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Production of gametangia by *Phytophthora ramorum in vitro*

Clive BRASIER and Susan KIRK

Forest Research Agency, Farnham, Surrey GU10 4LH, UK.
E-mail : clive.brasier@forestry.gsi.gov.uk

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Until now gametangia have not been obtained between paired European A1 and American A2 isolates of *Phytopthora ramorum in vitro*. Their production in artificial culture relies on interspecific pairings. Using *P. drechsleri* and *P. cambivora* testers, 51 of 110 *P. ramorum* isolates from across Europe were all shown to be A1s; while 32 of 38 American isolates from across California and southwest Oregon were shown to be A2s. However, these interspecific pairings are complex, unusually slow and unpredictable. A range of culture media and conditions are described that were tested, unsuccessfully, with a view to enhancing the efficiency of the interspecific pairings. In further tests, gametangia were obtained between A1 and A2 isolates of *P. ramorum* when juvenile, pre-chlamydospore producing mycelia were mixed together on carrot agar. The gametangia formed in 3–10 d, sparsely to frequently, initially only within the boundaries of the mixed inocula but subsequently in the extended mycelial growth. Chlamydospores were also produced. This inoculum-mixing method, though again sometimes unpredictable, should enhance efficiency of testing for compatibility types and facilitate further studies on whether the sexual outcrossing system of *P. ramorum* is functional. Differences between sexual reproduction of *P. ramorum* and that of other heterothallic *Phytophthora* species are discussed.

INTRODUCTION

Phytophthora ramorum (Werres *et al.* 2001) is responsible for 'sudden oak death,' a widespread mortality of tan oak and live oaks in the coastal fog belts of northern California and southern Oregon, first observed in *ca* 1995 (Rizzo *et al.* 2002). It has also been spreading on rhododendrons, viburnums and other woody ornamentals in the North American and European nursery trades (e.g. Werres *et al.* 2001, Moralejo & Werres 2002, Hansen *et al.* 2003, Lane *et al.* 2003). By late 2003, *P. ramorum* had appeared on beech, oak and horse chestnut trees at several sites with infected rhododendrons in southern England and on oak in The Netherlands. The pathogen is believed to have been recently introduced to both continents, and may pose a threat to many forest ecosystems worldwide. European and North American isolates of *P. ramorum* share the same unique ITS and isozyme profiles, confirming that they are conspecific (Ivors *et al.* 2002, Kelly Ivors, Peter Bonants & Willem Man in't Veld, pers. comm.). However, they fall into separate clusters on the basis of AFLP polymorphism (K. Ivors & P. J. M. Bonants, pers. comm.), exhibit significant differences in growth rate and aggressiveness (Brasier 2003) and are of predominantly opposite sexual compatibility types (see below). European and North American isolates therefore appear to comprise distinct sub-populations. In order to assess the potential for recombination between the European and American sub-populations and for a resulting emergence of novel genotypes, evidence is needed on whether or not the pathogen has a functional sexual breeding system (Brasier 2003).

Most *Phytophthora* isolates, whether homothallic or heterothallic, are bisexual and able to produce both oogonia and antheridia in culture. In heterothallic species the process of sexual development is overlain by a compatibility system involving two compatibility or compatibility types, termed A1 and A2 (Galindo & Gallegly 1960). Gametangia are formed in A1 × A2 pairings but not in A1 × A1 or A2 × A2 pairings. These two compatibility types are universal in pairings between A1 and A2 types of different species *in vitro* (though resulting zygotes may be inviable). Werres *et al.* (2001) obtained gametangia when European *P. ramorum* isolates were paired with A2 isolates of *P. cryptogea*, *P. drechsleri* or *P. cambivora* on carrot piece agar. European isolates were therefore assumed to be heterothallic and of A1 type. In further interspecific pairings, American *P. ramorum* isolates were

Table 1. Origins of principal *Phytophthora* isolates used in the experiments.

Isolate	Compatibility type	Host	Location	Isolated by (culture collection)[a]
P. ramorum				
P1356	A1	*Rhododendron* sp.	The Netherlands	*S. Werres*
P1358	A1	*Rhododendron* sp.	The Netherlands	*S. Werres*
P1361	A1	*Rhododendron* sp.	Germany	*S. Werres*
P1379	A1	*Viburnum tinus*	Fife, UK	*A. Schlenzig*
P1383	A1	*Viburnum* sp.	Lincolnshire, UK	*J. Rose*
P1347	A2	*Lithocarpus densiflorus*	California, USA	*D. M. Rizzo*
P1348	A2	*Quercus agrifolia*	California, USA	*D. M. Rizzo*
P1349	A2	*Rhododendron* sp.	California, USA	*D. M. Rizzo*
P1494	A2	*L. densiflorus*	Oregon, USA	*E. M. Hansen*
P1498	A2	*L. densiflorus*	Oregon, USA	*E. M. Hansen*
P. drechsleri[b]				
P537	A1	*Chrysanthemum roots*	Unknown	*M. J. Frezzi* (IMI 40499, ATCC 2548)
P540	A2	*Capsicum* sp.	Mexico	*G. Galindo* (IMI 149559)
P. cambivora				
P199	A1	*Fagus* sp.	Unknown, UK	*M. J. Griffin*
P1002	A2	*Quercus* soil	Surrey, UK	*J. Rose*

[a] Cultures are preserved in the isolators' institutional collections where no other culture collection is indicated.
[b] See also Brasier, Kirk & Sanchez Hernandez (2003).

designated as putative A2s (Brasier 2003, Werres & Zielke 2003).

Interspecific pairings involving *P. ramorum* take around 40–60 d to produce gametangia, usually result in only very small numbers, and the parentage of the gametangia is uncertain. Often, individual test pairings are unsuccessful (see below). In addition, direct pairings between European A1 and American A2 isolates of *P. ramorum* on artificial media have been consistently negative (Werres & Zielke 2003; C. M. B. & S. A. Kirk, unpubl.). However, gametangia were observed on outgrowing hyphae when a single A1 and a single A2 isolate of *P. ramorum* were jointly inoculated into live rhododendron stem sections (Werres & Zielke 2003). This paper describes attempts to enhance the interspecific sexual response of *P. ramorum in vitro* using a range of different media and culture conditions. Eventually, production of gametangia in direct pairings between A1 and A2 isolates of *P. ramorum in vitro* was achieved, but by an alternative method. Preliminary observations on the gametangia are presented and the status of the *P. ramorum* breeding system is discussed.

Table 2. Pairing tests of *Phytophthora ramorum* isolates against *P. drechsleri* and *P. cambivora* A1 and A2 type testers.

Source	No. of isolates tested	No. of A1 type	No. of A2 type
Europe:			
UK[a]	80	31	–
Germany[b]	16	13	–
Netherlands[b]	6	6	–
Poland[b]	3	1	–
Spain[b]	4	–	–
France[b]	1	–	–
Total	110	51	–
USA:			
California[c]	27	–	22
Oregon[d]	11	–	10
Total	38	–	32

[a] Isolates from *Rhododendron* and *Viburnum* spp. from 52 nurseries across 24 counties in the UK.
[b] Isolates mainly from *Rhododendron* and *Viburnum* spp.
[c] Isolates from native *Lithocarpus densiflorus*, *Quercus agrifolia*, *Umbellularia californica*, *Arbutus menzeisii*, *Vaccinium ovatum* and *Rhododendron* spp. from Marin, Sonoma, Napa and Santa Cruz Counties in California.
[d] Isolates from native *Lithocarpus densiflorus* in the Brookings area of south-west Oregon.

MATERIALS AND METHODS

The origins of key isolates used in pairing tests are shown in Table 1. The origins of other isolates studied are summarised in Table 2. For standard carrot agar (CA) 200 g washed carrots were liquidised in 500 ml tapwater and filtered through cotton sheet or multiple layers of cheesecloth; the liquid was then made up to 1 litre with tapwater, plus 15 g agar before autoclaving (Erwin & Ribeiro 1996). Strong CA (SCA) was standard CA reinforced with 50 g l^{-1} strained liquidised carrot residue before autoclaving. CA + sunflower oil and CA + mixed vegetable oil were prepared as CA but with the addition of 3 g of proprietary oil product. Red oak sapwood agar (ROSA) and viburnum sapwood agar (VSA) were prepared using 50 g of oven dried ground sapwood and 15 g agar l^{-1} as described previously for elm sapwood agar (Brasier 1981).

Interspecific pairings of *P. ramorum* isolates against standard A1 and A2 *P. drechsleri* or *P. cambivora* testers (Table 1) were carried out in 9 cm Petri dishes containing thinly poured (*ca* 10 ml per plate) CA. Isolates were inoculated *ca* 2 cm apart and incubated at 20 °C in the dark, with two replicates. Owing to the slower growth rate of *P. ramorum*, inocula of tester isolates of *P. drechsleri* or *P. cambivora* were added

2–3 d later. Pairings were examined for gametangia through the bottom of the plate (×100 magnification) at 10 d intervals.

The inoculum-mixing method involved *P. ramorum* isolates only. The isolates to be paired, for example presumptive A1 and A2 isolates, or an unknown plus an A1 or an A2 tester isolate, were grown initially on standard CA plates for 2–3 d at 20 ° in darkness. These cultures, being in a young pre-chlamydospore producing state, were then subcultured to very thin, *ca* 5–8 ml CA in order to set up a second set of 2–3 d old, pre-chlamydospore producing colonies. The second set of cultures was used for inoculum-mixing as follows. Three inoculum pieces *ca* 3 mm diam were removed from the edges of the second set of A1 colonies and transferred close to the centre of a plate of very thin (*ca* 5–8 ml), CA so that the inocula formed an equidistant triangle. Three similar-sized inoculum pieces were then taken from the edges of the second set of A2 colonies and placed on top of the three A1 inocula. The combined inoculum pieces were then gently sliced and mashed together with a sterile needle, resulting in three discrete closely mixed A1 + A2 inocula each of *ca* 0.5–1 cm in diam. Plates were incubated at 20 ° in darkness and examined daily.

RESULTS

Initial tests using the interspecific pairing method

Preliminary screening tests were initiated in June 2001. Fourteen European and six American *Phytophthora ramorum* isolates were paired with *P. drechsleri* A1 and A2 tester isolates P537 and P540 and with A1 and A2 *P. cambivora* tester isolates P199 and P1002. No gametangia had formed after 30 d, but sparse numbers were formed in *ca* 60 % of the pairings after 40–50 d. On this basis the 14 European isolates were identified as putative A1 and the 6 American isolates as putative A2 types. Subsequently, 51 of 110 *P. ramorum* isolates from across Europe were shown to be A1s by this method, while 32 of 38 American isolates from across California and southwest Oregon were shown to be A2s. The various hosts and geographic origins of these European and American isolates are summarised in Table 2.

Under the same conditions none of the European A1 and American A2 *P. ramorum* isolates identified above produced gametangia when paired directly together, although a variety of isolate combinations was repeatedly tested.

Effects of media and culture conditions

During 2002, the possibility was investigated of enhancing the rate or fecundity of the interspecific pairing response by adjusting media and conditions. Three European A1 *Phytophthora ramorum* isolates (P1356, P1358, P1361) that produced oogonia consistently with the *P. drechsleri* A2 tester and three American A2 isolates (P1347–P1349) that produced oogonia consistently with the or *P. cambivora* A1 tester were selected. These six interspecific A1 × A2 combinations were inoculated to a wide range of culture media, together with *P. drechsleri* × *P. drechsleri* and *P. cambivora* × *P. cambivora* controls. There were two replicates of each pairing. Plates were incubated at 20 ° in the dark for up to 60 d. The following media and conditions were used: standard CA (control); SCA; CA + sunflower oil and CA + mixed vegetable oil to enhance sterol uptake (sterols stimulate sexual reproduction in *Phytophthora*; Erwin & Ribeiro 1996); Red oak sapwood agar (ROSA) and viburnum sapwood agar (VSA); with a view to providing woody-host nutrients; CA incorporating 2, 5 and 10 % agar, with a view to reducing available free water; and CA overlain with a sterile cellophane membrane, with a view to providing a surface (leaf-like) effect and a higher oxygen environment for growth.

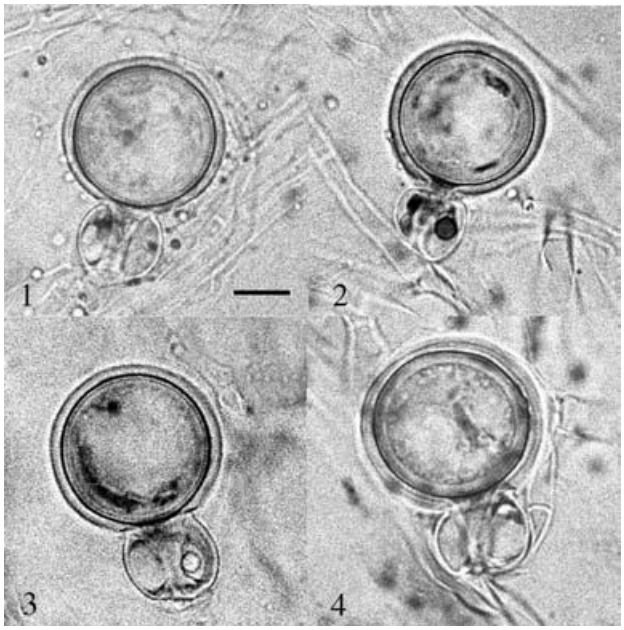
None of these tests resulted in any significant improvement in the rate or frequency of gametangial production in the interspecific pairings. The intraspecific control pairings with *P. drechsleri* or *P. cambivora* resulted in rapid and abundant gametangial formation on all media and conditions, except that *P. cambivora* did not produce gametangia on ROSA. On ROSA and VSA, but not on the other media, the oogonia were mainly golden-walled, resulting in readily visible dark lines at the junctions of the colonies. This may reflect uptake of polyphenols. Unexpectedly, growth of all the isolates was relatively unaffected on CA containing 5 or 10 g agar.

Gametangial production by paired isolates in vitro

Abundant chlamydospores (and some sporangia) were produced by *Phytophthora ramorum* on all the above artificial media (*cfr* Werres *et al*. 2001). This suggested that a diversion of energy resources into asexual reproduction might be one cause of the failure of *P. ramorum* to produce gametangia in intraspecific pairings and of the sparse gametangial production in the interspecific pairings. On CA at 20 ° chlamydospores usually appear in *P. ramorum* colonies after *ca* 7 d. An attempt was therefore made to pre-empt the tendency to produce chlamydospores by bringing juvenile mycelia, i.e. mycelia that might not yet be in 'chlamydospore-development mode', into close physical contact.

Two European *P. ramorum* A1 isolates (P1383 and P1379) and two American A2 isolates (P1494 and P1498) were selected and paired by the inoculum-mixing method. All four possible A1 × A2 isolate combinations were tested, with three replicates of each.

When assessed after 10 d, gametangia were found in all four A1 × A2 combinations directly beneath the mixed inoculum points, and continued to develop, slowly and very patchily, at these points. After 20 d they were rather variable in density, ranging from rare



Figs 1–4. Oogonia and amphigynous antheridia of *Phytophthora ramorum* produced in intraspecific pairings on CA medium. **Figs 2–3.** Dark bodies in the gametangia are probably lipid inclusions. **Fig. 4.** Oogonium containing a well formed oospore. Bar = 10 μm.

(*ca* 1–3 per ×100 field) to frequent (*ca* 10–80 per field). Subsequently, gametangia continued to form in the extending mycelial growth beyond the original mixed inoculum points. Chlamydospores developed rapidly throughout. In all four pairings the morphologies (Figs 1–4) and dimensions of the gametangia conformed closely to those described by Werres *et al.* (2001) and by Werres & Zielke (2003). Measurements of 10 gametangia (P1383 × P1498) were: oogonial diam average 30.5, range 25–35 μm; oospore diam average 25.5, range 22.5–27.5 μm; antheridial width average 17.3, range 15–20 μm; antheridial length, average 15, range 10–17.5 μm. Refractive globules, probably lipid inclusions, were common in antheridia (Figs 2–3). No gametangia formed in control A1 × A1 or A2 × A2 pairings.

In April 2003, the above *in vitro* 'inoculum mixing' method for intraspecific pairings was communicated to other laboratories working on *P. ramorum* for further assessment. Similar results were obtained by three laboratories in North America and another in the UK (Hansen *et al.* 2003; Nancy Osterbauer, Daniel Huberli, Patricia Giltrap & Charles Lane, pers. comm.). In all cases gametangial formation commenced relatively rapidly (*ca* 3–10 d). In one laboratory, 97 European isolates (70 from the UK and 27 from other countries) were all successfully identified as A1 compatibility types with no negative results and, in every test, gametangia were produced in only 3–7 d (Giltrap & Lane, pers. comm.). In another laboratory, the two inocula were placed side by side in direct contact rather than being inter-mixed, again with positive results (Everett M. Hansen, pers. comm.). However, though considerably faster than the interspecific pairing method, the inoculum-mixing method can be somewhat unpredictable: using previously fertile A1 × A2 *P. ramorum* isolate combinations, unexplained negative results sometimes occur, both in our own laboratory and elsewhere (Osterbauer & Huberli, pers. comm.).

DISCUSSION

The availability of a method for inducing gametangial production between *Phytophthora ramorum* isolates in artificial culture and in a reasonable time period, though still sometimes perfunctory, will hopefully enhance the efficiency of testing the sexual compatibility types of *P. ramorum* isolates. Indeed the method has already facilitated the first identification of the A1 type in North America (Hansen *et al.* 2003). The mixed inoculum method described here also avoids the uncertainty over whether gametangia produced in an interspecific pairing are of single or mixed parentage. Thus the gametangial morphology of *P. ramorum* was originally described from a pairing in which the *P. ramorum* isolate was separated from a *P. cryptogea* isolate by a cellophane membrane (Werres *et al.* 2001). It has long been considered that cellophane membranes are unsuitable for this purpose because they may be penetrated by *Phytophthora* hyphae (*cfr* Brasier 1972, Ko 1978). However, the morphology of the gametangia produced here, in direct intraspecific pairings, is very similar to those obtained in the cellophane membrane pairings by Werres *et al.* (2001). This suggests the latter were most probably formed as a result of selfing by the *P. ramorum* isolate in the presence of the other species.

The present method may also facilitate further *in vitro* studies on the breeding system of the pathogen. Indeed, with regard to the possible ecological threats posed by *P. ramorum*, the main reason for understanding its sexuality is not to determine sexual compatibility types of isolates *per se*. Rather, it is to determine whether the A1 × A2 outcrossing in *P. ramorum* process is functional, and to assess the risk that genotypes with novel fitness may emerge if genetic exchange occurs between the European and American subpopulations (Brasier 2003). Whether its sexual process is functional remains unclear, especially as there appear to be marked developmental differences between the process in *P. ramorum* and that of other heterothallic *Phytophthora* species. In most heterothallic *Phytophthora* species, pairing of A1 × A2 isolates (on CA or similar vegetable media) usually results in frequent to abundant gametangial production from 24–48 h of the colonies meeting. In contrast, the rate of production of gametangia by *P. ramorum* via the interspecific pairing method, at *ca* 40–60 d, is extremely slow. Even with the present inoculum-mixing method, the rate of gametangial formation, at *ca* 3–10 d, is comparatively slow, while the gametangia tend to be

produced more sparsely and their formation can be unpredictable.

Assuming these differences reflect the 'normal' behaviour of the *P. ramorum* sexual system, there are a number of possible explanations. One possibility is that they reflect a genetically pre-programmed balance between asexual and sexual reproduction. This is suggested by the ready and abundant production of chlamydospores by *P. ramorum* in culture and by present evidence that juvenile, non-chlamydospore producing mycelia may be more likely to produce gametangia. A strong developmental switch may exist that, in developing mycelia under appropriate environmental conditions, triggers intensive asexual reproduction and tends to override or limit sexual reproduction. Such a mechanism could reflect the unusual ecology of *P. ramorum*, for example it could be be an adaptation to its tree canopy-inhabiting lifestyle.

Another possibility is that the *P. ramorum* sexual mechanism is no longer genetically fully functional, due to a change in breeding strategy during its past evolutionary history. That, in interspecific pairings, compatible isolates of *P. ramorum* fail to induce rapid and abundant gametangial production by the other tester species, such as *P. drechsleri* or *P. cryptogea*, is in itself highly unusual. It could reflect a weak or dysfunctional compatibility system in *P. ramorum*. A third possibility is that, in nature, sexual reproduction of *P. ramorum* normally requires living host tissue (*cfr* Werres & Zielke 2003) and that *in vitro* conditions cannot fully reproduce this. However, this seems unlikely given the success of the inoculum-mixing method; and given the readiness with which asexual reproductive structures of *P. ramorum* are formed *in vitro*. Further studies on sexual development of *P. ramorum*, such as investigation of nuclear and chromosomal behaviour in the gametangia and of viability of the oospores, may help distinguish between these possibilities.

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REFERENCES

Brasier, C. M. (1972) Observations on the sexual mechanism in *Phytophthora palmivora* and related species. *Transactions of the British Mycological Society* **58**: 236–251.

Brasier, C. M. (1981) Laboratory investigation of *Ceratocystis ulmi*. In *Compendium of Elm Diseases* (R. J. Stipes & R. J. Campana, eds): 76–79. American Phytopathological Society, St Paul, MN.

Brasier, C. M. (2003) Sudden oak death; *Phytophthora ramorum* exhibits transatlantic differences. *Mycological Research* **107**: 257–259.

Brasier, C. M., Kirk, S. A. & Sanchez-Hernandez, E. (2003) *Phytophthora inundata* sp. nov., a part heterothallic pathogen of trees and shrubs in wet or flooded soils. *Mycological Research* **107**: 477–484.

Erwin, D. C. & Ribeiro, O. K. (1996) *Phytophthora Diseases Worldwide*. American Phytopathological Society Press, St Paul, MN.

Galindo, A. J. & Gallegly, M. E. (1960) The native of sexuality in *Phytophthora infestans*. *Phytopathology* **50**: 123–128.

Hansen, E. M., Reeser, P. W., Sutton, W., Winton, L. M. & Osterbauer, N. (2003) First report of A1 compatibility type of *Phytophthora ramorum* in North America. *Plant Disease* **87**: 1267.

Ivors, K., Hayden, K., Garbelotto, M. & Rizzo, D. (2002). Molecular population analyses of *Phytophthora ramorum*. *Abstract, Sudden Oak Death Science Symposium, Monterey, California,* 15–18 *December 2002*. http://danr.ucop.edu/ihrmp/sodsymp/paper

Ko, W. H. (1978) Heterothallic *Phytophthora*: evidence for hormonal regulation of sexual reproduction. *Journal of General Microbiology* **107**: 15–18.

Lane, C. R., Beales, P. A., Huges, K. J. D., Griffin, R. L., Brasier, C. M. & Webber, J. F. (2003) First outbreak of *Phytophthora ramorum* in England, on *Viburnum* trees. *Plant Pathology* **52**: 414.

Maralejo, E. & Werres, S. (2002) First report of *Phytophthora ramorum* on *Rhododendron* sp. in Spain. *Plant Disease* **86**: 1052.

Rizzo, D. M., Galbelotto, M., Davidson, J. M., Slaughter, G. W. & Koike, T. (2002) *Phytophthora ramorum* as the cause of extensive mortality of *Quercus* sp. and *Lithocarpus densiflorus* in California. *Plant Disease* **86**: 205–214.

Werres, S., Marwitz, R., Man in't Veld, W. A., de Cock, A. W. A. M., Bonants, P. J. M., de Weerdt, M., Themann, K., Ilieva, E. & Baayen, R. P. (2001) *Phytophthora ramorum* sp. nov., a new pathogen on *Rhododendron* and *Viburnum*. *Mycological Research* **105**: 1155–1116.

Werres, S. & Zielke, B. (2003) First studies on the pairings of *Phytophthora ramorum*. *Journal of Plant Diseases and Protection* **110**: 129–130.

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