Mating of *Phytophthora ramorum*: Functionality and Consequences[1]

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Introduction

Phytophthora ramorum (Werres, De Cock, Man in't Veld), which causes "sudden oak death" in the United States and dieback and leaf necrosis in ornamental plants (mainly *Rhododendron* and *Viburnum*) in Europe, is a heterothallic species with two mating types, A1 and A2 (Werres and others 2001, Rizzo and others 2002). Molecular studies on the population of *P. ramorum* isolates revealed a structure composed of three clonal lineages: one lineage from Europe (EU1) and two lineages from North America (NA1 and NA2) (Elliott and others 2009). Initial pairing studies revealed geographical separation of mating type isolates: all EU1 isolates were of A1 type whereas all NA1 and NA2 isolates were of A2 type (Werres and others 2001, Brasier 2003). However, since 2003 a few rare reports of A2 mating type isolates in Europe and reports of A1 mating type isolates in the United States (U.S.) have been made. In Europe, three EU1 A2 isolates were reported in Belgium (Werres and De Merlier 2003; Heungens, personal communication). In North America, some EU1 A1 isolates were reported in U.S. nurseries (Hansen and others 2003, Grünwald and others 2008). These findings suggest the potential for crossing between both mating types. However, attempts to produce oospores *in vitro* with classical methods were difficult compared to other heterothallic species (Werres and Zielke 2003, Brasier and Kirk 2004), therefore suggesting a weak functionality of the sexual system in *P. ramorum*. In a previous study, some critical parameters such as the gelling agent quality, the nutrient source, or the spatial arrangement of the two mating types in the Petri dish were optimized to produce a large amount of oospores *in vitro*. A particular EU1 A1 strain was found to be a better mating partner than other EU1 A1 strains when paired with some European (EU1) or American (NA1) A2 strains (Boutet and others 2009). The aims of this study were to investigate the functionality of *P. ramorum* sexual reproduction and to evaluate the characteristics of single-oospore isolates.

Materials and Methods

The *P. ramorum* strains used in this study are listed in table 1. Oospores resulting from the mating of EU1 x EU1 (2299 x 3237) and EU1 x NA1 (2299 x 3528 or 3638) strains were extracted from the agar plate after different maturation times (60, 110,

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250, 350, and 500 days) by using a newly developed method to separate oospores from mycelium and asexual spores (sporangia and chlamydospores) (Boutet and others 2010). Oospores were stained with tetrazolium bromide (MTT) as described by Sutherland and Cohen (1983) to evaluate their viability. Their germination ability was evaluated in parallel by embedding them in Soft Water-Agarose (water agarose 0.6 percent). Oospore progenies were characterized in terms of (1) genetic rearrangement using microsatellite markers (Vercauteren and others 2010) and (2) pathogenicity on *Rhododendron* "Cunningham's White" by inoculating leaves with a mycelium plug of each oospore offspring.

Table 1—List of *P. ramorum* isolates used in this study

Species	Isolate	Collection	Host	Mating Type	Lineage
P. ramorum	2299	CBS (101330)[1]	*Viburnum* sp.	A1	EU1
P. ramorum	3237	CRA-W[2]	*Viburnum bodnantense*	A2	EU1
P. ramorum	3638	ILVO (PRI 483)[3]	*Rhododendron* sp.	A2	NA1
P. ramorum	3528	USA (014)[4]	*Quercus* sp.	A2	NA1

[1] CBS: Centraal Bureau voor Schimmelcultures, Uppsalalaan 8, Utrecht, The Netherlands.
[2] CRA-W: Walloon Agricultural Research Centre, rue de Liroux 4, Gembloux, Belgium.
[3] ILVO: Instituut for Agricultural and Fisheries Research: Burg. Van Gansberghelaan 96 bus 2, Merelbeke, Belgium.
[4] PD: Plantenziektenkundige Dienst, Geertjesweg 15, Wageningen, The Netherlands.

Results

The viability test (staining with MTT) indicated that between 20 and 70 percent of the oospores possessed viable cellular activity. In contrast, only approximately 0.2 percent of these oospores were able to germinate (fig.1), suggesting that the MTT method can not be used to estimate the germination ability.

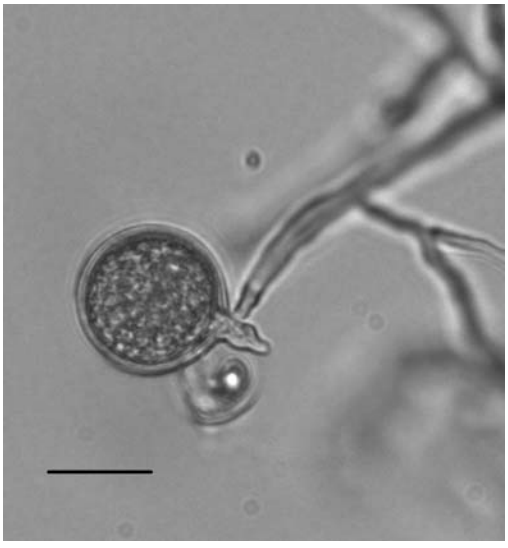

Figure 1—Germinated oospore from an EU1 x EU1 mating of *P. ramorum*. Scale bar represents 20 µm.

Among the isolates that were recovered from oospores maintained from 110 to 500 days in culture, 37 originated from the EU1 x EU1 pairing and 13 originated from EU1 x NA1 pairings. Microsatellite marker analysis showed that the progeny presented genetic rearrangements, either by allele combinations of both parents (EU1 x NA1 progenies) or by loss of one of the parental alleles due to a shift from heterozygosity to homozygosity (EU1 x EU1 progeny). Pathogenicity analysis revealed a large variability among the EU1 x EU1 single oospore isolates with more than 50 percent being significantly less aggressive on *Rhododendron* leaves than their EU1 parents (fig. 2) whereas no significant differences were found between EU1 x NA1 offspring and their parents.

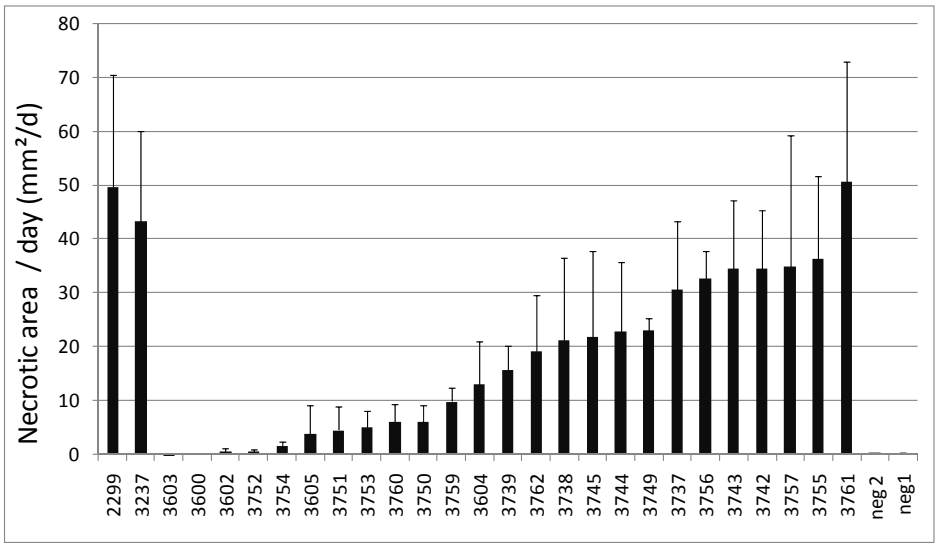

Figure 2—Pathogenicity of EU1 x EU1 offspring on *Rhododendron* leaves expressed by daily growth rate of necrotic area (three replicates). The parental strains are strains 2299 (EU1, A1) and 3237 (EU1, A2).

Conclusion

In a context of pest risk analysis, these data demonstrate the functionality of the *P. ramorum* mating system and the possibility of genetic exchange between the EU1 and NA1 populations, although the proportion of viable single-oospore isolates obtained was very low. The preliminary characterization of the progeny highlights an important phenotypical variability after only one generation. Nevertheless, there is still no data on the ability of *P. ramorum* to sustain a sexual cycle on host plants and in the natural environment. Moreover, a large proportion of EU1 or NA1 *P. ramorum* isolates seem unable to produce large amounts of oospores. Therefore, further experiments are required to fully assess the risk presented by sexual reproduction of *P. ramorum* in nature.

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