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Colonization of tree xylem by *Phytophthora ramorum*, *P. kernoviae* and other *Phytophthora* species

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The aetiology and frequency of *Phytophthora* spp. in discoloured xylem tissue beneath phloem lesions was investigated in a range of broadleaved trees infected with *P. ramorum, P. kernoviae, P. cambivora, P. citricola* and other species. Isolation was attempted from the inner surface of 81 sterilized discoloured wood panels (6 × 4 cm) from 53 trees. Discolouration mostly extended 1–5 mm into the xylem (75%), but incursions of 6–10 mm (10%) and 10–25 mm (15%) were frequent. Of the wood panels, 81% yielded *Phytophthora* spp. In 66 cases, both a wood panel and an overlying phloem panel were sampled. In 56% of these, a *Phytophthora* sp. was isolated from both the wood and the phloem panel. In 23% the *Phytophthora* sp. was isolated from the wood panel only and in 8% from the phloem panel only. Small 'island' phloem lesions, often in linear arrays adjacent to main lesions, were a common feature of *Fagus sylvatica* and *Quercus* spp. trees infected with *P. ramorum* or *P. kernoviae*. Island lesions were often connected by underlying strips or intermittent pits of discoloured xylem in line with the wood grain. *Phytophthora ramorum, P. kernoviae* and other *Phytophthora* spp. were successfully isolated from these connecting xylem features with *P. ramorum and P. kernoviae* also recovered from discoloured tissue 5–25 mm below exposed xylem surfaces 24–27 months after the overlying phloem was removed. These results show that these pathogens commonly occupy xylem beneath phloem lesions; that they can perennate in xylem tissue; that they can spread in xylem tissue ahead of phloem lesions; and indicate that they may initiate new phloem lesions in this way. Such colonization must lead to at least local xylem dysfunction. It is recommended that, if xylem discoloration is present, isolation of the *Phytophthora* sp. should be attempted from the xylem as well as the bark; also, that removal of infected outer sapwood should be undertaken during excision of bleeding lesions for disease control and in protocols aimed at preventing national or international spread of these tree stem pathogens.

Keywords: bark lesions, bleeding cankers, phloem, *Phytophthora*, tree stem diseases, wood

Introduction

In simple terms the outer stems of broadleaved trees consist of the non-living suberized outer bark, the living inner bark or phloem, a thin cambium layer, and outer rings of secondary xylem vessels or live sapwood also containing living parenchyma cells. A number of *Phytophthora* spp. are known to attack the phloem and cambial tissue of trees, causing stem necroses. They do so either by spreading upwards from the roots or collar or through aerial infections which penetrate the outer bark of the stem. The resulting stem necroses often give rise to a tarry exudate. In consequence, these infections are commonly described as stem bleeding lesions or stem bleeding cankers (e.g. Peace, 1962). Well-known examples in Europe include *P. cambivora* on *Castanea* and *Fagus*, *P. citricola* or *P. cactorum* on *Aesculus* and *Fagus*, *P. alni* and its subspecies on *Alnus*, *P. cinnamomi* on *Quercus suber* and *Q. rubra* and more recently, *P. ramorum* and *P. kernoviae* on *Fagus* and *Quercus* (Day, 1938; Peace, 1962; Brasier & Strouts, 1976; Brasier *et al*., 1993b, 2004a,b; Brasier, 1999; Gibbs *et al*., 2003; Jung *et al*., 2005; Vettraino *et al*., 2005).

Little information is available on the aetiology or frequency of *Phytophthora* spp. in xylem beneath natural stem infections, although the underlying xylem often shows a uniform dark discoloration. In the UK, for example, xylem discoloration is frequently observed in chestnut trees infected with *P. cinnamomi* or in beech trees infected with *P. cambivora* (R. G. Strouts & C. M. Brasier, unpublished). In northeastern USA and Germany, sapwood beneath phloem lesions on *Ulmus americana* and *Fagus sylvatica* caused by *P. inflata* is often discoloured red-brown or pink (Caroselli & Tucker, 1949; Jung *et al*., 2005). In California, Rizzo *et al*. (2002) observed black discoloration up to 3 cm into the xylem of *Lithocarpus densiflora* and

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Q. agrifolia infected with *P. ramorum*. Generally, xylem discoloration below phloem lesions has tended to be viewed as a host reaction to the presence of the pathogen in the phloem. *Phytophthora* spp. are often considered unable or unlikely to successfully inhabit secondary xylem tissue (e.g. Erwin & Ribeiro, 1996) and studies on natural infection of tree stems and attempts to isolate them from bleeding lesions have focussed mainly on phloem tissue (Smith *et al*., 1997).

Some information on *Phytophthora* spp. in tree xylem has come from studies involving wounding. Prior (1986) observed limited colonization of xylem of cocoa stems by *P. palmivora* around insect and beetle tunnels, and in artificial wound inoculations. Davison *et al*. (1994) isolated *P. cinnamomi* from the phloem and xylem of wound-inoculated *Eucalyptus marginata* and *Pinus radiata* stems; they considered that xylem invasion was confined to a narrow band adjacent to the cambium in *E. marginata*, whereas in *P. radiata* radial invasion of xylem occurred. They concluded that although disruption of xylem function, such as water transport, might result, evidence that this process played a role in the death of *E. marginata* trees infected by *P. cinnamomi* was inconclusive. Smith *et al*. (1997) showed that xylem tissue of wound-inoculated 2-year-old *Banksia brownii* saplings was invaded by *P. cinnamomi* and concluded that xylem responses of *B. brownii* were critical to understanding disease resistance.

In the UK, two invasive *Phytophthora* spp., *P. ramorum* (Werres *et al*., 2001) and *P. kernoviae* sp. nov. (Brasier *et al*., 2005), are currently spreading from heavily infected foliage of *Rhododendron ponticum* onto stems of *Fagus*, *Quercus* and other tree hosts, resulting in aerial stem bleeding lesions (Brasier *et al*., 2004b). During surveys on the natural host range and aetiology of the two pathogens in Cornwall, south-west England, a number of observations suggested the pathogens were active in xylem tissue underlying phloem lesions on naturally infected *F. sylvatica* and might also move from infected xylem into previously healthy bark. The presence of *P. kernoviae* or *P. ramorum* in the xylem of *Fagus*, *Quercus*, *Acer* and other hosts was therefore investigated by direct isolation. Similar investigations were carried out on trees in the survey areas with stem lesions caused by *P. cambivora*, *P. citricola*, *P. cinnamomi*, *P. gonapodyides* or *P. ilicis*. These observations and investigations are the subject of this paper.

Materials and methods

Media

The selective *Phytophthora* medium, SMA (synthetic mucor agar) + MRP, was prepared in the same way as the *Phytophthora* minimal medium (SMA) of Elliott *et al*. (1966) and then amended before autoclaving with 0·5 mL of a 4% MBC (benomyl hydrochloride) solution. The pH was adjusted to 6·5 with 1 M NaOH. After autoclaving at 121°C for 15 min the agar was cooled to 60°C then further amended with 0·4 mL of a 2·5% suspension of Pimaricin and 3 mL of a 1% w/v solution of Rifamycin SV. Carrot agar (CA) was prepared as described by Brasier (1969; details in Erwin & Ribeiro, 1996).

Sampling and isolation from phloem and xylem panels

Phytophthora spp. were isolated from necrotic inner bark or phloem tissue by initially removing an area of the outer bark with a mallet and chisel. A panel of the exposed necrotic phloem *c.* 4–6 × 4 cm was then removed and if necessary stored at 4–10°C. From this panel, 20 small pieces of necrotic phloem tissue (*c.* 3–20 mm^2), from the dead-live junction, were transferred directly onto SMA+MRP medium (10 pieces per Petri dish) within 4–24 h of collection of the sample.

To isolate from xylem, an additional panel of the same size was removed beneath the phloem panel (or from the exposed xylem surface if a larger area of inner phloem had already been removed). Wood panels were usually 10–12 mm deep, but occasionally additional layers were removed to 30 mm deep. As with the bark, isolations were undertaken within 4–24 h of removal from the tree after storage at 4–10°C.

With the exception of one preliminary test all wood panels were surface-sterilized before isolation using a standard method. This comprised immersing the wood panels in 70% ethanol for 20 s, rinsing in sterile water and then surface-drying on a paper towel. In the preliminary test on branch material of *F. sylvatica* tree BRN 11 a more stringent surface-sterilization method was used. This involved soaking the panels in tap water for 1 h, then sequentially rinsing for 15 s in 50% ethanol, 30 s in 10% sodium hypochlorite, a further 15 s in 50% ethanol, 3 min in sterile distilled water and 3 mins in fresh sterile distilled water. The panels were then surface-dried on filter paper.

After surface-sterilization thin slivers of discoloured xylem *c.* 5–10 × 10–15 mm were removed with a scalpel by working inwards from the unstained towards the discoloured area. From these slivers, 20 pieces 3–20 mm^2 were then plated onto SMA+MRP medium (10 per plate). All plates were incubated at 18–20°C in darkness and any developing colonies subcultured onto CA medium for identification.

ELISA tests

As a pre-isolation procedure, the presence of *Phytophthora* spp. was assessed in some samples using diagnostic lateral flow kits produced by Pocket Diagnostics. These kits incorporate a *Phytophthora*-specific antibody developed by Neogen. Three to four small pieces (*c.* 3 × 3 mm) of necrotic phloem or five to six slivers of discoloured xylem tissue were added to the commercial buffer and macerated. Two drops of the resulting suspension were added to the lateral flow device and the result read after 5–10 min. A blue line for the test material together with a blue line for the control indicated a positive result. Initially, the kits were tested with phloem material known to be infected with *Phytophthora* or, as negative controls,

Table 1 Recovery of *Phytophthora kernoviae* from surface-sterilized and non-surface-sterilized wood panels of *Fagus sylvatica* BRN 11a branch material

			Non-surface-sterilized		Surface-sterilized	
Lesion no.	Stem section	Quadrant	No. xylem pieces plated	No. positive for *P. kernoviae*	No. xylem pieces plated	No. positive for *P. kernoviae*
1	Middle	north-east	5	5	6	6
2		north-east	4	2	3	1
3		north-east	8	4	8	4
4		north-east	6	5	6	1
5		north-west	8	3	8	2
6	Bottom	north-east	10	10	10	7
Total no. positive isolations			41	31	41	21
Percentage positive isolations				76		51

[a]BRN, Burncoose, Cornwall.

other organisms such as *Armillaria* and *Ustulina*, and found to be a reliable indicator of *Phytophthora*.

Trees studied

Details of the species, size and location of the trees studied are given in Table 2, with the exception of trees BRN 09, 11 and 12, which are described below.

Results

Preliminary tests for *Phytophthora ramorum* of discoloured xylem

In February 2004, three mature *F. sylvatica* trees, BRN 09, 11 and 12, (50–140 cm diameter), exhibiting extensive aerial stem bleeding lesions caused by *P. kernoviae* and *P. ramorum*, were felled and their outer bark removed from ground level to *c.* 6 m to examine the distribution of lesions beneath. Subsequent removal of large areas of necrotic phloem revealed extensive discoloration of the cambial and xylem surface underlying the phloem lesions, in contrast to the surrounding healthy xylem. To investigate the xylem/phloem interface, a branch of BRN 11 *c.* 2·5 m long and 23 cm in diameter, with numerous discrete external bleed zones, was cut into three sections (top, middle and bottom). The sections were marked into north, south, east and west quadrants and the outer bark removed. Phloem lesions occupied about 40% of the surface area.

The south-east quadrant of the bottom section exhibited multiple small island lesions (Fig. 1a), designated lesions A-G. Seven phloem panels were removed, one for each lesion, revealing a discoloured xylem surface (Fig. 1b). Isolations were then made onto SMA+MRP, both from the phloem lesions and immediately beneath the discoloured xylem surface, but without surface-sterilization. *Phytophthora kernoviae* was isolated at high frequency from six of the seven phloem panels (panel E was negative), and at high frequency from all seven of the corresponding wood panels, including panel E. This demonstrated a significant *P. kernoviae* presence at or below the xylem surface.

Since the above wood panels also included the cambial layer, the result did not exclude the possibility that *P. kernoviae* isolated from the wood panels came from the cambium rather than the xylem. A further set of discoloured wood panels was therefore taken from the BRN 11 branch sections. These comprised six pairs of adjacent wood panels, taken from beneath phloem lesions at different positions on the branch (see Table 1). One panel of each pair was not surface-sterilized. Small pieces of xylem tissue were taken from the discoloration 1–3 mm below the surface and plated onto SMA+MRP. For the corresponding panels, the residual cambium was scraped off with a scalpel until the xylem fibres were clearly exposed. The panels were then subject to the stringent surface-sterilization protocol and isolation attempted from the discoloured xylem 1–3 mm below the surface. The results are shown in Table 1. All 12 of the wood panels yielded *P. kernoviae*. In terms of plated xylem pieces, 76% of the non-surface-sterilized and 51% of the surface-sterilized pieces yielded the pathogen, confirming that *P. kernoviae* was present to a significant level in the xylem beneath the phloem and cambium.

Phytophthora isolation frequency and other characteristics of xylem lesions

Following the successful isolation of *P. kernoviae* from xylem of *F. sylvatica*, attempts were made to isolate *Phytophthora* spp. from surface-sterilized discoloured wood panels beneath bleeding phloem lesions on a further 53 trees during surveys at 11 Cornish and one non–Cornish site. The results of these investigations are summarized in Table 2. In addition to *P. kernoviae* on *F. sylvatica*, the *Phytophthora*-host combinations for which the *Phytophthora* sp. was obtained from the xylem included *P. ramorum* on *Acer pseudoplatanus*, *F. sylvatica*, *Q. acuta*, *Q. cerris*, *Q. petraea* and *Schima argentea*; *P. cambivora* on *F. sylvatica* and *Q. robur*; *P. cinnamomi* on *Q. cerris*; *P. citricola* on *F. sylvatica* and *A. pseudoplatanus*; *P. gonapodyides* on *F. sylvatica*; and *P. ilicis* on *Ilex aquifolium*.

A total of 81 wood panels were taken from the 53 trees (Table 2). Of these 81% yielded a *Phytophthora* sp.

Table 2 Isolation of *Phytophthora* species from discoloured xylem

Phytophthora species	Host species	Tree no.[a]	Stem diam. (cm)[b]	Date	Wood panel no.	Discoloration depth (mm)	Xylem isolation (+/–)	Isolation fequency (%)[c]	Bark isolation (+/–)
P. cambivora	*Fagus sylvatica*	SCO 51	35	11/01/06	A	4	+	20	+
		WW 01	40	20/07/05	D	2	+	15	+
					E	2	–	0	+
		WW 03	80	20/07/05	D	2	–	0	–
		WW 05	40	20/07/05	A	2–3	+	5	+
	Quercus robur	TRE 15	65	24/08/05	C	3–6	+	30	–
P. cinnamomi	*Q. cerris*	LGH 14	30	08/12/05	A	10–12	+	20	+
P. citricola	*Acer pseudoplatanus*	CAE 63	60	20/02/06	A	4	+	25	+
		KIL 09	60	12/01/06	A	5	+	100	+
		MTE 17	30	07/12/05	A	2	+	80	+
		MTE 29	45	05/12/05	A	5–6	+	100	+
		MTE 40	40	07/12/05	A	2	+	100	+
					xylem streaks	1	+	5	n/a
		TRG 11	40	29/04/05	B	3–4	+	5	–
		TRG 28	30	12/01/06	A	4	+	15	+
	Aesculus indica	TRW 16	50	24/02/06	B	5	+	10	–
	F. sylvatica	KIL 04	75	30/06/05	A	3	–	0	+
		TRG 07	100	22/08/05	C	2–8	+	45	–
		TRW 10	100	09/01/06	D	2	+	40	+
					B	2	+	70	+
	Tilia sp.	LGH 06	80	26/08/05	C	3–8	–	0	–
P. gonapodyides	*F. sylvatica*	MTE 30	42	05/12/05	A	2	+	20	+
		MTE 36	120	07/12/05	A	10	+	40	+
		TRE 29	100	11/01/06	A	1	+	100	+
P. ilicis	*Ilex aquifolia*	PGP 06	18	26/05/05	B	1	–	0	+
		TRG 18	30	22/08/05	B	2	–	0	–
		TRG 19	20	22/08/05	B	2	–	0	–
		TRG 20	20	22/08/05	B	2	+	5	–
P. kernoviae	*F. sylvatica*	BRN 21	100	19/07/04	xylem pits	1–2	+	15	nd
					I	1–3	+	100	–
		CAE 57	120	21/02/06	B	5	+	15	nd
				21/09/06	C	5	+	75	n/a
		HOL 01	90	19/10/04	B	3–4	+	35	+
		HOL 07	100	23/08/05	A	2	+	10	+
		KIL 03	100	29/06/05	B	3–4	–	0	+
					C	3–4	+	25	+
				19/09/05	D	3–4	+	30	–
					E	12	–	0	–
					F	12	+	35	nd
		KIL 06	45	28/06/05	A	1–3	+	95	+
					B	1–3	+	35	+
					C	1–3	+	75	+
					D	6	+	40	+
				22/02/06	E xylem streaks	2	+	30	n/a
					F xylem streaks	2	+	10	n/a
					G	2	–	0	nd
					H	1	+	35	nd
		KIL 10	80	20/02/06	A	5	+	90	+
		KIL 11	80	21/02/06	A	1	+	45	+
		SCO 12	100	27/06/05	C	3	+	5	–[f]
		SCO 35	80	28/06/05	A	5	+	5	+
					B	2–3	+	10	–
					xylem pits	1–2	+	10	nd
		SCO 39	100	28/06/05	B	2	+	5	–
		TRG 21	100	25/04/05	A	2–3	+	45	+
		TRG 27	50	12/01/06	A	5	+	5	–
		TRG 29	80	12/01/06	A	2	+	15	+
		TRG 31	60	12/01/06	A	2	+	10	+

Table 2 *Continued*

Phytophthora species	Host species	Tree no.[a]	Stem diam. (cm)[b]	Date	Wood panel no.	Discoloration depth (mm)	Xylem isolation (+/–)	Isolation fequency (%)[c]	Bark isolation (+/–)
P. ramorum	*A. pseudoplatanus*	TRW 03	70	25/05/05	E1	20–25	+	67	nd
					E2	20–25	+	100	nd
					E3	20–25	+	100	nd
	F. sylvatica	BOL 02	50	12/09/05	A	2–3	+	55	–
	Q. cerris	BRN 58	120	25/08/05	A	6–8	+	10	+
					B	15	+	15	n/a
					C	20	+	60	n/a
					D	5–6	+	85	+
				11/12/06	E	25	+	25	n/a
		LGH 07	25	08/12/05	B xylem pits	1	–	0	+
					G xylem pits	2	+	75	+
					G extension	10	+	65	+
					Hi	20	+	5	–
					Hii	15	+	5	n/a
		BRN 05	100	04/10/04	E	10	+	5	+
	Q. acuta	TRW 19	40	09/12/05	A	2	+	70	+
	Q. petraea	BRN 62	45	13/09/05	E	4–5	+	10	–
	Schima argentea	TRW 13	18	09/12/05	A	3	+	85	+
Phytophthora sp.[d]	*Acer maximowiczianum*	TRW 15	50	09/01/06	A	5	+	5	–
Phytophthora sp.[e]	*F. sylvatica*	MTE 04	80	05/12/05	A	13	–	0	–
		SCO 25	70	28/06/05	C	3	–	0	–
		SCO 40	110	27/06/05	B	5	–	0	–
		TRW 10	100	26/08/05	C	2	–	0	–

nd, no data; n/a not applicable: no phloem lesion above the xylem pit or xylem streaking.

[a]All code letters except WW refer to sites in Cornwall. BOL, Bolitho; BRN, Burncoose; CAE, Caerhays; HOL, Holly Wood; KIL, Killifreth; LGH, Heligan; MTE, Mount Edgecombe; TRE, Trevince; TRG, Tregullow; TRW, Trewithen; SCO, Scorrier; WW, Waggoners Wells, Surrey.

[b]Stem diameter 1·3 m above ground level.

[c]Positive isolations of *Phytophthora* from 20 xylem pieces plated (%).

[d]Unidentified *Phytophthora* species.

[e]Positive ELISA test for *Phytophthora*, but *Phytophthora* not isolated on this attempt.

[f]No isolation achieved from phloem necrosis on three successive visits, but *P. kernoviae* obtained from underlying xylem.

Within-panel isolation frequencies (per 20 xylem pieces taken) ranged from 5 to 100%, the average being 32 ± 3·7(SE)%. For 66 wood panels a corresponding phloem panel was also sampled. Of these 66 instances, nine (14%), all from *F. sylvatica*, did not yield a *Phytophthora* sp. from either the wood or the phloem panel, despite the fact that the phloem gave a positive ELISA test for *Phytophthora*. These therefore represented either complete isolation failures or circumstances where the pathogen was no longer viable. Thirty-seven (56%) yielded a *Phytophthora* sp. from both the phloem and the wood panel. Five (8%) yielded a *Phytophthora* sp. from the phloem, but not from wood. Importantly, 15 (23%) yielded a *Phytophthora* sp. from the wood, but not from the phloem panel.

Isolation from the wood panel, but not from the associated phloem panel, occurred across a wide range of *Phytophthora*-host combinations, including *P. ramorum*, *P. kernoviae* and *P. cambivora* on *F. sylvatica* and *Quercus* spp; *P. citricola* on *F. sylvatica* and *A. pseudoplatanus*; and *P. ilicis* on *I. aquifolium*. A striking example was *F. sylvatica* tree SCO 12 (Table 2), where three successive attempts to isolate *Phytophthora* from phloem lesions on different dates failed, but the first isolation attempt from the wood (panel C) yielded *P. kernoviae*. Examples of a high frequency of isolation from the xylem, but zero from the phloem, included *F. sylvatica* BOL 02 panel A, where 55% (11/20) of the xylem pieces yielded *P. ramorum*; and *F. sylvatica* BRN 21 panel I (Table 2), where 100% (20/20) of the xylem pieces yielded *P. kernoviae*.

Among the 81 wood panels the depth of discoloration varied from 1 to 25 mm (Table 2). In most panels (*c.* 75%) the discoloration extended only 1–5 mm into the xylem. In 10% it extended 6–10 mm and in 15% it extended 11–25 mm. The overall mean depth of discoloration was *c.* 5·4 ± 0·6(SE) mm. The colour of the discoloration varied with the particular host-pathogen combination and the state of oxidation of the tissues. For example, xylem discoloration of fresh (unoxidised) *P. citricola* lesions on *A. pseudoplatanus* was usually a pale green to ochre colour, but after exposure to air often became a dark

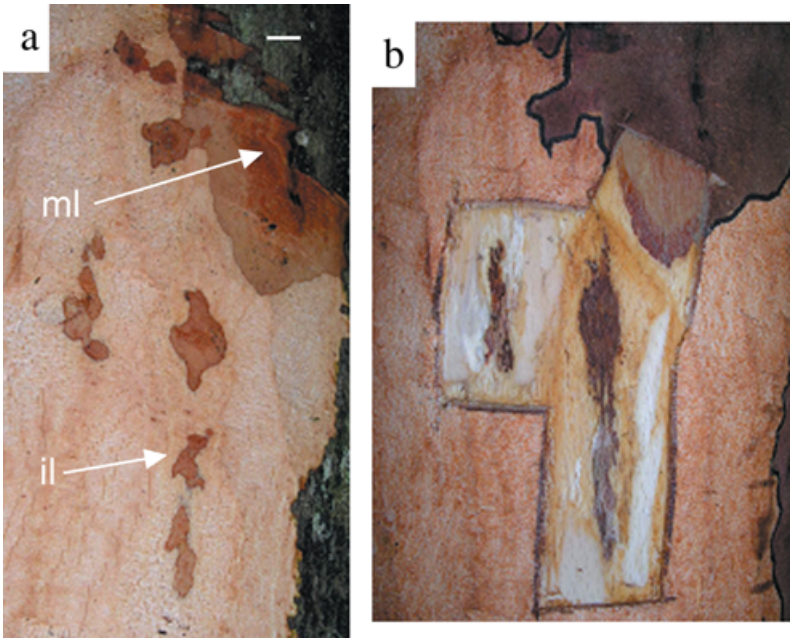

Figure 1 Phloem and xylem lesions on *Fagus sylvatica* BRN11 branch material caused by *Phytophthora kernoviae*. (a) Major and island lesions A-G in the phloem. (b) Partial removal of phloem to show xylem discoloration underlying island lesions and major lesion. ml, major lesion; il, island lesions. Scale bar 1 cm.

green-black. With *P. ramorum* on *Acer* fresh lesions were usually a pale tan, while those on *F. sylvatica* were commonly a pink or orange colour and oxidized lesions dark brown.

Detailed observations on *Phytophthora* in xylem

During studies on felled *F. sylvatica* trees BRN 09, 11 and 12 (see above), linearly distributed multiple island lesions were often observed in the phloem above or below major or coalesced lesions (Fig. 2a,b). Sometimes island lesions lay in line with an extension from a main lesion (Fig. 2b). In transverse section, some lesions appeared to be developing from the cambium/xylem surface rather than from the outer bark inwards (Fig. 4e). Usually, no connection was observed within the phloem either between island lesions, or between island lesions and main lesions. However, a possible connection via a distinct zone of discoloration on the cambial surface and within the outer sapwood vessels was frequently observed (Fig. 2b and 2c). To examine the aetiology of *Phytophthora*-infected xylem, including the possibility that connections were present between island lesions, detailed investigations were carried out on several trees listed in Table 2.

Phytophthora ramorum *in xylem of* Acer pseudoplatanus
On the east side of *A. pseudoplatanus* TRW 03 (Table 2), a large phloem lesion caused by *P. ramorum* extended from near ground level to *c.* 215 cm, and was 40 cm wide at the base (Fig. 3a). On the south-west side a second lesion extended from ground level to *c.* 255 cm, and was *c.* 55 cm wide at the base. A strip of fresh, pale ochre- to tan-coloured necrotic phloem 1–6 cm wide occurred around the entire perimeter of each lesion; larger areas of fresh necrosis occurred in the top 40–50 cm of each lesion (Fig. 3a). ELISA tests conducted on fresh necrotic phloem at 11 positions, five on the east lesion (Fig. 3a) and six on the south-west lesion, were all positive for *Phytophthora*.

Eight phloem panels were taken around the margin of the east lesion, five of which yielded *P. ramorum*. Three wood panels were also taken from beneath the necrotic bark: panel 1 at the top of the lesion (215 cm above ground level); panels 2 and 3 on each side of the lesion at *c.* 100 and 80 cm above ground level (Fig. 3a). The xylem was discoloured ochre-yellow to a depth of 20–25 mm (Fig. 3b). *Phytophthora ramorum* was isolated readily from the deepest stain on all three panels (Table 2), confirming its presence in xylem up to 25 mm beneath the bark/xylem interface.

Isolation of P. ramorum *and* P. kernoviae *from xylem connections between phloem lesions*
The possibility of xylem connections underlying island lesions in the phloem was examined in *F. sylvatica* trees BRN 21, SCO 35 and KIL 06 infected with *P. kernoviae*, and in *Q. cerris* LGH 07 infected with *P. ramorum* (Table 2). Removal of outer bark from bleeding areas (Fig. 4a) on all four trees revealed extensive lesions, together with arrays of island lesions, often with a slanted or sinuous linear distribution (Fig. 4b). Exposure of the xylem surface revealed general xylem discoloration beneath the larger lesions. In addition, the larger lesions and the small island lesions were often connected by discoloration in the xylem and cambium. Such connections included broader 1- to 2-cm wide strips of discoloration, continuous narrow streaks of discoloration <3 mm wide, and linear arrays of intermittent dark streaks, termed here xylem pits. Xylem pits comprised brown to black indents extending from the cambial surface into the xylem, surrounded by otherwise smooth, non-discoloured xylem

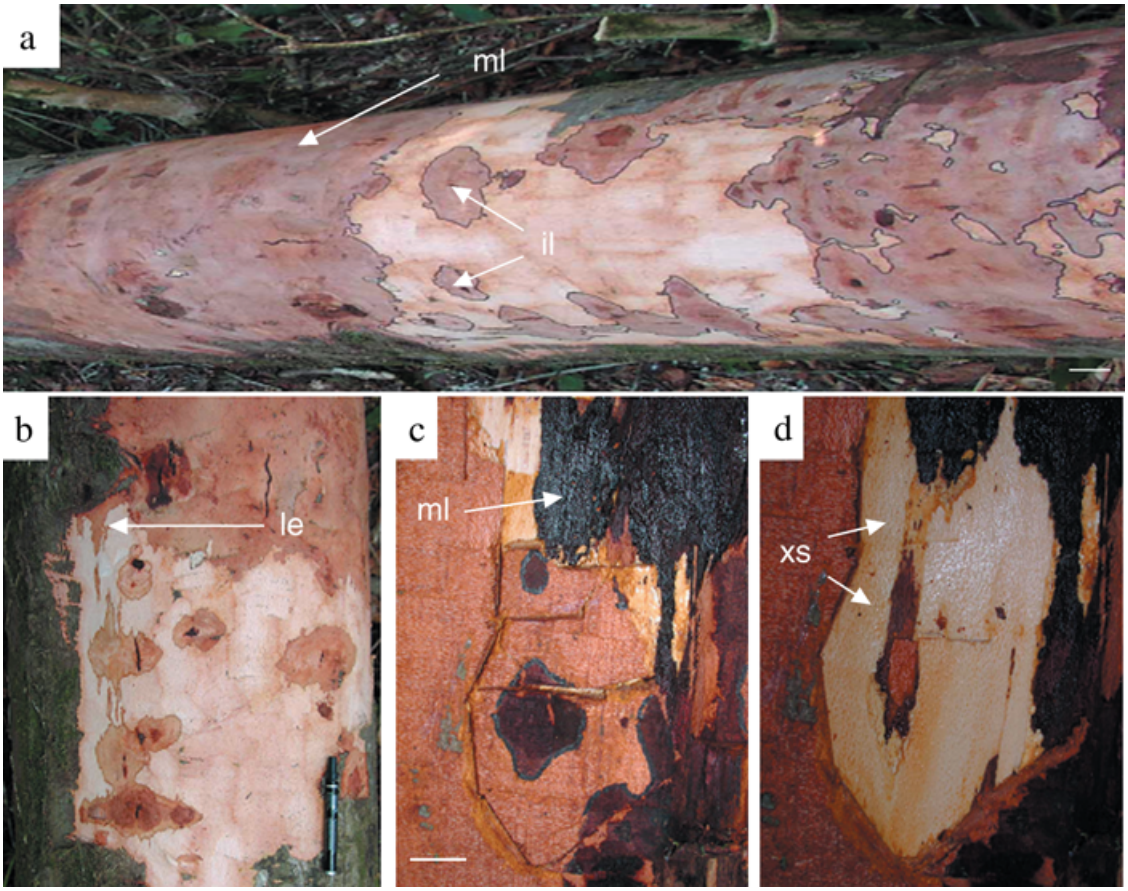

Figure 2 Phloem and xylem lesions on *Fagus sylvatica* BRN09, BRN21 and KIL06 caused by *Phytophthora kernoviae*. (a) Major lesions and island lesions in phloem of *F. sylvatica* BRN09 after removal of outer bark. (b) Linearly distributed island lesions in phloem of BRN09. (c) Island lesions below a major lesion in phloem of BRN21. (d) Xylem discoloration and xylem pitting beneath the lesions in (c), showing connections between the island lesions and also to the major lesion. ml, major lesion; il, island lesions; le, linear extension; xs, xylem stain or discoloration. Scale bars (a) 10 cm; (c) 1 cm.

tissue (Fig. 4 c). These features always followed the slant of the grain.

Shallow and deep xylem pits

Fagus sylvatica BRN 21 had heavy stem bleeding from the root flares to *c.* 2·5 m above ground level (Fig. 4a). Linear arrays of shallow (*c.* 1–2 mm) xylem pits were observed between small island lesions (Fig. 4 c). The phloem overlying the connecting xylem pits was visibly healthy. A wood panel with xylem pitting was taken and *P. kernoviae* was isolated at low frequency after standard surface-sterilization (Table 2). Isolation was also attempted from similar shallow xylem pits connecting two separate phloem lesions on *F. sylvatica* SCO 35 and again, *P. kernoviae* was isolated at low frequency (Table 2). These results confirmed that *P. kernoviae* was present in the shallow xylem pits between island lesions.

Quercus cerris LGH 07 (Table 2) had extensive bleeding caused by *P. ramorum* from ground level up to *c.* 2·5 m on the north-east to south-east side. Removal of outer bark revealed a large number of coalescing small lesions and occasional island lesions, corresponding to the bleed area. Most lesions were pale tan to dark pink-brown. When phloem was removed from near the base of the main lesion area xylem pits were observed extending *c.* 10 cm below this area. The pitting was in line with the grain (Fig. 4d). The pits were commonly in the form of cracks *c.* 20–50 mm long and 2–10 mm deep. *Phytophthora ramorum* was isolated at high frequency from beneath the xylem pits in two wood panels: panel G at 2 mm beneath the xylem surface and panel G extension at 10 mm below the surface (Table 2). *Phytophthora ramorum* was also isolated (but at lower frequencies) from the overlying lesions in the phloem. These lesions were mostly superficial, i.e. close to the outer bark surface. Often they were not visually connected with the pits in the xylem (Fig. 4d). However removal of the phloem above wood panel G revealed a fresh phloem lesion that was apparently a result of the pathogen breaking out directly from the pits in the xylem (Fig. 4e).

Below the main lesion area of LGH 07 was an array of small island lesions in the phloem (Fig. 4f). Removal of

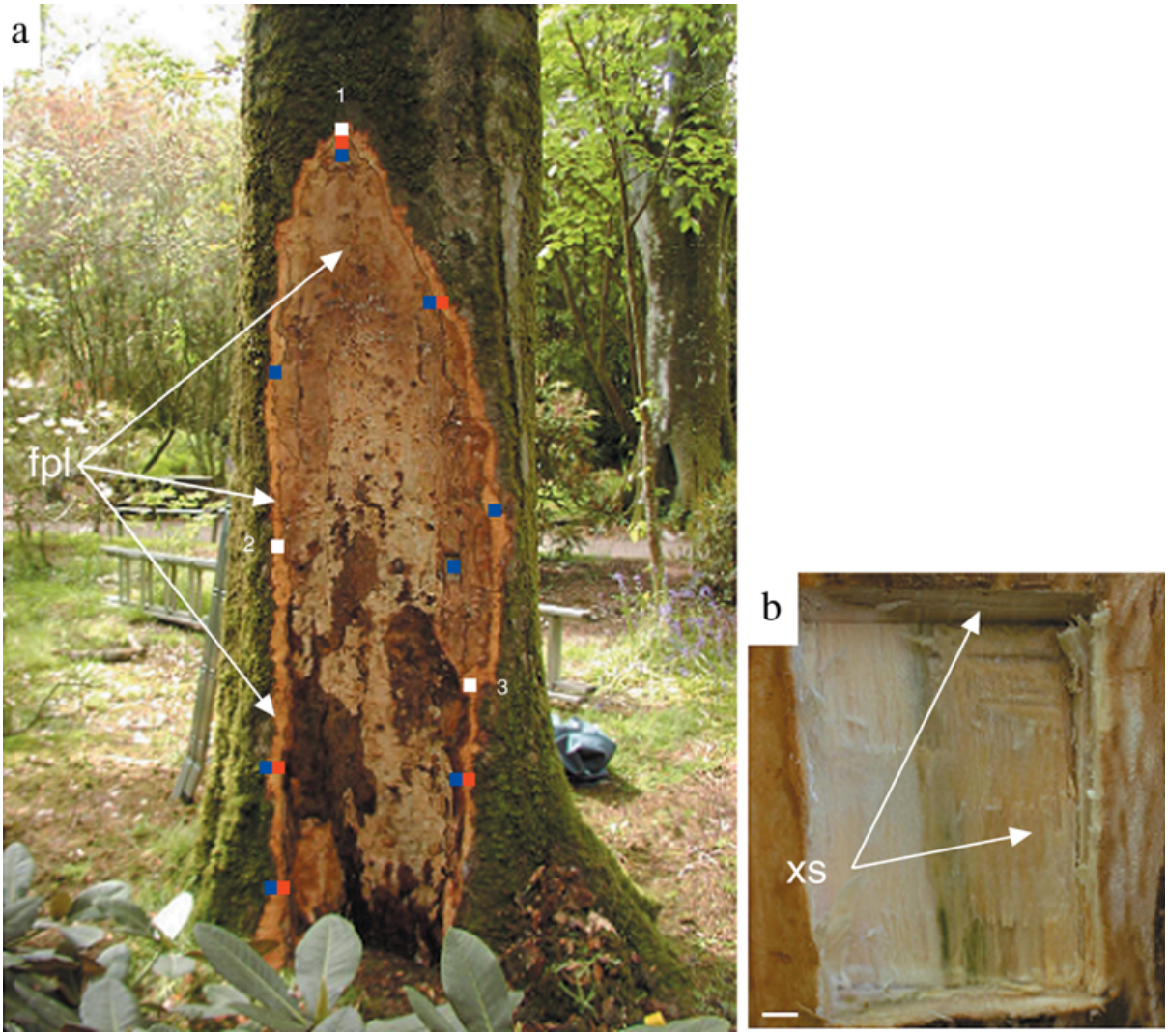

Figure 3 *Phytophthora ramorum*-lesions in phloem and xylem of *Acer pseudoplatanus* TRW03. (a) Large phloem lesion on east side, showing fresh necrosis around perimeter of lesion and large area of fresh necrosis at top of lesion. (b) Ochre-yellow, green-edged discoloration in xylem of wood panel 1 at a depth of *c.* 25 mm. fpi, fresh phloem lesion caused by *P. ramorum*; xs, xylem stain or discoloration; red square, ELISA test position; blue square, phloem sample; white squares, wood panels 1–3. Scale bar (b) 1 cm.

the phloem again revealed a number of xylem pits or cracks 5–20 mm deep. These pits were in line with the grain, running beneath and connecting the phloem lesions. The pits also ran downwards another 30 cm from the lesion array to a separate island lesion 10 cm below ground level (from which *P. ramorum* was later isolated). The phloem between these points was visibly healthy. One wood panel containing a deep xylem pit (panel Hi) was taken directly underneath a lesion in the array; another (panel Hii) was taken midway between the lesion array and the lesion 10 cm below ground level. *Phytophthora ramorum* was isolated (at very low frequency) from the xylem pits in both panels, at depths of 15 and 20 mm into the xylem, respectively (Table 2). In each instance the pathogen took 12–14 days to grow out of the xylem pieces.

These results confirmed that *P. ramorum* was present in deep xylem pits connecting otherwise apparently isolated phloem lesions.

Discoloured xylem strips and streaks

In June 2005 *F. sylvatica* KIL 06 (Table 2) exhibited two main bleeding zones: a lower zone 40–120 cm above ground level, and an upper zone 200–220 cm above ground level. In the phloem of the lower zone were five large island lesions, two of which were connected by a vertical strip lesion (Fig. 5a). There were also a number of small, often linearly arranged island lesions. Removal of the phloem containing these lesions revealed areas of discoloured xylem beneath all the island lesions and a narrow strip of discoloured xylem beneath the strip lesion (Fig. 5b). Two wood panels (A and C) were taken from

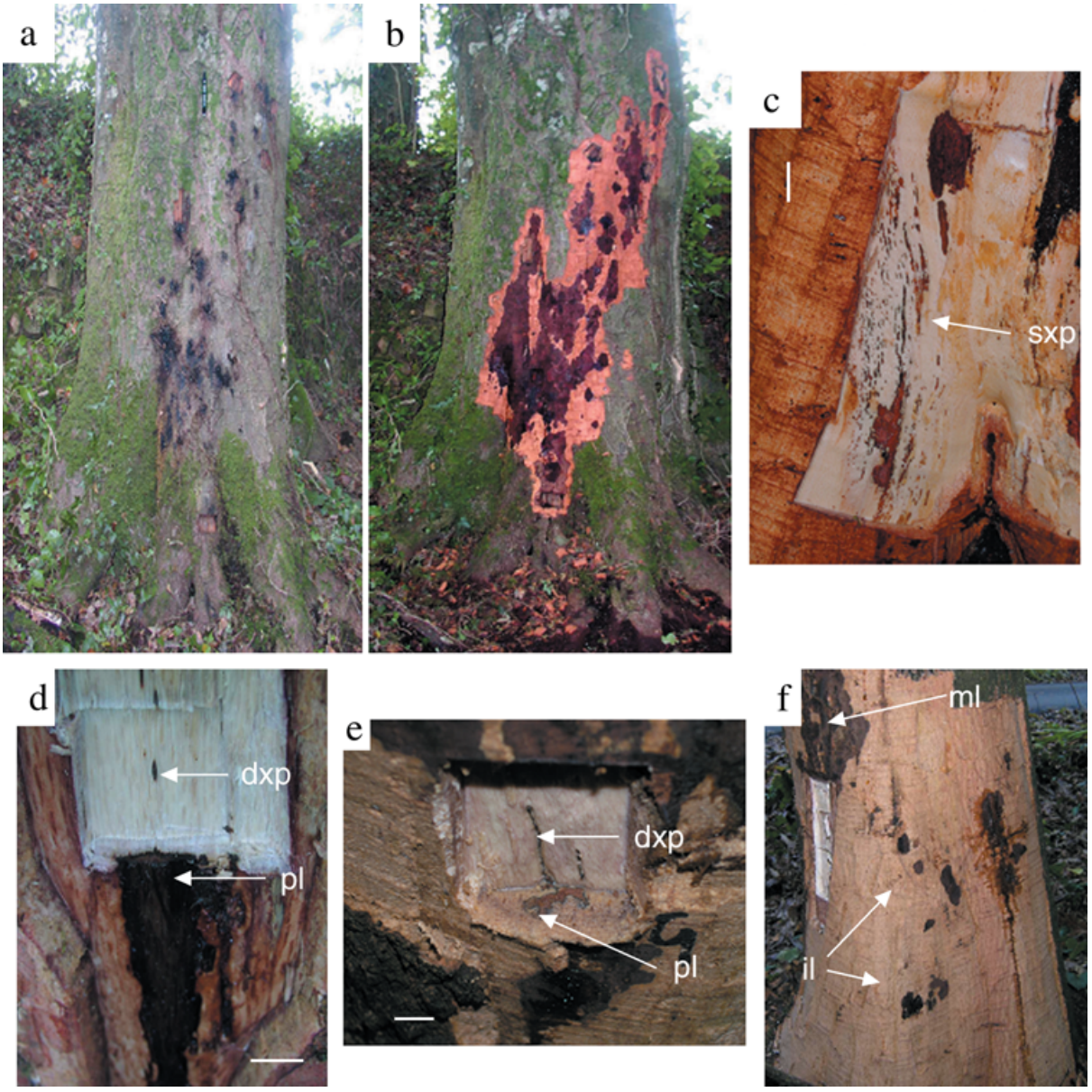

Figure 4 Xylem pitting in *Fagus sylvatica* BRN21 and *Quercus cerris* LGH07 caused by *Phytophthora kernoviae* and *P. ramorum*. (a) Bleeding on lower stem of *F. sylvatica* BRN21 caused by *P. kernoviae*. (b) Underlying lesions in phloem of BRN21. (c) Linear array of shallow xylem pits beneath two small island lesions in phloem of BRN21. (d) Deep xylem pits in *Q. cerris* LGH07 in line with the grain, caused by *P. ramorum*. (e) Pink phloem lesion spreading from deep xylem pits into the phloem of LGH07. (f) Array of small island lesions below main lesion on LGH07. sxp, shallow xylem pits; pl, phloem lesion; dxp, deep xylem pits; ml, main lesion; il, island lesions. Scale bars 1 cm.

beneath the main island lesions in the lower zone and a third panel (B) from the discoloured xylem beneath the vertical strip lesion (Fig. 5b). *Phytophthora kernoviae* was readily isolated from discoloration 1–3 mm below the xylem surface of all three panels (Table 2).

In the phloem of the upper bleeding zone a single 30 × 3 cm vertical strip lesion was present. Removal of this lesion revealed a 2-cm-wide strip of discoloured xylem that extended 7 cm above and 16 cm below the phloem lesion. A wood panel (D) was taken from the centre of the discoloured xylem strip and *P. kernoviae* was isolated 6 mm within the xylem (Table 2). These results confirmed the presence of *P. kernoviae* within the discoloured xylem strips as well as within the discoloured xylem beneath the main lesions.

In February 2006 fresh bleeding was observed around all the original lesion areas of KIL 06. Removal of outer bark adjacent to the lower bleeding zone revealed multiple new phloem lesions. Two new island lesions (Fig. 5c) and a small lesion extending upwards from the original lower bleeding zone were examined. Removal of the phloem revealed 4-cm-long faint grey xylem streaks connecting

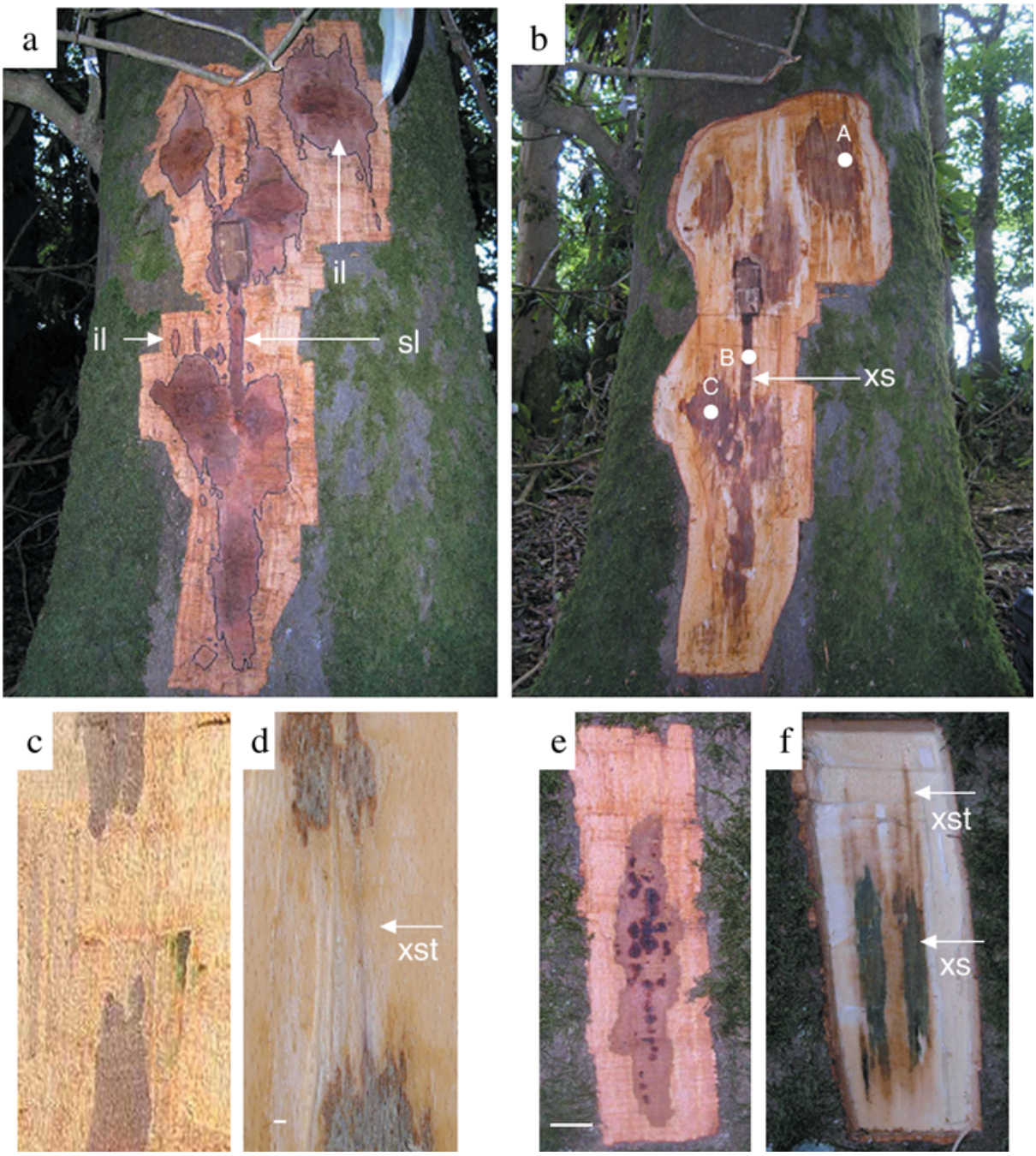

Figure 5 Xylem strips and streaks on *Fagus sylvatica* KIL06 and *Acer pseudoplatanus* MTE40 caused by *Phytophthora kernoviae* and *P. citricola*. (a) Multiple phloem lesions caused by *P. kernoviae* in the lower bleeding zone on KIL06, including a strip lesion and other linear features. (b) Xylem surface in lower zone showing discoloured xylem strip and positions of wood panels A-C. (c) New island lesions in phloem in February 2006. (d) Faint xylem streaks underlying island lesions shown in (c). (e) Phloem lesion in *A. pseudoplatanus* MTE40 caused by *P. citricola*. (f) Xylem discoloration and xylem streaks underlying phloem lesion in (e). sl, strip lesion; xs, stained or discoloured xylem strip; il, island lesions; xst, xylem streaks; white spots, positions of wood panels A-C. Scale bars 1 cm.

the new island lesions (Fig. 5d) and similar faint xylem streaks running vertically 25 cm above the lesion extension. Two wood panels, E and F, respectively, were taken from these two areas of xylem streaking. *Phytophthora kernoviae* was isolated at low frequency from both panels at *c.* 2 mm into the xylem (Table 2). This demonstrated that the pathogen was present in faint streaks of xylem both between and extending well beyond the phloem lesions.

A similar phenomenon was observed in *A. pseudoplatanus* tree MTE 40 infected with *P. citricola* (Table 2). This tree

had multiple bleed zones from 40 to 200 cm above ground level. Removal of outer bark from a zone at 100 cm revealed a pink-brown narrow vertical strip lesion *c.* 12 × 2 cm from which *P. citricola* was isolated (Fig. 5e). Removal of the phloem revealed two adjacent narrow discoloured areas on the xylem surface, moss-green to ochre in colour. The combined area covered by xylem discoloration, at *c.* 15 × 3 cm, was longer and wider than that of the phloem lesion (Fig. 5f). The green discoloration in the xylem was *c.* 2 mm deep. *Phytophthora citricola* was readily isolated from a wood panel taken from the discoloured xylem (Table 2). In addition, faint longitudinal brown streaks extended in the xylem *c.* 8 cm above the main xylem discoloration and 10 cm beyond the upper limit of the phloem lesion (Fig. 5f). These streaks penetrated only 1 mm into the xylem. Another wood panel was taken that included the faint streaking 5–8 cm above the main xylem lesions. *Phytophthora citricola* was isolated from the streaking at low frequency (Table 2), indicating that it was present in the xylem several centimetres ahead of the phloem lesion.

Perennation of P. ramorum *and* P. kernoviae *in xylem*

In June 2004, Turkey oak (*Q. cerris*) BRN 58 (Table 2) exhibited five external bleed spots on the east side of the trunk 160–230 cm above ground level caused by *P. ramorum*. Removal of the phloem revealed six extensive coalescing mottled pale to dark pink-brown lesions with black marginal zone lines, covering *c.* 3000 cm^2. In August 2004, as part of an experiment to control spread of the pathogen within the tree, all the necrotic phloem was excised to the level of the xylem surface and another 5 cm of healthy phloem was excised beyond the visible lesion edges. Removal of the phloem lesions revealed corresponding zones of pink to dark red-brown discoloration extending several millimetres into the xylem. No attempt was made at this time to isolate the fungus from the xylem.

In August 2005, 12 months after the phloem excision, two fresh bleeding spots were observed beyond the original excision area. Removal of outer bark revealed fresh lesion development (Fig. 6a). *Phytophthora ramorum* was isolated from these phloem lesions. The necrotic phloem was then removed and two wood panels, A and D, were taken from xylem discoloration up to 8 mm deep beneath these fresh phloem lesions. *Phytophthora ramorum* was obtained from both wood panels (Table 2).

The surface of the original August-2004 excision area was now dry and blackened. Two further wood panels, B and C, were removed from the edge and the centre of the original excision area, respectively (Fig. 6a). In panel B extensive dark brown to pinky-brown discoloration extended *c.* 15 mm into the xylem. Bright pink 'break out' tongues of discoloration were present at the deepest level, suggesting fresh pathogen activity. In panel C, similar discoloration extended up to 20 mm into the xylem, stripes of bright pink freshly colonized xylem alternating with pink-brown older colonized xylem (Fig. 6b,c). Again it appeared that *P. ramorum* might be surviving within the xylem. An ELISA test on the deepest discoloration in panel C was positive for *Phytophthora*. Isolation from the deepest parts of each panel yielded *P. ramorum* at low frequency in panel B and at high frequency in panel C (Table 2). On 11 December 2006, 27 months after the original excision, another wood panel, E, was removed close to the position of panel C. *Phytophthora ramorum* was again isolated (frequency 20%) 20 mm beneath the xylem surface. This confirmed that *P. ramorum* had remained active and was still penetrating into the xylem 27 months after the excision of the original phloem lesions.

A similar observation was made on *F. sylvatica* CAE 57 infected with *P. kernoviae*. In September 2005 extensive phloem lesions on this tree were excised down to the xylem surface, together with 5 cm of healthy phloem beyond the visible lesion edges. By February 2006 the surfaces of the original excision areas were dry and blackened. A wood panel (B) was removed from the centre of the main original excision area. Beneath the surface bright pink discoloration extended 5 mm into the xylem. *Phytophthora kernoviae* was isolated at low frequency from the deepest portion of the discoloration (Table 2). A similar wood panel, C, was taken close to panel B in September, 2006. Although the xylem had dried out, *P. kernoviae* was readily isolated from the deepest pale pink discolouration (Table 2). This demonstrated that *P. kernoviae* could survive at least 24 months within exposed xylem of *F. sylvatica*.

Lagoons

A common feature of phloem lesions below external bleed points was a cavity in the phloem where the tissue had broken down. In many cases the cavity extended into the xylem and occasionally it was entirely confined to the xylem. Often the cavity was filled with a clear, pale pink or orange liquid that quickly oxidized to red-brown on exposure. These cavities were therefore termed lagoons. Most lagoons were lenticular to oval (Fig. 7); they ranged in area from *c.* 1 cm^2 up to 150 cm^2 and were 2–15 mm deep. They were often associated with callusing (Fig. 7).

Sometimes a lagoon was found under a phloem lesion where no external bleeding was occurring. This was interpreted as an incipient bleed point: a point which would become an active bleed if sufficient pressure built up in the lagoon cavity, resulting in external release of the lagoon liquid. Not all external bleed points had a lagoon underneath. In these cases there was usually a more deeply discoloured area within the phloem lesion (Fig. 5e) and sometimes correspondingly darker discoloration on the xylem surface.

Lagoons were found frequently beneath external bleed points on *F. sylvatica* infected with *P. ramorum, P. kernoviae* (Fig. 7) or *P. gonapodyides*; *Q. cerris* infected with *P. ramorum*; *Q. robur* infected with *P. cambivora*; and *A. pseudoplatanus* infected with *P. citricola*. They were also observed with other, less common host-*Phytophthora* associations, including *S. argentea* infected with *P. ramorum* and *F. sylvatica* infected with *P. citricola*. They appear

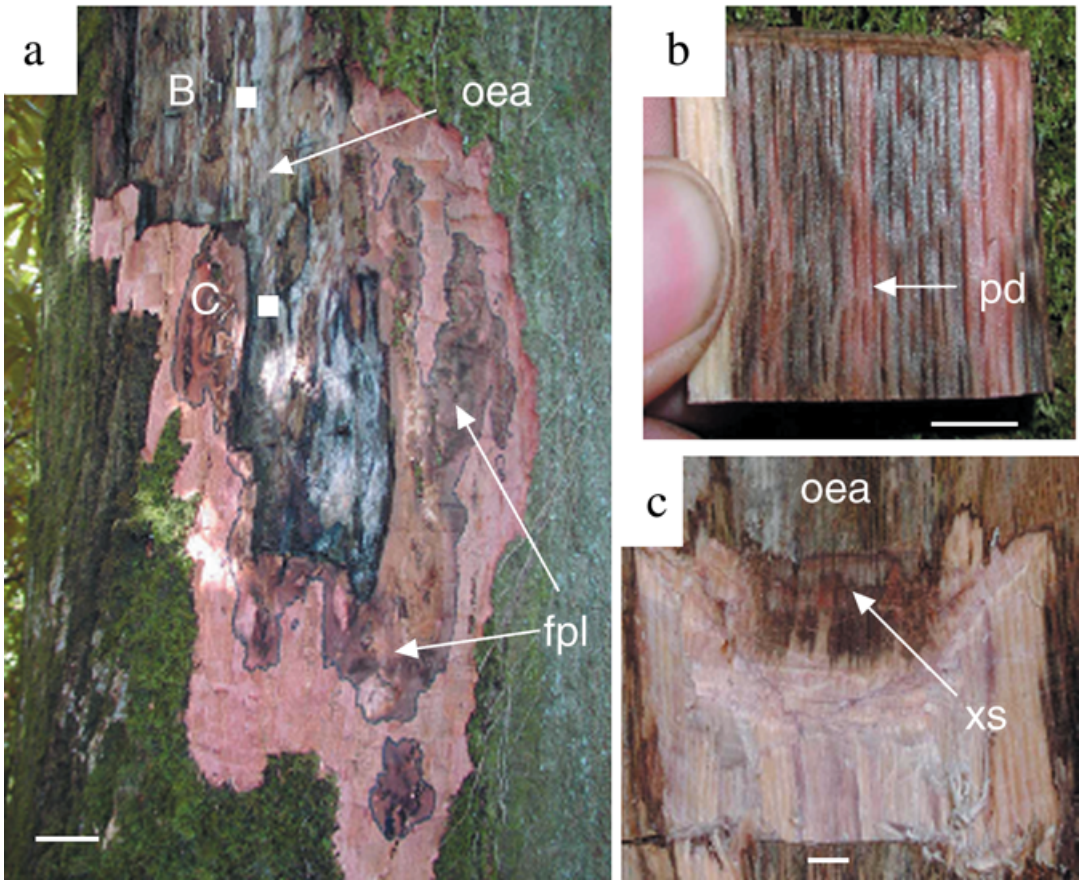

Figure 6 Perennation of *Phytophthora ramorum* and *P. kernoviae* in xylem of *Q. cerris* BRN58. (a) Fresh phloem lesions on *Q. cerris* BRN58 in August 2005 extending from the old lesion area excised in August 2004. (b) Inner surface of partially removed wood panel C showing stripes of pink, freshly colonized xylem tissue. (c) Depth of xylem discoloration penetration below excised surface after complete removal of wood panel C. fpl, fresh phloem lesions; oea, old lesion excision area; xs, xylem stain or discoloration; pd, pink discoloration; white squares, positions of wood panels B and C. Scale bars (a) 10 cm; others 1 cm.

therefore to be a common feature of broadleaved trees infected with *Phytophthora* spp. However, no lagoons were observed on stems of *I. aquifolia* infected with *P. ilicis*. This may have been because of the very thin (2–3 mm) phloem of this species and the tendency of the necrotic phloem to be very soft, quickly losing its structure.

In an attempt to isolate *Phytophthora* spp. directly from fresh lagoon liquid onto SMA, liquid was drawn off with a sterile 10-mL syringe and 0·2-mL aliquots were spread onto 10 plates of SMA+MRP. Fresh lagoon liquid from six *F. sylvatica* trees, two infected with *P. kernoviae*, two with *P. citricola* and two with *P. gonapodyides*, was examined. No colonies developed from five of the samples. One lagoon, with *P. kernoviae* present, produced *c.* five *Phytophthora* sp. colonies per mL lagoon liquid.

Discussion

These results demonstrate that *Phytophthora* spp. can be regularly isolated from the discoloured xylem underlying phloem lesions on stems of a wide range of broadleaved trees. This contrasts with the commonly held view that *Phytophthora* stem lesions tend to be confined to the phloem and cambial layers. Macroscopic characteristics of the xylem lesions were very variable. They included extensive areas of discoloured xylem; long narrow strips of discoloured xylem; multiple shallow pits and small streaks; and small to large liquid-filled cavities or lagoons. *Phytophthora* spp. were isolated from all such features (other than lagoon liquid) at depths of 1–25 mm below the xylem surface. Such colonization must lead to at least local disruption of xylem function. The colour of the stain varied markedly according to the host-*Phytophthora* combination and the level of oxidation. It seems likely that, as in phloem lesions, the discoloration is mainly caused by the host's reaction to the pathogen. In particular, it could reflect the diffusion of polyphenols from xylem parenchyma cells as part of the host defence mechanism.

A notable feature was that *Phytophthora* spp. were often recovered at a higher frequency from xylem tissue than from the overlying phloem lesions. Indeed, in *c.* 23% of cases isolation was only achieved from the xylem. Currently, most protocols used for isolation of *Phytophthora* spp. from bleeding stem lesions rely on isolation from

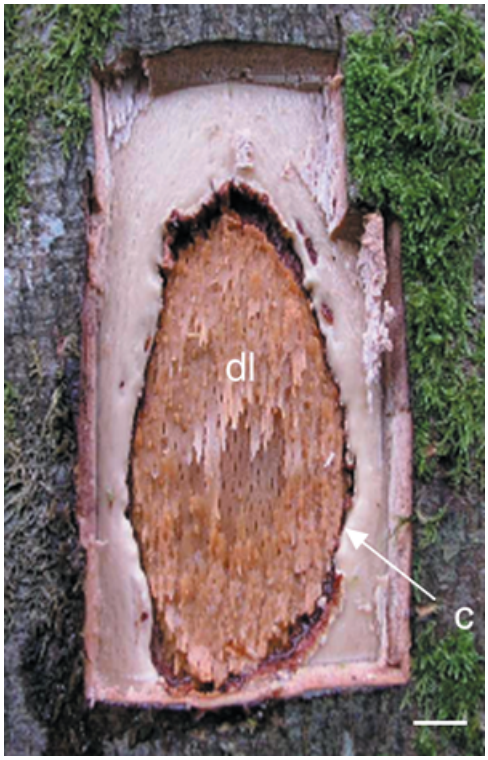

Figure 7 Lagoon cavity in xylem of *Fagus sylvatica* BRN53 caused by *Phytophthora kernoviae*. Note callusing around edge of cavity. dl, developing lagoon cavity; c, callusing. Scale bar 1 cm.

necrotic phloem tissue. On the evidence obtained here it is strongly recommended that isolations from the xylem as well as the phloem should be undertaken whenever xylem discoloration has occurred.

Phytophthora lesions in xylem were often found to extend well beyond the overlying phloem lesion. In two cases, the *Phytophthora* sp. was shown to have perennated successfully within xylem tissue for 24–27 months. In several cases, fresh phloem lesions were found to have arisen directly from the xylem surface. This suggests that the pathogen has the potential to break out from the xylem into uncolonized phloem tissue. The fact that isolation success per 20 pieces routinely taken from wood panels was often >70% also indicates that the pathogens are well dispersed within the affected xylem tissues.

An outstanding question is whether the *Phytophthora* spp. frequently penetrate and colonize the lignified xylem vessels or whether occupation of xylem tissue is mainly confined to the living parenchyma cells of the ray tissue and the auxiliary parenchyma cells around the vessels. Circumstantial evidence indicating the pathogen's ability to utilize xylem vessels comes from the distribution of non-contiguous or island lesions in the phloem. Island lesions were often observed in striking linear arrays, especially on stems of beech and oak infected with *P. citricola*, *P. cambivora* or *P. kernoviae*. Sometimes this linear sequence occurred from near ground level to 10 or 15 m up the tree (see also Jung *et al.*, 2005). In addition, linear connections between adjacent phloem island lesions were often observed in the underlying xylem, usually in the form of narrow strips of discoloration, streaking or pitting. Indeed the xylem streaks observed here were not unlike those observed in xylem of broadleaved trees infected with vascular wilt fungi.

Collectively, these features suggest that the linear pattern of phloem lesions may arise from the vertical movement of the *Phytophthora* sp. within the xylem and its subsequent, intermittent colonization of the bark. In many of the linear arrays of island lesions examined, even those separated by many metres, the phloem lesions appeared to be in a similar state of development, i.e. all the lesions appeared to have been initiated within a similar time frame. This suggests that a relatively sudden movement of the *Phytophthora* sp. in the xylem may have occurred. One possible explanation is that spores or mycelium are carried on an embolism developing in penetrated xylem vessels. However, it cannot be discounted that inoculum spread by water runoff on the bark surface can result in multiple simultaneous infections.

The process of lagoon cavity formation probably represents local breakdown of xylem and phloem tissue in the zone of an infection and often leads to external bleeding. Preliminary sampling of lagoon liquid indicated that, apart from the presence of a *Phytophthora* sp. in the host tissues, the liquid that had not breached the outer bark was generally microbially sterile. Since *Phytophthora* spp. are not known to constitutively produce ligninases or large quantities of cellulases, lagoon formation seems most likely to result from an autolytic breakdown of host tissue, i.e. a host reaction. Since callusing is often associated with lagoons, their formation may be part of a walling-off process by the host to restrict pathogen spread. This may cause local disruption of water and phloem transport, leading to pressure build up and ultimately to bark rupture and external bleeding.

Another issue is whether colonization of the xylem is simply a passive consequence of occupation of the phloem or whether it is an active trait that provides ecological advantage. Several arguments suggest it could be advantageous. As already indicated, the spread and perennation of a *Phytophthora* sp. in the xylem may give it greater potential for survival and the ability to break out and colonize fresh phloem elsewhere. This would clearly facilitate enlargement of the organism's substrate domain. As considered by Davison *et al.* (1994), occupation of the xylem is likely to lead to disruption of water transport and so to a weakening of host resistance. Since xylem usually has lower nutrient status than bark, it might offer protection from competitors or antagonists, as it is less likely to be colonized as rapidly by other microbes. If in some seasons the xylem has a higher water content, it may also provide a more favourable environment for survival of *Phytophthora* spp.

For most of the *Phytophthora* spp. listed in Table 2, apart from *P. cinnamomi* (Davison *et al.*, 1994; Smith *et al.*, 1997), this is apparently the first detailed record of

their presence in xylem of tree stems. Perhaps the most unexpected observation is that of *P. gonapodyides* in the xylem of *F. sylvatica*. For many years *P. gonapodyides* was considered to be a weak opportunistic pathogen of non-suberized tissue of trees and shrubs, such as the fine roots (Brasier *et al*., 1993a). Recently, however, it has been shown to cause strip cankers at the stem bases of mature *Q. robur* trees, and by artificial inoculation to cause limited lesions on seedlings and mature *Q. robur* and *F. sylvatica* stems after wounding (Jung & Blaschke, 1996; Jung *et al*., 1996; Brasier & Jung, 2003). In the present study (see also Brown *et al*., 2006; Jung *et al*., 2005) *P. gonapodyides* was isolated from (usually small) aerial bark lesions on *F. sylvatica* trees. Even more significantly, it was isolated up to 10 mm into the xylem beneath the lesions. Therefore, although *P. gonapodyides* may still be an opportunist in these circumstances, its tissue-colonizing abilities have been underestimated.

The presence of *Phytophthora* spp. in xylem has implications for disease control and quarantine protocols. In Cornwall, UK, experimental control of developing lesions on tree stems is being attempted by excision of infected phloem (AVB&CMB, unpublished). In the present study, *P. ramorum* and *P. kernoviae* were shown to have survived, and were probably still actively growing or viable, in the xylem of large *Q. cerris* and *F. sylvatica* trees at least two years after the overlying phloem had been excised. This may well involve the survival of resting spores, such as oospores of *P. kernoviae* and chlamydospores of *P. ramorum*. In some cases fresh phloem lesions were apparently initiated from a xylem inoculum source. It is therefore recommended that excision for control should include removal of affected xylem in addition to bark.

Total removal of phloem and outer bark from tree stems is a recommended protocol for preventing national and international spread of quarantine pathogens, such as *P. ramorum* and *P. kernoviae,* on transported wood products. Again, the evidence presented here that *Phytophthora* spp. are often active and can remain viable up to 25 mm into the xylem shows that a more stringent treatment is required to prevent risk of spread. As a minimum, removal of 3 cm of outer sapwood would be needed to address this issue, which may not be practicable. Where quarantine issues arise, therefore, it may be preferable to destroy the infected tree stems.

The present study was part of a field survey for *P. ramorum* and *P. kernoviae* and relied mainly on isolation and macroscopic observation. Nonetheless it has raised a number of questions about the physical and temporal nature of *Phytophthora* infections on tree stems. Outstanding issues include the physiological status of the *Phytophthora* mycelium in xylem vessels, such as enzymatic behaviour and whether vessels become occluded; the mode of any vertical movement within the xylem, including whether spores are involved; the process of lagoon formation; and the significance of xylem invasion for the wider biology of the pathogen. There is now a need to resolve these issues through research at a more microscopic and experimental level.

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