Biomarkers Identify Coast Live Oaks That Are Resistant to the Invasive Pathogen *Phytophthora ramorum*[1]

Brice A. McPherson,[2] Sylvia R. Mori,[3] Anna O. Conrad,[4] Stephen Opiyo,[4] Pierluigi Bonello,[4] and David L. Wood[2]

Abstract

California coast live oaks (*Quercus agrifolia*) trees have suffered large losses from sudden oak death, caused by the introduced oomycete *Phytophthora ramorum*. In this review paper, we discuss oak plant chemistry as a potential predictor of disease susceptibility. We have recorded an annual mortality rate of three percent in long-term monitoring plots in Marin County, resulting in greater than 40 percent loss since 2000. Despite this mortality rate, asymptomatic trees still persist in many heavily infected stands. We hypothesized that varying responses to *P. ramorum*, including apparent recovery from infections, reflected phenotypic differences in susceptibility. In a Marin County inoculation study, a logit model showed that external canker lengths measured 9 months following inoculation predicted both resistance and survival 7 years later. The distribution of canker length was consistent with quantitative resistance to *P. ramorum*. The role of plant chemistry in resistance was examined by quantifying soluble phenolics in phloem methanol extracts prepared from the surviving trees. A logistic regression model found that expression of resistance was associated with total phenolics and four phenolic compounds; ellagic acid, a partially characterized ellagic acid derivative, and two chromatographic peaks representing two uncharacterized phenolic compounds. *In vitro* tests showed that ellagic acid was fungistatic against *P. ramorum* and total phenolics were fungicidal at physiologically relevant concentrations. A subsequent inoculation study in Briones Regional Park, Contra Costa County, California, showed that some of the same compounds were correlated with resistance. The association of certain phenolics with resistance may facilitate the use of biomarkers in minimally invasive assays to predict the response of trees to *P. ramorum*, thereby increasing the options for managing threatened forests.

Key words: biomarkers, coast live oak, logistic regression, phenolic glycosides, *Phytophthora ramorum*

Introduction

Introduced pathogens characteristically encounter host plants with varying levels of resistance. Significant examples of such pathosystems affecting North American trees include chestnut blight [American chestnut (*Castanea dentata*) and *Cryphonectria parasitica*]; Dutch elm disease [American elm (*Ulmus Americana*) and *Ophiostoma ulmi* and related species]; butternut canker [butternut (*Juglans cinerea*) and *Sirococcus clavigignenti-juglandacearum*]; and beech bark disease [American beech (*Fagus grandifolia*) and *Neonectria faginata* and *N. ditissima*, which invade wounds caused by the scale insect *Cryptococcus fagisuga*]. Chestnut has been functionally eradicated from its original range in the eastern United States;

[1] An abbreviated version of this paper was presented at the Seventh California Oak Symposium: Managing Oak Woodlands in a Dynamic World, November 3-6, 2014, Visalia, California.

[2] Department of Environmental Science, Policy, and Management, University of California, Berkeley, CA 94705. (bmcpherson@berkeley.edu).
[3] USDA, Forest Service, Pacific Southwest Research Station, Albany, CA 94710.
[4] Department of Plant Pathology, The Ohio State University, Columbus, OH 43210.

American elms are now uncommon forest and urban trees; the uncommon butternut is becoming even less common and is considered endangered in certain areas; and beech is in decline across much of its habitat. This paucity of host resistance is likely due to the lack of coevolutionary history between the partners in each pathosystem.

Since the first observed tanoak mortality in Marin County in 1994, losses of tanoaks (*Notholithocarpus densiflorus*) and coast live oaks (*Quercus agrifolia*) to sudden oak death (SOD), caused by *Phytophthora ramorum*, have been estimated to be in the millions of trees. Despite concerns expressed early in the epidemic that coast live oak might become extinct in California, trees presently persist in heavily impacted stands that have been infested since the late 1990s (McPherson and others 2010). These survivors often exhibit evidence of previous infections, such as callus tissue at the margins of old cankers, but other trees show no evidence of infection (McPherson and others 2014, Swiecki and Bernhardt 2013). Lack of manifest infection suggests that those trees may be resistant, but until now it was not possible to establish if those individuals are truly resistant, and not just escapes, without resorting to invasive techniques based on artificial inoculation with the pathogen.

Efforts to stop the expansion of *P. ramorum* beyond its current geographic range in the coastal forests of California and southwestern Oregon have met with limited success (Goheen and others 2013). Within infested areas the options for affecting the course of the disease are quite limited. Removal of hosts that support sporulation, such as California bay laurel (*Umbellularia californica*) and tanoak, has been proposed and implemented in limited sites (Goheen and others 2013; Valachovic and others 2013), although the long-term efficacy of this approach has not been demonstrated and is only likely to slow an inevitable process, while altering the species composition and stand structure of those forests. In addition, such an approach does not account for other possible sources of inoculum. With a host range that encompasses most of the native woody species in these coastal forests, *P. ramorum* has a large array of potential hosts to support sporulation. It is also noteworthy that past efforts to control tree diseases by eliminating alternative hosts are not encouraging. Probably the best example is that of white pine blister rust (caused by *Cronartium ribicola*), which continues to expand its range despite a half century-long massive and costly project to remove the primary hosts, *Ribes* spp. (Maloy 1997).

Given the vast areas already infested and the large number of hosts that may support sporulation, the most practical, economical, and ecological approach to dealing with SOD in coast live oaks may be to let the pathogen cull the most susceptible individuals, thereby increasing the proportional representation of the survivors (Telford and others 2014). This approach is predicated on the existence of sufficient resistance within host populations. Here we present evidence for such resistance and consider the implications for managing forests and woodlands under the pressure of the SOD epidemic, based on the notion that "…genetic resistance is the cornerstone of plant protection against insect pests and diseases in conducive environments." (Conrad and others 2014).

Evidence for Resistance

Long-term studies

Long-term observation plots are the best source of information about such disease parameters as infection and mortality rates, survival times, and environmental influences on likelihood of infection. In plots monitored since March 2000, we have recorded the persistence of coast live oaks in three categories: trees that survived

infections, with thick callus growth on the margins of cankers; trees that previously expressed bleeding cankers but subsequently were no longer symptomatic; and trees that never showed symptoms (McPherson and others 2010). In addition, the apparent rate of new infections has been falling since 2000, which suggests that there are trees in the populations that are less susceptible to infection (McPherson and others 2010).

Mechanical Inoculation: long-infested stands

Mechanical inoculation has been the most reliable means of assessing resistance and susceptibility of trees to pathogenic microorganisms (for example, Gordon and others 1998). Although the direct introduction of *P. ramorum* culture into subcortical tissue likely represents an excessive dose, the resulting disease expression is consistent with long-term observations in stands that have been naturally exposed to the pathogen. In 2002, we inoculated 40 asymptomatic mature coast live oaks in each of two Marin County sites. Some trees died in less than 18 months, others still survive, although clearly in decline (displaying beetle attacks and secondary fungal colonization), while some trees never bled (McPherson and others 2014). Removal of the outer bark showed distinct reaction zones associated with the inoculation sites, confirming that all inoculated trees were infected. Across the populations of inoculated trees, canker size varied widely between individuals. In another Marin County inoculation study (unpublished), inoculations in trees with multiple stems produced cankers that were almost exactly the same size in the stems sharing the same base, suggesting strong genetic control for resistance.

Other evidence that is suggestive of genetically-controlled variation in resistance was shown in branch inoculations of coast live oaks. Dodd and others (2005) found significant variation in lesion length among individuals, but little evidence for population-level variation in response to inoculations.

The observation that trees differ in their response to both mechanical and natural inoculation suggested that genetically-controlled host chemistry might explain this difference. Ockels and others (2007) showed that several phenolic compounds varied in concentration between coast live oaks that were classified as asymptomatic and naturally infected. The tissue attacked by *P. ramorum* is primarily phloem, the nutrient-rich substrate that also serves as the defensive zone against pathogens and insects once the outer bark is breached. In oaks, the principal defensive response is believed to be due to phenolics, aromatic compounds with alcohol substituents on the benzene ring. Many if not most plant phenolic compounds occur *in vivo* as glycosides, in which the aromatic moiety is esterified to various saccharides (Hopkinson 1969). In naturally infected trees, phenolic compounds varied quantitatively between samples taken adjacent to canker margins and those excised 60 cm away from the canker margins (Ockels and others 2007). Phloem concentrations of two phenolic compounds, gallic acid and tyrosol, differed by infection status of the host tree. *In vitro* bioassays of these two compounds showed dose-dependent inhibition of *P. ramorum*, as well as of *P. cinnamomi*, *P. citricola*, and *P. citrophthora*. However, the relationships between disease status and compound concentrations were not consistent. This inconsistency was attributed to uncertainty about disease stage, such as time since infection, possible changes associated with the presence of beetles and fungi, as well as possible between-tree variability in phenolic profiles (Ockels and others 2007).

In 2009 we documented the progress of the disease for each tree in the Marin County inoculation study. Phloem samples were collected from inoculated trees in one of the two sites; the trees were categorized as **Symptomatic** (S; bleeding, with or

without beetle attacks), **In Remission** (IR; formerly recorded as bleeding, but subsequently asymptomatic by 2009), and **Putatively Resistant** (PR; never bled). IR and PR trees can be considered functionally resistant to the pathogen, and comparisons between phloem phenolics extracted from S and (IR + PR) trees showed consistent differences in ellagic acid, a partially characterized tyrosol derivative, and an unidentified compound (Nagle and others 2011).

We reanalyzed the data of Nagle and others (2011) using a logit model, based on 79 observations for survival and 64 observations for resistance from the 79 original randomly selected trees from two sites in 2002, to explore the relationship between external canker length measured early in the infection process (9 months after inoculation) and two measures of response to inoculation after 7 years: resistance and survival (McPherson and others 2014). We defined a tree as resistant if it never bled or ceased bleeding and was never attacked by beetles. Approximately 30 percent of the inoculated trees never developed the external bleeding cankers that are the most obvious symptom of *P. ramorum* infection, yet removal of bark at the inoculation sites showed reaction zones confirming that the inoculations had been initially successful. Canker length measured 9 months after inoculation predicted both resistance and survival after 7 years. For both, resistance and survival logistic regressions, the canker length was significant ($P < 0.0001$) (fig. 1).

Phloem concentrations of phenolic compounds predicted resistance, specifically ellagic acid, a partially characterized ellagic acid derivative, two putative uncharacterized compounds (represented by chromatographic peaks), and total phenolics (the phloem extract) (McPherson and others 2014). A logit model found that increasing concentrations of ellagic acid and two other compounds were associated with increased probability of resistance, and one compound was associated with reduced resistance (fig. 2). The model could be used to identify a threshold level corresponding to 80 percent probability of resistance. *In vitro* assays against *P. ramorum* confirmed that both ellagic acid and total phenolics were fungistatic at physiologically relevant concentrations and total phenolics were fungicidal at the highest concentration tested, which was within the range found in the phloem. These studies offer the prospect that the resistance status of a coast live oak can be predicted by analyzing biomarkers of resistance in small samples of phloem from trees prior to exposure to the pathogen. The logit model also helps to illustrate that biomarkers need not be positively associated with resistance to be valuable indicators of the host response.

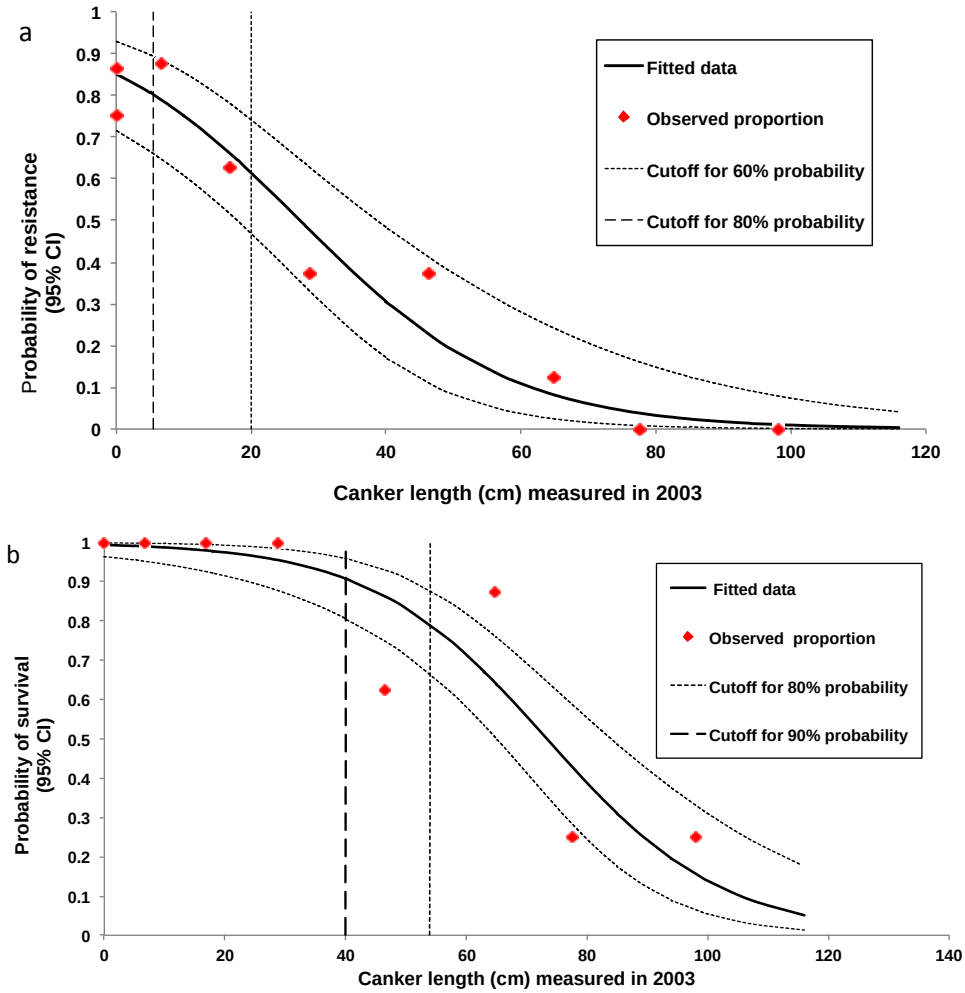

Figure 1—Probability that an infected coast live oak will (a) express resistance (either Putatively Resistant or In Remission) or (b) survive 7 years after inoculation. Observed proportions denote mean canker lengths binned into discrete intervals for illustration purposes. Dashed vertical lines in (a) show threshold canker lengths for 80 and 60 percent probabilities of resistance and in (b) show threshold canker lengths for 90 and 80 percent probabilities of survival (McPherson and others 2014).

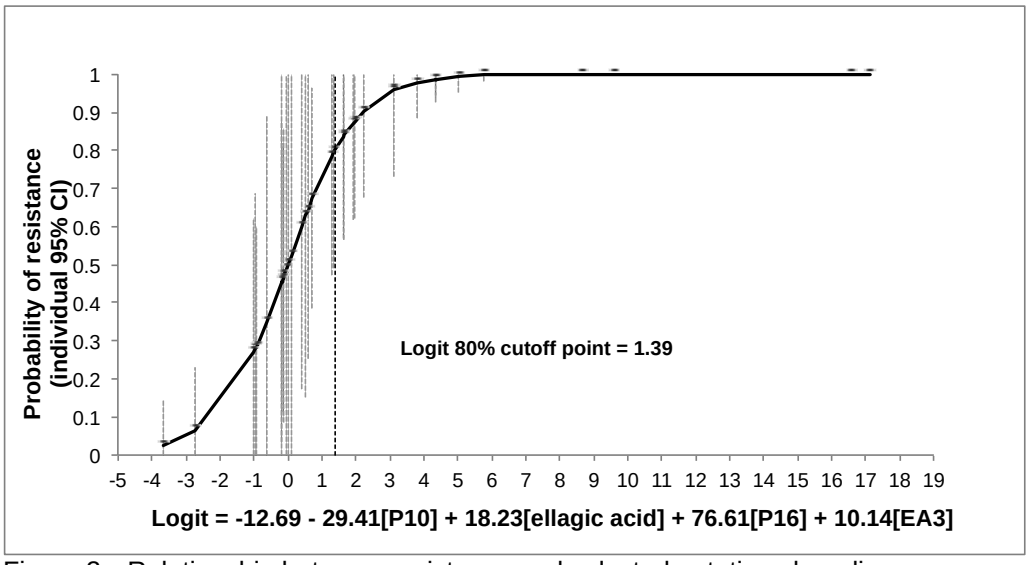

Figure 2—Relationship between resistance and selected putative phenolic biomarkers of resistance. The plot shows the estimated probability of resistance and logit values. The probability of resistance is greater than 80 percent when logit values are greater than 1.39 (dashed line) (McPherson and others 2014).

Mechanical inoculations: lightly or uninfested stands

We recognized that infection might change the phloem phenolics composition, particularly if defensive compounds are mobilized in response to the pathogen, bark and ambrosia beetles, or fungi that follow infection. In addition, the coast live oaks in Marin County already had been exposed to *P. ramorum* for an unknown time period when we inoculated them, likely 5 or more years, and thus the most susceptible trees probably had already been infected and a significant fraction killed. A better assessment of the validity of the relationship of phloem phenolic composition to resistance would be found in uninfested stands, where *P. ramorum* was still absent or in the earliest stages of invasion. The presence of *P. ramorum* was first confirmed in the East Bay in 2001 near Castro Valley and in Wildcat Canyon Regional Park. We chose to work in Briones Regional Park, which lies about 8 km east of the East Bay Hills. In 2010 the disease was only recorded in a small number of oak-bay stands at lower elevations. We reasoned that if the extensive coast live oak stands at higher elevations had yet to be exposed to *P. ramorum*, trees in these sites would provide a truer representation of the population-level response to this pathogen because the more susceptible coast live oaks would still be present and in addition, any possible induced defensive response to the pathogen would be unlikely.

At two sites 2 km apart, we randomly selected 600 asymptomatic mature coast live oaks and collected phloem from each tree, then inoculated 154 randomly selected trees at two locations with *P. ramorum* (courtesy of Dr. David Rizzo; initially isolated from California bay laurel), and left the other 450 trees for long-term monitoring. After 1 year we collected a second set of phloem samples from all the trees. Phloem from a group of 14 mature asymptomatic coast live oaks in a different part of the park was sampled at 3-month intervals over a 1-year period to determine if phenolic composition changed seasonally (Conrad and others, unpublished data). The un-inoculated trees will be revisited annually as the expected expansion of *P. ramorum* into these stands proceeds. In 2014 about 5 percent of this initially

asymptomatic cohort showed symptoms of *P. ramorum* infection (Conrad and others, unpublished data).

The inoculated trees were rated after 22 months as: **Susceptible** – brown leaves, or stem failure resulting from *P. ramorum* infection – dead and thus having no effective resistance; **Symptomatic** – long-term bleeding and/or beetle attacks and fungal fruiting observed – susceptible but with some ability to prolong survival; and **In Remission** – showing bleeding soon after inoculation, but no longer bleeding and without beetle attacks – the most likely to survive for the long-term. Consistently with our previous studies, trees classified as In Remission were considered resistant. The mean length of the two external cankers on each tree was determined 10 months after inoculation. Trees that were classified as In Remission or Susceptible, that is, those at the extremes of the symptom expression distribution, had significantly different canker lengths (independent t-test, $P < 0.001$) (Conrad and others 2014). For purposes of phenolics characterization, we limited the analyses to these two groups of trees.

The consistent relationship we found between phenolic composition and the In Remission phenotype requires high performance liquid chromatography (HPLC) chromatography to separate peaks for subsequent analysis (McPherson and others 2014). On the other hand, Fourier transform infra-red spectroscopy (FT-IR) is an analytical method for characterizing complex organic mixtures that can provide "chemical fingerprints". These are based on spectra that are unique to specific mixtures or individual compounds, and do not necessitate specific knowledge of the underlying chemical composition. Methanol extracts of phloem samples were analyzed using an Excalibur 3500GX FT-IR benchtop unit (Digilab, Randolph, MA). After signal processing and statistical analysis using soft independent modeling of class analogy (SIMCA) (Subramanian and others 2007), 100 percent of the FT-IR spectra from each category, Susceptible and In Remission (with outliers removed), were classified correctly (Conrad and others 2014). The FT-IR analysis also could predict the concentrations of both ellagic acid and an unidentified phenolic compound, as determined independently by HPLC analysis, that differ quantitatively between resistant and susceptible coast live oaks (Conrad and others 2014).

Discussion

As *P. ramorum* becomes established in a coast live oak stand, the early phase of the epidemic is characterized by the appearance of numerous dying trees where not long before the forest had appeared healthy: hence the term sudden oak death (Svihra 1999). We have recognized for some time that the sudden appearance of oak mortality is a function of our perception; although some infected trees die rapidly, more often the disease process is one of slow decline. The distribution of canker lengths following inoculation with the pathogen is consistent with quantitative, multi-gene, potentially durable resistance (Geiger and Heun 1989). We can now recognize that coast live oaks within a stand express a range of potential responses to *P. ramorum.* Realizing that a significant core of potentially resistant trees will remain after the primary impact of the epidemic has passed, new less destructive, and likely more successful management options become available.

Based on our spatially distributed, extensive longitudinal surveys, it appears that coast live oaks less than 10 to 15 cm diameter at breast height (1.37 m) only rarely become infected with *P. ramorum* (McPherson and other 2005, 2010). This phenomenon of age- or size-dependent susceptibility is presently not understood. If resistance is durable and heritable, the preservation of mature resistant trees may

maintain seed sources for future stands, even if this results in smaller mean tree size. To date there has been no practical way to assess the response of an individual coast live oak to natural infection by *P. ramorum*, other than by mechanical inoculation. Clearly, this is not useful other than as an experimental technique. The ability we have developed to predict the response of a specific tree to the pathogen provides a tool that may allow land managers to design proactive approaches to dealing with this epidemic. For example, one might wish to protect stands with large components of resistant trees from fires or development and to propagate resistant germplasm.

We have shown that even in mixed evergreen forests in which *P. ramorum* has been present for as long as 15 years, new infections continue to occur, although at lower rates than early in the epidemic (McPherson and others 2010). The survivors appear to have phloem chemistry that is correlated with resistance/susceptibility to the pathogen. Some of the compounds occurring in higher concentrations in resistant trees exhibit *in vitro* activities that are consistent with roles as anti-pathogen agents. Other compounds may not be physiologically active in defense against the pathogen, but are correlated with the tree's response to the pathogen and may serve as readily identifiable biomarkers of resistance. Unidentified compounds associated with resistant phenotypes (Conrad and others 2014, McPherson and others 2014) remain to be tested *in vitro*. The HPLC method requires skilled technical operators and laboratory space equipped with expensive instrumentation. On the other hand, while the FT-IR method also involves specialized instruments and skilled technicians, it requires much less lab preparation per sample and may be amenable to portable field operation. We are currently working to refine the methodology and to assess its reliability for detecting resistance in naturally exposed coast live oaks (McPherson and others, unpublished). If this method is shown to be consistently accurate, other forest tree species affected by introduced and co-evolved pathogens may be amenable to similar screening for resistance.

Acknowledgments

Funding has been provided by the USDA Forest Service, Forest Health Protection, and the Ohio Agricultural Research Development Center, Ohio State University. We gratefully acknowledge the East Bay Regional Park District for access to Briones Regional Park and to Marin County Open Space Department for access to Loma Verde and San Pedro Mountain Open Space Preserves.

References

Conrad, Anna O.; Rodriguez-Saona, Luis E.; McPherson, Brice A.; Wood, David L.; Bonello, Pierluigi. 2014. **Identification of *Quercus agrifolia* (coast live oak) resistant to the invasive pathogen *Phytophthora ramorum* in native stands using Fourier-transform infrared (FT-IR) spectroscopy.** Frontiers in Plant Science 5: 1–9.

Dodd, Richard S.; Huberli, Daniel; Douhovnikoff, Vlad; Harnik, Tamar Y.; Afzal-Rafii, Zara; Garbelotto, Matteo. 2005. **Is variation in susceptibility to *Phytophthora ramorum* correlated with population genetic structure in coast live oak (*Quercus agrifolia*)?** New Phytologist 165: 203–214.

Geiger, Hartwig H.; Heun, Manfred. 1989. **Genetics of quantitative resistance to fungal diseases**. Annual Review of Phytopathology 27: 317–341.

Goheen, Ellen M., Kanaskie, A., Hansen, E., Sutton, W, Reeser, P., Osterbauer, N. 2013 Monitoring the effectiveness of *Phytophthora ramorum* eradication treatments in Oregon tanoak forests. In: Frankel, S.J., Kliejunas, J.T., Palmieri, K.M., Alexander, J.M., eds. Proceedings of the Sudden Oak Death Fifth Science Symposium. Gen. Tech. Rep. PSW-

GTR-243. Albany, CA: U.S. Department of Agriculture, Forest Service, Pacific Southwest Research Station. 119.

Gordon, T.R.; Wikler, K.R.; Clark, S.L.; Okamoto, D.; Storer, A.J.; Bonello, P. 1998. **Resistance to pitch canker disease, caused by *Fusarium subglutinans* f sp. *pini*, in Monterey pine (*Pinus radiata*)**. Plant Pathology 47: 706–711.

Hopkinson, Shirley M. 1969. **The chemistry and biochemistry of phenolic glycosides**. Quarterly Review of the Chemical Society 23: 98–124.

Maloy, Otis C. 1997. **White pine blister rust control in North America: a case history.** Annual Review of Phytopathology 35: 87–109.

McPherson, Brice A.; Mori, Sylvia R.; Wood, David L.; Kelly, N. Maggi; Storer, Andrew J.; Svihra, Pavel; Standiford, Richard B. 2005. **Sudden oak death in California: disease progression in oaks and tanoaks.** Forest Ecology and Management 213: 71–89.

McPherson, Brice A.; Mori, Sylvia R.; Wood, David L.; Storer, Andrew J.; Svihra, Pavel; Kelly, N. Maggi; Standiford, Richard B. 2010. **Responses of oaks and tanoaks to the sudden oak death pathogen after 8 years of monitoring in two coastal California forests.** Forest Ecology and Management 259: 2248–2255.

McPherson, Brice A.; Mori, Sylvia R.; Opiyo, Stephen O.; Conrad, Anna O.; Wood, David L.; Bonello, Pierluigi. 2014. **Association between resistance to an introduced invasive pathogen and phenolic compounds that may serve as biomarkers in native oaks.** Forest Ecology and Management 312: 154–160.

Nagle, Annemarie M.; McPherson, Brice A.; Wood, David L.; Garbelotto, Matteo; Bonello, Pierluigi. 2011. **Relationship between field resistance to *Phytophthora ramorum* and constitutive phenolic chemistry of coast live oak**. Forest Pathology 41: 464–469.

Ockels, Frances S.; Eyles, Alieta; McPherson, Brice A.; Wood, David L.; Bonello, P. 2007. **Phenolic chemistry of coast live oak response to *Phytophthora ramorum* infection.** Journal of Chemical Ecology 33: 1721–1732.

Subramanian, A.; Ahn, J.; Balasubramanian, V.M.; Rodriguez-Saona, L. 2007. **Monitoring biochemical changes in bacterial spores during thermal and pressure-assisted thermal processing using FT-IR spectroscopy**. Journal of Agricultural and Food Chemistry 55: 9311–9317.

Svihra, P. 1999. **Sudden death of tanoak, *Lithocarpus densiflorus*.** Pest Alert No. 1. Novato, CA: University of California Cooperative Extension, Marin County. 2 p.

Swiecki, T.J.; Berhardt, E.A. 2013. **A reference manual for managing sudden oak death in California.** Gen. Tech. Rep. PSW-GTR-242. Albany, CA: U.S. Department of Agriculture, Forest Service, Pacific Southwest Research Station. 129 p.

Telford, A.; Cavers, S.; Ennos, R.A.; Cottrell, J.E. 2014**. Can we protect forests by harnessing variation in resistance to pests and pathogens?** Forestry. doi:10.1093/forestry/cpu012. (07 February 2015).

Valachovic, Y., Lee, C., Tweig, B., Rizzo, D., Cobb, R., Glebocki, R. 2013. Suppression of *Phytophthora ramorum* infestations through silvicultural treatment in California's north coast. In: Frankel, S.J., Kliejunas, J.T., Palmieri, K.M., Alexander, J.M., eds. Proceedings of the Sudden Oak Death Fifth Science Symposium. Gen. Tech. Rep. PSW-GTR-243. Albany, CA: U.S. Department of Agriculture, Forest Service, Pacific Southwest Research Station. 108-113.