramorum* (Eyre & Garbelotto, 2015).

When run-off water was used to irrigate two of the four plots, plants in the plots subjected to this treatment displayed an increase in foliar symptoms (data not shown). Such increase though was documented to be caused mostly by the recently described *P. chlamydospora*. *Phytophthora chlamydospora* has been previously recorded from the foliage of several horticultural nurseries' stock plants, including *Rhododendron*, in California (Yakabe *et al.*, 2009; Hansen *et al.*, 2015) and has been found causing root rot on *Calocedrus* and *Abies* spp. in nurseries and plantations (Hansen *et al.*, 2015). Notwithstanding the possible negative effect of *P. chlamydospora* on infection by waterborne *P. ramorum* inoculum, Tjosvold *et al.* (2008) also reported limited inoculation success of rhododendrons using irrigation water contaminated with *P. ramorum*. It is unclear why the viability of *P. ramorum* may be significantly reduced in circulated or recirculated irrigation systems, especially considering the significant infection levels caused by *P. chlamydospora* in infested water in the same recirculation irrigation system used in this experiment, but again it may suggest *P. ramorum* may not be a particularly competitive species in water, being mostly an aerial species. Conversely, there is a presumption of a good fitness of *P. chlamydospora* in water, given that it is commonly found in aquatic habitats (Garbelotto *et al.*, 2018). Unfortunately, even if this may not be a critical issue for *P. ramorum*, the dissemination of other *Phytophthora* species through the use of contaminated recirculation and irrigation water within nurseries, and even from nurseries to wildlands, is a further and relevant problem (Themann *et al.*, 2002; Hulvey *et al.*, 2010).

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Natural precipitation and temperature at the experiment site registered from January 2013 to May 2015 (https://www.usclimatedata.com/climate/san-rafael/california/united-states/usca1011) and percentage of disease incidence and severity at the different samplings.