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SHORT COMMUNICATION

Testing the pathogenicity of three *Phytophthora* species on California hosts commonly used in restoration

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Abstract

In California, soilborne *Phytophthora* species have been increasingly reported from diseased plants in nurseries and in failing restoration sites. To determine the role these pathogens may play in failing restorations and to better limit their introduction, the pathogenicity of *Phytophthora* species newly isolated from plant species used in restorations needs to be demonstrated. However, there is no consensus on the best and most cost-effective approach to confirm pathogenicity of *Phytophthora* species on putative hosts. The first objective of this study was to compare the efficacy of stem vs. root inoculations to test the pathogenicity of soilborne *Phytophthoras*, and to do so, we compared mortality and disease symptoms of *Diplacus aurantiacus* plants inoculated with *Phytophthora megasperma* and *P. crassamura* through root and stem techniques. The second objective was to assess the pathogenicity of *P. crassamura* and *P. megasperma* on *D. aurantiacus*, of *Phytophthora multivora* and *P. megasperma* on *Frangula californica* and of *P. multivora* on *Ceanothus thyrsi*florus. Root inoculations resulted in higher pathogen re-isolation and in greater leaf necrosis and mortality for all pathogen and host combinations. Considering both root and stem inoculations, Koch's postulates were completed for all pathogen and host combinations, except for *Frangula californica* inoculated with *P. megasperma*.

Keywords *Phytophthora crassamura* · *Phytophthora megasperma* · *Phytophthora multivora* · *Diplacus aurantiacus* · *Frangula californica* · *Ceanothus thyrsi*florus

In California, and for the past decade, soilborne *Phytophthora* species have been increasingly reported and isolated from diseased plants, both in nurseries providing plant stock for restoration and in failing restoration sites (Garbelotto et al. 2018; Rooney-Latham et al. 2019; Frankel et al. 2020). The introduction of *Phytophthora* spp. in restoration sites can happen either directly through the planting of infected plants (Jung et al. 2016; Sims and Garbelotto 2021), and, indirectly, through pathogen dispersion from infected sites (Sims and Garbelotto 2021). The presence of *Phytophthora* species in unrestored sites near restorations, and their absence in ecologically similar but spatially disjunct locations, suggest these pathogens are moving outside restoration sites following trails of animal, water and soil movement (Malewski et al. 2019; Sims and Garbelotto 2021) further threatening endangered plant species and natural ecosystems (Frankel et al. 2020).

Ideally, all *Phytophthora* species should be avoided in plant production facilities (Sims et al. 2019). However, when this is not possible, it is crucial for nurseries that supply plant stocks for restoration purposes to adopt best management practices and monitoring programs that help minimize the presence of *Phytophthora* species (Sims et al. 2019; Swiecki et al. 2021). Assessing the success of a pathogen mitigation program requires a knowledge of which *Phytophthoras* may be pathogenic to which plants species used in restoration efforts. Whenever possible, a formal verification of the pathogenicity of each *Phytophthora* species on novel hosts needs to be completed, so that appropriate pathogen surveys and regulations may be put in place.

Stem and/or root inoculations are the two most frequently used approaches to confirm pathogenicity of soilborne *Phytophthora* species (Aghighi et al. 2016; Jung et

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al. 2017). There is no clear consensus on which of the two may be better, and it may be that no single method works for all *Phytophthora* and host species combinations, given the complex nature of *Phytophthora*-induced disease (Weiland et al. 2010; Chandelier et al. 2016). Although wounded-stem inoculations have been successfully used to test the pathogenicity of some *Phytophthora* species and host combinations (Denman and Sadie 2001; Chandelier et al. 2016; Bregant et al. 2020), it is unclear whether this approach may work for all combinations. It is therefore important to test the pathogenicity by using both root and stem inoculation methods.

The bush monkeyflower (*Diplacus aurantiacus* Curtis), the blue-blossom (*Ceanothus thyrsiflorus repens* Eschw.) and the California coffeeberry (*Frangula californica* (Eschsch.) A. Gray) are Californian native species commonly used in restoration projects (Cione et al. 2002; Morandin and Kremen 2013; Hallett et al. 2017). In recent disease surveys in California plant nurseries, *D. aurantiacus* emerged as one of the species with higher *Phytophthora* incidence, closely followed by *F. californica* and by *C. thyrsiflorus* (Sims et al. 2019; Rooney-Latham et al. 2019). These three plant species have been identified as potential hosts for several *Phytophthora* species, and new potential plant-pathogen combinations identified included the following: *D. aurantiacus* infected by *P. megasperma* or by *P. crassamura*, *F. californica* infected by *P. megasperma* or by *P. multivora*, and *C. thyrsiflorus* infected by *P. multivora* (Rooney-Latham et al. 2019; Frankel et al. 2020; Sims and Garbelotto 2021). To date, a rigorous verification of the pathogenicity of the *Phytophthora* species listed above on these three host species has still not been completed.

The objectives of the present study were (i) to compare the efficacy of stem vs. root inoculations to test the pathogenicity of soilborne *Phytophthoras* by inoculating either the roots or the stems of *D. auranticus* with *P. megasperma* and *P. crassamura*, and (ii) to assess the pathogenicity of *P. crassamura*, *P. megasperma* and *P. multivora* on *D. aurantiacus, C. thyrsiflorus* and *F. californica* through either stem, root inoculations or both.

During the entire duration of the experiment, potted saplings of *D. aurantiacus, C. thyrsiflorus* and *F. californica* were maintained under well-watered conditions in a room at 24-25°C and 40–50% relative humidity. Stem inoculations were performed on *D. aurantiacus* and *F. californica* saplings using isolates of *P. megasperma, P. crassamura*, and *P. multivora* (Table 1). Stem inoculations were done by placing a 3-mm-diameter V8A disc taken from the margin of a 5-day old culture, onto a stem pricked ten times with the tip of a needle, with the mycelium side to the stem, 3 to 5 cm above the soil line (adapted from Sims and Garbelotto 2021). Negative controls consisted of the same procedure

Table 1 Mean values of disease severity metrics assessed in this experiment for all *Phytophthora* and host species combinations in control and inoculated treatments. Superscript annotation next to the numbers indicates significance level: *** is *p-value* < *0.001*, ** is *p-value* < 0.01, * is *p-value* < 0.05 and n/a is not observed

		n		Leaf necrosis			Root Damage		Mortality (%)		
Plant species	***Phytophthora* species**	**Stem**	**Root**	**Stem**	**Root**	**Control**	**Root**	**Control**	**Stem**	**Root**	**Control**
Diplacus aurantiacus	*Phytophthora megasperma*	8	8	1	9 **	1 in	1	1	0	100 ***	0
	Phytophthora crassamura	8	8	3.5	9 **	stem-inoculated 2.25 in root -inoculated	1	1	0	100 ***	0
Ceanothus thyrsiflorus	*Phytophthora multivora*	n/a	12	n/a	7.2 *	3.7	2.17 *	1	n/a	17	0
Frangula californica	*Phytophthora multivora*	12	n/a	2 *	n/a	0.75	n/a	n/a	25	n/a	0
	Phytophthora megasperma	12	n/a	2	n/a	0.75	n/a	n/a	0	n/a	0

but using a non-colonized V8A disc. The inoculated area was wrapped using Parafilm ® M sealing film and aluminum foil for 21 days.

Root inoculations were performed on *D. aurantiacus* and *C. thyrsi*flo*rus* saplings (Table 1) using a method adapted from Sims and Garbelotto (2018). A zoospore suspension was produced for each isolate using 5-mm-diameter plugs taken from the margin of a 5- to 7-day old cultures. A total of 9 plugs were put in 90-mm-diameter Petri plates with a mix of 1V8:4 soil tea for 24 h, after which the plugs were rinsed with distilled water and left on soil tea for the next 48 h. The plates were then put on a mix of water and ice for 1 h and left at room temperature while monitoring for zoospore release. Saplings of *D. aurantiacus* received a 50 mL zoospore suspension containing an estimated concentration of 15×10^6 /mL and 25×10^4 /mL zoospores for *P. megasperma* and *P. crassamura*, respectively, poured over a previously cut slit near the root collar. Saplings of *C. thyrsi*flo*rus* received a 60 mL suspension of *P. multivora* with an estimated zoospore concentration of 8×10^6 /mL. Negative controls were submitted to the same procedure, but using a soil tea suspension with no zoospores. Saplings were kept under waterlogging conditions for 48 h after inoculation and in well-watered conditions thereafter, until the end of the experiment at day 21 after inoculation. Each pathogen isolate used in stem and/or root inoculations had been obtained in California from the host on which it was tested (Sims et al. 2019; Sims and Garbelotto 2021).

Re-isolation was performed on inoculated and control saplings by plating the following two types of plant tissue onto selective medium V8-PARPH agar: (i) inner bark tissue from the outer edge of the stem necrosis or near the pin pricks on the stem if no necrosis was present, or (ii) necrotic roots from saplings whose roots were inoculated. Any *Phytophthora* spp colony observed in the selective medium was transferred to V8A, and species were confirmed by visual inspection of colony morphology.

Mortality and disease severity were evaluated on all saplings by tallying the number of dead plants and the percentage of necrotic leaves at 10, 14, 18 and 21 days post inoculation for stem-inoculated saplings, and at 8, 10 and 14 days post inoculation for root-inoculated saplings. The percentage of necrotic leaves was visually assessed and each plant was assigned a score between 0 (0% of necrotic leaves, when all leaves were healthy) and 13 (100% of necrotic leaves, when the plant was dead). Root damage scores between 1 (healthy roots) and 6 (complete root system rotten away) were assigned to all root-inoculated saplings at the end of the experiment (Sims and Garbelotto 2018). Necrotic area was calculated at the end of the experiment for all stem-inoculated saplings by measuring necrosis length and width starting from the inoculation point. The outer bark of each sapling was lightly scrapped to properly observe lesion size in the inner bark.

Koch's postulates were validated by applying a Chi-square (χ^2) test for every plant-pathogen combination. Mortality percentages were calculated using the Kaplan–Meier estimate, and the significance of the difference between controls and the inoculated treatments was examined using the log-rank test in the *survival* package (Thearneau and Grambsch 2000; Therneau 2021). Necrotic leaves score differences between saplings under control and inoculated treatments at each observation day were tested using *t*-test for all species and inoculation types. When data did not follow the normality assumption, a Mann-Whitney test was applied instead. Dunnett's test with a confidence interval of 95% was used to test for differences in mean values of necrosis area between *Phytophthora* species for each plant species and the control treatment. All statistical analyses were performed in R (v. 3.5.1; R Core Team, 2018).

Pathogen re-isolation (Fig. 1) was successful for all *D. aurantiacus* root-inoculated saplings (100% of 16 inoculated saplings, $p < 0.001$), for 67% of the *F. californica* saplings stem-inoculated with *P. multivora* (7 of 12 inoculated saplings, $p < 0.1$) and for 58% of the *C. thyrsi*flo*rus* saplings root-inoculated with *P. multivora* (8 of 12 inoculated saplings, $p < 0.1$). Pathogen re-isolation was successful in 42% of the *F. californica* saplings stem-inoculated with *P. megasperma* (5 of 12 inoculated saplings) and in 25% of *D. aurantiacus* saplings stem-inoculated with *P. crassamura* (2 of 8 inoculated saplings). Although these percentages were not statistically different from zero, *P. megasperma* was successfully reisolated from 5 *F. californica* saplings (Fig. 1). No *Phytophthora* was re-isolated from any of the mock-inoculated saplings, either stem- or root-inoculated.

Disease severity was similar for *D. aurantiacus* saplings root-inoculated with either *P. megasperma* or *P. crassamura*: mortality percentage was 100% and the average leaf necrosis score was 9 out of 13 (Table 1; Fig. 2a, b). At 8 days post root-inoculation, all inoculated *D. aurantiacus* saplings had symptoms of decline and all were dead by the 12th day ($p < 0.001$, Fig. 2a). On the contrary, saplings of *D. aurantiacus* that were stem-inoculated started showing leaf necrosis at the 18th day post inoculation but no stem-inoculated sapling died or showed stem lesions regardless of which *Phytophthora* spp. it was inoculated with (Table 1).

Declining symptoms for stem-inoculated *F. californica* saplings started 14 days after inoculation and were more pronounced on saplings inoculated with *P. multivora* ($p < 0.05$, Fig. 3a). A total of 25% of *F. californica* saplings inoculated with *P. multivora* died 18 days after stem inoculation but no saplings died from inoculation with *P. megasperma* (Fig. 3a). Stem necrosis was observed in 42% of the *F. californica* saplings inoculated with *P. multivora*

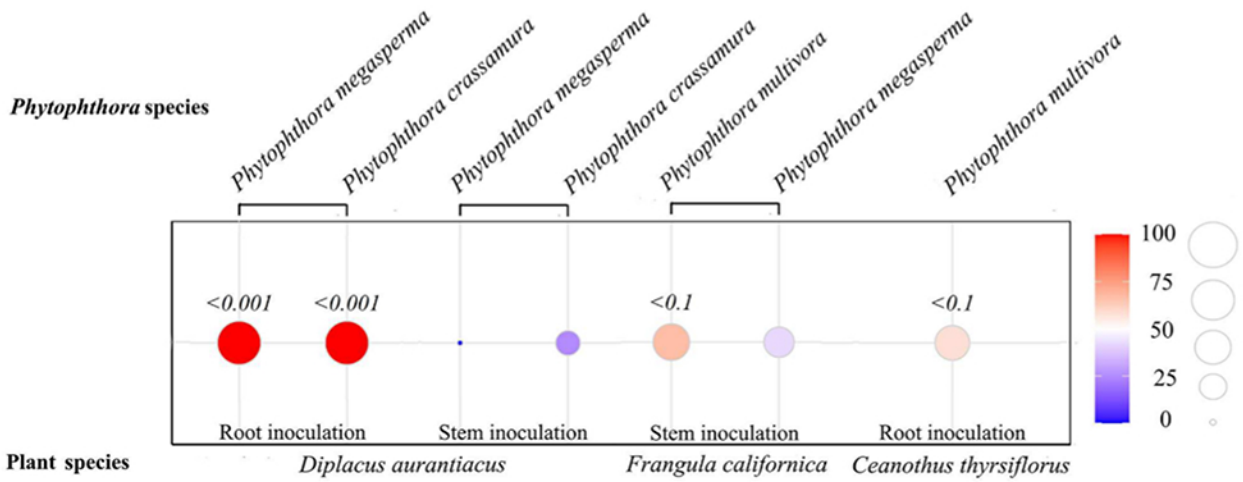

Fig. 1 Percentage of re-isolation for all *Phytophthora* and host species combinations. Annotations next to each circle indicates significance levels

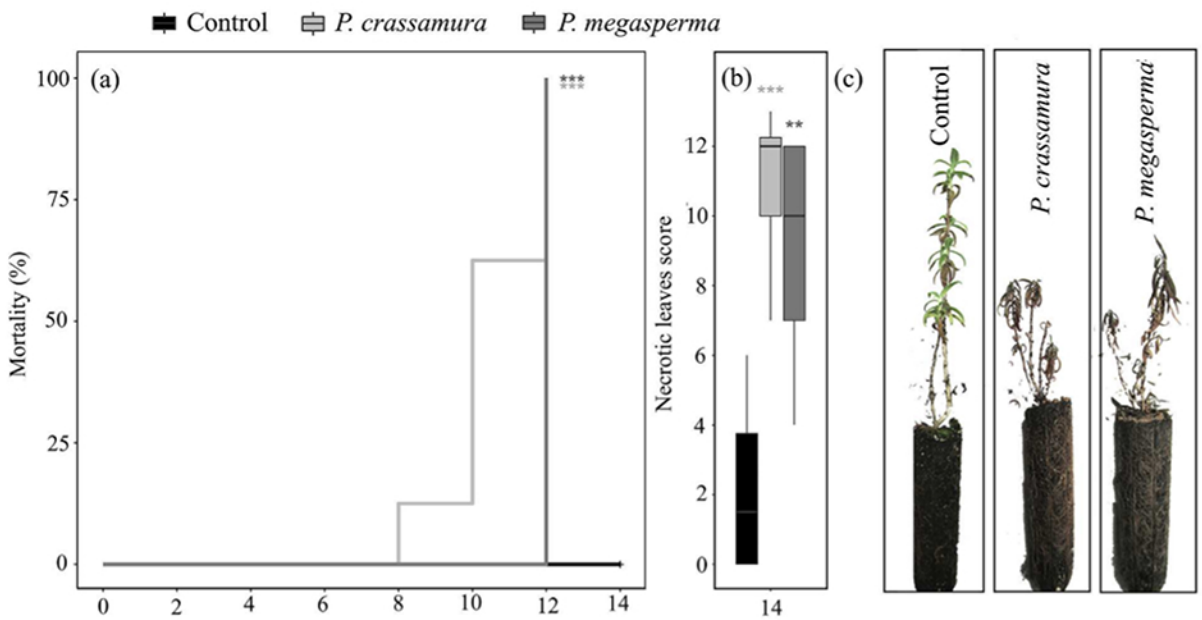

Fig. 2 Mortality percentage (a) and necrotic leaves scores (b) for *Diplacus aurantiacus* root-inoculated with *Phytophthora crassamura* and *P. megasperma* (colored in light grey and dark grey, respectively) in days after inoculation. Annotations indicate significance levels: *** is *p-value* < 0.001 and ** is *p-value* < 0.01. Photographs of saplings (c) were taken fourteen days post inoculation

and 25% of saplings inoculated with *P. megasperma*, and mean stem necrosis areas were of 90.34 mm^2 (±86.20) and 50.19 mm^2 (±148.97), respectively (Table 2). Saplings of *F. californica* whose stem were mock-inoculated had no stem lesions. Necrotic area was not significantly different between inoculated treatments and controls and between *Phytophthora* treatments (Table 2).

Saplings of *C. thyrsiflorus* that were root-inoculated with *P. multivora* had significantly higher leaf necrosis scores and root damage than controls ($p < 0.05$, Table 1) but the percentage of mortality in inoculated saplings was not significantly higher than that in controls (17%, Table 1).

All pathogens used in this experiment are mainly root rot pathogens, therefore, it is to be expected that root inoculations should result in higher infection success and greater disease severity than stem inoculations. Nonetheless, stem inoculation methods to test the pathogenicity of *Phytophthora* species are often used due to their less labor-intensive

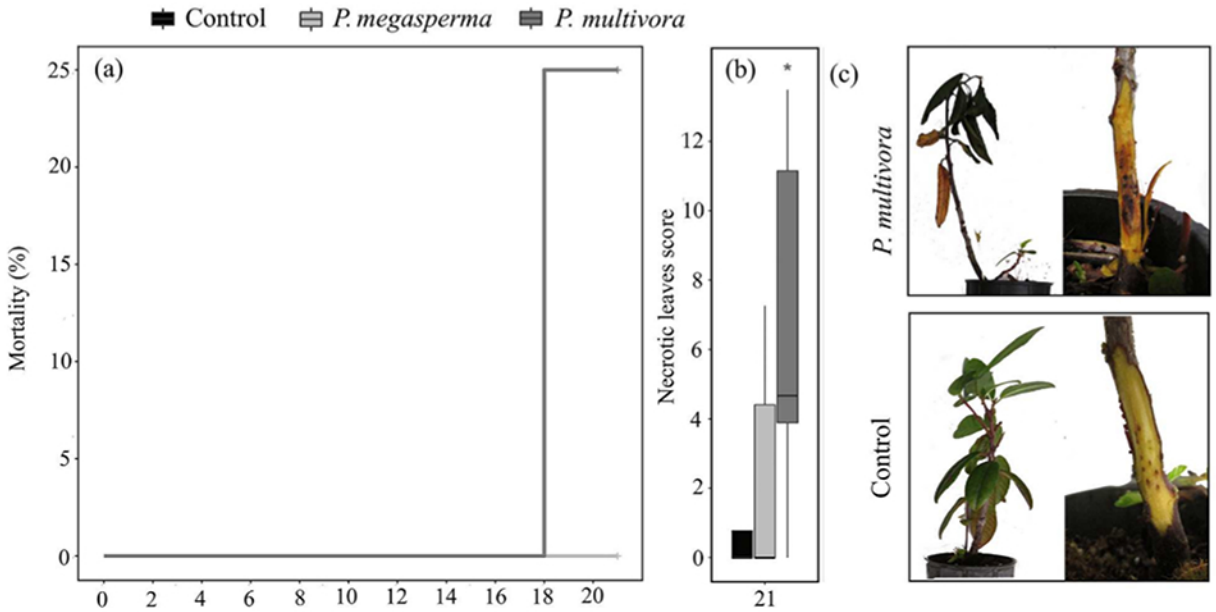

Fig. 3 Mortality percentage (a) and necrotic leaves scores (b) for *Frangula californica* stem-inoculated with *Phytophthora megasperma* and *P. multivora* (colored in light grey and dark grey, respectively) in days post inoculation. Annotations indicate significance levels: * is *p-value < 0.05*. Photographs of saplings (c) were taken twenty-one days post inoculation

Table 2 Results of the Dunnett's test for the stem necrosis area using the Control treatment as reference. Sd represents standard deviation and CI represents the 95% Confidence Interval

Plant Species	Treatment	Mean (mm^2)	Sd (mm^2)	Lower CI	Upper CI	*p*-value
Frangula californica	Control	0	0	-	-	-
	Phytophthora multivora	90.34	86.20	-299.71	629.81	0.46
	Phytophthora megasperma	50.19	148.97	-2.43	6.89	0.32

characteristics and because of their proven success in several pathogenicity trials for several *Phytophthora* and host species combinations (Hansen et al. 2005; Garbelotto et al. 2021). A comparison of results between stem and root inoculations of *D. aurantiacus* shows that the use of distinct inoculation methods can produce different pathogenicity results, deeming the use of just one inoculation method inconclusive for pathogenicity tests in some pathogen and host species combinations. In fact, there is the possibility that a failed stem inoculation may simply indicate that a soilborne *Phytophthora* species may not cause a stem disease but it may still be uncertain if the host is susceptible to root infection by the same pathogen. While stem inoculations of *P. megasperma* and *P. crassamura* on *D. aurantiacus* had nil or low re-isolation success and only caused minimal stem lesions or foliage symptoms, root inoculations showed the high pathogenicity of both *P. megasperma* and *P. crassamura* on this host. This result suggests that *P. megasperma* and *P. crassamura* are not likely to cause significant disease on stems of saplings of *D. aurantiacus* but are both root pathogens of this plant species and are both extremely pathogenic.

Likewise, results of stem-inoculations of *F. californica* were ambiguous, and while stem lesions were not statistically different between plants inoculated with either *P. megasperma* or *P. multivora* and mock-inoculated controls, lesions were present and foliage symptoms were statistically different between plants inoculated with *P. multivora* and controls. A pathogenicity trial using a root-inoculation method may instead be needed to clarify the pathogenicity of *P. megasperma* on *F. californica*.

Based on the presence of significant symptoms and/or mortality, combined with the success of pathogen re-isolation from 5 or more inoculated plants for each plant and pathogen combination, and given that 4 is a minimum number of plants used to test pathogenicity of putative pathogen on a new plant host (see Garbelotto et al. 2020), this study fulfills Koch's postulates and confirms the pathogenicity of the following *Phytophthora* species and host combinations: *D. aurantiacus* x *P. megasperma, D. aurantiacus* x *P. crassamura, C. thyrsi*floris x *P. multivora* and *P. multivora* x

F. californica. All three *Phytophthora* species and host combinations above are first reports, and the results presented here are consistent with disease and findings described in Sims et al. (2018), Sims and Garbelotto (2021) and Rooney-Latham et al. (2015, 2019). It should be further noted that our results do not account for the presence of external stressors, such as co-infection by other pathogens or environmental conditions, nor can they be used to predict the full range of disease symptoms on adult plants.

Plants of *D. aurantiacus* were extremely susceptible to *P. megasperma* and *P. crassamura*, as highlighted by the death of all inoculated saplings in less than 15 days. These results suggest that, whenever possible, managers should choose other plant species in restoration sites that are either infested by *Phytophthora* species or are adjacent to infested sites. Furthermore, recent studies have shown that *D. aurantiacus* plants are susceptible to other *Phytophthora* species, including the emergent species *P. tentaculata* (Rooney-Latham et al. 2015, 2019). Together, results of this and other studies suggest extreme caution should be used when growing *D. aurantiacus* in plant production facilities that supply plants for restoration and/or when using this plant species in restoration projects.

Saplings of *C. thrysiflorus* infected by *P. multivora* showed significant root damage and leaf necrosis, but mortality was low during the short duration of the experiment. Given the moderate severity of symptoms, it is possible that this species may play an important role in fostering, undetected, the survival and the spread of *P. multivora* in restoration sites or in nurseries. This is also in agreement with results of studies reporting the detection at somewhat low frequency of this *Phytophthora* species on *C. thrysiflorus* plants in nurseries (Sims et al. 2018; Rooney-Latham et al. 2019).

Finally, our experiment is the first to prove the pathogenicity of *P. crassamura*, a pathogen species recently described (Scanu et al. 2015) and only recently reported in California (Sims et al. 2019), on plant hosts outside of Europe. This study validates the possible role of *P. crassamura* as a primary pathogen in failing restorations, as hypothesized by Sims and Garbelotto (2021) based on isolation results from diseased plants in or immediately adjacent restoration sites. Thus, based on the results presented here, this *Phytophthora* species should be added to the list of plant pathogens that may have the potential to compromise the integrity of native California ecosystems.

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