
Biological and epidemiological aspects of the quarantine pathogen *Phytophthora ramorum*

Beatrice Ginetti, Stefano Carmignani, Alessandro Ragazzi, Salvatore Moricca

Dipartimento di Scienze delle Produzioni Agroalimentari e dell’Ambiente (DISPAA), Sezione di Patologia vegetale ed Entomologia. P.le delle Cascine 28, 50144 FIRENZE, Italy

Corresponding Author e-mail: beatrice.ginetti@unifi.it

Abstract

Phytophthora ramorum is a quarantine pathogen that causes leaf blight and shoot dieback of the crown, bark cankers and death on a number of both ornamental and forest trees, especially in North America and northern Europe, where it has produced severe outbreaks.

In Italy it was first reported in 2002, on *Rhododendron yakushimanum* in a Piedmont nursery; after that it seemed to have disappeared, only to re-emerge in 2013 when numerous isolates were detected on batches of *Viburnum tinus* plants growing in some nurseries in the Pistoia area (Tuscany), which is an important district in the trade of nursery plants world-wide. This work reports on a number of laboratory tests that were carried out on isolates from infected plant samples. The micromorphological and macromorphological characteristics of the pathogen growing on carrot agar (CA), corn meal agar (CMA), malt extract agar (MEA) potato dextrose agar, and V8 agar with added PARPNH (see text) were determined, as was the growth rate at 10°, 15°, 20°, 25°, 30°, 32° and 35°C. Molecular analysis was employed to identify the isolates more precisely. Inoculation trials under the bark were also carried out to ascertain the isolate virulence and the Koch’s Postulates.

The Plant Protection Service of the Tuscan Region (SFR, Servizio Fitosanitario Regionale) was alerted as soon as the pathogen infection was detected and it took the prescribed steps to eradicate the infection in the field and prevent the recurrence of an epidemic.

Keywords: leaf and shoot blights; bleeding cankers; nurseries; ornamental trees; control

Riassunto

Phytophthora ramorum è un patogeno da quarantena noto in letteratura per aver comportato diverse catastrofi ambientali, soprattutto nel Nord America e nel Nord Europa dove si è manifestato con disseccamenti delle chiome e cancri corticali su svariate specie di piante, sia ornamentali che forestali. In Italia un primo rinvenimento di questo organismo era stato effettuato nel 2002 in un vivaio piemontese su piante di rododendro; da allora la problematica è riemersa nel 2013, quando numerosi isolati di *P. ramorum* sono stati trovati associati a piante di *Viburnum tinus* in alcuni vivai del comprensorio pistoiese, rinomato centro commerciale per il vivaismo a livello internazionale. Numerose indagini di laboratorio sono state effettuate sugli isolati ottenuti dai campioni di tessuto sintomatico. I caratteri micro e macromorfologici accertati su diversi tipi di substrato [Carrot Agar (CA), Corn Meal Agar (CMA), Malt Extract Agar (MEA), Potato Dextrose Agar (PDA) e V8-agar+PARPNH (vedi testo)], il tasso di crescita a diverse temperature (10°, 15°, 20°, 25°, 30°, 32°, e 35° C) ed analisi di tipo molecolare sono stati impiegati per l’identificazione degli isolati. Sono state inoltre condotte prove di inoculazione artificiale sotto corteccia allo scopo di saggiare la virulenza degli isolati reperiti, nel rispetto dei Postulati di Koch.

Il Servizio Fitosanitario della Regione Toscana (SFR), immediatamente allertato, ha messo in atto le norme previste per eradicare il problema in campo e contenere ed arginare una eventuale epidemia.

Parole chiave: avvizzimenti fogliari e dei getti; cancri sanguinanti; vivaismo; specie ornamentali; misure di lotta

Introduction

Phytophthora ramorum Werres De Cock & Man in't Veld is a highly virulent quarantine pathogen that in 2013 and 2014 was isolated from some ornamental *Viburnum tinus* L. plants growing in nurseries in the Province of Pistoia (GINETTI et al., 2014). The discovery of this pathogen raised great concern since in other countries it has caused enormous damage: it is a polyphagous pathogen that attacks the phloem and the cambium of numerous tree species, causing necroses and cankers along the entire length of the trunk; in this it behaves like several other *Phytophthora* species on other trees: *P. alni* Brasier & S.A. Kirk, *P. cambivora* (Petri) Buisman, *P. cactorum* (Lebert & Cohn) J. Schröt., *P. cinnamomi* Rands, *P. citricola* Sawada, and *P. kernoviae* Brasier, Beales & S.A. Kirk. The symptoms caused by these species are produced either when the pathogen moves upwards from the root or root collar, or when it is spread by air to the bark of the trunks or branches. The resulting cankers often yield a sticky and tarry exudate of a blood-red colour, and hence they are called “bleeding lesions”, or “stem bleeding cankers”. They also give a blood-red colour to the cambium, as can be observed by removing the bark portion and the cambium underneath the canker (BROWN & BRASIER, 2007).

On shrubby species the symptoms are: brown spots and necrosis on the leaves, coalescing over time to cover the entire leaf surface; wilting of the buds with folding of their apical portion; discoloration with brown streaks running down the stem, visible after debarking the stems at cambium level.

Phytophthora ramorum is an oomycete that on oaks in the US causes a disease called ‘sudden oak death’ (WERRES et al., 2001). It was first isolated in California in 1995 and then spread rapidly, mainly infecting native oaks and *Notholithocarpus densiflorus* (Hook. & Arn.) Manos, Cannon & Oh causing very significant damage along ca. 300 km of coastline (RIZZO et al., 2002). Subsequently *P. ramorum* was reported in the nurseries of a number of European countries: Belgium, Denmark, France, Germany, Great Britain, Italy, Netherlands, Norway, Poland, Slovenia, Spain and Sweden, particularly on ornamental shrubs such as viburnum, rhododendron, and azalea (WERRES et al., 2001; RIZZO et al., 2002; GULLINO et al., 2003; MALONEY et al., 2004; BROWN AND BRASIER, 2007).

Other investigations have shown that *P. ramorum* also occurs on trees in forests and parks. Since 2002 the pathogen has been found in Great Britain and The Netherlands on horse chestnut, chestnut, beech, ash, and oak, all trees that grow in forests or parks (BRASIER et al., 2004a; BRASIER et al., 2004b; BRASIER et al., 2004c; DENMAN et al., 2005).

P. ramorum was first reported in Italy by GULLINO et al. (2003) on a batch of exotic *Rhododendron yakushimanum* F.C.C. 'Koichiro Wada. To counter its spread the European Union adopted Decision 2002/757/CE of 19 September 2002 ‘Provisional emergency measures to prevent the introduction and spread of *Phytophthora ramorum* in the EU’. The above decision was subsequently modified by decision 2004/426/CE of 29 April 2004, and decision 2007/201/CE of 27 March 2007; both of which prescribe mandatory regulations for its prevention and control. These measures require that: 1. All imported plants and timber susceptible to *P. ramorum* must be inspected at the moment of their introduction into one of the member states; 2. All plants and timber susceptible to *P. ramorum* may be imported from the US only if they meet the emergency plant health measures, and if they are also inspected at the point of entry; 3. All plants of *Viburnum* spp., *Camellia* spp. *Rhododendron* spp. (except *R. simsii* Planch, and the seeds of *R. simsii*), even from countries where *P. ramorum* is not (yet) known to occur, may be introduced into the EU only if they

bear a plant-health passport. Frequent inspections should also be undertaken by the plant health services to detect any plants infected with *P. ramorum*, which must be destroyed wherever they are found, as well as all susceptible plants found within a radius of 20 m from the infected plants. In the case of infected potted plants, the potted soil must also be sterilised.

The law also requires measures to be taken to prevent the spread of *P. ramorum* when it is first reported in a country: during the first two years after such a report, it is forbidden to plant, transplant or deposit any susceptible species in any area that was infected, and any susceptible plants that grow within a 10-m radius of a previously detected locus of infection should be inspected at least twice in the three months following the discovery of the infection (Decision of the European Community, 2002/757/CE). Due to its dangerousness, the pathogen was recently moved from the EPPO Alert List (pests possibly presenting a risk to EPPO member countries) to the EPPO A2 list (List of pests recommended for regulation as quarantine pests).

When these measures were taken in Italy after *P. ramorum* was first detected here in 2002 (GULLINO et al., 2003), they seem to have worked since at the time no further infections were found in either nurseries or natural ecosystems, and there were no environmental consequences. However, the problem re-emerged in 2013 when *P. ramorum* was discovered on a number of *Viburnum tinus* plants growing in a nursery in Pescia (Province of Pistoia) (GINETTI et al., 2014). About 20% of a batch of 700 plants exhibited the typical symptoms of the pathogen (Fig. 1 a). Symptoms included brown spots and lesions on the leaves (Fig. 1 b), withering and curving of the shoots (Fig. 1 c, d), and brown streaks along the stem, which were also visible on the cambium after the infected bark was removed (Fig. 1 e).



Fig. 1. Symptoms caused by *Phytophthora ramorum* on *Viburnum tinus*: a. *Viburnum* plant with the typical symptoms produced by *P. ramorum*; b. brown necrotic areas on the leaves; c, d. withered and curved shoots; e. brown streaks on the cambium.

Fig. 1. Sintomi causati da *Phytophthora ramorum* su *Viburnum tinus*: a. pianta di *Viburnum* con tipici sintomi determinati da *P. ramorum*; b. aree necrotiche fogliari; c,d. germogli appassiti e ricurvi; e. striature marroni a livello del cambio.

After the diagnosis made by the laboratory of the Sezione di Patologia vegetale ed Entomologia of the Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente (DISPAA) at the University of

Firenze, the Plant Protection Service (SFR) of the Tuscan Region was immediately alerted to enforce the regulations provided for under EU Decision 2516 of 26.06.2013, to contain the disease and eliminate the pathogen (RIZZO et al. 2013).

The main aim of this collaboration between the University of Firenze and the SFR was initially to prevent the further spread of *P. ramorum* beyond the nursery where it was first found. If the pathogen were to make the jump from the nursery to the natural environment outside, there would be a risk of its causing an epidemic that could no longer be controlled. In that case the disease would seriously impair the landscape and the ecology of the Italian flora, as well as have an adverse economic effect on the economy of the Province of Pistoia, which is largely based on the trade in nursery plants, and it would reduce both the yield of its forests, and its attractiveness as a tourist resort. Furthermore the roots of trees have an important role in stabilising mountain slopes; the death of hundreds of trees would destabilise these slopes, with negative effects on human settlements on this densely populated territory.

For all these reasons tests were carried out to determine some of the biological and epidemiological aspects of the *P. ramorum* isolated at Pescia, and to find the best way to prevent or if possible cure the disease it causes.

Materials and Methods

Sampling

Nurseries were sampled at Pescia (Lat. 43°53'52"80 N., Long. 10°41'29"04 E) in the Province of Pistoia from January 2013 to June 2014. Three nursery companies were monitored and samples were taken from 15 viburnum plants presenting the typical symptoms produced by *P. ramorum*. The presence of the pathogen was confirmed *in situ* by a lateral flow diagnostic kit (Pocket Diagnostic, York, UK). From each plant testing positive, five or more portions of xylem tissue were removed from the collar, at the interface between the advancing infection and healthy tissue. Soil samples (200 g) including root portions were also removed from the soil around infected plants. All samples were placed in thick polyethylene bags on which were noted the nursery data, batch data (plant species provenance, type of cultivation, plant age), and the date. The bags were transferred to the laboratory and stored at 5° C.

In watercourses adjacent to the nurseries immersed apple baits were used to capture the pathogen, as described below (Section "Isolation from watercourses").

Isolation

Due to difficulties in isolating *Phytophthora* spp. from the trees (leaves, stems, roots), soil or water (*in vitro* they tend to be much less competitive than fungi and bacteria) various isolation procedures have been attempted.

Isolation from tissue samples

Isolation from infected tissue (roots, stems, twigs and leaves) was carried out directly on a *Phytophthora*-specific V8-agar medium enriched with PARPNH (TSAO, 1983). The V8-agar medium itself consisted of 100 ml V8, 900 ml deionised water, 3 g/l CaCO₃, and 16 g/l agar, and was sterilised at 120°C for 15 min. To this medium was added a mix of antibiotics and fungicides: 20 mg/l pimaricin, 400 mg/l ampicillin, 20 mg/l rifampicin, 50 mg/l pentachloronitrobenzene (PCNB), 50 mg/l nystatin and 100 mg/l hymexazol. These ingredients were added after the sterilised V8-agar medium had cooled down to 45°C so that they would not be deactivated by the excessive heat of the medium.

The plant samples were washed in sterile water and dried on sterile filter paper. From each sample 3-4-mm fragments were removed from near the edge of the infection and transferred to Petri dishes each containing V8-agar+PARPNH (18 ml). The samples were incubated at a constant temperature of 20°C in the dark.

Colonies were inspected daily and all colonies with a shape resembling *Phytophthora* were transferred aseptically to PDA. Mono-hyphal cultures were obtained from subsequent colonies.

Isolations from soil

To isolate *Phytophthora* from the soil, unripe Golden Delicious apple baits were used. The apple peel was disinfected with 95% ethyl alcohol, and four holes, 1 cm in diameter and 2 cm deep, were made at the four compass points along the widest circumference of each apple standing up. Each hole was filled to the top with 10g soil taken from the pots in which infected plants had grown, and the holes were moistened with some drops of deionised sterile water to induce *Phytophthora* chlamydospores to germinate. Each apple was wrapped in transparent plastic wrap, incubated at 20°C in the dark for about seven days (Fig. 2 a); then the baits were cut up and no fewer than 5 bait portions per apple were removed from the interface between necrotic and healthy tissue, transferred to the selective V8-agar+PARPNH medium, and incubated at a controlled 20°C. The bait portions were inspected daily.

Isolations from water courses

Apple baits were placed in the water courses near the nursery farms in the Pistoia area to test the water for presence of *Phytophthora*.

The apples were cut in half, enclosed in a plastic net and immersed in the water (Fig. 2 b). After seven days the baits (apple halves) were transferred to the laboratory where the isolations were carried out on the specific V8-agar+PARPNH medium, as above described in section 2.2 above.

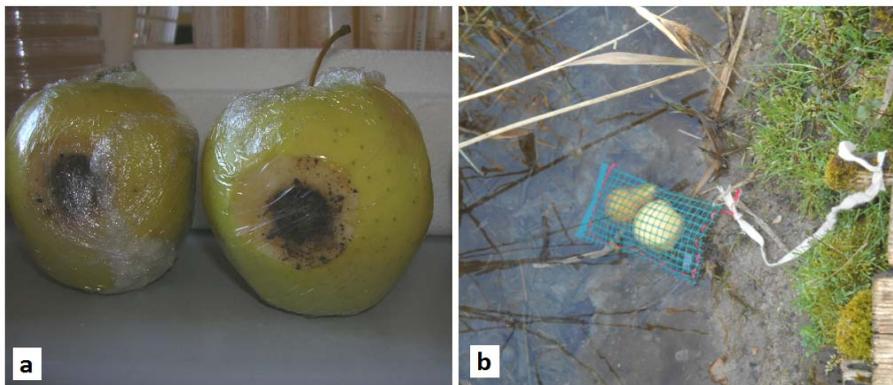


Fig. 2. Methods to trap *Phytophthora* sp.: a. apples inoculated with infected soil; b. apple baits immersed in water.

Fig. 2. Metodi per “catturare” *Phytophthora* sp.: a. mele inoculate con terreno infetto; b. mele “esca” immerse in acqua.

Identification

Phytophthora ramorum was identified by the shape and appearance of its colonies that grew on a variety of media: carrot agar (CA); corn meal agar (CMA), malt extract agar (MEA), potato dextrose agar (PDA), and V8-agar+PARPNH, and by the shape, appearance and biometric characteristics of the asexual reproductive structures (sporangia) and the chlamydospores, both of which *P. ramorum* produces in abundance. To induce sporangia production, 0.5-diam. plugs of mycelium from 7-day-old mother colonies were placed in Petri dishes containing filtered pond water, which was changed every 12 hours. The Petri dishes were incubated at 20°C for 24-36 hours, after which no fewer than 60 mature sporangia per isolate were examined and measured at 400x magnification under an optical microscope (ZEISS, Germany). The chlamydospores were examined and measured in the same way (400x magnification) by removing 1-cm-square plugs of mycelium from the margin of ca. 5-day-old colonies.

To identify *P. ramorum* at a molecular level, fresh isolate mycelium was collected with a sterile scalpel from seven-day-old colonies grown on PDA, and transferred to sterile 1.5-ml Eppendorf flasks. The samples were

placed in a freezer at -20°C until the mycelium was frozen. The mycelium DNA was then extracted with a GenElute plant genomic DNA miniprep kit (Sigma Aldrich), following the kit's instruction manual, and stored in the freezer at -20°C . The ITS region of the ribosomal DNA was amplified using the specific primers Ph1/Ph4.

Lastly, in collaboration with Prof. Sabine Werres at the Julius Kühn Institute (JKI), Federal Research Center for Cultivated Plants, Institute for Plant Protection in Horticulture and Forest (GF), Braunschweig, Germany, the mating type of the isolates found in the Province of Pistoia was determined.

***In vitro* growth tests**

The sequenced *P. ramorum* isolates SB05a and SB05b (GenBank accession no. KF181162 and KF181163) were grown on V8-agar+PARPNH for five days at 10° , 15° , 20° , 25° , 30° , 32° and 35°C to determine the optimum and the maximum growth temperatures. Radial growth was measured daily along two lines intersecting the centre of the inoculum at a right angle. Subsequently the average daily growth (mm/d) was calculated (HALL, 1993).

Pathogenicity test

The sequenced *P. ramorum* isolates SB05a and SB05b were used to test the pathogenicity of the microorganism. The isolates were grown on V8-agar+PARPNH for 4-7 days at 20°C in the dark. Portions of mycelium (5 mm diam.) were taken from the margin of actively growing colonies and used as inoculum.

The pathogenicity of *P. ramorum* was determined not only on the leaves and twigs of *V. tinus*, but also on twigs of *Fagus sylvatica* L. The latter tree is commonly used in pathogenicity tests in view of its long co-evolution with the genus *Phytophthora*.

The plant material for this test was obtained from the Centro Sperimentale per il Vivaismo (CeSpeVi, Experimental Nursery Centre) in Pistoia. Current-year twigs of *F. sylvatica* and *V. tinus* (ca. 1 cm diam.) were collected in April 2013, at the start of the growing season. The twigs were stripped of their leaves, cut to a length of ca. 12 cm, and washed in deionised water. A sliver of bark was lifted up from the cambium with a sterile scalpel and in an aseptic environment, and a plug of inoculum was placed between the bark and the cambium, after which the bark was replaced as before (Fig. 3 a), The wound was wrapped in cotton wetted with sterile water, and protected with parafilm and silver foil (Fig. 3 b). The twigs were then placed in sterilised 15-cm-diam. glass Petri dishes each containing two sheets of filter paper soaked in sterile water (Fig. 3 c). Ten twigs per isolate per species were tested; ten twigs per species inoculated with V8-agar+PARPNH but without *P. ramorum* were used as controls. The Petri dishes were incubated at 20°C for three weeks in the dark. At the end of this period the length of the lesions caused by *Phytophthora* was measured. To satisfy Koch's postulates necrotic material from the margin of actively growing cankers was randomly isolated and grown on V8-agar-PARPNH.

For the pathogenicity tests on the viburnum leaves a cut was made on the lower blade of a *V. tinus* leaf with a sterile scalpel and a 0.5-cm-diam. mycelium plug was placed in each cut. Twelve viburnum leaves per isolate were inoculated. Inoculated leaves were placed in glass Petri dishes in a wet chamber and kept at 20°C in the dark. After six days any lesions that appeared on the leaves were photographed and measured. Isolations were carried out on the V8-agar+PARPNH medium to satisfy Koch's postulates.

Results

Isolates

Sampling yielded 95 isolates of *P. ramorum*, of which 32 were from the cambium, 52 from the leaves, and 11 from the shoots of *V. tinus* plants. *P. ramorum* was not isolated from nursery soil or from any of the apple baits in the water courses.



Fig. 3. Example of pathogenicity tests on *Fagus sylvatica* twigs: a. twig with a sliver of bark raised to allow a plug of *Phytophthora ramorum* mycelium to be inserted underneath; b. twig wrapped in cotton moistened with sterile water; c. twigs placed in 15-cm-diam. glass Petri dishes.

Fig. 3. Test di patogenicità su rametti di *Fagus sylvatica*: a. rametto con corteccia sollevata per permettere l'inserimento di un tassello di micelio di *Phytophthora ramorum*; b. rametto avvolto da cotone inumidito con acqua sterile; c. rametti posti in piastre Petri di 15 cm di diametro.

Colony morphology and molecular analysis

The selected *P. ramorum* isolates SB05a and SB05b were incubated for seven days at 20°C in the dark on a variety of nutrient media in order to assess the colony morphology (shape and appearance). On CA colonies were uniform and moderately cottony in appearance, on CMA growth was poor and branched, on MEA and PDA colonies were petal-shaped and colonies moderately cottony, and on V8-agar colonies were from uniform to slightly stellate, and moderately cottony (Fig.4).



Fig. 4. Colony morphology of *Phytophthora ramorum* isolates grown at 20°C for seven days on a variety of substrates (from left to right: carrot agar, corn meal agar, malt extract agar, potato dextrose agar, and V8-agar+PARPNH).

Fig. 4. Morfologia delle colonie di *Phytophthora ramorum* cresciute a 20°C per sette giorni su substrati diversi (da sinistra a destra: agar carote, agar farina di mais, agar estratto di malto, agar patate destrosio, V8-agar+PARPNH).

Isolates grown in filtered pond water produced semi-papillate and deciduous sporangia, with a rounded or conical base (Fig. 5 a, b). Sporangia were borne singly or in pairs on sporangiophore hyphae. Sixty sporangia were measured. They averaged $56.2 \pm 9.5 \times 29.3 \pm 4.3 \mu\text{m}$, with a L/B ratio of 1.92 ± 0.19 . The ostioles of the zoosporangia averaged $7.0 \pm 1.0 \mu\text{m}$. Sporangium shapes were varied: 30% ellipsoid, 28.3% lemon-shaped, 20% ovoid, 16.7% obovoid, 3.3% ampulliform, and 1.7% peanut-shaped.

The isolates examined produced large amounts of globose chlamydospores both on V8-agar and on CA (Fig. 5c). These chlamydospores were borne on both the terminal and the intercalary portions; their size averaged $54.7 \pm 8.5 \mu\text{m}$. The average size of the chlamydospores distinguishes *P. ramorum* from the closely related *P. lateralis*, since chlamydospores of *P. ramorum* are much larger than those of *P. lateralis*: 46.4–60.1 μm vs. 33.8–39.4 μm (WERRES et al., 2001). In the present study at least 50 chlamydospores were measured, and their larger size indicated that they belonged to *P. ramorum*, and not to *P. lateralis*.

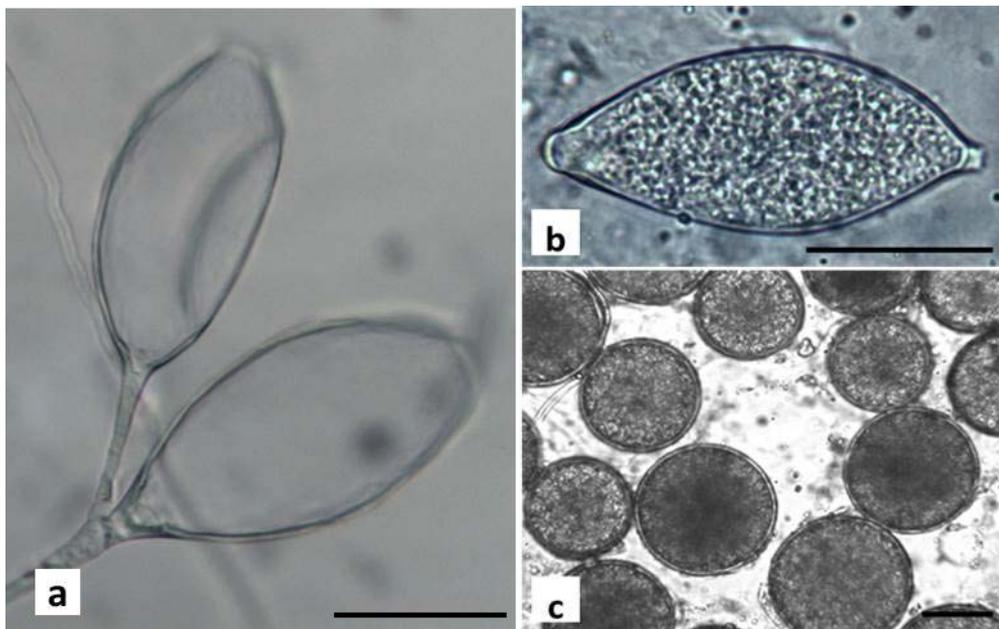


Fig. 5. a, b. Semi-papillate and deciduous *Phytophthora ramorum* sporangia borne singly or in pairs on sporangiophore hyphae with a rounded or conical base; c. abundant production of chlamydospores on V8-agar+PARPNH nutrient medium. (bar: 25 μm).

Fig. 5. a, b. Sporangii semi-papillati e decidui di *Phytophthora ramorum*, portati singolarmente o in coppia su ife sporangiofore, con base arrotondata o conica; c. abbondante produzione, su V8-agar+PARPNH, di clamidospore (bar: 25 μm).

Molecular analysis carried out in our laboratory also indicated that the isolates found were *P. ramorum*. Sequencing of the amplicons of the ITS region of ribosomal DNA yielded sequences having 100% homology with the *P. ramorum* sequences in the GenBank database.

Successful production of gametangia with mating type A2 isolates of *P. cambivora*, *P. cinnamomi*, *P. cryptogea* and *P. drechsleri* indicated that our *P. ramorum* isolates belonged to mating type A1.

***In vitro* growth tests**

Phytophthora ramorum isolates SB05a and SB05b were grown for five days in V8-agar at temperatures of 10°, 15°, 20°, 25°, 30°, 32°, and 35°C to determine the optimum and the maximum growth temperatures for this species. The results (mm/d) are shown graphically in Fig. 6.

Phytophthora ramorum grew very well at temperatures from 15° to 25°C, with an optimum growth temperature around 20°C, but it died *in vitro* when subjected to temperatures of 30°C and over.

Pathogenicity test

The pathogenicity of *P. ramorum* was determined by inoculating it on leaves and twigs of *V. tinus*, and on twigs of *F. sylvatica*. This test produced cankers that on *V. tinus* leaves averaged $2.5 \pm 0.7 \times 1.8 \pm 0.3$ cm after

six days of incubation at 20°C. On the *V. tinus* twigs *P. ramorum* caused cankers that averaged 6.4 ± 3.8 cm

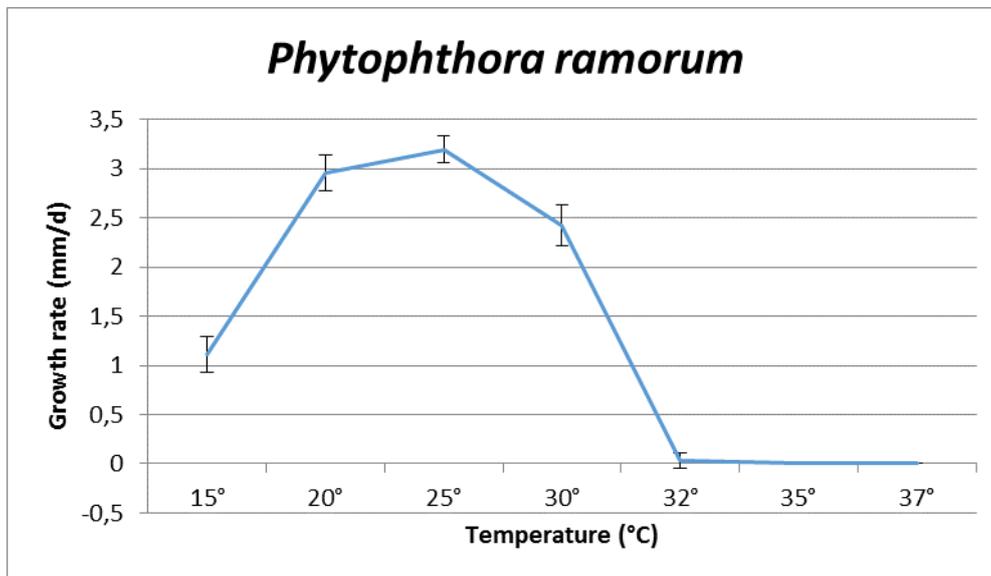


Fig.6. Daily growth rate (mm/d) of *Phytophthora ramorum* on V8-agar medium at various temperatures.
Fig. 6. Grado di crescita giornaliero (mm) di *Phytophthora ramorum* su V8-agar a temperature diverse.

after three weeks of incubation at 20°C in the dark. On *F. sylvatica* twigs the canker size averaged 2.72×0.66 cm after three weeks.

In the pathogenicity tests *P. ramorum* caused significant damage to both twigs and leaves of *V. tinus* (Fig. 7), the plant from which it was isolated, causing lesions on almost the whole leaf surface in only a few days, and cankers on the twigs of *F. sylvatica* (Fig. 8) (GINETTI et al., 2014).



Fig. 7. *Viburnum tinus* twigs (a) and leaves (b, c) inoculated with *Phytophthora ramorum*.
Fig. 7. Rametti (a) e foglie (b,c) di *Viburnum tinus* inoculati con *Phytophthora ramorum*.



Fig. 8. *Fagus sylvatica* twigs inoculated with *Phytophthora ramorum*.

Fig. 8. Rametti di *Fagus sylvatica* inoculati con *Phytophthora ramorum*.

Discussion

Phytophthora ramorum is an important quarantine pathogen that has caused enormous damage to natural ecosystems in various parts of the world, especially North America and northern Europe (UK) (BRASIER, 2003; HANSEN et al., 2003; WERRES & MERLIER, 2003). For this reason it must be controlled and if possible eradicated in accordance with the regulations laid down by the European Commission, which enforces a number of preventive measures to be taken in connection with the introduction, transport and certification of plants and plant materials susceptible to *P. ramorum*. It also requires the operators of nurseries growing species such as azalea, rhododendron and viburnum to be vigilant and destroy all such plants found to be infected with *P. ramorum*, as well as any susceptible plants growing in the vicinity of infected plants.

In the important nursery centre of the Pistoia area in which the pathogen was found, measures to eradicate the pathogen were immediately taken. The swiftness of the response was due to the close collaboration that exists between the Sezione di Patologia Vegetale ed Entomologia of the DISPAA and the Plant Protection Service of the Tuscan Regional Government. These bodies carried out further investigations on other samples and nurseries, applying standardised international protocols to identify the organism. After the occurrence of the pathogen was confirmed, the quarantine measures imposed by the EU were taken. Infected plant lots were marked with a ministerial seal, and in the immediate vicinity of the locus of infection a safety or buffer perimeter was set up with a width of 20 m, within which any plants susceptible to the pathogen were to be inspected for *P. ramorum* infection. The first detection of *P. ramorum* was on a batch of about 700 *V. tinus* plants growing in 30-cm-diam. pots. Infected plants and all plants thought to be at risk of infection were covered with polyethylene sheeting (Fig. 9 a) to prevent the further spread of *P. ramorum*, (Fig. 9 b). *Viburnum* plants with the soil shaken from their roots (Fig. 9 c) were dipped in 5% sodium hypochlorite for 12 h for a first disinfection, were then arranged on the ground on polyethylene sheet to dry (Fig. 9 d) and then burnt (Fig. 9 e). The soil was soaked in sodium hypochlorite and calcium hydroxide to kill the pathogen and its chlamydospores. This disinfected material was placed in baskets and taken to a protected refuse disposal site. The pots in which the infected plants had grown were thoroughly washed with sodium hypochlorite. The infected terrain was decontaminated by covering it with a thick layer (at least 1 kg/m²) of calcium hydroxide (Fig. 9 f).

The detection of *P. ramorum* in this nursery raised a number of questions: how the pathogen got there in the first place; how it managed to spread within the nursery; and whether it still posed a hazard to the natural environment outside.

The fact that *P. ramorum*, following the early report of Gullino et al. (2003), was later found in several nurseries in the Pistoia area means that the norms and regulations of the European commission had in effect failed, enabling the repeated introduction of the pathogen into Italy. Pistoia is an important centre for the trade in ornamental nursery plant material worldwide, and this explains why the pathogen was detected here rather than elsewhere. It is difficult to inspect great numbers of nursery plant lots especially if these checks are not carried out by qualified personnel fully trained to detect the pathogen. It should also be pointed out however that the pathogen can occur in nursery seedlings before any visible symptoms yet appear, and that particularly in the nursery the occurrence of pathogens such as *P. ramorum* is often masked by the use of fungicides, which keep it in a latent state for a time.

The spread of *P. ramorum* within individual nurseries, and the fact that where the disease occurred infected plants of the same batch were randomly interspersed with healthy plants, suggest that the infection spreads through the aerial parts of the plants and not through wounds on the leaves or the trunk. This supposition seems to be confirmed by the lack of any sign of the pathogen in the soil or in the roots. Infected plants produced copious amounts of sporangia from the leaf lesions, and these sporangia represented a massive

source of inoculum whereby the pathogen was spread both within the nursery and to adjoining areas. If this is so, the hazard posed by the pathogen is increased still more if we consider that it already is very polyphagous itself



Fig. 9. Measures to eliminate *Phytophthora ramorum*: a. infected *Viburnum tinus* plants covered with thick polyethylene sheeting; b. a batch of *V. tinus* plants infected with *P. ramorum*; c. *V. tinus* plants with the soil shaken from their roots ready to be treated with 5% sodium hypochlorite; d. plants left to dry for two days after being treated with sodium hypochlorite, before being burnt; e. infected *V. tinus* plants being burnt; f. area where the infection occurred being decontaminated with calcium hydroxide.

Fig. 9. Misure per eliminare *Phytophthora ramorum*: a. piante infette di *Viburnum tinus* coperte con polietilene; b. lotto di piante di *V. tinus* infettate da *P. ramorum*; c. piante di *V. tinus* con radici private dal terreno e pronte per essere trattate con ipoclorito di sodio al 5%; d. piante lasciate ad asciugare per due giorni dopo il trattamento, alle radici, con ipoclorito di sodio, prima di essere bruciate; e. piante infette di *V. tinus* che stanno bruciando; f. area dove si è presentata l'infezione decontaminata con idrossido di calcio.

and has a rapid spread. In the *P. ramorum* outbreak under study it was hypothesised that the spread of the pathogen was mainly caused by rain water and by watering of the crown, and in fact, the nursery plants on which the pathogen was first detected had been so watered.

If the pathogen has indeed spread out from the circumscribed place where it was initially found, to the wider area where many nurseries operate, it would have serious economic and ecological consequences and negative implications for tourism. Pistoia is located near the Appennines, a region of hills and mountains covered with extensive high-trunk forests consisting of hornbeam, Turkey oak, spruce, fir and beech, some of which are hosts, or potential hosts for *P. ramorum*. The hills around Pistoia are particularly important as a tourist resort, in both summer and winter, as the popular ski-slopes of Abetone (near Pistoia) are to be found here.

The pathogenicity tests carried out at the DISPAA indicate that the *P. ramorum* samples isolated from *V. tinus* in the laboratory also infect beech, which is one of the most common trees in the Pistoia Appennines. When the pathogen was inoculated on beech twigs and incubated for a few weeks in the dark, three-cm-diam. cankers were produced. In other contexts *P. ramorum* was found to infect other tree species (BRASIER et al., 2004b; BROWN & BRASIER, 2007; WEBBER et al., 2010). It would be interesting for future research to test *P. ramorum* on these tree species that grow also in the Pistoia area. The nursery trade in plants is the most important economic activity in the Pistoia area, with a market that has international dimensions, so much so that the town has been defined as the green capital of Europe. This trade has a vital role in the economy and in the landscape of the Province of Pistoia. The nursery trade is so large that it is important not only for the Province of Pistoia, but for Tuscany as a whole. For all these reasons it is essential to monitor

the territory around Pistoia, and in particular the nurseries, for any organisms that could hurt this trade. All potentially harmful organisms found should be promptly identified and controlled with the proper protective measures.

In this context, the genus *Phytophthora* assumes a vital importance on account of the danger posed by *P. ramorum* and its other members. It is therefore important to study the epidemiology of this pathogen closely, to prevent its spread, and, if it does so spread, to limit the damage it causes. The growth tests at various temperatures carried out in this study indicate that the growth temperature of *P. ramorum* ranges from 15° to 25°C. This range effectively corresponds to the average seasonal temperatures of March to April during which the isolates in our study were collected. Clearly the pathogen grows and produces symptoms on *V. tinus* at the temperatures tested. More growth tests will however be needed to determine more precisely both the minimum growth temperature and the optimal growth temperature, and the temperature threshold at which the pathogen is killed.

The *P. ramorum* isolates found at the nursery in Pescia probably arrived in a batch of asymptomatic but already infected plants, and it could jeopardise the flourishing and profitable trade in nursery plants. For this reason the SFR was promptly alerted and the infected plants were destroyed.

Thanks to the collaboration and the financial support offered by the Fondazione Cassa di Risparmio di Pistoia e Pescia to the Commune of Pescia and to the University of Firenze, further laboratory tests are now under way to study the pathogen more deeply and to find out more about its biological and epidemiological characteristics, and thus to develop better ways to manage nurseries, and, even more important, natural forests. This necessarily aims at the prevention of pathogens such as *P. ramorum*, but unfortunately sometimes must be a matter of their control, after they have already begun to spread.

References

- BRASIER C. (2003). Sudden oak death: *Phytophthora ramorum* exhibits transatlantic differences. *Mycological Research*, 107, 03, 257–259.
- BRASIER C.M., DENMAN S., ROSE J., KIRK S.A., HUGHES K.J.D., GRIFFIN R.L., LANE C.R., INMAN A.J., WEBBER J.F. (2004a). First report of ramorum bleeding canker on *Quercus falcata*, caused by *Phytophthora ramorum*. *Plant Pathology* 53, 804.
- BRASIER C.M., DENMAN S., BROWN A., WEBBER J.F. (2004b). Sudden oak death (*Phytophthora ramorum*) discovered on trees in Europe. *Mycological Research* 108, 1107–1110.
- BRASIER C.M., BEALES P.A., KIRK S.A., BARTON V.C., GILTRAP P., GRIFFIN R.L., HUGHES K.J.D., LAND C.R., ROSE J., DENMAN S., WEBBER J.F. (2004c). A new *Phytophthora* species affecting European beech (*Fagus sylvatica*) and rhododendron in the UK. COMTF poster, Sonoma, California, USA 9-10th Marzo 2004.
- BROWN A.V., BRASIER C.M. (2007). Colonization of tree xylem by *Phytophthora ramorum*, *P. kernoviae* and other *Phytophthora* species. *Plant Pathology* 56, 227–241.
- DENMAN S., KIRK S.A., BRASIER C.M., WEBBER J.F., HUGHES K.J.D., GRIFFIN R., HOBDON E. (2005). Foliar infection of sweet chestnut (*Castanea sativa*) by *Phytophthora ramorum* in the UK. *Plant Pathology* 54, 581.
- GINETTI B., CARMIGNANI S., RAGAZZI A., WERRES S., MORICCA S. (2014). Foliar blight and shoot dieback caused by *Phytophthora ramorum* on *Viburnum tinus* in the Pistoia area, Tuscany, central Italy. *Plant Disease* 98, 3, 423.
- GULLINO C., GAROFALO M.C., MORETTI F., GIANETTI G., MAINENTI E. (2003). Rinvenimento su rododendro di *Phytophthora ramorum*. *L'Informatore Agrario* 19, 87-89.
- HALL G. (1993). An integrated approach to the analysis of variation in *Phytophthora nicotianae* and a redescription of the species. *Mycological Research* 97, 559-574.
- HANSEN E. M., REESER P. W., SUTTON W., WINTON L. M., OSTERBAUER, N. (2003). First report of A1 mating type of *Phytophthora ramorum* in North America. *Plant Disease*, 87, 10, 1267.
- MALONEY P.E., LYNCH S., KANE S., RIZZO D.M. (2004). A comparison of infection by *Phytophthora ramorum* and *Botryosphaeria dothidea* on Pacific madrone. *Plant Disease*. 88, 852-857.

- RIZZO D.M., GARBELOTTO M., DAVIDSON J.M., SLAUGHTER G.W., KOIKE S.T. (2002). *Phytophthora ramorum* as the cause of extensive mortality of *Quercus* spp. and *Lithocarpus densiflorus* in California. *Plant Disease* 86, 205-214.
- RIZZO D., GILLI G., STEFANI L., PAOLI M., VANARELLI S. (2013). *Phytophthora ramorum*. Il vivaistaguardo trimestrale sul verde pistoiese-Estate 2013, 24-25.
- TSAO P.H. (1983). Factors affecting isolation and quantification of *Phytophthora* from soil. In *Phytophthora: Its Biology, Taxonomy, Ecology, and Pathology*. (Erwin D.C., Bartnicki-Garcia S., Tsao P.H., eds.) APS Press, The American Phytopathological Society, St. Paul, Minnesota, USA, 219-236.
- WEBBER J.F. , MULLETT M., BRASIER C.M. (2010). Dieback and mortality of plantation Japanese larch (*Larix kaempferi*) associated with infection by *Phytophthora ramorum*. *New Disease Reports* 22, 19.
- WERRES S., MARWITZ R., MAN IN'T VELD W.A., DECOCK A.W.A.M., BONANTS P.J.M., DE WEERDT M., THEMANN K., ILIEVA E., BAAYEN R.P. (2001). *Phytophthora ramorum* sp. nov., a new pathogen on *Rhododendron* and *Viburnum*. *Mycological Research* 105, 1155–1165.
- WERRES S., DE MERLIER D. (2003). First detection of *Phytophthora ramorum* mating type A2 in Europe. *Plant Disease*, 87, 10, 1266.