

Infectivity and sporulation potential of *Phytophthora kernoviae* to select North American native plants

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Phytophthora kernoviae exhibits comparable epidemiology to *Phytophthora ramorum* in invaded UK woodlands. Because both pathogens have an overlapping geographic range in the UK and often concurrently invade the same site, it is speculated that *P. kernoviae* may also invade North American (NA) forests threatened by *P. ramorum*, the cause of Sudden Oak Death. This paper addresses the susceptibility of select NA plants to *P. kernoviae*, including measures of disease incidence and severity on wounded and unwounded foliage. The potential for pathogen transmission and survival was investigated by assessing sporangia and oospore production in infected tissues. Detached leaves of *Rhododendron macrophyllum*, *Rhododendron occidentale* and *Umbellularia californica*, and excised roots of *U. californica* and *R. occidentale* were inoculated with *P. kernoviae* and percent lesion area was determined after 6 days. Leaves were then surface sterilized and misted to stimulate sporulation and after 24 h sporangia production was assessed. The incidence of symptomless infections and sporulation were recorded. All NA native plants tested were susceptible to *P. kernoviae* and supported sporangia production; roots of *U. californica* and *R. occidentale* were both susceptible to *P. kernoviae* and supported sporangia production. Oospore production was also observed in *U. californica* roots. The results highlight the vulnerability of select NA native plants to infection by *P. kernoviae*, suggest that symptomless infections may thwart pathogen detection, and underscore the importance of implementing a proactive and adaptive biosecurity plan.

Keywords: forest biosecurity, host susceptibility, invasive disease, *Phytophthora kernoviae*

Introduction

Phytophthora kernoviae is a recently emerged plant pathogen in the UK, threatening natural ecosystems including woodland and native heathland, as well as heritage gardens and national plant collections (Beales *et al.*, 2006; Defra, 2008). The pathogen was first isolated in late 2003 from a bleeding canker on European beech (*Fagus sylvatica*) and foliar lesions on *Rhododendron ponticum* during surveys designed to detect woodland invasion by *Phytophthora ramorum* (Brasier *et al.*, 2005). The known geographic distribution of *P. kernoviae* is limited in Britain, with the highest frequency of forest infestation occurring in southwest England (Defra, 2008). In 2008 the pathogen was also reported in the Republic of Ireland (Irish Department of Agriculture, Food & Fisheries, 2009); it has also been recovered from soils in New Zealand (NZ) forests and isolated from diseased custard apple (*Anona cherimola*) in an abandoned NZ orchard (Ramsfield *et al.*, 2009). Historical evidence suggests that *P. kernoviae* has been in NZ for over 50 years, although it has not been associated with forest decline (Ramsfield *et al.*, 2009).

In infested UK woodlands, *P. ramorum* and *P. kernoviae* share similar symptomology and epidemiology (Defra, 2008). *Rhododendron ponticum*, an invasive and undesirable understory woodland plant, supports sporangia production of both pathogens, thereby providing primary inoculum for infection of neighbouring broad-leaf trees (Defra, 2008; Webber, 2009). Symptoms of *P. ramorum* and *P. kernoviae* are almost indistinguishable, with infection generally resulting in foliar necrosis and shoot dieback on *R. ponticum* and bleeding cankers on *F. sylvatica* (Defra, 2008). Sporangia are only formed on hosts with susceptible foliage, whereas trunk cankers are not known to support sporulation and therefore do not transmit the pathogens (Defra, 2008). In mixed-species woodlands, the majority of *P. ramorum* and *P. kernoviae* infections are on *R. ponticum* and associated *F. sylvatica* (Brown *et al.*, 2006). Both pathogens cause symptomless root infections on *R. ponticum* (Fichtner *et al.*, 2011), and *P. kernoviae* infects *F. sylvatica* roots (C.M. Brasier, Forest Research, Farnham, UK, personal communication; E.J. Fichtner, unpublished data). *Phytophthora kernoviae* is currently known to cause disease on 17 plant genera in 12 families, and rhododendron is the main host involved in pathogen transmission (Defra, 2008; Fera, 2010). Because *P. kernoviae* was only recently described and has only been studied in invaded UK woodland and heathland, little is known about the

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potential ecology and epidemiology of the pathogen in uninvaded systems. Additionally, the fact that the disease cycles of *P. kernoviae* and *P. ramorum* are similar in UK woodlands highlights concern regarding the potential for *P. kernoviae* to inhabit similar niches to *P. ramorum* in North American (NA) forests.

To address the potential risk of *P. kernoviae* to selected NA native plants, the role of three plant species in supporting infection, sporulation and sexual reproduction of *P. kernoviae* was investigated. Foliage of two NA native rhododendrons, *Rhododendron macrophyllum* (Pacific rhododendron) and *Rhododendron occidentale* (Western Azalea), as well as *Umbellularia californica* (California bay laurel), a foliar host of *P. ramorum*, were compared with *R. ponticum* leaves for susceptibility to *P. kernoviae*. Plants were rated for disease incidence, severity and sporulation potential, as well as the degree of symptomless infection and sporulation. Additionally, the potential susceptibility, sporulation and oospore production of *P. kernoviae* on *U. californica* and *R. occidentale* roots was investigated. To address concerns that new introductions of *P. kernoviae* could evade detection in NA diagnostic labs, the growth habits of the organism were compared on the selective media typically used in the UK versus that used in the USA. The overall goal of this work was to elucidate the pathogenicity of *P. kernoviae* to three key native NA plants and assess the potential role of these plants in pathogen transmission and survival.

Materials and methods

Source of plants and pathogen isolates

Healthy, symptomless foliage of *R. ponticum* was collected from the woodland surrounding Alice Holt Lodge in Farnham, Surrey, England. The Alice Holt site has no history of infestation with either *P. ramorum* or *P. kernoviae*. Three potted plants of *R. occidentale* and five potted plants of *U. californica*, in approximately 4 L containers, were purchased from commercial nurseries. Because potted *R. macrophyllum* plants were not available, foliage of *R. macrophyllum* was collected from each of two healthy symptomless specimens, labelled 1 and 2, at Trellisick Gardens, a National Trust property located near Truro, Cornwall, England.

Two *P. kernoviae* isolates (ROS SD8 and ROS SD9) were used for all laboratory inoculations. Isolates were collected from infested leaf litter in heathland containing extensive mortality of *Vaccinium myrtillus* caused by *P. kernoviae*.

Inoculation of plant material

Phytophthora kernoviae isolates were grown for 1 week on carrot agar (CA) at 18°C under a continuous lighting regime of 12 h white light followed by 12 h black light (300–400 nm range in wavelength). Three millilitres deionized water (dH₂O) were pipetted onto the surface of each colony, and colonies were gently scraped with a

glass rod to dislodge sporangia. Sporangia were then rinsed from plates and the resulting spore suspension was incubated at 4°C for 45 min to induce zoospore release. Zoospores were enumerated with a haemocytometer and a zoospore suspension of 10⁵ zoospores mL⁻¹ was used for all inoculations.

A multifactorial treatment design with two inoculum levels (non-inoculated control and inoculated), and two tissue types (wounded and non-wounded) was used. Five replicate leaves were used in each treatment. Wounded leaves were cut cross-wise below the leaf tip with a sterile scalpel, removing approximately 25% of the leaf area. Leaves were inoculated by dipping the leaf tips or wounded edges in the zoospore suspension for 30 s, with 50% of the leaf submerged. Uninoculated control leaves were similarly dipped in dH₂O. Leaves were then incubated in moist chambers at 20°C and misted daily for 6 days with sterile dH₂O. Moist chambers were composed of clear plastic storage boxes lined with paper towels thoroughly moistened with sterile dH₂O. Plastic storage boxes were covered with plastic cling film to maintain humidity for the duration of incubation of plant materials.

Assessment of disease incidence and severity

Six days after inoculation, all leaves were surface sterilized in 70% ethanol for 30 s then rinsed three times in dH₂O and blotted dry. The number of leaves showing symptoms in each treatment was recorded to determine disease incidence. Individual leaves were traced onto semi-transparent paper with the lesion zone(s) carefully delineated. The lesion components of the tracings were then carefully excised with dissection scissors and a scalpel. The full leaf tracing as well as the excised lesion tracings were weighed on an analytical balance and the leaf and lesion areas determined by standardizing the weight per unit area of the paper. Disease severity was determined by calculating percent lesion area, and differences in disease severity between hosts and wounded/unwounded tissue were determined with ANOVA and a Waller–Duncan K-ratio test ($K = 100$). All statistical analyses were completed using SAS statistical software.

Assessment of sporangia production

After leaves were surface sterilized and traced, all leaves were returned to moist chambers, misted with dH₂O, and incubated for 24 h at 20°C. Using a modification of the protocol of Denman (2008), sporangia production was assessed on non-wounded leaves. One millilitre of dH₂O was pipetted onto each leaf and leaves were scraped 10 times on each side with a metal spatula. Leaves were rinsed with 750 µL of dH₂O and each sporangia suspension was collected in a 2 mL microcentrifuge tube. A drop of cotton blue was added and each suspension was centrifuged for 3 min at 1245 g. The supernatant was decanted and the spores were resuspended in 20 µL dH₂O and vortexed for 30 s. Two drops of the suspension (10 µL each)

were placed on a glass slide and sporangia were counted under the compound microscope. The number of sporangia produced per square centimetre leaf area and per square centimetre lesion area was calculated and statistical differences between sporulation on different plants were determined with ANOVA using square root transformed data, and means separated using a Waller-Duncan K-ratio test ($K = 100$). The square root transformation was used because variance in spore count increased with increasing means (Tukey, 1977).

Reisolation of *P. kernoviae* from leaves

After sporangia were scraped from leaves, tissue samples from each leaf were submerged into SMA selective medium (Elliott *et al.*, 1966) and incubated in the dark at 18°C. If lesions were present, samples were excised from lesion margins; however, in the absence of a lesion, symptomless tissues were sampled. Tissues were randomly sampled from the inoculated portion of symptomless leaves, with an aggregation of six small samples totalling approximately 2 cm² of the leaf. Colonies generally emerged from plated tissue within 5 days, and were then subcultured onto CA for confirmation of *P. kernoviae* recovery. Presence of sporangia on symptomless leaves, and isolation of *P. kernoviae* from symptomless tissue were recorded.

Assessing oospore presence

Concurrent with reisolation, tissue samples from each unwounded leaf were excised and each sample was incubated in 2 mL microcentrifuge tubes containing 1.5 mL of 2.5 M KOH. After 1 week, KOH was decanted from tubes and fresh KOH was added to facilitate clearing of tissue. Foliar tissue remained in KOH for approximately 1 month before observation under the compound microscope. Presence or absence of oospores in foliage was noted.

Root inoculations

Roots of *U. californica* and *R. occidentale* plants were obtained for susceptibility studies with *P. kernoviae*. Roots of *R. macrophyllum* could not be included in the trial because containerized plants of this species were unavailable in UK nurseries. Fine roots were excavated from pots and rinsed thoroughly with dH₂O to remove all soil and debris. Fine roots were then cut into 4-cm-long segments, with 20 segments prepared of each species. Ten root segments of each species were then inoculated by complete submergence in a zoospore suspension (10^5 spores mL⁻¹) for 30 s and the other 10 were similarly submerged in dH₂O to serve as an uninoculated control treatment. Root segments were then incubated in moist chambers for 7 days at 20°C. After 7 days, all root segments were scored for symptom development. All root segments were then surface sterilized in 0.025% NaOCl for 5 min (Shishkoff, 2007), and rinsed three times

in dH₂O. Each root segment was then cut in half with a sterile scalpel, and one half was placed on SMA to determine infection by *P. kernoviae* and the other half in 2.5 M KOH to render tissue translucent. Putative *Phytophthora* species emerging from root segments were subcultured onto CA for morphological identification. Roots were incubated in KOH for approximately 15 days, and then rinsed with dH₂O, stained with aniline blue, and examined under the compound microscope. Presence of sporangia and/or oospores on root segments was noted.

Isolation and identification of *Phytophthora kernoviae*

In September 2008, 20 *R. ponticum* leaves showing symptoms were collected from a *P. kernoviae* infested forest near Truro, Cornwall, England. Leaves were then returned to the laboratory and two circular samples were excised along the lesion margin using a 1 cm diameter cork borer. One sample was placed on SMA (Elliott *et al.*, 1966) and the other on PARP (Erwin & Ribeiro, 1996). As putative *Phytophthora* isolates emerged from samples, they were transferred to CA for morphological identification. The frequency of *P. kernoviae* recovery was compared between tissues placed on SMA and PARP. Furthermore, differences in macroscopic colony morphology and microscopic hyphal characteristics were noted. The width of hyphae growing on SMA and PARP was determined by placing colonized agar on glass slides, staining with cotton blue, and flattening the sample with a cover slip. Digital images were then captured at $\times 20$ magnification from 10 colonies on each medium. Images were digitally enlarged, and using ANALYSIS software (Olympus Soft Imaging Solutions), two measurements of hyphal width were taken of each colony and the average width per colony was recorded. A two-tailed *t*-test with equal variance was then employed to determine whether hyphal width was affected by selective culture medium.

Results

Lesions were observed on inoculated leaves of *R. ponticum*, *R. occidentale*, *R. macrophyllum* and *U. californica*, while uninoculated leaves all remained free of any symptoms (Fig. 1). The dark, black lesions on inoculated *R. occidentale* were difficult to discern visually due to the lack of contrast between the lesion and the healthy, glossy, dark green, wrinkled tissue (Fig. 1b). Similarly, symptoms on *R. macrophyllum* foliage could easily be overlooked because of the small lesion size (Fig. 1c). Foliar *P. kernoviae* lesions were well defined on *U. californica* (Fig. 1d).

Because disease severity in wounded and non-wounded foliage varied significantly between experimental runs, the results of each run were analysed separately (Fig. 2). An interaction between wound treatment and plant species significantly affected disease severity in both experimental runs ($P \leq 0.01$) (Fig. 2). In the first run, wounded *R. ponticum* leaves exhibited significantly higher disease severity than unwounded (Fig. 2a), but no difference was

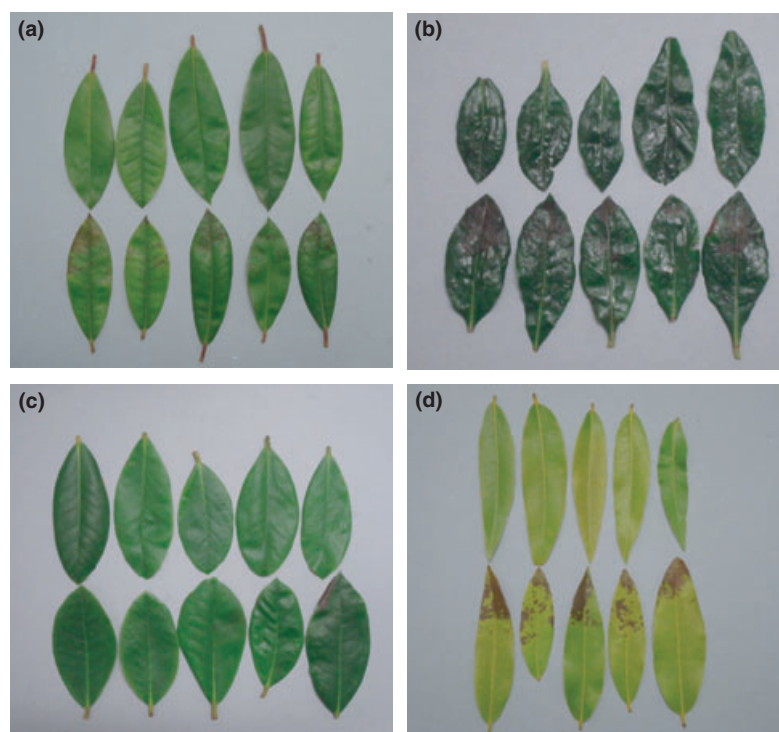


Figure 1 Inoculation of leaves of (a) *Rhododendron ponticum*, (b) *Rhododendron occidentale*, (c) *Rhododendron macrophyllum* and (d) *Umbellularia californica* with *Phytophthora kernoviae* resulted in foliar necrosis. Photographs illustrate uninoculated leaves in the top row and inoculated leaves in the bottom row. Leaves were inoculated by longitudinally submerging half the leaf in a zoospores suspension of *P. kernoviae* (10^5 spores mL^{-1}) and then incubating in moist chambers at 20°C and misting daily for 6 days.

observed between wounded and non-wounded *R. ponticum* leaves in the second run (Fig. 2b). Disease severity on wounded and non-wounded *U. californica* foliage also deviated between experimental runs (Fig. 2). In the first run no significant difference was observed in disease severity between wounded and unwounded foliage (Fig. 2a), whereas non-wounded leaves exhibited significantly higher disease severity in the second run (Fig. 2b). Disease severity on *R. macrophyllum* was significantly lower than on *R. ponticum*, and generally lower than on the other inoculated plants (Fig. 2a,b). The two *R. macrophyllum* individuals exhibited similar levels of disease severity (Fig. 2a,b).

The presence of symptomless infections was determined by comparing frequency of disease incidence with frequency of pathogen recovery (Table 1). Symptomless foliar infections were observed on both individuals of *R. macrophyllum* and *R. occidentale*. Higher disease incidence in wounded compared with non-wounded foliage was observed on *R. macrophyllum* (individual 2) over both experimental runs and on *R. occidentale* in the second experimental run. Inoculated foliage of *R. ponticum* and *U. californica* always exhibited 100% disease incidence and 100% pathogen recovery by isolation.

Sporangia production on individual plant species varied between the two experimental runs; therefore, sporulation data were analysed and presented separately for

run 1 (Fig. 3a,b) and run 2 (Fig. 3c,d). Foliar sporulation is presented on the basis of sporangia/leaf area (Fig. 3a,c) and on sporangia/lesion area (Fig. 3b,d). All three NA native plants supported sporangia production. *Rhododendron occidentale* and *U. californica* supported more sporangia/leaf area than *R. ponticum* or *R. macrophyllum* in both run 1 ($P \leq 0.01$) and run 2 ($P \leq 0.0001$). In the second experimental run, both individuals of *R. macrophyllum* supported fewer sporangia/leaf area than all other plants tested ($P \leq 0.0001$). Sporangia production/lesion area was significantly higher on *U. californica* and *R. occidentale* than on *R. ponticum* and *R. macrophyllum* in the first experimental run ($P \leq 0.0001$), but no significant differences in sporangia production/lesion area were observed in the second run. Sporangia were produced on symptomless leaves of *R. macrophyllum* in runs 1 and 2 and on a single leaf of *R. occidentale* in run 1. Because calculation of sporangia production per unit lesion area is impossible for symptomless infections, such values are not represented graphically when two or more leaves exhibited symptomless sporulation (Fig. 3b,d). *Phytophthora kernoviae* was always isolated from symptomless leaves that supported sporangia production. The pathogen was never isolated from uninoculated control leaves.

While enumerating sporangia produced on foliage, oogonia and associated antheridia were observed in the

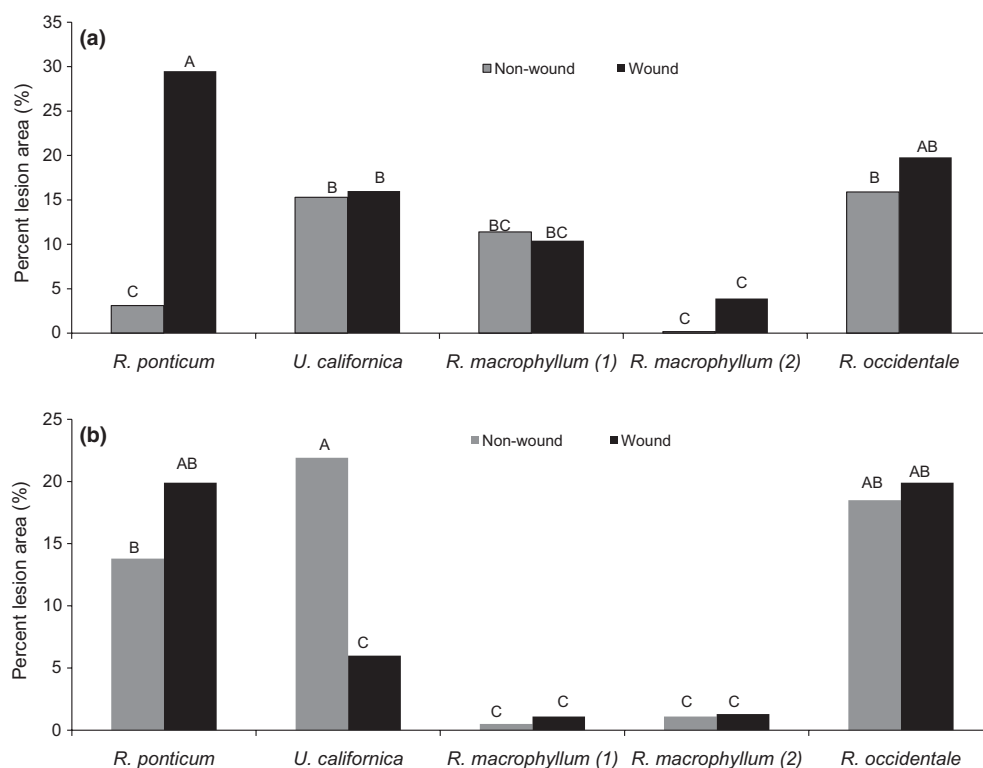


Figure 2 Disease severity on wounded and non-wounded North American plants is represented over two experimental runs (a and b).

A complete factorial treatment design was implemented, with two wounding treatments (wound and no wound) and two inoculation treatments (inoculated and uninoculated control). Five plant types were included in the experiment: *Rhododendron ponticum*, *Umbellularia californica*, *Rhododendron occidentale*, and two individuals of *Rhododendron macrophyllum*, (1) and (2). Wounds were created by removing the leaf tip with a sterile scalpel immediately prior to inoculation. Leaves were inoculated by dipping half the leaf in a zoospore suspension of *Phytophthora kernoviae* (10^5 spores mL^{-1}) and then incubating in moist chambers at 20°C and misting daily for 6 days. After 6 days, percent lesion area was determined as a measure of disease severity, and each disease severity datum represents the average of five leaves. Different letters above bars designate significant differences based on the Waller–Duncan K-ratio test ($K = 100$).

water rinsed from inoculated *U. californica* foliage (Fig. 4a,b). Mature oospores were not observed, but observations of foliar sporulation were only made 24 h after leaves were surface sterilized and misted to induce sporangia production. Oogonia were observed in the rinse water from two of five inoculated leaves in the first run of the experiment; no oogonia were observed in the second experimental run. Neither oogonia nor sporangia were observed in rinse water from uninoculated leaves. Additionally no oospores were observed in inoculated foliage of *R. ponticum*, *R. macrophyllum*, *R. occidentale* or *U. californica* that had been cleared with KOH.

Phytophthora kernoviae was isolated from all inoculated roots of *U. californica* and *R. occidentale*; no *Phytophthora* species were isolated from uninoculated roots. Sporangia were observed on inoculated roots of both plant species; however, oogonia and mature oospores were only observed in inoculated *U. californica* roots (Fig. 4c,d). The physical location of oospores within *U. californica* roots was not readily apparent, although oospore production appears to be both inter- and extra-cellular (Fig. 4c,d). Neither asexual nor sexual spores were observed on uninoculated roots.

Both SMA and PARP media were effective for isolating *P. ramorum* and *P. kernoviae* from rhododendron leaves showing symptoms. *Phytophthora ramorum* was isolated from 16 of the 20 rhododendron leaves with symptoms collected at the *P. ramorum*-infested site. Twelve of the 16 *P. ramorum*-infected leaves yielded *P. ramorum* colonies on both media; however, three positive isolations were detected only on SMA and one positive was detected only on PARP. *Phytophthora kernoviae* was also isolated from 16 of the 20 leaves with symptoms collected at the *P. kernoviae*-infested site. Of the 16 leaves yielding *P. kernoviae*, two were detected on PARP only. Colonies consisted of dense mycelial growth on SMA, but were thin and sparse on PARP (Fig. 5). Individual hyphae of *P. kernoviae* growing on SMA were significantly thicker than those growing on PARP ($t \leq 0.0001$), with a mean thickness of 4.79 and 3.08 μm on SMA and PARP, respectively. Though colonies of *P. kernoviae* were thin and sparse on PARP, the medium supports oospore production. Oospores of *P. kernoviae* were either rarely or sparsely produced on SMA medium.

Table 1 Summary of disease incidence, pathogen recovery and observation of symptomless infection and sporulation on several North American native plants inoculated^a with *Phytophthora kernoviae*

Plant ^b	Wound treatment ^c	Disease incidence ^d	Pathogen recovery ^e	Symptomless infection ^f	Symptomless sporulation ^g
Run 1					
<i>R. ponticum</i>	Non-wound	5/5	5/5	–	–
	Wound	5/5	5/5	–	na
<i>U. californica</i>	Non-wound	5/5	5/5	–	–
	Wound	5/5	5/5	–	na
<i>R. macrophyllum</i> (1)	Non-wound	5/5	5/5	–	–
	Wound	5/5	5/5	–	na
<i>R. macrophyllum</i> (2)	Non-wound	1/5	3/5	+	+
	Wound	5/5	5/5	–	na
<i>R. occidentale</i>	Non-wound	4/5	5/5	+	+
	Wound	5/5	5/5	–	na
Run 2					
<i>R. ponticum</i>	Non-wound	5/5	5/5	–	–
	Wound	5/5	5/5	–	na
<i>U. californica</i>	Non-wound	5/5	5/5	–	–
	Wound	5/5	5/5	–	na
<i>R. macrophyllum</i> (1)	Non-wound	1/5	1/5	–	–
	Wound	1/5	2/5	+	na
<i>R. macrophyllum</i> (2)	Non-wound	2/5	3/5	+	+
	Wound	3/5	4/5	+	na
<i>R. occidentale</i>	Non-wound	4/5	5/5	+	–
	Wound	5/5	5/5	–	na

na: not assessed.

^aFoliage of select plants was inoculated by dipping leaves in a zoospore suspension (10^5 zoospores mL⁻¹).

^b*Rhododendron ponticum* was used as a positive control. *U. californica*, *R. occidentale* and *R. macrophyllum* were selected as potential NA hosts. The two *R. macrophyllum* plants (1) and (2) were assessed separately. The specimens of *U. californica* and *R. occidentale* were container-grown and purchased from nurseries.

^cLeaves of each plant either remained intact or were wounded by cutting off the leaf tip with a sterile scalpel prior to inoculation.

^dAfter inoculation leaves were incubated in moist chambers at 20°C and misted daily. Disease incidence was assessed after 6 days.

Uninoculated control leaves remained symptomless and are therefore excluded from the table.

^ePathogen recovery was determined by submerging a portion of tissue in SMA selective medium.

^fPresence of symptomless infections was determined by assessing the difference between disease incidence and pathogen recovery.

^gA plant exhibited symptomless sporulation when sporangia were observed in leaf rinses derived from symptomless leaves. The presence (+) or absence (–) of symptomless sporulation was only assessed on unwounded leaves.

Discussion

The current body of knowledge on the host range of *P. kernoviae*, derived partly from naturally infected plants in infested UK systems and inoculation studies, suggests the susceptibility of select NA flora, both in natural and landscaped areas. Of the known foliar and canker hosts of *P. kernoviae* in the UK, several species are widely used as landscape plants in the United States. For example, *F. sylvatica*, the tree species sustaining the vast majority of canker infections in the UK, is available in a wide range of cultivars with diverse foliar colours and morphologies for planting in NA landscapes (Burnie *et al.*, 1999). American beech (*Fagus grandifolia*) extends in its native range across the eastern United States and parts of eastern Canada (Tubbs & Houston, 1990); however, its susceptibility to *P. kernoviae* is not yet known. *Quercus robur*, a known canker host in the UK, is widely used in NA landscape plantings in USDA hardiness zones 3–9 (Burnie *et al.*, 1999). *Liriodendron tulipifera*, both a foliar and canker host in the UK, is a North American

native, inhabiting forests from Ontario to Florida (Little, 1979). Infected foliage of *L. tulipifera* supports sporangia production (Defra, 2008), suggesting it could play a role in pathogen transmission in the event that *P. kernoviae* is introduced to eastern NA forests.

Because *P. kernoviae* has been found to infect 17 genera representing 12 families of plants (Fera, 2010), including several species used in NA landscapes, this paper seeks to further address the susceptibility of select NA native flora. Two western NA native rhododendron species, *R. occidentale* and *R. macrophyllum*, were included in the study because rhododendrons are integral in transmission of both *P. ramorum* and *P. kernoviae* in infested UK woodlands. California bay laurel, *U. californica*, was also included because of its role in the transmission of *P. ramorum* and consequent spread of Sudden Oak Death in California forests (Davidson *et al.*, 2005). The results of this work clearly illustrate that *U. californica*, *R. occidentale* and *R. macrophyllum* are susceptible to infection by *P. kernoviae*, regardless of whether foliage

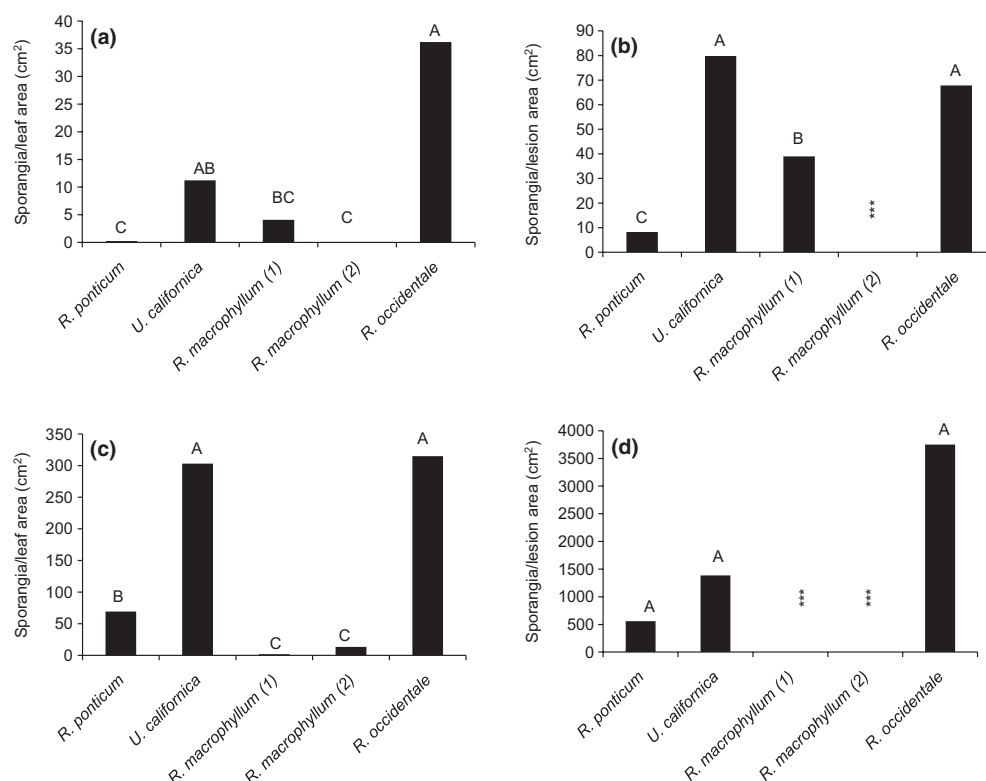


Figure 3 Sporangia production was determined on unwounded leaves inoculated with *Phytophthora kernoviae*. Sporangia production in Runs 1 and 2 are represented in a, b and c, d, respectively. After inoculation, leaves were incubated for 6 days in moist chambers at 20°C, then surface sterilized with 70% ethanol for 30 s, rinsed and then returned to moist chambers. After 24 h at 20°C, sporangia were scraped off leaves, counted, and then the average ($n = 5$) sporangia/leaf area (a and c) and sporangia/lesion area (b and d) were determined. Plant types exhibiting symptomless sporulation on two or more leaves are represented with asterisks (***), and were not included in the analysis of sporangia/lesion area due to the undefined values. Different letters above bars designate significant differences based on the Waller–Duncan K-ratio test ($K = 100$).

was wounded prior to inoculation. Disease severity on wounded and non-wounded *U. californica* leaves varied between experimental runs, with wounded leaves exhibiting either equal or lower lesion area than unwounded leaves. In a similar study focused on foliar susceptibility to *P. ramorum*, disease severity on wounded and non-wounded *U. californica* foliage also varied between experimental runs (Denman *et al.*, 2005). In the *P. ramorum* study, the observed variability on *U. californica* was hypothesized to result from differences in the physiological condition of the host tissue (i.e. leaf age and/or position in canopy), or genetic diversity in plants purchased from the nursery (Denman *et al.*, 2005). Such factors may have also influenced susceptibility of *U. californica* to *P. kernoviae*; however, further studies are needed to directly address factors influencing foliar susceptibility in the *P. kernoviae* pathosystem.

All three NA natives supported sporangia production, and *U. californica* and *R. occidentale* support at least the same level of sporulation seen in *R. ponticum*, the invasive plant associated with transmission of the pathogen in UK woodlands. Although the observed sporulation potential of *P. kernoviae* on *U. californica* and *R. occiden-*

tale is either similar or higher than on *R. ponticum*, infection of *R. ponticum* can result in massive inoculum loads at the ecosystem level simply because the invasive nature of the plant provides ample foliage as a platform for inoculum production. Consequently, *R. ponticum* removal is the primary strategy for inoculum and disease management in infested UK woodlands. Similarly, long-term investigations of thinning and removal of *U. californica* for management of *P. ramorum* in Sudden Oak Death-infested forests are currently underway (Valachovic *et al.*, 2008; Swiecki & Bernhardt, 2010).

Because introduction of *P. kernoviae* to the United States may result in a high-consequence plant disease outbreak, a recovery plan was produced as part of the National Plant Disease Recovery System (NPDRS) called for in Homeland Security Presidential Directive Number 9 (HSPD-9) (Benson *et al.*, 2009). Two of the five key initiatives outlined in the plan call for enhanced ability to detect pathogen introduction and accurately identify the pathogen, while another initiative calls for an assessment of the susceptibility of NA native species. The results of this work illustrate several factors that may curtail or impede rapid detection of an introduction of *P. kernoviae*

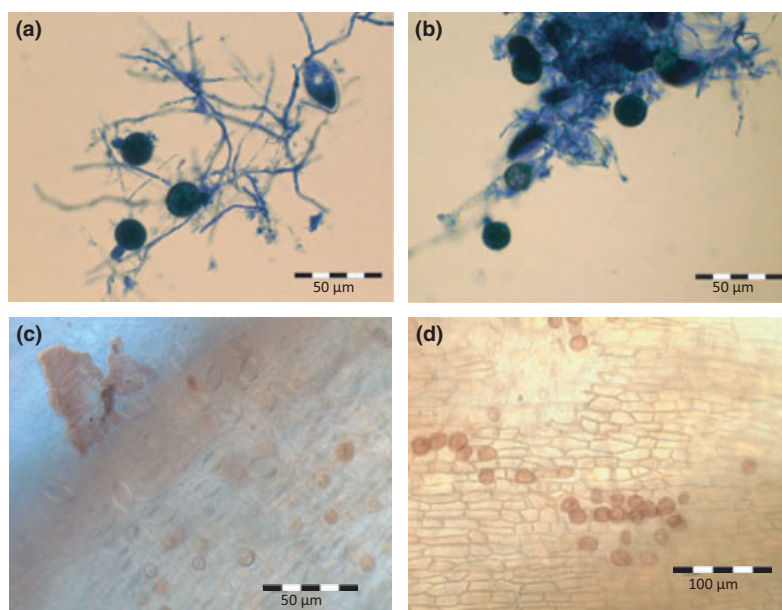


Figure 4 Production of sporangia, oospores and oogonia were observed on *Phytophthora kernoviae*-inoculated foliage (a and b) and roots (c and d) of *Umbellularia californica*. Foliar sporulation was assessed after 24 h in moist chambers at 20°C. Oogonia (a and b), sporangia (a and b), and zoospores (b) were observed in spore suspensions gathered from inoculated *U. californica* leaves. Sporangia and oospores were observed on the surface of inoculated *U. californica* roots (c). Mature intracellular oospores (d) were also observed in inoculated *U. californica* roots.

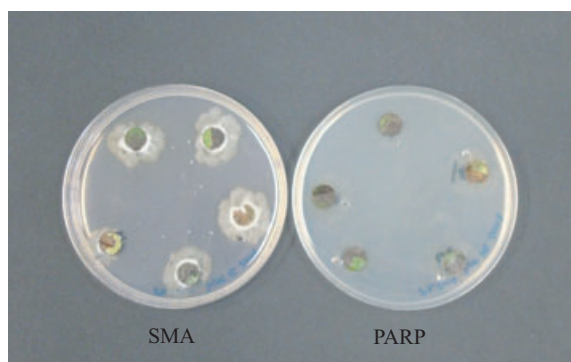


Figure 5 *Phytophthora kernoviae* colonies exhibit thick, dense growth on SMA medium (left) and sparse, thin growth on PARP medium (right).

to NA. Foliar symptoms of *P. kernoviae* on *U. californica* are identical to those of *P. ramorum* (Rizzo & Garbelotto, 2003). Consequently, an introduction of *P. kernoviae* to a well-documented, heavily infested *P. ramorum* site in California may escape notice while disease survey emphases are placed on documenting spread of Sudden Oak Death into new areas. Also, symptoms of *P. kernoviae* on *R. occidentale* are difficult to discern due to the colour and texture of the foliage, and lesions produced on *R. macrophyllum* are generally unnoticeable (<15% of the total leaf area). While there is no monitoring program in place for *P. kernoviae* in the United States, the most

likely opportunities for identification of *P. kernoviae* introduction is through samples sent for general diagnosis to the National Plant Diagnostic Network, or through the nationwide stream monitoring system for *P. ramorum* in US forests (Benson *et al.*, 2009). However, Benson *et al.* (2009) remark that *Phytophthora* samples isolated in diagnostic clinics are rarely identified to species level due to cost limitations. Additionally, streamwater is heavily infested with a diversity of *Phytophthora* species (Huang *et al.*, 2009; Sutton *et al.*, 2009; Reeser *et al.*, 2011) thereby diluting the relative concentration of each species on baits or on filter paper. Identification of new species may therefore be operationally limited by the volume of isolates and the goal of identifying and enumerating a specific pathogen (i.e. *P. ramorum*). Another limitation in confirming presence of *P. kernoviae* in the United States is the sparse growth pattern of the pathogen on PARP, the selective medium typically used for *Phytophthora* isolation in the United States. While *P. kernoviae* produces dense mycelial growth on SMA, it produces thin, sparse growth on PARP. The morphology of *P. kernoviae* on PARP resembles the sparse growth of *Pythium* species; consequently, positive isolations may be discarded as plate contaminants.

An additional major challenge to both intercepting *P. kernoviae*-infected plant material at NA points of entry and early detection of introductions is the potential for *P. kernoviae* to infect host tissues without symptoms. The high frequency of symptomless infections of both *P. ramorum* and *P. kernoviae* on Rhododendron

'Cunningham's White' trap plants was first reported in 2007 (Denman *et al.*, 2008; Denman *et al.*, 2009). Subsequently *P. kernoviae* was found to incite symptomless root infections on *R. ponticum* in UK woodlands (Fichtner *et al.*, 2011). The present study further demonstrates the potential for *P. kernoviae* to cause symptomless infections on foliage of *R. occidentale* and *R. macrophyllum*, and on roots of *U. californica* and *R. occidentale*. Additionally, sporangia were produced on the roots of both *U. californica* and *R. occidentale*, suggesting the potential for underground sporulation.

Phytophthora kernoviae is a recognized threat to NA native ecosystems, and the United States nursery industry, valued at approximately \$4.6 billion (Benson *et al.*, 2009). The results of this work demonstrate not only the susceptibility of select NA native plants, but also the potential for these plants to support pathogen sporulation and subsequent disease transmission. Additionally, *U. californica* foliage and roots support oospore production of *P. kernoviae* suggesting that the pathogen can reproduce sexually on this host. In invaded UK forests, *P. kernoviae* is considered more aggressive towards trees than *P. ramorum* (Defra, 2008), but the relative potential impact of each of these pathogens on NA ecosystems is not yet known. The competitive dynamics between the asexually reproducing *P. ramorum* and *P. kernoviae* on *U. californica* foliage is not known, but is a topic worthy of further investigation. Restrictions are in place for importation of nursery stock from infested areas (Benson *et al.*, 2009); however, the frequency of symptomless infections combined with the fact that the geographic origin of *P. kernoviae* is unknown may render point of entry detection difficult. As research findings fill gaps in knowledge of pathogen biology and disease epidemiology, the United States Recovery Plan and regulatory strategies implemented by APHIS must also evolve and adapt to enhance the efficacy of efforts to promote national biosecurity. Improved communication between the forestry, horticulture and regulator communities is key to successfully thwarting the introduction of *P. kernoviae*, and other as yet unknown invasive species, for protection of both NA's natural areas and the forestry and nursery industries.

Though the results of the current study underscore the potential risk of *P. kernoviae* to select NA native plants, the potential impact of introduction of *P. kernoviae* to native ecosystems worldwide should also be considered. While considering the extent of unknown hosts of *P. kernoviae*, it is also wise to highlight the risk of the pathogen to ecosystems where *R. ponticum*, a known host, is native. While *R. ponticum* is an invasive species in western Europe and the UK, it is a post Plio-Pleistocene relict and native understory component of Euxinian forests (south of the Black Sea) and riparian forests in the southern Iberian Peninsula (Mejías *et al.*, 2007). Native populations of *R. ponticum* on the Iberian Peninsula are in decline and riparian forests in the Aljibe Mountains constitute a refuge for the species. The persistence of *R. ponticum* and other relict species in this

meso-Mediterranean climate is dependent on maintenance of community structure in riparian forests (Mejías *et al.*, 2007); consequently, a disturbance caused by pathogen introduction may jeopardize the persistence of a unique ecosystem harbouring relict taxa. Additionally, it should be considered that *R. ponticum* is a member of the *Rhododendron* subsection *Pontica*, which contains four species native to southwest Eurasia (including *R. ponticum*), two species native to southeastern North America (including *R. catawbiense*, a known host of *P. kernoviae*), and two species in western North America, including *R. macrophyllum* (Milne, 2004). A phylogenetic analysis of *Rhododendron* subsection *Pontica* classified *R. ponticum* in the same monophyletic clade as other known hosts of *P. kernoviae*, including *R. macrophyllum* and *R. catawbiense* (Milne, 2004). Given the results of this work and the known susceptibility of members of the subsection *Pontica*, further directed host range testing should be conducted to better assess the potential risk of *P. kernoviae* introduction to as yet uninvaded ecosystems.

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References

- Beales PA, Lane CR, Barton VC, Giltrap PM, 2006. *Phytophthora kernoviae* on ornamentals in the UK. *EPPO Bulletin* 36, 377–9.
- Benson M, Ivors K, Koch F *et al.*, 2009. Recovery plan for *Phytophthora kernoviae*, cause of bleeding trunk cankers, leaf blight and stem dieback in trees and shrubs. *United States Department of Agriculture, Government Publication/Report* [<http://www.ars.usda.gov/SP2UserFiles/Place/00000000/opmp/P.%20kernoviae%2081100.pdf>].
- Brasier CM, Beales PA, Kirk SA, Denman S, Rose J, 2005. *Phytophthora kernoviae* sp. nov., an invasive pathogen causing bleeding stem lesions on forest trees and foliar necrosis of ornamentals in the UK. *Mycological Research* 109, 853–9.
- Brown A, Brasier C, Webber J, 2006. Aetiology and distribution of *Phytophthora kernoviae* and *P. ramorum* stem lesions on European beech in southwest England. In: Brasier C, Jung T, Osswald W, eds. *Progress in Research on Phytophthora Diseases of Forest Trees*. Proceedings of the 3rd International IUFRO Working Party 7.02.09 Meeting. Farnham, UK: Forest Research, 139–41.
- Burnie G, Forrester S, Greig D, 1999. *Botanica: The Illustrated A-Z of Over 10,000 Garden Plants and How to Cultivate Them*. New York, NY, USA: Welcome Rain Publisher LLC.

- Davidson JM, Wickland AC, Patterson HA, Falk KR, Rizzo DM, 2005. Transmission of *Phytophthora ramorum* in mixed-evergreen forest in California. *Phytopathology* 95, 587–696.
- Defra, 2008. *Consultation on Future Management of Risks from Phytophthora ramorum and Phytophthora kernoviae*. London, UK: Department for Environment, Food and Rural Affairs [<http://archive.defra.gov.uk/corporate/consult/phytophthoraram-kern/consultation.pdf>].
- Denman S, 2008. *RAPRA Protocols for Susceptibility Testing, Protocol 7: Assessment of the Sporulation Potential of Phytophthora ramorum on Detached Foliage* [http://rapra.csl.gov.uk/protocols/RAPRAProtocol_7.pdf].
- Denman S, Kirk SA, Brasier CM, Webber JF, 2005. *In vitro* leaf inoculation studies as an indication of tree foliage susceptibility to *Phytophthora ramorum* in the UK. *Plant Pathology* 54, 512–21.
- Denman S, Moralejo E, Kirk SA, Orton E, Whybrow A, 2008. Sporulation of *Phytophthora ramorum* and *Phytophthora kernoviae* on asymptomatic foliage and fruit. In: Frankel SJ, Kliejunas JT, Palmieri KM, eds. *Proceedings of the Sudden Oak Death Third Science Symposium*. Albany, CA, USA: USDA Forest Service: General Technical Report PSW-GTR-214, 201–7.
- Denman S, Kirk SA, Moralejo E, Webber JF, 2009. *Phytophthora ramorum* and *P. kernoviae* on naturally infected asymptomatic foliage. *EPPPO Bulletin* 39, 105–11.
- Elliott CG, Hendrie MR, Knights BA, 1966. The sterol requirement of *Phytophthora cactorum*. *Journal of General Microbiology* 42, 425–35.
- Erwin DC, Ribeiro OK, 1996. *Phytophthora Diseases Worldwide*. St Paul, MN, USA: APS Press.
- Fera, 2010. *Fera List of Natural Hosts of Phytophthora kernoviae with Symptoms*. London, UK: The Food and Environment Research Agency [<http://fera.defra.gov.uk/plants/plantHealth/pestsDiseases/phytophthora/documents/pKernoviaeHost.pdf>].
- Fichtner EJ, Rizzo DM, Kirk SA, Webber JF, 2011. Root infections may challenge management of invasive *Phytophthora* species in UK woodlands. *Plant Disease* 95, 13–8.
- Huang J, Oak SW, Jeffers SN, 2009. Monitoring occurrence and distribution of *Phytophthora* species in forest streams in North Carolina using baiting and filtration methods. In: Goheen EM, Frankel SJ, eds. *Phytophthoras in Forests and Natural Ecosystems*. Albany, CA, USA: USDA Forest Service: General Technical Report PSW-GTR-221, 93–5.
- Irish Department of Agriculture, Food and Fisheries, 2009. Annual Report 2009 on Ireland's National Control Plan [http://www.fsai.ie/uploadedfiles/about_us/service_contracts/Ireland_MANCP_annual_report_2009.pdf, 80].
- Little ELJ, 1979. *Checklist of United States Trees (Native and Naturalized)*. Washington, DC, USA: USDA Agriculture Handbook 541, 163.
- Mejías JA, Arroyo J, Marañón T, 2007. Ecology and biogeography of plant communities associated with the post-Pleio-Pleistocene relict *Rhododendron ponticum* subsp. *baeticum* in southern Spain. *Journal of Biogeography* 34, 456–72.
- Milne I, 2004. Phylogeny and biogeography of *Rhododendron* subsection *Pontica*, a group with a tertiary relict distribution. *Molecular Phylogenetics and Evolution* 33, 389–401.
- Ramsfield TD, Dick MA, Beever RE, Horner IJ, 2009. *Phytophthora kernoviae* – of southern hemisphere origin? In: Goheen EM, Frankel SJ, eds. *Phytophthoras in Forests and Natural Ecosystems*. Albany, CA, USA: USDA Forest Service: General Technical Report PSW-GTR-221, 47–53.
- Reeser PW, Sutton W, Hansen EM, Remigi P, Adams GC, 2011. *Phytophthora* species in forest streams in Oregon and Alaska. *Mycologia* 103, 22–35.
- Rizzo DM, Garbelotto M, 2003. Sudden oak death: endangering California and Oregon forest ecosystems. *Frontiers in Ecology and the Environment* 1, 197–204.
- Shishkoff N, 2007. Persistence of *Phytophthora ramorum* in soil mix and roots of nursery ornamentals. *Plant Disease* 91, 1245–9.
- Sutton W, Hansen EM, Reeser PW, Kanaskie A, 2009. Stream monitoring for detection of *Phytophthora ramorum* in Oregon tanoak forests. *Plant Disease* 93, 1182–6.
- Swiecki TJ, Bernhardt E, 2010. Assessing methods to protect susceptible oak and tanoak stands from Sudden Oak Death. In: Frankel SJ, Kliejunas JT, Palmieri KM, eds. *Proceedings of the Sudden Oak Death Fourth Science Symposium*. Albany, CA, USA: USDA Forest Service: General Technical Report PSW-GTR-229, 236–8.
- Tubbs CH, Houston DR, 1990. American Beech. In: Burns RM, Honkala BH, eds. *Silvics of North America, Vol. 2. Hardwoods*. Washington, DC, USA: USDA Forest Service: Agriculture Handbook 654, 406–16.
- Tukey JW, 1977. *Exploratory Data Analysis*. Reading, MA, USA: Addison-Wesley Publishing Co.
- Valachovic Y, Lee C, Marshall J, Scanlon H, 2008. Wildland management of *Phytophthora ramorum* in northern California forests. In: Frankel SJ, Kliejunas JT, Palmieri KM, eds. *Proceedings of the Sudden Oak Death Third Science Symposium*. Albany, CA, USA: USDA Forest Service: General Technical Report PSW-GTR-214, 305–12.
- Webber JF, 2009. Management of *Phytophthora kernoviae* and *P. ramorum* in Southwest England. In: Goheen EM, Frankel SJ, eds. *Phytophthoras in Forests and Natural Ecosystems*. Albany, CA, USA: USDA Forest Service: General Technical Report PSW-GTR-221, 177–83.