ELSEVIER

British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/mycres

Influence of the medium-solidifying agent, the nutrient, and the genotype on the production of gametangia by *Phytophthora ramorum in vitro*

*Xavier BOUTET, Frédéric LAURENT, Anne CHANDELIER**

Walloon Agricultural Research Centre, Department of Biocontrol and Plant Genetic Resources, Rue de Liroux, 4, B-5030 Gembloux, Belgium

ARTICLE INFO

Article history:
Received 19 June 2007
Received in revised form
17 June 2008
Accepted 1 September 2008
Corresponding Editor:
David E. L. Cooke

Keywords:
Agar
Gametangia
Oospore
Pairing
Phytophthora ramorum

ABSTRACT

The effect of different parameters, including the type of nutrients, the quality of the gelling agent, and the genotype of the strain, were evaluated in the production of gametangia by *Phytophthora ramorum in vitro*. By comparing different agar sources on a carrot-based medium, a delay or a failure in the production of oospores was observed in pairings carried out on media supplemented with technical agar. In contrast, oospores were produced on other agar types, the production on media supplemented with agarose being slightly higher. The formation of gametangia was also influenced by the genotype of the strains involved in the pairing. A European A1 strain producing very few chlamydospores was found to be a better mating partner than other A1 strains. Using a carrot–agarose medium and selected genotypes, all European isolates were characterized in terms of mating type. A macroscopic experiment highlighted a particular spatial distribution of *P. ramorum* oospores *in vitro*. A method using polycarbonate membrane was evaluated to assess the selfing ability of *P. ramorum*.

Introduction

Phytophthora ramorum, the causal agent of 'sudden oak death' in the USA (Rizzo *et al.* 2002), and of dieback and foliar necroses in several nursery plants in Europe, notably *Rhododendron* and *Viburnum* (Werres *et al.* 2001) is a heterothallic species, meaning that the sexual recombination process involves the pairing between strains of two compatibility types, termed A1 and A2. Initial pairing studies revealed that all European isolates were of A1 type, whereas all the American isolates were of A2 type (Brasier 2003; Werres & Zielke 2003). In 2003, a Belgian isolate was designated as a putative European A2 (Werres & De Merlier 2003), while some A1 isolates were reported in American nurseries (Hansen *et al.* 2003), therefore, suggesting a possible crossing between both mating types.

Considering there is a theoretical possibility that isolates of opposite mating types may come into contact, it is important to determine the mating efficiency and the proportion of viable progenies from such mating. Furthermore, in order to evaluate the risk of recombination, it is essential to determine the mating type of an isolate, and to produce a lot of oospores. Two methods have been described for the production of oospores *in vitro*. The first was developed by Werres & Zielke (2003) and consists in pairing the unknown *P. ramorum* strain with tester strains of other heterothallic *Phytophthora* species. Oospores are observed microscopically after *ca* 40–60 d. Although of great interest, this *in vitro* method is time-consuming and was not applicable in intraspecific pairings. Also, the origin of the oospore is uncertain due to potential selfing of the tester strain. A second method developed by Brasier &

* Corresponding author. Tel.:+32 81620320; fax: +32 81620349.
E-mail address: chandelier@cra.wallonie.be
0953-7562/$ – see front matter
doi:10.1016/j.mycres.2008.09.001

Kirk (2004) relies on the mixing of juvenile mycelia of complementary *P. ramorum* strains directly on a carrot agar (CA) medium. Oospores are observed within 10 d. This method is faster and does not require the use of tester strains from other *Phytophthora* species. However, it has proven to be sometimes unpredictable and is unsuccessful with some 'recalcitrant' *P. ramorum* isolates. The objective of this study was to enhance the production of oospores of *P. ramorum in vitro* by evaluating the influence of the medium-solidifying agent with different nutrient sources.

Materials and methods

Isolates

The origins of the *Phytophthora* isolates used in this study are listed in Table 1. The European isolates of *P. ramorum* originated mainly from Wallonia and were from *Rhododendron* and *Viburnum*. The infected plants came from nurseries and were analysed in the frame of the survey organized by the Belgian Plant Protection Service. American isolates (only A2) were provided by Dr. De Gruyter (Plantenziektenkundige Dienst, The Netherlands). Tester strains from other heterothallic *Phytophthora* species were provided by Dr. Werres (Julius Kuehn Institute, Germany). All isolates were hyphal tip cultures and were stored at 13 °C under sterilized water. The origin of the *P. ramorum* isolates (American or European) was determined using the molecular method of Kroon *et al.* (2004). All the *P. ramorum* isolates used in our study are permanently preserved in our collection (Walloon Agricultural Research Centre, Department Biocontrol and Plant Genetic Resources, laboratory of mycology).

Media

Isolates were cultured on standard CA as described by Erwin & Ribeiro (1996). Briefly, 200 g washed carrots blended in 500 ml distilled water were filtered through four layers of cheesecloth and added to 500 ml distilled water with 15 g gelling agent. The medium was sterilized at 121 °C for 20 min and then poured in 9 cm Petri dishes (7.5 ml plate^{-1}). For pairing studies, mycelial plugs from complementary strains were taken from the margin of actively growing CA cultures and mixed together onto CA medium as described by Brasier & Kirk (2004). Two different pairing media were evaluated: CA and tomato juice agar medium (TJA). The latter medium was prepared by mixing 300 ml tomato juice

Table 1 – Characteristics of the *Phytophthora* isolates used in this study

Strain no.	Species	Original host	Collection	Mating type	Origin/lineage[a]
2299	*Phytophthora ramorum*	*Viburnum* sp.	CBS[b] 101330 (PD98/5233)	A1	EU/1
2301	*P. ramorum*	*Rhododendron* sp.	CBS[b] 101332 (PD94/844)	A1	EU/na
3237	*P. ramorum*	*V. bodnantense*	CBS[c] 110901	A2	EU/1
2386	*P. ramorum*	*Rhododendron* sp.	CBS[c] 110900	A1	EU/1
2387	*P. ramorum*	*Rhododendron* sp.	CRAW[d]	A1	EU/na
2530	*P. ramorum*	*V. farreri*	CRAW[d]	A1	EU/na
3196	*P. ramorum*	*Rhododendron* sp.	CRAW[d]	A1	EU/na
3197	*P. ramorum*	*Rhododendron* sp.	CRAW[d]	A1	EU/na
3206	*P. ramorum*	*Rhododendron* sp.	CRAW[d]	A1	EU/na
3207	*P. ramorum*	*Rhododendron* sp.	CRAW[d]	A1	EU/na
3208	*P. ramorum*	*V. farreri*	CRAW[d]	A1	EU/na
3209	*P. ramorum*	*Rhododendron*	CRAW[d]	A1	EU/na
3211	*P. ramorum*	*V. bodnantense*	CRAW[d]	A1	EU/na
3528	*P. ramorum*	*Quercus* sp.	PD[e] 20017608 (USA014)	A2	US/na
3529	*P. ramorum*	*Lithocarpus* sp.	PD[e] 20018928 (USA13)	A2	US/na
3530	*P. ramorum*	*Rhododendron* sp.	PD[e] 20018930 (USA217, Pr52)	A2	US/1
3531	*P. ramorum*	*Vaccinium* sp.	PD[e] 20018931 (USA240, Pr58)	A2	US/na
3532	*P. ramorum*	*Arbutus* sp.	PD[e] 20018932 (USA351, Pr87)	A2	US/1
3533	*P. ramorum*	*Umbellularia* sp.	PD[e] 20018933 (USA354, Pr88)	A2	US/na
3229	*P. cambivora*	*Chamaecyparis lawsoniana*	BBA[f] 21/95-K2	A1	–
3230	*P. cambivora*	Soil (*Q. robur*)	BBA[f] 20/95-2b3	A2	–
3231	*P. cinnamomi*	*Camellia* sp.	BBA[f] 69094	A1	–
3232	*P. cinnamomi*	*R. simsii*	BBA[f] 62660	A2	–
3233	*P. cryptogea*	*Lewisia* sp.	BBA[f] 65909	A1	–
3234	*P. cryptogea*	*Begonia* sp.	BBA[f] 63651	A2	–
2542	*P. cactorum*	*Fragaria ananassa*	CRAW[d]	Homothallic	–

EU, European; na, not available; US, American isolate.
a Origin of the *Phytopthora ramorum* isolate (EU or US) determined by PCR-RFLP according to Kroon *et al.* (2004) and genetic lineage according to Ivors *et al.* (2006).
b CBS Collection, Utrecht, The Netherlands.
c These strains come from our collection and were deposited in the CBS Collection.
d Our collection.
e Plantenziektenkundige Dienst (PD) Geertjesweg 15, Wageningen, The Netherlands (De Gruyter).
f JKI (Julius Kuehn Institute), Messeweg 11/12, Braunschweig, Germany (Werres).

(Carrefour, Bruxelles, Belgium) with 5.6 g $CaCO_3$. The preparation was clarified by centrifugation, and diluted in 1.2 l demineralized water with 15 g gelling agent. Four agar sources were tested as gelling agent: technical agar (Difco BD, Erembodegem-Aalst, Belgium; two batches used, nos 5061698 and 5200597), bacto-agar (Difco BD, Erembodegem-Aalst, Belgium; batch no. 6080255), noble agar (Difco BD, Erembodegem-Aalst, Belgium; batch no. 6058031), and agarose (Invitrogen, Paisley, United-Kingdom batch no. 3102950). Incubation of the Petri dishes was carried out in the dark at 20 °C.

Evaluation of the quantity of gametangia

In order to evaluate the number of gametangia produced, the Petri dishes were observed microscopically (×125 magnification) after 6 and 20 d. Five classes were established according to the number of gametangia observed: 0 (no gametangia), 1 (less than ten gametangia per pairing), 2 (up to five gametangia per field), 3 (5–25 gametangia per field) and 4 (more than 25 gametangia per field). Pairings between the mating type A1 and A2 of other heterothallic *Phytophthora* species, and a pure culture of *P. cactorum*, a homothallic species, were used as experimental controls.

Selfing ability

A modified polycarbonate technique initially described by Ko (1978) was used to assess the selfing ability of *Phytophthora ramorum* isolates. A sterilized polycarbonate membrane (Isopore net filters 0.1 μm; Millipore) was sandwiched between hyphal plugs ($15 \times 10 \times 3$ mm^3) of complementary isolates taken from the margin of an actively growing carrot–agarose (CAose) culture. Four heterothallic *Phytophthora* species (*P. ramorum, P. cinnamomi, P. cambivora, P. cryptogea*) were compared and two replicates were considered for each intraspecific pairing. A control consisting of putting the complementary hyphal plugs in contact without membrane was also introduced in the assay. The plates were incubated for at least 10 d in the dark at 20 °C in a moist chamber. The number of gametangia was evaluated according to the scale previously described.

Results

Preliminary tests to select efficient 'mating partners'

As a preliminary experiment, eleven European *Phytophthora ramorum* isolates of A1 type were paired with the six American isolates, as well as the European A2 isolate, using the method of Brasier & Kirk (2004) without any modification. Technical agar was used as the gelling agent and carrot as the nutrient source. As shown in Table 2, isolate 3528 was found to be the best American A2 partner in all the tests carried out. Indeed, pairings between isolates 3528 and five European strains produced oospores. The pairings involving isolates 3531, 3532, and 3533 failed to produce any oogonia, whereas isolates 3529 and 3530 produced very few oospores with only one or two A1 strains. The European A2 isolate (3237) produced gametangia with isolates 2299 and 3196. When sexual reproduction occurred, the number of gametangia was generally very low in all crossings (less than ten oospores per pairing) and they were localized directly beneath the mixing point. When the experiment was repeated, the same results were observed except for some pairings that gave rise to one or two oospore(s) per mixing while they did not produce oospore in the first experiment (data not shown).

Table 2 – Intraspecific pairings between complementary strains of *Phytophthora ramorum* using the inoculum mixing method developed by Brasier & Kirk (2004) on a carrot agar medium (technical agar used as the gelling agent, batch 1, no. 5061698)

A1	A2						
	3528	3529	3530	3531	3532	3533	3237
2299	2	0	2	0	0	0	3
2301	0	0	0	0	0	0	0
2386	0	0	0	0	0	0	0
2387	0	0	0	0	0	0	0
2530	1	0	0	0	0	0	0
3196	0	0	0	0	0	0	1
3206	1	0	0	0	0	0	0
3207	0	0	0	0	0	0	0
3208	0	1	0	0	0	0	0
3209	1	0	0	0	0	0	0
3211	1	0	1	0	0	0	0

Observations were carried out after 10 d. The amount of oogonia was evaluated according to a 0–4 scale (three repetitions considered).

Identification of different parameters involved in the oospores production

In order to increase the oospore production rate, different media were tested with different *Phytophthora ramorum* isolates selected as 'efficient mating partners' according to the first experiment (2299 as A1 type paired with either 3528 or 3237, the American and European A2 types, respectively). The strains introduced in the pairing were taken from CA or TJA plates in order to evaluate the influence of the original medium on the pairing efficiency.

As shown on Table 3, after 6 d the production of gametangia from *P. ramorum* on carrot-based media was found to be high on medium complemented with agarose. In contrast, a weak production of gametangia appeared on medium complemented with technical agar for the combination 2299×3237 while no oospore was observed for the combination 2299×3528. After 20 d, the combination 2299×3528 produced some gametangia that were mainly in a development stage. On carrot-based media, the production of gametangia was not influenced by the origin of the mycelial plugs (TJA or CA) used as inoculum.

On tomato juice-based media, there were differences according to the origin of the mycelial plug as no gametangia were found when the mycelium plugs originated from TJA medium. In contrast, when the mycelial plugs were taken from a CA subculture, gametangia were produced on TJAose only. In this case, the gametangia were observed directly adjacent to the mycelial plugs used as inoculum. No gametangia were observed deeper in the medium. All the other *Phytophthora* species used as experimental controls produced a lot of

Table 3 – Production of gametangia from four heterothallic and a homothallic *Phytophthora* species on different pairing media

Species	Pairing	Pairing medium			
	(A1xA2)	CA	CAose	TJA	TJAose
Phytophthora ramorum	2299 (TJA)[b] × 3528 (TJA)[b]	0	4	0	0
P. ramorum	2299 (TJA)[b] × 3237 (TJA)[b]	2	4	0	0
P. ramorum	2299 (CA)[b] × 3528 (CA)[b]	0	4	0	1
P. ramorum	2299 (CA)[b] × 3237 (CA)[b]	2	4	0	2
P. cambivora	3229 (TJA)[b] × 3230 (TJA)[b]	4	4	3	3
P. cinnamomi	3231 (TJA)[b] × 3232 (TJA)[b]	4	4	4	4
P. cryptogea	3233 (TJA)[b] × 3234 (TJA)[b]	4[a]	4	4	4
P. cactorum	2542 (TJA)[b]	4	4	4	4

Observations carried out after 6 d. The amount of gametangia was evaluated according to a 0–4 scale (three repetitions considered). CA, carrot agar: batch 1 no. 5061698; CAose, carrot agarose; TJA, tomato juice agar: batch 1 no. 5061698, TJAose, tomato juice agarose.
a All oospores were immature.
b Media in brackets correspond to the origin of the mycelium plug.

Table 4 – Production of gametangia from four heterothallic and a homothallic *Phytophthora* species on a carrot-based medium (C) complemented with different gelling agents

Species	Pairing	Pairing media			
	A1 × A2	CBA	CA	CAN	CAose
Phytophthora ramorum	2299 × 3528	4	2	4	4
P. ramorum	2299 × 3237	4	2	4	4
P. ramorum	2387 × 3528	2	0	2	3
P. ramorum	2387 × 3237	2	1	2	3
P. ramorum	3206 × 3528	2	1	2	2
P. ramorum	3206 × 3237	3	2	2	2
P. cambivora	3229 × 3230	4	4	4	4
P. cinnamomi	3231 × 3232	4	4	4	4
P. cryptogea	3233 × 3234	4	0	4	4
P. cactorum	2542 (homothall.)	4	4	4	4

Observations were made after 6 d. The amount of gametangia was evaluated according to a 0–4 scale (three repetitions considered). BA, Bacto-agar; A, technical agar (batch 2, no. 5200597); AN, noble agar; Aose, agarose.

gametangia (equal or more than 50 gametangia per field) regardless of the medium used except for *P. cambivora*, which produced five to ten oogonia per field on tomato juice-based medium. For pairings of *P. cryptogea* carried out on CA (3233 × 3234), all the gametangia were in the granular stage of development.

All the pairings between *P. ramorum* complementary strains carried out on TJA or TJAose showed no increase in oogonia production after 20 d of incubation (data not shown).

Comparison between different medium-solidifying agents

Four different gelling agents (technical agar, bacto-agar, noble agar, and agarose) were added to a carrot-based medium. The European isolates 2299, 2387, and 3206 were selected as the A1 types and isolates 3528 (US) and 3237 (European) as the A2 types. The control species were the same as those used in the first experiment.

As shown on Table 4, gametangia were observed in all but one pairing (2387 × 3528 on CA). On CA, the production of gametangia from *Phytophthora ramorum* was very low (less than five gametangia per field). Moreover, the gametangia (oogonia and antheridia) displayed a granular appearance for all the combinations of genotypes, demonstrating a delay in the gametangia formation (Fig 1A). On the other media, the antheridia were not granular and the oospores were clearly visible in the oogonia (presence of a clear periplasmic space between the oogonium and the oospore), indicating a more advanced state in the maturation process (Fig 1B). The production of gametangia was also influenced by the strains used in the pairing. Strain 2299 was the best A1 partner, giving rise to many oogonia (more than 25 gametangia per field) except on CA. In the CAose medium, a high density of oospores was found in carrot aggregates, which appeared after the sterilization (up to 30–40 oospores per aggregate with the combination 2299 × 3237). These aggregates, which are composed of carrot micro-fragments, were not observed in the other media. All the other heterothallic *Phytophthora* species used as experimental controls produced many gametangia in all media except *P. cryptogea*. This species showed a delay in the production of oospores on CA (i.e. no gametangia after 6 d, gametangia in a development stage after 8 d, and a production of oospores similar to that of the other *Phytophthora* experimental control after 20 d).

Spatial distribution of Phytophthora ramorum *oospores in intraspecific pairings*

In the mating studies, *Phytophthora ramorum* gametangia were not evenly distributed in the plate, some sectors containing higher proportion of gametangia than others. This heterogeneous distribution was not observed for the other *Phytophthora* species. In order to visualize more easily the specific behaviour of both mating partners during the pairing, small agar plugs (*ca* 1 mm^2) of inoculum were placed linearly in close proximity to simulate the mixing point. Inoculum plugs from strains 2299 and 3237 were alternated in the middle of a CAose plate (Fig 2). Many gametangia and very few chlamydospores were produced mainly in the growth sector of strain 2299. In contrast, very few oospores and many chlamydospores appeared in the growth sector of strain 3237. This particular spatial distribution suggested further tests of the selfing ability of strain 2299. To this end, isolates 2299 (A1) and 3237 (A2) were paired across a polycarbonate membrane. Whereas oospores were produced for three other heterothallic *Phytophthora* species used as experimental controls, the *P. ramorum* isolates failed to produce oospores, even when opposite hyphal plugs were paired without membrane (data not shown).

Discussion

In this paper, we examined the influence of different parameters, including the agar type, the nutrient source, and the isolate on the production of oospores by *Phytophthora ramorum*.

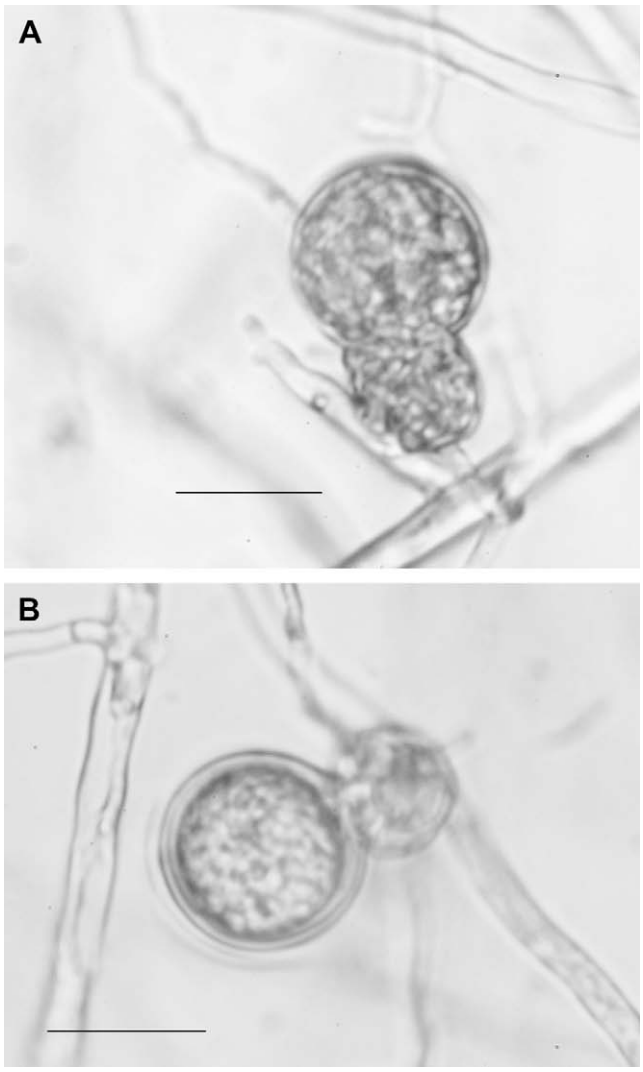

Fig 1 – Oogonium and antheridium of *Phytophthora ramorum* from intraspecific pairing: (A) in a granular stage; (B) with individualized oospore. Bar = 20 μm.

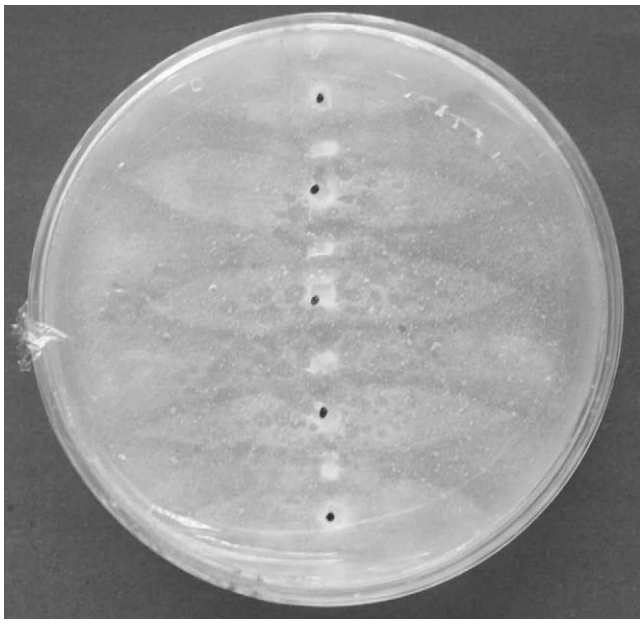

Fig 2 – Linear arrangement of inoculum plugs of *Phytophthora ramorum*. Plugs marked in black come from 2299 subculture (A1). Unmarked plug come from 3237 subculture (A2).

Agar is a generic term used to designate a mixture of galactose polymers produced by several species of algae. It is commonly used as a setting agent for nutrient media in many fields of microbiology and pairing studies are typically conducted on agar media. However, a critical effect of the agar source during *in vitro* experiments has already been reported by Ho & Ko (1980) working on the effect of antimicrobial substances on the germination of spores, by Borden *et al.* (1970) studying the capacity of some viruses to infect animal cells or by Beruto (1997) studying the influence of agar type on the *in vitro* growing of *Ranunculus* species. The results obtained in this study highlight the impact of the agar quality on the production of gametangia by *P. ramorum*. There are different types of agar proposed for microbiological purposes, those products differing notably by their chemical purity and their adsorption capacity linked to their molecular polarity. Moreover, as agar is a natural product, its quality can also vary between products from different brands or between batches from the same manufacturer (Beruto 1997; Perez 1993). In our experiments, we tested four agar types including technical agar (two batches), bacto-agar, noble agar, and agarose. When compatible genotypes were paired on a carrot-based medium, the production of oospores was low on a medium supplemented with technical agar regardless of the batch used. In contrast, the production was higher on the other media, especially on medium with agarose, a highly purified agar type. This suggests that failure of *P. ramorum* to produce oospores on technical agar might be due either to impurities inhibiting the production of gametangia or to the agar characteristics interfering with the diffusion of molecules involved in the sexual process. These molecules might be produced by the pathogen or present in the medium (plant extracts). The delay (rather than the absence) in gametangia production observed in CA suggests that a chemical adsorption of specific substances cannot be ruled out. In this context, Ko (1978) demonstrated a chemical regulation of the sexual reproduction in heterothallic *Phytophthora*. After placing complementary mating types on opposite sides of a polycarbonate membrane, he still observed gametangia in each side. He showed the existence of diffusible substances (or 'hormones') called alpha 1 (produced by A1 strains) and alpha 2 (produced by A2 strains) involved in the differentiation of vegetative hyphae in gametangia. The alpha 1 hormone has recently been isolated and characterized and seems conserved in the *Phytophthora* genus (Qi *et al.* 2005). Moreover, an isomeric mixture that could contain all possible stereoisomers of alpha 1 has been synthesized and its hormonal activity demonstrated (Yajima *et al.* 2007). The production of these hormones might be very low for *P. ramorum*, therefore, disturbing its sexual behaviour. In this way, any environmental condition interfering with the production or the diffusion of these hormones could result in a decrease in the production of gametangia. The hypothesis

of a deficient hormonal system in *P. ramorum* is reinforced by the fact that other heterothallic species introduced in the study are generally not affected by the agar source and display a normal sexual behaviour. The delay observed for *P. cryptogea* on CA is intriguing. This species was the most efficient in interspecific pairing with *P. ramorum* (Werres & Zielke 2003), suggesting a similarity in the sexual reproduction pathway of both *Phytophthora* species.

Besides the agar quality, the type of nutrients also seems to influence the production of gametangia by *P. ramorum in vitro*. As already observed with the agar source, *P. ramorum* seems more sensitive than other heterothallic species to the type of nutrient. The absence of oospores on a tomato juice-based medium except in close proximity of an agar plug that was taken from a carrot-based medium, as well as the high density of gametangia in carrot aggregates from a CAose medium demonstrate the influence of carrot extracts on the reproduction process. The influence of carrot pieces on the production of gametangia by *P. ramorum* has already been described by Werres & Kaminski (2005). The carrot aggregates that appeared in agarose medium could concentrate nutrients, which promote the sexual process. In this context, sterols are often described as critical nutrients for sexual reproduction (Elliott 1983; Sharma 1991). However, the addition of β-sitosterol (a plant sterol source) or cholesterol to the different media evaluated in this study did not improve the rate or frequency of gametangial production (data not shown).These results are in agreement with other studies demonstrating that most of the species of *Phytophthora,* which produce oospores in response to sterols, are homothallic (Hendrix 1970).

Finally, the genotype of the isolates involved in the pairings also has an impact on the reproduction. In this context, all pairings involving the A1 strain 2299 resulted in a higher quantity of oospores than with other European A1 strains. This strain produced very few chlamydospores compared with other *P. ramorum* strains, and this feature may enable the strain to allocate more energy to the production of sexual spores, as previously suggested by Brasier & Kirk (2004). It is interesting to notice that this strain was integrated in the genetic study carried out by Ivors *et al.* (2006) and classified among the EU1 clonal lineage, which represents the most important group of European strains. As a consequence, it seems that within a microsatellite clone, there is a phenotypic variation. As this variation is presumed to have arisen *via* asexual genetic events, other strains similar to the strain 2299 could, therefore, exist in the population of *P. ramorum* strains, increasing the probability of oospore production.

Sexual reproduction in *Phytophthora* species has a significant influence on the level of genetic diversity in the population. Concerning heterothallic species, the introduction of strains of the opposite mating type in geographic area where they were not present can promote sexual recombination with increased genetic diversity. This situation occurred with the introduction in Europe of new strains of *P. infestans* of the A2 mating type (Drenth *et al.* 1994). The mating of two isolates of opposite mating type results in either hybrid or selfed oospores. Although the crossing represents the main risk to generate new genotypes with a change in pathogenic fitness, selfed oospores could also lead to progenies with new phenotypical characters due to recombination events during meiosis. The recent report of a family of at least 350 infection-associated genes and the identification of an unusually large number of heterozygotic sites through the sequencing of the *P. ramorum* genome (Tyler *et al.* 2006) underlines the potential of this *Phytophthora* species for genetic rearrangements. Selfing could, therefore, represent an alternative mechanism (in addition to mitotic recombination or mutation) to generate diversity of the pathogen. In order to assess the selfing ability of the *P. ramorum* isolates, the mating partners were separated by a polycarbonate membrane as described by Ko (1978). Three reference heterothallic strains were introduced in the assay. A negative control, which consisted of putting the complementary mycelium plugs on contact without any membrane, was also considered. Oospores were produced for the three reference species that displayed a 'normal' breeding system, therefore, demonstrating that the experiment was conducted correctly and that these species were able to self under our experimental conditions. In contrast, the *P. ramorum* isolates failed to produce oospores, even without membrane (negative control). However, the conditions of this particular experiment (consisting in stacking complementary mycelium plugs) were very different from those used in experiments dealing with pairing ability. The addition of tryptophan, β-sitosterol, and $CaCl_2$ as proposed by Sharma (1991) did not increase the frequency of selfed oospores of *P. ramorum* (data not shown). These results suggest that the mixing of isolates or the close contact of small inoculum plugs before pairing is a prerequisite to the production of oospores by *P. ramorum* as already demonstrated by Brasier & Kirk (2004). The contact between strains could be required due to the low production of alpha hormone by this species as already discussed. Furthermore, our results suggest that the method using membrane is not suited to the evaluation of the selfing ability of *P. ramorum* isolates. As shown in *P. infestans*, the use of strains containing a β-glucuronidase (GUS) transgene could enable the assessment of whether oospores resulted from selfing (Judelson 1997). A genetic analysis of oospore progenies could also be used to trace the origin of oospores. However, this latest experiment requires the extraction of oospores from the medium and the activation of their germination. These experiments are in progress.

In conclusion, *P. ramorum* can generate oospores when specific strains are under suitable conditions. However, due to the low production of oospores *in vitro* and to the specific requirements of *P. ramorum* in terms of sexual reproduction, the test membrane generally used to assess the origin of oospores is questionable and other techniques should be evaluated. Nevertheless, our results suggest that new strains with altered disease potential could be generated. At an epidemiological level, oospores are known to be particularly resistant spores (Turkensteen *et al.* 2000). They could, therefore, enable the pathogen to survive more easily in unfavourable conditions. All these observations should be considered during pest risk analysis.

Acknowledgements

This research was funded by the Belgian Federal Government – Public Healthcare Service. We are grateful to Hans De Gruyter for sharing *Phytophthora ramorum* isolates from the US and to

Sabine Werres for providing tester strains from several heterothallic *Phytophthora* species. We also thank Kurt Heungens for his critical reading of the manuscript.

REFERENCES

Beruto M, 1997. *Agar and Gel Characteristics with Special Reference to Micropropagation Systems of Ranunculus asiaticus L.* PhD thesis, Gent University, Gent.

Borden EC, Gary GW, Murphy FA, 1970. Comparison of agar and agarose preparations for mengovirus plaque formation. *Applied Microbiology* **20**: 289–291.

Brasier CM, 2003. Sudden oak death: *Phytophthora ramorum* exhibits transatlantic differences. *Mycological Research* **107**: 257–259.

Brasier CM, Kirk S, 2004. Production of gametangia by *Phytophthora ramorum in vitro*. *Mycological Research* **108**: 823–827.

Drenth A, Tas ICQ, Govers F, 1994. DNA fingerprinting uncovers a new sexually reproducing population of Phytophthora infestans in the Netherlands. *European Journal of Phytopathology* **100**: 97–107.

Elliott CG, 1983. Physiology of sexual reproduction in *Phytophthora*. In: Erwin DC, Bartnicki-Garcia S, Tsao PH (eds), *Phytophthora, its Biology, Taxonomy, Ecology and Pathology*. APS Press, St Paul, pp. 71–80.

Erwin DC, Ribeiro OK, 1996. Culture, physiology and genetics of *Phytophthora* species. In: Erwin DC, Ribeiro OK (eds), *Phytophthora Diseases Worldwide*. APS press, St Paul, pp. 42–95.

Hansen EM, Reeser PW, Sutton W, Winton LM, 2003. First report of A1 mating type of Phytophthora ramorum in North America. *Plant Disease* **87**: 1267.

Hendrix JW, 1970. Influence of sterols on growth and reproduction of *Pythium* and *Phytophthora* spp. *Annual Review of Phytopathology* **8**: 111–130.

Ho WC, Ko WH, 1980. Agarose medium for bioassay of antimicrobial substances. *Phytopathology* **70**: 764–766.

Ivors KM, Garbelotto M, Vries IDE, Ruyter-Spira C, Hekkert BT, Rosenzweig N, Bonants P, 2006. Microsatellite markers identify three lineages of *Phytophthora ramorum* in US nurseries, yet single lineages in US forest and European nursery populations. *Molecular Ecology* **15**: 1493–1505.

Judelson HS, 1997. Expression and inheritance of sexual preference and selfing potential in *Phytophthora infestans*. *Fungal Genetics and Biology* **21**: 188–197.

Ko WH, 1978. Heterothalic *Phytophthora*: evidence for hormonal regulation of sexual reproduction. *Journal of General Microbiology* **107**: 15–18.

Kroon LPNM, Verstappen ECP, Kox LFF, Flier WG, Bonants PJ, 2004. A rapid diagnostic test to distinguish between American and European populations of *Phytophthora ramorum*. *Phytopathology* **94**: 613–620.

Perez R, 1993. *Ces Algues Qui Nous Entourent*. Ifremer, Plouzané.

Qi JH, Asano T, Jinno M, Matsui K, Atsumi K, Sakagami Y, Ojika M, 2005. Characterization of a *Phytophthora* mating hormone. *Science* **309**: 1828.

Rizzo DM, Garbelotto M, Davidson JM, Slaughter GW, Koike ST, 2002. *Phytophthora ramorum* as the cause of extensive mortality of *Quercus* spp. and *Lithocarpus densiflorus* in California. *Plant Disease* **86**: 205–214.

Sharma NK, 1991. Environmental control of selfing in *Phytophthora palmivora*: an aid to genetic analyses. *Current Science* **61**: 540–543.

Turkensteen LJ, Flier WG, Wanningen R, Mulder A, 2000. Production, survival and infectivity of oospores of *Phytophthora infestans*. *Plant Pathology* **49**: 688–696.

Tyler BM, Tripathy S, Zhang X, Dehal P, Jiang RH, Aerts A, Arredondo FD, Baxter L, Bensasson D, Beynon JL, Chapman J, Damasceno CM, Dorrance AE, Dou D, Dickerman AW, Dubchak IL, Garbelotto M, Gijzen M, Gordon SG, Govers F, Grunwald NJ, Huang W, Ivors KL, Jones RW, Kamoun S, Krampis K, Lamour KH, Lee M-K, McDonald WH, Medina M, Meijer HJG, Nordberg EK, Maclean DJ, Ospina-Giraldo MD, Morris PF, Phuntumart V, Putnam NH, Rash S, Rose JKC, Sakihama Y, Salamov AA, Savidor A, Scheuring CF, Smith BM, Sobral BWS, Terry A, Torto-Alalibo TA, Win J, Xu Z, Zhang H, Grigoriev IV, Rokhsar DS, Boore JL, 2006. *Phytophthora* genome sequences uncover evolutionary origins and mechanisms of pathogenesis. *Science* **313**: 1261–1265.

Werres S, De Merlier D, 2003. First detection of *Phytophthora ramorum* mating type A2 in Europe. *Plant Disease* **87**: 1266.

Werres S, Kaminski K, 2005. Characterisation of European and North American *Phytophthora ramorum* isolates due to their morphology and mating behaviour *in vitro* with heterothallic *Phytophthora* species. *Mycological Research* **109**: 860–871.

Werres S, Marwitz R, Veld W, De Cock A, Bonants PJM, De Weerdt M, Themann K, Ilieva E, Baayen RP, 2001. *Phytophthora ramorum* sp nov., a new pathogen on Rhododendron and Viburnum. *Mycological Research* **105**: 1155–1165.

Werres S, Zielke B, 2003. First studies on the pairing of *Phytophthora ramorum*. *Zeitschrift Fur Pflanzenkrankheiten Und Pflanzenschutz-Journal of Plant Diseases and Protection* **110**: 129–130.

Yajima A, Kawanishi N, Jianhua Q, Asano T, Sakagami Y, Nukada T, Yabuta G, 2007. Synthesis and biological activity of a stereoisomeric mixture of the mating type hormone of *Phytophthora*. *Tetrahedron Letters* **48**: 4601–4603.