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SHORT COMMUNICATION

Root and aerial infections of *Chamaecyparis lawsoniana* by *Phytophthora lateralis*: a new threat for European countries

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Summary

Phytophthora lateralis has been isolated from root and collar lesions in Port-Orford Cedar (POC) trees (*Chamaecyparis lawsoniana)* in north-western France (Brittany). These trees, planted in hedgerows, displayed symptoms similar to the typical symptoms of POC root disease. Until now, the disease has been found outside of the nurseries only in western North America. Aerial symptoms, not associated with root or collar infections, were also observed, and *P. lateralis* was isolated from branch lesions. Similar symptoms were previously observed only in one POC root disease site, located in the Pacific coast of Oregon where climatic conditions are similar to those occurring in Brittany. The reported aetiology as well as the morphological characteristics (deciduous sporangia) of *P. lateralis* suggests that this species could be air-dispersed, as described for *P. ramorum*, a closely related species. This outbreak of *P. lateralis* in Brittany in farming landscapes associated with the aerial spread of this pathogen represents a new threat for European countries.

1 Introduction

Phytophthora lateralis Tucker & Milbrath was first reported about 1920 on nursery stock near Seattle WA (USA) where it was causing mortality of Port-Orford Cedar (POC) or Lawson's cypress plants (*Chamaecyparis lawsoniana* [A. Murray]) Parl. (Tucker and Milbrath 1942). As a consequence of the pathogen's dissemination among nurseries in Oregon, Washington and California, the horticultural trade and use of POC as a landscaping tree were greatly curtailed. From initial detection outside of Seattle, it took about 30 years for this pathogen to establish in the south-western Oregon and northern California forests where POC is endemic (Zobel et al. 1985). However, after the first report in mixed conifer forests (near Coos Bay, Oregon, Roth et al. 1957), the spread of *P. lateralis* was rapid along coastal Oregon and slower into the drier inland forests (Jules et al. 2002). Today the disease occurs throughout the natural range of POC (Hansen et al. 2000).

Dissemination of chlamydospores in soil, linked to human activity, and dissemination of zoospores in streams account for its wide distribution. Although less susceptible, Pacific yew (*Taxus brevifolia* Nutt.) is also vulnerable to *P. lateralis* (DeNitto and Kleijunas (1991); Murray and Hansen 1997). Since POC and *T. brevifolia* are both key components of these ecosystems, *P. lateralis* is threatening the ecological and economical values of forests where the pathogen occurs. The low genetic diversity of the western North

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American populations of *P. lateralis* and the high susceptibility of its principal host, as well as the history of first identification in nurseries beyond the natural range of the tree, are arguments to suggest an exotic origin for this pathogen (Hansen et al. 2000). Previous reports had suggested a French origin to explain *P. lateralis* introductions to the USA, but this theory was probably due to misinterpretation of the disease history (Zobel et al. 1985). Although *P. lateralis* is occasionally isolated from *Chamaecyparis obtusa* (Hinoki cypress) in nurseries, (Tucker and Milbrath 1942), Asiatic species of *Chamaecyparis* are described as resistant to the pathogen (Sinclair et al. 1987). In 2009, *P. lateralis* was detected in soil of natural forests of *C. obtusa* in Taiwan (Ma-Kau Ecological Park, Chi-lan mountains), in agreement with a possible Asiatic origin for this species (Brasier et al. 2010).

The most frequent symptoms of disease caused by *P. lateralis* are root and collar lesions. Fine roots are the first target of zoospores. Then, hyphae develop in larger roots and into the root collar where they kill the inner bark (Oh and Hansen 2007). Upon removal of outer bark, a sharp margin is visible between necrotic phloem, discoloured to cinnamon-brown and healthy cortical tissues. The whole canopy of infected trees turns to pale green, yellow and then light-brown when the tree is dying. Trione and Roth (1957) also reported infrequent foliar infections, resulting in branch lesions. These symptoms have not been further observed in Oregon or California forests (E. Hansen, personal communication).

Until recently, the known distribution area of *P. lateralis* was limited to Oregon and California. The pathogen was detected in France in 1998 and in the Netherlands in 2004 (Hansen et al. 1999; Sansford 2009). In both cases, infected trees were young cedar plants grown in nurseries as ornamental plants. It has been suggested that *P. lateralis* had been introduced accidentally twice from unknown sources. This organism was considered as successfully eradicated from Europe. Pest risk analyses were conducted for the UK (Sansford 2009) and for member countries of the European Plant Protection Organisation (EPPO: http://www.eppo.org/QUARANTINE/Pest_Risk_Analysis/PRA_documents.htm). The probability of its establishment in Europe, and especially in the western coastal areas, was evaluated as high owing to the occurrence of the host plant, the climatic conditions and the horticultural trade. Because of its potential economic impact, *P. lateralis* was added in 2006 to the A1 list of exotic species that the EPPO recommends to regulate as quarantine organisms.

In Brittany (north-western part of France), *C. lawsoniana* trees were planted during the 1970s as windbreaks after removal of the traditional hedgerows and transformation of the historical low-intensity farming system to a more intensive system. Trees of this exotic species have rapid growth rates and thus provide excellent shelter from oceanic winds for plantations or cattle. From 2005 to 2008, the first signs of decline and mortality were observed at several sites separated in total by 60 km. Trees exhibited symptoms similar to the typical symptoms of POC root disease. Surprisingly, aerial infections were also detected. We report in this paper, the investigations carried out to confirm that *P. lateralis* is the pathogen responsible for these symptoms. To evaluate the factors that enabled the emergence of the disease, we retrieved the climatic data for the periods corresponding to the disease emergence or occurrence of *P. lateralis* in France, Oregon and Taiwan.

2 Material and methods

2.1 Study sites and sampling

In 2008 and 2009, disease severity assessments were performed in Brittany by the Department of Forest Health in four localities (Fig. 1a, Table 1). In September 2009, samples were collected from these sites. Bark samples from active cortical lesions were

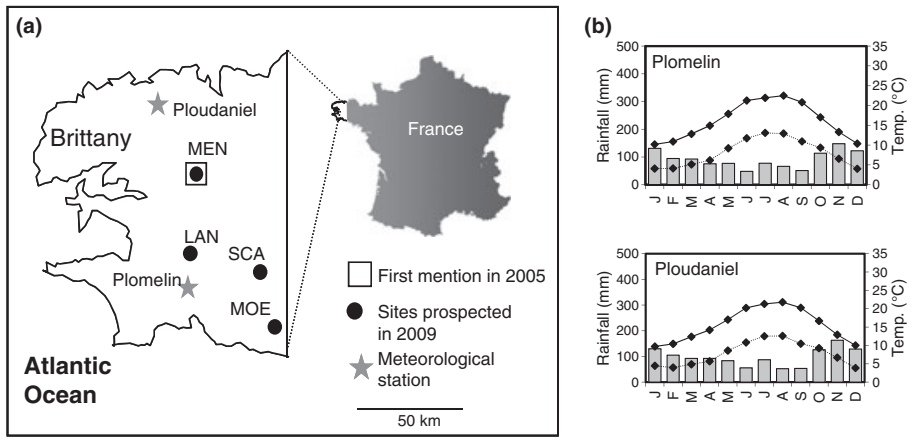

Fig. 1. (a) Location of study sites in western France. (b) Maximal (full line), minimal (dotted line) monthly temperatures and rainfall (bars) data (monthly means) for the 2000–2009 period in two meteorological stations.

Table 1. **Assessment of decline and sampling strategy in the studied sites.**

Locality	GPS lambert coordinates	Site code	Date of observation	Declining trees (%)	Type of symptoms	2009 sampling
Landrévarzec	123.1; 2363.3	LAN	21/10/2008	5	Root	S, R, LS
			14/09/2009	10		
Lopérec	127.2; 2391	MEN	25/06/2007	40	Root and aerial	S, R, LS, B
			15/09/2009	50		
Moëlan-sur-Mer	155.5; 2330.9	MOE	21/10/2008	10	Root	S, R, LS
			15/09/2009	15		
Scaër	148.7; 2354.4	SCA	15/10/2008	10	Aerial	S, B
			15/09/2009	20		

S, soil; LS, lower stem; R, root; B, branch.

removed from the lower stem. Symptomatic roots and branches were also harvested. In addition, in all sites, soil samples containing fine roots were also collected under the symptomatic trees (showing either root and collar infections or branch infection).

2.2 Isolation methods

All samples were kept at a cool temperature (10–20°C), before isolation (attempted in two different laboratories i.e. LNPV and INRA). Direct isolation was performed from tissue samples grown on a corn meal agar (CMA, Difco Laboratories, Detroit) selective medium supplemented with 20 ppm delvocid (50% natamycin salt), 200 ppm ampicillin sodium salt, 10 ppm rifampicin SV sodium salt and 30 ppm benlate (benomyl 50 WP) (Hansen et al. 2008). Small pieces of bark, removed from active lesion margins, were plated on CMA medium and incubated at 18–20°C in the dark.

Baiting from soils was performed at INRA, with *C. lawsoniana* foliage segments following the method described by Winton and Hansen (2001) and at LNPV, with

rhododendron leaves ('Cunningham's white'). Soil samples (50–200 g, depending on the samples) containing small roots were placed in a plastic box (24 × 18 × 9.5 cm). Sterile distilled water was added to allow healthy rhododendron leaves to be floated on the surface. After 1–3 weeks incubation, the leaves were checked for the presence of *Phytophthora* spp. by observation and isolation on CMA selective medium.

2.3 Morphological observations

Phytophthora-like isolates were grown on CMA and 1/3 strength V-8 agar for comparison of colony morphology and growth rate with known *P. lateralis* isolates collected from forest POC trees in Oregon. For induction of sporangia, three 5-mm diameter agar discs were cut from actively growing colony margins and transferred to pea broth (150 g split peas in 1 l dH_2O autoclaved for 4 min and filtered, 20 ppm β-sitosterol (Acros Organics, Morris Plains, NJ, USA) added to filtrate and the broth autoclaved for 25 min) in Petri dishes. The pea broth cultures were incubated for 7 days at 17°C, then drained, washed with distilled water, and flooded with 25 ml stream water and incubated for 2 days at 17°C to induce sporangia.

2.4 DNA extraction and amplification of rDNA region

Rapid DNA extraction was performed by placing a 5-mm diameter disc of *Phytophthora* agar culture into a 200-μl microtube with 100 μl Tris-EDTA and by boiling the sample for 5 min at 98°C followed by chilling on ice.

The internal transcribed spacer (ITS) including the 5.8S ribosomal gene was amplified using the universal primers ITS6 and ITS4 (White et al. 1990; Cooke et al. 2000). PCRs were carried out in a 30-μl reaction volume consisting of 1 μl of undiluted DNA template, 10 mM of each primer, 5 mM of each dNTPs (GE Healthcare UK & Ireland, Little Chalfont, UK), 50 mM $MgCl_2$ and 1 U of Platinum Taq DNA polymerase (Invitrogen, Cergy-Pontoise, France), with thermocycling conditions as follows: one cycle of 95°C for 10 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 90 s; and a final cycle of 72°C for 7 min. PCR products were visualized by ultra-violet fluorescence following electrophoresis on a 1% agarose gel in 0.5X TBE buffer and GelRed™ (Interchim, Montluçon, France) staining. Successfully amplified fragments were then sequenced using ABI Prism Bigdye Terminator Cycle Sequencing Ready Reaction Kit and analysed on an ABI 3730 automated DNA sequencer on the Genome-Transcriptome platform (INRA, Bordeaux, France, http://www.pierroton.inra.fr/biogeco/site_pole_agro/genoseq.html). Sequences were edited and manually aligned using BioEdit v.7.0.5 (Hall 1999). The best-fitting model of sequence alignment evolution was chosen using jModeltest v. 0.1.1 (Posada 2008), and then implemented in PAUP ver. 4.0b10 (Swofford 2003) for pairwise sequence distance matrix and NJ tree constructions.

2.5 Compilation of climatic data

Monthly means of minimum and maximum temperatures (in °C) and sum of precipitation (in mm) were collected from INRA database (https://intranet.inra.fr/climatik) over the 2000–2009 period for two sites in Brittany closest to the studied POC sites (Fig. 1b). The same climatic parameters were collected over the 1950–1990 period from the Oregon Climate Service database (http://www.ocs.orst.edu) for three sites in Oregon (Fig. 2a,b): Cave Junction (WGS84 coordinates: *ca* 42°53′N, 124°4′W) and Powers (*ca* 42°10′N, 123°38′W). These sites are located in the inland part of the POC distribution area. The third site, Bandon (*ca* 43°7′N, 124°24′W), is close to the coast and to the site where Trione

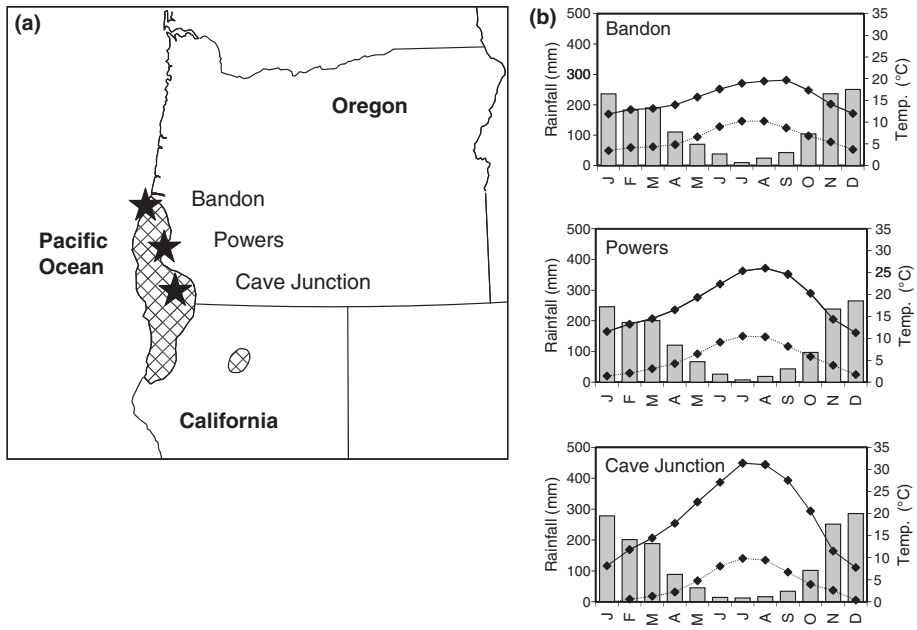

Fig. 2. (a) *Phytophthora lateralis* distribution area in western North America. (b) Maximal (full line), minimal (dotted line) monthly temperatures and rainfall (bars) data (monthly means) for the 1950–2000 period in three meteorological stations.

and Roth (1957) reported aerial spread of *P. lateralis*. Lastly, we retrieved climatic normals from Worldclim database (http://www.worldclim.org) for a longer period (1950–2000) for Ma-Kau Park (Taiwan) where *P. lateralis* was isolated from soil (latitude and longitude *ca* 24°32′N, 121°22′W, Fig. 3a,b; Brasier et al. 2010).

3 Results

3.1 Disease observations

In three sites (LAN, MEN, MOE), typical symptoms of POC root disease (i.e. discoloration and decline of the whole canopy associated with root and collar necrosis) were observed (Fig. 1a). Trees that showed the first signs of foliar discoloration of the entire canopy also exhibited root collars girdled by lesions. Lesions in outer bark cortical tissues appeared to come from the roots (as confirmed by excavating some major roots) and developed upwards in the trunk up to several tens of centimetres.

In sites MEN and SCA, another type of symptom was observed. Dead branches with necrotic lesions (same colour as in collar) were observed. The decline was localized in the middle or lower part of the canopy and seemed to be progressive, spreading from the foliage towards the trunk (Fig. 4). This type of decline could not be associated with any root or collar lesions. However, in some branches, small cankered areas were detected, and beneath them some brown cortical lesions were spreading downwards (Fig. 4). Bark samples of such aerial downward lesions in trunk and small branches were collected.

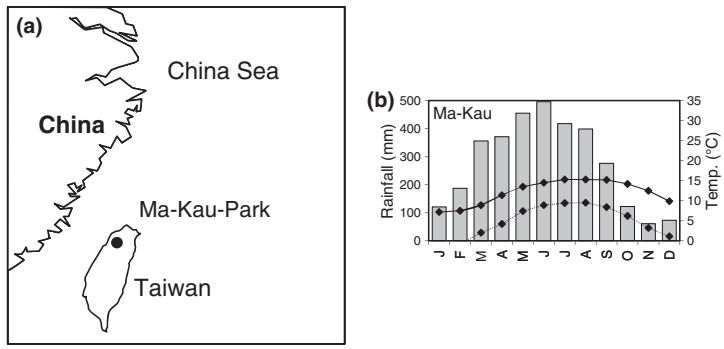

Fig. 3. (a) A location of *Phytophthora lateralis* discovery site in Taiwan. (b) Maximal (full line), minimal (dotted line) monthly temperatures and rainfall (bars) data (monthly means) for the 1950–2000 period in this site.

Fig. 4. Symptoms of aerial infection by *Phytophthora lateralis* in *Chamaecyparis lawsoniana*.

3.2 Isolation and identification

Direct isolations from bark samples yielded *Phytophthora*-like isolates, with a rate varying from 80 to 100% (Table 2). Only one of the symptomatic trees we sampled did not provide *Phytophthora*-like isolates. This failure could be accounted for by the inferior quality of the bark samples (older lesions, not sampled at the active margins). In two sites, (SCA and MEN) direct isolations from symptoms linked to an aerial infection also provided *Phytophthora*-like isolates. The rate of positive isolations from these lesions varied from 25 to 100%.

Table 2. **Results of direct isolations.**

Site	Tree	Lesion	Positive isolations (%)	Internal transcribed spacer sequence[1]
LAN	1	Trunk, going up	100	HM356021
	2	Trunk, going up	100	Not submitted
MEN	1	Trunk, going up	89	HM356018
	2	Trunk, going up	80	Not submitted
	2	Trunk, going down	75	
	3	Branch, going down	100	
	3	Branch, going down	60	
	4	Branch, going down	20	
SCA	1	Branch, going down	100	
	2	Branch, going down	0	
	2	Branch, going down	25	
	3	Branch, going down	60	
	4	Branch, going down	80	HM356019
MOE	1	Bark, going up	0	
	2	Bark, going up	80	HM356020

[1]Genbank accession number.

Soil baiting with cedar leaflets did not provide any *Phytophthora*-like isolate. However, in LAN site, baiting with rhododendrons leaves also yielded *Phytophthora*-like isolates.

All the *Phytophthora*-like isolates were stored on V8 agar in sterile water (at UMR BIOGECO, INRA). All these isolates were characterized by a slow growth rate at 20°C and morphological characteristics similar to those described for *P. lateralis* (Tucker and Milbrath 1942). However, deciduous sporangia with short pedicels were observed (Fig. 5). Sporangial apexes were sometimes swollen and appeared to be semipapillate or papillate. Under the same conditions, the Oregon isolates produced similar deciduous sporangia.

ITS1-5.8S-ITS2 sequences were obtained for eight isolates from Brittany sites. All were identical and shared a 100% homology with the ITS1-5.8S-ITS2 sequence of the type isolate of *P. lateralis* (GenBank Acc. No. AF266804), another American isolate (AF521581), and the isolate obtained in France in 1998 (AF287256), but differed at three nucleotide sites (two substitutions and one gap) from the isolate obtained in Taiwan (GQ381314, Fig. 6). Sequences of the eight *P. lateralis* isolates considered in this study (including the Taiwanese isolate) were grouped in a single monophyletic clade, which differed by 30 substitution polymorphisms (bootstrap value = 71%) with sequences of the closest related *Phytophthora* species (*P. hibernalis*, *P. foliorum* and *P. ramorum*) retrieved in the Genbank database (Fig. 6).

3.3 Climate comparisons

Optimum temperatures for *P. lateralis* are between 15 and 20°C, but the range of temperatures within which infection can occur is 3–25°C (Trione 1959). Common characteristics of climate in Brittany, coastal Oregon and Taiwan (cloudy zone of Chi-Lan mountains area) are mild winter temperatures. Lower and upper threshold temperatures were estimated to be 3 and 25°C (pest risk analysis EPPO). In the two French sites and in Bandon (Oregon), minimum temperatures were always above 3°C (Figs 1b and 2b). In Cave Junction and Powers (Oregon), minimum temperatures were lower than this threshold during five and three winter months, respectively (Fig. 2b). The ancient cypress forests of Ma-Kau Park are 1200–2000 m in elevation, and the climate is slightly colder than in Oregon and western France (Fig. 3b).

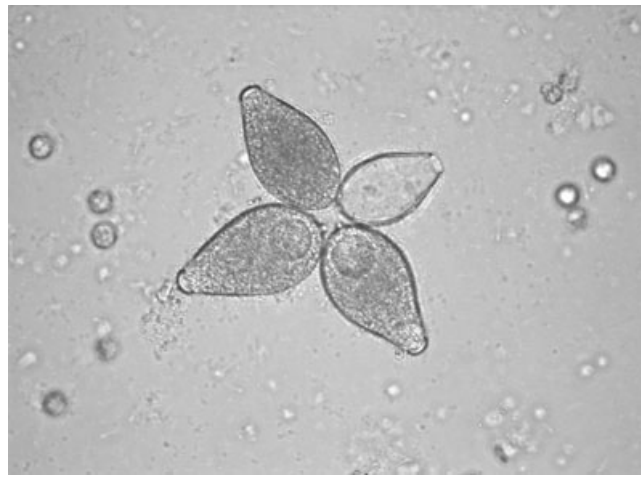

Fig. 5. Deciduous sporangia of *Phytophthora lateralis* (isolate MEN), produced from pea broth cultures incubated in natural stream water.

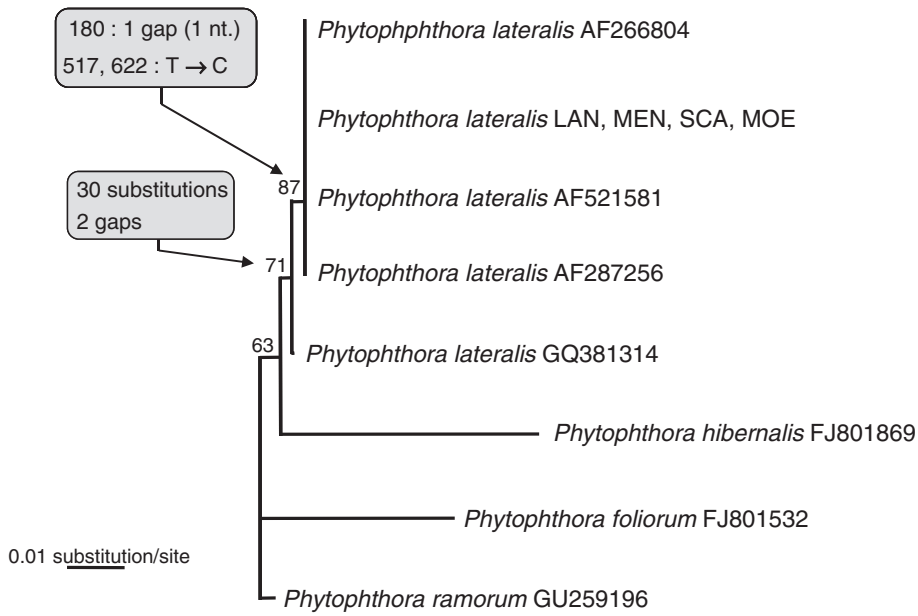

Fig. 6. Neighbour-joining tree based on a matrix of pairwise HKY85 distances obtained between internal transcribed spacer nucleotide sequences for *Phytophthora lateralis* isolates and closest related *Phytophthora* strains retrieved in the GenBank database. Bootstrap values estimated from 1000 replicates are indicated above branches. Boxes with arrow show the localization of polymorphisms observed in the alignment used to generate the distance matrix. *P. lateralis* strains named LAN, MEN, SCA and MOE corresponded to those isolated in this study at Landrévarzec, Lopérec, Scaër and Moëlan-sur-Mer, respectively.

Summer temperatures did not exceed 25°C in Ma-Kau Park, in western France and in Bandon, whereas this upper temperature threshold was reached in Powers and Cave Junction. In Brittany as in Ma-Kau Park, there is no dry season (minimal monthly rainfall >48 mm), whereas in Oregon summers are dry.

4 Discussion

Morphological and molecular characterization of all *Phytophthora*-like isolates obtained from infected tissues or soil were consistent with their assignation to *P. lateralis*. This is the first report of infection and decline of cedar trees caused by *P. lateralis* in several farming and forest environments in Europe. This report is independent of the two previous ones of *P. lateralis* made in French and Netherlander nurseries in 1998 and in 2004 (Hansen et al. 1999; Sansford 2009), and the establishment and spread of the pathogen might pose a significant threat for Europe. Indeed, disease incidence reached 50% of infected trees in some sites, and the pathogen was successfully isolated in four sites located in an area covering at least 400 km^2. Although *C. lawsoniana* is an exotic species, this disease emergence might have a significant ecological impact owing to the role of hedgerows in the agro-ecosystem in Brittany. Indeed, this pathogen has had a real impact on the landscape, because of the decline and mortality of several thousands of trees. Because this outbreak is quite recent and concerns private owners who do not receive any compensation, trees were cut down but have not yet been replaced.

The second notable finding is the description of aerial infections of *C. lawsoniana* by *P. lateralis*, not observed anywhere since the previous report by Trione and Roth (1957). In Oregon, aerial infections were associated with root infection, and it was suggested that this may have resulted from contact of lower branches with contaminated soil or by splashing rain. In Brittany, at one site, we observed that all infected trees exhibited only aerial infections, with no sign of root infection. At the second site, we found only one tree showing both root and aerial infections. Our results and observations raise several questions about the ecology, the origin and the potential impact of *P. lateralis* in France and by extrapolation, in Europe.

It was first suggested by Ostrofsky et al. (1977) that *P. lateralis* did not behave as a real soil-borne pathogen, as its survival in soil depends on the presence of organic matter and the presence of host roots. Aerial infections are usually associated in *Phytophthora* species with caducous sporangia with short pedicels. *P. lateralis* was originally described as a non-deciduous species, with non-papillate sporangia. However, under certain conditions, isolates obtained in Brittany produced caducous sporangia. Similar observations were made on the first isolate collected in France (Hansen et al. 1999) and on Taiwanese and American isolates (Brasier et al. 2010). As suggested by these authors, *P. lateralis* could have retained the ability to disperse aerially. Our observations of aerial infections without root infections suggest that the inoculum of this pathogen could be air-dispersed, as described for *P. ramorum,* a closely related species (Hansen et al. 2008). This last hypothesis fits with the phylogenetic information provided by ITS sequences, as *P. lateralis* is clustered within a clade that includes *Phytophthora* species with potential for aerial dispersal (Cooke et al. 2000; Martin and Tooley 2003).

In good agreement with this hypothesis is the climate of the supposed Asian centre of origin, characterized by mild and rainy summers to which *P. lateralis* is thought to be adapted. Aerial infections and rain-splash dispersal could be promoted by mild temperatures and a humid environment. According to Trione and Roth (1957), formation of sporangia and leaf infections would occur under temperatures varying from 10–20°C and 10–25°C, respectively. In Taiwan, no symptoms were observed on foliage of *C. obtusa,* but neither was infection evident on roots or stems. If this host species had co-evolved with *P. lateralis*, aerial infections may be very subtle, especially in tall and old trees. Climatic

conditions, which occur along the Pacific Coast (but not in drier inland forests) in Oregon and in Brittany, seem conducive for aerial infections and foliage infections. Moreover, the absence of root symptoms suggests that in Brittany aerial dispersal could occur via deciduous sporangia, without involvement of soil plash.

Given the climate of its supposed centre of origin, *P. lateralis* is likely adapted to mild summers. However, *P. lateralis* has developed a different host life strategy to escape unfavourable climatic conditions in the introduced areas. For example in Oregon, the pathogen behaves as a root pathogen and can stay inactive during warm and dry summers, which might explain the difficulty to isolate it from infected bark (Trione 1959). In the pest risk analysis performed for EPPO, Atlantic coasts of Portugal, Spain, France, south of Great Britain and Ireland were considered suitable for the establishment of the species (http://www.eppo.org/QUARANTINE/Pest_Risk_Analysis/PRA_documents.htm). Taiwan and the Himalaya region also appeared as highly suitable for this pathogen.

Epidemiological consequences of an aerial dispersal strategy are important. As a root pathogen, *P. lateralis* is well known to be effectively disseminated by water, soil and plants. As a result of this ability, it spread rapidly in the native range of Lawson's cedar trees (Hansen et al. 2000; Jules et al. 2002). In environments conducive to aerial spread, the dissemination of the pathogen might be even more effective. Moreover, once established outside a nursery, soil- or air-borne pathogens are much more difficult to eradicate. This aerial dispersal had not been considered in the pest risk analyses that have been conducted to date. As the first two steps of biological invasions (introduction and establishment) have been completed by *P. lateralis* in Brittany, this exotic species is likely to threaten extensive areas planted with *C. lawsoniana*, notably in Britain where *C. lawsoniana* is not a forestry species but is largely planted in amenity situations (Woodhall and Sansford 2006). This last point justified the inscription of *P. lateralis* into the A1 list of EPPO.

This outbreak report highlights the limitations of the European quarantine regulation.

First, although imports of *C. lawsoniana* plants are prohibited in Europe, *P. lateralis* was introduced, at least in two countries, and the sources of these first introductions have not yet been elucidated. It is highly likely that *P. lateralis* introduction pathways could be diverse even if the pathogen is a specialist. Moreover, plants infected by *Phytophthora* spp. can appear non-symptomatic if they have been treated with chemicals and simple visual inspection may not detect the disease (Brasier 2008). Plants for planting will represent a risky pathway until appropriate quarantine procedures with inspections before importations, effective diagnostic methods and quarantine periods are developed. Second, although *P. lateralis* was considered as eradicated in the nurseries, this organism could have escaped before the first report or remained unnoticed during several years before being recognized in farming environments. A more intensive control of the area surrounding the site of the first reports might have been useful. Concerning the outbreak in Brittany, it seems unlikely that trees were infected when planted (in the 1970s). Indeed, infected *C. lawsoniana* seedlings can die within a few weeks after infection and discoloured trees within a year (Hansen et al. 2000). Supplementary observations are required to assess the emergence of the disease in Brittany. However, we have already noticed that the symptom evolution can be rapid because pale green trees turned yellow within the summer period. The inoculum might have been transported after the planting of the hedges, through soil (potting mix of non-host plants may carry the pathogen) and/or water courses as has been described in American forests (Hansen et al. 2000), or by wind, in the hypothesis of an aerial dispersal.

As has been shown for *P. ramorum* (Goss et al. 2009), multiple introductions of *P. lateralis* might have occurred in Europe and in North America. Analysis of ITS sequences did not show any differences between *P. lateralis* strains from Oregon and those from France, but we cannot exclude the possibility of different introductions in Brittany.

Further genetic diversity analyses using more informative molecular markers of French and American isolates, in comparison with isolates from Taiwan or other Asiatic countries, might provide clues to understand the source of *P. lateralis* foci in France.

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