Plant Pathology (2014) Doi: 10.1111/ppa.12253

Phytophthora austrocedrae emerges as a serious threat to juniper (*Juniperus communis*) in Britain

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From 2011 to 2013, *Phytophthora austrocedrae* was isolated from diseased *Juniperus communis* exhibiting dieback and mortality at eight geographically separate sites in Scotland and northern England. The pathogen was also confirmed present either by standard PCR of the ITS locus and sequencing or by real-time PCR on *J. communis* with symptoms at a further 11 sites in northern Britain. Out of 167 *J. communis* sampled across the 19 sites, 154 had foliage dieback over all or part of the crown as a result of basal lesions, which extended up the stem. Thirteen sampled trees had aerial branch lesions or discrete stem lesions with no apparent connection to the base of the tree. At 13 sites, dieback was concentrated in areas of poor drainage and/or alongside streams and other watercourses. In artificial inoculation experiments, *P. austrocedrae* caused rapidly extending stem and root lesions on *J. communis* and was reisolated from these lesions. Lesions also developed on *Chamaecyparis lawsoniana* and *Chamaecyparis nootkatensis* but the pathogen was not reisolated. All *P. austrocedrae* isolates obtained from *J. communis* in Britain shared 100% identity across the ITS locus but were distinct at one sequence position from *P. austrocedrae* isolates collected in Argentina from diseased *Austrocedrus chilensis*. This study provides clear evidence that *P. austrocedrae* is a primary pathogen of *J. communis* and now presents a significant threat to this species in Britain. Pathways for the emergence of *P. austrocedrae* in Britain, and possible ways in which the pathogen may have spread within the country, are discussed.

Keywords: disease symptoms, field survey, juniper, pathogenicity testing, *Phytophthora austrocedrae*

Introduction

Juniperus communis (common juniper) is a dioecious evergreen conifer and one of the most widely distributed conifer species in the world, with a broad circumpolar boreo-temperate distribution stretching to 30°N throughout northern Asia, North America and Europe (Preston *et al.*, 2002; Thomas *et al.*, 2007). In Britain *J. communis* is one of only three native conifer species and can be found right across the country, with one population centre on the chalk downlands of southern England, another in northern England and Scotland, and scattered populations in between (Thomas *et al.*, 2007). In Scotland and northern England, *J. communis* occurs predominantly in mesic conditions on heather moorlands, oceanic heaths, rocky slopes and as a component of *Betula*, *Quercus* and *Pinus* woods (Preston *et al.*, 2002). In Scotland in particular, *J. communis* is highly valued as an important constituent of the woodland ecosystem and for this reason is listed as a priority species in the UK Biodiversity Action Plan (http://jncc.defra.gov.uk/ukbap).

Over the last 10 years there have been increasing reports of dying *J. communis* at mature, upland sites in northern Britain, including conservation Sites of Special Scientific Interest (SSSI) forming part of the European network of protected habitats. Trees with symptoms display foliage browning or bronzing over all or part of the crown and appear to die rapidly. This is in addition to a general population decline already apparent in Britain over the last 60–70 years due to overgrazing, burning, population fragmentation and lack of regeneration (Preston *et al.*, 2002; Thomas *et al.*, 2007). Investigations carried out in the early–mid 2000s at two *J. communis* woodlands displaying severe dieback – one located in Perthshire, Scotland and another in Cumbria, northern England – found that root damage was the reason for the observed mortality of trees. At the time a causal agent was never identified and the damage thought most likely due to poor drainage in the worst affected areas of the sites.

In late 2010, the Tree Health Advisory Service of Forest Research was asked to investigate dieback and mortality of *J. communis* at the Upper Teesdale National Nature Reserve in northern England. There, a decline of trees had been observed expanding outwards from a flat, boggy area on Holwick Moor since the mid-2000s and was initially presumed to be due to site wetness (M. Furness, Natural England, UK, personal communication). Following site visits to Holwick Moor in July and November 2011, a slow-growing *Phytophthora* species was isolated from a phloem lesion on the upper root of a *J. communis* growing adjacent to a stream (Green *et al.*, 2012). This *Phytophthora* was identical in terms of

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culture morphology and ITS sequence to a *Phytophthora* species isolated a few months earlier from a single declining *Chamaecyparis nootkatensis* (Nootka cypress) and a single declining *Chamaecyparis lawsoniana* (Lawson cypress) located separately in a public park and private garden, respectively, in the Glasgow area (S. Green & G. A. MacAskill, Forest Research, Roslin, UK, unpublished). Based on culture morphology and analysis of its partial ITS1–5·8S–ITS2 (ITS) locus, this unknown *Phytophthora* was identified as *Phytophthora austrocedrae* (Green *et al.*, 2012), a pathogen first described in 2007 from southern Argentina where it is associated with widespread dieback and mortality of the native cypress *Austrocedrus chilensis* (Cupressaceae) (Greslebin *et al.*, 2007; Greslebin & Hansen, 2010). Subsequently, in February 2012, *P. austrocedrae* was confirmed infecting *J. communis* at the site in Perthshire, Scotland (Green *et al.*, 2012). These were the first confirmed findings of *P. austrocedrae* outside Argentina.

In response to these findings, dieback of *J. communis* was investigated at 19 sites in Scotland and northern England from 2011 to 2013, and at all sites *P. austrocedrae* was either isolated or detected in phloem lesions by standard PCR and sequencing or by real-time PCR. The disease aetiology and site characteristics observed in this survey are reported here. As *P. austrocedrae* has now been isolated from diseased *A. chilensis* (Greslebin *et al.*, 2007; Greslebin & Hansen, 2010), *C. nootkatensis* and *C. lawsoniana* (S. Green & G. A. MacAskill, Forest Research, Roslin, UK, unpublished), and *J. communis* (Green *et al.*, 2012) a preliminary investigation into host range was undertaken. The implications of the findings in terms of the current and potential impact of *P. austrocedrae* on *J. communis*, and the possible ways in which the pathogen may have spread to and within Britain, are discussed.

Materials and methods

Field survey and isolation

Nineteen sites in Scotland and northern England (Fig. 1) at which decline and mortality of mature upland juniper had been reported were investigated between July 2011 and December 2013 (Table 1).

For isolation of *Phytophthora* from necrotic phloem on the stem or branches of *J. communis*, tissue samples of *c.* 5 mm^2 were excised from the margins of freshly exposed phloem lesions in the field and plated directly on to synthetic mucor agar (SMA) + benomyl hydrochloride, rifamycin and pimaricin (MRP), a *Phytophthora*-selective medium (Brasier *et al.*, 2005), in 9-cm-diameter Petri dishes. Plates were incubated at room temperature in darkness; the resulting colonies were subcultured after *c.* 2 weeks onto V8 agar and isolates were maintained at 17°C in darkness with further subculturing every 4–6 weeks.

DNA extraction and amplification

To obtain DNA of *Phytophthora* species from phloem tissue, *c.* 100 mg of chopped phloem from the lesion margins was placed in an Eppendorf tube, frozen in liquid nitrogen, ground to a powder in a bead mill (Retsch) and the DNA extracted using the DNeasy Plant Mini kit (QIAGEN). Prior to the development of a real-time PCR assay specific to *P. austrocedrae* (Mulholland *et al.*, 2013), standard PCR was performed using 2 μL of both the *Phytophthora*-specific forward primer Ph2 (Ippolito *et al.*, 2002) and the universal reverse primer ITS4 (Eurofins MWG Operon) at a concentration of 10 mM. Total reaction volume was 25 μL comprising 1·5 μL $MgCl_2$ (at 0·45 mM), 5 μL of 5× buffer (TaqMan Environmental Mastermix 2·0; Applied Biosystems), 0·5 μL dNTPs (at 0·2 mM), 0·125 μL U *Taq* DNA polymerase (Applied Biosystems), 15·5 μL molecular grade water and 1 μL template DNA. Amplification was performed in a Biometra Tgradient thermocycler (Thistle Scientific) with initial denaturation at 95°C for 5 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension of 72°C for 7 min. PCR products were purified and sequenced in both directions with the BigDye v. 3.1 Ready Reaction kit on an ABI Prism 3730 capillary sequencer (Applied Biosystems). Raw sequences were aligned and edited using SEQUENCHER v. 4.8 FOR WINDOWS, and aligned with published ITS sequences in GenBank using BLAST (Altschul *et al.*, 1990). Real-time PCR was conducted using *P. austrocedrae*-specific primers Paus-481-F (5′-TGGTGAACCGTAGCTGTATTTAAGC-3′), Paus-554-R (5′-GGAACAACCGCCACTCTACTTC-3′) and probe Paus-507-TM (5′-TGGCATTTGAACCGRCGATGTG-3′) following the protocol described by Mulholland *et al.* (2013).

For sequencing from pure cultures, 50 mg of mycelium was scraped from each agar colony into a 1·5 mL Eppendorf tube. A small scoop of autoclaved fine commercial garden sand and 400 μL AP1 buffer (DNeasy Plant Mini kit; QIAGEN) was added and the sample homogenized by grinding for 1 min with a micropestle. The homogenized sample was then heated at 65°C for 1 h before the extraction was carried out using the DNeasy Plant Mini kit protocol. Amplification, sequencing and editing of the ITS region was carried out as described above. Edited sequences from pure cultures were aligned using CLUSTAL OMEGA and deposited in GenBank as KJ490659–KJ490668.

Pathogenicity and host range testing

Pathogenicity was tested on nine tree species (Table 2) using a method similar to that described by Greslebin & Hansen (2010) in which healthy potted young saplings (2-years-old; stem diameters 0·8–1·5 cm) were inoculated with 6 mm diameter mycelial plugs taken from the margins of 4–5-week-old cultures of *P. austrocedrae* isolate 5038 (Table 3) growing on V8 agar. For eight of the tested species (Table 2), the stem bases were inoculated by cutting a small flap (*c.* 5–8 mm long) into the bark 2 cm above soil level using a sterile scalpel and the mycelial plug placed into the flap, mycelial side down. A droplet of sterile water was placed on to the plug and a muslin strip wetted with sterile water was wrapped several times around the inoculation site and secured in place with Parafilm. Due to the difficulties of obtaining whole plants of *C. nootkatensis*, 35-cm-long shoots were excised from a mature tree growing in the Royal Botanic Gardens of Edinburgh, inoculated at a point 5 cm from the shoot bases as described above and the shoot ends clipped and sealed with Parafilm. For each tree species six replicate plants/shoots were inoculated with *P. austrocedrae* and two plants/shoots inoculated with plugs of sterile V8 agar as controls. To test for colony viability, two mycelial plugs were cut from the colony margins of each *P. austrocedrae* culture plate used in the inoculations, transferred to fresh V8 agar and incubated at 17°C

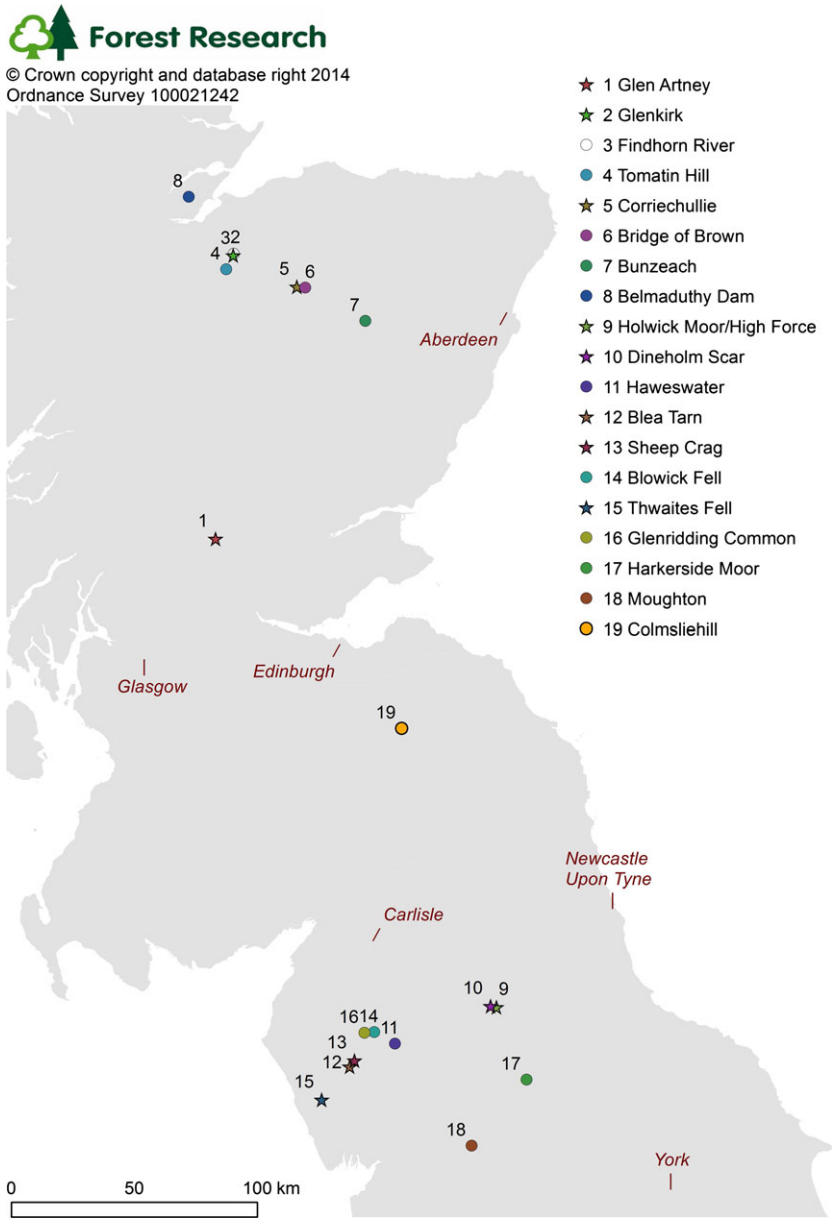

Figure 1 Location of 19 *Juniperus communis* sites in northern Britain surveyed and found to be infected with *Phytophthora austrocedrae*. Stars indicate sites where *P. austrocedrae* was isolated, circles indicate sites where the pathogen was detected by standard PCR or real-time PCR.

in darkness. All tree species were inoculated twice in separate trials; trial 1 was conducted in February–April and trial 2 in April–June.

Inoculated plants and shoots were maintained in a quarantine-licensed greenhouse at 17–25°C with natural lighting. Whole plants were placed on trays and watered daily to maintain continuous soil wetness with 2–3 cm of standing water in each tray. The C. *nootkatensis* shoots were maintained inside sealed incubation trays containing water to provide high humidity. Six weeks after inoculation the plants/shoots were harvested, the outer bark removed, and the length of each lesion in the phloem measured. Isolations were made onto SMA + MRP medium to confirm the presence of *P. austrocedrae*. Analysis of variance (ANOVA) was used to identify significant effects of tree species and trial on lesion length. Lesion lengths were log transformed to standardize their variability and Tukey's multiple comparison test (95% confidence) used to identify differences between tree species.

Results

Field survey and isolation

Juniperus communis trees with symptoms were observed and sampled at all 19 sites surveyed in this study (Table 1). Both upright and prostrate forms of *J. communis*, the latter tending to have multiple stems growing

Table 1 Details of *Juniperus communis* sites in Scotland and England at which *Phytophthora austrocedrae* was found to be present

Site name and whether grazed by livestock	Dates investigated	Approximate area of site and no. of *J. communis*	Approximate percentage of *J. communis* with symptoms on site	No. of *J. communis* from which *P. austrocedrae* was isolated/total sampled	No. of *J. communis* which tested positive for *P. austrocedrae* by phloem PCR/total tested	Basal (B) or aerial (A) lesions observed	*J. communis* planted at site?	Pattern of dieback relative to site drainage
Glen Artney, Perthshire, Scotland Sheep grazed	February, May and September 2012, May 2013	102 ha >3000 trees	40	2/27	16/27	B + A	No	Dieback occurs all across site, particularly in wet flushes and along seepages and streams
Glenkirk, Inverness-shire, Scotland Not grazed	October 2012	15 ha >500 trees	<1	1/2	1/1	B	No	Single, small pocket of dieback in wet flush among otherwise healthy trees
Findhorn River Sheep grazed in areas	December 2013	25 ha >500 trees	<5	0/5	4/5	B + A	Unknown	Scattered dieback of individual trees across site, not necessarily related to wet flushes
Tomatin Hill Sheep grazed	December 2013	20 ha >500 trees	1	0/2	1/2	B	Unknown	Scattered dieback of individual trees across site, mainly in wetter flushes
Corriechullie, Inverness-shire, Scotland Sheep grazed	October 2012 and July 2013	1 ha >100 trees	10	1/4	3/3	B	Unknown	Scattered dieback alongside a stream
Bridge of Brown, Inverness-shire, Scotland Sheep grazed	July 2013	>50 ha >1000 trees	10	0/6	3/6	B	Unknown	Dieback scattered about site but not necessarily associated with wet areas
Bunzeach, Aberdeenshire, Scotland Not grazed	October 2012, December 2013	56 ha >500 trees	<5	0/1	7/14	A + B	Unknown	Scattered dieback across predominately well-drained slopes
Belmaduthy Dam, Ross & Cromarty, Scotland Cattle grazed	October 2012	19 ha >150 trees	80	0/6	6/6	A + B	Unknown	Dieback all across wet, marshy site
Holwick Moor/ High Force, Co. Durham, England Sheep grazed until 2006	July and November 2011, March and November 2012, September 2013	70 ha 6000 trees	25–30	3/33	14/25	B + A	Yes	Dieback concentrated in wet flushes and along seepages and streams
Dineholm Scar, Co. Durham, England Sheep grazed until 2006	March 2012	20 ha 4000 trees	<5	1/4	1/3	B + A	Yes	Scattered dieback adjacent to river
Haweswater, Cumbria, England Sheep grazed	June and November 2012, June and October 2013	15 ha >1000 trees	15	0/22	20/22	B + A	Yes	Dieback concentrated at northern end of site in wet flushes and along seepages and streams

(continued)

Table 1 (continued)

Site name and whether grazed by livestock	Dates investigated	Approximate area of site and no. of *J. communis*	Approximate percentage of *J. communis* with symptoms on site	No. of *J. communis* from which *P. austrocedrae* was isolated/total sampled	No. of *J. communis* which tested positive for *P. austrocedrae* by phloem PCR/total tested	Basal (B) or aerial (A) lesions observed	*J. communis* planted at site?	Pattern of dieback relative to site drainage
Blea Tarn, Cumbria, England Sheep grazed	November 2012	40 ha 3600 trees	50–60	1/6	5/5	B + A	Yes	Extensive dieback all across site which is predominantly wet, and along seepages and streams
Sheep Crag, Cumbria, England Sheep grazed	May 2013	16 ha >1850 trees	<10	1/2	2/2	B + A	Unknown	Dieback concentrated in wet flushes and along seepages and streams
Blowick Fell, Cumbria, England Sheep grazed	June and October 2013	114 ha >10 000 trees	10	0/10	10/10	B	Unknown	Dieback concentrated in wet flushes and along seepages and streams
Thwaites Fell, Cumbria, England Open to sheep grazing	May 2013	55 ha >3000 trees	20	1/4	3/3	B	Unknown	Dieback scattered all across site which is predominantly wet and boggy
Glenridding Common Sheep grazed	October 2013	20 ha >1000 trees	<5	0/3	3/3	B	Yes	Scattered dieback mainly in small wet flushes or beside streams
Harkerside Moor, North Yorkshire, England Sheep grazed	September 2013	21 ha 1000 trees	<1	0/5	3/5	B	Unknown	Two small pockets of dieback on apparently well drained site
Moughton, North Yorkshire, England One section still sheep grazed, other section not sheep grazed since 1995	September and November 2013	151 ha >1500 trees	90	0/9	5/9	B	Yes	Dead and dying trees across entire site, few healthy trees visible
Colmsliehill, Borders, Scotland Sheep and cattle grazed	November 2013	3·3 ha 300 trees	70	Isolations not done	5/5	B	Yes	Dieback throughout site which is predominantly well drained with one small wet flush

Table 2 Pathogenicity of *Phytophthora austrocedrae* isolate 5038 to *Juniperus communis* and other tree species with lesion lengths measured 6 weeks after inoculation (data combined from two inoculation trials)

Tree species	Tested material	Mean length of lesions (mm) ± standard error	
		Inoculated with *P. austrocedrae*[a]	Controls
Juniperus communis	Whole plants, 50–60 cm height	71·4 ± 18·8 d	1·3 ± 1·3
Chamaecyparis lawsoniana	Whole plants, 70–80 cm height	11·3 ± 3·2 bc	2·5 ± 1·7
Chamaecyparis nootkatensis	Excised shoots, 35 cm length	51·9 ± 14·3 cd	4·0 ± 2·3
Thuja occidentalis	Whole plants, 90–100 cm height	7·4 ± 2·4 ab	1·3 ± 1·3
Thuja plicata	Whole plants, 30–35 cm height	4·8 ± 1·4 ab	1·8 ± 1·8
Sequoiadendron giganteum	Whole plants, 40–50 cm height	3·8 ± 1·4 ab	2·0 ± 1·3
Sequoiadendron sempervirens	Whole plants, 50–60 cm height	1·4 ± 0·8 a	1·8 ± 1·8
Cuppressocyparis leylandii	Whole plants, 30–35 cm height	4·4 ± 1·6 ab	0·0 ± 0·0
Taxus baccata	Whole plants, 20–30 cm height	7·0 ± 3·2 ab	0·0 ± 0·0

[a]Analysis of variance (ANOVA) was used to identify significant effects of tree species on lesion length. Lesion lengths were log transformed to standardize their variability and Tukey's multiple comparison test (95% confidence) was used to identify differences between tree species. Mean lesion lengths that share the same letter are not significantly different at $P < 0{\cdot}05$.

Table 3 Details of *Phytophthora austrocedrae* isolates obtained from *Juniperus communis* in England and Scotland

Tree code and location	Date isolated	Sample material	*P. austrocedrae* isolate code[a]
TDJ3, Holwick Moor, Co. Durham, England	November 2011	Phloem, lesion on upper root	5038
TDJ6, Dineholm Scar, Co. Durham, England	March 2012	Phloem, aerial branch lesion	5039
TDJ20, Holwick Moor, Co. Durham, England	November 2012	Phloem, basal lesion	5040
GA3, Glen Artney, Perthshire, Scotland	February 2012	Phloem, basal lesion	5036
GAT6, Glen Artney, Perthshire, Scotland	February 2012	Phloem, basal lesion	5037
GK2, Glenkirk, Inverness-shire, Scotland	October 2012	Phloem, basal lesion	5042
SS2, Corriechullie, Inverness-shire, Scotland	October 2012	Phloem, aerial branch lesion	5043
BT3, Blea Tarn, Cumbria, England	November 2012	Phloem, aerial branch lesion	5041
SC1, Sheep Crag, Cumbria, England	May 2013	Phloem, basal lesion	5045
TF1, Thwaites Fell, Cumbria, England	May 2013	Phloem, basal lesion	5046

[a]Isolates deposited in the Forest Research Northern Research Station culture collection.

out from one central basal region, exhibited a range of symptoms including subtle foliage discolouration over all or part of the crown (Fig. 2a,b), browning of all foliage associated with an individual stem (Fig. 2c), and overall reddening or bronzing and desiccation of the foliage on recently dead trees (Figs 2d & 3a,c). The generally uniform discolouration of foliage over all of the crown, or sections of the crown emanating from a single stem, suggested that the trees were dying due to root dysfunction and death and girdling damage of phloem at the root collar. Other symptoms included bronzing of all needles on smaller diameter branches to a point part way down the branch (Fig. 2i,k). Resinosis on the outer bark was occasionally observed on these smaller diameter branches but rarely on main stems.

Overall, 167 *J. communis* trees were examined and sampled across the 19 sites (Table 1). Of these, 154 were found to have necrotic orange-brown lesions in the phloem on the upper roots closest to the soil surface, root collar or stem base (Fig. 2e–h), which extended up the stem, often to more than 50 cm above the collar. The majority of trees with basal lesions had some degree of foliage discolouration. Occasionally, trees without obvious symptoms, adjacent to trees with symptoms, were sampled to determine whether these neighbouring trees were indeed infected, and four were found to have basal lesions, including one apparently symptomless tree which had a non-girdling basal lesion extending to 60 cm up one side of the stem. At one site, a healthy-looking tree (adjacent to dying trees) sampled in November 2012 was found to have a basal lesion extending to 10 cm up the stem. A year later, in October 2013, the same tree was still alive with green foliage, but the lesion had extended 20 cm above the previous year's sampling point. Lesions occasionally had resin pockets in the phloem (Fig. 2e,l) not visible in the outer bark, and a diffuse yellow discolouration was frequently observed in the phloem at the lesion edges or in advance of the lesion margin (Fig. 2f–h). The diameters of sampled stems ranged from 5–50 cm.

Thirteen trees sampled across eight of the sites exhibited dieback of individual branches at heights up to 1·5 m (Fig. 2i,k) and were found to have girdling orange-brown phloem lesions in which the margins appeared to be extending down towards the stems with no apparent connection to the base of the tree (Fig. 2j,l).

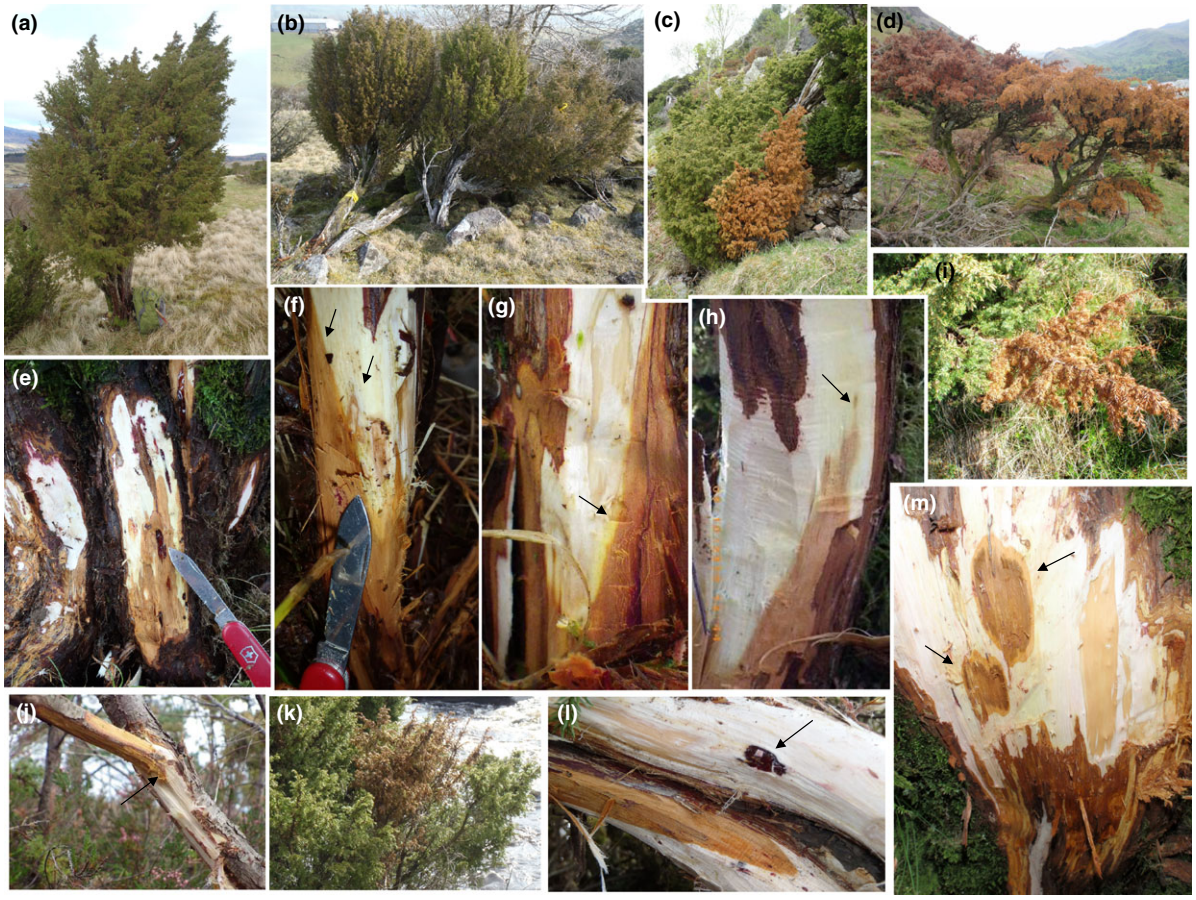

Figure 2 *Juniperus communis* with phloem lesions caused by *Phytophthora austrocedrae* at sites in Scotland and northern England. (a) Subtle discolouration of foliage over the entire crown. This tree was found to have the basal lesion shown in (e). (b) Early stage discolouration of foliage on individual stems with basal lesions. (c) Browning of part of the crown associated with a single diseased stem on a multistemmed individual. (d) Overall foliage bronzing of two infected trees with basal lesions. (e) Lower stem of tree shown in (a) with outer bark cut away to reveal an orange-brown phloem lesion extending up from below ground level. The penknife blade points to a resin pocket within the lesion. Note that healthy phloem is white. (f) Basal phloem lesion on a small diameter tree showing yellow colouration at the lesion margin (arrows). (g) Bright yellow discolouration of phloem (arrow) at the margin of a basal lesion. (h) Orange-brown basal lesion with lesion extending into healthy phloem (arrow). Note yellowing of healthy phloem in advance of lesion margin. (i) Dieback of a small diameter branch. Real-time PCR confirmed the presence of *P. austrocedrae* in a phloem lesion on this branch with no connection to the base of the tree. (j) Orange-brown phloem lesion on a branch. This lesion had no connection to the base of the tree. Arrow indicates the margin of the lesion in which *P. austrocedrae* was detected by PCR and sequencing. (k) Branch dieback on a tree growing on a riverbank. (l) Orange-brown phloem lesion on the branch shown in (k) extending downwards with no connection to the base of the tree. *Phytophthora austrocedrae* was isolated from this lesion. Arrow indicates resin pocket. (m) Two discrete lesions (arrows) in the phloem near the stem base of a tree. DNA of *P. austrocedrae* was detected in these lesions by real-time PCR.

Several trees also exhibited discrete lesions in the phloem, often on the lower stem (Fig. 2m) or centred on the junctions of stems lying close to the ground. These discrete lesions (i.e. in Fig. 2m) appeared to be the result of independent multiple stem infections which had penetrated directly through the outer bark.

Phytophthora austrocedrae was isolated from a total of 10 trees across eight of the 19 sites (Tables 1 & 3). Two isolates were obtained from aerial branch lesions and the rest from basal lesions (Table 3). Out of the remaining 157 sampled trees not yielding an isolate of *P. austrocedrae*, the pathogen was detected in DNA extracted from necrotic phloem in 117 trees either by standard PCR and sequencing of the ITS region or by real-time PCR (Table 1). This included 105 trees with basal lesions, 10 trees with aerial branch lesions and two trees with discrete stem lesions. Thus, taking into account both isolation and phloem PCR, *P. austrocedrae* was detected on 127 trees across the 19 sites (Table 1). The basidiomycete fungus *Amylostereum laevigatum* was occasionally isolated from basal lesions and *Phomopsis juniperovora* was isolated from small diameter shoots showing dieback. Snow breakage and bark stripping by mammals were other causes of dieback observed at some of these sites.

At 18 of the sites, *J. communis* was the dominant tree species with an understorey of heathland vegetation comprising mainly acid or wet grassland, heathers (*Erica*

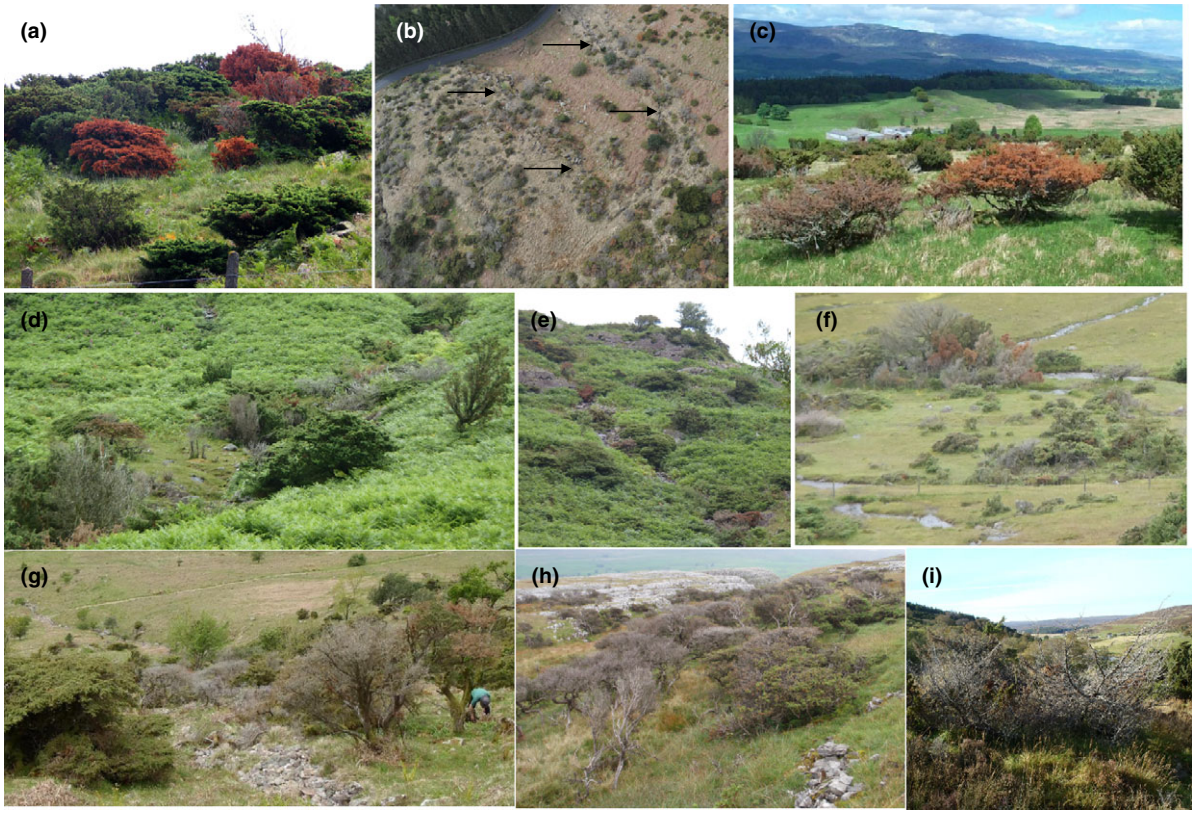

Figure 3 Symptoms caused by *Phytophthora austrocedrae* on mature upland stands of *Juniperus communis* in Scotland and northern England. (a) Trees showing striking red/bronze colouration. (b) Aerial photograph showing extensive mortality (grey trees) throughout image. Arrows indicate location of two streams with mortality occurring alongside. (c) Foliage discolouration. (d–f) Dead and dying trees along two streams (d,e) and in flat, marshy ground (f). (g) Dieback adjacent to a stream. (h) Extensive dieback across site. (i) Pocket of dieback in wet flush among otherwise healthy trees.

spp. and *Calluna vulgaris*) and bracken (*Pteridium aquilinum*) (Fig. 3a–i). At one site (Table 1; Fig. 3i) *J. communis* was present in a mix of *Betula* (birch) and *Salix* (willow). The extent of damage observed at the sites varied from individual pockets of dieback involving less than 10 trees within an otherwise healthy population of *J. communis* (Table 1; Fig. 3i) to extensive dieback and mortality occurring throughout the site (Table 1; Fig. 3c, d–f,h). All except two of the sites had areas of wet heath and mire in which *J. communis* was growing among an understorey of marsh grasses and rushes (*Juncus* spp. and *Carex* spp.), and mosses (*Sphagnum* spp. and *Hylocomium splendans*). Streams and/or wet seepages ran through all sites. At all except four sites, which were predominantly well drained, the damage to *J. communis* appeared to be greatest alongside streams or seepages (Fig. 3b,d,e,g,), in areas of wet mire (Fig. 3f,h) and within isolated wet flushes (Fig. 3i) (Table 1). Fifteen of the sites were open to stock grazing (Table 1) and none were fenced to exclude rabbits or deer. Thirteen of the sites had public footpaths or public roads running either directly through or adjacent to the *J. communis*. Young *J. communis* raised in plant nurseries had been planted in at least seven of the sites (Table 1) in regeneration schemes dating from the late 1990s through to the 2000s.

Pathogen identification

All 10 isolates fitted morphologically within the description given by Green *et al.* (2012). Colonies were very slow growing (<0·5 mm per day at 17°C) forming dense, white mycelia on V8 agar with distinctive, highly coralloid hyphae at the colony margins. Globose oogonia with amphigynous antheridia were occasionally observed in older colonies. Irregularly shaped sporangia were also observed on SMA but rarely on V8 agar. All isolates shared 100% sequence similarity across their ITS locus and were identical to *P. austrocedrae* isolates RG04 and 10_113_100 isolated in Scotland from ornamental *C. nootkatensis* and *C. lawsoniana*, respectively (GenBank accession numbers JQ346530 and JQ346531). BLAST analyses of the ITS sequences from British *P. austrocedrae* isolates showed that they share 99% identity with the ITS sequences from Argentinian *P. austrocedrae* isolates AG195 (DQ995185), AG203 (DQ995184), AG270 (JX121855) and AG309 (JX121857) in GenBank, with a G at position 519 on the alignment

whereas the Argentinian isolates are heterozygous for both A and G at this position. The closest other sequence matches in GenBank were *Phytophthora syringae* and *Phytophthora obscura* at 96 and 97% identities across the same ITS sequence. ITS sequences obtained from DNA extracted from phloem lesions collected at the sites also shared 100% identity with the British isolates.

Pathogenicity and host range testing

Overall significant differences in lesion lengths were observed between trial dates (trial 1 > trial 2, $P < 0{\cdot}001$) and between control and isolate lesions (control < isolate, $P < 0{\cdot}001$). No significant overall interaction was observed. Maximum temperatures in the greenhouse were higher for trial 2, regularly reaching 25°C, and this may have reduced pathogen activity. Control lesions were similar across tree species and showed no significant interaction over the two trials. Lesions varied significantly ($P < 0{\cdot}001$) across the nine tree species inoculated with *P. austrocedrae* (Table 2). On *J. communis*, lesions extended in the phloem either side of the inoculation point to an overall mean length of 71·4 mm 6 weeks after inoculation, significantly longer than for all other tree species except *C. nootkatensis* (Table 2). The length of lesions on *J. communis* varied from plant to plant and ranged from 5 to 178 mm. Four of the 12 inoculated *J. communis* had started to exhibit foliage browning after 6 weeks and were found to have lesions extending to more than 100 mm. These lesions could be traced down into the fine root system from the inoculation point, as well as extending up the stem. On the remaining inoculated *J. communis*, in which the extending lesions had not girdled the stem, the foliage looked outwardly healthy. Lesions on excised shoots of *C. nootkatensis* extended to a mean length of 51·9 mm (Table 2) although the pathogen was not successfully reisolated. *Chamaecyparis lawsoniana* also showed susceptibility to *P. austrocedrae* with lesions extending to a mean length of 11·3 mm (Table 2). There were no significant differences in lesion lengths among all other tree species tested (Table 2). On these species lesions were significantly shorter than for *J. communis*, *C. nootkatensis* and *C. lawsoniana*, and were limited to a surrounding necrosis of the inoculation site (Table 2). No extending lesions developed on any of the control plants and *P. austrocedrae* was only reisolated from *J. communis*. For other tree species there was insufficient lesion material remaining after sampling for isolation to test for the presence of *P. austrocedrae* with standard PCR and sequencing. Host range tests were conducted before the real-time PCR assay had been developed.

Discussion

This study provides evidence that *P. austrocedrae* is a primary pathogen of *J. communis* and occurs on this host across a range of geographically disparate sites in northern England and Scotland. The dieback and mortality observed at many of these sites is severe, and more suspect outbreak sites are being reported following aerial surveys in 2013. It is therefore clear that *P. austrocedrae* presents a significant threat to *J. communis* in Britain.

The main symptom observed at the sites was foliage dieback over all or part of the crown as a result of basal lesions originating from below the ground and extending up the stem, killing phloem and cambial tissues. This is similar to the damage observed on *A. chilensis* in which lesions caused by *P. austrocedrae* extend in the phloem from killed roots up to 1 m up the tree bole (Greslebin & Hansen, 2010). The observations of the present study are thus consistent with the disease originating predominantly in the root systems. The severity of crown symptoms on *J. communis* did not necessarily reflect the extent to which lesions had extended in the stem. For example, some trees with outwardly healthy foliage adjacent to dying trees had sizable basal lesions. Such trees will almost certainly succumb when the collar is girdled, or the root system killed, causing fatal disruption of phloem and xylem transport. Lesions found on live infected *J. communis* were a bright orange-brown (cinnamon) colour whereas on recently killed trees all phloem was dull brown and desiccated in appearance, making it difficult to identify a lesion margin. On *A. chilensis*, hyphae of *P. austrocedrae* also invade the xylem ray parenchyma and fibre tracheids below phloem lesions, blocking water transport and thus contributing to foliage decline (Vélez *et al.*, 2012). Xylem infection of *J. communis* was not investigated in this study but should be examined in a more detailed analysis of individual infected trees in which the roots are excavated further to confirm the origin and extent of infection relative to crown symptoms.

One notable feature of many lesions on *J. communis* was the yellow discolouration of phloem at lesion edges, which often extended up to 30 cm or more in advance of the lesion margin. Following an in-depth study of the impact of *P. austrocedrae* on the physiological status of *A. chilensis*, Vélez *et al.* (2012) suspected the involvement of effectors such as elicitins and toxins secreted by the pathogen ahead of the infection front. Whether such a mechanism is responsible for the yellowing of otherwise healthy phloem in infected *J. communis* needs to be clarified. The resin pockets occasionally seen in lesions on *J. communis* are a common feature of *P. austrocedrae* infections on *A. chilensis* and are produced by xylem ray parenchyma as a defence against the pathogen (Greslebin & Hansen, 2010; Vélez *et al.*, 2012).

Lesions on branches with no apparent connection to the base of the tree, from which *P. austrocedrae* was either isolated or confirmed present by PCR, were observed on *J. communis* at eight of the sites, albeit at a much lower frequency than basal lesions. Aerial infections by *P. austrocedrae* have not been reported on *A. chilensis*, but the pathogen was isolated from an aerial branch lesion on a young, hedgerow *C. lawsoniana* in Scotland (S. Green & G. A. MacAskill, Forest Research, Roslin, UK, unpublished). It is not known whether

P. austrocedrae is capable of true aerial dispersal and further work is planned to determine this. As *J. communis* is a low, spreading species and all branch infections occurred at less than 1·5 m from ground level, a feasible explanation for the aerial lesions is that inoculum was splashed upwards from the soil during heavy rain. Such splash dispersal from soil to aerial plant parts is a major means of dispersal for a number of soil-inhabiting *Phytophthora* species (Ristaino & Gumpertz, 2000). The presence of discrete lesions on the stems unconnected to the roots does suggest that inoculum of *P. austrocedrae* is able to penetrate the outer bark of *J. communis* directly, and independently of a soil medium.

Out of 151 trees sampled for culturing, only 10 isolates were obtained, despite the fact that isolations were done in the field from freshly exposed lesions and plated directly onto SMA + MRP. Isolates were obtained in October, November, February, March, April and May, and so time of year is not thought to be a limiting factor. The very low rate of isolation may be explained by the exceptionally slow growth rate of British isolates, a rate even slower than that reported for Argentinian isolates by Greslebin *et al.* (2007). It is also possible that incubation of isolation plates at room temperature (17–24°C) may have provided higher than optimal temperature conditions for pathogen growth. Colony growth studies are currently underway to determine the optimal temperature requirements for British isolates of *P. austrocedrae*. Due to the low isolation rate, PCR was carried out on DNA extracted from diseased phloem and enabled detection of the pathogen at all sites. The real-time PCR assay in particular was a rapid and reliable tool for confirming field symptoms, and often yielded results on samples for which a band could not be obtained in standard PCR due to very low quantities of pathogen DNA being present.

The majority of sites surveyed harboured, to varying degrees, areas of standing or moving water, for example wet flushes, mire, streams and seepages. Dieback of *J. communis* occurred predominantly within these wet areas, and whereas at some sites dieback extended to the drier, steeper slopes away from streams or bog, the extent of symptoms and pattern of dieback at each site could be linked to the degree of site wetness and/or the proximity of streams. This is not surprising because *Phytophthora* species disseminate via free-swimming zoospores and the presence of watercourses and/or waterlogged soil conditions provide favourable conditions for disease spread (Lamour, 2013). In Argentina, the extensive decline of *A. chilensis*, known as mal del ciprés ('cypress sickness'), for which *P. austrocedrae* is strongly implicated as the main causal agent, is clearly associated with areas of high soil moisture and poor drainage (La Manna & Rajchenberg, 2004a,b). As *P. austrocedrae* grows best in culture at cool temperatures (10–20°C), with optimum growth at 17·5°C (Greslebin *et al.*, 2007), the cool, wet climatic conditions prevalent in Scotland and northern England would appear to be very suitable for the pathogen. An extended, systematic survey of *J. communis* is now needed in Britain, involving sites both with and without symptoms, to determine whether the presence of the pathogen is clearly linked to site factors such as soil moisture conditions and stock management regimes.

Given the high level of susceptibility of *J. communis* to *P. austrocedrae* and the fact that all British isolates from geographically distinct sites were identical across the ITS locus, including those isolated from *C. lawsoniana* and *C. nootkatensis*, it is suspected that the pathogen is an exotic introduction into Britain. The widespread distribution of the pathogen across northern Britain and the extensive nature of the dieback observed at some of the sites, including the presence of many long-dead trees, indicates that *P. austrocedrae* may have been present in the country for some time. Until 2007, *P. austrocedrae* was not known to exist as a species and PCR tools were not available for its detection. Therefore, given its extremely slow growth rate and difficulty of isolation, it is unsurprising that *P. austrocedrae* was not discovered in Britain during earlier investigations. It is hoped that through an in-depth study of the genetic diversity of isolates and DNA samples collected from lesions at each site, using a range of nuclear and mitochondrial loci as well as microsatellite markers, useful information may be gained on the recent evolutionary history of *P. austrocedrae* in Britain.

The ITS sequence of British isolates differs consistently from that of the Argentinian isolates, which themselves have very low levels of genetic diversity (Vélez *et al.*, 2013). Indeed the clonal nature and the aggressive behaviour of the pathogen on its host in Argentina indicate strongly that it has been introduced there (Vélez *et al.*, 2013). Thus the evidence so far points to *P. austrocedrae* having an unknown origin in a different location to the current known geographical areas of disease outbreak. The question arises as to how the pathogen might have spread internationally. In addition to the field outbreaks in Britain reported here, DNA of *P. austrocedrae* matching both UK and Argentinian ITS genotypes has over the last few years been identified in a small number of diseased *J. communis* plants located in nurseries or private gardens in England and Wales (Denton, 2014; B. Henricot, Royal Horticultural Society, Wisley, UK, personal communication; J. Barbrook, Food and Environment Research Agency, York, UK, personal communication) and Scotland (A. Schlenzig, Science and Advice for Scottish Agriculture, Edinburgh, UK, personal communication). A thorough investigation of *Juniperus* nursery material is now required in order to determine the risk of spread of *P. austrocedrae* in the plant trade. The host range tests conducted here, although unable to satisfy Koch's postulates for *C. lawsoniana* and *C. nootkatensis*, do nonetheless indicate that *P. austrocedrae* presents most risk to these two species among the other hosts inoculated. Therefore, *C. lawsoniana* and *C. nootkatensis* should also be included when surveying nursery material for the presence of *P. austrocedrae*.

Acknowledgements

The authors wish to thank Barnaby Wylder, Mick Biddle, Ben Jones, Richard Thompson, Giles Brockman and Ian Murgatroyd of The Forestry Commission; Martin Furness, Chris McCarty and Colin Newlands of Natural England; Dave Shackleton of the RSPB; Michael Douglas of the Cumbria Wildlife Trust; Frances Graham of The Yorkshire Dales National Park Authority; and Helen Taylor, Karen Rentoul and Denise Exton of Scottish Natural Heritage for their valuable assistance in reporting sites with symptoms, and in providing site information and samples. The authors also wish to thank Niamh Britton, Heather Steele, Grace MacAskill and Bridget Laue for assistance with collecting and processing field samples, Alexandra Schlenzig and Rachel Campbell of Science and Advice for Scottish Agriculture (SASA) for assistance with pathogenicity and host range testing, The Royal Botanic Gardens of Edinburgh for offering material of *C. nootkatensis*, Andy Peace for assistance with statistical analyses and Ana Perez-Sierra for a critical review of the manuscript. This work was funded by the Forestry Commission and performed under Scottish Government Plant Health Order 2005 Licence PH/27/2013.

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