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Association between resistance to an introduced invasive pathogen and phenolic compounds that may serve as biomarkers in native oaks

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ABSTRACT

California coast live oaks (*Quercus agrifolia* Nee) have suffered large losses from sudden oak death, caused by the introduced oomycete *Phytophthora ramorum*. Infected mature coast live oaks exhibit cankers on the main stem that produce a viscous sap-derived exudate, referred to as bleeding. Subsequent attacks by ambrosia and bark beetles, followed by the activity of fungi introduced by these insects, have led to mortality levels greater than 50% since the mid-1990s. Despite an infection rate of 5% year^{-1}, asymptomatic trees still persist in many heavily infected stands after more than 15 years of exposure to the pathogen. We hypothesized that varying responses to *P. ramorum*, including apparent recovery from infections, reflected phenotypic differences in susceptibility. In this study we evaluated the relationship between the early development of symptoms in mature trees that were experimentally inoculated with *P. ramorum* and long-term survival. A logit model showed that external canker lengths measured 9 months following inoculation predicted survival 7 years later. We defined resistance to *P. ramorum* in the surviving trees as absence or cessation of bleeding after the 7 years of the study and absence of beetle attacks on bleeding trees. Probability of resistance was also predicted by external canker length measured 9 months after inoculation. Canker length distribution 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 four phenolic compounds; ellagic acid and a partially characterized ellagic acid derivative, and two chromatographic peaks that represent two uncharacterized phenolic compounds. Ellagic acid and a crude methanol extract from coast live oak phloem (total phenolics) were fungistatic when assayed *in vitro* at physiologically relevant levels and total phenolics were fungicidal at the highest concentration tested. 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.

1. Introduction

One consequence of rapidly increasing international trade is the introduction of forest pathogens and insects into habitats where they lack an evolutionary history with their hosts (Langor and Sweeney, 2009). These introductions constitute long-term threats to the ecological integrity of forests worldwide. Forests are particularly vulnerable to non-native organisms because trees have long generation times, which limit their capacity to be selected for resistance. In addition, early detection is difficult, and complete eradication is usually impossible, because forests tend to be spatially and structurally heterogeneous and the pests tend to spread rapidly and unimpeded, which is particularly true for air dispersed pathogens. Thus, invasive organisms may escape detection until considerable range expansion has occurred. The accelerating introductions of alien invasive pathogens and insects in North American forests include such iconic examples as *Ophiostoma ulmi* (Buisman) Melin & Nannf and *O. novo-ulmi* Brasier (cause of Dutch elm disease) (Brasier, 2000), *Cryphonectria parasitica* (Murrill) Barr (chestnut blight) (Tainter and Baker, 1996), *Lymantria dispar* L. (gypsy moth) (Elkinton and Liebhold, 1990) and, more recently, *Agrilus planipennis* Fairmaire (emerald ash borer) (Cappaert et al., 2005), *Raffaelea lauricola* (T.C. Harr. Fraedrich & Agheyeva) (laurel wilt)

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(Harrington et al., 2008), and *Phytophthora ramorum* Werres et al. (sudden oak death) (Rizzo et al., 2002). Despite abundant evidence of the ecological and economic consequences of the inadvertent transfer of organisms between continents, prevention of importation of exotic pests through the proven pathways of solid wood packing and live horticultural material remains elusive (Campbell, 2001). Given these evident trends, the next best option for responding to these invasions is to identify, protect, and propagate resistant genotypes of susceptible tree species (Schoettle and Sniezko, 2007).

Sudden oak death is changing the stand structure and composition of forests in California (Rizzo et al., 2005) and threatens forests worldwide (Brasier and Webber, 2010). In northern and central California, more than 45 woody and herbaceous species are known hosts (APHIS, 2010), an unknown number of which may serve as sources of inoculum. California bay laurel (*Umbellifera californica* Hook & Arn. Nutt.), which is abundant throughout the region, is considered to be the primary source of new infections in coast live oaks (*Quercus agrifolia* Nee) (Davidson et al., 2005). Structurally and ecologically dominant tree species are among the relatively few hosts killed by the pathogen: coast live oaks, California black oaks (*Q. kelloggii* Newberry), canyon live oaks (*Q. chrysolepis* Liebm.), Shreve oaks (*Q. parvula* var. Shrevei (C.H. Muller)), and tanoaks (*Notholithocarpus densiflorus* Hook & Arn. (Manos, Cannon, & Oh)).

Coast live oak is a major component of many coastal forests within the range of the *P. ramorum* epidemic. Long-term monitoring plots in Marin County, California, documented coast live oak infection and mortality rates of 5% year^{-1} and 3% year^{-1}, respectively (McPherson et al., 2010). Infected trees develop main stem phloem cankers that exude a viscous sap, the external symptom known as bleeding, which closely tracks internal canker expansion (Rizzo et al., 2005). These cankers may eventually girdle and kill trees, but mortality is characteristically preceded by bark and ambrosia beetle attacks that are focused on bark overlying cankers, which can accelerate mortality and lead to trees breaking on the main stem while still alive (McPherson et al., 2008; 2010).

Since the first observed mortality of coast live oaks due to *P. ramorum* infections in 1997 (Svihra, 2001), more than half the mature individuals in some monitored populations have died, with as many as 25% of the remaining trees exhibiting symptoms in any year (McPherson et al., 2010). Survival analysis of bleeding coast live oaks provided estimated median survival times of 7.5–11.7 years, which were further reduced to about 2–4 years if infected trees were attacked by beetles (McPherson et al., 2010). Despite this rapid loss, individual trees without any evidence of infection still persist in these populations. Observations in long-term monitoring plots document previously symptomatic trees without any active cankers, but with distinct callus formation at the margins of quiescent cankers (McPherson et al., 2005). Also, the infection rate in long-term monitoring plots appears to have slowed in recent years (McPherson et al., 2010). Presently there is no way to determine if an asymptomatic tree is expressing resistance or simply has escaped exposure to *P. ramorum* spores.

Resistance to canker pathogens in trees can be assessed by measuring the size of cankers induced by inoculation of the pathogen into mechanical wounds. In resistant trees canker sizes are typically not significantly different from wounds alone or are below a critical threshold (Gordon et al., 1998). Resistance in coast live oaks can also be expressed as the absence of attacks by the beetles that accelerate disease progression and mortality (McPherson et al., 2008; 2010). To date, evidence for resistance of coast live oaks to *P. ramorum* has been observational and poorly documented. We report here that coast live oak populations artificially inoculated with *P. ramorum* express a range of responses that are consistent with quantitative genetic resistance. Furthermore, we report a new analysis of previously published data on the association of coast live oak phloem phenolic compounds with resistance to *P. ramorum* (Nagle et al., 2011) in the context of the resistance data presented here.

2. Material and methods

2.1. Study sites and inoculation procedure

The study was conducted in two forested sites on Marin County Open Space District lands. The Nike site, near China Camp State Park, occupied 0.96 ha, with the center at 37° 59′ 41.04″ N, 122° 30′ 4.26″ W. The Novato site encompassed 0.7 ha, centered at 38° 3.1′ 31″ N, 122° 32′ 20″ W. These stands had extensive *P. ramorum* infestations in coast live oaks prior to 2002. In each site we inoculated asymptomatic mature coast live oaks (39 in Nike and 40 in Novato, mean DBH = 31.1 cm) in July 2002, by using a 2-cm diameter hole saw to remove bark and phloem, inserting a 5 × 5 mm plug of *P. ramorum* culture grown on V8 agar culture, then replacing the bark section. We also wounded 20 trees in each site without inoculation (mock-inoculated) to test the effects of wounding alone on beetle attraction and lesion development (McPherson et al., 2008). An additional 20 asymptomatic trees per site were monitored for development of *P. ramorum* infection. Trees were monitored monthly for symptoms of *P. ramorum* infection (bleeding cankers) and beetle attacks for the first 18 months, then several times per year thereafter.

2.2. Relationship between canker length and survival

Canker length was estimated in April 2003, 9 months after inoculation, by measuring the maximum longitudinal length of the area showing external bleeding. Subcortical cankers and wounds (for mock-inoculated trees) were evaluated 7 years after inoculation, in July 2009, by removing the exterior bark around the inoculation site, photographing the exposed area with a digital camera with a reference length marker, and using ASSESS (American Phytopathological Society) image analysis software to quantify the wound or canker length.

We estimated the probability of survival to 2009 using a logit model (McCullough and Nelder, 1991) to regress on maximum external canker lengths measured in April 2003, as shown:

$$P(Y=1) = \exp(a + b\,\text{Canker length})/[1 + \exp(a + b\,\text{Canker length})] \quad (1)$$

where P is the probability of a tree surviving (Y = 1, if alive in 2009; Y = 0, if dead in 2009) and a and b are parameters to be estimated.

To create a reliability diagram, which plots the observed frequency against the predicted frequency, we binned the canker lengths into eight intervals by grouping the estimated probabilities of survival, and calculated the actual proportion of surviving trees to visually compare them against the survival predicted by the logit model. Parameters were estimated using SAS. 9.3 (Cary, North Carolina, USA) GLIMMIX procedure for over-dispersed data (to account for location variability over two sites).

2.3. Relationship between canker length and resistance

We consider as resistant those trees that, despite becoming infected, survive attack by *P. ramorum* and secondary organisms in the long run. Such resistant trees are characterized by healed cankers following artificial inoculations, or from contained or healed natural infections (Nagle et al., 2011). Because ambrosia and bark beetle attacks on *P. ramorum* cankers may considerably reduce survival times (McPherson et al., 2010) and introduce diverse fungi into trees that may contribute to tree death (McPherson et al.,