Sporulation of *Phytophthora ramorum* and *P. kernoviae* on Asymptomatic Foliage and Fruit[1]

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Abstract

Phytophthora ramorum and *P. kernoviae* are newly discovered invasive *Phytophthoras* causing leaf necrosis, shoot tip dieback (mostly on ornamental and forest understorey host species) and bleeding cankers on tree trunks of a wide range of plant species. Both pathogens are now present in south-west England. Sporulation occurs on infected shoots and foliage but not on bleeding stem cankers; thus foliar hosts are key in disease epidemiology. During evaluation of a two-year field trial established to assess infection periods and infection incidence, we discovered that naturally infected, asymptomatic leaves supported sporulation of both pathogens. Asymptomatic leaves of 79 percent of rhododendron trap plants exposed to natural inoculum yielded *P. kernoviae* in the first year of field trials and 53 percent in the second year, whereas 36 percent of trap plants yielded *P. ramorum* in the first year and 33 percent in the second. We realized that sporulation occurred because asymptomatic leaves subjected to baiting remained asymptomatic for the duration of the baiting period, but the baits were positive for the pathogen. The foliage of approximately 20 percent of the positive trap plants remained asymptomatic for the duration of the trial and baiting period, indicating that asymptomatic infection can endure for at least 8 days but may be as long as 22 days. In laboratory trials artificially-inoculated leaves and fruits supported *P. ramorum* sporulation, sporangia were consistently observed on asymptomatic leaves and fruit of several Mediterranean species.

Key words: *P. kernoviae, P. ramorum*, asymptomatic, artificially inoculated, foliage, fruit, natural inoculum, trap plants.

Introduction

P. ramorum and *P. kernoviae* are two recently discovered invasive *Phytophthoras* affecting a wide range of ornamental trees and shrubs as well as forest species (Brasier and others 2004). *Phytophthora ramorum* initially associated with shoot tip dieback and foliage necrosis of ornamental plants in nurseries in Europe (Werres and others 2001) is now known to be the cause of high mortality of native oak species and tan oaks (*Lithocarpus densiflorus*) in the coastal forests of California (Rizzo and

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others 2002). *Phytophthora kernoviae* was first discovered in 2003 in the U.K. on bleeding cankers of beech trees (*Fagus sylvatica*) and wild *Rhododendron ponticum* in woodlands and heritage gardens (Brasier and others 2005) but has recently been reported from soil and diseased *Annona cherimoya* (custard apple) fruit in New Zealand (http://www.maf.govt.nz/mafnet/press/240306fungus.htm). The diseases these pathogens cause include leaf necrosis and shoot tip dieback on foliar hosts which comprise mostly ornamental and forest understory species, and bleeding cankers on tree trunks, sometimes resulting in the death of trees.

Both pathogens share commonality in several biological aspects, including the production of deciduous sporangia adapted for aerial dispersal, which is thought to be the main mode of dissemination. Sporangia and zoospores produced from sporangia of *P. ramorum* are considered the primary inoculum propagules responsible for causing epidemics in California (Davidson and others 2005, Rizzo and others 2005). Neither pathogens form sporangia on bole cankers but do produce inoculum on infected foliage and twig cankers of certain host species (Brasier and others 2004, Davidson and others 2005, Rizzo and others 2005). Thus, for both of these *Phytophthoras* foliar hosts are a crucial component of the disease epidemiology because they are the platforms from which epidemics are driven.

Information about the comparative sporulation potential of susceptible hosts as well as prime infection periods and infection intensity under field conditions is essential to assess the risk posed by these pathogens. Thus, field trials were set up in Cornwall, U.K., to investigate this and *in vitro* tests were carried out to assess the comparative sporulation potential of *P. ramorum* on various host species. While processing plant material exposed to natural inoculum it became apparent that sporulation was occurring from asymptomatic foliage. Closer inspection of artificially inoculated material revealed that sporulation occurred consistently on fruit and foliage of certain host species in the absence of any visible symptoms. This paper gives a summarized account of the incidence of sporulation on asymptomatic foliage of plants exposed to field inoculum of *P. kernoviae* and *P. ramorum* during two seasons of observation, and the extent of sporulation on asymptomatic fruit following artificial inoculation with *P. ramorum*.

Materials and Methods

A detailed account of the methods employed in both the *in vivo* and *in vitro* trials are unpublished (Denman unpublished). Here we give a summary of the field trial methods then briefly describe the laboratory methods used in the artificial inoculations.

Field Trials

In the field trials trap plants were used as indicators of pathogen activity. Field study sites were set out at three woodland locations in south-west England, one naturally infected with *P. ramorum*, and the other two naturally infected with *P. kernoviae*. A single infected wild *Rhododendron ponticum* plant approximately 3 m high was selected at each site to serve as an inoculum source plant. Three 2-year-old, potted rhododendron ‘Cunningham’s White’ plants that had been raised in polytunnels were placed in the drip line of the canopy of the inoculum source plant at each site and replaced with new plants every two weeks. The exposed plants were processed in the

field by separating and bagging symptomatic and asymptomatic leaves from each plant. In the laboratory all leaves were surface sterilised by washing in 70 percent ethanol for 30 seconds, rinsing in sterile distilled water for 1 minute and then air-drying. Thereafter isolations were made from the dead-live junctions of symptomatic leaves, and all asymptomatic leaves were baited as described by Denman unpublished). Seven days after asymptomatic leaves were baited the baits were plated on synthetic mucor agar (SMA, Brasier and Kirk 2002) and the leaves in the bait box were examined for symptoms that might have developed during the 7 day incubation period. When this occurred isolations were made from the leaves to determine the cause of the symptoms (Denman and others 2005). If the baits were positive it indicated that sporulation occurred during baiting since the baits were floating approximately 5 to 10 mm above the surface of the leaves, inferring that zoospores swam to the baits and infected them. Data were gathered on whether or not trap plants had symptomatic and asymptomatic leaves on the same plant, the cause of the symptoms on symptomatic leaves, whether asymptomatic leaves were infected with the pathogen, and on the fate of the asymptomatic leaves during baiting as well as whether baits tested positive for the pathogen or not.

In vitro Fruit Inoculation

The artificial inoculation studies carried out with *P. ramorum* are described in detail (Denman unpublished; Moralejo and others 2006). An isolate of the EU1 lineage and one of the NA1 lineage were used to inoculate fruit of *Crataegus monogyna* (Hawthorn), *Laurus nobilis* (European bay tree), *Quercus ilex* (holm oak), *Rosa sempervirens* (rose) and *Smilax aspera* (rough bindweed). A 20 µl drop of 4×10^4 zoospores/ml was placed on the top of each of the fruits. Controls were inoculated with sterile distilled water. Fruit were incubated in moist chambers for 10 days, the first 48 hours in darkness followed by 12 hour/day exposure to fluorescent white light for the remaining 8 days. The percentage of asymptomatic fruit bearing sporangia in each host-isolate combination was recorded 10 days after inoculation. The numbers of asymptomatic fruit bearing sporangia were analysed using a generalised linear model with binomial error and logit link. The predicted proportions from the model were compared using a t-test.

Results and Discussion

Field Trials

Results are presented for two seasons data from June to November 2005 and 2006. There were 11 fortnightly observation frames per year, representing 132 rhododendrons at the *P. kernoviae* sites and 63 at the *P. ramorum* site (which only had 10 observation frames in 2006).

None of the trap plants had 100 percent infected foliage at any of the sites. Most trap plants had both symptomatic and asymptomatic foliage on the same plant but in some cases entire trap plants were visually disease-free at the end of the exposure period (table 1). For plants with both symptomatic and asymptomatic foliage, in the first year of the field trial 79 percent of plants exposed to *P. kernoviae* inoculum yielded the pathogen from asymptomatic foliage, and in the second year the corresponding figure was 53 percent (table 2). For plants exposed to *P. ramorum* inoculum 33 percent or more yielded the pathogen from non-symptomatic foliage in both years.

Table 1—Number of plants showing symptoms after being exposed to natural inoculation events occurring in 14 d exposure periods

Pathogen	*P. kernoviae*		*P. ramorum*	
Year	**2005**	**2006**	**2005**	**2006**
Appearance: Plants completely symptomatic	0	0	0	0
Appearance: Plants completely asymptomatic	$^{3}/_{66}$	$^{15}/_{66}$	$^{1}/_{33}$	$^{7}/_{30}$
Appearance: Plants with both symptomatic and asymptomatic leaves*	$^{63}/_{66}$	$^{51}/_{60}$	$^{32}/_{33}$	$^{23}/_{30}$

*Note: Isolations need to be made to confirm that symptoms are caused by the pathogens (results of this are unpublished).

Table 2—Number of trap plants with asymptomatic leaves that tested positive for *P. kernoviae* or *P. ramorum* after being subjected to a baiting treatment

Pathogen	*P. kernoviae*		*P. ramorum*	
Year	**2005**	**2006**	**2005**	**2006**
Appearance: Plants completely asymptomatic after exposed to potential natural inoculum for 14d	$^{2}/_{3}$	$^{6}/_{15}$	$^{0}/_{1}$	$^{2}/_{7}$
Appearance: Plants with both symptomatic and asymptomatic leaves on the same plant (testing asymptomatic leaves only)	$^{50}/_{63}$	$^{29}/_{51}$	$^{12}/_{32}$	$^{8}/_{23}$

The fate of the asymptomatic leaves that were baited and baits is summarized in *table 3*. For plants that had both symptomatic and asymptomatic leaves on the same plant, 12 to 20 percent of the baited asymptomatic leaves remained asymptomatic for the duration of the baiting period (regardless of pathogen), but gave rise to positive baits. This indicated that sporulation had occurred in the absence of any visible symptoms on leaves. Generally positive baits were obtained from about 60 to 70 percent of plants that had leaves that went on to develop symptoms during baiting process indicating that sporulation also occurred from these leaves (table 3).

More than 50 percent of plants that had completely asymptomatic foliage after exposure in the field to *P. kernoviae* inoculum yielded the pathogen from asymptomatic leaves, while the incidence was lower for those exposed to *P. ramorum* (table 2).

There was a higher incidence of asymptomatic infection and sporulation in year 2 than in year 1. This appears to correlate with weather conditions prevailing in year 2, where there were periods of unusually high temperature and dryness. Additionally, there had been very severe disease and consequent defoliation of the inoculum source plant in year one. With less foliage, inoculum production is likely to have been reduced. Thus the effect of both these factors (inoculum density thresholds and

environment factors, namely, temperature and humidity) on triggering asymptomatic infection and sporulation needs further investigation.

Table 3—Response of asymptomatic leaves and baits to the baiting treatment

			Response		
	Pathogen	**Year**	**Bait only positive**	**Leaves develop symptoms in bait box and baits positive**	**Leaves develop symptoms in bait box, baits negative**
Appearance: Plants completely asymptomatic after exposure to potential natural inoculum	Pk[a]	2005	$^{0}/_{3}$	$^{1}/_{3}$	$^{1}/_{3}$
		2006	$^{0}/_{15}$	$^{6}/_{15}$	$^{0}/_{15}$
	Pr[b]	2005	$^{0}/_{1}$	$^{0}/_{1}$	$^{0}/_{1}$
		2006	$^{2}/_{7}$	$^{0}/_{7}$	$^{0}/_{7}$
Appearance: Plants with both asympomatic and symptomatic leaves on the same plant (testing asymptomatic leaves only)	Pk	2005	$^{10}/_{50}$	$^{35}/_{50}$	$^{5}/_{50}$
		2006	$^{6}/_{29}$	$^{20}/_{29}$	$^{3}/_{29}$
	Pr	2005	$^{2}/_{12}$	$^{6}/_{12}$	$^{4}/_{12}$
		2006	$^{1}/_{8}$	$^{2}/_{8}$	$^{5}/_{8}$

[a] Pk = *Phytophthora kernoviae*
[b] Pr = *P. ramorum*

In vitro fruit inoculation

Sporulation occurred between 3 to 10 days on all fruit types tested. At one end of the scale almost all the rose hips (98 percent) supported sporulation with no indication of lesion development, compared with about 50 percent of hawthorn and European bay berries (table 4). Based on our results we believe that infected fruit may be an important source of inoculum in natural disease systems and could be significant in the nursery trade if fruit is present. Furthermore, in natural environments where disease is present, inoculum transmission through frugivorous birds may be an explanation for the emergence of new long-distance foci in disease outbreaks, the source of which are otherwise difficult to understand. There has been little research carried out on this aspect and more attention is merited.

Although our work has demonstrated that fruit and foliage can be infected and support sporulation without any visual symptoms much more work is required to get a better understanding of the range of conditions and thresholds under which these pathogens exist and behave in this way. Information on the incubation period (the time between infection and symptom development, Shurtleff and Averre 1997) and the latent period (time between infection and that infection producing infectious propagules; M.J. Jeger, Imperial College, personal communication) of both *P. kernoviae* or *P. ramorum* is required. Additionally the effects that inoculum density and environmental conditions, chiefly temperature and humidity, have on disease expression and inoculum production need investigation. Once more is known about the phenomenon of asymptomatic infection and sporulation its significance

with regard to the potential for introduction and spread by these pathogens through plant trade can be evaluated.

Table 4—Sporulation on asymptomatic detached fruit 10 d after artificial inoculation

Host	Common name	No. fruit tested	Fruit asymptomatic and sporulation (%)
Crataegus monogyna	Hawthorn	64	55 c*
Laurus nobilis	European bay tree	80	48 c
Quercus ilex	Holm oak	64	70 b
Rosa sempervirens	Rose	80	98 a
Smilax aspera	Rough bindweed	80	73 b

* Numbers followed by the same letters do not differ significantly (P<0.5).

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