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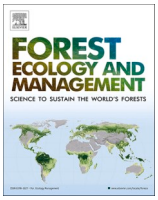

Phlebiopsis gigantea biocontrol efficacy against *Heterobasidion irregulare* infection in California Jeffrey pine forests

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ABSTRACT

Heterobasidion irregulare causes a widespread and long-term root disease in some of the most common Pacific western US coniferous forests including those with important trees such as Ponderosa pine (*Pinus ponderosa*), Jeffrey pine (*P. jeffreyi*), Western juniper (*Juniperus occidentalis*) and Incense cedar (*Calocedrus decurrens*). We conducted a series of disease-control efficacy trials in the field and laboratory using California isolates of *Phlebiopsis gigantea*, a well-known biocontrol for *Heterobasdion* spp. that has yet to be developed in Western North America. We conducted two laboratory evaluations of *P. gigantea* including growth and sporulation performance trials and three independent tests of efficacy in preventing pathogen establishment: a field trial on stumps; a field trial on vertically positioned logs; and a laboratory trial on wood discs. In the laboratory we first assessed 1) *in vitro* growth rate of California *P. gigantea* isolates across a range of temperatures and conidial formation by the same isolates at 20 C° and 2) evaluated the effect of Hi-Lite Blue Dye, a common additive used to track treatments in the field, on isolate growth and conidial germination for both *P. gigantea* and *H. irregulare*. These trials identified several *P. gigantea* isolates with biocontrol potential and found no evidence that dye additives impact growth or sporulation. The subsequent three biological control efficacy trials demonstrated the best performing *P. gigantea* isolates from the laboratory trials reduced *H. irregulare* establishment by 83.4 % although the common US chemical treatment borate reduced colonization by $<$ 99.99 %. Variation in disease response, another measure of biocontrol performance, was also significantly lower for *P. gigantea* in the field stump trial (58.4 %) vs reference treatments, although borate variance was 97.6 % lower than the reference. Our experiments evaluated *P. gigantea* efficacy against *Heterobasidion* inoculum levels which are unrealistically high compared to ambient inoculum and yet still indicate they have potential to suppress pathogen colonization and thus disease emergence.

1. Introduction

Heterobasidion root disease (HRD) is the most widespread and important native root disease of conifers in the northern hemisphere (Garbelotto and Gonthier, 2013). The disease is caused by several *Heterobasidion* species which vary in their most common hosts, geographic distribution, and environmental associations. In California and much of the western United States, two species cause this disease in native forests: *Heterobasidion irregulare* and *H. occidentale*, formerly referred to as the "pine type' and "fir type" for their primary respective preferred host genera (Otrosina and Garbelotto, 2010). A third, yet unnamed hybrid species affects alpine larch and was recently documented in Montana (*Larix lyallii*; Sillo et al., 2019). The two widespread North American *Heterobasidion* species have several epidemiological similarities to the genus as a whole: their establishment is most often associated with forest harvest, specifically freshly-cut stumps and also stem wounds for some *Heterobasidion* species (Bendz-Hellgren and Stenlid, 1998; Möykkynen et al., 1997; Rishbeth, 1959). Once established within a stump or within a wounded tree, *Heterobasidion* pathogens can spread to adjacent living trees via root contacts, root-to-root grafts, or in sandy soils by

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ectotrophic growth on the outside of woody roots that are in contact with one another, albeit not necessarily grafted (M. Garbelotto et al., 1999; Slaughter et al., 1991). *Heterobasidion* mycelium cannot grow freely in soil although basidiospores can percolate through sandy soils with low organic matter content (Garbelotto and Gonthier, 2013).

In California, pathogen growth slows in the decade after establishment but, tree mortality can extend up to ~20 m from the point of infection and can remove between 25 % and 45 % of host basal area (Flores et al., 2023; Rizzo et al., 2000; Rizzo and Slaughter, 2001). More importantly, although local pathogen spread and disease emergence eventually reaches a state of quiescence, pathogen survival and persistence within disease centers can maintain canopy openings for periods exceeding 50 years and plausibly persist locally and infect newly established trees for several decades longer via inoculum in buried coarse roots (Flores et al., 2023; Garbelotto and Gonthier, 2013). Regeneration within these disease centers can remain low suggesting the long-lived nature of this pathogen creates similarly long-term changes in forest structure that can reduce timber growth over long time periods (Flores et al., 2023; Rizzo and Slaughter, 2001).

Heterobasidion root disease (HRD) is notable among forest diseases in that several low cost and particularly effective chemical and biological control treatments are commercially available. The disease is managed in European spruce forests and pine forests of the Southeastern US through chemical treatment using urea (in Europe) or borates (Eastern and Midwestern US pine forests). Alternatively, mitospore suspensions of *Phlebiopsis gigantea* can be applied to reduce *Heterobasidion* pathogen establishment on individual stumps (Blomquist et al., 2020; Oliva et al., 2010; Pratt and Redfern, 2001; Zaluma et al., 2021). In European and Eastern US production forest systems, locally collected *P. gigantea* isolates have been developed into a commercial spray-on product, commercialized under the name Rotstop® followed by letters to specify the world region appropriate application. However, the biocontrol fungus isolates used in the product originally available in the US and Canada (Rotstop® C) are genetically distinct from those collected in California and may even be different (cryptic) species. The evolutionary and taxonomic divergence between Eastern and Western *Phlebiopsis* strains in the US has led to prohibition on the use of available commercial *Phlebiopsis* products and their source isolates in the US national forests (Dovana et al., 2021). Furthermore, as of 2025, Rotstop® C is no longer available in North America. Borate chemical treatments are used to treat this disease in both western and southeastern forests using the product Cellu-Treat®, but these applications can lead to a simplification of the microbial communities in sites that are treated (Pratt, 2000; Varese et al., 2003). This simplification combined with some concern regarding potential human reproduction harm, (Bolt et al., 2012) and a broader push to reduce chemical usage in California suggests borate products could be phased out of the state's permitted fungicides. This threatens to leave forest managers in California without a clear set of post-cutting *Heterobasidion* prevention tools.

To increase treatment options for this disease, we evaluate *Phlebiopsis gigantea* isolates from California for their efficacy in preventing *H. irregulare* establishment on Jeffrey pine stumps, one of the most abundant pine species on the eastern slope of the Sierra Nevada and Cascade ranges where *H. irregulare* is widespread and locally impactful (Flores et al., 2023; Garbelotto et al., 1996; Slaughter and Parmeter, 1995). Our effort builds on a previous study comparing the use of two *Phlebiopsis* isolates with urea and borate treatments and documenting significant pathogen growth suppression on stumps for *Phlebiopsis gigantea* spore suspensions (Poloni et al., 2021). Here, we evaluate the efficacy of a wider range of *P. gigantea* isolates and evaluate potential contraindication with the common blue dye additives used to track treatment applications on stumps and across stands. Jeffrey pine forests are valuable as timber resources and are often prioritized for fuels reduction treatments due their occurrence with environmental conditions associated with intense wildfire (Stephens, 2004; Vaillant and Stephens, 2009). Our study had three goals: 1) rank a set of California-collected *P. gigantea* isolates in terms of their growth rate and conidial formation relative to two aggressive isolates of *H. irregulare* in controlled conditions to select top performing biocontrol strains; 2) evaluate deleterious interactions between the *Phlebiopsis* biocontrol treatment and commonly used blue dye markers if they occur; and 3) compare *P. gigantea* with borate treatment efficacy in slowing *H. irregulare* growth and reducing pathogen growth rate deviance on Jeffery Pine in multiple, independent laboratory and field efficacy trials. A reduction in average disease severity of a population is expected for effective treatment (slowing *H. irregulare* growth rate). However, a decrease in deviance, that is variability in disease severity among individuals within a population, is expected for a treatment that is effective on a large segment of the population. Each goal represents individual steps necessary to develop an approved biological control for this widespread root disease in western US forests.

2. Methods

2.1. Objective 1: laboratory evaluation of Phlebiopsis gigantea performance

We conducted a laboratory growth rate evaluation of eight *P. gigantea* and two *H. irregulare* (Hi 25 and Hi 1590) isolates across the typical growing season temperature range for Jeffrey Pine forests (15, 20, and 25 ℃) between 5/24/21 and 6/30/21. Petri dishes 9 cm in diameter were filled with full-strength malt extract agar (MEA; 12.5 g malt extract, 5.0 g dextrose, 17.5 g agar per Liter of deionized water) amended with sawdust at a rate of 15 g pine sawdust and 12 g agarose per Liter of medium; this method was originally devised to achieve a similar growth-comparison for European *H. annosum* and invasive *H. irregulare* isolates (Giordano et al., 2018). Measurements of colony diameter along two perpendicular axes were recorded every two days for 14 days post inoculation (7 temporal measurements) or until the individual plate reached 100 % of colonizable area.

Sporulation studies were performed in controlled conditions at 20 ℃ on unamended MEA. Our collection of *P. gigantea* isolates were inoculated in the center of 6 cm -diameter Petri dishes on 7/5/21 with each isolate inoculated on five replicate petri plates. Fourteen days post inoculation plates were flooded with 5 mL sterile water and gently shaken twenty times before the suspension was poured into a sterile conical centrifuge tube. From each sample, six 10uL aliquots were removed and conidia concentration was determined using a hemocytometer and a 10uL draw from gently shaken samples to calculate average conidia concentration.

2.2. Objective 2: effect of blue dye on pathogen and biocontrol growth

We evaluated the effect of the blue dye, a common treatment additive which aids in evaluation of contract compliance and enhances treatment coverage, on the germination and mycelial growth rate of *P. gigantea* and *H. irregulare*. Here, we repeated the laboratory growth rate trials but amended the media with 3.96 mL/L of Hi-Light Blue spray indicator dye (BASF CORP, 100 Park Avenue Florham Park, NJ 07932, USA) and compared this with unamended media (control treatment). This concentration of blue dye was applied in all experiments and is also the recommended concentration for field use. We grew the top two performing *P. gigantea* (Pg) isolate from Objective 1, Pg 39 and Pg 41-Victor (see below), on four 60 mm MEA plates for seven days at 18 ℃ and then used the colonies to test both the germination rate of conidia and growth rates of *P. gigantea*, in the presence and absence of the dye. Conidia suspensions were created with the same procedure employed in our laboratory trial. To evaluate conidial germination, a conidial suspension of 7000 conidia per mL was gently shaken and 50uL aliquots were pipetted onto ten dye amended and ten unamended MEA plates and distributed evenly to cover the plate using a sterile glass rod. To measure cross-contamination in the laboratory, we also include a

negative control treatment where all plates were treated identically, except a sterile water solution was used in place of the conidial suspension. All plates were incubated for five days, and conidial germination was assessed as the number of colonies growing on each plate. A small-scale proof of concept test was performed in March 2022 that included two sets of treatments and two sets of controls; each set included two sets of ten unamended plates (no dye) with one set was sprayed with conidial suspensions and the other with sterile water (negative control). In September 2024, a second follow-up experiment was conducted with the two *P. gigantea* isolates alongside the *H. irregulare* isolate Hi 25 in four sets of ten plates per isolate and treatment while retaining uninoculated controls in a single set of ten plates. Negative controls were reduced in this second experiment given the plates treated with sterile water (negative controls) in the first experiment were contamination free.

2.3. Objective 3: biocontrol efficacy trials

2.3.1. Laboratory efficacy trial

Fresh Monterey pine (*Pinus radiata*) wood discs, approximately 8 cm in diameter, were used in a lab efficacy trial (Pellicciaro et al., 2021a). Monterey pine was used in the wood disc trial because it is easy to acquire, and its wood characteristics (growth ring diameter, growth rates) are relatively uniform. Monterey pine stems were debarked and sprayed with 70 % Ethanol, before being sliced in 1cm-thick discs using sterile technique. We excluded sections with branch nodes. Discs were placed on top of filter paper imbibed with sterile water and placed on the bottom of a deep Petri dish, 9 cm in diameter. *P. gigantea* and *H. irregulare* spores were applied at a concentration of 10^2 conidia/mL and a final inoculum density of 10.500 conidia per square meter was applied for each fungus. To compare effects of borate vs *P. gigantea* treatments on *H. irregulare* growth rates we prepared a 5 % disodium octaborate tetrahydrate (Cellutreat $^{(R),}$ Nisus Corp, 100 Nisus Drive, Rockford, TN, USA) solution according to the manufacture label. An identical volume to that used for the conidial suspensions was applied to a set of randomly selected wood discs with the final amount of active ingredient applied at a rate of 105 mL/m^2. *Heterobasidion irregulare* spores (isolate Hi 1590) were applied 24 h after individual *P. gigantea* isolate inoculations (Pg 21, Pg 22, Pg 39, or Pg 41-Victor) or borate applications. Treatments were initiated 10/27/2021 with *H. irregulare* inoculation performed 24 h post treatment; after 10 days discs were analyzed for the presence of conidiophores at 20x and 40x magnification using a dissecting scope (for pathogen confirmation) and surface area colonized was measured.

2.3.2. Log field efficacy trial

We next performed a trial using vertically oriented Jeffrey pine logs. Given that each tree provides multiple logs, this approach allows for good replication without the harvest of large numbers of trees and working within inherently dangerous active timber harvest operations. Furthermore, using multiple logs per tree also allows measurement of the effect of individual trees on treatment efficacy. Our log study was performed at two sites (41.607841°, −120.975821° and 41.619728°, −120.988492°) within the Modoc National Forest. At each site, four adult Jeffrey pine trees were felled and delimbed with a sanitized chainsaw. We intentionally avoided trees with obvious or frequent branch nodes and when necessary, we removed any section with a branch stub and discarded it. Logs were sectioned into 17 sections per tree, debarked, and sprayed with ethanol 70 % before cutting; the chainsaw was also sprayed with 70 % ethanol between cuts. Within 72 h from felling, logs were sectioned in 50 cm length sections and the basipetal part of each section (e.g. more proximate to the roots) was sealed using Nel-Seal Log and Lumber End Sealer (The Nelson Paint Company, Kingsford, MI, USA). Log sections were stood up vertically with an end buried at a shallow 5 cm soil depth and braced to remain standing. Logs were established in groups of three with each log randomly assigned a treatment (sterile water, *P. gigantea*, or borate) and treated immediately as each log-triplet was established. At each site, treatments were replicated within 63 log sections (N = 21 per treatment). The remaining logs (five) were employed to collect preliminary measures to optimize sampling ("monitoring logs"). Two and three uninoculated monitoring logs were established at each site, one treated with *P. gigantea* at each site and one log treated with water at site 1 and two logs treated with water at site 2. Water (control), *P. gigantea* (isolate Pg 41-Victor) and borate treatments were performed on 10/27/21 at site 1 and on 10/28/21 at site 2. Twenty-four hours post treatment, all logs were inoculated using a conidial suspension of *H. irregulare* (isolate Hi 1590). *Heterobasidion irregulare* and *P. gigantea* conidial suspensions were 10^3 conidia mL^{-1} and, for either fungus, the total amount of conidial suspension applied was 105.mL/m^2 for a total of 105.000 conidia/m^2. The total liquid volume applied was equal across each treatment (105 mL/m^2). On 3/16/22, after eighteen weeks, monitoring logs were taken to the lab and dissected at 15 cm intervals. Each log section was incubated in a moist chamber and stored in a cold room at 5ºC. Discs were inspected for the presence of *H. irregulare* conidiophores using a dissecting scope at 20x or 40x magnification 5, 9, and 12 days after removal from the cold room and incubation at 20 ℃. A marker was used to delineate the edge of the area colonized by *H. irregulare*, based on presence of conidiophores. Photos were taken of each disc and analyzed using the Image Analysis tool in Adobe Photoshop to determine the area of each disc and areas bounded by the red marker. Metrics recorded were presence/absence of *H. irregulare*, area colonized by *H. irregulare*, and percentage of stem section colonized by *H. irregulare*. Monitoring logs showed that *H. irregulare* colonization varied by section and that area colonized by *H. irregulare* was maximized in the section that was 5–10 cm from the top of the log after a 12-day incubation at room temperature in a moist chamber (Supplemental information; Figure S1).

On 4/28/22, after 23 weeks from the beginning of the trial, all study logs were transported to U.C. Berkeley. From each log, one 5 cm thick section was extracted 10 cm from the top of the log and was incubated and analyzed as described above for the monitoring logs. In this forest system, *H. irregulare* causes disease but the morphologically similar *H. occidentale* – which causes disease in true-fir (*Abies*) forests – is part of the broader saprotrophic fungal community (M. Garbelotto et al., 1999; Otrosina and Garbelotto, 2010; Poloni et al., 2021). Differentiating between the two *Heterobasidion* species is essential to quantifying efficacy in the Jeffrey pine forest context. Conidiophores characteristic of *Heterobasidion* were isolated once from sample using 18-gauge sterile hypodermic needles to carefully lift individual conidia and hyphae off the wood disc and transfer them to Khulman's media (5 g bacto-peptone, 20 g agar, 0.25 g $MgSO_4$, 0.5 g KH_2PO_4, 190 ppm pentachloronitrobenzene (PCNB), 100 ppm streptomycin, 2 mL lactic acid 50 %, 20 mL ethyl alcohol 95 %, per L deionized water). Isolates were grown in liquid malt extract (described above), mycelium was harvested using filter paper, and isolate DNA was extracted using the Qiagen DNA Plant Extraction kit per manufacturer's instructions and a PCR assay specific for *Heterobasidion* (see Shamoun et al., 2019). The PCR assay was applied on a 1:100 dilutions of culture DNA extracts to identify each isolate as either *H. irregulare* or *H. occidentale*.

2.3.3. Stump efficacy trial

Lastly, we tested the same treatments on Jeffrey pine stumps in two sites within the Inyo National Forest (37.709848° −118.957595° and 37.713451° −118.955435°). A week before treatment, 119 high stumps were created in two sites (61 at site one, 58 at site two) in the Inyo NF in conjunction with a stand harvest operation; stumps averaged 140 cm in height. On 10/14/22 (site one) and 10/25/22 (site two), the top 10 cm of each stump in site one was cut, to expose fresh and uncolonized wood (130 cm average height). Stumps were grouped in blocks of three and one of the three treatments (water, *P. gigantea* conidial suspension and borate) was randomly assigned and treatments were performed using concentrations, isolates, and volumes identical to those described for the

log study described above. We created 19 complete triplets at site one and 16 complete triplets at site 2; we applied *P. gigantea* on one and two additional stumps at site one and two (respectively), and borate treatments on one and three additional stumps at site one and two (respectively). Seven stumps, three at site one and four at site two were retained as monitoring stumps (to optimize sampling); this resulted in a total of 38 stumps treated with *P. gigantea*, 38 with borates, and 36 with water (controls) across the two sites. Twenty-four hours post-treatment, all stumps were inoculated with a *H. irregulare* (Hi 1590) conidial suspension created with the same methods as the log study. On 9/18/23 the seven monitoring stumps were sampled and returned to the laboratory. Stump sampling was conducted with a chainsaw by cutting the stump 100 cm below the stump surface and returning this 100 cm section to the laboratory. The chainsaw was cleaned with 70 % ethanol and vigorous use of a stiff brush between samples. At the lab, each monitoring stump was subsequently sectioned every 15 cm after removing and discarding the top 5 cm. Sections were incubated in a moist chamber and placed for 4–7 days in a cold room at 5 ℃. Discs were then placed at 20 ℃ and *H. irregulare* colonization was measured after 5, 9, and 12 days after they were taken out of the cold room. The monitoring logs showed that the area colonized by *H. irregulare* varied by stump section with the maximum at 15 cm below the surface after a 12-day incubation in moist chambers (Figure S2). We returned to the Inyo sites and, on 10/10/23, all stumps were sampled and the optimal stump section 15 cm below the surface was returned to the laboratory at U.C. Berkeley. This created a field incubation lasting just under one calendar year. Stump sections were treated and analyzed as described for log sections including one *Heterobasidion* isolate per disc subcultured and identified using the PCR analysis.

2.4. Growth rate and efficacy calculations

For objectives 1 and 2, we calculated the growth rate for each *P. gigantea* or *H. irregulare* isolate for each plate and across the three incubation temperatures: 15, 20, and 25 ℃ (N = 5 per each isolate x temperature combination). Growth rates were measured as changes in colony surface area over 12 days of incubation with three measurement dates and rendered as the proportion of plate area colonized (up to 100 % colonization). Change in the proportion of area colonized was approximately linear, this allowed us to calculate linear growth rates for each replicate and temperature. We summarized the ranked growth performance of each isolate across temperatures by calculating the area of the curve using the trapezoid rule. The ideal *P. gigantea* biocontrol should perform well across the range of temperature variability in a real stand, thus we interpret the highest area under the curve to indicate the most consistent and greatest total growth rate among isolates. Conidial concentration data did not require any additional data processing or calculations. Objective 3 was evaluated by relativizing all pathogen establishment rates using a simple proportion of colonizable area for the basal or surface area of each treatment subject (stump, log, or wood disc surface area). We further evaluated treatment efficacy by calculating the deviance of disease responses; a reduction in deviance indicates a more uniform population-level response to treatment when overall (average) disease responses also decline with treatment (Poloni et al., 2021). Lower levels of efficacy variation are desirable across disease systems (Gibson et al., 1999; Posteraro et al., 2014). By contrast high deviance levels suggest the treatment is effective on some individuals and ineffective on a substantial number of other individuals. We used a simple measure of $\text{Deviance}_{trt} = \text{Abs}(\mu_{trt}\text{-}y_i)$, where μ_{trt} is the respective treatment mean and y_i is an observation within the same treatment.

2.5. Statistical analysis

For the *P. gigantea* growth rate hypothesis tests, we applied a linear model with isolate as a factor, temperature, a factor indicating species (*P. gigantea* or *H. irregulare*) and the interaction term for species and temperature (Table 1). These models were used to evaluate growth rates and conidial production in our full range of *P. gigantea* isolates. We then applied similar models to evaluate blue dye impacts on *P. gigantea* and *H. irregulare* growth rates and employed a paired *t*-test on the simpler experimental design of the test for blue dye impacts conidial germination (*P. gigantea* and *H. irregulare* isolates; Table 1). For the laboratory wood disc, field log, and stump trials, colonization and colonization deviance were assessed with linear mixed models and structured the random variables in each model with respect to each trial (Table 1). The Inyo NF stump study included two sites as random intercepts. The Modoc NF log study (log study) included two sites as random intercepts and a second random intercept for each tree (log source). No random variables applied for the laboratory wood disc study, therefore we here employed a simple linear model. Although the laboratory trial included the four best performing *P. gigantea* isolates, only the control discs had any *Heterobasidion* detected, so isolate identity was dropped from the model.

For the mixed model analysis, an individual model was deemed suitable for inference when the models converged (were non-singular) and met the model assumptions. Our initial models included stump or log diameter as a fixed effect, but these models often violated our criteria for convergence and were dropped either because of this problem or because they appeared to explain little or no variance in the response. Similarly, a preliminary analysis showed no evidence of interactions between stump/log surface area and treatment efficacy; thus this variable was also dropped from the hypothesis tests. Each model was examined in terms of their adherence to the model assumptions and occasional square root or logit transformations were required. All analysis was conducted using R 4.4.1 (© 2024 The R Foundation for Statistical Computing) with the package NLME employed for mixed models. For all analysis, we use the decision rule of $p \geq 0.05$, for determining statistical significance.

3. Results

3.1. Objective 1: Phlebiopsis gigantea isolate performance

Phlebiopsis gigantea growth rates increased with temperature and, in absolute terms, were faster than our two *H. irregulare* cultures (Fig. 1). Our *P. gigantea* growth rate experiments did not demonstrate a statistically significant difference compared to *H. irregulare* however, isolates Pg 21, Pg 39, and Pg 41 grew significantly more rapidly than the other

Table 1
Statistical model structure for a series of Heterobasidion control experiments with diameter ranges and mean diameter where applicable.

Trial (diameter cm range; mean cm)	
Response	*Model*
Objective 1: Isolate performance analysis	
Growth rate Pg†	= *time (days)* + *temperature* * *Pg isolate*
Growth rate Hi	= *time (days)* + *temperature* * *Pg isolate*
Conidia	= *Pg isolate*
Objective 2: Blue dye experiment	
Growth rate	= *time (days)* + *treatment* + *isolate (Pg or Hi)*
Difference (conidia)	$= \overline{Blue_i} - \overline{Control_i}$
Objective 3: Field trials	
Inyo stumps – (20.5–45.4; 31.95)	
Colonization	= *treatment* + *(1|site)*
Deviance	= *treatment* + *(1|site)*
Modoc vertical logs – (22–34; 26.4)	
Colonization‡	= *treatment* + *(1|site)* + *(1|tree)*
Deviance	= *treatment* + *(1|site)* + *(1|tree)*
Laboratory wood discs – (5–8.8; 6.8)	
Colonization†	= *treatment* + *isolate*
Deviance	= *treatment* + *isolate*

†Parameter logit transformed
‡Parameter square root transformed

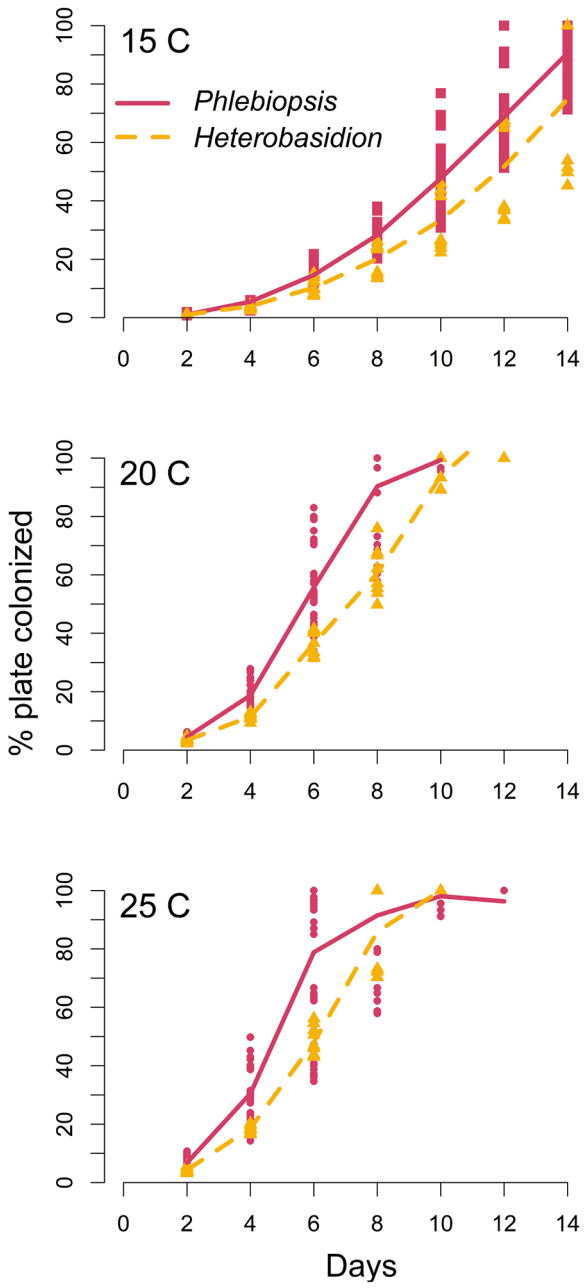

Fig. 1. *Phlebiopsis gigantea* vs *Heterobasidion irregulare* growth rates in controlled temperature environments and three temperatures (C°). Eight *P. gigantea* isolates and two *H. irregulare* isolates, each with ten replicate trials were included in the experiment.

P. gigantea isolates or *H. irregulare* (Table 2). The relative performance of *P. gigantea* and *H. irregulare* isolates were not consistent, several *P. gigantea* grew slower at all temperatures (isolates 8 & 9) while others demonstrated notability slower or faster growth rates at one temperature or another (Pg 24 & Pg 26, interaction $p < 0.001$, Table 2). Of the best performing isolates, Pg 21, Pg 22, Pg 39, and Pg 41-Victor were statistically indistinguishable in terms of their growth rates (Table 2), and none of the interactions with temperature were significant. Isolate Pg 41-Victor also had the greatest area under the curve after integrating growth rate across all three temperatures and significantly greater conidial concentration compared to the rest of our collection (Table 2). Isolates Pg 21, Pg 24 and Pg 39 also demonstrated strong conidial production although somewhat lower than Pg 41-Victor. All other isolates were characterized by limited conidial production (Table 2). When combining the two metrics of isolate performance, growth rate and conidia production, Pg 21, Pg 39 and Pg 41-Victor, were characterized by good or excellent biological control potential relative to the rest of our *P. gigantea* collection.

3.2. Objective 2: evaluation of marking dye on *H. irregulare* and *P. gigantea* growth rates

Our blue dye experiments demonstrated no effect of this additive on the growth rate of *P. gigantea* or *H. irregulare* at 20 ^{0}C (Fig. 2). Likewise, germination of conidia of *P. gigantea* at 20 ^{0}C was not affected by the blue dye (Table S1). This result was consistent across the isolates we tested, including Pg 39 and Pg 41-Victor and *H. irregulare* isolate Hi 25. That is, within the experiment, all isolates, regardless of species, grew and germinated at the same rate in the presence and in the absence of the dye in our MEA growth medium.

3.3. Objective 3: *Phlebiopsis gigantea* laboratory and field efficacy trials

Each field and laboratory efficacy trial demonstrated potentially useful and consistent *P. gigantea* effects in terms of both colonization and colonization deviance (treatment consistency across individuals; Fig. 3). For each trial, either stump (Inyo), log (Modoc), or wood disc (Laboratory), *H. irregulare* colonization was significantly higher for subjects treated with DI water (Control) compared to either *P. gigantea* or borate treatments (Fig. 3). However, in both field trials (Inyo stump and Modoc log studies), *P. gigantea* treatments resulted in significantly higher *H. irregulare* colonization relative to the borate treatments. In the laboratory wood disc trial, no *H. irregulare* was recovered from either the *P. gigantea* or borate treatments and unsurprisingly the estimated efficacies for both treatments were indistinguishable ($p = 0.99$). Colonization deviance was mostly consistent across measures of colonization; the deviance of *P. gigantea* and borate treatments was lower compared to that of the control treatments, but it was higher for *P. gigantea* relative to borate treatments (Fig. 3). An important exception was the Modoc log trial where the deviance of *P. gigantea* was significantly greater than the borate treatment and not statistically different from the control treatments ($p = 0.93$).

4. Discussion

Although specific *P. gigantea* strains have been selected and are already marketed as biocontrols in Europe and in the Eastern and Midwestern USA, the potential use of this biocontrol in the western US hinges on the identification of *P. gigantea* strains that meet seven criteria: 1) are native to the western US, 2) are prolific producers of conidia, given that the biocontrol has to be applied as a conidial suspension, 3) have growth rates higher than or equal to *Heterobasidion* pathogens across an ecologically relevant range of temperatures, 4) can be mixed with a commercial available dye marker to ensure its application is visible, 5) are competitive against the target pathogen in terms of growth rates *in vitro*, 6) can control pathogen infection in the lab, and, finally, 7) can control pathogen infection in the field. The basis for the current study is previous work by Dovana et al. (2021), who compiled a collection of *P. gigantea* isolates from California, and thus fulfilled the first of these seven requirements. This study is the first to investigate whether *P. gigantea* isolates available from California may meet the rest of the above requirements for a viable, effective, and marketable biocontrol product. The results are largely positive outcomes that could advance the development of a *P. gigantea* biocontrol with a California origin that is a suitable alternative to the use of borates and may be used in all U.S. Western forests.

The comparative analysis of eight *P. gigantea* California isolates performed *in vitro* in this study identified significant variation among isolates in both growth and sporulation rates, here measured as conidial production and gemination. Interestingly, the two metrics – growth rate

Table 2
Phlebiopsis gigantea and *Heterobasidion irregulare* isolate performance dynamics (n = 5 for each estimate) as measured by growth rate (% of colonizable plate area) in culture at three temperatures, the area under the curve for all three temperatures, and conidial mass (concentration); rank of performance for each parameter is included in the parenthesis for *Phlebiopsis* with results of a Tukey's HSD post-hoc means comparison indicated by different letters ($p \leq 0.05$); the same parameters are shown for *H. irregulare* for comparison where applicable.

Isolate	% plate day^{-1} 15 ℃	% plate day^{-1} 20 ℃	% plate day $^{-1}$ 25 ℃	Average colonization†	Area under curve	Conidia (10^4 mL^{-1})
Pg_8	6.75 (6)	11.85 (8)	10.74 (8)	30.49^{c}	102.95^{c} (8)	4.2^{c} (8)
Pg_9	6.74 (7)	14.73 (7)	11.84 (7)	32.47^{c}	120.08^{bc} (7)	6.9^{c} (7)
Pg_21	8.93 (3)	16.59 (3)	21.97 (3)	55.04^{a}	160.22^{a} (2)	48.1^{b} (2)
Pg_22	9.0 (2)	16.27 (6)	19.30 (5)	48.34^{a}	152.09^{a} (4)	8.6^{c} (6)
Pg_24	6.2 (8)	15.98 (1)	22.23 (2)	39.69^{b}	150.97^{a} (5)	43.7^{b} (4)
Pg_26	8.0 (4)	12.45 (4)	16.19 (6)	39.98^{b}	122.61^{b} (6)	14.3^{c} (5)
Pg_39	7.5 (5)	16.32 (5)	23.04 (1)	50.8^{ab}	157.9^{a} (3)	47.9^{b} (3)
Pg_41-"Victor"	9.63 (1)	16.79 (2)	21.1 (4)	59.17^{a}	160.76^{a} (1)	87.7^{a} (1)
	Heterobasidion irregulare					
Hi 25	4.08	11.36	12.16	42.5^{a}	97.4^{a}	NA
Hi 1590	8.07	12.53	16.11	32.8^{b}	123.07^{b}	NA

† Tukey's HSD mean percent of colonizable plate from Logit transformed data (inverse Logit estimates reported); interactions effects are included with temperature effects at 18.9 ℃ with each species modeled separately.
NA – not applicable

and sporulation – were not necessarily collinear, similar to other studies of *P. gigantea* with larger isolate collections (Kļaviņa et al., 2023; Sun et al., 2009). Given that temperatures in our target California forests can experience ~25 ℃ for weeks and months during the field season, we were interested in isolates with high growth rates at this temperature. These temperatures are in the higher range of many European forests where *P. gigantea* is deployed as a disease mitigation and previous work has shown these relatively high temperatures do not interfere with these biocontrol treatments (Oliva et al., 2015). Three isolates appear to have superior biocontrol potential: Pg 41-Victor, Pg 39, Pg 21 which were characterized by excellent (Pg 41-Victor) or good (Pg 39, Pg 21) sporulation rates, and growth rates superior to those of *H. irregulare* across temperatures, particularly at 25 ℃. The availability of three isolates with high sporulation potential is helpful for 1) synthesis of concentrated spore suspensions to be used in treatments and 2) secondary natural local spread following forest treatments. A fourth isolate, Pg 22, also met these growth requirements, but we see this isolate as less ideal due to its lower sporulation rate. We found no evidence that germination of conidia from isolate Pg 41-Victor was affected by the presence of the blue dye (Table S1); we also found no evidence that growth rates of Pg 41-Victor and Pg 39 were affected by this common additive (Fig. 2). Based on our results, at least three strains of *P. gigantea*, and possibly four, meet our biocontrol criteria 2, 3 and 4. Unlike a recent analysis of 60 *P. gigantea* isolates, we found no evidence of tradeoffs between growth rate and sporulation however, the current California *P. gigantea* isolate collection is much smaller than collections in Europe (Kļaviņa et al., 2023). Increasing the *P. gigantea* isolate collection for California is needed to understand if these tradeoffs occur outside of Europe as well as identify potentially better biocontrol isolates.

Growth rates of *P. gigantea* isolates were comparable or above the growth rates of the two *H. irregulare* isolates at 15 ℃, but they were consistently higher at 20 and 25 ℃, especially when considering the four top-performing isolates (Table 2). In the *in vitro* trial on wood discs, all *P. gigantea* isolates were able to prevent colonization by *H. irregulare*, suggesting suitability as biocontrol agents. The fact that 100 % control was achieved, independent of *P. gigantea* or pathogen isolate, could be a shortcoming of the oversimplified lab trial which was performed at a constant room temperature of 20 ℃ and on small surfaces (Fig. 3). We should also note that, given the small size of discs fitting inside 90-cm Petri dishes, we were able to generate enough spore suspensions for all eight *P. gigantea* strains, but with an experiment at a larger scale, only five strains may produce enough conidia to make them viable candidates in an experimental setting. However, the results of these two trials satisfy criteria 5 and 6.

Of course, the last requirement of efficacy in the field is the most important (criteria 7): can these isolates serve as a management tool to reduce disease? The California *P. gigantea* isolates we tested in the field (Pg 39 and Pg 41 – Victor) in this study and in Poloni et al. (2021) show promise as biological controls for Heterobasidion Root Disease (HRD) on eastern-slope Jeffrey Pine forests. This is an encouraging result in light of a previous study which demonstrated *P. gigantea* biological control for *H. occidentale* on white fir and in western-slope forests (Poloni et al., 2021; Table 3). Furthermore, it suggests *P. gigantea* can help effect management aimed at preventing or reducing HRD in ways and to degrees which may be comparable to its use in commercial products (Blomquist et al., 2020; Nicolotti and Gonthier, 2005; Pratt et al., 2000; Zaluma et al., 2021). While our trials to date suggest that *P. gigantea* treatments do not fully prevent stump infection and are not as efficacious as borate applications in terms of preventing pathogen establishment (Fig. 3), the consistent decrease in *H. irregulare* growth in *P. gigantea* treatments suggests the biocontrol could have long-term disease-prevention benefits, especially if borates are phased out as approved fungicides. Our results are largely consistent with treatment outcomes from previous experiments in which *Phlebiopsis gigantea* reduced stump colonization by *Heterobasidion* spp. but did not fully prevent stump infection when stumps are artificially inoculated, particularly pine stumps (Covert et al., 2011; Pellicciaro et al., 2021b). Other published results report 100 % efficacy in preventing stump infection when using formulations that include *P. gigantea*, however those studies were performed in more mesic environments and, more importantly, were based on natural infection (Dumas and Laflamme, 2013). Likewise the better performance of chemical treatment vs treatments using *P. gigantea* have been reported (Blomquist et al., 2020). Our trials may be strongly biased in favor of the pathogen, given that we intentionally employed *H. irregulare* strains with relatively rapid growth rates and applied one hundred thousand spores per square meter 24 hrs after treatment vs. the 240 spores per square meters per 24 h (10 spores per square meter per hour) are regarded as being sufficient to cause disease (Gonthier et al., 2005). Trials using a much lower and arguably, more realistic *H. irregulare* inoculum density – particularly ambient inoculum – are needed to better determine the relative efficacy of our best performing *P. gigantea* isolates, such as Pg-41 Victor, in comparison to borates. Trials with a greater range of *H. irregulare* isolates, including those considered to have slower growth rates in wood, as well as increasing the overall isolate collection of *P. gigantea* will improve our overall understanding of this biocontrol efficacy, durability, and probably also help tailor its application in the field (Redfern et al., 2010; Sun et al., 2009; et al., 2021)

Heterobasidion irregulare wood colonization was consistently lower in *P. gigantea* treatments relative to controls in each trial, including the Modoc stump surrogate (log) trial where the variation in disease response (deviance) was notably high (Fig. 3). In contrast, in the

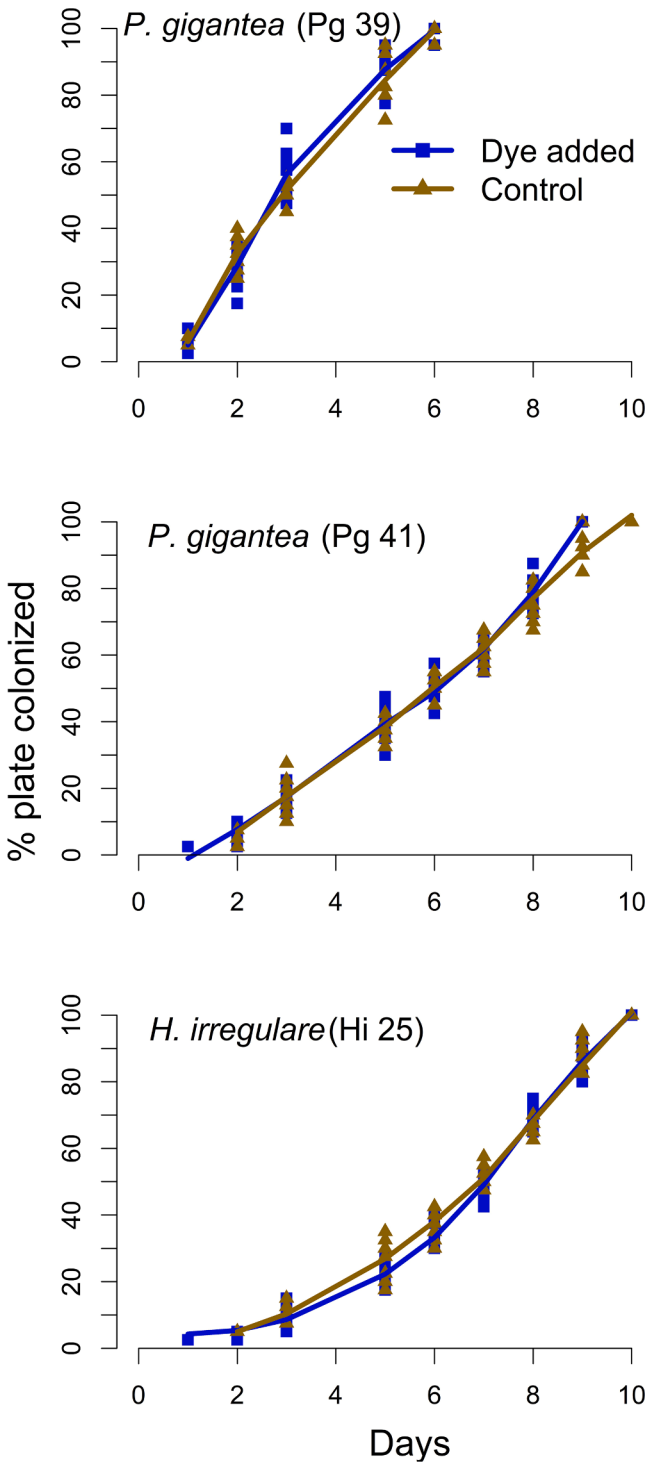

Fig. 2. Experimental evaluation of blue marking dye on *Phlebiopsis gigantea* (isolates Pg 41 and Pg 39) and *Heterobasidion irregulare* (isolate Hi 25) growth rates at 20 °C employing media with blue dye added (Dye added) vs unamended media (Control). Each trial/treatment included ten replicates.

laboratory wood disc trial *P. gigantea* and borate treatments completely prevented *H. irregulare* growth. Although the reason for the difference in response between the field and lab trials is not clear, it is possible that a short (i.e. 10 days) *in vitro* test under complete environmental control and with the lack of natural antagonists may be strongly biased in favor of the biocontrol. The lab trial was designed to verify the potential of each *P. gigantea* isolate as a biocontrol agent and the laboratory approach may be insufficient to differentiate efficacy among isolates. More importantly, the field trials on logs and stumps are more realistic and are arguably a much better measure of overall biocontrol efficacy; these measurements are only limited by the substantial effort required to undertake them.

The Modoc log trial also attempted to constrain the effect of host genotype by including log source (e.g. tree) as a random variable to encompass this variation. Genetically influenced wood anatomy characteristics including vessel occlusion, repair, and embolism may vary by individual and with log origin relative to the complete tree bole, which in turn could influence the rate of moisture loss for each log and thus infection (Bendz-Hellgren and Stenlid, 1998). This variability may be significantly less in stumps given any height influence on wood anatomy would be minimum given the variation in stump height in our experiments and that each stump retains an intact root system that are often grafted with the roots of living neighbor trees. Our deviance measure was particularly high for *P. gigantea* in the Modoc trial where study subjects originated from several individuals and were sourced from different locations on the standing bole (e.g. different heights above the base of the tree). As higher variability in treatment efficacy (here measured as deviance) is undesirable (Gibson et al., 1999; Hart, 1949; Posteraro et al., 2014), some caution as well as follow-up testing is justified in light of this result. In contrast, deviance (variation in treatment efficacy across subjects) was reduced by *P. gigantea* treatments on stumps in this study (Fig. 3) and for *H. occidentale* in white fir (*Abies concolor*) stumps (Table 3; Poloni et al., 2021). Overall, the results from stumps should carry the most weight as treatments given stumps are the treatment targets in real-world forestry (Garbelotto et al., 1999; Redfern et al., 2010).

As previously discussed, we found significant variability of growth rates and conidial production across our *P. gigantea* isolates, a pattern consistent with other studies of *P. gigantea* isolate collections (Kļaviņa et al., 2023; Sun et al., 2009). The range of isolate response to variation in temperature may suggest environmental adaptation across this fungal species in California. This is a sensible expectation, given that *P. gigantea* is distributed across a wide range of forest types and environments including the drier Jeffery pine ecosystems, wetter western-slope forests and pine forests in the California coastal ranges (Dolanc et al., 2014; Dovana et al., 2021; Thorne et al., 2008). While, as mentioned above, we found several isolates that stood out for their performance measures (Table 2), it is important to acknowledge that the optimal growth characteristics for *P. gigantea* biocontrol may vary across the range of stands and host species which are at risk to infection (Flores et al., 2023; Redfern et al., 2001; Shaw et al., 1995). Thus, an optimal *P. gigantea* product for disease control may very well be a mix of isolates that maximize overall biocontrol colonization across a range of temperatures or provide some degree of genetic variability to hedge against environmental change and susceptibility to fungal viruses (see Pratt et al., 2000). Additionally, it is almost certain that these fungi will respond to variation in precipitation and precipitation form (snow vs rain; Pratt et al., 2000, Nicolotti and Gonthier, 2005, Zaluma et al., 2021). Additional field tests, including those with different isolate mixtures may be able to resolve the importance of additional environmental factors on biocontrol efficacy. Additional evaluation of mixtures would also be needed to test for dilution impacts to efficacy which could occur if a slower growing isolate at one environmental condition causes lower efficacy overall.

Given that our tested *P. gigantea* isolates are indigenous to California forests (Dovana et al., 2021), evaluation of their biocontrol could justifiably move to an adaptive management framework for further evaluation and improvement. This would also satisfy criteria #7 to an even greater degree than our stump trials. California is in the midst of a multi-year and multi-billion USD investment into forest fuels reduction, an investment in direct response to a wildfire crisis which threatens public health, agricultural production, and infrastructure on or near the wildland-urban interface, and forest productivity, resilience to climate change, and resilience to wildfire (Charnley et al., 2023; Forest

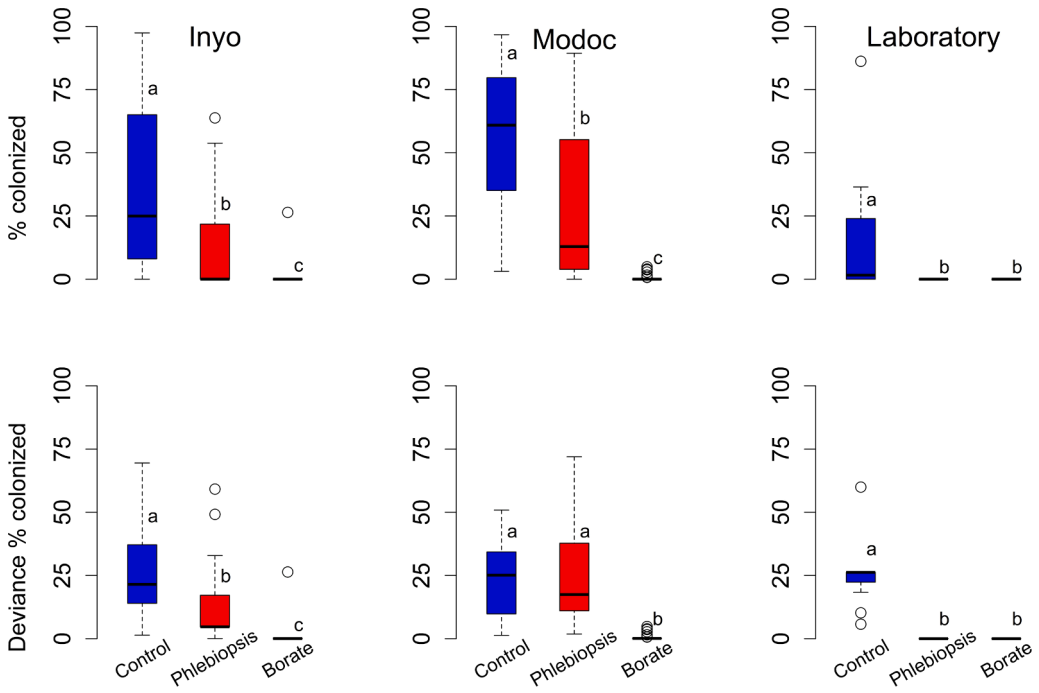

Fig. 3. Treatment effects and assessment of treatment means for three trials comparing control (DI water, blue), California-native *Phlebiopsis gigantea* (red), and borate (black) as measured by % of colonizable area by *Heterobasidion irregulare*. The independent trials included a field stump trial (Inyo), a trial on installed vertical logs (stump surrogate; Modoc), and a trial conducted at 20 °C on wood discs (Laboratory). Different letters indicate statistical significance of a Tukey's post-host means comparison test.

Table 3
Estimated treatment efficacy (difference from control) of California isolates of *Phlebiopsis gigantea* against Heterobasidion Root Disease, drawn from all currently available efficacy trials.

	Infection		Deviance	
Trial	*Phlebiopsis*	Borate	*Phlebiopsis*	Borate
Inyo stump	46.9 %	97.2 %	58.4 %	97.6 %
Modoc log trials	23.8 %	81 %	−4.3 %	97.6 %
Laboratory wood disc	100 %	100 %	100 %	100 %
Poloni et al. stump†	11.5 %	63.5 %	55.6 %	84.7 %
Poloni et al. lab trial (wood blocks)†	29.4 %	52.9 %	–	–
Mean	43.3 %	78.9 %	52.4 %	95 %

† Trial conducted against Heterobasidion occidentale; Poloni et al., (2021).
– not reported

Management Task Force, 2021). While climate change-driven increases in annual temperature and the severity and frequency of drought are major contributors to these wildfire problems (Williams et al., 2022, 2019), increased stand densities and shifts to more fire-sensitive species in response to fire suppression are additional contributors to the crisis (Dolanc et al., 2014; Knapp et al., 2013; MacDonald et al., 2023). The major aim of the current investment is to substantially increase application of thinning, mastication, and prescribed fire (Forest Management Task Force, 2021). Thinning of stands, especially the relatively flat, accessible, and valuable Jeffery Pine forests of the eastern Sierra Nevada slope will often be targets of these treatments, especially when they are located near population centers (Safford et al., 2009). If past forest responses to management are accurate forecasts of future disease conditions, then a period of stump creation from fuels reduction treatments in these forests will lead to emergence of Heterobasidion root disease in many California forests (Rizzo and Slaughter, 2001; Slaughter and Rizzo, 1999). Widespread emergence of the disease could diminish the benefits of fuels treatments overall as the pathogen will reduce host growth rates and limits forest reestablishment in disease centers for decades (Flores et al., 2023; Rizzo et al., 2000). As the only legal treatment for this disease in California – borates – could face regulatory phase-out, there is a clear need to test and improve *P. gigantea* as a biocontrol for California's *Heterobasidion* pathogens.

CRediT authorship contribution statement

Cobb Richard: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Investigation, Funding acquisition, Formal analysis, Data curation. **Edoardo Scali:** Writing – review & editing, Validation, Project administration, Methodology, Investigation, Data curation. **Schmidt Douglas:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Tina Popenuck:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Data curation. **Adrian Poloni:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Matteo Garbelotto:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

We declare no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.foreco.2025.122930.

Data availability

Data will be made available on request.

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