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Characterization of Alder *Phytophthora* Isolates from Wallonia and Development of SCAR Primers for their Specific Detection

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Abstract

Isolates of alder *Phytophthora* were collected in the southern part of Belgium on riverbanks planted with *Alnus glutinosa* and *A. incana*. They were compared with strains isolated in other European countries in terms of maximum temperature for growth, oogonia shape, pathogenicity on *Alnus* seedlings and genetic traits. Using both molecular techniques [random amplified polymorphic DNA (RAPD) and random amplified microsatellite (RAMS)], two groups of isolates were identified, the first group being further divided into two subgroups, Ia and Ib, using RAPD. Most of the Walloon alder *Phytophthora* isolates as well as the standard type from UK (formally designated *P. alni* subsp. *alni*) fell into group Ia. One isolate was classified in group Ib with the German and Dutch variants (*P. alni* subsp. *multiformis*), while three isolates were placed with the Swedish variant (*P. alni* subsp. *uniformis*) in group II. In terms of morphological properties, isolates from groups Ia and Ib developed colonies with a felt-like appearance and usually produced numerous oogonia, varying from wavy to warty after 1 week (group Ia) or 2–3 weeks (Ib) in darkness. In contrast, colonies from group II isolates were generally irregular, and smooth oogonia were produced in low quantities after approximately 1 month in culture. A polymerase chain reaction (PCR) using sequence-characterized amplification region (SCAR) primers derived from a polymorphic amplification product generated with a RAPD primer was developed for the specific detection of alder *Phytophthora*. The specificity and sensitivity of this test are discussed here.

Introduction

In 1993, a severe decline of riparian alders (*Alnus* spp.) was observed in southern Britain (Gibbs et al., 1994). Affected trees displayed crown dieback, inner bark lesions at the stem base with sometimes tarry or rusty exudations. A high level of mortality was observed in trees from some regions of England (Gibbs, 1995). A *Phytophthora* which resembles *P. cambivora* in its gametangial and sporangial morphology, but presented homothalism (self-fertility) rather than outcrossing and a high frequency of zygotic abortion, was identified as the causal agent (Brasier et al., 1995). Brasier et al. (1999) showed that the causal organism comprises a range of heteroploid isolates generated by hybridization, probably between *P. cambivora* and a taxon closely related to *P. fragariae*. By comparing isolates from different European countries, they demonstrated that the 'alder *Phytophthora* population' could be divided into two groups, the standard and the variant isolates. The standard isolates, recently designated *P. alni* subsp. *alni* (Brasier et al., 2004) are near tetraploid ($n = 18–22$) and unable to complete meiosis. In contrast, the variant type comprises a range of isolates, some of them being close to diploid and normal in their nuclear behaviour, and others exhibiting intermediate chromosomes numbers and irregularities similar to standard genotypes regarding meiosis. An analysis of the internal transcribed spacer (ITS1 and ITS2) region revealed that *P. alni* subsp. *alni* isolates display dimorphic ITS arrays while the variants are either monomorphic for the same genome region (Dutch, German and UK variant – formally designated *P. alni* subsp. *multiformis*; Brasier et al., 2004) or monomorphic at some sites and dimorphic at other sites (Swedish variant – formally designated *P. alni* subsp. *uniformis*; Brasier et al., 2004). Further studies showed that the mean oospore viability in *P. alni* subsp. *alni* isolates is very low (approximately 30%), while it varies between 25 and 75% in *P. alni* subsp. *multiformis* and *P. alni* subsp. *uniformis* isolates respectively. Moreover, attempts to induce oospore germination were unsuccessful in all cases (Delcan and Brasier,

2001). Since its first identification, the disease has been recorded in several European countries (for a review, see Streito, 2003). In Belgium, this 'alder *Phytophthora*' was isolated for the first time in 1999 from a stem lesion on a common alder (*A. glutinosa*) (Cavelier et al., 1999). A survey carried out in 2002 in the southern part of Belgium showed that the disease occurred along the vast majority of rivers and had attacked approximately 28% of the riverside alders (Debruxelles et al., 2003). The objectives of this study were to compare genetic and phenotypic traits of alder *Phytophthora* isolates found in Wallonia with those of reference isolates from other countries, as well as to develop a polymerase chain reaction (PCR) for the specific detection of alder *Phytophthora* isolates.

Materials and Methods

Isolates and culture conditions

The isolates used in this study are described in Table 1. The Belgian alder *Phytophthora* isolates (17

Table 1
List of isolates used in this study

Isolate	Species	Host	Country, location	Sampling date	Reference
2198	*Phytophthora* sp.	*Alnus glutinosa*	Belgium, Vielsalm	9/1999	–
2199	*Phytophthora* sp.	*Alnus glutinosa*	Belgium, Vielsalm	9/1999	–
2200	*Phytophthora* sp.	*Alnus glutinosa*	Belgium, Vielsalm	8/2000	–
2201	*Phytophthora* sp.	*Alnus glutinosa*	Belgium, Vielsalm	8/2000	–
2202	*Phytophthora* sp.	*Alnus glutinosa*	Belgium, Vielsalm	8/2000	–
2270	*Phytophthora* sp.	*Alnus glutinosa*	Belgium, Stoumont	2/2001	–
2271	*Phytophthora* sp.	*Alnus glutinosa*	Belgium, Stavelot	2/2001	–
2272	*Phytophthora* sp.	*Alnus glutinosa*	Belgium, Chiny	3/2001	–
2273	*Phytophthora* sp.	*Alnus glutinosa*	Belgium, Vielsalm	3/2001	–
2274	*Phytophthora* sp.	*Alnus glutinosa*	Belgium, Fosses-la-Ville	4/2001	–
2275	*Phytophthora* sp.	*Alnus glutinosa*	Belgium, Quiévrain	4/2001	–
2276	*Phytophthora* sp.	*Alnus glutinosa*	Belgium, Mons	4/2001	–
2277	*Phytophthora* sp.	*Alnus incana*	Belgium, Habay-la-Neuve	4/2001	–
2278	*Phytophthora* sp.	*Alnus glutinosa*	Belgium, Rochefort	4/2001	–
2279	*Phytophthora* sp.	*Alnus glutinosa*	Belgium, Esneux	5/2001	–
2280	*Phytophthora* sp.	*Alnus glutinosa*	Belgium, Rochefort	7/2001	–
2286	*Phytophthora* sp.	*Alnus glutinosa*	Belgium, Tellin	8/2001	–
2287	*Phytophthora* sp.	*Alnus* sp.	Netherlands, De Wieden	ND	Clive Brasier, UK[a] (P972)
2288	*Phytophthora* sp.	*Alnus glutinosa*	UK, South Yorkshire	1994	Clive Brasier, UK[a] (P772)
2289	*Phytophthora* sp.	*Alnus* sp.	Sweden, Gothenburg	ND	Clive Brasier, UK[a] (P875)
2291	*Phytophthora* sp.	*Alnus incana*	Germany, Freising	1995	Clive Brasier, UK[a] (P889R)
2295	*Phytophthora* sp.	*Alnus glutinosa*	Belgium, Wellin	11/2001	–
2303	*Phytophthora* sp.	*Alnus incana*	Belgium, Grez-Doiceau	1/2002	–
2304	*Phytophthora* sp.	*Alnus incana*	Belgium, Grez-Doiceau	1/2002	–
2305	*Phytophthora* sp.	*Alnus incana*	Belgium, Grez-Doiceau	1/2002	–
2306	*Phytophthora* sp.	*Alnus incana*	Belgium, Grez-Doiceau	1/2002	–
2349	*Phytophthora* sp.	*Alnus incana*	Belgium, Grez-Doiceau	6/2002	–
2354	*Phytophthora* sp.	*Alnus incana*	Belgium, Grez-Doiceau	6/2002	–
2216	*P. fragariae*	ND	ND	ND	J. C. Streito, France[b] (34′)
2207	*P. cambivora*	*Castanea* sp.	France, Corrèze	1994	C. Robin, France[c] (450)
2208	*P. cambivora*	*Quercus petraea*	France, Haute Vienne	1999	C. Robin, France[c] (599)
2284	*P. cambivora*	ND	Hungary	1997	MUCL, Belgium[e] (43488)
2290	*P. cambivora*	*Quercus* soil	UK, Alice Holt Forest	1998	Clive Brasier, UK[a] (P1002)
2292	*P. cambivora*	*Quercus* soil	UK, Northamptonshire	1998	Clive Brasier, UK[a] (P1010)
2542	*P. cactorum*	*Fragaria* sp.	Belgium, Gembloux	2003	CRA-W, Belgium
2282	*P. cactorum*	*Pyrus communis*	Belgium, Hacquegnies	1956	MUCL, Belgium[e] (213)
2204	*P. cinnamomi*	*Castanea sativa*	France, Dordogne	1989	C. Robin, France[c] (299)
2205	*P. cinnamomi*	*Quercus rubra*	France, Dordogne	1995	C. Robin, France[c] (571)
2206	*P. cinnamomi*	*Quercus suber*	France, Pyrénées	1996	C. Robin, France[c] (578)
2353	*P. cinnamomi*	Soil	Australia	2001	MUCL, Belgium[e] (43491)
2355	*P. gonapodyides*	ND	ND	1967	CBS[d] (554.67)
2294	*P. citricola*	Soil	Belgium, Gembloux	2001	CRA-W, Belgium
2356	*P. citricola*	*Pinus resinosa*	ND	1925	CBS[d] (181.25)
2385	*P. europae*	*Quercus* soil	France, Mersuay	1999	C. Delatour, France[c] (2AV2)
2338	*P. ramorum* A2	*Viburnum* sp.	Belgium, Gembloux	2002	CRA-W, Belgium
2387	*P. ramorum* A1	*Rhododendron*	Belgium, Gembloux	2002	CRA-W, Belgium
2283	*P. syringae*	*Cymbidium* sp.	Australia	2001	MUCL, Belgium[e] (43495)
2281	*P. cryptogea*	*Gerbera* sp.	The Netherlands	1971	MUCL, Belgium[e] (28777)
2138	*Verticillium* sp.	*Acer campestre*	Belgium	1998	CRA-W, Belgium
2217	*Fusarium* sp.	*Triticum aestivum*	Belgium	2001	CRA-W, Belgium

ND, not determined.
[a]Forest Research Station, Farnham, UK.
[b]Laboratoire National de la Protection des Végétaux, Nancy, France.
[c]INRA, Bordeaux, France.
[d]Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands.
[e]Belgian Coordinated Collection of Microorganisms (BCCM), Louvain La Neuve, Belgium.

from *A. glutinosa* and seven from *A. incana*) were collected from trees located along the rivers in Wallonia (the southern part of Belgium). They were obtained from bark tissue displaying necrosis symptoms. Pieces of bark and cambium were excised at the margin of the necrosis, surface-disinfested with a 1 : 10 dilution of commercial bleach (NaOCl 1%) and water, rinsed with sterile distilled water and placed onto MPH medium [agar 5.5 g/l, malt extract 10 g/l, $Ca(NO_3)_2$ 2 mM, $MgSO_4$ 0.6 mM, KH_2PO_4 1 mM and KCl 0.7 mM, amended with polymixin B sulphate (62 μg/ml; Sigma Chemicals), penicillin B (600 μg/ml; Sigma-Aldrich Chemie GmbH, Germany), virolex (30 μl/l, Carbendazime, Protex s.a., Belgium), quintozene (100 μg/ml, PCNB; Sigma Chemicals) and cholesterol (10 mg/ml)]. The plates were incubated for 5–7 days at 20°C under 12 h daylight using culture tables maintained at 18°C in order to avoid condensation. *Phytophthora* isolates were then subcultured on corn meal agar (CMA) medium (Difco, Becton Dickinson, France). For hyphal tips production, mycelium plugs from actively growing colony on CMA were placed onto water agar (agar 15 g/l; Difco Laboratories) for 1 week at 22°C. Hyphal tips were excised with a fine needle and transferred individually onto CMA for all *Phytophthora* isolates listed in Table 1. For long-term storage of hyphal tip isolates, cultures on CMA were stored in sterile distilled water at 4°C. In order to determine the growth rate at different temperatures, pieces of actively growing CMA colony were cut out at the margin of the culture and placed onto CMA. The plates were incubated in the dark at two temperatures (27 and 30°C). Measures (radial growth of the culture) were carried out after 4 and 8 days respectively. One measure was carried out per plate and three plates were considered per isolate. The daily growth rate (mm/day) was calculated from the following formula: (R_2-R_1)/the number of days between R_1 and R_2, R_1 being the colony radial growth 4 days after the inoculation, and R_2 being the colony radial growth 8 days after the inoculation (Erwin and Ribeiro, 1996). The colony pattern (mycelium appearance, and culture stability) was determined on mycelium grown on carrot agar (CA) in darkness at 25°C, as described previously (Brasier et al., 1995). These macroscopic observations were combined with microscopic observations in order to evaluate oogonial frequencies and shape for the various isolates studied.

DNA extraction

Pieces from the edge of a growing CMA culture were added to Erlenmeyer flasks containing liquid V8 juice medium (354 ml of V8 juice buffered with 5 g $CaCO_3$, centrifuged 20 min at 1500 g and diluted to 1 l with distilled water; Erwin and Ribeiro, 1996). After 4–5 days on an orbital shaker at 22°C, the mycelium was harvested. DNA was extracted from mycelium or from plant tissue following the recommendations of the High Pure PCR Template Preparation Kit (Roche Diagnostics, GmbH, Mannheim, Germany) after grinding the tissues in liquid nitrogen. DNA concentrations were estimated by electrophoresis on 2% agarose gel in TAE buffer [40 mM Tris-acetate (pH 8.0); 1 mM ethylenediaminetetraacetic acid (EDTA)] stained with ethidium bromide and visualized under UV light.

Phylogenetic analyses

All the PCR were conducted on in a Perkin-Elmer device (GeneAmp PCR System 9700, Perkin-Elmer, Norwalk, CT, USA) using *Taq* Platinum from Invitrogen Life Technologies Ltd products (The Netherlands). Thermocycling profiles for random amplified microSatellite (RAMS) primers (Table 2) have been described (Hantula et al., 1997). random amplified polymorphic DNA (RAPD) reactions were carried out with primers from OPC and OPD sets (Operon Technology, Alameda, CA, USA) using the manufacturer's recommendations for the thermocycling profile. Polymorphic RAPD primers are listed in Table 2. The amplification reactions (50 μl) consisted of 2 μM (RAMS) or 1 μM (RAPD) primer, 200 μM (RAMS) or 100 μM (RAPD) of each dNTP, 1.5 mM (RAMS) or 2 mM (RAPD) $MgCl_2$, 5 μl of 10X reaction buffer, 1 U of *Taq* DNA polymerase and fungal DNA. Amplification products were visualized under UV light after electrophoresis in 2% agarose gels stained with ethidium bromide. The length of the amplification products was estimated by comparing them with a 100 bp DNA ladder (Invitrogen Life Technologies Ltd). Molecular analyses carried out on the 24 Walloon alder *Phytophthora* isolates characterized in this study were repeated at least twice. Reproducible, polymorphic RAMS or RAPD bands were converted in binary data [scored as 1 (band present) or 0 (band absent)]. A similarity matrix was

Table 2
List of PCR primers

Name	Type	Sequence[a]
OPC01	RAPD	TTC GAG CCA G
OPC02	RAPD	GTG AGG CGT C
OPC03	RAPD	GGG GGT CTT T
OPC04	RAPD	CCG CAT CTA C
OPC05	RAPD	GAT GAC CGC C
OPD01	RAPD	ACC GCG AAG G
OPD02	RAPD	GGA CCC AAC C
OPD03	RAPD	GTC GCC GTC A
OPD04	RAPD	TCT GGT GAG G
OPD05	RAPD	TGA GCG GAC A
OPD07	RAPD	TTC GCA CGG G
OPD08	RAPD	GTG TGC CCC A
OPD16	RAPD	AGG GCG TAA G
OPD18	RAPD	GAG AGC CAA C
OPD19	RAPD	CTG GGG ACT T
OPD20	RAPD	ACC CGG TCA C
RAMS1	RAMS	DDB CCA CCA CCA CCA CCA
RAMS2	RAMS	DHB CGA CGA CGA CGA CGA
RAMS3	RAMS	VHV GTG TGT GTG TGT GTG
ITS1	Generic PCR	TCCGTAGGTGAACCTGCGG
ITS4	Generic PCR	TCCTCCGCTTATTGATATGC
D16F	Specific PCR	AGGGCGTAAGGGTGCGAAATA
D16R	Specific PCR	AGGGCGTAAGCCTGGACCG

[a]Sequence from 5′ to 3′.
PCR, polymerase chain reaction; RAPD, random amplified polymorphic deoxyribonucleic acid; RAMS, random amplified microsatellite.

constructed using the Jaccard coefficient. Unweighted pair group method cluster analysis (UPGMA) of binary data was performed with FREETREE software (Pavlicek et al., 1999).

Design of SCAR primers and PCR conditions

The DNA band of interest (OPD16-marker, 366 bp) was cut from gel, purified using the QiaExII Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into the plasmid pCR® 2.1 using the TA Cloning Kit and InvαF′ competent cells (Invitrogen Life Technologies Ltd). Ampicillin-resistant (100 μg/ml) clones were checked by PCR using the M13 direct and reverse primers. One recombinant clone was submitted to the Eurogentec s.a. (Belgium) for sequencing in both directions. From the sequence data, a pair of sequence-characterized amplification region (SCAR) primers (D16F and D16R, Table 2) was designed. Genomic DNA was amplified in PCR mixtures (50 μl) containing 0.4 μM of D16F, 0.4 μM of D16R, 200 μM of each dNTPs, 1 mM $MgCl_2$, 5 μl of 10X reaction buffer, 2.5 U of *Taq* DNA polymerase (*Taq* Platinum, Invitrogen Life Technologies Ltd products) and fungal DNA. The thermocycling regime consisted of a first denaturation step at 94°C for 5 min, followed by 35 cycles with DNA denaturation at 94°C for 30 s, primers annealing at 64°C for 1 min and primer extension at 72°C for 1 min. The final extension step was performed at 72°C for 10 min. Amplified fragments were visualized after electrophoresis in agarose gel (2%) as already described. For the different isolates used in this study, the quality of the DNA extracted from mycelium was evaluated by performing a PCR with the universal primers ITS1 and ITS4 (Table 2) described by White et al. (1990) according to the procedure proposed by Chowdappa et al. (2003).

Pathogenicity tests

All alder *Phytophthora* isolates described in Table 1 except two (isolates 2306 and 2291) were tested for aggressiveness on 2-year-old seedlings of *A. glutinosa* from two provenances (one from Germany, the other from Belgium) placed in plastic pots filled with a compost mixture. Two successive experiments were conducted in July (first experiment, provenance Germany) and August (second experiment, provenance Belgium) 2003 in a greenhouse maintained at 20°C, with natural daylight. The plants were inoculated by inserting a 5 mm agar plug from the margin of an actively growing colony on V8 agar (V8 juice liquid medium plus 15 g agar technical) into bark wounds on the stem of seedlings. Control plants were treated with sterile agar discs. The plants were evaluated visually every 4 days for 16 days for disease severity (lesion length in mm). The area under the disease progress curve (AUDPC) was calculated for each plant using a formula described by Shaner and Finney (1977). The experiment was organized as a randomly complete block design with three replicates per isolate. AUDPC values transformed to square roots as a normalizing transformation were evaluated using ANOVA and mean values were compared using the Tukey test. A *t*-test was carried out to evaluate the differences between the two inoculation trials. All statistical analyses were performed using the statistical SAS software (SAS Institute, Cary, NC, USA, version 8.2).

Results

Characterization of the Belgian alder *Phytophthora* spp.

Isolates of *Phytophthora* were collected from *Alnus* trees at various points covering the main hydrographic basins of Wallonia, where *Alnus* species are distributed mainly along streams and rivers. Both *A. glutinosa* and *A. incana* were infected (Table 1). Under the above-mentioned experimental conditions, isolations were successful provided that the ex-plant was taken from fresh inner bark lesions. Out of the 20 RAPD primers evaluated, 16 primers gave polymorphism between isolates. A phylogenetic tree constructed from these data (266 RAPD considered) and representing the relationship between alder *Phytophthora* isolates, *P. cambivora* (isolate 2208) and *P. fragariae* (isolate 2216) revealed that alder *Phytophthora* clustered outside the *P. cambivora*/*P. fragariae* cluster. On the contrary, alder *Phytophthora* were separated into two groups (I and II), the first group being further divided into two subgroups (Ia and Ib) (Fig. 1). Most of the Walloon isolates (nine of the 13 collection sites), as well as the reference isolate 2288 from the UK, were classified in group Ia. The reference isolates 2291 and 2287 from Germany and the Netherlands, respectively, as well as the Belgian isolate 2274, were classified in group Ib, while the reference isolate 2289 from Sweden and Belgian isolates 2271, 2276 and 2277 were classified in

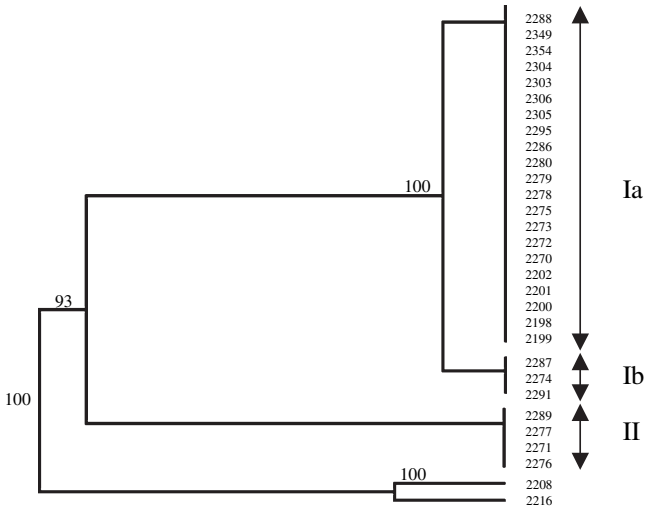

Fig. 1 Dendrogram derived from cluster analysis (UPGMA, Jaccard's coefficient) obtained from genetic similarity indices of 30 *Phytophthora* isolates (28 alder *Phytophthora*, one *P. fragariae* and one *P. cambivora*) based on random amplified polymorphic DNA (RAPD) analysis. The figures indicate the frequencies (in %) with which a given branch appeared in 300 bootstrap replications. The three molecular groups identified in the collection of alder *Phytophthora* isolates (Ia, Ib and II) are shown

Table 3
Comparison between Walloon and reference alder *Phytophthora* isolates in terms of biological properties according to their molecular classification

		Growth rate (°C)[1]		Oogonia[2]					Aggressiveness[3]	
RAPD	Isolate	27	30	Size (μm)	Shape	Frequency	Time (week)	Colony type	1	2
Ia	2198	2.3	0.0	44.6 ± 2.7	O	+ +	1	Felty	1211 ± 155^{a}	ND
	2199	4.5	0.3	42.8 ± 2.8	O	+ +	1	Felty	1284 ± 778^{a}	1507 ± 140^{a}
	2200	2.4	0.0	46.2 ± 3.0	O	+	1	Felty	814 ± 65^{a}	751 ± 109^{a-c}
	2201	1.3	0.2	47.8 ± 3.5	O	+ +	1	Felty	1097 ± 298^{a}	1298 ± 390^{a,b}
	2202	3.8	0.0	49.2 ± 1.7	O	+	1	Felty	872 ± 372^{a}	1420 ± 58^{a,b}
	2270	0.8	0.2	44.5 ± 2.5	O	+ +	2	Felty	976 ± 211^{a}	510 ± 253^{b-d}
	2272	1.5	0.2	43.5 ± 2.2	O	+ + +	1	Felty	1254 ± 751^{a}	1067 ± 392^{a-c}
	2273	2.4	0.1	40.3 ± 2.8	O	+	1	Felty	1000 ± 561^{a}	951 ± 332^{a-c}
	2275	4.3	0.3	43.3 ± 2.1	O	+	1	Felty	739 ± 346^{a}	830 ± 154^{a-c}
	2278	2.6	1.1	42.5 ± 2.9	O	+ + +	1	Felty	525 ± 128^{a,b}	889 ± 151^{a-c}
	2279	2.2	0.1	48.2 ± 3.5	O	+ + +	1	Felty	599 ± 47^{a,b}	396 ± 262^{c,d}
	2280	4.2	0.0	47.2 ± 2.7	O	+	1	Felty	690 ± 140^{a}	1113 ± 446^{a-c}
	2286	1.3	0.0	44.2 ± 3.0	O	+ + +	1	Felty	1127 ± 282^{a}	1404 ± 217^{a,b}
	2288	*2.2*	*0.2*	*42.2 ± 2.6*	*O*	+	*2*	*Felty*	*584 ± 171*^{a}	*639 ± 431*$^{a-d}$
	2295	3.3	0.0	–	–	–	–	Wolly	1279 ± 252^{a}	1493 ± 157^{a}
	2303	3.7	0.4	43.7 ± 3.9	O	+ +	1	Felty	1194 ± 445^{a}	1263 ± 314^{a,b}
	2304	3.3	0.1	44.6 ± 1.0	O	+	1	Felty	1011 ± 396^{a}	679 ± 219^{a-c}
	2305	2.5	0.3	44.1 ± 2.0	O	+ +	1	Felty	985 ± 292^{a}	1139 ± 313^{a-c}
	2306	1.5	0.0	40.3 ± 1.4	O	+ +	1	Felty	ND	ND
	2349	1.9	1.8	42.4 ± 2.8	O	+	1	Felty	1347 ± 253^{a}	898 ± 107^{a-c}
	2354	2.7	0.8	44.3 ± 2.9	O	+	1	Felty	1141 ± 455^{a}	1345 ± 278^{a,b}
Ib	2274	4.3	1.8	–	–	–	–	Wolly	922 ± 429^{a}	702 ± 160^{a-c}
	2287	*4.3*	*1.1*	*41.6 ± 2.0*	*O*	+	*2*	*Felty*	*743 ± 301*^{a,b}	*848 ± 400*$^{a-c}$
	2291	*3.4*	*0.8*	*38.6 ± 6.0*	*O*	+ +	*3*	*Felty*	*ND*	*ND*
II	2271	1.5	0.0	42.6 ± 3.3	S	+	4	Felty	620 ± 192^{a,b}	852 ± 132^{a-c}
	2276	0.2	0.0	38.8 ± 4.1	S	+	4	Variable	366 ± 73^{a,b}	348 ± 262^{c,d}
	2277	1.7	0.2	42.9 ± 2.4	S	+	5	Rings	656 ± 147^{a}	718 ± 159^{a-c}
	2289	*0.4*	*0.1*	*44.9 ± 3.6*	*S*	+	*5*	*Rings*	*109 ± 188*^{b}	*135 ± 135*^{d}

[1]Daily growth rate (mm/day) on CMA was calculated from the following formula $[(R_{i+1} - R_i)/d_{i+1} - d_i]$, where R, colony radial growth (mm); d, the number of days from the inoculation date; i, the observation). Measures were carried out on three different Petri dishes per isolate, 4 and 8 days after inoculation.
[2]Measures carried out on 30 oogonia per isolate. Size, mean ± SD; Shape – O: Ornamented, S: Smooth.
[3]Aggressiveness expressed as the area under disease progress curve (AUDPC) after inoculation of stem with inoculum agar plugs (necrosis size measured every 4 days after the inoculation, three plants per isolate). Two kinds of plants were used as successive tests (1, German provenance and 2, Belgian provenance). Average AUDPC values followed by a same letter are not significantly different at the 95% level ($P > 0.05$) using the Tukey test.
–, not available; ND, not determined; rows in italics, foreign reference isolates; CMA, corn meal agar.

group II. RAMS analysis carried out with three different primers on the same samples confirmed the existence of two groups of isolates (I and II), but did not discriminate between subgroups Ia and Ib (data not shown). In order to evaluate the possible relationships between these molecular groupings and biological/morphological properties, the different isolates were examined for a range of characters. First, the maximum temperature for growth was evaluated. At 27°C, the growth rate was approximately 1 mm/day for all the isolates except for two from group II (2276 and 2289) and one from group Ia (2270). At 30°C, most of the isolates displayed a growth rate close to zero, except for all isolates from group Ib and three isolates from group Ia (2278, 2349 and 2354) (Table 3). On CA, isolates from groups Ia and Ib displayed uniform colonies with an appressed felt-like appearance, and produced at the margin of the culture oogonia with walls ranging from extremely ornamented to wavy (except for isolates 2274 and 2295, which did not produce any oogonia in our culture conditions). For most of the isolates from group Ia, oogonia were observed after 7 days in culture, while 2–3 weeks were needed for the isolates of group Ib. All but one isolate from group II were characterized by irregular growth (successive rings of cotton- and felt-like mycelium for isolates 2277 and 2289, and a petaloid colony appearance for isolate 2276) and by the production of few smooth-walled oogonia which appeared after 4 or 5 weeks in culture (Table 3). Pathogenicity tests were carried out by inserting a mycelium plug into a wound in the stem of alder seedlings. A t-test did not reveal any significant difference between the two inoculation trials ($P < 0.05$), which were conducted on two different provenances. As soon as 4 days after the inoculation, a dark-stained necrosis was observed in both experiments on the alder stems of the three alder plants inoculated, while the control plants remained symptomless. As revealed by the AUDPC values, the vast majority of isolates from group Ia were moderately to highly aggressive pathogens of alder seedlings. Two isolates from group II (Swedish isolate 2289 and

Belgian isolate 2276) proved to be the least aggressive isolates. The other isolates from group II (2271 and 2277), as well as both isolates from group Ib (2274 and 2287), displayed intermediate behaviour. However, in spite of the significant effect of isolates (ANOVA, $P < 0.001$), the Tukey test did not reveal groupings according with the molecular classification.

Specific detection of alder *Phytophthora* spp.

SCAR primers were developed based on sequence information of a 366 bp polymorphic amplification product generated with the RAPD primer OPD16 (Fig. 2). A BLAST search did not reveal any homology with published sequences. By performing PCR with the SCAR primers on total DNA from mycelium of the alder *Phytophthora* listed in Table 1, all the isolates from groups Ia and II were detected, while those from group Ib were not (Fig. 3). Specificity was tested on a range of *Phytophthora* species (including *P. cambivora*, a species closely related to alder *Phytophthora*), as well as on two fungi from other genera. On agarose gel, an expected 366 bp signal was detected for the alder *Phytophthora* isolate used as a control (isolate 2200 – group Ia). None of the other samples evaluated gave an amplification signal (Fig. 4a). In order to screen for the presence of inhibitors contained in the DNA samples used to evaluate the test specificity, a PCR was carried out with the universal primers ITS1 and ITS4. An expected signal at approximately 900 bp was amplified for all the *Phytophthora* samples

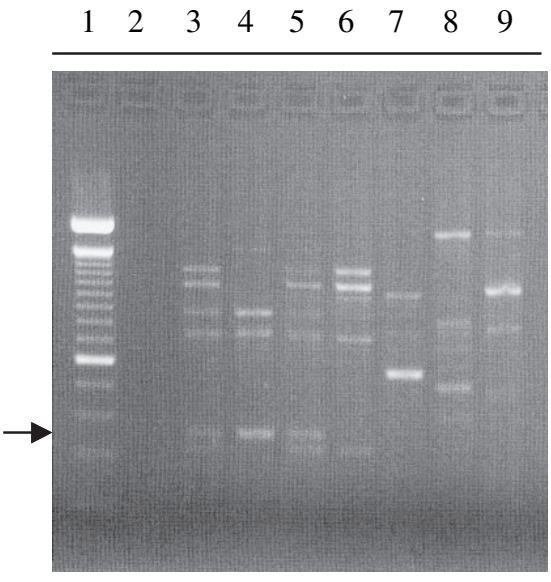

Fig. 2 Gel electrophoresis of DNA products generated with the random amplified polymorphic DNA (RAPD) primer OPD16. Lane 1, molecular weight marker (100 bp; Invitrogen); lane 2, negative control; lanes 3 and 5, alder *Phytophthora* group Ia isolates (*P. alni* subsp. *alni*); lane 4, alder *Phytophthora* group II isolate (*P. alni* subsp. *uniformis*); lane 6, alder *Phytophthora* group Ib isolate (*P. alni* subsp. *multiformis*); lane 7, *P. fragariae*; lane 8, *P. cambivora*; lane 9, *P. cinnamomi*. Arrow indicates the 366 pb RAPD band used for the design of sequence-characterized amplification region (SCAR) primers

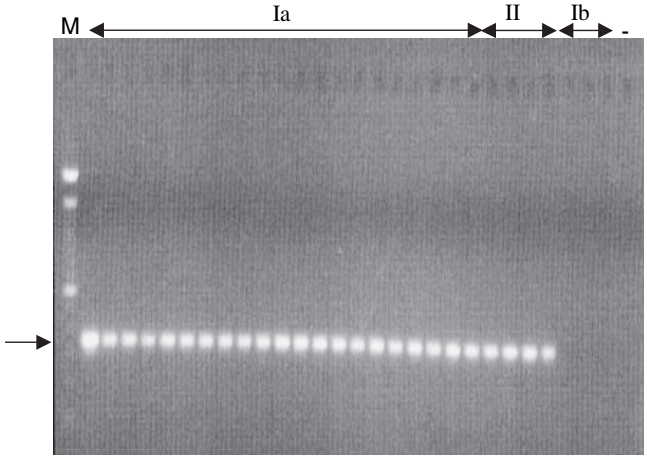

Fig. 3 Gel electrophoresis of DNA products after polymerase chain reaction (PCR) with primers D16F and D16R on total DNA from alder *Phytophthora* isolates. Lane 1, molecular weight marker (100 bp; Invitrogen); lanes 2–22, alder *Phytophthora* group Ia; lanes 23–26, alder *Phytophthora* group II; lanes 27–29, alder *Phytophthora* group Ib; lane 30, negative control. The arrow indicates the position of the expected signal (366 pb)

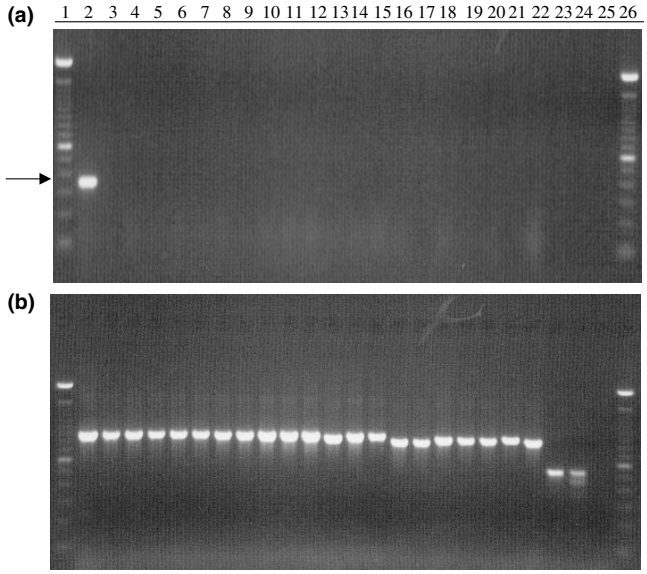

Fig. 4 Gel electrophoresis of DNA products after polymerase chain reaction (PCR) with primers D16F and D16R (a) or primers ITS1 and ITS4 (b) on total DNA from different *Phytophthora* species. Lanes 1 and 26, molecular weight marker (100 bp; Invitrogen); lane 2, alder *Phytophthora* (isolate 2200); lane 3, *P. fragariae*; lanes 4–8, *P. cambivora*; lanes 9–12, *P. cinnamomi*; lane 13, *P. cryptogea*; lane 14, *P. syringae*; lane 15, *P. europae*; lane 16 and 17, *P. citricola*; lanes 18–20, *P. ramorum*; lane 21, *P. gonapodyides*; lane 22, *P. cactorum*; lane 23, *Verticillium dahliae* (Belgian isolate); lane 24, *Fusarium graminearum* (Belgian isolate); lane 25, negative control. The arrow indicates the position of the expected signal (366 pb)

(Fig. 4b). Test sensitivity was evaluated on various dilutions of total DNA extracted from mycelium. Amplification products were reproducibly detected when 20 pg of total DNA was put into the PCR mixture (data not shown). From a practical point of view, the PCR assay was evaluated on DNA extracted from

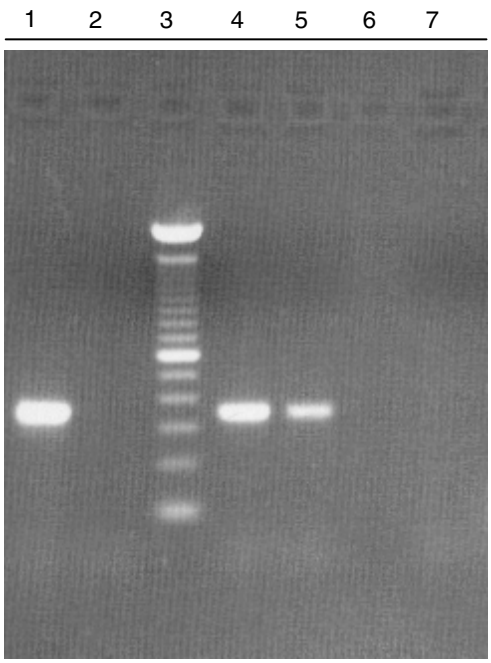

Fig. 5 Gel electrophoresis of DNA products after polymerase chain reaction (PCR) with primers D16F and D16R. Lane 1, positive control (total DNA extracted from mycelium, isolate 2200); lane 2, negative control; lane 3, molecular weight marker (100 bp; Invitrogen); lanes 4 and 5, total DNA extracted from infected plant tissue; lane 6, no sample; lane 7, total DNA extracted from healthy plant

infected and healthy plants. Consistent amplification was reproducible for infected samples (Fig. 5).

Discussion

The main objective of this work was to compare genetic and phenotypic features of isolates of the alder *Phytophthora* complex found in Wallonia with those of isolates from other European countries. Previous studies have demonstrated that the various types of 'alder *Phytophthora*' could behave as different *Phytophthora* species, making it difficult to develop specific control strategies.

Cluster analysis of 266 RAPD bands from 16 primers showed that *P. cambivora* and *P. fragariae* grouped outside the alder *Phytophthora* complex. The same results were obtained when RAMS (34 additional bands) were taken into account (data not shown). In contrast, amplified fragment length polymorphism (AFLP) analysis carried out with four primers combinations placed interspecific hybrids between *P. cambivora* and *P. fragariae* (Brasier et al., 1999). These different results suggest that phylogenetic trees involving interspecific hybrids must be interpreted with a degree of caution and that different approaches should be used to determine the origin of alder *Phytophthora*. RAPD analyses have proven to be reliable in the characterization of natural *Phytophthora* hybrids isolated from *Primula* and *Spathiphyllum* as the banding patterns of the hybrids consisted mainly in bands that were present in either *P. cactorum* or *P. nicotianae*, the parental species (Man In't Veld et al., 1998). The situation is not so obvious with alder *Phytophthora* as the parental species involved in the hybridization process have not been clearly identified.

Concerning the grouping of alder *Phytophthora*, our results were consistent with those of Brasier et al. (1999) based on AFLP analysis. Alder *Phytophthora* were clustered into three groups, corresponding respectively to the *P. alni* subsp. *alni* isolates (first group, called Ia), the *P. alni* subsp. *multiformis* isolates (second group, called Ib) and the *P. alni* subsp. *uniformis* isolates (third group, called II). Of the 24 Walloon isolates tested, 20 were classified in the first group, one in the second group and three in the third group. A survey carried out since 1993 on 280 samples from various European countries has demonstrated the predominance of the isolates from the first group (*P. alni* subsp. *alni*), the two other groups being generally less frequent (Brasier, 2003). In Wallonia, the distribution of isolates belonging to RAPD group Ib or II was not restricted to a particular region (data not shown).

As already shown by Brasier et al. (1999), our results confirm that the different groups are closely related as the RAPD banding pattern of the *P. alni* subsp. *alni* isolates was generally a composite of the bands amplified in isolates from the two other subspecies (data not shown). However, the origin of the different forms is so far unclear. Alder *Phytophthora* isolates could represent transient forms in the process of fixation of a new species, the *P. alni* subsp. *alni* isolates (standard type) corresponding to the first products of the hybridization process, and the two other subspecies (variant types) being recombination products of the standard type. Support for this viewpoint comes from evidence that standard isolates appear as 'aberrant forms', near tetraploid and unable to complete meiosis, while some variant forms are close to diploid and normal in their nuclear behaviour. Nonetheless, the possibility that variant forms could be the first products of the hybridization process cannot yet be excluded, the standard hybrids resulting in that case from 'accidental events' due to environmental stresses. In this context, the suddenness of the appearance in Europe of this new disease on 10 years only suggests that a common event is at the origin of the development of the infections following a modification of the host–pathogen relationship or a modification of the aggressiveness of the population of the pathogenic agent. The molecular analysis of interspecific hybrids generated by zoospore fusion between compatible *Phytophthora* species could result in a better understanding on the hybridization process. Such 'laboratory made' interspecific hybrids have already been produced within the genus *Phytophthora* (Goodwin and Fry, 1994; Ersek et al., 1995). A long-term evaluation of the proportion of the different subspecies in European countries should also provide insight in their respective evolution rate.

The characterization of isolates on the basis of morphological and biological properties proved to be less reliable than molecular tests, probably because of the

instability of the hybrid genotypes in culture. This is clearly illustrated by the behaviour of isolates 2274 and 2295, collected in 2001, which displayed an altered colony pattern (woolly appearance) and a complete loss of oogonia production, making it difficult to classify them as standard/variant types. In addition, unlike RAPD profiles, most of the parameters used to characterize the isolates found in Belgium were not typical of one group. In this respect, some isolates from RAPD group Ia (*P. alni* subsp. *alni*) were found to grow at 30°C, like isolates from RAPD group Ib (*P. alni* subsp. *multiformis*). With regard to oogonia shape, all isolates from RAPD group II (including the reference Swedish isolate) produced smooth-walled oogonia. This result accords with previous studies (Brasier et al., 1999; Nagy et al., 2003), but some isolates from both other groups were also found to produce a small proportion of smooth oogonia and weakly ornamented oogonia. The appressed felt-like appearance described elsewhere for *P. alni* subsp. *alni* isolates was also observed for some isolates from the other subspecies, including the German reference isolate, while the chimaeric fertile sectors characterizing the Dutch isolate described by Brasier et al. (1999) have not been observed in our culture conditions. These results suggest that molecular tools are more reliable than morphological characters when classifying an isolate as the *P. alni* subsp. *alni*, *P. alni* subsp. *multiformis* or *P. alni* subsp. *uniformis*.

In terms of aggressiveness, all isolates evaluated in the pathogenicity tests were able to infect alder seedlings, some of the *P. alni* subsp. *alni* isolates inducing the more severe symptoms, as described previously (Brasier and Kirk, 2001). There was no significant difference between two alder provenances, one from Belgium and the other from Germany. In contrast, great variability was observed between repetitions. This might reflect variable resistance levels. It must be stressed, however, that the inoculation technique used in this study (stem wound) cannot be used to evaluate the infection capacity. In the context of plant breeding for disease resistance, inoculation techniques that mimic natural infection (by submerging plants in zoospores suspension, for example) should be carried out.

Due to the low success of isolation when fresh active inner-bark lesions are unavailable (Streito et al., 2002) and to the time needed to isolate *Phytophthora* species in culture, the PCR using species-specific primers appeared to be a good alternative to an identification procedure based on microbiological technique. Species-specific PCR is rapid to carry out, and does not require any mycological expertise. Such molecular tests have already been developed for detecting *Phytophthora* disease in trees (Lilja et al., 1996; Schubert et al., 1999). The primers designed in this study were found to detect the *P. alni* subsp. *alni* (group Ia) and the *P. alni* subsp. *uniformis* (group II) isolates of the alder *Phytophthora*, but failed to detect isolates from the *P. alni* subsp. *multiformis* (group Ib). The risk of false-negatives resulting from the non-detection of some isolates cannot be excluded. However, our results (one isolate belonging to group Ib of the 24 Walloon isolates characterized) and the studies carried out in other countries (Brasier, 2003; Nagy et al., 2003) suggest that isolates from the *P. alni* subsp. *multiformis* do not represent a great proportion of isolates in the European population of alder *Phytophthora*. The proposed specific PCR could be a promising diagnostic tool in certification schemes in order to confirm the presence of the pathogenic agent in plants where isolation methods are unsuccessful. As it has proved to be specific to alder *Phytophthora*, the PCR could also be used to evaluate the infection potential of river water or the soil of riparian ecosystems where other *Phytophthora* species are found (Werres et al., 2001). The present work has concentrated mainly on Belgian alder *Phytophthora* isolates. Further validation of the PCR assay should include a great number of strains from various geographic origins.

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