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Genotypic and phenotypic characterization of the European A2 isolates of *Phytophthora ramorum*

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Abstract *Phytophthora ramorum,* a recently described North American and European pathogen, has three clonal lineages. The NA1 and NA2 lineages are found in North American forests and nurseries, while the EU1 lineage appears mainly in European nurseries. *P. ramorum* is heterothallic, having two mating types A1 and A2. All NA1 and NA2 isolates are of A2 mating type. When first collected, all EU1 isolates were of A1 mating type, with the exception of one A2 isolate collected in Belgium in 2002. Screening 410 other Belgian isolates for mating type revealed two additional EU1-A2 isolates collected in 2002 and 2003. PCR-RFLP, AFLP and SSR markers were used to determine the nature of the mating type change. The three isolates show no indications of sexual recombination or mitotic crossing over, indicating that mutation or mitotic gene conversion is the most likely explanation for the mating type change. We compared the pathogenicity and sporulation characteristics of the EU1-A2 isolates to those of EU1-A1 and NA1-A2 isolates on four host plants. Despite small differences in pathogenicity on some hosts, the EU1-A2 isolates were similarly aggressive to each other and to the EU1-A1 isolates and more aggressive than the NA1-A2 isolates. Sporulation characteristics were also comparable among EU1-A2 isolates and between EU1-A1 and EU1-A2 isolates, except for EU1-A2 isolate BBA 26/02. The limited genotypic and phenotypic differences between EU1-A2 isolates probably evolved after the mating type change, which may have occurred several years before the isolates were detected. There are strong indications that the EU1-A2 population has been eradicated from Belgium.

Keywords Aggressiveness · Mating type · Oomycetes · Sporulation · Sudden Oak Death

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Introduction

In 1993, *Phytophthora ramorum* was first found to cause leaf blight, stem canker, and tip dieback on nursery-grown *Rhododendron* and *Viburnum* spp. in Germany and the Netherlands (Werres and De Merlier 2003). In 1995, a non-European lineage of *P. ramorum* started an epidemic disease on tanoaks (*Lithocarpus densiflorus*) and oaks (especially *Quercus agrifolia*) in California, now commonly referred to as Sudden Oak Death (SOD) (Rizzo et al. 2002). SOD has caused

mortality of more than one million trees on the west coast of the US (Meentemeyer et al. 2008). In Europe, *P. ramorum* has been detected in 21 countries, primarily on nursery plants of *Rhododendron*, *Viburnum,* and *Camellia*, with sporadic findings on other host plants such as *Pieris* and *Kalmia.* As of 2008, only approximately 30 trees had died from *P. ramorum* in the (semi-)natural environment (Tracy 2009). Bleeding cankers have been observed on several oak species as well as on beech, horse chestnut, and sycamore, while leaf necrosis was observed on sweet chestnut, ash, and other species. Infected rhododendrons, especially *R. ponticum*, were found in close proximity to most of the infected trees in Europe and were serving as the most epidemiologically important understory plant (Tracy 2009). In the UK in 2009, *P. ramorum* was also found causal to infections of *Vaccinium myrtillus* in heathland and more serious leaf and stem infections of *Larix kaempferi* in plantations (http://www.forestry.gov.uk/forestry/infd-7xvewh). On several of these sites, little or no *R. ponticum* is present. These recent findings indicate a possible change in the dynamics of the disease in Europe.

Three clonal lineages of *P. ramorum* have been recognized based on microsatellite profiles, AFLP and mitochondrial sequences (Grünwald et al. 2009; Ivors et al. 2004, 2006; Martin 2008). The North American lineages (NA1 and NA2) have been detected in forests and nurseries in North America only, whereas the EU1 lineage has mainly been found in Europe and occasionally on nursery stock in California, Oregon, Washington and British Columbia (Grünwald et al. 2008c; Hansen et al. 2003). *P. ramorum* is heterothallic and has two mating types A1 and A2. So far, all NA1 and NA2 isolates are of A2 mating type. Originally, all isolates of the EU1 lineage were identified as A1 mating type (Rizzo et al. 2002; Werres et al. 2001). In 2003, a single isolate of the EU1 lineage with A2 mating type was detected in Belgium (Werres and De Merlier 2003). Both mating types co-existing at one site can lead to sexual recombination as the sexual cycle in *P. ramorum* is functional (Boutet et al. 2010). The occurrence of a sexual cycle in the population may have an important impact on the genetic diversity and pathogenic properties. After recombination of A1 and A2 mating types in *P. infestans,* a population resulted that is now more aggressive and harder to control (Goodwin 1997). Sexual recombination also leads to the production of oospores, an extra source of inoculum that allows the pathogen to survive for extended periods of time outside its host.

To estimate the risk of sexual recombination of *P. ramorum* in the EU1 population, the presence and distribution of isolates of both mating types needs to be known. Belgium was the only country where an EU1-A2 isolate of *P. ramorum* was found. The first objective of our research was to determine if any other EU1-A2 isolates were present in Belgium. The second objective was the genetic characterization of the A2 isolates to discriminate between the following hypotheses: that the A2 mating type in Europe arose 1) by sexual recombination or mitotic crossing over, 2) by mutation or mitotic gene conversion of a EU1-A1 isolate, or 3) it was introduced by migration. As the risk for the creation of progeny with an increased fitness is larger if the mating isolates have different epidemiological characteristics (such as aggressiveness and inoculum production), the third objective was the phenotypic characterization of the European A2 isolates on different host plants. Last, to help determine if the different European A2 isolates have evolved out of a single mating type change, the data on the phenotypic and genotypic characterization of the EU1-A2 isolates were interpreted and combined with the data on isolation year, site, and host.

Materials and methods

Phytophthora ramorum isolates

All *P. ramorum* isolates from the samples collected by the Belgian Plant Protection Service (FAVV) between 2002 and 2008 (n=411) were used in this study (including BBA 26/02, the first EU1-A2 isolate). The majority (370 isolates) originated in Flanders, where most of the Belgian ornamental nurseries are located. The isolates were collected from either *Rhododendron* (90.5%) or *Viburnum* (9.5%) in nurseries (97.3%), public parks (1.5%), and private gardens (1.2%). Diagnosis and isolation was done as described in Vercauteren et al. (2010). After isolation, cultures were stored on V8 (Erwin and Ribeiro 1996) and corn meal agar (CMA, BBL™, France) at 4°C and transferred annually onto fresh medium. *P. ramorum* PRI480 (NA1-A2), PRI483 (NA1-A2), and PRI549 (EU1-A1) were included as non-Belgian reference

strains. Details of the primary strains used in this study are listed in Table 1. Of these, the EU1-A1 isolates were selected based on isolation date (mostly from the same time period as the EU1-A2 isolates), genotype (three multilocus genotypes), and country of origin (two). The NA1 isolates were selected based on host (two).

Mating type screening and oospore production

The mating type of each isolate was determined by first pairing it with *P. ramorum* reference isolate PRI483 (NA1-A2) on carrot agar (CA) (Erwin and Ribeiro 1996). Agar from ForMedium™ (England) was used as gelling agent (the type of agar has been shown to influence the mating behaviour in Boutet et al. (2009)). Based on Brasier and Kirk (2004), young 5 mm-diameter mycelium plugs from both the test isolate and the reference A2 isolate were brought into close contact by mixing the mycelial plugs on very thinly poured CA medium. After 5–15 days incubation at 20°C in the dark, the test isolate was classified as A1 when oogonia appeared. If none formed, the test isolate was paired with Belgian EU1-A1 *P. ramorum* isolate PR/D/02/2084, using the same method.

The EU1 isolates of A2 mating type that were identified in this study as well as all reference A2 isolates (Table 1) were paired with 20 EU1-A1 isolates to determine the relative mating efficiency of the EU1-A2 isolates. The number of oogonia in the mycelium mix was counted under a compound microscope at 100× magnification. Five classes were established according to the number of oogonia per pairing observed: 0 (no oogonia), 1 (up to five oogonia), 2 (5–20 oogonia), 3 (20–50 oogonia) and 4 (more than 50 oogonia).

Genotypic characterization

Genotypic characterization was performed using PCR-RFLP, AFLP, and microsatellite analysis. The *cox*1 polymorphism between the EU1 and NA1 lineage isolates was verified in each isolate using the PCR-RFLP method of Kroon et al. (2004). AFLP analysis was performed as described in Vercauteren et al. (2010), which is based on Ivors et al. (2004). SSR analysis was performed with 119 primer pairs that amplified EU1/NA1-polymorphic microsatellites using the methods described in Vercauteren et al. (2010).

Phenotypic characterization in vitro

In vitro growth rate and sporulation of the EU1-A2 isolates were compared to those of selected EU1-A1 and NA1-A2 reference isolates (Table 1). Growth rate was determined on V8 agar by measuring the colony diameter after 7 days' incubation at 20°C in the dark. Sporangia were dislodged from 10-day-old cultures on diluted (10% clarified juice) V8 agar using 20 ml of sterile MilliQ water and a glass rod, then quantified

Table 1 *Phytophthora ramorum* isolates used for pathogenicity and sporulation tests

Isolate	Isolation year	Genotype[a]	Mating type	Host plant	Origin	Resistance to metalaxyl	in vitro growth rate (mm day^{-1})	Sporulation in vitro (10^4 cm^{-2})
PR/D/02/880	2002	EU1MG1	A1	*Rhododendron* sp.	Belgium	R	3.0±0.2	6.8±0.6
PR/D/02/4294	2002	EU1MG2	A1	*Viburnum x bodnantense*	Belgium	S	3.0±0.2	5.6±0.4
PR/D/03/336	2003	EU1MG1[b]	A1	*Viburnum tinus*	Belgium	S	2.5±0.3	4.4±0.9
PR/D/06/08	2006	EU1MG24	A1	*Rhododendron* sp.	Belgium	R	3.4±0.1	5.9±0.3
PRI 549	2001	EU1MG1	A1	*Viburnum* sp.	the Netherlands	S	2.9±0.3	7.0±1.5
BBA 26/02	2002	EU1MG1	A2	*Viburnum x bodnantense*	Belgium	S	3.4±0.2	6.3±0.7
PR/D/02/2340	2002	EU1MG1	A2	*Viburnum x bodnantense*	Belgium	S	3.2±0.3	5.3±0.8
PR/D/03/424	2003	EU1MG5	A2	*Rhododendron* sp.	Belgium	S	3.1±0.2	6.9±1.0
PRI 480	–	NA1	A2	*Quercus agrifolia*	USA, CA	R	3.0±0.2	3.0±0.7
PRI 483	2000	NA1	A2	*Rhododendron* sp.	USA, CA	R	3.3±0.1	2.8±0.9

[a] Lineage according to Grünwald et al. (2009). Multilocus genotype according to Vercauteren et al. (2010)

[b] Different from EU1MG1 with an AFLP marker (Vercauteren et al. 2010)

[c] *R* resistant; *S* sensitive. Resistance profile of BBA 26/02, PRI549, PRI480 and PRI483 determined in this study. Resistance profile of other isolates determined in Vercauteren et al. (2010).

with a haemocytometer. For each isolate, five replicates were used. *P. ramorum* isolates BBA 26/02, PRI549, PRI480 and PRI483 were evaluated for resistance to metalaxyl as described in Vercauteren et al. (2010).

Pathogenicity tests

Pathogenicity of the EU1-A2 isolates was compared to the pathogenicity of EU1-A1 and NA1-A2 reference isolates (Table 1) on four tree species (*Fagus sylvatica*, *Quercus rubra*, *Castanea sativa,* and *Fraxinus excelsior*), two forest understory shrubs (*Rhododendron ponticum* and *Viburnum opulus*), and two ornamental nursery plants (*Rhododendron* 'Cunningham's White' and *Viburnum x bodnantense* 'Dawn'). In total, three inoculation methods were used: wounded leaves, non-wounded leaves and wounded stems. Inoculation of non-wounded leaves was used to estimate the ability of the pathogen to penetrate the tissue while the tests with wounded tissue were used to evaluate the growth capacity inside leaf or stem tissue.

In the test with non-wounded tissue, zoospores were used to inoculate 20 detached and washed leaves of the host plants as described in De Dobbelaere et al. (2010). In this test, the relative lesioned leaf area (RLLA) is used as the measure of pathogenicity. Preliminary tests determined that leaves of *Viburnum opulus, Castanea sativa, Quercus rubra,* and *Fagus sylvatica* had low to very low susceptibility when using this inoculation method, independent of the isolate. As the main purpose was to compare the pathogenicity of the isolates, these hosts were not included in the final experiment using this inoculation method. In the wounded-leaf test, 20 leaves were punctured with a needle and inoculated with a mycelium plug as described in De Dobbelaere et al. (2010). Tests with wounded leaves were conducted three times.

The wounded-stem test was performed as follows. For the tree species, 20 freshly-cut 1.2-m-long twigs or stems of each species were wounded at 11 equidistant points (10 cm apart) with a cork borer and a 5-mm-diameter mycelium plug was placed in the resulting hole (Brasier and Kirk 2004). For the shrubs and ornamental plants, wounds were made with a drill (1.5 mm diameter) and a mycelium plug was packed into the wound. All inoculated wounds were taped tightly with Parafilm (Pechiney Plastic Packaging Company, USA). Inoculated twigs or stems were wrapped with moist paper towels, aluminum foil, and two polyethylene bags to maintain suitable humidity, and incubated at 17°C. After 1–5 weeks (depending on the host plant), the bark was removed and the length of the lesion was measured parallel to the axis of the twig or stem. Growth rate was calculated based on this length and the number of days post inoculation.

Sporulation

The *in planta* sporulation characteristics of the 10 isolates listed in Table 1 were determined on *Viburnum x bodnantense* 'Dawn' and on *Rhododendron* 'Cunningham's White'. For each isolate, sporulation was studied in 20 leaves that were inoculated and incubated as described in the pathogenicity test with non-wounded leaves.

Sporangia and chlamydospores were quantified as described in De Dobbelaere et al. (2010). The experiment was conducted twice. Sporangia and chlamydospore production of isolate BBA 26/02 and EU1-A1 reference isolate PR/D/02/880 were also examined on the six other host plants described in the pathogenicity tests and on three extra host plants (*Rosa canina*, *Sambucus nigra* and *Quercus robur*) that were selected based on a preliminary screening.

Data analysis

Analyses of aggressiveness and sporulation data were conducted using the general linear model in Statistica 8.0 (StatSoft). Similar to Elliott et al. (2009) and Suassuna et al. (2004), the model consisted of $Y_{ijklm} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \gamma_{k(i)} + \beta\gamma_{jk(i)} + \delta_l + e_{ijklm}$ where μ = overall mean, α_i = fixed effect of (sub)lineage (EU1-A1, EU1-A2 and NA1), β_j = fixed effect of host, $\alpha\beta_{ij}$ = interaction term of (sub)lineage and host, $\gamma_{k(i)}$ = fixed effect of isolates nested within lineages, $\beta\gamma_{jk(i)}$ = interaction term of host and isolates within (sub)lineages, δ_l = effect of blocks and e_{ijklm} = random experimental error. This model allowed testing for differences between lineages as well as among isolates within a lineage. Unequal N HSD tests ($P = 0.05$) were used for multiple comparisons. Differences between individual isolates were tested within each host. The data on RLLA are expressed as proportions, and were transformed using the arcsine

square root transformation before statistical analysis. Data that were not normally distributed were analyzed using the non parametric Mann-Whitney test in Statistica 8.0 (StatSoft). Throughout the document, averages are reported together with the corresponding standard error (avg±SE).

Results

Mating type screening and production of oogonia

Formation of oogonia generally started 3–10 days after pairing. In approximately 25% of the cases, multiple mating experiments were required, but ultimately formation of oogonia was observed for all isolates. Oogonia were most abundant in zones with relatively few chlamydospores. Among the 410 isolates, two new A2 isolates were identified. These were PR/D/02/2340, isolated in 2002 from *Viburnum x bodnantense* 'Dawn', and PR/D/03/424, isolated in 2003 from a *Rhododendron* hybrid. Together with the previously identified EU1-A2 isolate BBA 26/02 from *Viburnum x bodnantense* 'Dawn' (Brasier and Kirk 2004; Werres and de Merlier 2003), this brings the total number of EU1-A2 isolates to three, or 0.7% of all Belgian isolates. The EU1-A2 isolates originated from three nurseries, a minimum of 37 km apart, two plant species (*Viburnum* and *Rhododendron*), and 2 years (2002 and 2003). There is no obvious commercial link between the nurseries (Belgian Plant Protection Service, personal communication). The plants in at least two of the three cases were imported from two EU countries during the year before they tested positive for *P. ramorum*.

Figure 1 shows the number of oogonia produced when pairing the three European A2 isolates and the two NA1-A2 isolates with 20 EU1-A1 isolates. Isolate BBA 26/02 produced significantly more oogonia than PR/D/02/2340 ($P= 0.001$).

Genotypic characterization

All European A2 isolates had RFLP and AFLP banding patterns matching those of the main EU1 genotype group. Microsatellite analysis also confirmed that the three Belgian A2 isolates belong to the EU1 lineage. Out of the 119 microsatellite markers tested, only one (ILVOPrMS145c) showed a polymorphism due to a single stepwise mutation (6-bp microsatellite repeat) from 277 to 271 bp in isolate PR/D/03/424. As a result, this isolate belongs to multilocus genotype EU1MG5, while the other two belong to the main genotype EU1MG1 (Vercauteren et al. 2010).

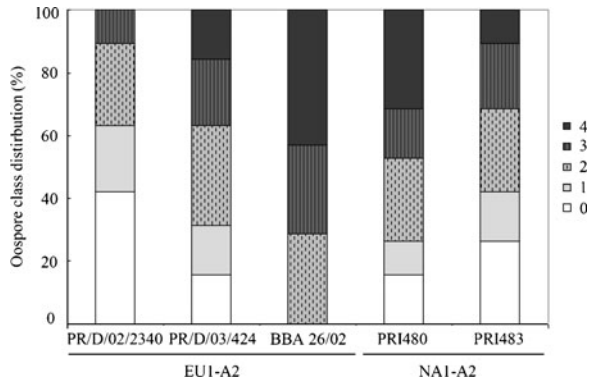

Fig. 1 Oogonia production of the A2 isolates of *Phytophthora ramorum* listed in Table 1 when paired with 20 EU1-A1 isolates. The numbers of oogonia produced were evaluated visually and divided into 5 classes (0 = no oogonia to 4 = more than 50 oogonia per pairing). Isolates marked with the same letter are not significantly different (Mann-Whitney; $P= 0.05$)

Phenotypic characterisation in vitro

No differences in in vitro growth rate were observed between lineages (3.1±0.1 mm day^{-1} versus 3.1 ± 0.2 mm day^{-1}, $P= 0.42$) or between EU1-A1 and EU1-A2 isolates (3.0±0.3 mm day^{-1} versus 3.2± 0.6 mm day^{-1}, $P= 0.065$). The in vitro growth rate was highest for EU1-A2 isolate BBA 26/02 and EU1-A1 isolate PR/D/06/08 (both 3.4 mm day^{-1}), but it was not significantly different from the other EU1-A2 isolates (Table 1). The smallest growth rate was observed for PR/D/03/336 (EU1-A1), which was significantly different from all other isolates.

In vitro sporangia production (in 10^4 sporangia cm^{-2}) was not significantly different between EU1-A1 and EU1-A2 isolates (6.2±0.5 versus 5.9±0.5, respectively; $P= 0.52$) or among EU1-A2 isolates ($P= 0.42$), but the EU1 lineage isolates produced significantly more sporangia than the NA1 lineage isolates (6.0±0.3 versus 2.9±0.6, $P< 0.0001$).

The three EU1-A2 isolates were sensitive to metalaxyl. Resistance was observed in the two NA1-A2 isolates, and in two of the EU1-A1 isolates (Table 1).

Pathogenicity tests

The pathogenicity assays on non-wounded leaves showed a significant effect of host ($P< 0.001$), (sub) lineage ($P= 0.033$), isolate nested within (sub)lineage ($P< 0.001$), and both interaction terms ($P< 0.01$). The NA1 isolates were significantly less aggressive than both EU1 groups ($P< 0.0001$). Within the EU1 lineage, EU1-A2 isolates were as aggressive as EU1-A1 isolates, except on *Rhododendron ponticum*, on which they were less aggressive ($P= 0.023$). Within the EU1-A2 isolates, no significant differences were detected ($P=0.99$). Among the hosts tested, *Rhododendron ponticum* and *Viburnum x bodnantense* 'Dawn' had the most susceptible leaves (Table 2).

Inoculation of wounded leaves showed fewer differences in aggressiveness between the isolates than inoculation of non-wounded leaves. No significant effect of (sub)lineage was observed ($P= 0.24$) and the interaction between (sub)lineage and host plant was also not significant ($P=0.32$). Among the isolates, some significant differences were detected ($P=0.029$), which were mostly host dependent ($P< 0.001$). Only a single significant difference was detected among the EU1-A2 isolates on a single host: on *Viburnum x bodnantense* 'Dawn', BBA 26/02 was significantly more aggressive than PR/D/02/2340 ($P= 0.015$) (Table 3). Using the means of all host plants for each isolate, a significant linear correlation was detected between the RLLA (non-wounded leaves) and the average lesion diameter (wounded leaves) ($R^2= 0.53$; $P= 0.017$).

For growth inside stem tissue only a significant effect of host plant was detected ($P< 0.001$). *Rhododendron ponticum* and *Rhododendron* 'Cunningham's White' had the highest lesion length. Stem tissue of *Fraxinus excelsior* was almost resistant to the *P. ramorum* isolates used. The other host plants had an intermediate sensitivity (Table 4).

Sporulation

For sporangia production, there was only a significant isolate effect nested within (sub)lineage ($P= 0.024$) and a significant interaction effect of isolates within (sub)lineage and host ($P=0.031$). BBA 26/02 produced significantly more sporangia per leaf than all other isolates ($P< 0.001$). Similar results were observed for sporangia per cm² of lesion area, with a significant effect for isolate nested within (sub)lineage ($P< 0.001$) and a significant interaction term with host ($P< 0.001$). On *Rhododendron* 'Cunningham's White', BBA 26/02 and PR/D/02/4294 were the

Table 2 Average[a] relative lesioned leaf area (in%) on five host plant species after inoculation with the EU1-A2 isolates or reference EU1-A1 and NA1-A2 isolates of *Phytophthora ramorum* (see Table 1) when using the assay with non-wounded leaves

Isolate	*Viburnum x bodnantense*	*Fraxinus excelsior*	*Rhododendron* C. White	*Rhododendron ponticum*	Average hosts
EU1-A1					
PR/D/02/880	75.4±2.7 bc	62.9±4.2 cd	38.1±4.2 abcd	83.2±2.7 cd	65.2±2.1 cd
PR/D/02/4294	79.8±2.4 c	69.9±3.8 d	43.2±4.6 bcde	86.1±2.2 d	69.7±2.1 cd
PR/D/03/336	64.7±4.5 ab	32.0±5.2 b	26.0±3.5 ab	75.3±4.4 bcd	52.0±2.8 ab
PR/D/06/08	86.2±1.8 c	55.5±5.5 cd	51.7±4.4 de	86.8±2.3 d	72.1±2.1 d
PRI 549	83.7±1.9 c	68.3±4.4 c	53.4±4.6 de	81.2±3.2 bcd	72.2±2.0 d
EU1-A2					
BBA 26/02	76.7±2.7 bc	62.1±4.4 cd	49.3±4.3 de	71.6±3.7 ab	64.0±2.1 bc
PR/D/02/2340	81.9±2.3 c	70.5±4.4 cd	32.2±3.8 de	76.4±3.4 bcd	64.5±2.2 cd
PR/D/03/424	75.2±2.9 bc	52.0±4.6 c	42.9±4.2 cde	72.8±3.6 bc	62.0±2.1 bc
NA1-A2					
PRI 480	61.1±3.5 a	23.2±4.1 ab	46.6±4.1 cde	69.3±3.9 ab	53.9±2.2 b
PRI 483	59.9±3.8 a	8.5±1.8 a	24.8±3.2 a	60.7±4.5 a	42.7±2.4 a
Average	74.8±1.0	51.1±1.8	41.3±1.4	76.5±1.1	62.1±0.7

[a] Data are averages (± SE) of three experiments. Within each host, isolates marked with the same letter are not significantly different (GLM post hoc Unequal N HSD; $P= 0.05$).

Table 3 Average[a] lesion diameter (in mm) of eight host plant species after inoculation with the EU1-A2 isolates or reference EU1-A1 and NA1-A2 isolates of *Phytophthora ramorum* (see Table 1) when using the assay with wounded leaves

Isolate	*Fraxinus excelsior*	*Rhododendron* C. White	*Rhododendron ponticum*	*Viburnum x bodnantense*	*Castanea sativa*	*Quercus rubra*	*Viburnum opulus*	*Fagus sylvatica*	Average hosts
EU1-A1									
PR/D/02/880	12.7±0.4 b	13.3±0.5 ab	19.8±0.6 a	14.6±0.5 bcd	3.5±0.3 a	3.9±0.2 ab	6.8±0.4 a	2.6±0.1 ab	10.3±0.3 a
PR/D/02/4294	12.4±0.3 ab	14.1±0.5 b	19.2±0.8 a	14.4±0.6 bcd	3.1±0.2 a	3.6±0.1 a	8.3±0.5 a	2.7±0.2 bc	10.2±0.3 a
PR/D/03/336	12.4±0.4 ab	12.1±0.4 ab	17.9±0.7 a	12.3±0.4 ab	4.0±0.3 a	3.3±0.2 a	7.1±0.4 a	3.1±0.2 ab	9.6±0.3 a
PR/D/06/08	12.8±0.3 b	14.7±0.5 b	25.3±1.2 b	14.5±0.7 bcd	3.9±0.4 a	3.3±0.2 a	8.1±0.5 a	3.2±0.2 bc	9.7±0.4 a
PRI 549	12.7±0.3 b	12.8±0.4 ab	20.0±0.9 a	16.8±0.4 d	3.5±0.3 a	4.5±0.2 b	7.9±0.3 a	3.0±0.1 bc	10.8±0.3 a
EU1-A2									
BBA 26/02	13.0±0.3 b	13.2±0.5 ab	18.9±0.7 a	14.9±0.5 cd	4.1±0.3 a	3.8±0.1 ab	7.1±0.6 a	2.8±0.1 ab	10.3±0.3 a
PR/D/02/2340	12.5±0.3 ab	12.0±0.5 ab	18.9±0.8 a	11.3±0.9 a	3.4±0.2 a	3.3±0.2 a	7.7±0.5 a	3.0±0.2 abc	9.6±0.3 a
PR/D/03/424	13.5±0.5 b	13.5±0.3 b	18.6±0.6 a	13.3±0.4 abc	3.8±0.2 a	3.6±0.1 a	7.7±0.6 a	2.3±0.1 a	10.0±0.3 a
NA1-A2									
PRI 480	11.0±0.3 a	12.1±0.5 ab	20.7±0.8 a	13.2±0.7 abc	3.0±0.2 a	3.4±0.1 a	7.6±0.6 a	3.5±0.2 c	10.0±0.3 a
PRI 483	11.0±0.3 a	11.3±0.4 a	18.4±0.7 a	14.0±0.5 bc	3.5±0.2 a	3.6±0.2 a	6.7±0.5 a	2.9±0.1 ab	9.4±0.3 a
Average	12.4±0.1	12.7±0.2	19.4±0.2	13.9±0.2	3.6±0.1	3.6±0.1	7.5±0.2	2.9±0.0	10.0±0.1

[a] Data are averages (± SE) of three experiments. Within each host, isolates marked with the same letter are not significantly different (GLM post hoc Unequal N HSD; $P = 0.05$).

Table 4 Average[a] lesion length (in mm day^{-1}) of eight host plant species after inoculation with the EU1-A2 isolates or reference EU1-A1 and NA1-A2 isolates of *Phytophthora ramorum* (see Table 1) when using the assay with wounded stems

Isolate	*Fraxinus excelsior*	*Rhododendron* C. White	*Rhododendron ponticum*	*Viburnum x bodnantense*	*Castanea sativa*	*Quercus rubra*	*Viburnum opulus*	*Fagus sylvatica*	Average hosts
EU1-A1									
PR/D/02/880	0.6±0.1	6.2±0.7	8.5±0.7	2.1±0.2	3.1±0.2	2.7±0.3	3.5±0.2	1.4±0.0	3.3±0.2
PR/D/02/4294	0.8±0.2	5.7±0.5	8.4±0.7	2.2±0.1	2.9±0.2	2.7±0.2	3.4±0.2	1.4±0.0	3.3±0.2
PR/D/03/336	0.8±0.1	5.4±0.3	7.1±0.5	2.6±0.2	2.8±0.2	2.5±0.3	3.6±0.2	1.4±0.0	3.1±0.2
PR/D/06/08	nd[b]	nd	nd	1.8±0.1	nd	nd	3.2±0.2	1.3±0.0	nd
PRI 549	0.7±0.1	5.9±0.5	7.6±0.5	2.3±0.2	3.1±0.2	2.5±0.2	3.5±0.2	1.5±0.1	3.3±0.2
EU1-A2									
BBA 26/02	0.6±0.1	6.5±0.7	8.3±0.7	2.2±0.1	3.0±0.2	2.7±0.3	3.5±0.2	1.5±0.1	3.3±0.2
PR/D/02/2340	0.7±0.1	6.1±0.5	8.0±0.6	2.2±0.1	2.9±0.2	2.8±0.3	3.4±0.2	1.3±0.1	3.3±0.2
PR/D/03/424	0.9±0.1	6.7±0.7	8.0±0.5	2.1±0.2	3.0±0.2	2.5±0.3	3.5±0.2	1.5±0.0	3.4±0.2
NA1-A2									
PRI 480	0.6±0.1	5.5±0.7	8.5±0.5	2.1±0.1	2.8±0.2	2.7±0.3	3.4±0.2	1.4±0.1	3.1±0.2
PRI 483	0.6±0.1	6.7±0.6	8.2±0.7	2.0±0.1	3.0±0.2	2.7±0.3	3.3±0.2	1.2±0.1	3.2±0.2
Average	0.6±0.0	6.1±0.2	7.7±0.2	2.2±0.1	2.8±0.1	2.6±0.1	3.4±0.1	1.4±0.0	3.1±0.1

[a] Data are averages (± SE) of 20 stems. Within each host, no significant differences were observed between the isolates (GLM post hoc Unequal N HSD; $P = 0.05$).

[b] *nd* not determined

highest sporangia producers with a significant difference between BBA 26/02 and PR/D/03/424 ($P= 0.025$) (Table 5). On *Viburnum x bodnantense* 'Dawn', BBA 26/02 produced significantly more sporangia than all other isolates ($P< 0.001$). Using the 10 isolates, no significant linear correlation between in vitro and in vivo sporangia production was detected for *Viburnum x bodnantense* 'Dawn' ($P= 0.722$; $R^2= 0.0193$) or *Rhododendron* 'Cunningham's White' ($P= 0.662$; $R^2= 0.0289$). The average number of sporangia on the 10-day-old inoculated leaves was 15.8 cm^{-2} lesioned tissue in *Rhododendron* 'Cunningham's White' and 23.9 cm^{-2} lesioned tissue on *Viburnum x bodnantense* 'Dawn'.

When comparing sporangia production between BBA 26/02 and PR/D/02/880, BBA 26/02 produced significantly more sporangia per cm^2 of lesion on *Fraxinus excelsior*, *Rosa canina*, *Quercus rubra*, *Castanea sativa,* and *Rhododendron ponticum* (Table 6). These host plants all produced large numbers of sporangia per cm^2 of lesion. Fewer sporangia were produced per leaf in *Rosa canina* and *Quercus rubra*, owing to the smaller size of the leaf lesions. In contrast, *Rhododendron ponticum* and *Fraxinus excelsior* have highly susceptible leaves and can support abundant sporulation under optimal laboratory conditions. No sporangia were formed on the surface of the leaves of *Fagus sylvatica*.

For chlamydospore production, the average number produced per unit of lesion area was much higher and less variable than the number of sporangia produced per unit of lesion area. It ranged from 1,497 in *Viburnum x bodnantense* 'Dawn' to 1,759 chlamydospores per cm^2 lesion in *Rhododendron* 'Cunningham's White'. Using all 10 isolates, there was only a significant effect of host ($P< 0.001$) and its interaction with isolate nested within (sub)lineage ($P= 0.003$). On *Viburnum x bodnantense* 'Dawn', BBA 26/02 produced significantly fewer chlamydospores than five other isolates, including the other two EU1-A2 isolates (Table 5). The smaller number of chlamydospores produced by BBA 26/02 was also observed on the nine extra hosts (Table 6). This difference was significant for three host species (Table 6).

Using the individual leaf data and all isolates, a significant negative linear relationship was detected between the number of sporangia and the number of chlamydospores produced on *Viburnum x bodnantense* 'Dawn' ($R^2= 0.066$; $P< 0.001$). Averaged per isolate the results were even more pronounced ($R^2= 0.55$, $P= 0.013$). However, this significant linear relationship is the result of isolate BBA 26/02 only, which produces more sporangia and fewer chlamydospores than the other isolates on *Viburnum x bodnantense* 'Dawn'. Without this isolate, there was no linear relationship ($R^2= 0.005$; $P= 0.86$).

No significant linear relationship was observed between the production of the two spore types on *Rhododendron* 'Cunningham's White' using the individual leaf data ($P= 0.79$) or averaged per isolate ($P=0.93$).

Discussion

Mating type distribution in the Belgian *P. ramorum* population

Monitoring of A1 and A2 mating type distribution is essential when estimating the possibility and extent of sexual recombination (Brasier and Kirk 2004). Mating type assays with 126 European isolates of *P. ramorum* were first reported by DEFRA Science and Research (2004). In 60 of these, oogonia were produced and all isolates were identified as EU-A1. About 300 European isolates were later screened in the framework of the RAPRA project (RAPRA 2009; Werres and Kaminski 2005). This led to the finding of one EU1-A2 isolate that had been collected in 2002 on *Viburnum x bodnantense* 'Dawn' in a Belgian nursery (RAPRA 2009; Werres and De Merlier 2003). This finding prompted our screening of the entire Belgian population of *P. ramorum*, and resulted in the identification of two additional EU1-A2 isolates in this study.

The mating-type assay, based on the pairing of an unknown isolate with known A1 and A2 tester strains and screening for oogonia production is reliable, as selfing has not been reported in field isolates of *P. ramorum* (Boutet et al. 2009; Boutet et al. 2010). However, formation of oogonia is sometimes unsuccessful, requiring multiple mating experiments with some isolates. Oogonia production can be affected by nutrients (e.g., the type of carrots used in the medium, data not shown) and the strain (Boutet et al. 2009).

Table 5 Average[a] numbers of sporangia produced per leaf and per cm^2 of lesion and number of chlamydospores produced per cm^2 of lesion with the EU1-A2 isolates or reference EU1-A1 and NA1-A2 isolates of *Phytophthora ramorum* (see Table 1) on two host plant species

Isolate	Sporangia per leaf			Sporangia per cm^2 lesion			Chlamydospores per cm^2 lesion		
	Viburnum x bodnantense	*Rhododendron* C. White	Average	*Viburnum x bodnantense*	*Rhododendron* C. White	Average	*Viburnum x bodnantense*	*Rhododendron* C. White	Average
EU1-A1									
PR/D/02/880	126.5±21.1 a	137.4±39.5 a	131.9±22.3 a	15.4±2.5 a	10.8±2.7 a	13.2±1.8 a	1,393.2±96.1 ab	1,754.1±72.6 a	1,564.1±67.2 ab
PR/D/02/4294	89.1±20.2 a	447.4±159.9 ab	261.2±79.7 a	17.8±5.2 a	36.2±11.7 b	26.4±6.2 a	1,732.1±100.5 a	1,875.9±69.1 a	1,802.0±62.0 b
PR/D/03/336	160.6±26.3 a	105.6±46.4 a	133.4±26.5 a	21.8±3.1 a	7.7±2.4 a	14.9±2.1 a	1,348.0±74.9 ab	1,886.2±61.9 a	1,617.1±64.5 ab
PR/D/06/08	107.8±19.5 a	9.7±3.3 a	58.8±11.3 a	13.5±2.1 a	1.6±0.4 a	8.9±1.5 a	1,687.0±94.0 a	1,812.2±85.6 a	1,749.6±63.5 b
PRI 549	61.5±13.1 a	301.6±126.4 a	181.5±64.6 a	8.7±2.5 a	16.2±6.1 ab	12.2±3.1 a	1,584.3±114.4 a	1,728.2±62.9 a	1,656.3±65.4 ab
EU1-A2									
BBA 26/02	685.5±100.9 b	828.2±248.1 b	756.8±133.3 b	94.9±12.4 b	38.6±9.7 b	63.4±8.4 b	1,095.1±83.0 b	1,723.4±77.0 a	1,401.2±75.7 a
PR/D/02/2340	160.3±21.4 a	291.2±113.1 a	225.7±57.7 a	21.0±3.5 a	19.1±6.2 ab	20.1±3.4 a	1,605.7±104.7 a	1,620.7±64.4 a	1,613.2±60.6 ab
PR/D/03/424	200.1±39.8 a	97.8±33.3 a	149.0±26.4 a	25.2±5.4 a	6.7±1.3 a	16.0±2.9 a	1,595.3±92.5 a	1,811.5±81.2 a	1,703.4±63.2 b
NA1-A2									
PRI 480	83.4±13.2 a	83.0±19.6 a	83.2±11.7 a	12.2±2.1 a	7.1±1.6 a	9.7±1.3 a	1,435.3±108.0 ab	1,711.1±83.9 a	1,573.2±71.2 ab
PRI 483	168.4±35.7 a	109.8±36.7 a	139.1±25.7 a	26.2±5.6 a	11.6±2.4 a	19.4±3.3 a	1,519.1±107.0 ab	1,656.4±91.3 a	1,582.2±71.4 ab
Average	184.3±15.1	239.9±36.3	212.0±19.6	23.9±1.9	15.8±1.9	20.1±1.3	1,496.9±32.9	1,758.9±24.1	1,625.5±21.5

[a] Data are averages (± SE) of two experiments. Within each column, isolates marked with the same letter are not significantly different (GLM post hoc Unequal N HSD; $P = 0.05$).

Table 6 Average[a] numbers of sporangia and chlamydospores produced per cm^2 of lesion with the EU1-A2 *Phytophthora ramorum* isolate BBA 26/02 and a reference EU1-A1 isolate (PR/D/02/880) on nine host plant species

Host plant	Sporangia per leaf		Sporangia per cm^2 lesion		Chlamydospores per cm^2 lesion	
	PR/D/02/880	BBA 26/02	PR/D/02/880	BBA 26/02	PR/D/02/880	BBA 26/02
Castanea sativa	697.1±182.0	845.6±242.8	172.0±61.3	247.2±61.3 *	111.4±23.0	92.7±15.9
Fagus sylvatica	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Fraxinus excelsior	721.8±271.7	497.0±156.6	102.1±35.9	247.2±61.6 *	298.9±33.3	145.5±24.9 *
Quercus robur	4.4±2.6	1.5±1.5	5.6±2.8	233.1±86.1	37.6±21.6	2.4±2.4
Quercus rubra	115.5±33.0	129.9±33.3	208.7±40.1	2.0±2.0	168.6±29.7	51.7±18.9 *
Rhododendron ponticum	1871.3±353.0	5420.0±826.1 *	96.3±19.3	393.9±106.1 *	555.2±62.8	459.3±52.6
Rosa canina	6.3±3.0	7.6±4.9	171.3±46.6	279.8±46.3	167.9±64.4	49.4±22.0
Sambucus nigra	24.8±11.7	1.4±0.9	49.3±14.9	13.9±10.5	0.0±0.0	0.0±0.0
Viburnum opulus	4.2±2.0	0.3±0.3 *	11.8±5.7	9.9±9.9	94.1±25.9	12.1±4.8 *

[a] Data are averages (± SE) of 20 leaves. Within each host, the isolates that are significantly different (Mann-Whitney; $P < 0.05$) are marked with *.

Genotypic characterization of the EU1-A2 isolates

One of the purposes of the genotypic characterization was to test whether the Belgian A2 isolates were introduced by a separate migration event or whether they were the result of sexual recombination, mitotic crossing over, mutation, or mitotic gene conversion events in the original EU1 population. Characterization of the three A2 isolates with the *cox* RFLP, AFLP, and SSR markers revealed that all three belong to the EU1 lineage and show only one polymorphism at the markers tested. Therefore, the A2 isolates were probably not introduced by separate migration. This contrasts with *P. infestans,* where the migration hypothesis was upheld for the occurrence of the A2 mating type in Europe and Japan (Goodwin 1997). There is no support for the hypothesis of sexual recombination or mitotic crossing over in the EU1-A2 isolates either. Given the large amount of heterozygosity in *P. ramorum* (Vercauteren et al. 2010), marker recombination would be easy to detect, but of the 119 microsatellite primer pairs tested, all alleles were present in the three EU1-A2 isolates and no allelic rearrangements were found. The EU1-A2 isolates were not the result of selfing, as this would also have resulted in marker recombination, which was not observed. The hypothesis that the EU1-A2 isolates originated by mutation or mitotic gene conversion from the EU1-A1 mating type population is the remaining, and most likely, explanation. The question then arises whether the three isolates were clonally derived from a single mating-type-changed ancestor, or whether the mutation(s) or mitotic gene conversion(s) leading to the mating type change happened independently in the three isolates. Genotyping of the EU1-A2 isolates based on the 119 microsatellite primer pairs showed no differences between the three isolates, except for a single stepwise mutation in the hypervariable locus ILVOPrMS145c in isolate PR/D/03/42, which places this isolate in genotype group EU1MG5 (Vercauteren et al. 2010). EU1MG5 is the second largest genotype group in Belgium, representing 7.3% of the Belgian population. Given the relatively high frequency of this microsatellite mutation (12.7% of all Belgian isolates) compared to the very low frequency of the mating type change mutation, it is likely that the EU1MG1 to EU1MG5 genotype switch in PR/D/03/424 occurred after the mating type change.

Phenotypic characterization of the EU1-A2 isolates

Phenotypic characterisation showed a limited variation in growth rate, aggressiveness and sporulation potential. Differences between the isolates were not the result of differences in culture storage time: tests with isolate PR/D/02/880 over a 3-year period showed no negative effect of culture storage on growth rate in vitro or on pathogenicity to leaves of *Rhododendron* 'Cunningham's White' (data not shown).

Brasier et al. (2006) found no differences in in vitro growth rate and colony type between the

Belgian A2 isolate BBA 26/02 and other EU1 isolates. However, Werres and Kaminski (2005) observed a higher growth rate (3.4 mm day^{-1}) for this isolate compared to the other EU1 isolates (3.1 mm day^{-1}), which is in accordance with our study.

No single inoculation method can predict the full range of symptoms observed in the field, but the combination of the three pathogenicity tests used on multiple hosts can give a good indication of the aggressiveness of the isolates. Of the three tests used, most differences between isolates were observed with the assay with non-wounded leaves. Averaged over all host plants, no differences were detected within EU1-A2 or between EU1-A1 and EU1-A2 isolates. However, some variation was detected on specific hosts. EU1-A1 isolates PR/D/06/08 and PRI549 seem to be the most aggressive isolates on *Viburnum* and *Rhododendron*, whereas PR/D/03/336 had a low pathogenicity compared to the other EU1 isolates tested. Some differences in aggressiveness among the EU1-A2 isolates and between EU1-A2 and EU1-A1 were also detected on specific hosts. However, the number and the nature of the differences in pathogenicity among the EU1-A2 isolates seem comparable to those between EU1-A1 isolates. NA1 isolates PRI483 and PRI480 were on average less aggressive than the EU1 isolates. The growth rates of the EU1 isolates were also higher and more uniform than those of the NA1 isolates. Previous studies showed similar phenotypic differences between European and North American isolates (Brasier 2003; Brasier et al. 2006; DEFRA Science and Research 2004; Grünwald et al. 2008a; Hüberli et al. 2006; Tooley et al. 2004; Werres and Kaminski 2005). However, other studies did not report any differences on a variety of other host species (Denman et al. 2005; Grünwald et al. 2008b; Hüberli et al. 2006; Tooley et al. 2004; Werres and Kaminski 2005). Variation in aggressiveness among isolates within both NA1 and EU1 lineages has been documented on coast live oak seedlings, detached bay laurel leaves and *Q. rubra* cut stems (Brasier et al. 2006; Hüberli et al. 2006). Differences in pathogenicity were only observed on specific hosts. The host of origin was not indicative of higher or lower aggressiveness on other hosts. Host preference seems rare in *P. ramorum* (Vercauteren et al. 2010). In the few cases where specific genotypes are associated with a specific host, this is probably because the host is grown and traded in specialized single-host nurseries and/or because the genotype has not yet had the chance to spread widely rather than increased pathogenicity of that genotype on the specific host.

No significant differences were observed in in vitro sporangia production. On leaves, sporangia production ranged from 10^1 to 10^3 units per cm^2 of leaf lesion, which is in accordance with previous studies (McDonald et al. 2006; Tooley et al. 2004). Due to the large variability in sporulation between replicate leaves, significant differences in sporulation among the isolates were limited. The main difference related to EU1-A2 isolate BBA 26/02, which produced significantly more sporangia per cm^2 on five out of eleven host plants. Based on these data, in vitro sporulation was not a good predictor of the in vivo sporulation.

Chlamydospore production was less variable between leaves of the same host, ranging from 1 to 2×10^3 units cm^{-2} in *Viburnum x bodnantense* 'Dawn' and *Rhododendron* 'Cunningham's White', similar to the levels reported by Tooley et al. (2004). The number of chlamydospores seems to be negatively correlated with the sporangia production. This was especially noted in BBA 26/02 on *Viburnum x bodnantense* 'Dawn'. It would seem that this isolate allocates its finite resources to the production of sporangia, and not chlamydospores.

The phenotypic characterization would have been more powerful when based on a larger number of isolates, especially those of the EU1-A1 group. However, genotyping of the EU1 lineage isolates using AFLP and 119 SSR markers in this study, of 411 Belgian isolates with polymorphic SSR and AFLP markers in Vercauteren et al. (2010), and of more than 800 non-Belgian EU1 isolates using the same polymorphic SSR markers in an ongoing study (data not shown), all indicate very limited genetic diversity in this lineage, reducing the need to use a large number of isolates. Given that differences in pathogenicity were few and host dependent, we had to include a relatively large number of hosts. As the differences between the means were small, while in some cases the variability was not, we also had to use a large number of replicates and needed to replicate the experiments in time. These needs, together with a relatively small availability of EU1-A1 isolates from the same time period as the EU1-A2 isolates and from

different hosts, limited our ability to use a larger number of EU1-A1 isolates.

Origin of *P. ramorum* EU1-A2 population

Overall, a very limited number of genotypic and phenotypic differences was observed among the EU1-A2 isolates and between the EU1-A2 and EU1-A1 isolates. The only genotypic difference observed with the applied analytical methods was a single stepwise mutation in a hypervariable microsatellite marker of one isolate. Using three pathogenicity assays, only a single difference was observed on a single host among the three EU1-A2 isolates and none was observed between the EU1-A2 and EU1-A1 isolate groups. The sporulation data indicated few differences between the isolates: only BBA 26/02 produced significantly more sporangia and fewer chlamydospores on selected hosts compared to some of the other isolates. As the level of differences within and between the EU1-A1 and EU1-A2 populations is similar, it is difficult to determine whether the EU1-A2 isolates evolved out of one mating type change from a EU1-A1 isolate several years ago, allowing a similar amount of divergence as the EU1-A1 population, or whether several mating type changes took place independently. Presumably, the mutation or mitotic gene conversion responsible for the mating type change is a very rare event, which would favour the first hypothesis. However, the genetic basis of the mating type of *Phytophthora* is not yet known (Judelson 1996). As a result, the identification and subsequent comparison of many more molecular markers in these isolates, especially the specific mutations that are responsible for the mating type change, is needed to help distinguish between the hypotheses. This may become possible once high-throughput sequencing is more affordable.

Belgium is the only country where EU1-A2 isolates were detected, but these isolates may have been present in other European countries. The host plants for two of the three EU1-A2 isolates were imported into Belgium the year before detection, from two EU countries. Also, the three EU1-A2 isolates were found in three nurseries that have no obvious commercial link, on two plant species and in 2 years. If the EU1-A2 isolates were all derived from a single mutation or gene conversion event, then the extent of spread in space, time, and host may indicate that the isolates were spread internationally via the commercial nursery circuit several years before being identified. Given the low frequency of A2 isolates (0.73%) in Belgium, the only reason why three EU1-A2 isolates were identified in this country and not in other EU countries may be due to coincidence and the large number of Belgian isolates that were screened. Determining the true distribution of the EU1-A2 isolates would require the screening of all European isolates, but only a limited number of EU isolates has been collected.

Current risk for sexual recombination in Europe

No EU1-A2 isolates were found after 2003, even though 81.3% of the isolates in this study were collected during 2004 or later. This suggests that the EU1-A2 isolates may have been eliminated from the Belgian population due to control and quarantine measures. There are several arguments to support this hypothesis. First, all EU1-A2 isolates were sensitive to metalaxyl. In Vercauteren et al. (2010), a high selection pressure for metalaxyl-resistant isolates in 2004 and 2005 was shown, which had a high impact on genetic and genotypic diversity. Second, eradication efforts had a higher chance of eliminating the relatively small EU1-A2 population (genetic drift). Third, Mascheretti et al. (2008) hypothesized that during an unfavourable season, populations of the pathogen crash to a basal level and individual isolates need to recolonize infested sites at the beginning of the growing season. These seasonal variations offer an advantage to the already established EU1-A1 isolates during the recolonization process and may also cause the extinction of the EU1-A2 isolates through drift. In this scenario, the European A2 isolates detected could also represent the relics of a more important A2 presence in an earlier period.

In the unlikely case that EU1-A2 isolates would still exist and co-occur in nurseries where EU1-A1 isolates are also present, then there is a chance for recombination, considering that the mating system is functional (Boutet et al. 2010). However, even though progeny could be created in in vitro matings, viable oospores could only be generated at a very low frequency (Boutet et al. 2010). Oogonia could not be detected in leaves when two point inoculations were made with both mating types (Boutet, personal communication). Therefore, presence of both mating

types at a single site may still pose a limited risk. This is supported by the situation in North America, where isolates of EU1-A1 and NA1-A2 have been detected at one site but where no indications of sexual reproduction could be found (Grünwald et al. 2008c; Prospero et al. 2009).

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