



# Detection of the *Phoma* pathogens *Plenodomus biglobosus* subclades ‘*brassicae*’ and ‘*canadensis*’ on wasabi, and ‘*canadensis*’ in Europe

Kevin M. King · Jonathan S. West

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**Abstract** Phoma stem canker / blackleg is an internationally important disease of *Brassicas* including *B. napus* (oilseed rape, OSR), caused by multiple genetic subclades of the fungi *Plenodomus lingam* (formerly *Leptosphaeria maculans*) and *P. biglobosus* (*L. biglobosa*). In Spring 2021, Phoma-like disease symptoms were observed on leaves and stems of *Eutrema japonicum* (wasabi) crops at three UK sites (Northern Ireland, Southern England and the West Midlands). Fungal isolation from wasabi leaf spots yielded colonies with two distinct phenotypes on potato dextrose agar (PDA). Isolates from the Northern Ireland and Southern England sites had white colonies with abundant pink cirri that were confirmed (based on ITS rDNA, beta tubulin and actin sequences) as *P. biglobosus* subclade ‘*canadensis*’ (Pbc). Those from the West Midlands site, however, had yellow pigmented colonies and were confirmed by sequencing as *P. biglobosus* subclade ‘*brassicae*’ (Pbb). Greenhouse pathogenicity testing showed that Pbb and Pbc wasabi isolates were pathogenic not only to this host but also OSR, *B. oleracea* (cabbage), and *B. rapa* (pak choi). Re-isolation of the fungi was attempted and confirmed from lesions that developed on inoculated OSR and wasabi, thus completing

Koch’s postulates. These findings represent new discoveries for both Pbb and Pbc on wasabi, plus for Pbc in Europe. The crop health implications of these results are briefly considered.

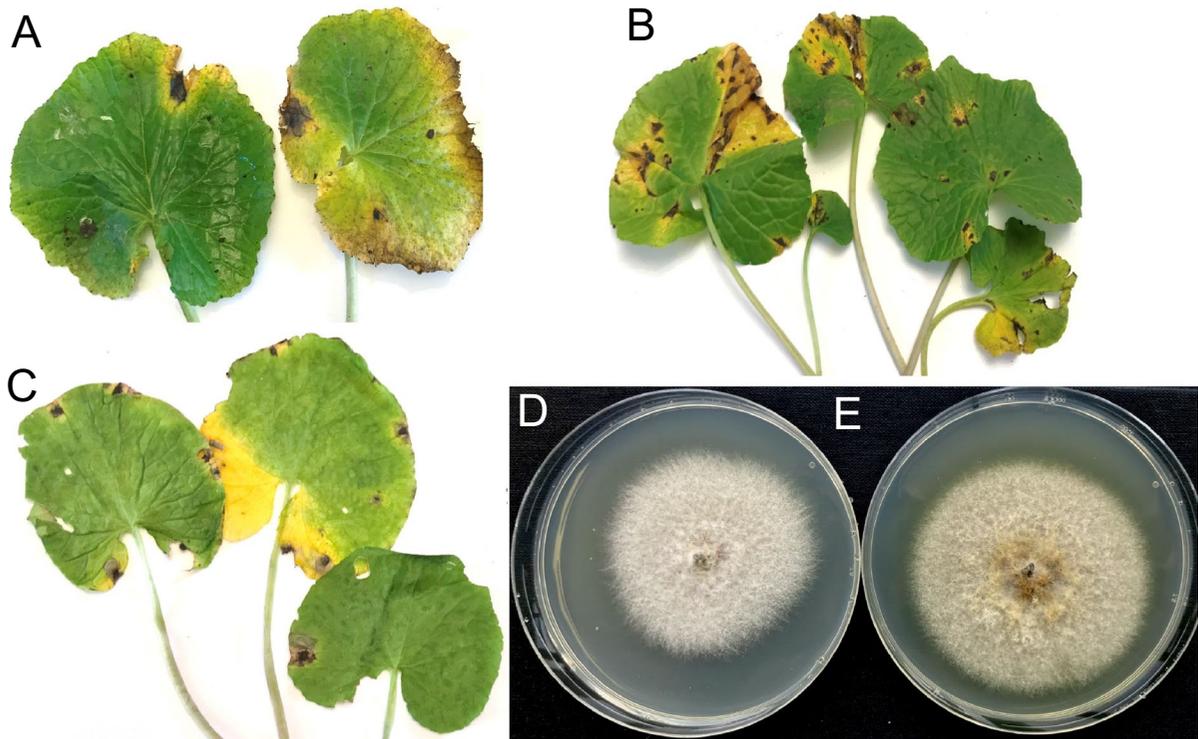
**Keywords** Blackleg · *Brassica* · *Leptosphaeria* spp. · Phoma · *Plenodomus* spp. · Subclade

In Spring 2021, Phoma-like leaf spot symptoms were observed on *Eutrema japonicum* (wasabi) crops at three geographically distinct UK locations (Northern Ireland, Southern England, and the West Midlands). Lesions were dark brown / black often with chlorotic yellow margins, with larger lesions often coalescing together (Fig. 1A–C). Occasionally, black elongate lesions were present on some petioles, and when stems (rhizomes) were cut open black streaks were sometimes observed running alongside the vascular bundles with black internal lesions evident.

Fungal isolation from lesion margins was attempted by surface sterilization of ~3mm<sup>2</sup> leaf fragments (by rinsing in 70% ethanol for 30 s, 5% (w/w) sodium hypochlorite solution for 30 s, and three times in sterile distilled water (SDW)). Leaf fragments were subsequently blotted dry on sterile paper, transferred to potato dextrose agar (PDA) plates (containing 50 µg / mL each of penicillin plus streptomycin sulphate) and incubated at 20 °C in the dark for 7 days. After this time, emergent colonies were examined under a stereomicroscope, and single hyphal strands

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K. M. King (✉) · J. S. West  
Biointeractions and Crop Protection Department,  
Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ,  
UK  
e-mail: Kevin.king@rothamsted.ac.uk



**Fig. 1** Isolation of *Plenodomus biglobosus* subclades ‘brassicae’ (Pbb) and ‘canadensis’ (Pbc) from diseased *Eutrema japonicum* (wasabi) leaves collected from three UK field sites in Spring 2021. Shown are diseased wasabi leaves from (A) Southern England (from which Pbc was isolated), (B) Northern Ireland (Pbc isolated), and (C) the West Midlands (Pbb

isolated). Representative isolates obtained from the material and grown on potato dextrose agar (PDA) plates for 12 days growth are (D) 21WAS7-1 (from Northern Ireland, white colony with pink oozing cirri evident, Pbc), and (E) 21WAS8-4 (from the West Midlands, yellow pigmented colony, Pbb)

were transferred using a sterile needle to fresh PDA plates. After 12 days growth on PDA plates, colonies displayed two distinct phenotypes. When viewed from above, the isolates from Northern Ireland and southern England produced white colonies with abundant oozing pink cirri and no yellow pigment (Fig. 1D). By contrast, isolates from the West Midlands had distinctive yellow pigmented colonies (Fig. 1E). These isolates were all provisionally considered, based on fungal colony morphologies and disease symptomologies, to probably be a *Plenodomus* species, most likely the *Brassica* Phoma pathogens *P. lingam* (formerly *Leptosphaeria maculans*) or *P. biglobosus* (formerly *L. biglobosa*) (de Gruyter et al., 2013).

Genomic DNA was extracted from lyophilized mycelium of five of the newly cultured wasabi isolates (Table 1) with a MasterPure Yeast DNA Purification kit (Epicentre, USA). Using the Easy A cloning enzyme kit (Agilent Technologies), fragments

were amplified via PCR (with a 55 °C annealing temperature in all cases) for the ITS rDNA (primers ITS4/5), beta tubulin ( $\beta$ -tubulin F/R), and actin (Actin F/R) loci (White et al., 1990; Van de Wouw et al., 2008). PCR amplicons were purified and sent for bidirectional sequencing to MWG Eurofins (Germany). Phylogenetic analyses were based on concatenated ITS rDNA, beta tubulin, and actin sequences derived from isolates collected in this study (Table 1), and also reference sequences of all known genetic subclades of *P. lingam* (subclades ‘brassicae’ and ‘lepidii’) and *P. biglobosus* (subclades ‘americensis’, ‘australensis’, ‘brassicae’, ‘canadensis’, ‘erysimii’, ‘occiaustralensis’ and ‘thlaspii’) sourced from GenBank (see Fig. 2 legend for full details). The analyses revealed that isolates from Southern England and Northern Ireland (21WAS1-2, 21WAS7-1, 21WAS7-7), that had produced white non-pigmented colonies on PDA, as *P. biglobosus* subclade ‘canadensis’

**Table 1** Details of *Plenodomus biglobosus* isolates from *Eutrema japonicum* (wasabi) in 2021 examined in this study

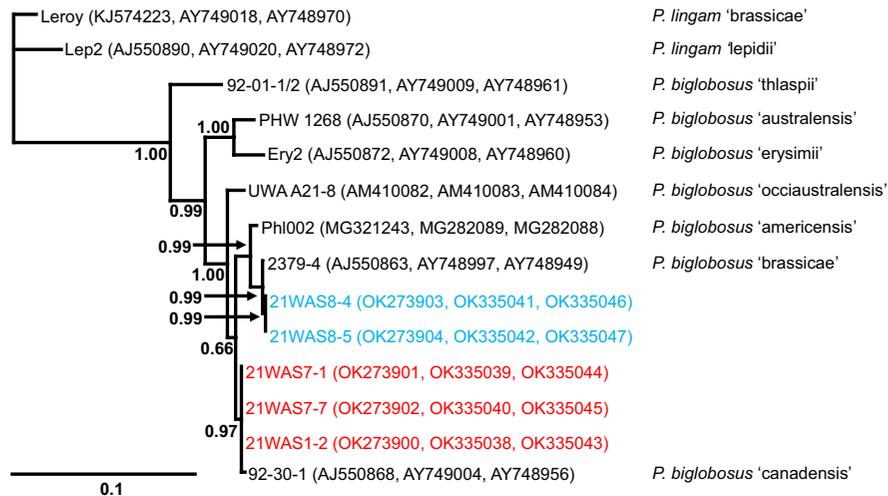
Isolate code	Geographic origin	Genetic subclade	Yellow pigmented colonies <sup>a</sup>	Newly obtained GenBank accession Nos			Live culture deposit Nos	
				ITS rDNA	Beta tubulin	Actin	CABI <sup>b</sup>	CHAP <sup>c</sup>
21WAS1-2 <sup>d</sup>	Southern England, UK	'canadensis'	No	OK273900	OK335038	OK335043	-	-
21WAS7-1 <sup>d</sup>	Northern Ireland, UK	'canadensis'	No	OK273901	OK335039	OK335044	IMI 507211	CB00116
21WAS7-7	Northern Ireland, UK	'canadensis'	No	OK273902	OK335040	OK335045	-	-
21WAS8-4 <sup>d</sup>	West Midlands, UK	'brassicae'	Yes	OK273903	OK335041	OK335046	IMI 507212	CB00117
21WAS8-5	West Midlands, UK	'brassicae'	Yes	OK273904	OK335042	OK335047	-	-

<sup>a</sup>After incubation on potato dextrose agar plates for 10–12 days at 20 °C in the dark

<sup>b</sup>CABI GRC: <https://www.cabi.org/services/microbial-services/culture-collection-microorganism-supply/grc/>

<sup>c</sup>CHAP NRC: <https://chap-solutions.co.uk/nrc-portal/>

<sup>d</sup>Included in pathogenicity testing



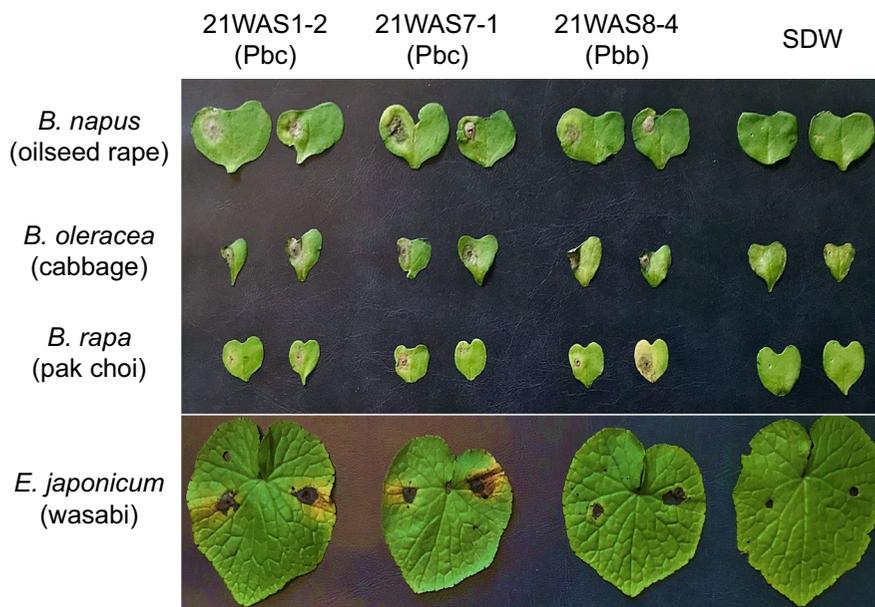
**Fig. 2** Bayesian phylogenetic tree (constructed with MrBayes) inferred from concatenated partial ITS rDNA (456 bp), beta tubulin (375 bp), and actin (415 bp) sequences of *Plenodomus* species. Isolates newly obtained from *Eutrema japonicum* (wasabi) in the UK in this study were identified as either *P. biglobosus* subclade 'brassicae' (blue) or subclade 'canadensis' (red); newly obtained sequences were deposited onto GenBank (see brackets). Reference sequences from isolates of known genetic subclades of *P. lingam* and *P. biglobosus*

used in the analyses are indicated to the right, with sequences downloaded from GenBank (see brackets). The tree shown was based on the on the GTR+I+G model (determined as optimal via JModelTest), with 1,000,000 MCMC generations and a 25% burn in. Bayesian posterior probabilities are indicated in bold at nodes. The outgroup for this tree was *P. lingam* subclade 'brassicae'. The scale bar represents the number of nucleotide substitutions per site

(Pbc). However, isolates from the West Midlands (21WAS8-4, 21WAS8-5), that instead yielded yellow pigmented colonies on PDA, were resolved as *P. biglobosus* subclade ‘brassicae’ (Pbb). Newly obtained sequences were deposited to GenBank, and reference isolates were deposited into both the CABI (IMI) and CHAP live culture collections (Table 1).

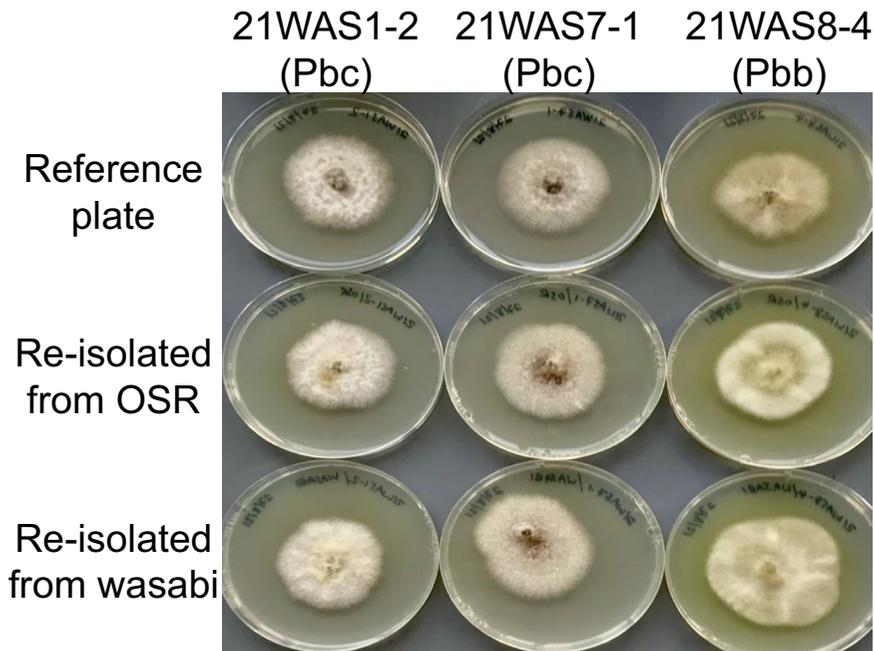
The pathogenicity profiles of three newly obtained tester isolates (Pbc: 21WAS1-2, 21WAS7-1; Pbb: 21WAS8-4) were evaluated on live plants under greenhouse conditions in Surrey in July/August 2021, with OSR (*Brassica napus*, cv. Westar), cabbage (*B. oleracea*), and pak choi (*B. rapa* cv. Yuushou F1) plants 14 days old at time of testing, and wasabi plants having leaves ~10 cm diameter. Conidial suspensions were harvested from PDA cultures (grown at 20 °C in the dark for 8 – 12 weeks) and adjusted to 10<sup>7</sup> conidia / mL using SDW. Four cotyledons of each of the *Brassica* hosts (left lobes only), and three wasabi leaves (both left and right sides) were gently wounded (with a sterile cocktail stick) and point inoculated with either 10 µl isolate conidial suspension or treated with 10 µl SDW. Plants were sealed

in polyethylene bags to maintain high humidity for 48 h. After one week for OSR, cabbage and pak choi cotyledons, and two weeks for wasabi leaves, Phoma lesions were evident on all hosts at points inoculated with Pbc or Pbb isolates, but not SDW-treated controls. Representative pathogenicity testing results are shown in Fig. 3. Subsequently, re-isolation of Pbb/Pbc was attempted from surface sterilized lesion margins (i.e. fungal inoculated) or SDW treatment points (i.e. controls) from OSR cotyledons and wasabi leaves (Fig. 4, see legend for additional information). Isolates, the species and subclade identities of which were confirmed as either Pbb/Pbc, based initially on colony morphology and subsequently by ITS rDNA sequencing were successfully cultured from lesions that developed after inoculation. Cultures of Pbc were obtained from lesions that developed after inoculation with Pbc isolates (i.e. 21WAS1-2, 21WAS7-1); cultures of Pbb were obtained from lesions that developed after inoculation with the Pbb isolate (i.e. 21WAS8-4). Last, no fungi grew from SDW-treated controls, thus completing Koch’s postulates.



**Fig. 3** Greenhouse pathogenicity testing of *Plenodomus biglobosus* subclades ‘brassicae’ (Pbb) and ‘canadensis’ (Pbc) originally obtained from diseased *Eutrema japonicum* (wasabi) leaves in the UK. Two representative *Brassica napus* (oilseed rape), *B. oleracea* (cabbage), and *B. rapa* (pak choi) cotyledons are shown at 1 week post inoculation (wpi) for each iso-

late or sterile distilled water (SDW) treatment tested (note that cotyledons were tested on left lobes only). A representative wasabi leaf is shown at 2 wpi for each isolate or SDW treatment tested (note both sides of wasabi leaves were tested). All plants were wounded prior to either isolate inoculation or SDW treatment



**Fig. 4** Re-isolation of *Plenodomus biglobosus* subclades ‘brassicae’ (Pbb) or ‘canadensis’ (Pbc) from lesion margins of artificially inoculated *Brassica napus* (oilseed rape, OSR) and *Eutrema japonicum* (wasabi) leaves. The three isolates used for pathogenicity testing were cultured from diseased wasabi leaves (tester isolate reference plates, top row), caused disease on artificially inoculated leaves of both oilseed rape and wasabi (see Fig. 2), and were subsequently re-isolated on

potato dextrose agar (PDA) plates from lesions that had developed inoculated OSR (middle row) and wasabi (bottom row) leaves. Plates shown were incubated at 20 °C in the dark for 10 days. Note the production of bright yellow pigmented colonies for Pbb isolate 21WAS8-4 but not Pbc isolates 21WAS1-2 or 21WAS7-1. Species and subclade identities of the re-isolated cultures were confirmed by sequencing of the ITS rDNA region (data not shown)

To date, Pbc has been reported from *Brassica* species (and also *Thlaspii arvense*) in Australia, Canada, China, Mexico and the USA (e.g. Mendes-Pereira et al., 2003; Van de Wouw et al., 2008; Dilmaghani et al., 2009, 2010; Luo et al., 2021). However, the present study extends the known geographic range of Pbc to now include Europe, having been found at two geographically distinct UK sites (Southern England and Northern Ireland). Moreover, this study also represents, to the best of the authors’ knowledge, new discoveries for both Pbb and Pbc as causal agents of Phoma disease on wasabi plants. Prior to this study, based on available sequence data, *P. biglobosus* subclade ‘occiaustralensis’ appears to be the predominant subclade on Phoma-symptomatic wasabi, with reports from Canada, New Zealand and Taiwan (de Gruyter et al., 2013; Johnston et al., 2017; Punja et al., 2017). Thus, it is evident that multiple genetic subclades of *P. biglobosus* are pathogenic to wasabi.

In the present study, petiole and stem lesions were also observed occasionally on naturally infected wasabi plants suggestive of systemic infection of this host by *P. biglobosus*. However, as fungal isolation was attempted in the present study only from foliar lesions, and not from those on petioles/stems, further work is required to investigate the infection strategy of *P. biglobosus* on wasabi. Additional research is also now needed to explore the geographic distribution, comparative epidemiology, taxonomic status and *Brassica* crop health implications of the *P. biglobosus* subclades. In recent years, there is evidence that *P. biglobosus* has become an increasingly problematic important pathogen of UK OSR crops (Huang et al., 2014). Previously, the only *P. biglobosus* subclade reported in Europe has been Pbb (e.g. Liu et al., 2014; Mendes-Pereira et al., 2003). One hypothesis for the reported increase in *P. biglobosus* importance is that additional genetic subclades, including Pbc, may now

be present. Additional monitoring surveys are now required to understand the geographic distribution of the *P. biglobosus* subclades present in current pathogen populations, both on wild and cultivated (particularly OSR) brassicas from throughout the British Isles and continental Europe. Molecular-based approaches will be required, as although some previous studies have used pigment production in agar culture as a criterion for discrimination of *P. lingam* / *P. biglobosus* (Williams & Fitt, 1999), given that only some *P. biglobosus* isolates appear to produce such pigment, this is insufficient for species / subclade discrimination.

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#### Declarations

The authors declare that the research complies with ethical standards.

**Conflicts of interest** The authors have no conflicts of interest to declare that are relevant to the content of this article.

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