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Differential response of four Californian native plants to worldwide *Phytophthora cinnamomi* genotypes: implications for the modeling of disease spread in California

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Abstract Multiple introductions in California of *Phytophthora cinnamomi* are severely threatening its native ecosystems. However, little is known about the consequences of outbreaks caused by different genotypes on endemic hosts. Virulence of 10 genotypes representative of the California and worldwide genetic variability of *P. cinnamomi* was studied on roots and stems of four native California plant hosts. Hosts differed in type of response and susceptibility level, and individual pathogen genotypes differed in virulence. Pathogen variability was clear when testing hosts of moderate susceptibility (*Pseudotsuga menziesii* and *Umbellularia californica)*, but not so on highly susceptible hosts. Some genotypes are better adapted as root rather than stem pathogens, causing a different disease. *Arbutus menziesii* and *Arctostaphylos viscida* are hosts with aerial and root susceptibility, while *U. californica* acts mainly as root host. Four isolates representing three genotypes caused the highest level of disease: two of them were from a genetic lineage only recently identified in California and Mexico, but nowhere else. The results presented here are preliminary, however if confirmed, they would suggest that novel invasive genotypes of *P. cinnamomi* may require regulatory action to prevent their further spread. It also proves that highly susceptible hosts should not be used to identify phenotypic variability in pathogens.

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Introduction

The California Floristic Province is classified as a global biodiversity hotspot due to its varied climate, diverse topography, and unusual range of endemic species (Calsbeek et al. 2003). It has recently been determined that several natural California ecosystems are being seriously threatened by the soilborne oomycete *Phytophthora cinnamomi* Rands. This is a devastating plant pathogen registered among the 100 World's Worst Invasive Alien Species (Lowe et al. 2004). *Phytophthora cinnamomi* mainly causes rot of feeder roots that occasionally may extend into the stem base (below ground infection). Additionally, it often causes stem cankers (above ground infection) (EPPO/CABI 2011). In California, *P. cinnamomi* was first isolated in 1942 from avocado and in 1966 from *Pseudotsuga menziesii* (Douglas fir) in orchards (Wager 1942; Roth and Kuhlman 1966). However, its presence has been recorded in California wildlands only recently, causing root rot on native species such as *Arctostaphylos myrtifolia* (Ione manzanita), *Ar. viscida* ssp. *viscida* (whiteleaf Manzanita) (Swiecki et al. 2003), *Quercus agrifolia* (Coast live oak) (Garbelotto et al. 2006), *Arbutus menziesii* (Pacific madrone), and, *Umbellularia californica* (California bay laurel) (Fichtner et al. 2009).

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Phytophthora cinnamomi is a heterothallic (e.g. sexually outcrossing) species with two mating types (A1 and A2) (Erwin and Ribeiro 1996), both necessary for sexual reproduction. Although selfing can occur naturally in plants (Jayasekera et al. 2007; Crone et al. 2013), the vast majority of worldwide infestations are caused by a single mating type, and there are no reports of autogamous sexual reproduction from regions exotic to the pathogen such as California, South Africa (Linde et al. 1997), the Iberian Peninsula (Caetano et al. 2009), the Eastern USA (Eggers et al. 2012) or Australia (Dobrowolski et al. 2002). Overall, this lack of recombination results in low genetic variability, so that assignment to a certain group is straightforward and depends on the genotype and geographic source of founder individuals for each exotic outbreak (Linde et al. 1999a). Furthermore, lack of recombination among isolated populations has resulted in groups that are genetically clearly distinct, due to their independent evolution. These groups have also been defined as clades or lineages based first on multilocus isozyme genotypes, and then on microsatellites or amplified fragment length polymorphisms analysis (Old et al. 1988; Oudemans and Coffey 1991; Linde et al. 1997; Dobrowolski et al. 2003; Pagliaccia et al. 2013). A DNA microsatellites analysis of 159 isolates of *P. cinnamomi* of Californian, Mexican and worldwide origin identified over 75 multilocus genotypes from 3 clearly distinct STRUCTURE groups (Table 1) (Serrano et al. 2019). A putative ancestral Group WW1 included both A1 and A2 mating types and all isolates from Papua New Guinea, a region regarded as endemic to *P. cinnamomi* (Arentz and Simpson 1986). Group WW1 genotypes were also present worldwide on different commodities but were rare in California wildlands. Groups WW2 and WW3 genotypes, carrying the A2 mating type, have been frequently isolated from agricultural commodities and natural settings in Mexico and California and included some clonal isolates, that is, isolates belonging to the same genotype and isolated from different hosts, sites and regions. These results suggest multiple introductions have occurred in wildlands of the "Golden State" (California) (Serrano et al. 2019), and this study also indicates introductions into California natural ecosystems are more recent than those in commodity hosts. The presence of widespread clonal genotypes in Groups WW2 and WW3 can be indicative of a recently young age of such genotypes, combined with their effective long-distance movement. However, the presence of clonal genotypes could also be interpreted as the result of their higher fitness and virulence: this interpretation implies clonal genotypes may represent a higher threat to the ecosystems they inhabit.

Table 1 Main characteristic of the three distinct STRUCTURE Groups (WW1, WW2 and WW3) obtained based on a DNA microsatellites analysis of 159 isolates of *P. cinnamomi* of Californian, Mexican and worldwide origin: hosts and locations where each of the three groups was isolated and the mating types described in each group (Serrano et al. 2019)

	STRUCTURE GROUPS		
	WW1	WW2	WW3
Hosts	*Ananas comosus* *Arctostaphylos myrtifolia* *Arctostaphylos viscida* *Camellia japonica* *Castanopsis* sp. *Eucalyptus* sp. *Nothofagus* sp. *Persea americana* *Rhododendron* sp. *Vitis vinifera*	*Abies spp.* *Arctostaphylos sp.* *A. myrtifolia* *A. viscida* *Pseudotsuga menziesii* *Quercus* sp.	*Abies sp.* *Abies concolor* *Abies nordmanniana* *A. myrtifolia* *A. viscida* *Arbutus menziesii* *P. americana* *Pinus radiata* *Quercus agrifolia* *Vaccinium corymbosum*
Locations	Australia California, USA China Madagascar Papua New Guinea South Africa Taiwan	California, USA (Amador, El Dorado) Mexico (Colima)	California, USA (Alameda, Amador, El Dorado, Marin, San Diego, San Mateo, San Rafael, Santa Cruz, Sonoma) Florida, USA
Mating types	A1 and A2	A2	A2

Previous studies on pathogenic variability of *P. cinnamomi* have not confirmed the presence of significant differences based on pathogen provenance, host origin, or genotype (Dudzinski et al. 1993; Robin and Desprez-Loustau 1998; Linde et al. 1999b; Caetano et al. 2009), but those studies included only a limited representation of the overall genetic variability and history of this pathogen. Furthermore, and based on the results of the present study, previous approaches designed to identify intraspecific variability may have been faulty due to the use of highly susceptible host species as tester species (see results and discussion). Currently, little is known

about the variation in pathogenicity among Californian populations of *P. cinnamomi*, about the relative pathogenicity of genotypes present in natural California ecosystems vs. that of genotypes limited to agricultural settings in California, and on the virulence of genotypes still unreported from California. Additionally, several soilborne *Phytophthora* spp. have recently been reported for the first time as pathogens of native vegetation in several restoration sites across California (Garbelotto et al. 2018). Thus, a broad study on what is probably is the best known soilborne *Phytophthora* is not only timely, but it may also help predict and understand the potential for future spread and disease caused by this and also by other novel and less known *Phytophthora* spp.

The main aims of the current work were to:

1- Assess variation in levels of pathogenicity of *P. cinnamomi* in correlation with its genetic variability on four important Californian native plant species, all recently described as being naturally infected by this oomycete (Roth and Kuhlman 1966; Swiecki et al. 2003; Fichtner et al. 2009).
2- Determine whether intraspecific variability in pathogenicity and preference for distinct plant parts (above vs. below ground) by *P. cinnamomi* may be more effectively assessed in moderately, rather than in highly susceptible host species.
3- Test whether genotypes from two newly described widespread young clonal genotypes of *P. cinnamomi* isolated both in California and Mexico, may be more pathogenic than genotypes that are either ancestral or represented by one or a few isolates.
4- Determine whether genotypes of *P. cinnamomi* with a limited distribution range, e.g. present in commercial California facilities, but absent in California natural ecosystems, or present only in the native range of the pathogen, may represent a threat to California plant species.

Materials and methods

Isolates

Ten *P. cinnamomi* isolates were selected based on a microsatellite (Simple Sequence Repeat or SSR) DNA analysis that grouped them in three different STRUCTURE groups (Table 1) (Serrano et al. 2019). Table 2 lists the collector and date, host, demographic origin, mating type, SSR clade and multilocus genotype (Serrano et al. 2019) of the isolates of *P. cinnamomi* employed in the study. The 10 isolates were passaged through Granny Smith apples to ensure they had not lost their pathogenicity due to prolonged storage (Erwin and Ribeiro 1996), and then re-isolated on PARPH for use in later inoculations. These isolates are stored in the culture collection of Garbelotto's lab, University of California, Berkeley (UCB).

Plant material

Three Californian native evergreen trees and one native shrub were included in the study: California bay laurel (*Umbellularia californica*), Pacific madrone (*Arbutus menziesii*), Douglas-fir (*Pseudotsuga menziesii*), and the shrub whiteleaf manzanita (*Arctostaphylos viscida ssp. viscida*). Plants provenance is shown in Table 3. All the seedlings were maintained in the UCB greenhouse and regularly watered for two months before starting the inoculations. Additionally, every plant was tested to be free of other *Phytophthora* or *Pythium* root pathogens as follows: segments (3–4 mm long) of roots obtained from ten plants per species randomly selected were plated on PARPH culture medium (Jeffers and Martin 1986) as well as baited (Robin et al. 1998; McConnell and Balci 2014), using young tender and old *Q. agrifolia* leaves as baits.

Root inoculation

One-year-old seedlings of the four plant species were inoculated with an aqueous suspension of *P. cinnamomi* chlamydospores, prepared as described in Sánchez et al. (2002). In brief, isolates were separately grown in Petri dishes containing carrot broth (20 ml per plate) at 20 °C in darkness. After 1 month of growth, the liquid medium was filtered in sterile conditions. Mycelium obtained per each isolate was washed, put into sterile water, shaken and mixed for 3 min. All the inocula were adjusted to 1.5×10^4 chlamydospores ml^{-1} (Romero et al. 2007; Serrano et al. 2012a).

Each plant was removed from its container (forestry trays) and each root ball was inoculated using 50 ml of inoculum (Serrano et al. 2012b). Inoculated plants were then re-potted in free-draining plastic pots (~1 gal) containing sand. Five seedlings (replicates) per plant

Table 2 Characteristic of the *P. cinnamomi* isolates selected

SSR-clade[a]	Isolates	Collector[b], Date	Host	Origin	Mating type	W/C[c]	MG[a]
WW1	P3662 (CSIROA126)	FA, 1988	*Castanopsis* sp.	PNG[d]	A1	W	MG19
WW1	P2183 (ATCC38581)[e]	JR/MJ, 1970	Soil	PNG[d]	A1	W	MG06
WW1	P6379[e]	PJA, 1989	*Ananas comosus*	Taiwan	A1	W	MG20
WW1	P2170	GAZ,1972	*Camellia japonica*	California	A1	C	MG13
WW1	P2301 (ATCC46686)[e]	GAZ,1976	*Rhododendron* sp.	California	A2	C	MG10
WW1	Mc33 = 17a1	TS, 2003	*Arctostaphylos viscida*	California	A2	W	MG10
WW2	Mc14[e]	MG, 2003	*Pseudostuga menziesii*	California	A2	W	MG08
WW2	Mc 09[e]	MG, 2003	*Arctostaphylos* sp.	California	A2	C	MG08
WW3	Mc 12	MG, 2003	*Abies nordmanniana*	California	A2	C	MG03
WW3	Mc 08[e]	MG, 2001	*Quercus agrifolia*	California	A2	W	MG16

[a] Serrano et al., 2019

[b] Collectors: FA = F. Arentz, GAZ = G.A. Zentmyer, JR = J. Reid, MG = M. Garbelotto, MJ = M. Jackson, PJA = P-J. Ann, TS = T. Swiecki

[c] Collected in the wild (W) or from an agricultural commodity (C)

[d] PNG = Papua New Guinea

[e] Isolates selected for testing on Whiteleaf Manzanita

species were inoculated with each isolate and the same number of replicates per species served as controls (not inoculated). All the pots were placed in plastic trays (43 × 36 × 15 cm) and incubated in a greenhouse (22 ± 2 °C during the day and 10 ± 2 °C at night). To avoid cross-contamination, pots containing seedlings infected with the same isolate were placed in separate trays and watered as required. One-week post inoculation, all trays including the controls were partially filled with de-ionized water to flood the soil for 2 days each week (Serrano et al. 2012a, b). Severity of crown symptoms (foliar wilting and/or defoliation) was assessed once a week for each plant on a 0–4 scale, according to the percentage of symptomatic foliage (0 = 0–10%, 1 = 11–33%, 2 = 34–66%, 3 = more than 67% and 4 = dead foliage) (Sánchez et al. 2002; Serrano et al. 2012a, b). At the end of the experiment, after carefully washing the roots to eliminate the sand, the severity of root symptoms was assessed on the same 0–4 scale according to the percentage of root necrosis and presence/absence of feeder roots (Sánchez et al. 2002, Serrano et al. 2012a, b).

To confirm symptoms were caused by *P. cinnamomi,* segments (3–4 mm long) from inoculated and control roots were plated on PARPH culture medium (Jeffers and Martin 1986) for re-isolation of the pathogen.

Stem inoculation

Pathogenic variability was also tested by stem inoculations on 1-year-old seedlings of California Bay laurel, Douglas fir and Pacific madrone (Table 3). Inoculum consisted of 4-mm plugs of V8 juice agar bearing mycelium from the margin of a 7-day-old culture

Table 3 Provenance of each plant species included in the two experiments (root and stem inoculations) and the duration (weeks) of each one

	Root inoculations		Stem inoculations	
	Provenance	Time[a]	Provenance	Time[b]
Pacific madrone	San Luis Obispo County, California	10	Contra Costa County, California	3
Douglas fir	Josephine County, Oregon	10	Josephine County, Oregon	4
California bay laurel	Sacramento County, California	12	Josephine County, Oregon	9
Whiteleaf manzanita	San Luis Obispo County, California	5	–	–

[a] Weeks after the first flooding

[b] Weeks after the stem inoculation

of the same *P. cinnamomi* isolates previously used in the root inoculations. A 4 mm diameter bark plug was removed with a cork borer approximately 5 cm above the collar, plugs containing *P. cinnamomi* mycelium were applied to exposed phloem/cambial surface, and the bark plug was replaced afterwards (Balci and Halmschlager 2003). The wound was covered with wet, autoclaved, cotton wool, wrapped in cheesecloth and then Parafilm, before being entirely covered with aluminum foil (Balci et al. 2008). Ten seedlings (replicates) per plant species were inoculated per isolate. The same number of plants (replicates) per species were inoculated with sterile agar plug and served as mock-inoculated controls. All plants were incubated and regularly watered in the greenhouse where recorded temperatures ranged between 22 ± 2 °C and 10 ± 2 °C.

The severity of foliar symptoms was assessed weekly according to the 0–4 scale (Sánchez et al. 2002) described in the section above. At the end of the experiment, lesion length was measured on the cambial surface after removing the bark (internal lesion) to calculate the percentage of stem affected (PSA) by *P. cinnamomi* lesion as:

$$\mathrm{PSA} = ((\text{Total plant length} - \text{Internal lesion length})/\text{Total Plant Length}) \times 100$$

To confirm symptoms were caused by *P. cinnamomi*, wood chips from the margin of the lesion were plated on PARPH selective medium (Jeffers and Martin 1986).

Statistical analyses

For each plant species and *P. cinnamomi* isolate, the relative area under the disease progress curve (rAUDPC) was estimated as a percentage with regard to the potential maximum value, as follows (Campbell and Madden 1990):

$$\mathrm{rAUDPC} = \frac{100}{(S_{max} + t_e)} \times \sum_{i=1}^{n} \frac{(S_i \times S_{i+1})}{2} \times (t_{i+1} \times t_i)$$

where s_i = disease severity value for observation number i, s_{max} = maximum value of severity (4), t_i = number of days between planting and observation i, t_e = total evaluation period, and n = number of observations.

Values of rAUDPC, severity of root symptoms and PSA were analyzed by ANOVA and mean values equally compared among them and with the controls by the Tukey's HSD test for $P < 0.05$. Data were tested for homoscedasticity using the Levene's test and, when heterogeneity was detected squared root (rAUDPC and PSA of madrone) and $(\text{squared root})^{-1}$ (PSA of bay laurels) transformations were applied (Statistix 10.0 software, Analytical Software, Tallahassee, FL, USA).

Results

Both experiments ended when one or more inoculated plants died. Death occurred at different times depending on plant species and experiment (Table 3), so variability among pathogen isolates on each host was considered and analyzed independently. However, comparisons were made among hosts by: a)- comparing foliar symptoms for all species 5 weeks after root inoculations and 3 weeks after stem inoculations, and b)- comparing percentage of stem lesioned at the end of each experiment. Note that length of trials evaluating stem lesions differed among species (see below and Table 3).

Root inoculations

Over the course of the experiment, every inoculated plant developed aerial crown symptoms typically associated with root disease. Control plants growing in uninfested soil reached a slightly severity of crown symptoms possibly associated with watering conditions. Symptoms included foliar yellowing, wilting, and defoliation following a similar pattern across species: in ten weeks average severity values were 3.2 ± 0.1 for Pacific madrone (Fig. 1a) and 2.3 ± 0.2 for Douglas fir (Fig. 1b), while average severity was scored as 3.2 ± 0.2 for Whiteleaf manzanita (Fig. 1d) after only 5 weeks. By contrast, twelve weeks after the first flooding, foliar symptoms were yet to fully develop on California Bay laurels, except for the highly symptomatic seedlings inoculated with isolate Mc12 (Fig. 1c).

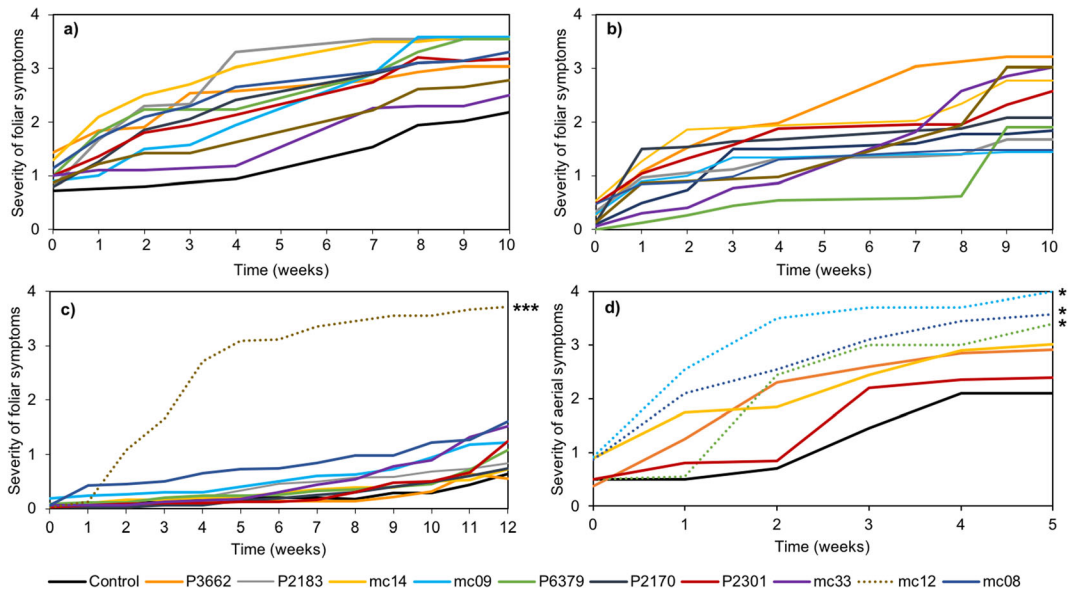

Fig. 1 Average values of foliar symptoms over the experiment for Pacific madrone (**a**), Douglas-fir (**b**), California Bay laurel (**c**) and Whiteleaf manzanita (**d**) recorded by root inoculation. In panel c and d, isolates with dashed lines significantly differ according to the Tukey's HSD test. For California Bay laurel, isolate Mc12 significantly differs from the other genotypes and uninfected control for $P < 0.0001$ (***). Isolate Mc09 infecting Whiteleaf manzanita significantly differs from the isolate P2301 and control for $P < 0.05$ (*)

Five weeks after the first flooding, no significant differences among rAUDPCs of foliar symptoms on Douglas fir and Pacific madrone ($F = 0.65$, $P = 0.7657$ and $F = 1.83$, $P = 0.0862$, respectively) (Table 4) between inoculated and mock inoculated plants, but differences were observed on the other species. *Phytophthora cinnamomi* isolates Mc08 on whiteleaf Manzanita ($F = 6.73$, $P = 0.0004$) and Mc12 on California Bay laurel ($F = 6.69$, $P = 0.0000$) showed rAUDPCs significantly higher than the un-infected control (Table 4).

At the end of the experiment, the severity of root symptoms (root necrosis and lack of feeder roots) for Pacific madrone ($F = 10.56$, $P = 0.0000$), Douglas fir ($F = 15.27$, $P = 0.0000$), Whiteleaf manzanita ($F = 11.89$, $P = 0.0000$) and California Bay laurel ($F = 13.67$, $P = 0.0000$) was significantly higher than that of the controls (Table 4), however statistical differences were not obtained among *P. cinnamomi* isolates for the first three species. Conversely, on California Bay laurel, mean comparisons allowed to separate the isolates in three groups: very virulent (P2170, Mc12 and Mc08), virulent (P3662, P2183, Mc09, P6379, P2301 and Mc33), and moderately virulent (Mc14). *Phytophthora cinnamomi* was always re-isolated from necrotic roots of plants infested with every isolate (ranging 12.2–92.6% of isolation) and never from roots of control plants.

Stem inoculations

Stem inoculations using the pathogen resulted in foliar symptoms analogous to those described above, e.g. wilting, chlorosis, and defoliation. In addition, bark necroses were observed on stems inoculated with *P. cinnamomi*, but not on mock-inoculated controls. Three weeks post inoculation, foliar symptoms became apparent and significant ($F = 3.29$, $P = 0.0013$) on Pacific madrones inoculated with isolates P3662, Mc14, P6379, P2170 and Mc08 (Fig. 2a) and on Douglas firs ($F = 2.34$, $P = 0.0169$) inoculated with isolate Mc12 (Fig. 2a, b; Table 5). However, symptom development on California Bay laurel was marginal (around 0.8 on a 0–4 scale) and insignificant when compared to the controls (Fig. 2c; Table 5).

On Pacific madrones, percentages of stem affected by internal lesion (PSA values) (Table 5), were different between inoculated and mock-inoculated trees, but there were no differences among isolates. On Douglas-firs, *P. cinnamomi* isolates Mc14, P2170, P2301, Mc33 and Mc12 caused a PSA significantly higher than that of the controls ($F = 3.82$, $P = 0.0002$). On California Bay

Table 4 Average severity of foliar symptoms progress curve (rAUDPC) five weeks after the first flooding and average severity of root symptoms per isolate recorded by root inoculation at the end of the experiment for each plant species. For each row, values with different letters differ significantly according to the Tukey's HSD test for $P < 0.05$ (Steel and Torrie 1985). For the last column, values of rAUDPC and severity of root symptoms with different capital and capital-italic letters, respectively, differ significantly according to the Tukey's HSD test for $P < 0.05$. Lower case in bold is used to identify groups across different genotypes of the pathogen, while bold upper case was used to identify different groups among host species

			WW1						WW2		WW3		
		Control	P3662	P2183	P6379	P2170	P2301	Mc33	Mc14	Mc09	Mc12	Mc08	Average
Pacific madrone	rAUDPC[a]	31.8 ± 9.3	42.5 ± 14.8	80.6 ± 16.5	93.8 ± 11.1	80.6 ± 10.1	59.0 ± 14.8	66.9 ± 16.6	52.3 ± 14.6	74.7 ± 21.4	91.8 ± 7.9	71.4 ± 15.4	71.4 ± 5.2 **A**
	Severity of root symptoms	0.8 ± 0.3 **b**	3.4 ± 0.1 **a**	3.5 ± 0.2 **a**	3.5 ± 0.2 **a**	3.5 ± 0.3 **a**	3.1 ± 0.3 **a**	3.5 ± 0.2 **a**	3.5 ± 0.2 **a**	3.5 ± 0.2 **a**	3.3 ± 0.4 **a**	3.3 ± 0.2 **a**	3.4 ± 0.2 ***AB***
Douglas-fir	rAUDPC[a]	40.4 ± 22.9	60.4 ± 21.9	39.7 ± 23.2	13.6 ± 2.6	53.5 ± 22.4	54.0 ± 23.4	25.0 ± 6.1	60.6 ± 21.9	40.5 ± 19.5	32.8 ± 7.5	37.8 ± 23.6	41.8 ± 4.9 **B**
	Severity of root symptoms	1.4 ± 0.2 **b**	3.6 ± 0.2 **a**	3.8 ± 0.1 **a**	3.8 ± 0.1 **a**	3.8 ± 0.1 **a**	3.6 ± 0.2 **a**	3.5 ± 0.4 **a**	3.8 ± 0.1 **a**	3.5 ± 0.1 **a**	3.8 ± 0.1 **a**	3.6 ± 0.2 **a**	3.7 ± 0.1 ***A***
California bay laurel	rAUDPC[a]	3.3 ± 0.4 **b**	1.8 ± 0.6 **b**	4.1 ± 0.3 **b**	4.3 ± 0.5 **b**	1.4 ± 0.8 **b**	2.2 ± 0.9 **b**	2.6 ± 0.9 **b**	3.9 ± 1.2 **b**	7.1 ± 3.2 **b**	35.7 ± 7.9 **a**	12.1 ± 7.8 **b**	7.5 ± 3.3 **C**
	Severity of root symptoms	0.5 ± 0.2 **c**	2.7 ± 0.3 **ab**	3.1 ± 0.3 **ab**	3.2 ± 0.4 **ab**	3.7 ± 0.0 **a**	2.8 ± 0.4 **ab**	3.1 ± 0.2 **ab**	2.4 ± 0.3 **b**	3.5 ± 0.1 **ab**	3.7 ± 0.0 **a**	3.6 ± 0.1 **a**	3.2 ± 0.2 ***B***
Whiteleaf manzanita	rAUDPC[a]	30.2 ± 5.2 **c**		53.2 ± 7.9 **abc**	54.7 ± 2.9 **abc**		38.2 ± 4.2 **bc**		54.6 ± 11.9 **abc**	79.6 ± 1.7 **a**		67.1 ± 4.8 **ab**	57.9 ± 5.7 **AB**
	Severity of root symptoms	1.4 ± 0.3 **b**		3.2 ± 0.3 **a**	3.5 ± 0.1 **a**		2.9 ± 0.3 **a**		3.2 ± 0.3 **a**	3.8 ± 0.1 **a**		3.6 ± 0.1 **a**	3.4 ± 0.2 ***AB***

[a] Severity of foliar symptoms progress curve (rAUDPC) recorded by root inoculations five weeks after the first flooding

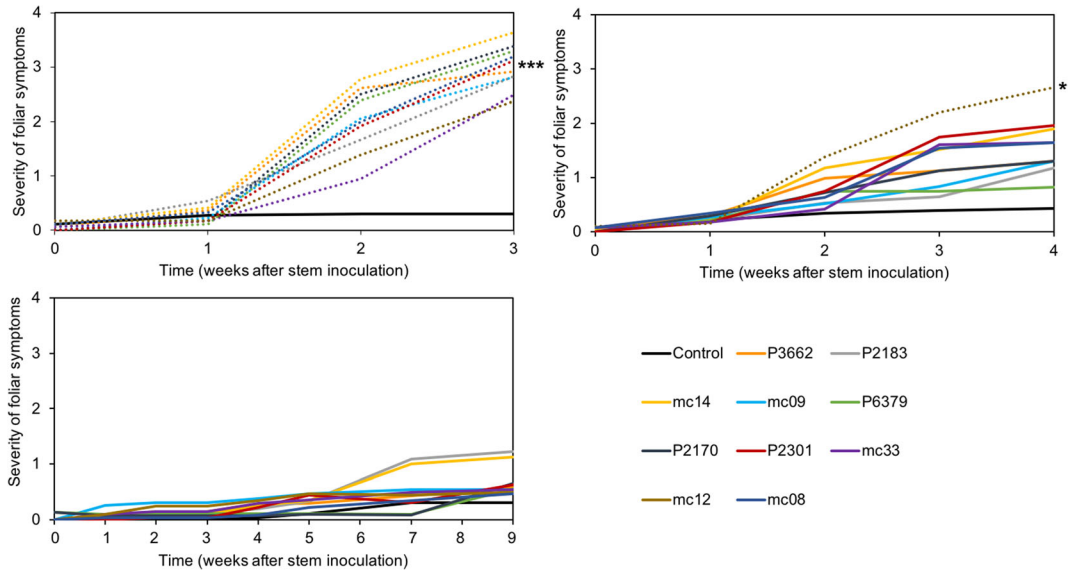

Fig. 2 Average values of foliar symptoms over the experiment for Pacific madrone (**a**), Douglas-fir (**b**) and California Bay laurel (**c**) recorded by stem inoculations. In panels a and b, genotypes with dashed lines significantly differ according to the Tukey's HSD test. For Pacific madrone every genotype significantly differs from the mock-inoculated plants for $P < 0.0001$ (***). For Douglas-fir, genotype Mc12 significantly differs from P6379 and control plants for $P < 0.05$ (*)

laurels, all isolates but P3662 had PSA values different than those caused by the controls, and isolate Mc14 had a PSA value higher than that of other isolates. Interestingly, *P. cinnamomi* isolates, Mc33 (Spearman's correlation of −1.000; $P = 0.0000$) and P2183 (Spearman's correlation of −0.9487; $P = 0.0167$) showed a negative correlation between the severity of root symptoms and PSA on California Bay laurel and Douglas-Firs, respectively. Both genotypes in fact displayed low virulence on stems and high virulence on roots. Conversely, isolate P6379 showed a positive correlation (Spearman's correlation of 1.0000; $P = 0.0000$) between symptoms caused on roots and stems of California Bay laurels. No correlations could be established for the other isolates and plant species.

All *P. cinnamomi* isolates were able to girdle seedlings of each host species, with the following exceptions: P3662, P2183, Mc33 and Mc08 on California Bay laurel and Mc12 on Pacific madrone. Finally, significant mortality was recorded for Pacific madrone, while percentages of mortality of Douglas fir and California Bay laurel were very low (Table 5).

When comparing STRUCTURE groups, mating types, and provenances of *P. cinnamomi* (Table 2) for pathogenicity on either roots or stems, significant differences were found only for California Bay laurel root and stem inoculation and for Douglas fir stem inoculations. The severity of root symptoms developed by isolates belonging to WW3 on California Bay laurel was significantly higher than those caused by WW2, although group WW2 reached a PSA significantly higher than the other two STRUCTURE groups. No differences were observed among Structure groups on Douglas fir, Whiteleaf manzanita and Pacific madrone. Moreover, statistical differences were obtained between the two isolates representing an identical multi locus genotype within STRUCTURE group WW2. Isolates Mc14 caused a severity of root symptoms significantly higher than Mc09 on Douglas firs, while the results were opposite for California Bay laurels. When analyzing results by mating type, isolates with mating type A2 caused PSA values significantly higher on inoculated Douglas-firs than isolates with mating type A1.

Phytophthora cinnamomi was re-isolated with varying success from inoculated madrones (31.5–88.9%), Douglas firs (38.3–55%) and bay laurels (1.7–28.3%). *Phytophthora cinnamomi* was never re-isolated from the stems of control plants.

Discussion

The genotypes of *P. cinnamomi* studied in this work included 7 isolates from California, 2 from Papua New

Table 5 Average severity of foliar symptoms progress curve (rAUDPC) three weeks after the stem inoculation, average percentage of stem affected (PSA) by internal lesion, average percentage of plant girdled and mortality per isolate at the end of the experiment for each plant species. For each row, values with different letters differ significantly according to the Tukey's HSD test for $P < 0.05$ (Steel and Torrie 1985). For the last column, values of rAUDPC and PSA with different capital and capital-italic letters, respectively, differ significantly according to the Tukey's HSD test for $P < 0.05$. Lower case in bold is used to identify groups across different genotypes of the pathogen, while bold upper case was used to identify different groups among host species

			WW1						WW2		WW3		
		Control	P3662	P2183	P6379	P2170	P2301	Mc33	Mc14	Mc09	Mc12	Mc08	Average
Pacific Madrone	rAUDPC [a]	6.5 ± 1.9 **b**	37.2 ± 7.1 **a**	30.3 ± 7.9 **ab**	34.5 ± 5.3 **a**	36.3 ± 4.3 **a**	30.7 ± 5.1 **ab**	19.8 ± 4.3 **ab**	42.2 ± 4.5 **a**	30.7 ± 5.1 **ab**	23.6 ± 6.5 **ab**	33.0 ± 4.1 **a**	31.8 ± 5.5 **A**
	PSA	3.9 ± 0.1 **b**	87.7 ± 0.3 **a**	68.6 ± 0.7 **a**	92.2 ± 0.2 **a**	89.3 ± 0.2 **a**	79.9 ± 0.3 **a**	76.0 ± 0.4 **a**	91.1 ± 0.3 **a**	85.6 ± 0.3 **a**	66.9 ± 1.1 **a**	80.6 ± 0.4 **a**	81.8 ± 0.5 ***A***
	% girdling [b]	0	33.3	33.3	22.2	11.1	22.2	11.1	22.2	11.1	0*	11.1	17.5 [d]
	% mortality [c]	0.0	44.4	22.2	44.4	44.4*	33.3	11.1	55.6*	33.3	33.3	44.4	36.6 [e]
Douglas-fir	rAUDPC [a]	6.5 ± 1.8 **b**	15.7 ± 4.9 **ab**	8.9 ± 1.5 **ab**	10.9 ± 2.6 **ab**	13.1 ± 2.1 **ab**	15.6 ± 3.2 **ab**	11.9 ± 2.2 **ab**	18.2 ± 3.0 **ab**	10.1 ± 1.2 **ab**	22.3 ± 3.8 **a**	14.5 ± 3.6 **ab**	14.1 ± 2.8 **B**
	PSA	0.6 ± 0.1 **b**	18.6 ± 9.3 **ab**	10.5 ± 2.3 **ab**	10.9 ± 1.8 **ab**	21.5 ± 2.6 **a**	27.4 ± 4.9 **a**	21.9 ± 3.5 **a**	26.0 ± 4.7 **a**	16.4 ± 2.5 **ab**	27.6 ± 3.3 **a**	17.7 ± 4.6 **ab**	19.8 ± 3.9 ***B***
	% girdling [b]	0.0	10.0	10.0	10.0	10.0	50.0	50.0	30.0	30.0	70.0*	30.0	30.0 [d]
	% mortality [c]	0.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0 [e]
California bay laurel	rAUDPC [a]	0.0 ± 0.0	2.4 ± 1.5	2.0 ± 1.3	5.2 ± 1.9	2.8 ± 1.4	0.4 ± 0.4	2.7 ± 1.5	1.6 ± 1.4	6.0 ± 2.1	3.9 ± 1.4	0.6 ± 0.6	2.8 ± 1.4 **C**
	PSA	1.9 ± 0.0 **a**	3.8 ± 0.0 **ab**	25.7 ± 0.1 **bc**	8.7 ± 0.1 **bc**	7.4 ± 0.1 **bc**	14.9 ± 0.1 **bc**	8.7 ± 0.1 **bc**	35.7 ± 0.1 **c**	13.4 ± 0.1 **bc**	11.5 ± 0.1 **bc**	8.5 ± 0.1 **bc**	13.8 ± 0.1 ***C***
	% girdling [b]	0.0	0.0	0.0	10.0	10.0	20.0	0.0	20.0	10.0	10.0	0.0	8.0 [d]
	% mortality [c]	0.0	0.0	20.0	0.0	0.0	0.0	0.0	20.0	0.0	0.0	0.0	4.0 [e]

[a] Severity of foliar symptoms progress curve (rAUDPC) recorded by stem inoculations three weeks after the inoculation

[b] Based on Pearson's chi-squared test at $P = 0.01$ and excluding mock inoculations, average percentages of girdling were different across isolates for Pacific madrone and Douglas-firs, but not for California bay laurels (Chi-square = 17.53, $P = 0.0002$ for isolate Mc12). Asterisks indicate values significantly different according to the multiple comparison for proportions test

[c] Based on Pearson's chi-squared test at P = 0.01, average percentages of mortality was different across isolates, and excluding mock inoculations, for Pacific madrones only (Chi-square = 9.97, $P = 0.0068$ for isolate P2170 and Chi-square = 8.13, $P = 0.0172$ for isolate Mc14). Asterisks indicate values significantly different according to the multiple comparison for proportions test

[d] Based on Fisher's exact values (P = 0.01), average girdled values were significantly different between Pacific madrones and California Bay Laurels (F = 9.80, $P = 0.0020$) and between California Bay Laurels and Douglas-firs (F = 26.09, $P = 0.0000$), but not between Douglas firs and Pacific madrones

[e] Based on Fisher's exact values (P = 0.01), average mortality values were significantly different between Pacific madrones and California Bay Laurels (F = 26.58, $P = 0.0000$) and between Pacific madrones and Douglas-firs (F = 51.72, $P = 0.0000$), but not between Douglas firs and California bay laurels

Guinea and 1 from Taiwan belonging to three clearly distinct STRUCTURE groups previously described based on an SSR analysis (Serrano et al. 2019). Together, these 10 genotypes can be considered representative of the deep worldwide genetic variability within the species. Although genetic variation was high among the isolates employed, differences in pathogenicity were recorded at the STRUCTURE group level only for California Bay laurel in the two tests performed. The response of California Bay laurel was opposite depending on whether trees were inoculated with Group WW2 or Group WW3 isolates and depending on the inoculation method. On this species, isolates of Group WW2 are better adapted as a stem rather than a root pathogen, while the best performers on roots were isolates from Group WW3. Additionally, differences were detected at the individual genotype level for MG08 (isolates Mc14 and Mc09) but only in root inoculation tests on California Bay laurel and Douglas fir.

Correlation between *P. cinnamomi* genetic variability and pathogenicity

Lack of differences in pathogenicity has been previously reported for *Eucalyptus* spp., but results have been biased by the use of only three Australian isolates (Dudzinski et al. 1993). The two genetic clades identified within the Iberian Peninsula populations were also reported not to differ in pathogenicity on *Quercus* spp. (Caetano et al. 2009). However, in agreement with our work, several studies reported significant variability among South African genotypes (Linde et al. 1999b); on holm and cork oak in Europe (Robin et al. 1998); and on *Eucalyptus marginata* and *Corymbia calophylla* in Australia (Hüberli et al. 2001). Differences among studies are to be expected when a pathogen species, or subpopulations within it such as lineages or isolated demes, comprise genotypes with highly variable phenotypes as discussed specifically for Phytophthoras in Eyre et al. (2014). Such variability may depend either on different genetic make-up (Eyre et al. 2014) or on genomic level factors affecting either gene regulation or genome structure (Kasuga et al. 2012, 2016). Nonetheless, independent of the mechanisms underlying the high phenotypic variability among genotypes or even among isolates belonging to the same genotype, different outcomes may be expected when disease is initiated by different genotypes of *P. cinnamomi*. Hence, lack of variability reported when looking at pooled results of individuals belonging to different evolutionary lineages, mating types, provenances, or affecting different hosts, may be erroneously interpreted as lack of variation within the species, unless data for individual genotypes are analyzed. Additionally, genotypes may differ in their ability to cause disease on different plant parts, so a complete evaluation should include tests performed both on roots and aerial portions of plants. Finally, when hosts are highly susceptible to a pathogen or a pest, their high susceptibility may mask possible variation in virulence among different genotypes of the disease causal agent.

Our study attempted to take into account all of the aspects above. Namely, it investigated variation in individual virulence among a subset of isolates representative of: a) the deep genetic variability among lineages of *P. cinnamomi*; b) the two different mating types; and c) a variety of hosts and provenances. Virulence was studied both on roots and stems of four important natural California hosts for this pathogen. By selecting an experimental length common to all species tested, we were able to compare the relative susceptibility among hosts; in addition, the length of time required for the development of symptoms and/or to reach death can also be used for comparative purposes. Even if the four different species were not inoculated concomitantly, they were inoculated within a short time frame. Additionally, the number of disease metrics measured (including speed at which symptoms appeared, the overall severity of foliar symptoms and of root necrosis, the size of stem lesions, and whether mortality occurred or not), as well as the number of pathogen genotypes used (10 in total), allowed us to safely rank the susceptibility of the four hosts. Based on the time until onset of symptoms (Figs. 1 and 2) and on results summarized in Tables 4 and 5, Pacific madrones were extremely susceptible, closely followed by whiteleaf Manzanita, with Douglas firs and California Bay laurels being a distant third and fourth in susceptibility, respectively. Our data suggest that when susceptibility is high (such as in the first two hosts), both roots and stems will be highly susceptible and differences among virulence of genotypes may be hard to detect with the methods currently employed. These methods have been and are currently being used routinely to test pathogenic variability (Dudzinski et al. 1993; Hüberli et al. 2001), and to evaluate susceptibility level of woody hosts (Robin and Desprez-Loustau 1998; Garbelotto et al. 2006; Elliott et al. 2010; Serrano et al. 2012b; McConnell and Balci 2014). Based on the results presented here, it is evident that additional methods must

be included when performing within-species comparison among genotypes of the pathogen if host species are very susceptible to a pathogen (Linde et al. 1999b). Our presumption was that better information on intraspecific variability can be gathered when testing hosts that are not highly susceptible to the pathogen. This hypothesis was proven to be correct, as both California Bay laurel and Douglas fir were only moderately susceptible to *P. cinnamomi,* and data obtained from inoculating them allowed to better identify differences among genotypes of the pathogen, as well as among STRUCTURE groups. Significant variation obtained in controlled experiments, even if small, often highlights very significant differences in disease etiology, progression, severity and incidence (Brasier 1978): thus, introduction of isolates proven to differ phenotypically in laboratory tests may have a very different outcome on ecosystem health or on productivity of commercially-run businesses.

Intraspecific variability in pathogenicity

While there was no generalized correlation between virulence of genotypes on both roots and stems, there was some evidence from two genotypes (Mc33 and P2183) of adaptation as root pathogens, meaning that their virulence on stems was negatively correlated to their virulence on roots. One genotype instead (P6379), showed the opposite trend, suggesting it is better adapted as a pathogen of stems. This has important implications for diseases progression: a) with genotypes adapted to infect roots one expects disease to progress mostly underground, with typical soilborne transmission pathway through contaminated soil and water, and with aerial symptoms being the consequence of a progressively smaller functional rhizosphere; b) with genotypes better adapted to infect stems, disease will be determined by the rate at which the stem is girdled, direct symptoms of infection such as gummosis may be visible above ground (EPPO/CABI 2011), and a splash transmission mode (Ristaino and Gumpertz 2000) may play a relevant role in the overall epidemiology of the disease; and c) co-infection by a root adapted and a stem adapted genotype could potentially greatly hasten disease severity as the two genotypes will not be competing for the same physical space and resource but may be simultaneously affecting two different parts of the tree. When looking at the progression of foliar symptoms on Douglas-fir, the genotypes resulting in fastest and highest disease severity were Mc12 and P3662, in root and stem inoculations, respectively. This result suggests that, depending on the plant part infected, disease progression will differ depending on the isolate that is introduced. Furthermore, the discovery that individual genotypes of *P. cinnamomi* differ in virulence on some hosts have some additional practical implications. For instance, the genotype Mc12 belonging to Structure group WW2 was more aggressive on Douglas-fir, however such a genotype is still absent from the Coastal region of California, where this host is extremely abundant. The movement of this genotype to the California coastal region, thus, could lead to a new disease outbreak on Douglas-fir.

Overall, our results show that some species act more as root-hosts (California Bay laurel), while others (whiteleaf Manzanita) act as general hosts being susceptible both below and above ground. This has been previously shown to be true for other hosts affected by *Phytophthora* species. For instance, *Phytophthora psychrophilla* and *P. quercina*, two soilborne pathogens never isolated from bark tissues, shown to be pathogenic on roots on Mediterranean oaks by root inoculation, but not on cut stems (Seddaiu et al. 2014). The conclusion that some genotypes may lead to drastically different rate and severity of disease on different parts is also supported by results of inoculations on bay laurels and Manzanita. On bay laurel, genotype Mc12 caused the greatest amount of disease when inoculated in the roots, and very rapidly so. Mc12 was also part of a group of three isolates that colonized roots more effectively. When analyzing results of stem inoculations on bay laurels, instead, isolate Mc14 was the most aggressive. Once again, not only do different genotypes display different levels of virulence, but, additionally, the best performing genotypes on roots may be different from those on stems. Genotype Mc09, ranking among the best colonizers of Manzanita root systems, caused significantly higher levels of foliar symptoms on that host than any of other genotype, providing additional data in support of intraspecific variation in virulence among isolates of *P. cinnamomi.*

Results obtained with isolates Mc09 and Mc14, both from WW2, showed some possible signs of host specificity, suggesting some isolates may be better pathogens on the host they were originally isolated from. Although Mc09 and Mc14 belonged to the same multilocus genotype (Serrano et al. 2019), they caused a different response on root-inoculated California Bay laurel and Douglas fir. Mc14, isolated from Douglas fir, caused a severity of root symptoms significantly higher than isolate Mc09 in Douglas fir. Response was the opposite when using the same two isolates on California Bay

laurel. Further studies are needed to confirm the presence of host specificity but, although results presented here are preliminary, if confirmed, they would provide evidence of a significant *pathogen genotype x host* interaction, with some genotypes being more aggressive than other genotypes, but only on specific hosts.

Differential pathogenicity of STRUCTURE groups in California

According to our findings, four isolates out of 10 showed the potential to cause higher levels of disease. Two of these isolates belonged to STRUCTURE group WW2 (Serrano et al. 2019): this is a genetically distinct group only recently identified (Serrano et al. 2019), and known to be associated with three dramatic and recent disease outbreaks, namely, the massive oak mortality in the Mexican State of Colima (Tainter et al. 2000), the outbreaks of *P. cinnamomi* in California Christmas tree farms (personal communication G. Browne, U.C Davis, Garbelotto et al. 2018), and the massive mortality of Manzanita documented in the foothills of the Sierra Nevada in California (Swiecki et al. 2003). According to Serrano et al. (2019), the introduction of Group WW2 in California may have been through infected Christmas trees or Christmas tree stock from Mexico. In California, Group WW2 is represented by a single genotype with geographically limited distribution in two adjacent counties; in contrast, Group WW2 is extremely widespread and genetically highly diversified in Mexico. Although there is no available information on the worldwide distribution of Group WW2, it appears plausible that the novelty and the high severity of the three disease outbreaks listed above may be associated with the recent emergence and spread of isolates belonging to this new group. The minimum spanning network suggests Group WW2 may have emerged through mutation of an isolate belonging to Group WW1. The emergence of Group WW2 may have occurred in Mexico or in a source area not yet sampled or genetically analyzed, followed by transport of one or more Group WW2 genotypes into Mexico.

Mc12 (Group WW3) was particularly aggressive on roots of bay laurels and on stems of Douglas fir. Interestingly, this genotype belonging to Group WW3 (Serrano et al. 2019) was originally isolated from Norman fir (*Abies nordmanniana*) in a California Christmas tree farm. The association of an aggressive genotype with a Christmas tree production facility is worrisome because it may imply that pathogen genotypes may be selected for increased virulence in these artificial settings. It may also indicate that in these production facilities there may be an ongoing adaptation of *P. cinnamomi* toward becoming a better aerial pathogen (Sims et al. 2018; Hunter et al. 2018).

Finally, P2183 (Group WW1) showed high virulence on roots of Douglas fir. P2183 is an A1 mating type genotype isolated in Papua New Guinea, a region considered to lie within the native range of the pathogen (Arentz and Simpson 1986). An introduction of such a genotype in California wild lands could cause significant new disease outbreaks. Currently, Douglas firs are known to be infected by *P. cinnamomi* in plant production facilities, mostly Christmas tree farms, but this host is not known to be significantly affected by this pathogen in the wild. If an isolate similar to P2183 were to escape from plant production facilities into forests, it is possible that infection and disease of Douglas firs in natural settings may follow. This last point highlights again how multiple introductions of different genotypes of the same pathogen may result in clearly different disease outbreaks and should be avoided.

Our experiment also shows that if *P. cinnamomi* or new genotypes of *P. cinnamomi* were to be introduced in a novel ecosystem or on a novel host, some hosts although infected may not show symptoms for a relatively long time. In fact, aerial symptoms recorded in both inoculation experiments were not correlated with the severity of root symptoms or with the percentage of stem affected by internal lesion, regardless of the plant species tested. This result was especially significant for California Bay laurel, a species that showed very low foliar symptoms even when most of the feeder roots were necrotic or had disappeared. Our results are in good agreement with many studies of the pathogenicity of *P. cinnamomi* on *Quercus* spp. (*Q. ilex*, *Q. suber*, *Q. rubra* or *Q. alba*), in which no evidence of a correlation between foliar and root/stem symptoms could be found (Robin et al. 1998; Serrano et al. 2012a; McConnell and Balci 2014).

Conclusion

In conclusion, the pathogenicity of *P. cinnamomi* varies mostly at the isolate level, regardless of its host and genetic make-up, however some notable differences were also found when looking at results by mating type and STRUCTURE group. It has been suggested that history of an isolate may affect its phenotype by causing chromosomal copy number and other genomic structural variations

(Kasuga et al. 2012, 2016). Because genotypes within a genetic subgroup, such as a clade or a deme, may undergo different spread histories and encounter different hosts and environments, phenotype may thus be highly isolate-, rather than being clade- or deme- dependent.

Here, we have shown that in order to better describe the potential phenotypic variation at the intraspecific level of a pathogen, experiments need to include hosts with moderate susceptibility. Our results using hosts with moderate susceptibility show that genotypes differ in their overall virulence, in their affinity to hosts and to different parts within hosts. For this reason, the introduction of multiple genotypes of the same pathogen can cause marked shifts in the intensity and type of disease outbreak, even when other genotypes of the same pathogen are already present. Our work additionally shows that two isolates, both belonging to the emergent and newly discovered genetic Group WW2, are particularly aggressive. The high virulence of these two genotypes may thus explain at least three new types of severe outbreaks both in Mexico and California. Two additional genotypes were strikingly aggressive on at least two native California tree species, namely Mc12 and P2183: one such genotype is already present in California tree farms, while the other comes from the genetically diverse population of Papua New Guinea, assumed to be part of the native range of this pathogen. This result highlights that the release in nature of strains established in agriculture and the introduction of new strains from elsewhere may both have severe outcomes on the health and even on the survival of natural populations of important California tree species. In particular, we have shown here that Pacific madrone and Manzanita are extremely susceptible to this pathogen, and thus likely to be severely impacted by its release in nature, even in the short time. The results presented here are preliminary, however if confirmed, they would suggest that invasive genotypes of *P. cinnamomi* may require regulatory action to prevent their further spread.

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