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Characterisation of European and North American *Phytophthora ramorum* isolates due to their morphology and mating behaviour *in vitro* with heterothallic *Phytophthora* species

Sabine WERRES and Katrin KAMINSKI

Federal Biological Research Centre for Agriculture and Forestry, Institute for Plant Protection in Horticulture, Messeweg 11/12, D – 38104 Braunschweig, Germany.
E-mail: S.Werres@BBA.de.

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Vegetative growth rate, and size of sporangia, chlamydospores and oospores from 94 *P. ramorum* isolates were measured and the isolates were paired *in vitro* with four different heterothallic *Phytophthora* species isolated from infected nursery plants in Germany. *P. ramorum* isolates originated from different European countries and from Canada and the USA. 66 of the 67 European isolates were determined as mating type A1; only one isolate was of mating type A2. Of the 27 North American isolates tested, seven (all from nurseries) were determined to be the A1 mating type and 17 to be the A2 mating type. Three isolates did not produce gametangia during the incubation period. Discriminant analysis of all data allowed a grouping based on the vegetative growth rate. The two groups corresponded with the mating type no matter whether the isolates originated from Europe or North America. The A1 isolates were much more homogeneous in their morphology than the A2 isolates. They grew faster, had larger chlamydospores and did not produce gametangia with *P. cambivora*. Within the A2 group, the single European isolate of mating type A2 (BBA 16/02) and three US isolates showed intermediate characters and were classified with the discriminant function into that of the opposite mating type. The morphological characters and the mating behaviour of the isolates will be discussed.

INTRODUCTION

The fungal-like organism *Phytophthora ramorum* (Werres *et al.* 2001a) is a severe pathogen on hardy ornamentals and trees in Europe and in North America. At the beginning of the 1990s, it was first detected in Germany and The Netherlands in nurseries as the causal agent of an unusual twig blight on *Rhododendron* (Werres & Marwitz 1997, Werres *et al.* 2001a). Since then, the pathogen has been found in Belgium, Denmark, Ireland, Italy, France, Norway, Slovenia, Spain, Sweden, Switzerland, the UK, and Poland. In some of these countries the pathogen was isolated only from imported plants. Up until 2003, *P. ramorum* was only isolated from *Rhododendron* and *Viburnum* sp. in Europe, and it occured mainly in nurseries, parks and private gardens. Recently, however, the pathogen has been isolated from additional herbaceous species (Hüberli *et al.* 2003, 2005), woody ornamental species (Inman *et al.* 2003, Beales *et al.* 2004a, b, Giltrap *et al.* 2004, Hüberli *et al.* 2004, Lane *et al.* 2004) and from diseased *Quercus rubra* in The Netherlands (Hans de Gruyter, pers. comm.), and from *Quercus falcata* and *Q. ilex*, *Castanea sativa*, *Fagus sylvatica* and *Aesculus hippocastanum* in the UK (Brasier *et al.* 2004, EPPO Reporting Service 2004/024). In North America during the mid-1990s, unusual oak deaths were noted in natural habitats along the California coast; *P. ramorum* was subsequently shown to be the underlying cause of this mortality (Rizzo *et al.* 2002). In the last few years, the pathogen has been isolated from a wide range of tree and shrub species in California (Davidson *et al.* 2003). As in Europe, the geographical distribution of *P. ramorum* in North America is no longer limited to the California coast: It has been detected in other areas in the USA and in Canada (Goheen *et al.* 2002a, b, Hansen *et al.* 2003).

In the first few years since its detection and description relatively few isolates of *P. ramorum* were collected and only isolates of mating type A1 were detected in Europe, while in the USA only isolates of mating type A2 were found. However, in 2003 the first isolate of the A2 mating type was isolated from *Viburnum* in a nursery in Belgium (Werres & De Merlier 2003) and the first A1 isolate in North America was detected, also from a horticultural nursery (Hansen *et al.* 2003). Initial studies indicated that there was genetic and phenotypic variation between the European and North

Table 1. Hosts and sources of the isolates.

Isolate no.	Host	Year of isolation	Source	Country
Europe[a]				
BBA 27/02	*Rhododendron* sp.	2002	Nursery	Belgium
BBA 26/02[b]	***Viburnum bodnantense***	**2002**	**Nursery**	**Belgium**
BBA 10/02	*Rhododendron* sp.	2002	Nursery	France
BBA F.002	*Rhododendron* sp.	2002	Nursery	France
BBA F.003	*Rhododendron* sp.	2002	Nursery	France
BBA F.004	*Rhododendron* sp.	2002	Nursery	France
BBA F.005	*Rhododendron* sp.	2002	Nursery	France
BBA F.006	*Rhododendron* sp.	2002	Nursery	France
BBA 2R0033/1	*Rhododendron* sp.	2002	Nursery	France
BBA 2N0362/2	*Rhododendron* sp.	2002	Nursery	France
BBA 2N0389	*Rhododendron* sp.	2002	Nursery	France
BBA 2N0586/2	*Rhododendron* sp.	2002	Nursery	France
BBA 2N0589/1	*Rhododendron* sp.	2002	Nursery	France
BBA 9/95 (ex-type strain)	*Rhododendron catawbiense*	1995	Nursery	Germany
BBA 15/01-29b	*R. ferrugineum* × *hirsutum*	2001	Nursery	Germany
BBA 15/01-2a	*R.* hybrid cv. 'Roseum Elegans'	2001	Nursery	Germany
BBA 15/01-2d	*R.* hybrid cv. 'Roseum Elegans'	2001	Nursery	Germany
BBA 15/01-3a	*R.* cv. 'Catawbiense Grandiflorum'	2001	Nursery	Germany
BBA 15/01-28a	*R.* cv. 'Catawbiense Boursault'	2001	Nursery	Germany
BBA 15/01-46a	*R.* cv. 'Mrs. P. den Dew Ouden'	2001	Nursery	Germany
BBA 15/01-25A	*R.* cv. 'Fidelius'	2001	Nursery	Germany
BBA 15/01-1c	*R. wardii* cv. 'Ehrengold'	2001	Nursery	Germany
BBA 37/01-a	*R. wardii*	2001	Nursery	Germany
BBA 15/01-13a	*R. repens* hybrid cv. 'Baden-Baden'	2001	Nursery	Germany
BBA 15/01-24a	*R. yakushimanum* hybrid cv. 'Polaris'	2001	Nursery	Germany
BBA 15/01-45b1	*Rhododendron* sp.	2001	Nursery	Germany
BBA 35/01-1	*Rhododendron* sp.	2001	Nursery	Germany
BBA 1/02-7	*Rhododendron* hybrid	2002	Nursery	Germany
BBA 14/02	*Rhododendron* hybryd	2002	Nursery	Germany
BBA 15/02-a	*Rhododendron* sp.	2002	Nursery	Germany
BBA 15/02-b	*Rhododendron* sp.	2002	Nursery	Germany
BBA 20/02	*Rhododendron* sp.	2002	Nursery	Germany
BBA 14/03	*Rhododendron* sp.	2003	Nursery	Germany
BBA 15/01-18	*V. bodnantense* cv. 'Dawn'	2001	Nursery	Germany
BBA 15/01-19B	*V. bodnantense* cv. 'Dawn'	2001	Nursery	Germany
BBA 15/01-5b	*V. fragans*	2001	Nursery	Germany
BBA 15/01-39b	*V. plicatum*	2001	Nursery	Germany
BBA 23/01	*V. tinus*	2001	Nursery	Germany
BBA 15/01-8a	*Viburnum* sp.	2001	Nursery	Germany
BBA 15/01-11a	*Viburnum* sp.	2001	Nursery	Germany
BBA 15/01-14	*Viburnum* sp.	2001	Nursery	Germany
BBA 15/01-38a	*Viburnum* sp.	2001	Nursery	Germany
BBA 19/02	*Viburnum* sp.	2002	Nursery	Germany
BBA 24/02	*R. yakushimanum*	2002	Nursery	Italy
BBA PD 20025728-1	*Rhododendron* sp.	2002	Private garden	The Netherlands
BBA PD 20026419-1	*Rhododendron* sp.	2002	Park	The Netherlands
BBA PD 2003/5548-1	*Quercus rubra*	2003	Park	The Netherlands
BBA PD 20023604	*Viburnum bodnantense* cv. 'Dawn'	2002	Nursery	The Netherlands
BBA PD 20025489	*Viburnum bodnantense* cv. 'Dawn'	2002	Nursery	The Netherlands
BBA 22/01-1	*Rhododendron* sp.	2001	Nursery	Poland
BBA 22/01-5	*Rhododendron* sp.	2001	Nursery	Poland
BBA 22/01-6	*Rhododendron* sp.	2001	Nursery	Poland
BBA 4/02-1	*Rhododendron* cv. 'Catawbiense Grandiflorum'	2002	Garden centre	Spain (Mallorca)
BBA 4/02-3	*Rhododendron* sp.	2002	Garden centre	Spain (Mallorca)
BBA 4/02-4	*Rhododendron* sp.	2002	Garden centre	Spain (Mallorca)
BBA 7/02	*Rhododendron* sp.	2002	Garden centre	Spain (Mallorca)
BBA 2/03	*Rhododendron* sp.	2002	Garden centre	Spain (Mallorca)
BBA 3/03	*Rhododendron* sp.	2002	Garden centre	Spain (Mallorca)
BBA 1/03	*Viburnum tinus*	2002	Garden centre	Spain (Mallorca)
BBA CSL 1604o	*R. grandiflora*	2002	Garden centre	UK
BBA CSL 1604m	*Rhododendron* sp.	2002	Garden centre	UK
BBA CSL 1612	*Rhododendron* sp.	2002	?	UK
BBA CSL 1653	*Rhododendron* sp.	2002	Nursery	UK

Table 1. (*Cont.*)

Isolate no.	Host	Year of isolation	Source	Country
BBA CSL 1622	*Viburnum* × *bodnantense*	2002	Nursery	UK
BBA CSL 1614	*Viburnum farreri*	2002	Nursery	UK
BBA CSL 1623	*Viburnum plicatum*	2002	Nursery	UK
BBA CSL 1560	Viburnum tinus	2002	Nursery	UK
North American				
BBA MSOD 03-002	*Rhododendron* sp.	2003	Nursery	Canada
BBA MSOD 03-0105	*Rhododendron* sp.	2003	Nursery	Canada
BBA MSOD 03-0107	*Rhododendron* sp.	2003	Nursery	Canada
BBA MSOD 03-0110	*Rhododendron* sp.	2003	Nursery	Canada
BBA Pr 86	***Arbutus menziesii***	**2001**	**Forest**	**USA[1)]**
BBA Pr 87	***A. menziesii***	**2001**	**Forest**	**USA[1)]**
BBA Pr 05 m	***Lithocarpus densiflora***	**2000**	**Forest**	**USA[1)]**
BBA Pr 05 j	***L. densiflora***	**2000**	**Forest**	**USA[1)]**
BBA Pr 03	***L. densiflora***	**2000**	**Forest**	**USA[1)]**
BBA Pr 01	***Q. agrifolia***	**2000**	**Forest**	**USA[1)]**
BBA Pr 06	***Q. agrifolia***	**2000**	**Forest**	**USA[1)]**
BBA Pr 71	***Q. agrifolia***	**2001**	**Forest**	**USA[1)]**
BBA Pr 04	***Q. kelloggii***	**2000**	**Forest**	**USA[1)]**
BBA Pr 65	***Q. parvula***	**2001**	**Forest**	**USA[1)]**
BBA Pr JL 3.5.3	***Sequoia sempervirens***	**2002**	**Forest**	**USA[1)]**
BBA Pr 88	***Umbellularia californica***	**2001**	**Forest**	**USA[1)]**
BBa Pr 110m	***U. californica***	**2001**	**Forest**	**USA[1)]**
BBA Pr 110a	***U. californica***	**2001**	**Forest**	**USA[1)]**
BBA Pr 84	**Soil**	**2001**	**Forest**	**USA[1)]**
BBA Pr 70	*Vaccinium ovatum*	2001	Forest	USA[1)]
BBA Pr 58	*Vaccinium* sp.	2001	Forest	USA[1)]
BBA 1820WA	***Camellia japonica***	**2003**	**Nursery**	**USA[2)]**
BBA 1743WA	*Rhododendron* cv. 'Jean Marie'	2003	Nursery	USA[2)]
BBA 1747WA	*Rhododendron* cv. 'Jean Marie'	2003	Nursery	USA[2)]
BBA Pr 52	***Rhododendron* sp.**	**2000**	**Nursery**	**USA[1)]**
BBA Pr 93	*Rhododendron* sp.	2001	Nursery	USA[1)]
BBA 03-74-2	*Viburnum* × *bodnantense* cv. 'Dawn'	2003	Nursery	USA[3)]

[a] All isolates are hyphal tip cultures. Isolates were supplied by Daphné De Merlier (Belgium), Claude Delatour and Carole Saurat (France), Clotilde Gullino (Italy), Hans deGruyter (The Netherlands), Grazyna Skuta (Poland), Eduardo Moralejo (Spain), Alan Inman (UK), Stéphan Brière (Canada), Jenny Davidson/Dave Rizzo (1, USA), Kelly Ivors (2, USA), and Everett Hansen (3, USA).

[b] Normal type = mating type A1; **bold type** = mating type A2, shading: no successful mating.

American *P. ramorum* populations (de Gruyter *et al.* 2002, Pogoda & Werres 2002, Brasier 2003, Hansen *et al.* 2003, Werres & Zielke 2003, Ivors *et al.* 2004). To date, however, there have been no detailed studies with a wide range of isolates from both continents and none with regard to differences between the two mating groups. In the present studies, *P. ramorum* isolates were characterized by morphology and their mating behaviour *in vitro* with other heterothallic *Phytophthora* species. These data should help to understand the population dynamics of this emerging pathogen. A possible grouping for the *P. ramorum* isolates is suggested and discussed.

MATERIAL AND METHODS

Isolates

We studied 94 *Phytophthora ramorum* isolates, 67 from Europe, and 27 from North America (Table 1). The European isolates originated from Belgium (2), France (11), Germany (30, including the ex-type strain), Italy (1), The Netherlands (5), Poland (3), Spain (7), and the UK (8). Isolates from Europe were from *Rhododendron* (48), *Viburnum* sp. (18) and *Quercus rubra* (1). The infected plants came mainly from nurseries, but some also from garden centres, parks, or private gardens. The first oak isolate in Europe was from a public garden. The North American isolates originated from Canada (4) and the USA (23). All Canadian isolates were from *Rhododendron* in nurseries. The USA isolates came mainly from trees such as *Quercus* sp. (5), *Lithocarpus densiflora* and *Umbellularia californica* (3 each), *Arbutus menziesii* (2) and *Sequoia sempervirens* (1) but also from *Rhododendron* (4), *Viburnum* sp. (1) and from *Vaccinium* sp. (2). One isolate originated from a soil sample. The US tree isolates and the *Vaccinium* isolates came from forest trees, while all North American *Rhododendron* and the single *Viburnum* and *Camellia* isolates originated from nurseries. All isolates used in these studies were collected between 1995 and 2003. In all studies the ex-type strain isolate BBA 9/95 (Werres *et al.* 2001a) was included. All isolates were hyphal tip cultures and stored under liquid nitrogen.

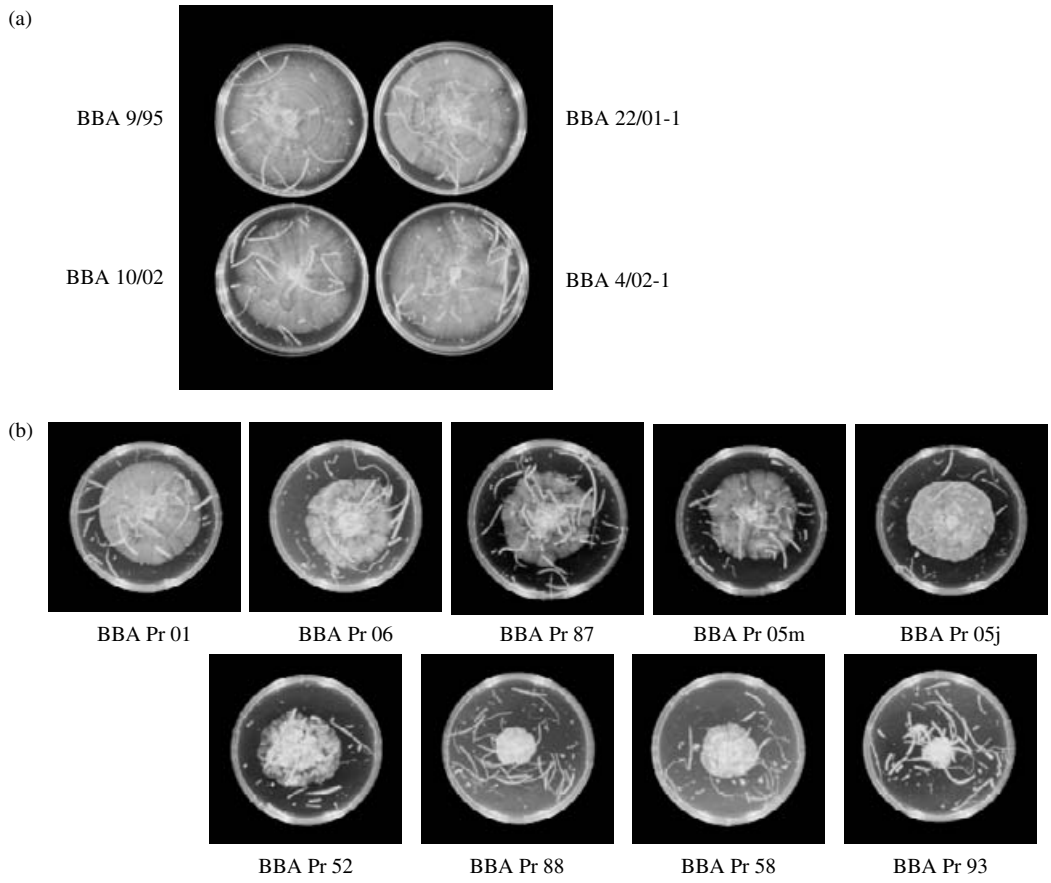

Fig. 1. Colony pattern of *Phytophthora ramorum* isolates of mating type A1 (*a*) and A2 (*b*) on CPA (13 d incubation at 20 °C in the dark).

For the mating studies four heterothallic *Phytophthora* species of mating type A1 and A2 were used (Werres *et al*. 2001a). All tester *Phytophthora* species originated from woody plants from nurseries and all isolates were hyphal tip cultures.

Medium

Morphological characters were measured on carrot piece agar (CPA) made with 15 g carrot pieces and 22 g agar in 1000 ml DH_2O. The agar was prepared with carrots that had not been sprayed with fungicides.

Colony pattern and vegetative growth rate

To test for colony pattern and growth rate, each isolate was plated on CPA and incubated in the dark for 24 h at 20 °C, and followed by 2, 5, 10, 15, 20, 25, 28, 30, 32, 35 or 37 ° according to Werres *et al*. (2001a). Two Petri dishes (90 mm diam) per isolate and temperature were prepared.

Sporangium and chlamydospore sizes

On CPA, *Phytophthora ramorum* can produce sporangia without flooding. But preliminary tests showed that some isolates produced more sporangia in a shorter time after flooding with azalea soil extract (data not shown). Therefore, all isolates were flooded with azalea soil extract according to Werres *et al*. (2001a), and incubated for 24 h in the dark at 20 °. Fifty sporangia per isolate were measured at 500× magnification.

For the determination of the size of the chlamydospores, pieces of a growing colony (at 20 °, in the dark) were taken out and placed on a slide; 50 fully developed chlamydospores per isolate were measured at 500× magnification.

Mating studies and measurements of oogonium size

All isolates were paired on CPA with mating type A1 and A2 of four different heterothallic *Phytophthora* species. From the edge of a growing colony, a mycelium disc was cut out and placed on CPA about 1 cm from the edge of the Petri dish. A similar disc from the mating partner was placed on the opposite side, also about 1 cm from the edge of the Petri dish. Petri dishes were incubated for 6 wk at 20 ° in the dark. Preliminary tests using this method (data not shown) had shown that most *P. ramorum* isolates produce oogonia within 6 wk of incubation. Agar discs with oogonia where cut out for size measurement under the microscope at 500× magnification. The measurements of *P. ramorum* oogonia were prepared from the mating

Table 2. Vegetative growth of *Phytophthora ramorum* isolates on CPA.

Country	Number of isolates	Temperature (°C) (mean, range)			Growth rate at optimum temperature (mm d^{-1}) (mean, range)
		Minimum	Optimum	Maximum	
Europe					
Belgium	1 (BBA 27/02)	2	20	28	3.1
	1 (BBA 26/02[a])	**2**	**20**	**30**	**3.4**
France	11	2	20 (15–25)	30 (28–32)	3.2 (2.6–3.4)
Germany	30	2	20 (15–25)	28 (26–30)	3.1 (2.5–3.5)
Italy	1	2	20	30	3.4
The Netherlands	1 (BBA PD 2003/5548-1)	2	20	28	3.4
	4	2	20–25	30 (28–30)	3.4 (3.0–4.4)
Poland	3	2	20	28	3.1 (2.9–3.4)
Spain	7	2	20	28 (28–30)	3.1 (2.8–3.3)
UK	8	2	20 (20–25)	30 (28–30)	3.2 (2.9–3.4)
North America					
Canada	4	2	20 (20–25)	30	3.1
USA	**17**	**2 (2–5)**	**20 (15–25)**	**25–30**	**2.2**
	3	2	20–25	28–30	3.3
	1 (BBA Pr 70)	2	10	28	1.1
	1 (BBA Pr 58)	2	20–25	25	1.1
	1 (BBA Pr 93)	5	15–20	25	1.8

[a] Normal type = mating type A1; **bold type** = mating type A2; shading: no successful mating.

with *P. cryptogea*, except for those isolates which did not produce oogonia with these mating partners. For these isolates oogonium sizes from the mating studies with *P. cambivora* were calculated; between 20–30 oogonia per isolate and pairing were measured.

Statistical calculation

To test for any significant separation of groups within the *Phytophthora ramorum* isolates, discriminant analysis was used (Mann-Whitney Rank Sum Test in SIGMA STAT 3.0). The discriminant analysis determines which variables and how many variables account for most of the differences in mean profiles of the groups.

RESULTS

Colony pattern

The European isolates showed no distinct colony pattern on CPA (Fig. 1a). However, many produced concentric zones even when incubated in the dark. The colony pattern of the Canadian isolates was identical to that of the European ones. But the US isolates showed a variety of colony patterns: those of the fast growing isolates looked similar to those of the European isolates, but the very slow growing ones showed more compact colony morphology, a flattening colony edge, or grew with sectors (Fig. 1b).

Vegetative growth

The minimum temperature for vegetative growth for all 94 isolates was 2–5 °, the optimum temperature was between 15–25 ° with 20 ° for most of the isolates, and the maximum temperature varied between 26 and 30 °. Growth rate at optimum temperature was between 1.1–4.4 mm d^{-1} with a mean of 2.9 mm d^{-1} (Table 2). The first European isolate from *Quercus rubra* (BBA PD 2003/5548-1) did not differ from those from *Rhododendron* and *Viburnum*.

There was a significant difference ($P = < 0.001$) between the two mating types: for the 73 A1 isolates the mean of the vegetative growth rate at optimum temperature was 3.1 ± 0.3 mm d^{-1}, the minimum growth rate was 2.5 mm d^{-1}, and the maximum growth rate was 3.5 mm d^{-1} (Table 2, Fig. 2a). The data for the single European A1 isolate from *Quercus rubra* (BBA PD2003/5548-1) was as high as those from nursery isolates. The 17 A2 isolates showed lower growth rates on CPA: 2.3 ± 0.15 mm d^{-1} mean, 1.6 minimum and 3.4 mm d^{-1} maximum (Table 2). The high growth rate of 3.4 mm d^{-1} could be reached only by the single European A2 isolate (BBA 26/02). Fifteen of the 17 A2 isolates had a growth rate below 3.0 mm d^{-1} and one isolate at 3.0 mm d^{-1}. The three US isolates which did not produce gametangia in the mating studies had the lowest growth rates (1.1 mm d^{-1} and 1.8 mm d^{-1} m, respectively).

Sporangia

The mean sporangium length of the 93 isolates was 43.6 μm ± 5.3 with a range from 20–79 μm and the mean sporangium width was 23.9 μm ± 2.6, with a range from 12–40 μm (Table 3). The mean length : breadth ratio was 1.8 : 1 ± 0.12, the minimum was 1.5 : 1; and the maximum was 2.2 : 1 (Table 3).

There was no significant difference between the sporangia sizes of A1 and A2 isolates (Table 3, Fig. 2a, b).

Table 3. Sizes of sporangia, chlamydospores, and oogonia of *Phytophotora ramorum* isolates.

		Sporangia ($n=50$)				Chlamydospores ($n=50$)		Oogonia ($n=20$–30)[b]	
Country	Number of isolates	Length range (μm)	Width range (μm)	Average (μm)	L:B ratio	Range (μm)	Average (μm)	Range (μm)	Average (μm)
Europe									
Belgium	1 (BBA 27/02)	32–64	16–32	43.2 × 22.4	1.9	50–74	61.0	26–34	29.8
	1 (BBA 26/02[a])	**34–58**	**20–32**	**45.4 × 24.8**	**1.8**	**46–72**	**56.4**	**24–34**	**29.6**
France	11	22–68	12–38	43.1 × 23.5	1.8	34–88	57.3	22–34	28.8
Germany	30	20–79	14–34	45.2 × 24.5	1.9	22–78	52.4	22–46	29.3
Italy	1 (BBA 24/02)	24–56	16–34	36.6 × 19.6	1.9	22–74	49.2	24–32	28.4
The Netherlands	1 (BBA PD 2003/5548-1)	32–54	18–28	42.0 × 22.2	1.9	42–66	53.3	28–36	29.8
	4	26–60	18–32	43.4 × 23.6	1.9	38–78	57.6	26–36	28.7
Poland	3	36–66	20–34	46.9 × 26.0	1.8	38–68	48.6	26–36	31.1
Spain	7	26–72	16–38	45.7 × 24.0	1.8	32–72	51.5	24–36	29.9
UK	8	28–60	14–38	42.3 × 23.3	1.8	38–78	57.5	24–34	28.4
North America									
Canada	4	24–46	16–24	32.4 × 20.4	1.6	40–80	54.2	24–32	28.0
USA	**11**	**22–74**	**14–40**	**44.0 × 24.3**	**1.8**	**22–78**	**49.2**	**22–44**	**30.0**
	3	28–50	16–30	37.1 × 22.6	1.7	28–80	59.1	24–32	30.9
	1 (BBA Pr 70)	26–64	14–30	45.0 × 25.4	1.8	32–72	51.8	None produced	
	1 (BBA Pr 58)	30–64	18–28	42.6 × 23.4	1.8	38–68	55.6	None produced	
	1 (BBA Pr 93)	32–60	18–30	42.6 × 23.8	1.8	42–76	61.4	None produced	

[a] Normal type = mating type A1; **bold type** = mating type A2; shading = no successful mating.

[b] Presented are only data from the mating experiments with *P. cryptogea* BBA65909 (mating type A1) for the A2 isolates and with *P. cryptogea* BBA63651 (mating type A2) for the A1 isolates, respectively.

When the number of oogonia was very low or when a precise measurement was not possible because the oogonia were only situated directly in the carrot pieces, the size was not measured: France ($n=11$), Germany ($n=13$), The Netherlands ($n=4$), Poland ($n=4$), Spain ($n=7$), UK ($n=8$), Canada ($n=3$), and USA (for A1 $n=2$, for A2 $n=11$).

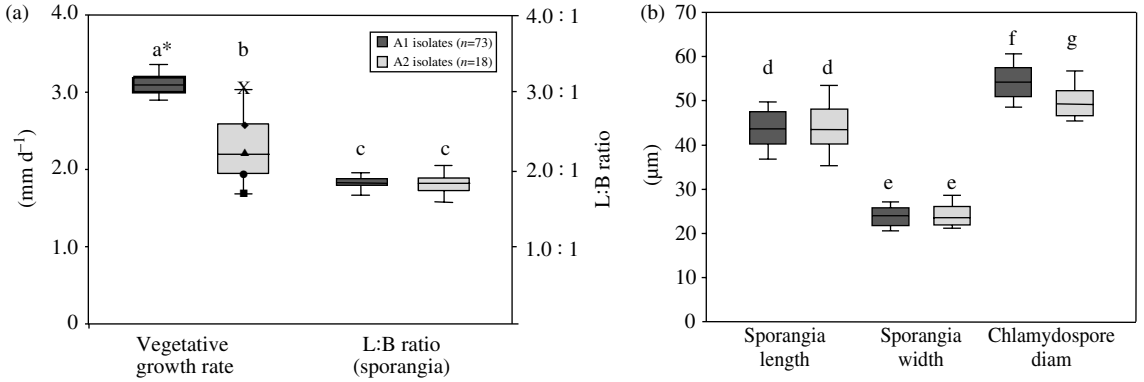

Fig. 2. Comparison of the morphological characteristics of the *Phytophthora ramorum* mating type groups (box contains the middle 50% of the data: •, q1 = 25%, ◆, q2 = 75%; whiskers indicate the minimum (■, 5%) and maximum, (X, 95%) data values; ▲, median (Mann-Whitney Rank Sum Test; *, boxes with the same letter are not significantly different at $P=0.05$).

But the data showed a high variation in sporangium size. The shape of the sporangia from European and from the US isolates of mating type A1 were very uniform and very similar to those of the type strain BBA 9/95. But some of the US isolates of mating type A2 had a much higher number of sporangia with distorted shapes or abnormal sizes (Fig. 3). There was no clear pattern to the production of these sporangia. They mainly occurred in single colony segments, which could be visually separated on the agar, but sometimes they could be observed in all parts of the colony. Some isolates never had abnormal sporangia, while others produced them in one subculture but not another. The four Canadian isolates had the lowest length:breadth ratio (1.6:1, Table 3).

Chlamydospores

All 94 *Phytophthora ramorum* isolates produced intercalary and terminal chlamydospores on CPA. Chlamydospore size ranged from 22–88 μm with a mean size of 53.4 μm ± 4.7 (Table 3).

There was a significant difference ($P=<0.001$) between the chlamydospore size of isolates from mating type A1 and A2: The A1 isolates produced larger chlamydospores (mean 54.3 μm ± 4.3) than the A2

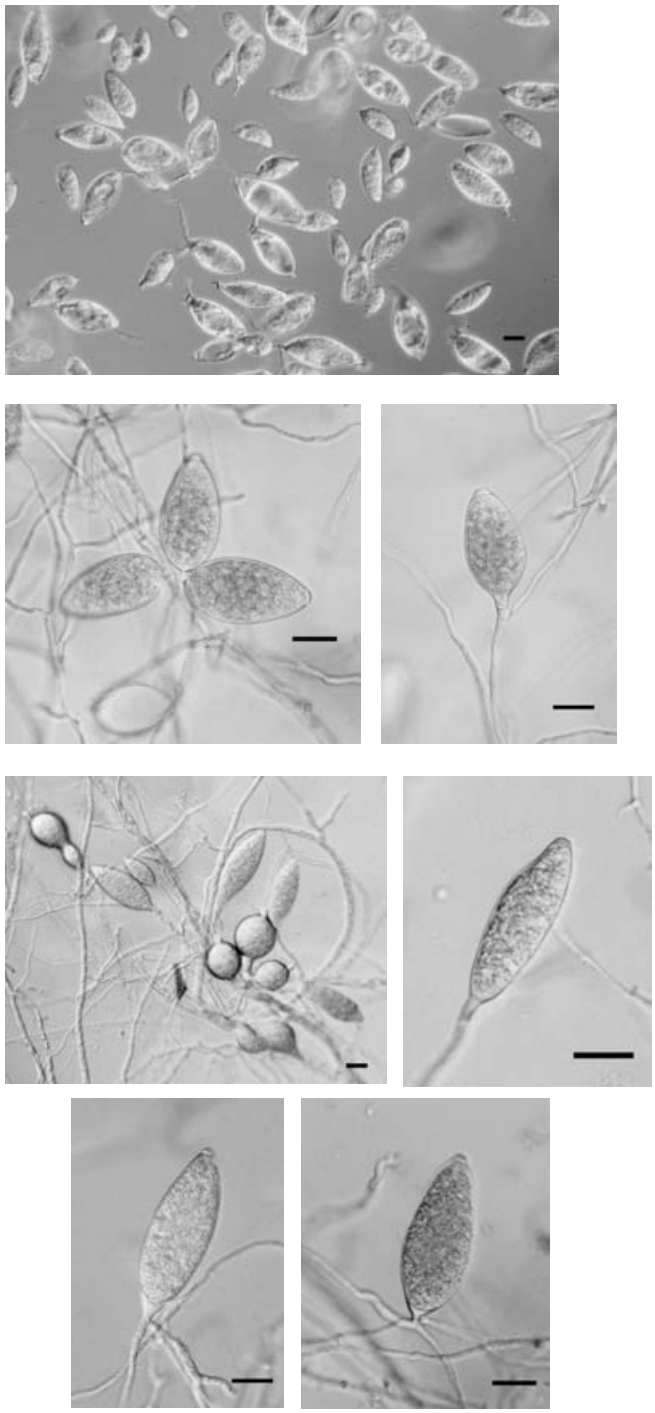

Fig. 3. Sporangia of *Phytophthora ramorum* mating type A1 (BBA 35/01-1) and A2 (BBA Pr01) isolates with normal size and of mating type A2 (BBA Pr 88) with abnormal length. Bar = 20 μm.

isolates (mean 49.6 μm ± 4.4). Only the single European isolate of mating type A2 (BBA 26/02) produced chlamydospores (mean value 56.4 μm) as big as the A1 isolates (Table 3, Fig. 2b). The single European isolate from *Quercus rubra* (BBA PD 2003/5548–1) did not differ in its chlamydospore size from the nursery isolates.

Mating type and oogonium size

All European *Phytophthora ramorum* isolates produced gametangia (Table 4), except Belgian isolate BBA 26/02, when paired with heterothallic *Phytophthora* species of mating type A1. Nearly all European isolates were classified as the A1 mating type, including the single isolate from *Quercus rubra* (BBA PD 2003/5548-1); BBA 26/02 was the only one of mating type A2. From the 27 North American *P. ramorum* isolates only 24 were paired successfully. Three of the US isolates (BBA Pr 93, BBA Pr 70, BBA Pr 58) did not produce gametangia within 6 wk with any of the tester mating partners (Table 4). Seven isolates could be determined as mating type A1 and 17 as mating type A2. In total 73

Table 4. Mating of *Phytophthora ramorum* isolates on CPA (incubation 20 °C in the dark; incubation period 6 wk).

		Mating partners (mating type A1)			
Country	Number of isolates	*P. cambivora* (BBA21/95-KII)	*P. cinnamomi* (BBA69094)	*P. cryptogea* (BBA65909)	*P. drechsleri* (BBA65172)
Belgium	1 (BBA 26/02)	X[b]	X	X	X
USA	**15**[a]	**12**[c]	**4**	**13**	**5**[c]
	2	2	Ø	2	2
		Mating partners (mating type A2)			
		P. cambivora (BBA20/95-2bIII)	*P. cinnamomi* (BBA62660)	*P. cryptogea* (BBA63651)	*P. drechsleri* (BBA62679)
Belgium	1 (BBA 27/02)	Ø	Ø	X	Ø
France	11	0	9	11	3
Germany	30	0	24	30	4
Italy	1 (BBA 24/02)	Ø	X	X	Ø
The Netherlands	**1 (BBA PD 2003/5548-1)**	**Ø**	**X**	**X**	**Ø**
	2	**0**	**0**	**2**	**1**
	2	0	1	2	0
Poland	3	0	2	3	0
Spain	7	0	4	7	0
UK	8	0	4	8	1
Canada	4	0	3	3	1
USA	3	0	1	3[c]	1

[a] Normal type = isolates from nursery or garden centre; **bold type** = isolates from park, private garden or forest.

[b] X = production of gametangia; Ø = no production of gametangia; numbers present number of isolates producing gametangia with the mating partner.

[c] = one isolate with only a single degenerated oospore after 8 wk.

No successful mating nor with mating partners of A1 nor with partners of mating type A2: BBA Pr 93, BBA Pr 70, BBA Pr 58.

of the 94 *P. ramorum* isolates were determined as mating type A1 and 18 as mating type A2.

The most successful mating partner was *P. cryptogea* (Table 4). In total 79.2% of the 91 successfully mated isolates produced sex organs after mating with *P. cryptogea*. Only three of the North American isolates (BBA Pr 88, BBA Pr 110a, and BBA MSOD 03–002), could not be paired with *P. cryptogea* within 6 wk. The first two isolates originated from US forests, the third from a Canadian nursery. These three isolates belonged to mating type A2. *P. cinnamomi* was a successful partner for 50%, *P. drechsleri* for 17.3% and *P. cambivora* for 13.6% of the isolates.

There was a clear separation between isolates of mating type A1 and A2. A1 and A2 isolates both preferred *P. cryptogea* as the most favourable partner, except two US isolates of mating type A2 (BBA Pr 88 and BBA Pr 110a). However, none of the A1 isolates could be paired with *P. cambivora* while 83.3% of the 18 A2 isolates formed gametangia with this mating partner (Table 4). On the other hand 68.5% of the 73 A1 isolates accepted *P. cinnamomi* while only 27.8% of the 18 A2 isolates produced gametangia with this *Phytophthora* species, among others the single European isolates BBA 26/02 of mating type A2. *P. drechsleri* was successful in the mating studies for 15.1% of the A1 isolates but for 44.4% of the A2 isolates.

More than half (52.7%) of the 91 isolates produced gametangia with two different mating partners (Table 4). It was mainly *P. cryptogea* and *P. cinnamomi* for the A1 isolates and *P. cryptogea* and *P. cambivora* for the A2 isolates. More than a quarter (27.5%) of the 91 isolates could be paired with only one mating partner which was always *P. cryptogea* for the A1 isolates or *P. cambivora* for the A2 isolates. For 17.6% of the isolates three of the four mating partners were successful and only two isolates of mating type A2 (the single European A2 isolate BBA 26/02 and the US isolate BBA Pr 65) gave positive results with all four *Phytophthora* species.

The total number of gametangia produced in the experiments was very low. Highest numbers of gametangia were observed with *P. cryptogea* for isolates of both mating types and lowest with *P. drechsleri*. Oogonia/antheridia were produced directly in the carrot pieces or surrounding the pieces. Most of the oospores looked degenerated. Mainly the pairings with *P. cryptogea* gave apparently 'healthy' looking oospores with some of the *P. ramorum* isolates. The size and shape of the oospores in the pairings with *P. cryptogea* looked identical to those presented in the original description of *P. ramorum* (Werres *et al.* 2001a). In the pairings with *P. cryptogea* the size of the oogonia of the 68 isolates of which the oogonia were measured, was 29.4 µm ± 2.0 diam (Table 3) with 29.2 µm ± 1.6 diam for the 56 isolates of mating type A1 and 29.9 µm ± 3.4 diam for the 12 isolates of mating type A2.

Discriminant analysis

The discriminant analysis indicated that the vegetative growth rate at the optimum temperature (mm d^{-1}) was the variable that most clearly discriminated the isolates into groups; inclusion of further variables like size of chlamydospores etc. did not further improve the discrimination (R squared). The two groups which were discriminated corresponded with the isolates mating types. Therefore the vegetative growth rate was used to create the discriminant function for separation of the mating types:

$D = 3.156 - 1.264 \times$ vegetative growth rate

$D < 0.0$ classified the isolate as mating type A1, $D > 0.0$ as mating type A2 (Fig. 4). $D = 0$ presented a growth rate of 2.5 mm d^{-1}. So, the faster the growth of an isolate the more likely it belonged to mating type A1. Two isolates of mating type A1 (BBA 19/02, BBA 20/02) and one isolate of mating type A2 (BBA Pr 65) could not be classified with the discriminant function because their growth rates were exactly 2.5 mm d^{-1} resulting in $D = 0$. All isolates of mating type A1 could be classified correctly according to the mating studies. In the group with mating type A2, the single European isolate (BBA 26/02) and three US isolates (BBA Pr 04, BBA Pr 01, BBA Pr JL3.5.3) were determined as mating type A1, based on the discriminant analysis D while the mating studies showed these to be A2 types. These four isolates had vegetative growth rates between 3.0 and 3.4 mm d^{-1} similar to the A1 isolates. The three North American isolates which did not produce gametangia in the mating studies (BBA Pr 93, BBA Pr 70, BBA Pr 58) belonged to mating type A2 according to this discriminant function. They had very low growth rates (1.8, 1.1, 1.1 mm d^{-1}).

DISCUSSION

The morphological data of the studied *Phytophthora ramorum* isolates were similar to those presented in the original description (Werres *et al.* 2001a). However, there were differences within the 94 isolates. Regarding only the morphological data, the European isolates were much more homogenous than the North American. That confirms the results of some of the original studies but is discordant with the results from molecular studies (Brasier *et. al.* 2002, Brasier 2003, Werres & Zielke 2003, Brasier & Kirk 2004, Ivors *et al.* 2004). However, these studies were focused predominantly on differences between the European *and* North American populations. That was because in the past most A1 isolates could be only detected in Europe while A2 isolates occurred in North America. But now the opposite mating type has been detected in both continents, although in Europe up to now there is only one finding of an isolate of mating type A2 (Hansen *et al.* 2003, Werres & De Merlier 2003), and in North America mating type A1 has only been found in a few nurseries in Oregon, Washington, and British Columbia, never in forests.

The morphological data and the discriminant analysis showed that a grouping within the studied *P. ramorum* isolates was possible. The grouping corresponded better with mating type than with the origin of the isolate (Europe, North America). Furthermore it seems as if there are group-specific preferences concerning the mating partners. The isolates of mating type A1 were characterised by a high uniformity in phenology, faster growth rate, larger chlamydospores and by the inability to pair with *P. cambivora in vitro*. These criteria were characteristic for all European *and* North American isolates of this mating type. The only exceptions were the four Canadian isolates that had very small sporangia. The morphological characters of the group of A2 mating type isolates were much more heterogeneous; most were slow growing and had smaller chlamydospores, and nearly all produced gametangia in pairings with tester isolates of *P. cambivora*. The data confirmed previous studies with vegetative growth rates (Brasier *et al.* 2002, Brasier 2003). Concerning the preferences for single mating partners it should be noted that mating success was inconsistent from test to test, primarily when the number of oospores produced was very low, for example with *P. drechsleri*. Studies with other isolates of the *Phytophthora* species used as mating partners must be used to verify these mating group specific preferences for *P. ramorum*. Nevertheless there is the question whether the previous characterisation 'European' or 'North American' type is still useful since both mating types are now found on both continents.

Within the isolates of the mating type A2 group (according to the mating studies), some showed morphological and mating characteristics atypical for this group. First there was the single European A2 isolate BBA 26/02 which had the mating behaviour of most of the North American A2 isolates, but was as fast growing as the A1 isolates. The discriminant function predicted it as A1 mating type. Molecular studies also assigned the isolate BBA 26/02 to the group of the A1 isolates ('European group', Peter Bonants, pers. comm.). It appears to represent a recent 'mating type switch'. In addition, three US isolates (BBA Pr01, BBA Pr04, BBA Pr JL3.5.3) were assigned to the A1 group with the discriminant analysis due to their high growth rate but were classified as A2 by the mating studies. Two of these (BBA Pr01 and BBA Pr04) produced gametangia with *P. cambivora* as most of the A2 isolates did; the isolate BBA Pr JL3.5.3 did not. Molecular studies classified all three isolates as members of the predominantly A2 population (Peter Bonants pers. comm.). The data indicate that natural variation is much higher within the A2 type than within the A1 type.

For the three US isolates BBA Pr58, BBA Pr70 and BBA Pr93 which did not produce gametangia with the tester strains, the grouping result with the discriminant

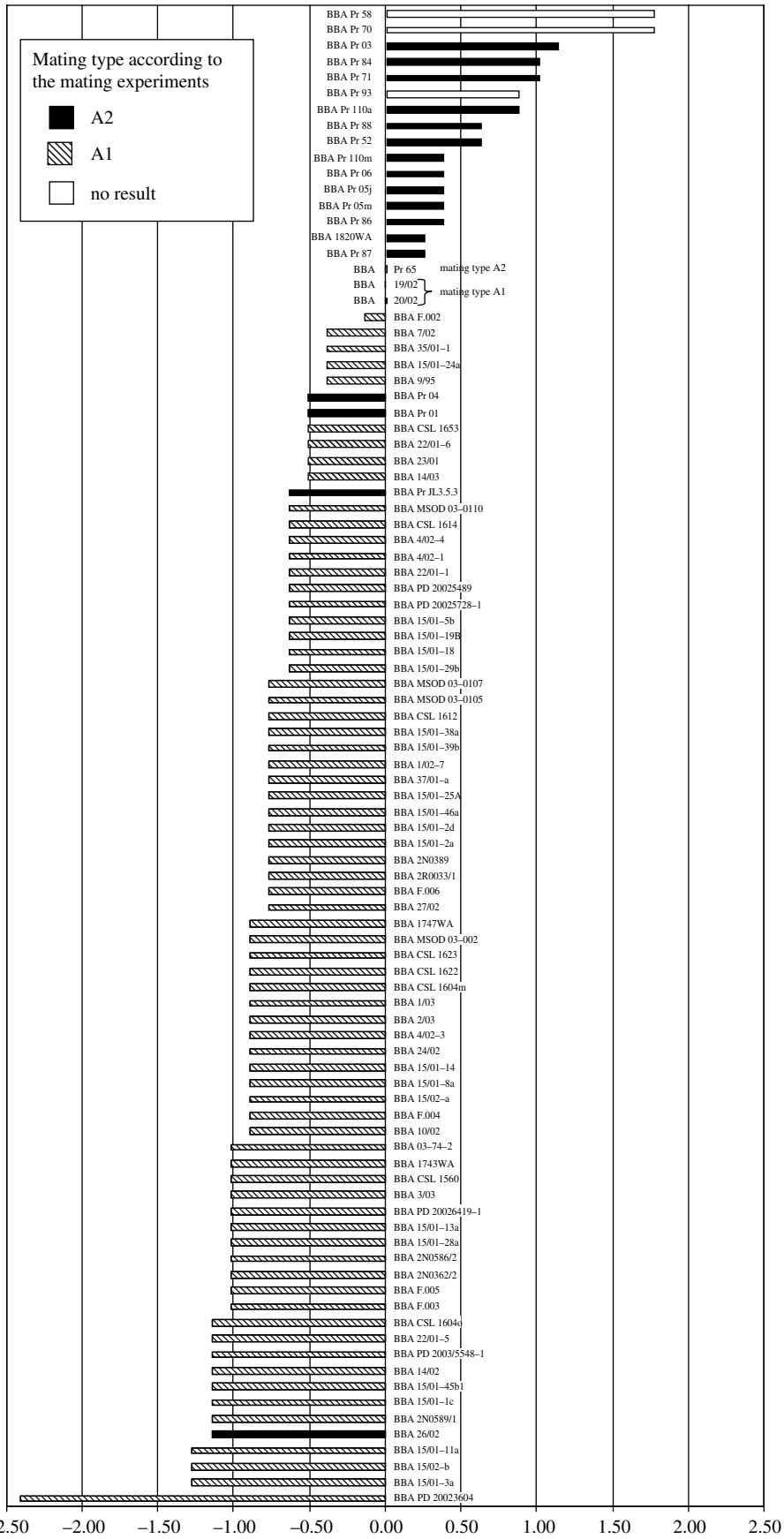

Fig. 4. Characterisation of *Phytophthora ramorum* isolates according to the mating studies and to the discriminant function ($D = 3.156 - 1.264 \times$ vegetative growth rate).

function was confirmed in mating experiments with *Rhododendron* twigs (B. Zielke & S. Werres, unpubl.). They belong to mating group A2. These three isolates grew very slowly. First studies with subcultures indicate that the morphological character of these slow growing isolates can change very quickly (S. Werres & K. Kaminski, unpubl.). So it may be that the heterogenicity, in morphological characters within the A2 isolates is perhaps caused by subculturing. If so this highlights the importantance of storing the isolates under well defined conditions and the passage through plant material before using in infection tests.

To verify the differences between and within the A1 and A2 mating group and to verify the character of colony changes it would be helpful not only to have more molecular studies but also to compare the pathogenicity of the isolates and subcultures, respectively. Previous studies showed that there are differences in the aggressiveness of North American and European isolates (Brasier *et al*. 2002, de Gruyter *et al*. 2002). In infection studies on *Rhododendron* twigs with (North American) isolates of mating type A2 and (European) isolates of mating type A1, the pathogenicity within the isolates of the mating type A1 was high and nearly uniform across the range of isolates used (F. Pogoda & S. Werres, unpubl.). Only the very slow growing A2 isolates showed low virulence while the aggressiveness of the faster growing isolates was similar to the A1 isolates. These first results indicate that with *Rhododendron* over all the aggressiveness of the A1 isolates is higher and more homogeneous than that of the A2 isolates. But there is the question whether the pathogenicity of the very slow growing A2 isolates is influenced by subculturing. More studies are necessary to validate this hypothesis. Differences in the pathogenicity of North American isolates were also observed in infection studies with *Quercus* sp. (Brasier *et al*. 2002, Hüberli *et al*. 2005b).

The sparse and erratic oospore production in the *in vitro* studies is discussed in detail by Brasier & Kirk (2004) who obtained similar results with *P. cambivora* and *P. cryptogea*. But *in vivo* mating studies with *P. cryptogea* in *Rhododendron* twigs showed that the oospores on the mycelium emerging from infected twig tissue looked healthy and vital (B. Zielke & S. Werres, unpl. data). These results were contradictory to those obtained in the *in vitro* studies, where most of the oospores looked damaged. This suggests that living plant material provides an environment more favourable for gametangial production.

Brasier & Kirk (2004) suggested that in the *in vitro* pairings *P. ramorum* gametangia production is 'a result of selfing of the *P. ramorum* isolates in the presence of the other species'. In terms of the epidemiology and evolution of *P. ramorum* it is important to know whether oospore production is as a result of selfing or true crossing and to know their role as resting spores. A serious danger is that A1 and A2 of *P. ramorum* may produce fertile offspring. An isolate suspected to be a recombinant has been recently detected in the USA (Garbelotto *et al*. 2005).

To prevent adaption and spread of more aggressive isolates, movement of infected plants or plant material should be avoided and quarantine regulations should be followed strictly to prevent the introduction of new genes and gene exchange.

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