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Sexual recombination in *Phytophthora cinnamomi in vitro* and aggressiveness of single-oospore progeny to *Eucalyptus*

C. Linde[a]*†, S. H. Soo[b] and A. Drenth[b]

[a]*Institute of Plant Sciences/Phytopathology, ETH-Zentrum, Universitätstrasse 2, CH-8092 Zürich, Switzerland; and* [b]*Cooperative Research Centre for Tropical Plant Pathology, Level 5, John Hines Building, The University of Queensland, Brisbane, 4072, Australia*

Fifty single oospore progeny were established from an *in vitro* mating of A1 and A2 mating type isolates of *Phytophthora cinnamomi* from South Africa. Forty-nine progeny were identified as F_1 hybrids using seven random amplified polymorphic DNA (RAPD) primers, and one was a selfed isolate of the A1 mating type parent. Among the hybrid progeny, 24 and 25 were A1 and A2 mating type, respectively. Aggressiveness of progeny and parental isolates was assessed on 1-year-old seedlings of *Eucalyptus smithii*. The mean aggressiveness of hybrid oosporic isolates, expressed as lesion length, was significantly ($P = 0{\cdot}0001$) lower than that of the parental isolates. No significant difference in aggressiveness of A1 and A2 mating type F_1 hybrid isolates was observed. This is the first report demonstrating sexual recombination *in vitro* in *P. cinnamomi*.

Keywords: aggressiveness, *Eucalyptus smithii*, F_1 hybrid, *Phytophthora cinnamomi*, RAPD, sexual recombination

Introduction

Phytophthora cinnamomi is a soilborne pathogen of worldwide importance, and is particularly important as a pathogen of *Eucalyptus* in South Africa (Wingfield & Knox-Davies, 1980; Linde *et al*., 1994). *Phytophthora cinnamomi* belongs to the Oomycota, whose members are diploid in the vegetative stage (Brasier & Sansome, 1975); it is heterothallic, with A1 and A2 mating types (Galindo & Zentmyer, 1964) which sexually interact to form oospores.

Both mating types of *P. cinnamomi* have been introduced into South Africa (Linde *et al*., 1997), and opportunities for sexual reproduction are presumed to exist. However, population analysis using isozymes has indicated that sexual reproduction occurred rarely, if at all, in Australian (Old *et al*., 1984, 1988) and South African *P. cinnamomi* populations (Linde *et al*., 1997). Significant genetic differentiation among the South African *P. cinnamomi* mating type populations was indicative of the lack of sexual reproduction (Linde *et al*., 1997). Various hypotheses for the apparent lack of sexual reproduction in these populations have been put forward. These include: (i) inability to mate and produce viable oospores because of genetic barriers between mating types such as different ploidy levels or chromosome numbers (Whittaker *et al*., 1991); (ii) oospore abortion preventing reproduction of nonparental genotypes (Rutherford & Ward, 1985; Old *et al*., 1988); (iii) F_1 hybrids are nonpathogenic and therefore never make up a significant part of the population and thus escape detection; and (iv) failure of oospores to germinate (Chang & Ko, 1991; Chang *et al*., 1996). Because of the lack of genetic studies in *P. cinnamomi*, unambiguous evidence to test these hypotheses is, unfortunately, not available.

The importance of sexual reproduction in enhancing genotypic diversity in *P. cinnamomi* populations, and the importance of the formation of long-term survival structures in the form of oospores, have not been determined. In a recent analysis of fixation index values in *Phytophthora* species, Goodwin (1997) showed that *P. cinnamomi* had intermediate values indicating a mixed mating system, that is, mostly clonal reproduction with some outcrossing. Abundant oospore production occurs when opposite mating types of *P. cinnamomi* cultures are paired *in vitro* (Galindo & Zentmyer, 1964; Ribeiro *et al*., 1975), whereas oospores produced *in vivo* have been observed only sporadically in soil and naturally infested host tissue (Mircetich & Zentmyer, 1966). Germination rates of between 1 and 45% have been reported for oospores produced *in vitro* (Ribeiro *et al*., 1975); however, previous studies (Ribeiro *et al*., 1975; Zentmyer, 1980) have not established whether or not oospores were selfs, hybrids, or products of apomixis.

*To whom correspondence should be addressed.

†E-mail: celeste.linde@ipw.agrl.ethz.ch

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Table 1 RAPD markers used to identify F_1 hybrid progeny by the presence of bands in the parental South African *Phytophthora cinnamomi* isolates CP513 and CP508

RAPD primer[a]	Sequence	Marker	Mol. size (bp)	Band presence		F_1 ratio[b] (1 : 0)
				CP513	CP508	
OPG-16	AGCGTCCTCC	G16–1	580	0	1	38 : 8
		G16-2	640	1	0	30 : 19
OPS-14	AAAGGGGTCC	S14–1	580	0	1	49 : 0
OPS-20	TCTGGACGGA	S20–1	600	0	1	41 : 7
		S20-2	660	1	0	25 : 23
OPX-12	TCGCCAGCCA	X12-2·1	1050	1	0	25 : 23
		X12-2·2	660	1	0	27 : 22
OPG-11	TGCCCGTCGT	G11-2·2	1120	1	0	4 : 6
		G11-2·2	790	1	0	9 : 3
OPG-14	GGATGAGACC	G14–1	1320	0	1	11 : 1
		G14–2	1730	1	0	3 : 7
		G14–3	1080	1	0	6 : 4
OPG-15	ACTGGGACTC	G15–1	960	0	1	8 : 2
		G15-2·1	2200	1	0	3 : 6
		G15-2·2	1950	1	0	7 : 1
		G15-2·3	1580	1	0	5 : 4

[a]Operon Technologies.

[b]Based on the complete dominance of RAPD markers, a segregation of 1 : 0 is expected for homozygous markers, while for heterozygous RAPD markers a 1 : 1 ratio is expected. Observed ratios do not total 49 for some markers as these data were not needed to verify the hybrid nature of the F_1 progeny.

Sexual reproduction in *Phytophthora* species has a significant influence on the levels of genotypic diversity in pathogen populations. For example, the introduction of new strains of *Phytophthora infestans* into Europe consisting of both the A1 and A2 mating type, has promoted sexual recombination with increased genotypic diversity (Drenth *et al.*, 1994).

The aims of the current investigation were to: (i) test for *in vitro* sexual reproduction in *P. cinnamomi* and determine mating type segregation among F_1 hybrids; (ii) determine the hybrid nature of F_1 progeny using genetic markers; and (iii) determine the aggressiveness of F_1 hybrids as compared to the aggressiveness of the parental isolates. This information is vital in order to test the ability of *P. cinnamomi* to outbreed and produce viable hybrid progeny. Levels of aggressiveness in the progeny, compared to the parents, will provide information on the pathogenicity of oosporic progeny.

Materials and methods

P. cinnamomi isolates

Over a period of 2 years, 15 crosses (CP513 × CP508, CP513 × CP470, CP518 × CP550, CP551 × CP548, CP551 × CP510, CP233 × CP468, CP233 × CP494, CP531 × CP468, T13 × CP80, T34 × CP481, T37 × CP504, T27 × T38, T34 × C504, T35 × T36, C410 × C9) were made using 11 A1 and 14 A2 mating type isolates of *P. cinnamomi* of South African origin. Details pertaining to the original host, random amplified length polymorphic DNA (RAPD) phenotype, and restriction fragment length polymorphism (RFLP) genotype of isolates have been published elsewhere (Linde *et al.*, 1999). All the crosses produced oospores, but the oospores failed to germinate. Viable single-oospore progeny were established, however, in a repeat mating between isolates CP513 (A1) and CP508 (A2), both of which originated from *Ocotea bullata*, a native South African forest tree species. These parental isolates are deposited as UQ2923 and UQ2919 in the culture collection of the Cooperative Centre for Tropical Plant Pathology, the University of Queensland.

Isolation and RAPD analysis of F_1 progeny

Mycelial discs from both parental mating types were placed on opposite sides of five Petri dishes containing carrot agar (Ribeiro, 1978), which were incubated at 20°C in the dark for 3 months. Oospores were harvested and isolated by maceration of agar strips in a cooled domestic blender at full speed for 10 min. The oospores were sieved through a nylon mesh (pore size 75 μm to remove hyphal fragments), plated on 1·5% water agar, and allowed to germinate in the dark at 25°C. Germinating oospores were obtained after 3 days. They were transferred to 20% clarified V8 agar (Ribeiro, 1978) using microdissection, and incubated in the dark at 25°C. Subcultures were established by taking hyphal tips from mycelial cultures of single-oospore progeny. Aerial mycelium was used to inoculate two Petri dishes containing 20% clarified V8 broth and incubated in the dark at 25°C for 5–6 days. Mycelium was harvested using a Büchner funnel and freeze-dried for DNA extraction according to Drenth *et al.*,

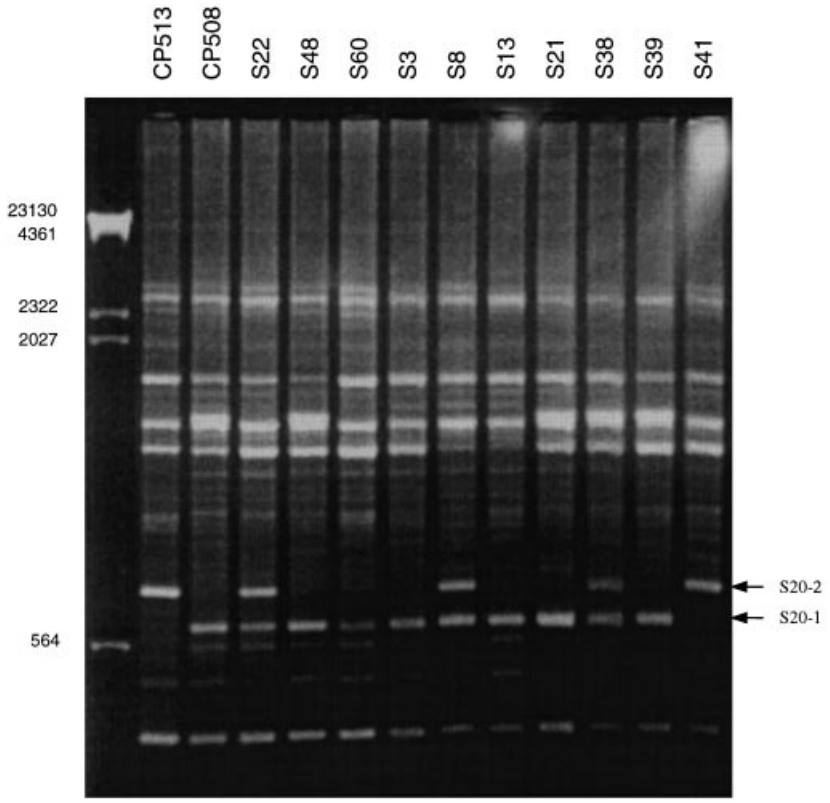

Figure 1 Identification of F_1 hybrids in *Phytophthora cinnamomi* using RAPD primer OPS20. Parental isolates CP513 (A1 mating type) and CP508 (A2 mating type) together with 10 F_1 progeny isolates are shown. DNA fragment size calibrations from λ phage/*Hin*dIII in the first lane are indicated on the left. RAPD primer OPS20 could not distinguish S48 from parent CP508 and S41 from CP513, but these could be differentiated using additional primers as outlined in Table 1.

1993). Seven primers (Table 1) were selected from a total of 55 RAPD decanucleotide primers from Operon kits (Operon Technologies, Alameda, CA, USA) OPG, OPS and OPX for their ability to distinguish hybrids and selfs from parental isolates. RAPD procedures were as described by Whisson *et al.* (1995), except that 1% instead of 1·5% agarose gels were used. RAPD analysis was repeated to confirm the reproducibility of bands.

Determination of mating type

Mating type of all F_1 isolates was determined by pairing with isolates of known mating type on carrot agar plates (Ribeiro, 1978). Plates were incubated at 20°C in the dark and examined for the presence of oospores after 4 weeks.

Aggressiveness tests

Eucalyptus smithii seedlings (1 year old), growing in a shadehouse, were inoculated with parental isolates CP513 and CP508 and all single-oospore progeny isolates to determine if they were pathogenic and to compare their aggressiveness. Eight seedlings per isolate were artificially inoculated in a completely randomized design with each seedling considered as a replicate. Mycelial discs of *P. cinnamomi* isolates grown on potato dextrose agar (PDA) for 7 days at 25°C were inoculated into 10 mm diameter wounds made with a cork borer in the stem of each seedling. For control inoculations eight seedlings were inoculated with a sterile disc of PDA. Wounds were sealed with parafilm to prevent desiccation. The length of lesions formed in the secondary phloem (Tippett *et al.*, 1983; Shearer *et al.*, 1987) were measured 2 weeks after inoculation. Re-isolations onto a selective medium (Tsao & Guy, 1977) were made from control and inoculated seedlings. To prevent the release of *in vitro*-produced hybrid F_1 isolates, seedlings and seedling pots used in aggressiveness tests were autoclaved after measurements were made. Stem lesion data were analysed using analysis of variance (ANOVA) (Snedecor & Cochran, 1980). After log transformation to achieve normal distribution, comparisons were made between lesion lengths of F_1 hybrid and parental isolates, and between F_1 hybrid isolates of different mating types (Snedecor & Cochran, 1980).

Results

Although oospores formed within 3–4 weeks after incubation of A1 × A2 mating type isolates, they were

not harvested for 3 months to allow maturation of oospores. Germinating oospores were observed 3 days after harvesting and for a further 15 days, with a peak 5–10 days after harvesting. Sixty-seven germinated oospores were transferred, with 50 single oospore progeny being established as mycelial cultures after transfer and subculturing of hyphal tips. These progeny and parents were screened with RAPD primers (Table 1). All but one of the progeny contained one or more RAPD bands from each parent, indicating that they were likely to be true hybrids (Fig. 1). One of the progeny, S14, was a putative self of the A1 parent isolate CP513.

Because RAPD bands may be homo- or heterozygous, multiple RAPD primers must be used to distinguish hybrids from selfs among single-oospore progeny of diplontic organisms such as *P. cinnamomi*. Seven such primers were used in this study, and segregation analysis of the absence and presence of bands suggested that all bands except those produced by primer OPS14-1 were heterozygous (Table 1).

Among the 49 F_1 hybrids identified, 24 were A1 and 25 A2 mating type. The single selfed isolate (S14) was A1 mating type, like isolate CP513, its putative parent.

All 49 F_1 hybrids and both parental isolates were pathogenic on *E. smithii and* the lesions produced differed significantly ($P = 0{\cdot}0001$) from that of control inoculations and from each other. Contrast analysis showed that the average lesion length, and thus the aggressiveness of F_1 hybrid isolates as a group, was significantly ($P = 0{\cdot}0001$; mean square $= 11{\cdot}342$; error mean square $= 0{\cdot}190$) lower than both parental isolates. However, the average lesion lengths of some F_1 hybrid progeny did not differ significantly from those of the parents. The average lesion length of the selfed isolate, S14, was significantly ($P = 0{\cdot}0001$) lower than that of the parental isolates, but did not differ significantly from the average lesion length of the F_1 isolates as a group. Average lesion lengths of A1 and A2 mating type F_1 isolates did not differ significantly from each other ($P = 0{\cdot}0001$; mean square $= 1{\cdot}487$; error mean square $= 0{\cdot}190$). Inoculated *P. cinnamomi* isolates were successfully re-isolated from all inoculations. Control inoculations did not develop any lesions.

Discussion

Using RAPD markers, this study provides the first evidence for sexual recombination *in vitro* between A1 and A2 mating type isolates of *P. cinnamomi*. The use of RAPD markers revealed the hybrid nature of progeny and distinguished hybrids from selfs. Previously, biochemical and other molecular markers have been used successfully in genetic studies of homothallic species such as *Pythium ultimum* (Francis & St. Clair, 1993) and *Phytophthora sojae* (Förster *et al*., 1994; Whisson *et al*., 1994), and in heterothallic species such as *P. infestans* (Shattock *et al*., 1986; Shattock, 1988; Shaw & Shattock, 1991; Judelson *et al*., 1995) and *P. nicotianae* (syn. *parasitica*) (Förster & Coffey, 1990).

Mating type segregated in our cross in a close to perfect 1 : 1 ratio. Mating type in Oomycota, and in *P. infestans* in particular, is postulated to be controlled by one locus: the A1 type is thought to be determined by the heterozygous genotype *Aa* and the A2 type by the homozygous genotype *aa* (Judelson, 1996). However, mating type segregation in *P. infestans* frequently is not Mendelian (Timmer *et al*., 1970; Khaki & Shaw, 1974; Shattock *et al*., 1986; Judelson *et al*., 1995; Judelson, 1996).

The inability to germinate oospores in the first attempt with 15 crosses is probably due to our lack of understanding of the conditions favourable for the germination of oospores. The parental isolates in our successful cross had been used once before without obtaining any germinating oospores. Oospores are believed to have some form of constitutive dormancy, and although conditions for oospore germination have been investigated in detail (Erwin & Ribeiro, 1996), no reliable inducers of synchronous germination have been identified.

In heterothallic Oomycota two mating types are required for sexual reproduction and outcrossing, although inbreeding has also been observed in *P. infestans* (Shattock *et al*., 1986; Shattock, 1988; Goodwin *et al*., 1992; Goodwin & Fry, 1994), *P. nicotianiae* (syn. *parasitica*) (Förster & Coffey, 1990), and *Pythium sylvaticum* (Martin, 1989). Inbreeding in heterothallic species will lead to an increase in the frequency of homozygous clones and will result in a higher fixation index. In some populations of *P. cinnamomi* a slightly higher than expected fixation index has been identified (Goodwin, 1997), which may indicate either the occurrence of some selfing, or more probably the predominance of particularly fit clonal genotypes, as found in the South African population by Linde *et al*. (1997).

In addition to generating genotypic diversity, oospores are also long-term survival structures (Duncan & Cowan, 1980). Oospores of *P. infestans* have been shown to survive for over a year (Pittis & Shattock, 1994; Drenth *et al*., 1995; Turkensteen *et al*., 2000), while oospores of onion downy mildew (*Peronospora destructor*) have been shown to survive for over 25 years in soil (McKay, 1957). This ability to survive in the absence of the host for long periods may be important in the dispersal and spread of the pathogen, and in surviving cropping cycles in agricultural rotation crops.

In this study F_1 hybrids were pathogenic to *E. smithii*, although their level of aggressiveness was significantly reduced on this particular host. Complete loss of pathogenicity in the sexual progeny of *P. infestans* has been reported previously (Al-Kherb *et al*., 1995) and in the interspecific cross between *P. infestans* and *P. mirabilis* (Goodwin & Fry, 1994).

The discovery of sexual recombination *in vitro* in *P.*

cinnamomi suggests that oospores may be produced and outbreeding may take place under field conditions. There is a need to determine to what extent oospores contribute to disease, and which factors influence the production, survival and germination infection potential of oospores.

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