



HAL
open science

Assessment of inoculation methods for screening black alder resistance to *Phytophthora xalni*

A. Chandelier, Claude Husson, P. Druart, Benoit Marçais

► **To cite this version:**

A. Chandelier, Claude Husson, P. Druart, Benoit Marçais. Assessment of inoculation methods for screening black alder resistance to *Phytophthora xalni*. *Plant Pathology*, 2016, 65 (3), pp.441-450. 10.1111/ppa.12418 . hal-01312106

HAL Id: hal-01312106

<https://hal.science/hal-01312106>

Submitted on 27 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Assessment of inoculation methods for screening black alder resistance to *Phytophthora* × *alni*

A. Chandelier^{a*}, C. Husson^{bc}, P. Druart^a and B. Marçais^{bc}

^aLife Sciences Department, Walloon Agricultural Research Centre, Gembloux B-5030, Belgium; ^bUMR1136 Interactions Arbres-Microorganismes, INRA, Champenoux F-54280; and ^cUMR1136 Interactions Arbres-Microorganismes, Université de Lorraine, Vandoeuvre-les-Nancy F-54500, France

Identification of resistance to *Phytophthora* × *alni* could provide the basis for a management strategy against alder decline in riparian ecosystems in Europe. This study aimed to test methods to evaluate the resistance of riparian alders to the disease, and to screen alder genotypes for resistance. *Phytophthora* × *alni* isolates were compared for their aggressiveness (lesion length on stem) and sporulation capacity (sporangia). While no difference in lesion lengths was found between isolates, sporangia production was dependent on isolate, highlighting the need for careful selection of isolates used for zoospore inoculation methods. Inoculation tests carried out at different periods of the year revealed a seasonal change in susceptibility to the disease, with the period from June to September being the most efficient for inoculation tests. Stem-wounded inoculations, carried out on excised shoots, were unreliable for evaluating the level of resistance of alder genotypes to *P.* × *alni* infection, with divergent results between two successive years or between two inoculation periods during the same year. In contrast, a method that mimics the natural conditions of infection, based on flooding of rooted cuttings in artificially infected river water, was found promising. Another method, based on the inoculation of foliated terminal shoots with zoospore suspensions, was found to be repeatable and could be used for high-throughput analyses. Altogether, the results show a continuous resistance response from highly susceptible to moderately resistant genotypes. This suggests that breeding might be a useful strategy to manage alder decline caused by *P.* × *alni*.

Keywords: alder, flooding, inoculation method, *Phytophthora alni*, resistance

Introduction

The oomycete *Phytophthora alni* is a soilborne pathogen infecting alders in Europe, mostly in riparian ecosystems. Black (or common) alder (*Alnus glutinosa*), grey alder (*Alnus incana*) and Italian alder (*Alnus cordata*) are susceptible to the disease (Gibbs *et al.*, 1999). Infected trees exhibit tarry spots on the lower bole, with extensive necrosis in the inner bark (Gibbs, 1995). The soilborne pathogen affects fine and major roots, leading to abnormally small, sparse and yellowish foliage and dieback of the crown. Every class and age of tree can be infected, although young seedlings are affected more strongly (Jung & Blaschke, 2004; Elegbede *et al.*, 2010). A study carried out in Germany demonstrated that the pathogen was first introduced along watercourses via infected young alder plantations. Subsequent spread of the disease along rivers resulted mainly from direct infection of the collar during flooding (via the lenticels) or infection of the fine roots by motile zoospores released from infected trees into river water (Jung & Blaschke, 2004).

This emerging lethal disease has been a major threat to alder populations in Europe since its first report in the

UK in 1993 (Brasier *et al.*, 1995; Streito *et al.*, 2002; Jung & Blaschke, 2004) and it is still continuing to widen its distribution area (Solla *et al.*, 2010). The death of alders along rivers, in particular *A. glutinosa*, jeopardizes riverbank stability, has a negative impact on the riparian ecosystem by modifying the local environmental conditions, and makes the management of riverbanks difficult (Claessens, 2005).

Alder decline in Europe is due to a *P. alni* species complex composed of *P.* × *alni*, *P. uniformis* and *P.* × *multiformis* (Brasier *et al.*, 2004). *Phytophthora* × *alni* arose through interspecific hybridization between *P. uniformis* and *P.* × *multiformis* (Ioos *et al.*, 2006, 2007). It is an allotriploid species containing half of the genome of both *P.* × *multiformis* (allotetraploid) and *P. uniformis* (diploid) (Husson *et al.*, 2015). *Phytophthora uniformis* has been isolated in alder riparian ecosystems in Alaska and Oregon and genetic studies demonstrated that it is very probably native to North America (Adams *et al.*, 2008; Aguayo *et al.*, 2013). *Phytophthora* × *multiformis* has so far only been found in Europe, and its origin is still unknown. Although the three species are pathogenic to alders, *P.* × *alni* is the most aggressive (Brasier & Kirk, 2001). Moreover, surveys carried out in different countries have shown that

*E-mail: a.chandelier@cra.wallonie.be

P. ×alni is the most frequently recovered and is responsible for the bulk of the disease outbreaks, whereas both *P. uniformis* and *P. ×multiformis* are comparatively rare (Nagy *et al.*, 2003; Santini *et al.*, 2003; De Merlier *et al.*, 2005; Thoirain *et al.*, 2007; Aguayo *et al.*, 2013).

Few control measures are available for management of this disease. Coppicing has proven to be partly effective for disease control over a short-term period (Gibbs, 2003). However, coppicing may be inefficient in the long term because it can be applied only to trees with symptoms, and the strongest producers of inoculum are infected trees that do not yet show symptoms such as canker or crown decline (Elegbede *et al.*, 2010). In these conditions, stems that resprout from roots that are still alive will probably be reinfected from continuing spore production by surrounding infected trees. Planting resistant alders along watercourses could be a more durable option to manage the disease. The presence of symptomless alders in close proximity to declining trees in highly infested riparian stands has been reported, notably in Germany (Jung & Blaschke, 2004) and France (Streito *et al.*, 2002), which could suggest presence of resistance in black alder.

Alnus glutinosa is the most widespread *Alnus* species in Europe. It is a monoecious tree, adapted to allogamy. The species can colonize large areas via seeds dispersed over long distances by watercourses (Claessens, 2005). Molecular markers have recently been used to characterize the genetic diversity of this species in Europe (Mingeot *et al.*, 2010, 2013; De Kort *et al.*, 2014). Both studies highlighted a high allelic diversity but a very low level of genetic differentiation among populations of *A. glutinosa* from Belgium, France, Denmark and Italy. These results are promising for the screening of resistant material usable at the European level.

Stem-wound inoculations have commonly been used to assess the susceptibility of woody plants to *Phytophthora* infections (Brasier & Kirk, 2001; Santini *et al.*, 2003; Hansen *et al.*, 2005; Robin *et al.*, 2006; Jung & Nechwatal, 2008). This method consists of wounding the stem bark to the cambium and inserting a mycelial plug into the wound. The level of susceptibility is inferred from the lesion length induced by the pathogen (or induced lesion length). Although this method is widely used, its reliability might be called into question for soilborne *Phytophthora* species such as *P. alni*, as it does not accurately mimic the infection process through the roots, where infection most frequently occurs.

The present study aimed to provide an efficient tool for the identification of the most promising alder genotypes for long-term trials in natural settings along watercourses. First evaluated was the influence of the season and the *P. ×alni* isolates on inoculation efficiency and susceptibility of alders to infection. Then, inoculation methods were compared using mycelium and/or zoospore suspension on excised shoots or entire plants. In order to compare inoculation techniques with minimal host genotype effects, alder progenies and clones produced by vegetative cuttings were used.

Materials and methods

Tree orchards

Seed orchard

Seedlings were produced from seeds collected from 35 symptomless trees (32 *A. glutinosa* and three *A. incana*) located close to declining alders along 11 rivers in Wallonia. The progeny of each mother tree consisted of two to five trees (open pollinated families) depending on the genotype. They were transferred to Orchard 1 in 2005.

Clonal orchards

Alder stems were collected in spring in Wallonia (Belgium, along 34 watercourses) and in Lorraine (France, along one watercourse). The sampled alders were selected without consideration of their behaviour towards *P. ×alni*. Rooted cuttings were produced from stem sections as described by Druart *et al.* (2013). Altogether, 336 genotypes from Belgium, each one represented by four ramets, were planted in Orchard 2 (Gembloux) in 2006 while 79 genotypes from France, each one represented by two to five ramets, were planted in Orchard 3 (Roville-aux-Chênes) in 2006 and 2007.

Collection of *Phytophthora ×alni* isolates

The *P. ×alni* isolates used in this study are listed in Table 1. They were isolated from inner bark of infected black alders on PARP selective medium (Jeffers & Martin, 1986) and stored on V8 agar plugs in vials of sterile water at 13°C. Each isolate was identified to species based on PCR tests (Ioos *et al.*, 2005; Husson *et al.*, 2015). Before each inoculation assay, the isolates were isolated from inoculated alder stem to ensure that they retained full pathogenicity. Working cultures were maintained on V8 agar at 20–22°C in the dark until use. Not all isolates described in Table 1 were used in all the inoculation tests carried out.

Seasonal influence on alder receptivity to *P. ×alni* (test 1)

Three different types of plant material were used to evaluate the susceptibility of alders to infection at different periods of the year. All inoculations for this test were carried out with the *P. ×alni* isolate 2295 (Table 1).

Excised shoots

In 2006, shoot sections were collected from ten 8-year-old *A. glutinosa*, of unknown origin, from a hedge and were inoculated in the laboratory. Three shoots were sampled from each tree at five different periods of the year (February, April, June, August and October). Stem sections (length 10–15 cm and diameter 0.5–1 cm) were cut from the trees 24 h before the experiment and were stored at 2–8°C after having removed the leaves. A c. 40 mm² wound, reaching the stem cambium, was made at the centre of the section. A mycelial plug (6 mm diameter) from the margin of a 4- or 5-day-old *P. ×alni* culture was inserted into the wound and the inoculated area was sealed with Parafilm. Control inoculations were carried out on an additional shoot section for each genotype by replacing the mycelial plugs of *P. ×alni* by uncolonized V8 plugs. Hereafter, this method will be referred to as the wound-inoculation method. The four sections (three inoculated with *P. ×alni* and one control) were

Table 1 List of isolates of *Phytophthora ×alni* used in this study

Isolate	Origin	Isolation year	Pathogenicity ^a		Sporulation ^b (mean ± SD)
			Mean ± SD	Max.	
2295	Belgium, Wellin	2001	16.3 ± 2.6	20	8.5 ± 0.6
3534	Belgium, Seneffe	2006	15.4 ± 3.2	20	6.9 ± 0.4
3539	Belgium, Spa	2006	14.8 ± 3.6	18	1.7 ± 0.7
3542	Belgium, Bizet	2006	14.9 ± 3.6	25.3	6.1 ± 0.7
3544	Belgium, Saint-Léger	2006	16.2 ± 2.7	20.5	4.5 ± 0.8
PAA325	France, Dossenheim-sur-Zinsel	2008	17.4 ± 0.4	20.6	–
PAA301	France, Baerendorf	2008	–	–	–
PAA314	France, Gorhey	2008	15.2 ± 0.3	18.1	–
PAA315	France, Allarmont	2008	16.5 ± 0.3	20	–
PAA115	France, Gosselming	2008	16.1 ± 0.4	20.1	–

^aLesion length (in mm day⁻¹) on stem sections. The isolates from France and from Belgium were tested in separate experiments and their pathogenicity cannot be compared from these data.

^bProduction of sporangia on mycelial plugs according to a 0–10 scale (30 replicates).

put into a plastic box on a moist paper towel and incubated in a growth chamber at 20–22°C. The length of the resultant lesions was measured 4 days post-inoculation, after removing the periderm to expose the phloem.

Shoot inoculation in situ

In 2006, an inoculation test was performed in the field on five trees of Orchard 1 from different open pollinated families. Trees were selected distant from a river to avoid any contamination due to flooding. Two stems per tree were wound inoculated, as described above. Observations were carried out after 2 weeks. Maximum daily air temperature during the inoculation test was recorded.

Seedling inoculation in greenhouse

In 2011, inoculation tests were conducted on 2-year-old seedlings produced from *A. glutinosa* seeds purchased at the Comptoir à Graines (Marche-en-Famennes, Belgium). Three seedlings were inoculated each month, from April to December. Wound inoculations were carried out on the main stem at approximately 5–10 cm above ground level as described before. The inoculated plants were kept in a greenhouse (20–22°C, ambient daylight). Observations were made after a 2-week incubation period. Because seedlings were girdled in periods of high susceptibility, lesion severity was not scored by measuring lesion length but according to a 0–5 level scale (0 = no necrosis, 1 = necrosis length <5 cm, 2 = necrosis length between 5 and 10 cm, 3 = necrosis length >10 cm or girdling, 4 = dead plant).

Comparison of *P. ×alni* isolates for inoculation trials (test 2)

Pathogenicity on excised shoots

Excised shoots collected from 1-year-old branches of 79 alder genotypes (Orchard 3) were inoculated in June 2011 using four isolates, PAA115, PAA314, PAA315 and PAA325 (Table 1, test 2a). Two excised shoots per isolate × alder genotype combination were inoculated following the wound-inoculation method previously described, and the length of the induced lesion was

assessed 7 days post-inoculation. Diameter of each shoot, measured the day of inoculation, was used as a covariate in the analysis. In another test in June 2006, excised shoots from three 8-year-old alders were wound inoculated, as described above, with five isolates of *P. ×alni* collected at different sites in Belgium (2295, 3435, 3539, 3542, 3544); lesion length was assessed 4 days post-inoculation (five replicates each isolate × alder tree combination; Table 1, test 2b). The stability of the pathogenicity of isolate 2295 over time was then assessed by carrying out inoculations in August 2009 and August 2010 on four excised shoots from the same tree using the wound-inoculation method.

Evaluation of sporulation capacity

Thirty mycelial plugs (6 mm diameter) from the growing margin of *P. ×alni* isolates 2295, 3534, 3539, 3542 and 3544 (Table 1) were put in Petri dishes containing river water and incubated at 20–22°C in the dark. After an incubation period of 24 h, the abundance of sporangia on each plug was estimated using a stereomicroscope and scored according to a 0–10 level scale (0 = no sporangia, 1 = 1–5 sporangia, 2 = 6–10 sporangia, 3 = 11–30 sporangia, 4 = 31–50 sporangia, 5 = 51–70 sporangia, 6 = 71–100 sporangia, 7 = 101–150 sporangia, 8 = 151–200 sporangia, 9 = 201–300, 10 = >300 sporangia).

Assessment of the wound-inoculation method (test 3)

The repeatability of the wound-inoculation method was assessed in two tests carried out on excised shoots and entire plants using plant material from Orchard 1 and 2. In test 3a, excised shoots were collected twice in 2010, in July and September, from 20 genotypes: six from Orchard 1 (originating from six different open pollinated families) and 14 from Orchard 2 (rooted cuttings originating from 14 different mother trees). In test 3b, excised shoots were sampled from 21 genotypes from Orchard 2 in July 2010 and in August 2011. The trees were selected throughout Wallonia, representing 18 different rivers in test 3a and 16 in test 3b. Four 1-year-old shoots were sampled per genotype in both tests, either from four branches of single trees in Orchard 1 (test 3a) or from the four ramets available per

clone in Orchard 2 (test 3b). In both tests, excised shoots were then wound-inoculated in the laboratory.

A comparison between inoculations carried out on excised shoots and on entire plants in the field was also performed using 29 alders from Orchard 1 (test 3c). The trees were selected from 21 different open pollinated families and from 11 rivers of Wallonia. In August 2011, inoculations were carried out using the wound-inoculation method described previously: for each tree, four stem sections were sampled from four branches for inoculation in the laboratory and four stems were inoculated in the field. Lesion lengths were measured 4 days post-inoculation for the test carried out on stem sections in the laboratory (corresponding to the maximum period to avoid complete invasion of the phloem by the pathogen), and 18 days post-inoculation for the test carried out directly on the tree in the field. All the inoculations were performed with isolate 2295 (test 3a, 3b and 3c).

Inoculation by flooding of rooted cuttings (test 4)

Two different root inoculation methods using cloned material were compared to test the resistance of entire seedlings to *P. ×alni*. The results of both methods were then compared to the wound-inoculation test. Twenty-one alder genotypes in Orchard 2, originating from 13 different rivers of Wallonia, were used (20 *A. glutinosa* and one *A. incana*). Seedlings were kept in a greenhouse in daylight at 20–22°C. A first test took place in August 2011 with 2-year-old rooted cuttings. Two treatments were used, i.e. inoculation of either bare root or of potted seedlings, with four replicates per genotype × treatment combination. Potted seedlings were in 1 L plastic pots filled with a potting soil (peat 25%, Baltic black peat 30%, German black peat 45%, PG mix fertilizer 14-16-18 (Yara) at 1.5 kg m⁻³). Potted and bare-root seedlings were inoculated by immersion in plastic boxes containing 20 L river water and 60 agar plugs of *P. ×alni* isolate 2295 collected at the margin of a 5-day-old culture with a punch (6 mm diameter) and evenly distributed in the box. The sporulation capacity of isolate 2295 was previously evaluated on a subset of 10 mycelial plugs as explained above. Inoculation tests were performed only in the case of an average sporulation score of 8. Three days after inoculation, the water was stirred to keep the zoospores in suspension. After 7 days' incubation, potted plants were removed from water while bare root plants were potted in 1 L plastic pots filled with potting soil. Both types of plants were grown for 3 months in the greenhouse (20–22°C, ambient daylight). A negative control was introduced to each trial by immersing plants from each genotype in river water without inoculum. Resistance was then assessed as the proportion of plants that had survived. The experiment was repeated in July 2012 with the same 21 genotypes, but with 1-year-old seedlings and only bare-root inoculated plants (six replicates per genotype).

In 2011, shoots were excised from the 21 genotypes (four replicates, one for each of four ramets). They were inoculated in the laboratory with the wound-inoculation method described previously (measurement of lesion length 4 days post-inoculation). The inoculations were carried out with the isolate 2295 (Table 1).

Excised foliated terminal shoots inoculated with zoospore suspension (test 5)

A resistance assay on foliated shoots inoculated with zoospore suspension was carried out and compared to the wound-inoculation method on excised shoots. For this, 29 alder genotypes of

Orchard 3 were used. Eight excised shoots per genotype were inoculated according to the wound method described above, using four isolates (two shoots per isolate; Table 1). In 2010, shoots from that year were used, whereas in 2011, shoots were 1 year old. The length of the induced lesion was assessed 5 and 7 days post-inoculation in 2010 and 2011, respectively. In parallel, 16 cm long foliated terminal shoots were collected from the same trees in 2010 and 2011 and kept in a moist plastic bag a few hours before further processing. The tips were inoculated with zoospore suspensions as follows. Thirty mycelial plugs of *P. ×alni* were incubated 2–3 days in Petri dishes containing filtered river water (0.45 µm pore size filter) that was changed every day. As soon as sporangia were produced, plugs were placed in sterilized 4°C water. Zoospores released were counted with a haemocytometer and the suspension was calibrated to 2000 zoospores mL⁻¹. The eight excised tips per genotype were incubated in 14 mL Falcon tubes containing 3 mL of the zoospore suspension and placed in a growth chamber with a 16 h light cycle and a 20°C stable temperature. Every day, sterile water was added to the tubes to compensate for transpiration. Shoot wilting (presence/absence) was assessed 11 days after inoculation. This experiment was performed in June 2010 using isolates PAA301 and PAA325 (four tips inoculated per genotype) and, in June 2011 using PAA314.

A summary of all inoculation tests carried out is presented in Table 2.

Data analysis

One-way analysis of variance (ANOVA) was carried out to compare mean lesion length after bark inoculation on excised shoots (test 1 and 2a). As the data were not normally distributed, even after transformation, differences between *P. ×alni* isolates in terms of pathogenicity and sporulation capacity (test 2b) were analysed using the nonparametric Kruskal–Wallis test, with the lesion lengths (pathogenicity test) and sporulation scores (sporulation capacity test) used as dependent variables, respectively. Isolate was introduced as a fixed effect. Lesion lengths from

Table 2 Characteristics of the inoculation methods tested in this study

Test	Inoculation	Measurement ^a	Comparison	N ^b
1	Stem wound	Lesion length	Seasonal receptivity of alder to <i>Phytophthora ×alni</i>	10
2	Stem wound	Lesion length and amount of sporangia	Pathogenicity and sporulation capacity of <i>Phytophthora ×alni</i>	4 or 5
3	Stem wound	Lesion length	Excised shoots and entire plants over different inoculation periods	20, 21 or 29
4	Zoospore suspension	Survival rate	Bare root versus potted cuttings over 2 years	21
5	Zoospore suspension and stem wound	Wilting/lesion length	Foliated terminal shoots versus excised shoots over 2 years	29

^aLesion length (stem wound-inoculation method); survival rate (flooding with suspension of zoospores on rooted cuttings); presence/absence of wilting (foliated terminal shoot tip inoculation).

^bNumber of alder genotypes considered in each experiment.

each wound-inoculation method on the same genotypes for different treatments (different inoculation period or excised/*in situ*) were compared with the Pearson's r . Logistic regression (χ^2 test) was used to compare survival or wilting rates using the GLM function of R. To determine whether the flooding test with rooted cuttings (test 4) or the test with excised shoots and zoospore suspension (test 5) gave similar results in the two tested years, a logistic regression was performed, with the effect of year, genotype and their interaction. The broad sense heritability was computed as the 'Clone' deviance drop divided by the total deviance. All statistical analyses were carried out with R software (R Core Team, 2012).

Results

Seasonal influence on alder receptivity to *P. ×alni* (test 1)

In the 2006 experiment, mean lesion length significantly depended on the month of the inoculation regardless of the type of material (Fig. 1a,c; ANOVA, $P < 0.001$ in both cases). For the test carried out on excised shoots in the laboratory, host susceptibility was high in June and August and strongly suppressed in February and April, and to a lesser extent in October (Fig. 1a). For the test carried out outdoors, the highest susceptibility was observed in August. In contrast, lesion lengths were very small in June and did not differ significantly from the lesion lengths observed in winter and spring (Fig. 1c). Maximum daily air temperature was particularly high at that time with values of about 34°C during the inoculation test (June 2006; Fig. 1d). The test carried out in 2011 on potted seedlings in the greenhouse confirmed the influence of sea-

son on the susceptibility of alders (Kruskal–Wallis test, $P < 0.001$), with a higher susceptibility in summer compared to the other seasons (Fig. 1b). None of the control plants developed necrosis (data not shown).

Comparison of *P. ×alni* isolates for inoculation trials (test 2)

In test 2a, a significant correlation (Fisher's test, $P = 0.004$) between stem segment diameter and lesion length was found, the largest stem section developing the smaller lesions. The effect of isolate on lesion length was significant (Fisher's test, $P = 0.0000$) but inter-isolate differences were of limited magnitude (Table 1).

No significant difference was observed in test 2b between the five isolates inoculated on excised shoots in June 2006 (Kruskal–Wallis test, $P = 0.634$; Table 1). By contrast, the production of sporangia in Petri dishes containing river water showed highly significant differences between isolates (Kruskal–Wallis test, $P = 0.000$). Isolate 2295 produced the highest amount of sporangia (Table 1). This isolate retained the same aggressiveness in 2009 and 2010 as in 2006 (lesion increment in mm day^{-1} of 14.8 ± 3.2 in 2006, 15.6 ± 6.8 in 2009 and 14.4 ± 1.4 in 2010).

Assessment of the wound-inoculation method (test 3)

In test 3a, the lesion lengths measured on excised shoots of the 20 studied genotypes inoculated in July and in September 2010 were significantly correlated, although the correlation was low (Pearson $r = 0.338$, $P = 0.002$).

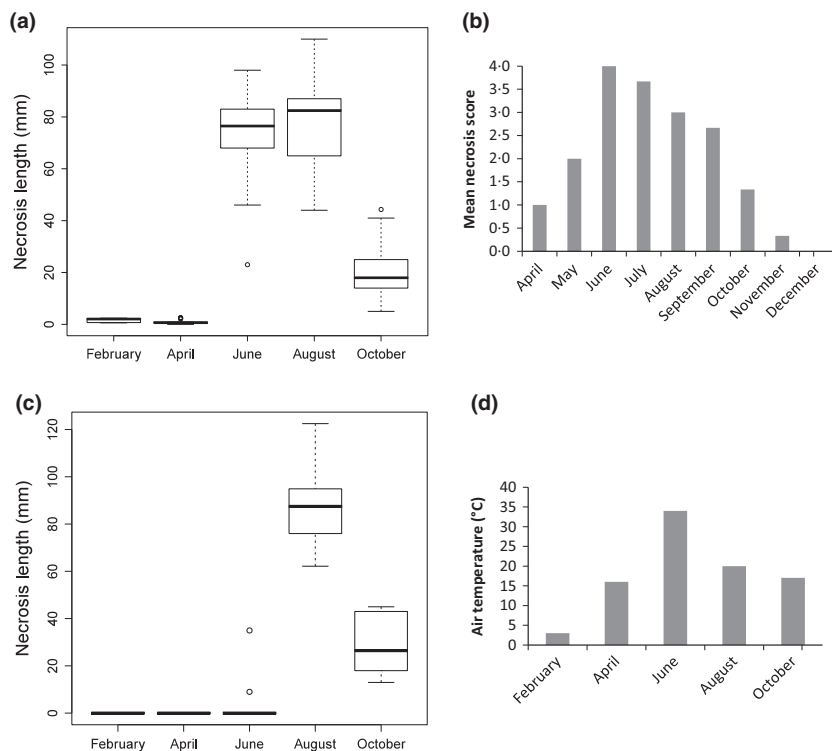


Figure 1 Susceptibility of black alder to *Phytophthora ×alni* (test 1). (a) Inoculation of stem sections in the laboratory (20–22°C), in 2006; (b) inoculation of seedlings in the laboratory (20–22°C), in 2011; (c) inoculation of entire plants (1-year-old) in the field, in 2006; (d) maximum air temperature in the field during the inoculation period (°C) in 2006. Whiskers of the box–whisker plots represent the minimum and maximum values excluding outliers, circular points indicate the outliers.

The mean lesion length was significantly different between the 2 months, with a higher mean in July (Kruskal–Wallis test, $P < 0.0001$). By contrast, in test 3b, no significant correlation was observed between the length of lesions induced on the excised shoots from the 21 tested alder genotypes in July 2010 and in August 2011 (Pearson $r = 0.083$, $P = 0.453$).

In test 3c, wound inoculations on excised shoots and on entire alder in the field were compared among 29 genotypes in August 2011. No significant correlation was found between lesion length obtained on the same genotype *in vitro* (4 days post-inoculation) and *in vivo* (18 days post-inoculation, Pearson $r = 0.024$, $P = 0.800$).

Inoculation by flooding of rooted cuttings (test 4)

Twenty-one alder genotypes in the form of both bare root and potted seedlings were inoculated using the flooding inoculation method in 2011 and in 2012 (bare root plants only in 2012). Significant mortality occurred 3 months after inoculation in the inoculated seedlings, but none in the control plants, whether in 2011 or 2012. The mortality rate was influenced neither by the year ($\chi^2 = 0.080$, $P = 0.777$) nor by the inoculation method used ($\chi^2 = 0.000$, $P = 1.000$). In contrast, there were significant differences between alder genotypes (from 0 to 100% mortality, $\chi^2 = 45.638$, $P < 0.001$). The broad sense heritability was 0.80 (computed using only the *A. glutinosa* clones). The only *A. incana* genotype used in the test was among the most resistant (clone LiriaW247; Fig. 2a,b). The susceptibility rank of the alder genotypes was very similar for potted or bare root seedlings, as indicated by a nonsignificant method \times clone interaction ($\chi^2 = 1.902$, $P = 0.999$; Fig. 2a). The method based on inoculation of bare root seedlings showed consistent results across years ($\chi^2 = 17.851$ for the year \times clone interaction, $P = 0.597$; Fig. 2b).

By contrast, the resistance estimated by lesion length on wound-inoculated excised shoots was not related to resistance estimated by the root inoculation tests on bare root or potted root inoculated seedlings ($P = 0.819$ and 0.470, respectively; Fig. 3a,b).

Excised foliated terminal shoot inoculated with zoospore suspension (test 5)

Wilting likelihood, assessed by logistic regression, was significantly different among the 29 genotypes tested ($\chi^2 = 109.9$, $P < 0.001$), and between 2010 and 2011 ($\chi^2 = 47$, $P < 0.001$). However, despite the fact that different *P. ×alni* isolates were used, the frequency of foliated terminal shoots that wilted for a given genotype was similar in 2010 and in 2011, as indicated by a non-significant genotype \times year interaction ($\chi^2 = 34.1$, $P = 0.200$; Fig. 4a). None of the eight control shoots that were immersed in sterile water wilted. By contrast, no correlation was observed between lesion lengths measured on the excised shoots in 2010 and in 2011 on the same genotypes (Pearson $r = 0.131$, $P = 0.489$; Fig. 4b).

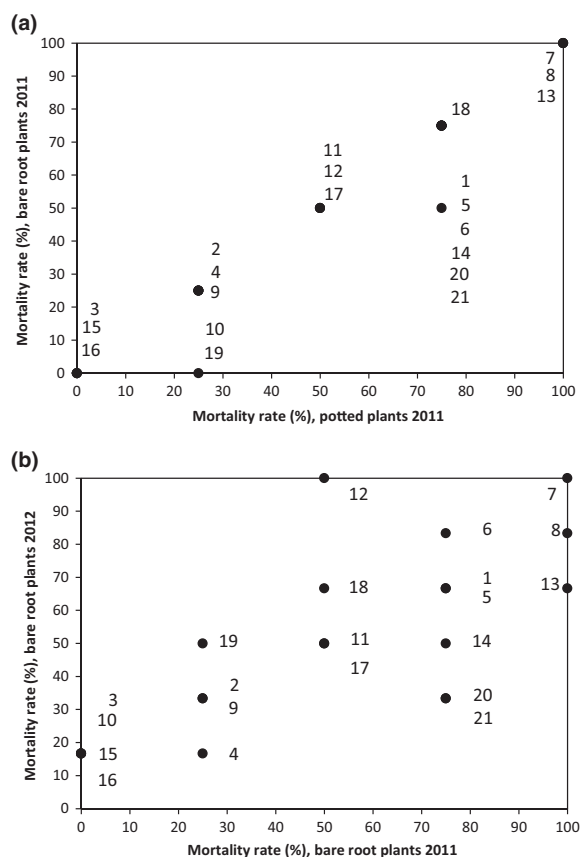


Figure 2 Mortality rate (percentage of plants), 3 months after flooding with river water artificially contaminated with *Phytophthora ×alni* (test 4). (a) Comparison between potted plants and bare root plants in 2011; (b) comparison between flooding on bare root plants in 2011 and 2012. The clone numbers are indicated on each graph (1 = LiriaW183, 2 = LiriaW29, 3 = LiriaW247, 4 = LiriaW59, 5 = LiriaW60, 6 = LiriaW61, 7 = LiriaW62, 8 = LiriaW69, 9 = LiriaW79, 10 = LiriaW124, 11 = LiriaW123, 12 = LiriaW127, 13 = LiriaW137, 14 = LiriaW251, 15 = LiriaW252, 16 = LiriaW2, 17 = LiriaW256, 18 = LiriaW154, 19 = LiriaW156, 20 = LiriaW167, 21 = LiriaW171).

Finally, the two methods (% wilting in zoospore suspension versus lesion length after wound inoculation) gave significantly correlated results when the assay was performed in the same year ($\chi^2 = 7.45$, $P = 0.006$ in 2010 and $\chi^2 = 4.56$, $P = 0.033$ in 2011). This relationship was not as strong when percentage wilting in zoospore suspension in 2011 was compared to the lesion length obtained by the wound-inoculation method in 2010 ($\chi^2 = 5.040$, $P = 0.025$) or the reverse ($\chi^2 = 0.447$, $P = 0.500$). The broad sense heritability computed for the zoospore inoculation of foliated shoots was of 0.577.

Discussion

In this study, the reliability and repeatability of several inoculation methods on different types of plant materials is reported. The method that proved the most reliable, close to the root-dip inoculation method described by

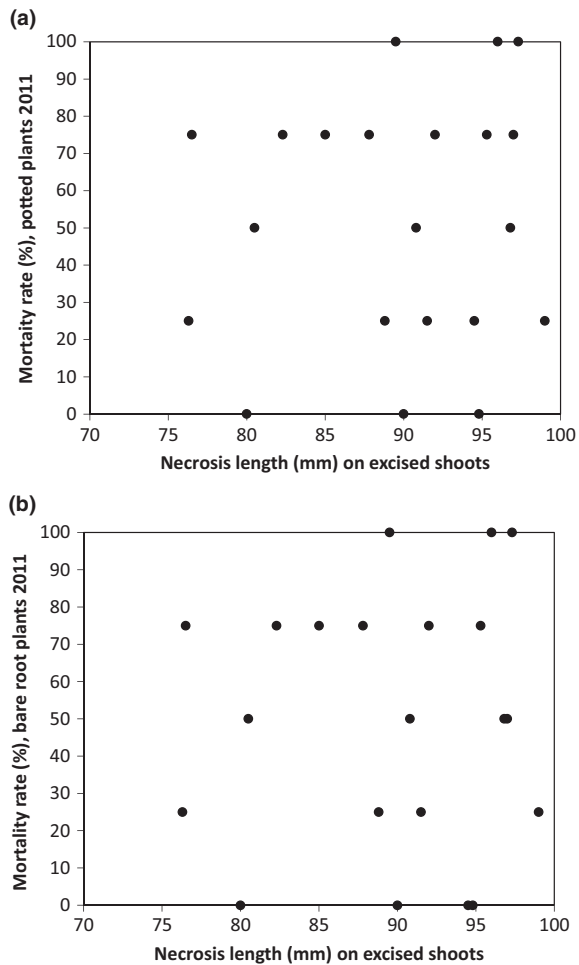


Figure 3 Comparison of results from wound inoculation of excised shoots of 21 genotypes of alder, expressed in mean necrosis length in mm, with results from inoculation by flooding of potted plants (a) or bare root plants (b) of the same genotypes, expressed as a mortality rate (percentage of plants).

Oh *et al.* (2006) for inoculation of Port-Orford-cedar with *P. lateralis*, mimics natural conditions of infection for a soilborne *Phytophthora* species. This method consists of flooding the root system of alder seedlings for a few days after inoculation with zoospore suspensions. Although labour-intensive, the method was repeatable (correlation between years) and robust (correlation between 1-year-old and 2-year-old plants, and between bare roots and potted plants as well). By using unwounded plants, the method measures both the plant's ability to escape infection and the spread of the pathogen post-infection. Moreover, inoculated plants developed all the symptoms typical of the disease, including wilting, fine root necrosis and stem canker. Root system flooding during long periods (7 weeks) was found to stress alders and to decrease their resistance to *P. ×alni* (Strnadová *et al.*, 2010). However, the duration of flooding in the present experiments was limited to 7 days and no mortality was observed in control plants. The measurements

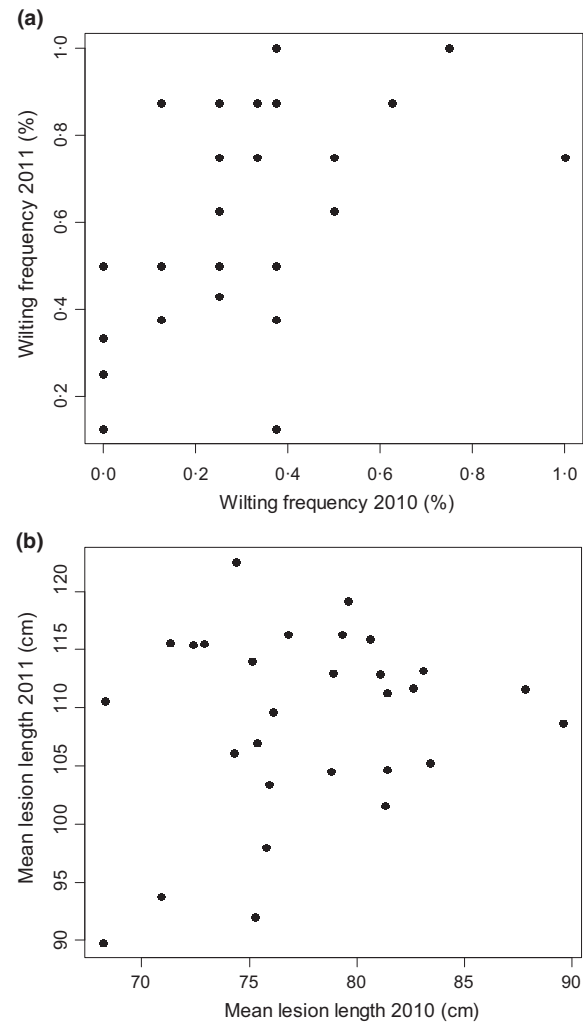


Figure 4 Repeatability of the two tested excised terminal shoot assays over years (test 5). (a) Wilting frequencies after inoculation of foliated terminal shoots of 29 genotypes of alder with a zoospore suspension of *Phytophthora ×alni*, in 2010 and 2011; (b) mean lesion length per genotype obtained using the wound-inoculation method.

allowed progressive disease development and the categorization of the alder genotype's resistance. The only *A. incana* genotype used in this test was among the most resistant genotypes, which is in accordance with the lower susceptibility of that species to *P. ×alni* (Webber *et al.*, 2004).

Inoculation of entire trees with *Phytophthora* species in a natural environment conducive to the disease is not recommended, due to the risk of spreading the pathogen, especially when regulated or emergent pathogens are concerned; also, uncontrolled environmental conditions may influence inoculation efficiency (this study; Hansen *et al.*, 1989). For these reasons, inoculations on detached material (leaves, stem pieces) are often carried out (Denman *et al.*, 2005; Iwano *et al.*, 2005). These methods are rapid, appropriate to high-throughput studies and offer the possibility of directly testing adult trees (Hansen

et al., 1989). The use of under-bark inoculation with mycelium plugs on detached plant material has also often been used either to evaluate the aggressiveness of *Phytophthora* isolates (Brasier & Kirk, 2001) or to evaluate the pathogenicity of *Phytophthora* on different tree species (Moralejo et al., 2009). However, the results of the present study demonstrated the lack of repeatability of this method to assess susceptibility of alder genotypes to *P. ×alni*, with no correlation between tests carried out in two successive years. They also highlighted its lack of robustness, with no correlation between results obtained on excised or *in situ* shoots. These results differ from those of Hansen et al. (1989) and Robin et al. (2006) working respectively on *P. lateralis* on *Chaemecyparis lawsoniana* and *P. cambivora* on *Castanea sativa*, but are consistent with those of Hayden et al. (2013) on *P. ramorum* on *Notholithocarpus densiflorus*. Due to the lack of reliability of the stem inoculation method for *P. ×alni* in the present investigation, another method was developed, based on inoculation of foliated terminal shoots with a zoospore suspension. This method proved repeatable over 2 years. It was also robust, with similar results being obtained using different isolates, and is appropriate for high-throughput studies. Nevertheless, further experiments comparing this method with inoculation of entire seedlings, using the same alder genotypes, would be useful to confirm these results.

Wound inoculation, either on detached or *in situ* stems, has been used to test seasonal changes in susceptibility of black alder to *P. ×alni* (Stochlová et al., 2012) and of red oaks to *P. cinnamomi* (Robin et al., 1994). In agreement with these studies, the present investigation showed that the highest susceptibility was in summer. The absence of necrosis observed in June 2006 might result from the exceptionally high maximum daily temperature that was recorded during the test (34°C). Previous studies showed that high summer temperature negatively affects *P. ×alni*-induced alder decline (Aguayo et al., 2014). This observation suggests the need to work in standardized conditions of temperature for carrying out inoculation trials. As no necrosis development was observed in winter, even in controlled conditions (20–22°C), temperature alone cannot account for the seasonal changes observed in alder susceptibility to *P. ×alni*. Other climatic parameters such as day length (Tsrör (Lahkim), 2004); physiological changes in the host plant (Browne & Mircetich, 1996) or abiotic stress (Bostock et al., 2014) might modify disease susceptibility.

This study focused on *P. ×alni* because of the higher aggressiveness of this species compared to the parental species *P. uniformis* and *P. ×multiformis* (Brasier & Kirk, 2001), as well as its higher frequency of occurrence in natural conditions. The objective was to identify isolates suitable for testing alder resistance, i.e. that were able to cause inner bark necrosis, that sporulated well, and were stable over years of long-term storage at 13°C. Very little variation in the ability to cause bark necrosis was found in *P. ×alni*. Although test 2a found significant difference, the resultant lesion lengths were very similar

between tested isolates, with a ratio of 1:1 between the more and the less aggressive isolate in both tests 2a and 2b. In contrast, production of sporangia was more variable between the isolates. In this context, selection of an isolate that produces a high amount of sporangia or use of calibrated zoospore suspensions needs to be carried out before performing flooding or foliated terminal shoot inoculations.

The results showed that genetic variation for susceptibility to *P. ×alni* exists in natural *A. glutinosa* populations. The estimated broad sense heritability was high at 0.60–0.80, which can be explained by the fact that the tests were conducted in controlled conditions (laboratory, greenhouse), minimizing the effect of environment. Although a limited number of alder genotypes were tested, a wide variation in resistance was observed, with some clones displaying a survival rate after inoculation of more than 80%, while others showed high susceptibility. This degree of variation is very encouraging from the perspective of selecting for alder resistance to *P. ×alni*. Nevertheless, several factors can cloud the evaluation of trees' resistance to a disease, including the risk of different responses between juvenile and mature trees, the effect of tree physiology and/or environmental conditions on the disease development, in particular the seasonal influence, and the effect of pathogen isolate on resistance evaluation. As a result, the next step of the programme will be to assess the behaviour of alder genotypes of known resistance over several years under natural conditions of infection. In this context, a set of six genotypes ranging from highly susceptible to potentially resistant will be planted along a watercourse where *P. ×alni* is present. The objective will be to compare the level of susceptibility estimated under laboratory conditions to the growth, presence of canker and crown decline measured in a naturally infected stand, with climatic conditions that are not always conducive to the disease (Thoirain et al., 2007; Aguayo et al., 2014).

Acknowledgements

This research was funded by European (Feder-Interreg programme) and regional fundings (France, Agence de l'Eau Rhin-Meuse; Walloon Region – DGO3, Direction des Cours d'eau non navigables; projects ECOLIRI and ECOLIRIMED). The authors are grateful to R. Gruselle for the production of alder clones, and to Martine Leclercq, Frédéric Fauche, Claire Kraenner, Flora Bourguès and Olivier Caël for technical assistance. The Nancy UMR1136 is supported by a grant overseen by the French National Research Agency (ANR) as part of the 'Investissements d'Avenir' programme (ANR-11-LABX-0002-01, Lab of Excellence ARBRE). The authors also thank Dr Katherine Hayden (UC Berkeley) for critical review of the manuscript.

References

- Adams GC, Catal M, Trummer L, Hansen EM, Reeser P, Worrall J, 2008. *Phytophthora alni* subsp. *uniformis* found in Alaska beneath

- thinleaf alders. *Plant Health Progress*. doi:10.1094/PHP-2008-1212-02-BR.
- Aguayo J, Adams GC, Halkett F *et al.*, 2013. Strong genetic differentiation between North American and European populations of *Phytophthora alni* subsp. *uniformis*. *Phytopathology* **103**, 190–9.
- Aguayo J, Elegbede F, Husson C, Saintonge FX, Marçais B, 2014. Modeling climate impact on an emerging disease, the *Phytophthora alni* induced alder decline. *Global Change Biology* **20**, 3209–21.
- Bostock RM, Pye MF, Roubtsova TV, 2014. Predisposition in plant disease: exploiting the nexus in abiotic and biotic stress perception and response. *Annual Review of Phytopathology* **52**, 517–49.
- Brasier CM, Kirk SA, 2001. Comparative aggressiveness of standard and variant hybrid alder phytophthoras, *Phytophthora cambivora* and other *Phytophthora* species on bark of *Alnus*, *Quercus* and other woody hosts. *Plant Pathology* **50**, 218–29.
- Brasier CM, Rose J, Gibbs JN, 1995. An unusual *Phytophthora* associated with alder mortality in Britain. *Plant Pathology* **44**, 999–1007.
- Brasier CM, Kirk SA, Delcan J, Cooke DEL, Jung T, Man In 't Velt WA, 2004. *Phytophthora alni* sp. nov. and its variants: designation of emerging heteroploid hybrid pathogens spreading on *Alnus* trees. *Mycological Research* **108**, 1172–84.
- Browne GT, Mircetich SM, 1996. Effects of month of inoculation on severity of disease caused by *Phytophthora* spp. in apple root crowns and excised shoots. *Phytopathology* **86**, 290–4.
- Claessens H, 2005. *L'Aulne Glutineux. Ses Stations et sa Sylviculture*. Gembloux, Belgium: ASBL Forêt Wallonne.
- De Kort H, Vandepitte K, Bruun HH, Closset-Kopp D, Honnay O, Mergeay J, 2014. Landscape genomics and a common garden trial reveal adaptive differentiation to temperature across Europe in the tree species *Alnus glutinosa*. *Molecular Ecology* **23**, 4709–21.
- De Merlier D, Chandelier A, Debruxelles N *et al.*, 2005. Characterisation of alder *Phytophthora* isolates from Wallonia and development of SCAR primers for their specific detection. *Journal of Phytopathology* **153**, 99–107.
- Denman S, Kirk SA, Brasier CM, Webber JF, 2005. *In vitro* leaf inoculation studies as an indication of tree foliage susceptibility to *Phytophthora ramorum* in the UK. *Plant Pathology* **54**, 512–21.
- Druart P, Leclercq M, Joussemet MA *et al.*, 2013. Construction et développement d'une filière de production d'écotypes ligneux pour l'application des techniques végétales à la renaturation des berges de cours d'eau. In: Druart P, Husson C, Paul R, eds. *Renaturation des Berges de Cours d'Eau et Phytoremédiation. Utilisation d'Écotypes Ligneux de Ripisylves pour l'Ingénierie Végétale*. Gembloux, Belgium: Les Presses Agronomiques de Gembloux, 121–55.
- Elegbede CF, Pierrat JC, Aguayo J, Husson C, Halkett F, Marçais B, 2010. A statistical model to detect asymptomatic infectious individuals with an application in the *Phytophthora alni*-induced alder decline. *Phytopathology* **100**, 1262–9.
- Gibbs JN, 1995. *Phytophthora* root disease of alder in Britain. *EPPO Bulletin* **25**, 661–4.
- Gibbs JN, 2003. *Phytophthora* disease of alder: management and control. In: Gibbs JN, Van Dijk C, Webber JF, eds. *Phytophthora Disease of Alders in Europe*. Edinburgh, UK: Forestry Commission Bulletin No. 126, 73–8.
- Gibbs JN, Lipscombe MA, Peace AJ, 1999. The impact of *Phytophthora* disease on riparian populations of common alder (*Alnus glutinosa*) in southern Britain. *European Journal of Forest Pathology* **29**, 39–50.
- Hansen EM, Hamm PB, Roth LF, 1989. Testing Port-Orford-cedar for resistance to *Phytophthora*. *Plant Disease* **73**, 791–4.
- Hansen EM, Parke JL, Sutton W, 2005. Susceptibility of Oregon forest trees and shrubs to *Phytophthora ramorum*: a comparison of artificial inoculation and natural infection. *Plant Disease* **89**, 63–70.
- Hayden KJ, Garbelotto M, Dodd R, Wright JW, 2013. Scaling up from greenhouse resistance to fitness in the field for a host of an emerging forest disease. *Evolutionary Applications* **6**, 970–82.
- Husson C, Aguayo J, Revellin C, Frey P, Ioos R, Marçais B, 2015. Evidence for homoploid speciation in *Phytophthora alni* supports taxonomic reclassification in this species complex. *Fungal Genetics and Biology* **77**, 12–21.
- Ioos R, Husson C, Andrieux A, Frey P, 2005. SCAR-based PCR primers to detect the hybrid pathogen *Phytophthora alni* and its subspecies causing alder disease in Europe. *European Journal of Plant Pathology* **112**, 323–35.
- Ioos R, Andrieux P, Marçais B, Frey P, 2006. Genetic characterization of the natural hybrids species *Phytophthora alni* as inferred from nuclear and mitochondrial DNA analyses. *Fungal Genetics and Biology* **43**, 511–29.
- Ioos R, Barrès B, Andrieux A, Frey P, 2007. Characterization of microsatellite markers in the interspecific hybrid *Phytophthora alni* spp. *alni* and cross-amplification with related taxa. *Molecular Ecology Notes* **7**, 133–7.
- Iwano AD, Thévenin JM, Butler DR, Eskez AB, 2005. Usefulness of the detached pod test for assessment of cacao resistance to *Phytophthora* pod rot. *European Journal of Plant Pathology* **113**, 173–82.
- Jeffers SN, Martin SB, 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. *Plant Disease* **70**, 1038–43.
- Jung T, Blaschke M, 2004. *Phytophthora* root and collar rot of alders in Bavaria: distribution, modes of spread and possible management strategies. *Plant Pathology* **53**, 197–208.
- Jung T, Nechwatal J, 2008. *Phytophthora gallica* sp. nov., a new species from rhizosphere soil of declining oak and reed stands in France and Germany. *Mycological Research* **112**, 1195–205.
- Mingeot D, Baleux R, Watillon B, 2010. Characterization of microsatellite markers for black alder (*Alnus glutinosa* [L.] Gaertn.). *Conservation Genetic Resources* **2**, 269–71.
- Mingeot D, Husson C, Mertens P, Druart P, 2013. Évaluation par marqueurs moléculaires microsatellites de la diversité d'une collection d'aulnes glutineux provenant de Wallonie (Belgique), de Lorraine (France) et du Grand-Duché du Luxembourg. In: Druart P, Husson C, Paul R, eds. *Renaturation des Berges de Cours d'Eau et Phytoremédiation. Utilisation d'Écotypes Ligneux de Ripisylves pour l'Ingénierie Végétale*. Gembloux, Belgium. Les Presses Agronomiques de Gembloux, 49–58.
- Moralejo E, Garcia-Muñoz JA, Descals E, 2009. Susceptibility of Iberian trees to *Phytophthora ramorum* and *P. cinnamomi*. *Plant Pathology* **58**, 271–83.
- Nagy ZA, Bakonyi J, Ersek T, 2003. Standard and Swedish variant types of the hybrid alder *Phytophthora* attacking alder in Hungary. *Pest Management Science* **59**, 484–92.
- Oh E, Hansen EM, Sniezko RA, 2006. Port-Orford-cedar resistant to *Phytophthora lateralis*. *Forest Pathology* **36**, 385–94.
- R Core Team, 2012. *R: a language and environment for statistical computing*. Vienna, Austria: R foundation for Statistical Computing. [http://www.R-project.org/]. Accessed 28 May 2015.
- Robin C, Dupuis F, Desprez-Loustau ML, 1994. Seasonal changes in Northern red oak susceptibility to *Phytophthora cinnamomi*. *Plant Disease* **78**, 369–34.
- Robin C, Morel O, Vettraino AM, Perlerou C, Diamondis S, Vannini A, 2006. Genetic variation in susceptibility to *Phytophthora cambivora* in European chestnut (*Castanea sativa*). *Forest Ecology and Management* **226**, 199–207.
- Santini A, Barzanti GP, Capretti P, 2003. Susceptibility of some mesophytic hardwoods to alder *Phytophthora*. *Journal of Phytopathology* **151**, 406–10.
- Solla A, Perez-Sierra A, Corcobado T, Haque MM, Diez JJ, Jung T, 2010. *Phytophthora alni* on *Alnus glutinosa* reported for the first time in Spain. *Plant Pathology* **59**, 798.
- Stochlová P, Novotná K, Cerný K, 2012. Factors affecting the development of *Phytophthora alni* spp. *alni* infections in *Alnus glutinosa* L. *Journal of Forest Science* **58**, 123–30.
- Streito JC, Legrand P, Tabary F, Jarnouen de Villartay G, 2002. *Phytophthora* disease of alder (*Alnus glutinosa*) in France: investigations between 1995 and 1999. *Forest Pathology* **32**, 179–91.

- Strnadová V, Cerný K, Holub V, Gregorová B, 2010. The effect of flooding and *Phytophthora alni* infection on black alder. *Journal of Forest Science* 56, 41–6.
- Thoirain B, Husson C, Marçais C, 2007. Risk factors for the *Phytophthora*-induced decline of alder in Northeastern France. *Phytopathology* 97, 99–105.
- Tsrer (Lahkim) L, 2004. Effect of light duration on severity of black dot caused by *Colletotrichum coccodes* on potato. *Plant Pathology* 53, 288–93.
- Webber J, Gibbs JN, Hendry S, 2004. *Phytophthora* disease of alder. Edinburgh, UK: Forestry Commission: Forestry Commission Information Note 6.