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Control of *Phytophthora* species in plant stock for habitat restoration through best management practices

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Emergent plant pathogens represent one of the most significant threats to biodiversity, and exotic *Phytophthora* species have recently emerged as a serious problem in restored habitats in California and in nurseries producing the plant stock. It is hypothesized that 'best management practices' prescribed through a *Phytophthora* Prevention Programme (PPP) could be useful in minimizing phytophthora disease incidence. To understand the magnitude of the problem and the efficacy of the PPP, plants in restoration nurseries were evaluated for (i) the *Phytophthora* species assemblage present in the absence of the PPP, and (ii) the effectiveness of the PPP to reduce them. Sampling included 203 plants grown in the absence of the PPP, and 294 grown implementing the PPP. Only samples collected in the absence of the PPP were *Phytophthora*-positive, and cumulatively yielded 55 isolates from 13 different taxa, including 1 putative interspecific hybrid genotype. There were 21 novel *Phytophthora*–plant species combinations. The most common *Phytophthora* species was *P. cactorum*. Four plant species had the highest disease incidence, namely: *Diplacus aurantiacus* (50 ± 11.2%), *Heteromeles arbutifolia* (33 ± 9.6%), *Ceanothus thyrsiflorus* (30 ± 8.4%), and *Frangula californica* (30 ± 8.4%). Disease incidence in nurseries after the implementation of the PPP dropped to zero ($P < 0.001$), and was unaffected to any significant degree by nursery differences, or plant species tested. This study identifies a large number of novel 'plant species × *Phytophthora* species' combinations, and provides for the first time strong evidence that the PPP significantly reduced *Phytophthora* in plant stock for habitat restoration.

Keywords: best management practices, biological contamination, emergent pathogens, *Phytophthora* species, restoration plant stock

Introduction

The introduction of invasive species threatens the survival of endemic species. While the literature on invasive plants and animals is rich (Lowe *et al.*, 2000), the same cannot be stated for invasions by emerging pathogens. The relative paucity of research on this topic is in striking contrast with the extremely significant economic and environmental impacts associated with the severe and irreversible die-offs caused by introduced plant and animal pathogens (Pimentel *et al.*, 2005). *Phytophthora* is a genus of fungus-like microorganisms notable among plant pathogens in wild settings because of its consistent human-mediated distribution (Redondo *et al.*, 2018), and because of the serious diseases it can cause in wild plants (Rizzo *et al.*, 2002). Restoration plantings are also implicated in the spread of *Phytophthora* species and have been studied in Europe (Jung *et al.*, 2015). For instance, notable *Phytophthora* species released during restoration efforts include the aggressive alder pathogen *Phytophthora alni* subsp. *alni* found throughout Europe (Webber *et al.*, 2004), and it is also possible *Phytophthora austrocedri* threatening *Juniperus communis* in Scotland and northern England may have been introduced in a similar way (Green *et al.*, 2015).

Studies have investigated the connectivity between the ornamental plant industry and the introduction of exotic *Phytophthora* species into wildlands in North America (Garbelotto & Hayden, 2012). In addition, a great number of previously unreported plant species × *Phytophthora* species combinations have been recently identified in nursery-grown ornamental and fruit crops (Prigigallo *et al.*, 2015). However, no in-depth studies have been done regarding nurseries producing native plant stock for restoration, nor have any prevention programmes been used to try and stop the inadvertent spread of phytophthora disease through restoration efforts. In 2014, the introduction of *Phytophthora tentaculata* in a restored site in California through the outplanting of infected nursery stock was reported (Rooney-Latham & Blomquist, 2014). Around the same time, many other *Phytophthora* species, including hybrids (authors' unpublished data), were recovered from native California plant

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stock, and from heavily infested, mainly riparian, restoration sites in California (Bourret, 2018).

The most likely way to prevent the introduction of *Phytophthora* species into wildlands through infected plant stock is with the implementation of 'best management practices' (BMPs) designed to prevent nursery-grown plants from becoming infected in the first place (Baker, 1957). Consequently, there is an urgent need to identify proper management strategies that may successfully prevent the spread of the many diverse *Phytophthora* species found in nursery plant production (Hardy & Sivasithamparam, 1988, 2002; Davison *et al.*, 2006; Prigigallo *et al.*, 2015). There are manuals describing BMPs designed to reduce the chances of establishment and spread of *Phytophthora* species in nurseries producing ornamental or other commercial crop plants (Griesbach *et al.*, 2011). While in theory the same principles are likely to be effective in the production of plants to be used in restoration projects, the efficacy of BMPs implemented as a programme in facilities producing restoration stock has not been tried or evaluated in practice. Here, a *Phytophthora* Prevention Programme (PPP) has been designed that targets improved water management, soil management and nursery sanitation. These three areas have been long identified as key for the spread of *Phytophthora* species in plant production facilities (Baker, 1957). The principal objectives of this study were to work with restoration nurseries, during regular production cycles, and using available plant stock to: (i) examine assemblages of *Phytophthora* species in northern California production facilities focusing on restoration efforts, and identify any new host × *Phytophthora* species combinations; (ii) determine plant species with the greatest disease incidence and evaluate the number of *Phytophthora* species infecting them; (iii) institute a PPP based on BMPs and then examine its efficacy to reduce disease incidence; and (iv) model the health of restoration crops factoring in presence or absence of a PPP, heterogeneity in plant species, and variation among nurseries.

Materials and methods

Sampling design

The trial described in this paper took place between January 2015 and September 2016 and involved five nurseries that had variable levels of BMPs instituted prior to the trial and grew different volumes of plants and numbers of species (Table 1). The nurseries ranged in size from 0.2 to 1.6 ha, produced 25 to 330 different plant species, and had a stock size ranging from 7000 to 75 000 plants.

Samples for the study were collected from 497 plants belonging to 24 species being produced in the five restoration nurseries (Table 2). Phytophthora disease incidence was determined in three of these nurseries before (CG-1; control group 1) and after implementation of the PPP (TG-2; treatment group 2), while

Table 1 The restoration nurseries sampled including the size of the site, the number of plants, and details of best management practices prior to implementation of the *Phytophthora* Prevention Programme.

Nursery	Size of nursery site (ha)	No. of plant species	No. of plants	Plant stock buy-ins	Key management success areas based on a systems approach prior to Best Management Practice (BMP) intervention[a]
1	1.6	135	75 000	Yes	Roads are paved, gravelled or rocked (although nursery floors are not), plants are regularly weeded, water is from a municipal source, propagation areas are mostly free of noticeable debris (weeds, moss, plant waste), most plants are maintained on benches, there is some traceability in plant processing. Some plants are evaluated once a year for *Phytophthora ramorum* foliar symptoms and testing.
2	0.2	50	10 000	Yes	Roads are paved, gravelled or rocked, plants are regularly weeded, water is from a municipal source, propagation areas are kept free of noticeable debris (weeds, moss, plant waste) for the most part, plants are maintained on benches, occasionally plants are professionally inspected for pests, there is some traceability in plant processing.
3	0.2	25	7000	No	Roads are paved, gravelled or rocked, plants are regularly weeded, water is from a municipal source, propagation areas are kept free of noticeable debris (weeds, moss, plant waste) for the most part, plants are maintained on benches, occasionally plants are inspected for pests, there is some traceability in plant processing. The site is signed, fenced and locked after hours to exclude intruders and some animals.
4	0.8	100	25000	Yes	Roads are paved, gravelled or rocked, plants are regularly weeded, water is from a municipal source, propagation areas are kept free of noticeable debris (weeds, moss, plant waste) for the most part, plants are maintained on benches or elevated off the ground and site is gravelled, occasionally plants are inspected for pests, there is some traceability in plant processing. The site is signed, fenced and locked after hours to exclude intruders and some animals
5	1.2	330	50 000	Yes	Water is from a municipal source, some plants are on benches or maintained gravel, there is a list of plants on site.

[a]Griesbach *et al.* (2011).

Table 2 Number of individual plants of each species sampled in each of the restoration nurseries, to examine the potential treatment effects of the *Phytophthora* Prevention Programme (PPP treatment).

	Pre-PPP (CG-1) Nursery			PPP Treatment (TG-2) Nursery			Control (CG-3) Nursery	
Plant species[a]	1[b]	2	3	1	2	3	4	5
Aesculus californica				7			10	
Ceanothus thyrsiflorus	20			21			10	
Diplacus aurantiacus				5				20
Frangula californica	10			14			10	10
Heteromeles arbutifolia	14			21			10	
Morella californica	10			12				
Quercus agrifolia	5	13		10	10			
Acer circinatum								10
Achillea millefolium				16	25			
Alnus rubra	6							
Arctostaphylos uva-ursi								10
Artemisia douglasiana					5			
Baccharis pilularis	7			22	21	9		
Elymus glaucus				3		3		
Eriophyllum stoechadifolium				18	11	4		
Gaultheria shallon	6							
Hesperocyparis macrocarpa				4				
Juncus patens						10		
Lonicera hispidula				6				
Lonicera involucrata				13				
Quercus lobata							10	
Rubus ursinus		3	3	4	8	5		
Stachys ajugoides				4				
Symphyotrichum chilense	6			3				

[a]Upper portion was used to compare treatment effects.
[b]Each nursery has a unique numerical code (1–5).

two nurseries (CG-3; control group 3) did not implement the PPP and were sampled as controls during the same time interval as the nurseries sampled after PPP implementation. BMPs included in the PPP were based on standard guidelines to reduce *Phytophthora* (Griesbach *et al.*, 2011).

The key elements of the PPP were aimed at achieving proper water management, improved soil management (especially handling and pasteurization), and enforcing nursery sanitation. All three general management practices were not properly enforced in nurseries prior to participating in the study (Table 3). Training was provided to all staff involved in growing plants in participating nurseries, and site and management improvements were made before participating nurseries were allowed to be resampled. No significant changes in policies or procedures beyond the BMP programme were noted.

Sampling plants for Phytophthora *species*

The five participating nurseries all grew native plants to be used in habitat restoration projects. Plant species were selected for the study based on availability in stock and were even aged and well established in containers. Plants were sampled by inspecting them for symptoms, and were then placed either into the group with or without symptoms, and an equal number of plants from each group was randomly selected to be baited. Symptoms were recorded regarding the general appearance of the entire plant, and more specifically regarding the health of the foliage, the stems, and the roots. In the absence of overt symptoms in above-ground parts, roots were checked for necrotic flecking, dead, broken roots, and water-soaked lesions. Plant species were either regional to the west coast (native range) or naturally widespread across North America (USDA NRCS, 2017). The number of *Phytophthora* species isolated from each plant species was also recorded, and the results along with the list of symptoms are provided (Table S1).

Sampling from control and PPP intervention groups

Sampling was split into three groups: CG-1 included 103 plants from three nurseries collected before implementation of the PPP, TG-2 included 294 plants from the same three nurseries collected after implementation of the PPP, and CG-3 included 100 plants from two additional 'control' nurseries that never implemented the PPP and were sampled at the same time as TG-2 samples (Table 2). The sampling of CG-1 was performed in 2015, lasted approximately 6 months and occurred between 1 January and 22 June. The sampling of TG-2 and CG-3 lasted 1 year and occurred between 22 September 2015 and 22 September 2016. The post-PPP sampling of TG-2 was intentionally designed to be more extensive (and thus longer) than the sampling of CG-1, to maximize the likelihood of identifying low levels of phytophthora disease incidence.

Determining phytophthora disease incidence and *Phytophthora* species assemblages on a variety of hosts

Phytophthora species were isolated from each plant and identified to species-level (see below). This information was then used to determine disease incidence for each of the three sampling groups CG-1, TG-2 and CG-3. *Phytophthora* species assemblages by host species, and disease incidence by host species were computed for every group.

Phytophthora spp. isolations were attempted once from all of the 497 plants by baiting the roots, base of stem and nursery potting mix that was submerged, followed by plating baits on selective medium [1/2-strength VARP+: V8 stock agar (V8A), with 25 ppm hymexazol (97%), 10 ppm pimaricin, 200 ppm ampicillin, 10 ppm rifampicin and 15 ppm benomyl]. Some differences might be expected between direct isolation and baiting (Jung & Blaschke, 1996; Sims *et al.*, 2015); however, direct isolation normally includes plating of tissue with symptoms while baiting does not. Because baiting allowed the study to be performed independent of the presence of disease symptoms, it was selected as the main isolation approach for the entire study. Flooding and baiting were done with bait pieces placed in a tea bag filter paper with a foam packing peanut, stapled shut, and floated on the surface of the water over the flooded soil/roots of the nursery plant. Baits consisted of D'anjou pear pieces and occasionally also included rhododendron leaf pieces and oregano stem and leaf pieces. Baits remained in place for 5 days. Bait bags were then removed and opened with forceps. The contents of each bait bag was thoroughly blotted dry, and then partially submerged into the medium, with forceps and tools cleaned and sterilized with a flame between each sample. In three cases where stem cankers were observed, tissue at the outer edge of cankers was excised, outer bark removed, and remaining tissue placed in VARP medium (above medium without hymexazol). Additionally, roots were thoroughly washed from a subset of 100 plants and placed in a 0.5% sodium hypochlorite solution (made at each interval plants were processed) for 30 s and then rinsed and blotted dry. Six 1 cm-long root pieces were plated each onto three different media: V8A, malt extract (ME) and 1/2-strength VARP+.

Table 3 Best management practices that were the key focus of the *Phytophthora* Prevention Programme (PPP). Changes made in nurseries were to improve nursery sanitation, water management and soil management.

Aim	Practice
Improved nursery sanitation	
General	After every crop rotation, disinfect (pressure wash and sanitize): propagation areas, greenhouses and shadehouses
	If plants are placed on the ground, they must be discarded
	Do not place dirty tools (gloves, buckets, trowels etc.) on a growing bench
	Remove and dispose of leaf and soil debris from plant production areas
	Control weeds
From field to the nursery	Clean vehicle, clothes, boots and tools before entering the nursery. Sanitize shoes upon entry and exit of the nursery and only use clean and sanitized tools at the nursery
	Park vehicles in designated areas away from growing areas
	Clean plant propagules as much as possible before bringing them to the nursery
	Enclose propagules brought to the nursery in a container, only opening them in designated areas
	Use clean gloves when at the nursery (no field gloves)
	Shoes and tools must be cleaned and sanitized upon departing the nursery
Improved water management	
General	Plants checked regularly for proper watering and plants grouped on benches based on their watering needs
Movement of water	Do not allow the accumulation of standing water in the nursery
	Nursery and shadehouse floors need to be finished and even, so that water does not collect or mud form
	Divert any runoff away from crops and grow areas
	Ensure no runoff from the cull pile bins
Improved soil management	
General	Use only pasteurized potting soil (= media and components mix) stored in enclosed sanitized or sterile container
	Only use new or clean and properly sanitized containers
Movement of soil/debris	Protect potting soil (= media and components) from outside soil and water
	Keep culled material including used potting soil enclosed and away from the growing area
	Do not bring buy-in plants into the nursery
	Plants grown on open mesh top benches at least 3 feet above the nursery floor and away from potential soil contamination sources
Propagation material	Seed is preferred
	Make collections from healthy plants in healthy field sites or healthy nursery beds
	Collect seed away from the ground and contamination sources using tarp traps
	Treat low growing seeds and all cuttings according to treatment recommendations
Additional steps	
	Document procedures
	Attend routine training

Phytophthora spp. were identified by culture morphology (Sims *et al.*, 2015) and by internal transcribed spacer (ITS) DNA barcoding (Schoch *et al.*, 2012). Morphological characterization to genus was done on culture plates (subcultured onto V8A). Morphological characters used to distinguish *Phytophthora* species from other fungi growing on the plates included rate, timing and pattern of growth, accompanied by the hyphal branching and colony pattern and absence of spore types typical of other genera. In addition, hyphal refraction of light was compared to *Pythium* species. Other features were used as well, but absence did not eliminate them as a possible *Phytophthora* species, and each independently did not confirm them as *Phytophthora* either, but instead character traits were used in tandem as were generally observed for particular species. These included hyphal pinching at branch point, presence of mainly globose, darkly pigmented chlamydospores, and presence of typical *Phytophthora*-type sporangia (Erwin & Ribeiro, 1996) either with or without papillae, presence of hyphal swellings, and/or oospores either with amphigynous or paragynous attachment of antheridia at the base of the stalk.

DNA was extracted using the DNeasy Tissue Extraction kit (QIAGEN) following the manufacturer's instructions. ITS amplification was performed using 2 μL of each primer DC6 (5′-GAGGGACTTTTGGGTAATCA-3′; Cooke *et al.*, 2000) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; White *et al.*, 1990) at a concentration of 10 μM. Other PCR cocktail ingredients were 29 μL double distilled sterile water, 1 μL dNTPs (10 mM), 4 μL $MgCl_2$ (25 mM), 10 μL 5 × Green GoTaq reaction buffer and 1 U GoTaq polymerase (Promega), and 1 μL template DNA. The PCR cycling (T100 machine; Bio-Rad) was set to 94 °C for 2 min; 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; followed by a final 72 °C for 7 min, and 24 °C for 1 min. All PCR products (850 ng per 10 μL double distilled sterile water) were submitted for Sanger sequencing to the Sequencing Facility at the University of California, Berkeley. Raw sequence traces were assembled, and quality processed by evaluation of chromatograms and saved as consensus fasta files (Staden *et al.*, 1999). Sequences were aligned using CLUSTALX (Larkin *et al.*, 2007) with references from local in-house, and type sequences from GenBank. One representative (and consensus) sequence of each *Phytophthora* species from each plant species was deposited in GenBank, and the closest reference including sequences of the ex-type material is provided (Table S1).

Identifying new Phytophthora × *plant species combinations*

All *Phytophthora* species isolated and identified are listed (Table S1). Available information was accessed using the USDA

host–fungus databases to determine whether a plant species × *Phytophthora* species combination was indeed novel (Farr *et al.*, 2009).

Genus-level phytophthora disease incidence by plant species

Each plant of each plant species was tallied as either *Phytophthora*-positive or -negative. Disease incidence was defined as the number of *Phytophthora*-positive plants with symptoms/total number of plants within each sampling group (CG-1, TG-2 and CG-3). Disease incidence by plant species was assigned to those adequately sampled (at least 20 samples) and based on the sample proportion ($\hat{P}$):

$$\hat{P} = \text{diseased plants/total plants of a species sampled}$$

An 'adequately sized' sample was determined to be 20 based on (i) the general statistical inference that 20 was the smallest number in which the effect of a single replicate can be regarded as statistically negligible (Fugard & Potts, 2015); and (ii) results based on a complete destructive sampling of a diseased *Ceanothus thyrsiflorus* crop from nursery one, indicating that 10 was the minimum number of samples necessary to capture *Phytophthora* from a crop with 95% confidence (Garbelotto *et al.*, in press). Thus, the study conservatively used a minimum sample size of 20 plants to estimate the binomially distributed sample proportion ($\hat{P}$) where the standard deviation (SD) for $(\hat{P}) = \sqrt{(\hat{P} \times (1 - \hat{P})]/n}$ is used as an estimate of the population proportion (P).

Evaluating the efficacy of the PPP

Evaluating the efficacy for all Phytophthora *species across multiple plant species*

The PPP is intended to reduce or eliminate phytophthora disease incidence independent of which *Phytophthora* species may be present or which plant host may be infected. Thus, to examine the overall efficacy of the PPP, the overall phytophthora disease incidence was first determined in the three experimental groups (CG-1, TG-2 and CG-3) by calculating the proportion of all plants within each group that was infected by any *Phytophthora* species. Then, two sets of comparisons were performed using Fisher's exact tests, and chi-square analysis with Yates continuity correction (Crawley, 2007; R Core Team, 2018). The first set compared disease incidence of plants within the same nurseries before and after the implementation of the PPP (CG-1 and TG-2). This comparison examined whether phytophthora disease incidence significantly decreased post-implementation of the PPP. The second set compared disease incidence recorded in nurseries after they fully implemented the PPP (TG-2) with disease incidence recorded in two control nurseries (CG-3) that did not implement the PPP but were sampled at the same time as TG-2. This comparison examines whether the trend in the change in disease incidence observed in TG-2 was replicated (or not) in control nurseries that did not implement the PPP. If there was no trend, and no difference between CG-1 and CG-3 (or CG-3 was larger than CG-1) then the second comparison would provide stronger support for causality between the implementation of the PPP and change in disease incidence, thus helping to exclude that changes were caused by other unknown factors occurring in the later time interval.

Evaluating the efficacy for individual plant species

The analyses described above were also used to compare disease incidence for five individual plant species paired in nurseries before and after the implementation of the PPP. The paired species were: *C. thyrsiflorus*, *Frangula californica*, *Heteromeles arbutifolia*, *Morella californica* and *Quercus agrifolia*. In addition, the efficacy of the PPP was evaluated for five individual plant species across different nurseries but sampled in the same time interval, by comparing disease incidence species by species in the three TG-2 nurseries (post-implementation of the PPP) and in the two CG-3 nurseries (no implementation of the PPP). The species analysed in this second analysis were: *C. thyrsiflorus*, *F. californica*, *H. arbutifolia*, *Aesculus californica* and *Diplacus aurantiacus*.

PPP effectiveness factoring variability among nurseries and plant species

Restoration nurseries in California are inevitably very heterogeneous. Although this study was interested in overall effects of the PPP on phytophthora disease regardless of plant species or nursery, by necessity nurseries are not fully comparable due to the fact they are located in different sites and grow different selections of plants. Variability among nursery and plant species might confound the pooled Fisher's and chi-square results (Crawley, 2007). Thus a model of the categorical effects (explanatory variables: nursery, plant species and treatment) was designed to measure the programme effectiveness in terms of the proportion of healthy plants following the implementation of the PPP (response). In the model, nurseries and plant species were considered as blocking factors and the overall effect of treatment was determined. The same data set from 242 plant samples was used as that employed for the Fisher and chi-tests described above. Specifically, the number of uninfected plants expected from a crop of 100 was set as the response from each treatment nursery, compared to no treatment control nurseries, and accounting for each plant species.

Results

The *Phytophthora* species assemblage

A total of 55 unique *Phytophthora* isolates were obtained from the 203 plants produced without BMPs in groups CG-1 and CG-3. One of these was only obtained by direct plating of roots. Not a single *Phytophthora* isolate was obtained from the 294 samples taken from the PPP treatment group (TG-2) sampled post-BMP intervention.

Phytophthora species were isolated from nine of the 24 plant species evaluated. *Phytophthora cactorum* was the most common species and was isolated from the greatest number of plant species (18 times and six plant species). Overall, there were 13 *Phytophthora* taxa identified, including one putative interspecific hybrid taxon (Table S1). Seven of the taxa had ITS sequences identical to the ex-type ones of known species: *P. cactorum*, *P. hedraiandra*, *P. multivora*, *P. occultans*, *P. crassamura*, *P. thermophila* and *P. pseudocryptogea*. *Phytophthora* ITS clades 1, 2, 6, 7 and 8 (Cooke *et al.*, 2000) were all present in the samples: *Phytophthora* species isolates came from clades 1 (21 isolates), 8 (11), 2 (11), 6 (6) and 7 (5). Both morphology and ITS sequence information was used to define species, and most isolates were 100% match to the ex-type isolate sequences, with any differences listed in Table S1.

There were 20 novel *Phytophthora* species × plant species combinations according to a USDA fungal-host

database search. However, the most common *Phytophthora* species × plant species combination was not novel: *P. cactorum* on *H. arbutifolia* (eight times). Symptoms and percentage of plants showing at least one symptom observed in each nursery for each *Phytophthora* × host combination are given (Table S1).

Phytophthora disease incidence based on plant species

Four plant species were highest for the observed phytophthora disease incidence and were considered to have enough samples for evaluation (Fig. 1). These four species were: *D. aurantiacus* (50 ± 11.2%), *H. arbutifolia* (33 ± 9.6%), *C. thyrsiflorus* (30 ± 8.4%) and *F. californica* (30 ± 8.4%). Figure 1 displays genus-level phytophthora disease incidence by plant species, and the portion of that disease incidence made up by each *Phytophthora* taxon (bar shading).

Efficacy of the PPP

Effect on overall phytophthora disease incidence

Overall disease incidence values were as follows: 22 ± 4% in CG-1 nurseries before implementation of the PPP, 0% in TG-2 samples from the same nurseries after implementation of the PPP, and 32 ± 5% in CG-3 samples from two control nurseries that did not implement the PPP and were sampled at the same time as TG-2. There were no significant differences comparing CG-1 and CG-3 ($P = 0.112$); however, CG-1 and TG-2 had significantly different disease incidence ($P < 0.001$). These results suggest the PPP was highly effective in reducing disease incidence in the three nurseries that implemented the PPP.

Effect on disease incidence at the plant species level

In all single species comparisons, the PPP reduced disease incidence to zero (Table 4). For six of 10 of the plant species × nursery comparisons, the drop to zero in disease incidence was validated as statistically significant by both Fisher's exact test and chi-square analysis. Significance was not obtained in four cases, namely one comparison between CG-1 and TG-2 for *M. californica*, and three comparisons between CG-3 and TG-2 for *A. californica*, *D. aurantiacus* and *F. californica*. Lack of significance was due to the low sample size and/or low initial disease incidence levels.

Effectiveness factoring variability in nursery and plant species

Overall modelled percentage of healthy plants post-implementation of PPP resulted in an estimated average across all species of 100% [confidence interval (CI) ± 15.7] for a crop of 100 plants. This translates into an estimated treatment effect of 33 additional healthy plants per crop no matter the plant species ($P = 0.65$) and to a marginal statistically valid effect of nursery (0.06). Implementation of the PPP was significantly different compared to no implementation of the PPP ($P = 0.0001$), independent of blocking effects 'nursery' and 'plant species'.

Discussion

In the present study, more than a quarter of all of the samples taken from nurseries that had not implemented the PPP produced *Phytophthora* isolates. Successful isolation of these pathogens was overwhelmingly associated with disease symptoms, and symptom severity was rather non-uniform throughout a crop, as expected for plants with infectious diseases whose symptoms can be strongly modulated by host genotype, microclimatic conditions, and time since infection (Kennelly *et al.*, 2012). *Phytophthora*-positive plants that did not display significant symptoms above ground had necrosis or water-soaked lesions on the outer roots. Although symptoms alone cannot be considered diagnostic, the symptoms recorded in this study were '*Phytophthora*-like' and always accompanied by isolation of the pathogen.

Some *Phytophthora* species were found to be prevalent in some nurseries, and absent or seemingly so in others.

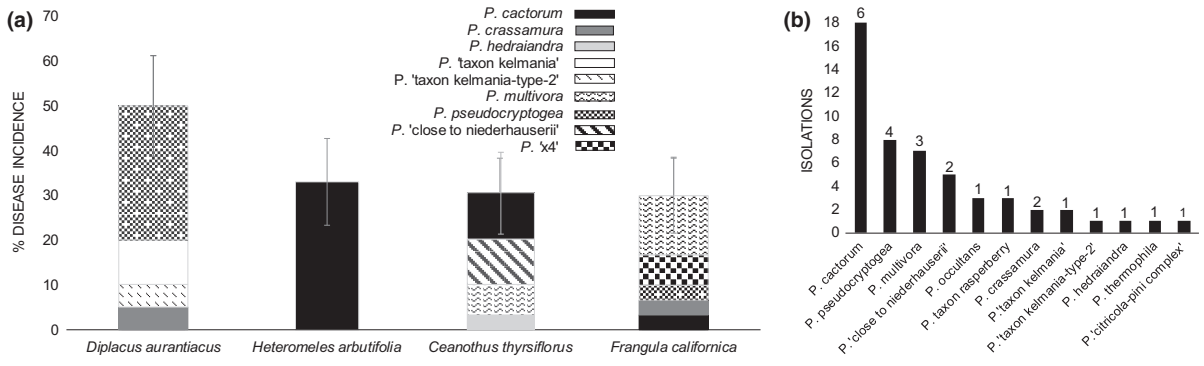

Figure 1 (a) Phytophthora disease incidence for the four plant species with the highest disease incidence. The relative proportion of each *Phytophthora* species is shown by different shading patterns inlaid in the figure. Error bars are for the overall genus level disease incidence. (b) *Phytophthora* species abundance based on the number of successful isolations. The number of hosts from which each *Phytophthora* species was isolated is shown above the bar.

Table 4 Phytophthora disease incidence with and without *Phytophthora* Prevention Programme (PPP)

Plant species	Pre-PPP (CG-1)		PPP treatment (TG-2)		Fisher exact	Chi-square[e]
	Disease incidence	*n*	Disease incidence	*n*	*P*-value	*P*-value
Frangula californica	0.5	10[a]	0	14[a]	0.006	0.007
Heteromeles arbutifolia	0.4	14[a]	0	21[a]	0.006	0.007
Ceanothus thyrsiflorus	0.3	20[a]	0	21[a]	0.009	0.011
Quercus agrifolia	0.4[a], 0.2[b]	5[a], 13[b]	0[a], 0[b]	10[a], 10[b]	0.017	0.020
Morella californica	0.2	10[a]	0	12[a]	0.198	0.189
Overall	0.3	72[f]	0	88[f]	<0.001	<0.001
	Control nurseries (CG-3)		PPP treatment (TG-2)			
Diplacus aurantiacus	0.5	20[d]	0	5[a]	0.061	0.063
Ceanothus thyrsiflorus	0.3	10[c]	0	21[a]	0.026	0.023
Heteromeles arbutifolia	0.3	10[c]	0	21[a]	0.026	0.023
Aesculus californica	0.3	10[c]	0	7[a]	0.228	0.171
Frangula californica	0.2[c], 0.2[d]	10[c], 10[d]	0	14[a]	0.126	0.107
Overall	0.3	70	0	68[f]	<0.001	<0.001

Statistical test *P*-values are included for Fisher's exact and chi-square for individual plant species and for comparisons overall.
[a]Nursery 1; [b]nursery 2; [c]control nursery 4; [d]control nursery 5; [e]statistical test with Yates continuity correction; [f]pooled samples across species.

For instance, it was noted that while *P. cactorum* was dominant in nursery 1, *P. pseudocryptogea* was dominant in nursery 5 and apparently absent in nursery 1. A different history of contagion may explain variation in prevalence of *Phytophthora* species across nurseries. Independent of the reason behind the variability across nurseries, using plant stock from different nurseries may result in the introduction of different *Phytophthora* species, increasing unpredictability of outcomes when using plant stock from nurseries without an adequate BMP programme in place.

No significant differences were found in the assemblages of *Phytophthora* species obtained through baiting compared to those obtained through direct isolations from roots on a subset of 100 plants. Only a single additional *Phytophthora* species × host combination was found from direct plating of roots (*P. pseudocryptogea* × *A. californica*), no additional *Phytophthora* species were obtained through direct isolation (potentially missed by baiting), and the same species was generally collected from both methods. However, considering the large number of new host × *Phytophthora* species combinations identified in this study, it is surmised that pathogenicity on new hosts would be ascertained with more confidence using the set of *Phytophthora* isolates obtained through direct isolation from plant tissue with symptoms, as these would also have the qualitative character of having certainly infected the host, whereas baited isolates may possibly have arisen from dormant structures in the rhizosphere outside the root system.

The co-mingling of different plant species in the same facility and the polyphagous nature of most *Phytophthora* species isolated in this study could explain the great degree of novelty in host × pathogen combinations. Although it is impossible to reconstruct with confidence how these plant production facilities became infested in the first place, the lack of infestation in the only facility (CG-3) not trading plant stock with others provides some evidence that the trade or purchase of plants from other facilities may represent a major pathway of introduction of *Phytophthora* species in plant production facilities. Alternative pathways may be provided by the use of contaminated soil, green waste and tools. Examples of such contaminated substrates were recently identified during the course of this and other studies (data not shown).

Four plant species had the highest disease incidence, namely *Diplacus aurantiacus*, *Heteromeles arbutifolia*, *Frangula californica* and *Ceanothus thyrsiflorus*. No matter the reasons behind such high disease incidence, extra caution should be taken when using these four species in restorations. It should also be noted that three of the four species (all but *H. arbutifolia*) were infected by multiple *Phytophthora* species, thus using infected plants belonging to these species could result in the release of multiple species in the wild.

The relevance of producing plant stock with as few *Phytophthora* species as possible for restoration projects needs to be properly emphasized, given recent results showing an excellent match between the lists of *Phytophthora* species found in restoration projects (Bourret, 2018) and the list produced here from plant production facilities providing plant stock for restoration. Such overlap is in fact strongly suggestive that nurseries producing plant stock for restorations are the source of infestations detected in some restored wildland sites in California.

Phytophthora cactorum, *P. multivora*, *P. crassamura*, *P. pseudocryptogea* and *P.* 'taxon raspberry' were found both in wildlands (Bourret, 2018) and in nurseries surveyed in this study. In both cases, *P. cactorum* was the dominant species and its most common host was *H. arbutifolia*. This *Phytophthora* species occurs worldwide, and it is known to parasitize over 200 different plant species across many different plant families, particularly in regions with temperate climates (Erwin & Ribeiro, 1996). Yet, many of the plant hosts of *P. cactorum*

reported here are new, and novel genotypes of this pathogen appear to display increased pathogenicity, at least on some hosts (Sims & Garbelotto, 2018), suggesting this pathogen is emerging as a greater threat than previously predicted. *Phytophthora cactorum* has been reported as absent or rare in North America outside of restoration sites (Balci *et al.*, 2007; Reeser *et al.*, 2011; Sims *et al.*, 2015), while its incidence has been reported as high in both restoration sites (Bourret, 2018) and restoration nurseries (this study). Cumulatively, these findings support the notion that *P. cactorum* may be a pathogen associated with human activity, recently emerged in North American wildlands, and may not yet have spread very broadly away from planted areas.

The other four species prevalent both in restored wildlands and restoration nurseries (this study) are either newly described taxa, such as *P. crassamura* (Scanu *et al.*, 2015), or they have been described from wild settings in North America only recently (Martin *et al.*, 2014; Bourret, 2018). This indicates they also are likely to be emerging new pathogens, and may have a greater impact than expected, as indicated by the large number of hosts for these species identified in this study.

The PPP was found to be effective in reducing overall phytophthora disease incidence in facilities that implemented the programme. There was no strongly significant effect of nursery or plant species on disease incidence following the implementation of the PPP, indicating that implementing a PPP is the most important approach to minimize phytophthora disease, no matter the nursery or plant species involved. This study may be one of the first that shows a short series of specific and relatively straightforward BMPs implemented together as a programme can reduce the incidence of disease in production nurseries focused on native plants used for restoration projects. Some of the nurseries participating were already implementing some BMPs, but the PPP described here was meant to break the disease cycle by simultaneously performing a series of tasks including adequate sanitation (clean up of entire facility by pressure washing, sanitation of benches with 70% ethanol or isopropyl alcohol, detection and elimination of infected crops, pasteurization of soil and used pots, resanitation of surfaces in between crops), designing adequate watering regimes by placing plant species with comparable water requirements close to one another rather than grouping them by project, reducing the chance of plant infestation occurring by providing proper drainage, ensuring all plants are grown on raised open mesh top benches, and controlling entry onto grounds to minimize infection from outside landscape areas or infected plants. Thus, it should be clear that adhesion to the PPP required a significant effort: it is believed that success was possible thanks to strong educational programmes instituted in each of the nurseries to clarify the importance of the PPP, and to justify the increased effort to workers by enhancing their involvement and participation. While the change was difficult, once instituted, the procedures became routine and overall crop health improved significantly, which also improved worker morale, potentially extending the benefits even beyond healthy plants.

Finally, this study exemplifies how robust results can be obtained even without the possibility of a perfectly balanced and fully factorial experimental design, but working together with nurseries' management and staff, and within the constraints of a real plant production cycle. It should be added that working in a real production situation provides the added benefit of immediately testing results in the 'real world'. The results presented here have been reverified, by both the California regulatory agency (the California Department of Food and Agriculture) who are sent any suspicious bait material (Christa Conforti, Presidio Trust and Alisa Shor, Golden Gate National Parks Conservancy, San Francisco, USA, personal communication) and by using a monitoring system from an independent contractor (Tedmund Swiecki, Phytosphere Research, Vacaville, USA, personal communication), which is purportedly more rigorous. These have confirmed zero *Phytophthora* detections in follow-up years (2017–18) over multiple crops in the same nurseries participating in this study and implementing the PPP.

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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.

Table S1. The plant species from nurseries, the symptoms on each, and the *Phytophthora* species isolated. GenBank accessions are from isolates in this study, and from published GenBank sequence references with the percentage homology and the number of reference identities. The number (*n*) of isolates for each plant species × *Phytophthora* species combination is also given.