Session 12C

Phytophthora ramorum and Related Pathogens

Chairman & Session Organiser:	Dr David Slawson *Plant Health & Seeds Inspectorate, Defra, York, UK*
Platform Papers:	12C-1 to 12C-4
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The threat from invasive *Phytophthora* species: flaws in international biosecurity

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Nature of the threat

The genus *Phytophthora* is probably the world's most destructive group of plant pathogens. Historical examples of damaging Phytophthoras include *P. infestans*, cause of potato blight; *P. cinnamomi*, cause of enormous damage to wild flower communities, forests and horticulture; and *P. megakarya*, seriously damaging to cocoa crops in west Africa.

Until the 1990s, considerable emphasis was placed on the role of Phytophthoras in agricultural systems, but in the past decade the spotlight has also focused increasingly on forests and natural ecosystems. Many unidentified *Phytophthora* species are believed to occur in natural ecosystems around the world, causing limited damage to their natural environment. Recent events show that previously unknown, invasive *Phytophthora* pathogens are being spread via the international trade in nursery stock or via private plant collecting. Examples include *P. ramorum*, responsible for the death of tens of thousands of trees and shrubs in northern California in the past decade, now introduced and spreading onto trees in the UK and the Netherlands; *P. kernoviae*, currently spreading in south west England and killing rhododendrons and beech trees; *P. alni*, currently spreading and causing serious damage to native alders in some European countries; and *P. ilicis*, damaging *Ilex* species in the UK and recently recorded in the Netherlands. Also representative of the problem is the widespread establishment of *P. palmivora*, in nurseries in Italy; and known dangerous incursions, such as the arrival of *P. lateralis* in France and the Netherlands in the last 10 years. *P. lateralis* currently threatens stands of native 40m *Chamaecyparis lawsoniana* in the Pacific Northwest. Other *Phytophthora* species recently recorded for the first time in the UK include *P. hibernalis, P. citrophthora, P. quercina, P. inundata, P. pseudosyringae* and *P. inflata.*

The geographical origins of many invasive Phytophthoras remain unknown. Probably they are native to as yet unexplored ecosystems, whether accessible or remote. If one accepts estimates that only 7-10% of fungi are known to science then there may be 200-600 extant *Phytophthora* species, with some 150-450 still unknown. Of these, based on current evidence, roughly 10% (i.e. some 30 unknown species), could be seriously damaging to forests, natural ecosystems and horticulture outside their natural ranges.

P. kernoviae has recently been found at several sites in New Zealand and is now believed to have been present there since at least the 1950s. Whether it is endemic or introduced is still unclear. Two other new *Phytophthora* species have just been described from trees in New Zealand and several more are currently being described. Whether we are net exporters or importers of plants or have sophisticated or unsophisticated biosecurity frameworks, we are often unaware of what is in our own backyards.

There is evidence that nurseries in Europe and elsewhere are infested with multiple exotic and introduced Phytophthoras. Up to five *Phytophthora* species have been isolated from around a single nursery alder seedling in Bavaria (Jung, personal communication). Such multiply infested European nurseries are potential breeding grounds for new, interspecific hybrid pathogens that may be more aggressive, or have host ranges unknown in the parent

species. A prominent example is the swarm of newly evolved *Phytophthora* interspecific hybrids assigned to the new species *P. alni* presently killing native alders across Europe and considered an emerging natural disaster in southern Bavaria and Northern France. A link has been established between the spread of *P. alni* to Bavarian rivers and infested alder nursery stock. Two other new, hybrid Phytophthoras have recently appeared in glasshouses in the Netherlands. The intermixing of related but previously geographically isolated pathogens as a result of the international plant trade can promote novel and dangerous evolutionary risk to the environment.

Climate change will also influence the behaviour of invasive pathogens. *Phytophthora* pathogens in particular are likely to show enhanced activity under the conditions of greater climatic perturbance, such as more extreme rainfall and drought episodes, predicted in current UK Climates Impact Programme scenarios. *P. cinnamomi,* introduced into the UK around 150 years ago, continues to cause widespread economic and cultural damage here and around the world. Modelling using CLIMEX suggests its activity in the western UK and parts of Europe will increase significantly with only a modest +1.5°C average warming and greatly with +3°C warming. The same can be expected for many other Phytophthoras.

Main drivers of the threat

A major factor in the threat is the increasing intensity and globalisation of the trade in plants. Plant imports into the UK have more than doubled in the past decade, the drivers being the availability of cheap plants from Europe and developing countries; the novelty of exotic plants and the desire for 'instant trees'. Plants are being shipped from exotic locations such as China and South Africa. Imports of trees of up to 10m in height with roots and soil attached are also increasing. All these factors increase the biosecurity risk.

The problem is exacerbated by international plant health protocols which are characterised by serious scientific and system weaknesses. A major problem is that the protocols are list-based, allowing regulation only of named organisms. Because they were unknown to science, *P. ramorum, P. kernoviae* and *P. alni* were not on any prohibited list before they began to inflict serious damage. Other factors contributing to biosecurity breakdown include problems with protocol implementation e.g. failure to conduct surveys or to publicise security breaches; loopholes in regulation e.g. export of certificated stock that turn out to be infested on arrival; reliance on solely visual inspection of a small percentage of stock; and a lack of official follow-up to security breaches, such that there is little feedback on the market to encourage more effective policy and practice. In the author's view the current biosecurity approach is outmoded, having been overtaken by changes in scientific knowledge and changes in patterns of world trade and development. To address the problem, more awareness and debate is needed among regulators and international agencies, forestry and horticultural professionals, the plant trade and the public.

Many of the above issues are not confined to *Phytophthora* pathogens. They apply to the whole gamut of introduced pests and pathogens turning up around the world. *Phytophthora* just happens to be one of the more insidious and threatening pathogen groups. In agricultural systems, such organisms may be controlled by technological innovation. In complex 'natural' ecosystems such as forests, eradication often proves technologically impossible or environmentally unacceptable. Yet their impact is unpredictable and can lead to long lasting ecosystem and habitat degradation. Each invasion is an uncontrolled, open-ended experiment in evolution; and a failure of international biosecurity.

Phytophthora ramorum – development of field and laboratory diagnostic strategies for effective disease management

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Introduction

Phytophthora ramorum is a recently described pathogen that has resulted in emergency EC phytosanitary legislation since November 2002. The Department for Environment, Food and Rural Affairs Plant Health and Seeds Inspectorate (PHSI) initiated a survey in England and Wales in autumn 2001 to ascertain whether the organism was present. In April 2002, *P. ramorum* was found for the first time in the UK and for the first time in Europe since the initial reports in the Netherlands and Germany. Effective disease management and implementation of plant health legislation is reliant upon rapid and accurate disease diagnosis based upon recognition of symptoms in the field and identification of the cause by testing. Other diseases and disorders prevent reliable identification of the problem at the time of inspection with typically only 20-30% of samples submitted testing positive for *Phytophthora* species. It has been necessary to develop a range of diagnostic strategies suitable for both laboratory and on-site testing to permit rapid, cost-effective and reliable testing for plant material by plant health inspectors, researchers and diagnosticians. This paper presents evaluation data for a real-time PCR method, *Phytophthora* lateral flow devices in comparison to isolation and discusses evolution of techniques.

Diagnostic methods

Symptomatic material was tested according to methods developed at CSL and now part of the EPPO Diagnostic protocol (Anon., 2006). Methods included isolation on semi-selective media (PARP-H) and assessment of cultural morphology after five days incubation, PCR using species–specific primers and probes (Hughes *et al.*, 2006) and on-site serological test kits called lateral flow devices (LFDs) (Lane, *et al.*, 2007).

Interpretation of results

The diagnostic sensitivity and specificity were calculated using a 2x2 contingency table with letters assigned to the four quadrats as follows: A both negative, B false positives - comparative test (LFD or PCR) positive, but the standard laboratory method negative, C false negative - comparative test negative but a *Phytophthora* detected using the standard laboratory method, D both positive. The diagnostic sensitivity (A/A+C) and specificity (D/D+B) were calculated using these formulae.

Results

Lateral flow devices

The LFD performed well in comparison to laboratory based methods with 536 out of 634 samples (84.5%) giving the same result. The diagnostic sensitivity was 87.6% and the diagnostic specificity was 82.9%. False positives occurred on 70 occasions (11.0%) in addition to some false negatives (4.4%). They were shown to be highly specific reacting to a wide range of *Phytophthora* species and with no cross reaction with *Pythium* spp, downy mildews or other true fungi. They were found to be very sensitive detecting at little as 1.5 mg wet weight of infected rhododendron leaf tissue.

Real time PCR

Three hundred and seventy nine samples were tested but amplifiable DNA was not extracted from 59 samples. Most of the samples tested were from rhododendron (219) and viburnum (54) but DNA was successfully extracted from a further 47 samples from 17 other plant taxa. *P. ramorum* was identified only on rhododendron and viburnum samples. When the two methods were compared for all 320 samples tested there was an agreement of 316/320 (98.8%) giving a diagnostic sensitivity of 92.9% and specificity of 99.3%.

Discussion

Due to the variability in symptoms, and the as yet, undetermined extent of the pathogen's host range, field identification of *P. ramorum* based on symptoms alone is unreliable therefore requiring some form of testing. Routine well-established diagnostic techniques for the identification of *Phytophthora* were initially applied but as the number of samples increased it became necessary to develop and evolve the strategy to cope with the range of genera, outbreak sites, expertise of the samplers and demands for water and soil testing. Initially this led to development of a conventional PCR assay primarily to confirm the identity of the organism following plating but could also be used to test plant material directly. This in turn led to the development a real-time PCR assay more suited for high-volume testing in the laboratory or on-site testing using Smartcycler technology. In these studies comparative testing showed that direct real-time PCR of plant material gave equivalent results to isolation. This has led to direct PCR testing of all rhododendron samples without a requirement to isolate from existing outbreak sites in England and Wales.

This diagnostic approach has successfully been accredited to ISO17025 demonstrating the validity of the method. Further diagnostic assistance was achieved with the implementation of a *Phytophthora* LFD for plant health inspectors following extensive evaluation studies. The level of agreement (84.5%) between the LFD and the standard laboratory method was not as stringent as the comparison to real-time PCR, however, for statutory purposes as all positive LFD results require laboratory testing to determine the species of *Phytophthora* involved, the presence of false positives is negated, so an overall efficiency of 95.6% was achieved. The routine use of LFDs has resulted in at least a 50% reduction in samples submitted for laboratory testing saving both time and money for growers and plant health services. In summary, diagnostic techniques have been developed to aid both laboratory and field based staff with a range of experience and expertise resulting in an efficient, robust and reliable service able to process in excess of 10000 samples per annum.

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Spatio-temporal analysis of *Phytophthora ramorum* cases in the UK

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Phytophthora ramorum was first described and identified in 2001 as the causal agent of a twig blight affecting nursery and garden *Rhododendron* spp. in Germany and the Netherlands. Sudden oak death syndrome was first observed in 1994 and associated with widespread mortality of oaks (*Quercus* spp.) and tanoaks (*Lithocarpus densiflorus*) along the coastal ranges of California, USA.

Since 2002 when *P. ramorum* was first discovered on container-grown *Viburnum* in a nursery in the UK, Defra's Plant Health and Seeds Inspectorate (PHSI) and the Forestry Commission have been carrying out extensive surveys to check for the presence of this disease. Statutory action is being taken whenever the pathogen is found, including destruction of affected plants and tracing of related stocks. Initially the disease was mainly found in a small number of large private gardens and nurseries predominantly in the south of England. In the survey, all positive *P. ramorum* cases were documented irrespective of their location. These data were analysed to evaluate the successfulness of the containment policy and the spatio-temporal disease development.

In total, there were 3828 positive cases on live plant samples during 2003-2005. Of these cases, 2568 referred to *P. ramorum* and the others to *P. kernoviae*. Excluding duplicate cases within the same site led to 1312 and 264 unique combinations of sites and host species that were positive for *P. ramorum* and *P. kernoviae*, respectively. The incidence of *P. ramorum* in nurseries/garden centres remained stable over the past three years but appeared to have increased in the semi-natural environment (Table 1). There were also indications of a possible annual cycle peaking in the period from late-spring to early summer. Most *P. kernoviae* cases were found in the semi-natural environment with its incidence remaining stable. There is evidence that the current containment policy is being effective in limiting the spread of *P. ramorum* in nurseries/garden centres.

In addition to the spatial co-ordinates for each infected site, we have also obtained spatial co-ordinates for all nurseries and garden centres, which were used as a 'control' data set when testing hypotheses on spatial relations amongst infected sites. We focused on determining spatio-temporal relationships among infected nurseries/garden centres and spatial relationships between infected nurseries/garden centres and outbreaks in the semi-natural environment. Univariate and bivariate random labelling was used as a null model for testing the above spatial relationships. Significant departure from the null hypothesis was determined on the basis of 99 permutations (randomisations). Ripley's K and

L-function, and Wiegand-Moloney's *O*-ring statistic were calculated using the *Programita* software (Wiegand & Moloney, 2004).

Table 1. Total number of new cases with either *P. ramorum* or *P. kernoviae* in nurseries (including garden centres) and semi-natural environment in each quarter, excluding duplicates of positive cases, during the same inspection, within the same site

	2003				2004				2005			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
P. ramorum												
Nurseries	104	131	58	24	69	59	29	16	103	21	26	23
Environment	30	18	62	33	38	42	39	46	48	90	87	58
P. kernoviae												
Nurseries						0	5	6	0	7	10	2
Environment						5	64	21	20	25	41	58

P. ramorum cases in nurseries/garden centres were significantly aggregated spatially within the distance of ca. 50 km in 2003 and 2004, i.e., infected nurseries/garden centres were closer to each other than expected for the given disease level and the spatial location of nurseries and garden centres. The degree of spatial aggregation appeared to have decreased over the three years to such an extent that no more significant aggregation was observed for 2005 cases. There were some indications of local spread from infected nurseries/garden centres to neighbouring ones, particularly to those within the distance of ca. 60 km, when the infected sites in 2003 and 2004 were considered. In contrast, no such significant relationship was found when 2004 and 2005 cases were considered. Because the overall incidence of infected sites did not increase over the three years, these results suggest that the current containment policy is succeeding in limiting disease spread. Data from 2006 are currently being analysed to confirm whether this trend continues.

It was not possible to determine whether *P. ramorum* outbreak sites in the general environment were spatially aggregated among themselves. This is because spatial locations of all susceptible hosts in the environment are not known and it is inappropriate to assume that these susceptible plants are either randomly or homogeneously distributed over the general environment. Instead, we tried to infer from spatial locations whether infected nurseries/garden centres could act as an inoculum source of those infected plants in the semi-natural environment. In all three years, analyses suggest that *Phytophthora ramorum* outbreak sites in the general environment were closer to infected nurseries/garden centres (within the distance of ca 40 km) than expected if there were no substantial disease spread amongst them. Now, we are investigating the converse problem, i.e. whether there was any evidence that infections in the natural environment acted as an infection source of nurseries.

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Phytophthora kernoviae – a new emerging problem on woodland ornamentals

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Introduction

Phytophthora kernoviae (Brasier *et al.* 2005) is a new and potentially serious pathogen of woodland environment plants. It was first discovered in historic woodland gardens in Cornwall in 2003 during routine inspections for the sudden oak death pathogen, *Phytophthora ramorum*. Since then it has been found at woodland and garden sites throughout Cornwall, a few garden sites in South Wales and at two sites in North West England. Only two findings have been made on nurseries.

The organism appears to have a fairly restricted host range (nine families so far) and to date has been found on 4 genera of ornamental plants (*Rhododendron* spp., *Pieris* spp., *Hedera helix* and a variety of *Magnolia* species) and 8 genera of trees (*Drymis winteri, Fagus sylvatica, Gevuina avallena, Ilex aquifolium, Liriododendron tulipifera, Michelia doltsopa, Prunus laurocerasus, Quercus ilex* and *Q. robur*).

Symptoms

The disease causes a range of symptoms depending on the host, similar to those caused by *P. ramorum* or some other *Phytophthora* spp. The most commonly infected ornamental host accounting for 95% of plants tested in CSL's plant clinic is rhododendron (principally *R. ponticum*). Early leaf symptoms include a blackening of the leaf petiole that extends down the midrib and into the leaf lamina often resulting in a dagger like appearance. *P. kernoviae* infects both old and young leaves throughout the bush causing early leaf abscission. Leaf tip blackening is also observed. Later symptoms include the presence of cankers and shoot dieback. Severe infection may result in shrub death.

Symptoms on other ornamental hosts, although less severe than those on rhododendron, are very similar and indistinguishable from those caused by *P. ramorum*. The most notable symptom on trees is the presence of bleeding lesions on the trunk. These can be found anywhere from ground level up to 12m. The bleed is usually dark brown to blue-black and older lesions may become sunken. On some hosts, leaf and/or bud necrosis is the only symptom of infection e.g. *Magnolia*, *Q. ilex*.

Disease development and spread

P. kernoviae only infects aerial parts of susceptible host plants. Under suitable conditions, asexual sporing structures (sporangia) emerge from necrotic lesions. These detach from sporangiophore supports and are carried on wind-driven rain to neighbouring leaves or shrubs. Infective zoospores mature within each sporangium and are released through a small papilla. These swim in surface water, encyst and germinate infecting via stomata or wounds on the leaf. As the organism grows throughout the leaf, it degrades tissue enzymatically and produces further sporangia and sexual oospores (*P. kernoviae* is homothallic). Unlike *P. ramorum*, *P. kernoviae* does not produce chlamydospores.

Disease diagnosis

Diagnosis of the disease begins in the field with recognition of potential leaf or stem symptoms. The presence of the genus *Phytophthora* associated with the symptoms can be determined using an antibody based field test kit (lateral flow device; Forsite) but, laboratory examination is necessary to determine the species. Here, suspect samples are treated according to a standard diagnostic protocol involving both cultural and molecular (real-time PCR) techniques. Diagnostic features of *P. kernoviae* are looked for on P_5ARP[H] medium (Jeffers & Martin, 1986)) onto which pieces of symptomatic, washed plant material excised from the leading edge of the necrosis have been transferred and allowed to incubate (six days at 20°C on the laboratory bench). Molecular analysis (real-time PCR) with primers and probes developed at CSL (Hughes, 2005) can be used on both cultures or on symptomatic plant tissue. In addition, real-time PCR can be undertaken in the field using a portable Smartcycler.

Current status

P. kernoviae was added to the EPPO Alert List in October 2005 having been formally named as a new species (Brasier *et al.*, 2005). It is a notifiable disease in the UK and as part of the Plant Health & Seeds Inspectorate (PHSI) and the Forestry Commission Woodland Survey for *P. ramorum*, all samples submitted to CSL are tested for *P. kernoviae*.

Between October 2003 and December 2006 *P. kernoviae* was found at 39 sites in England and Wales. In the UK, statutory action to eradicate and contain *P. kernoviae* is taken in line with action for *P. ramorum.* This includes the destruction of the infected plant and all susceptible plants in a 2m radius and the restriction of movement of susceptible plants in a 10m radius from the infection. In gardens, eradication and containment measures are taken as appropriate to the site and a 'disease management zone' has been established in Cornwall to restrict the spread from infected areas. *P. kernoviae* has not been detected for over a year in two of the outdoor sites subject to eradication and containment measures.

To date, New Zealand is the only other country the pathogen is known to occur in where it has been found in unmanaged wild area and nurseries. No direct link has been established but examination of imports of known host plants shows that there are potential pathways of entry for *P. kernoviae* from New Zealand to the UK and vice versa.

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Evaluation of a rapid diagnostic field test kit for identification of *Phytophthora ramorum*, *P. kernoviae* and other *Phytophthora* species at the point of inspection

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Introduction

Plant health regulations to prevent the introduction and spread of *Phytophthora ramorum* and *P. kernoviae* require rapid, cost effective diagnostic methods for screening large numbers of plant samples at the time of inspection. Current on-site techniques require expensive equipment, considerable expertise and are not suited for plant health inspectors. Therefore, an extensive evaluation of a commercially available lateral flow device (LFD) for *Phytophthora* species was performed.

Lateral flow device (LFD)

Phytophthora LFD kits designed to recognise all species of *Phytophthora,* including *P. ramorum* and *P. kernoviae,* were supplied by Forsite Diagnostics Ltd, York, UK. Detailed instructions on LFD use were supplied with the kits.

In summary, several small pieces of leaf showing symptoms were broken up between the thumb and fingers before being placed in a plastic bottle containing five small (approximately 3mm) ball bearings and extraction buffer. Pieces of suspected diseased tissue were transfered to the extraction bottle. The bottle was shaken vigorously for 60 seconds and then the extract was taken up in a small disposable dropper. Two to four drops were placed onto an absorbent pad within the kit and left for at least two minutes but no longer than 10 minutes before reading. A single blue line developed to indicate the test kit was working (control line) whilst the development of a second blue (target line) indicated the presence of *Phytophthora* spp. A larger sample from the same part of the plant with identical symptoms was submitted for laboratory testing according to a protocol developed at CSL and now part of the EPPO Diagnostic protocol (Anon., 2006).

The sensitivity of the LFDs was evaluated using naturally infected rhododendron leaves previously tested as positive for *P. ramorum* by isolation and real-time PCR. A small square of necrotic tissue (approx. 12 x 12 mm) was excised from the leaf and the wet weight determined. It was then dissected further into smaller portions, the wet weights determined and then tested with an LFD. The specificity of the LFD was evaluated using a range of cultures. A small piece of agar (1 cm^2) was excised from the centre of the colony, placed in an extraction bottle, shaken vigorously for 10-15 seconds and then tested with a LFD as described above.

Results

Sensitivity

A positive reaction was clearly obtained with just a few mg of necrotic rhododendron leaf tissue (equivalent to a few square millimetres) either alone or when mixed with healthy leaf tissue permitting detection in leaf tissue which was less than 1% infected by *P. ramorum*.

Specificity

The negative control (agar plug), true fungi (*Alternaria alternata, Botrytis cinerea, Cylindrocarpon sp., Monilinia laxa, Pleospora herbarum, Trichoderma harzianum, Rhizopus sp.)* and isolates of the oomycete *Pythium* also all tested negative. All 13 species of *Phytophthora,* including *P. ramorum* and *P. kernoviae* tested positive.

Comparative testing

A total of 634 samples were tested with agreement on 536 occasions (84.5%). False positives and negatives were encountered on 70 (11.0%) and 28 times (4.4%) occasions respectively. The diagnostic sensitivity was 87.6% and the diagnostic specificity was 82.9%.

Discussion

A commercially available lateral flow device for *Phytophthora* identified the presence of *P. ramorum, P. kernoviae* and other *Phytophthora* species on a wide range of plant material as part of plant health inspection and disease management work. The assay was demonstrated to identify a broad range of *Phytophthora* species and did not cross react with other true or lower fungi. The LFD was shown to be very sensitive and able to detect *P. ramorum* in less than 1% infected rhododendron leaf tissue. The assay was simple to use, provided results in 3-5 minutes and on every occasion a control line appeared confirming the validity of the test. LFD results were compared to those from testing a parallel, but not always identical, sample using well-established laboratory methods. These trials demonstrate that LFDs offer a useful decision support tool for the detection of *Phytophthora* spp. at the point of inspection. For statutory purposes, as positive LFD results require laboratory testing to determine the species of *Phytophthora*, the presence of false positives is overcome.

Therefore, in this study where all LFD positives were submitted for laboratory testing, an overall efficiency of 95.6% was achieved which is a substantial improvement on relying on visual assessment alone. The LFD kits for detecting *Phytophthora* spp. cost from £6 per test so are significantly cheaper than laboratory testing. They have been of considerable value in instructing new plant health inspectors in disease recognition, helping to convince growers and land-owners of the need to sample and hold plants. The simplicity and robustness of these kits makes them ideally suited for all skill levels and their size and weight ideal for varying sites and conditions to obtain a rapid assessment of whether *Phytophthora* may be present. They have the potential to assist plant health organisations manage their disease campaigns in a new way by helping to optimise and target the use of field inspectors, highly skilled diagnostic staff and centralised laboratory services. In England and Wales they have resulted in at least a 50% reduction in samples submitted for laboratory testing saving both time and money for growers and plant health services.

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Mating type of Belgian *Phytophthora ramorum* isolates

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Abstract
Phytophthora ramorum was first identified in 1993 in Germany and the Netherlands on *Rhododendron* and *Viburnum* spp., on which it causes leaf blight, stem canker, and tip dieback. Around the same period, *P. ramorum* started causing mortality of tanoaks (*Lithocarpus densiflorus*) and oaks (*Quercus* spp.) in California, where the disease is called "Sudden Oak Death" and has since reached epidemic proportions. In Europe, the pathogen has been observed in nurseries in several countries, primarily on *Rhododendron*, *Viburnum* and *Camellia*, but mortality of trees is limited to a few instances in the UK and the Netherlands. *P. ramorum* is heterothallic and originally the two mating types were considered to be genetically and geographically distinct. All US isolates are A2 mating type and belong to genetic lineages that are distinct from a single EU lineage, except for some US isolates of EU lineage that were probably imported. All EU isolates were considered to be A1 mating type, except for a single A2 isolate of EU lineage, collected from a nursery plant in Belgium (Werres & De Merlier, 2003).

A hypothesis was formulated that this single EU A2 isolate had undergone a mating type switch as a result of a mutation. Mating type screening of the Flemish isolates that were collected from 2002 to 2006 resulted in the identification of two new A2 isolates. These isolates were confirmed to belong to the European most common genotype based on polymorphic markers (polymorphic microsattelites and a COX gene polymorphism). Inoculation tests on several host plants indicate no significant difference in pathogenicity between the European A1 and A2 mating type isolates.

Mating type screening of additional isolates

Mating type tests (Brasier & Kirk, 2004) involving 285 isolates from the Northern part of Belgium (Flanders), collected from 2002 to 2006, resulted in the identification of two extra A2 isolates. AFLP and SSR profiles (Ivors *et al.*, 2004; 2006), as well as the COX polymorphism (Kroon *et al.*, 2004) confirmed that these isolates belonged to the EU lineage. Mating type was confirmed by two research groups and involved mating experiments with several A1 and A2 isolates. Together with the data from the EU-funded project "Risk Analysis of *Phytophthora* ramorum" (RAPRA), which showed only A1 mating type isolates in over 250 EU isolates tested, it is intriguing that only three isolates were found to be of A2 mating type, and all were isolated in Belgium.

The hosts originated from three different nurseries and two different plant species (*Viburnum* and *Rhododendron*), with no obvious commercial link between the nurseries. Considering there are no indications of a relationship between these three isolates, it could be that the EU-A2 genotype is rare but not as exceptional as originally hypothesized. Alternatively, the three EU-A2 isolates may still be the result of clonal propagation after a mutation event. Phenotypic or genotypic differences between these isolates would clarify the situation. Therefore, the isolates were subjected to genotyping and to pathogenicity tests.

Genotyping

The simple sequence repeat (SSR) technique was used to fingerprint *P. ramorum* isolates. Ivors *et al.* (2006) identified three markers (loci 18, 64 and 82) that were polymorphic within European isolates. Using these markers, they identified 7 EU genotypes. However 86% of the isolates showed no polymorphism (the EU1 genotype). Screening of the three A2 isolates with these markers showed no genotypic differences between the Belgian A2s and the main European genotype group (EU1). Another 90 microsatellite markers were identified and used to screen for polymorphism between EU-A1 and the three EU-A2 isolates using universal labelling (Prospero *et al.*, 2004). Again, the three Belgian A2 isolates belonged to the most common genotype. Also, amplified fragment length polymorphism (AFLP) fingerprinting of the isolates (Ivors *et al.*, 2004), using five primer combinations, could not distinguish the EU-A2 isolates from the genotype that dominates the European population.

Pathogenicity test

Several host species were inoculated with 3 EU-A1 isolates, 3 EU-A2 isolates and 2 US-A2 isolates. A first inoculation method consisted of a leaf dip in a zoospore suspension. It was used to estimate the ability of the host to resist pathogen penetration. There was no significant difference between the EU-A2 and EU-A1 isolates. One American isolate was less virulent. Using a second inoculation method, leaves were pin-wounded and inoculated with a mycelium plug to evaluate the resistance to pathogen growth inside the leaf tissue. This test also indicated no significant difference between the European A2 and A1 isolates.

Conclusion

No genotypic difference could be found between the three European A2 isolates. They all belonged to the main genotypic group of EU isolates. Also, no pathogenic differences could be observed between the three A2 isolates and between the EU-A2 and EU-A1 isolates. Therefore, given the limited amount of genotypic markers available within the EU population of *Phytophthora ramorum*, the question of the clonal nature of these EU-A2 isolates remains.

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