

The role of paragynous and amphigynous antheridia in sexual reproduction of *Phytophthora cinnamomi*

DANIEL HÜBERLI¹, I. C. TOMMERUP^{2*} AND GILES E. ST J. HARDY¹

¹ Murdoch University, School of Biological Sciences, Perth, Western Australia, 6150

² CSIRO Forestry & Forestry Products, Private Bag PO Wembley, Western Australia, 6014

The morphology of gametangia was examined in 43 pairs of isolates (mating types A1 × A2; 11 A1 and 24 A2 isolates; five isozyme/electrophoretic types) of *Phytophthora cinnamomi*. An amphigynous antheridium always formed with each oogonium. However, in 41 of the crosses a proportion (39 had 0.2–10% and two had > 30%) of oogonia also consistently had single or multiple paragynous antheridia. Single or multiple paragynous antheridia formed concurrently with amphigynous ones during the period of gametangial production in paired colonies. Where there were multiple paragynous antheridia associated with an oogonium, sometimes additional antheridia formed after fertilization or even after oospores were visible. Developmental studies showed that when meiosis in amphigynous and paragynous antheridia was simultaneous, fertilization tubes developed synchronously from both. However, cytological examination indicated that either a nucleus from an amphigynous or a paragynous antheridium fertilized the oosphere. Observations of paragynous and amphigynous, and amphigynous-only associations suggested that fertilization from either type of antheridium only occurred when meiosis in the oogonium was nearly synchronous with that of the antheridium. Asynchronous meiosis between oogonia and antheridia may contribute to failed fertilization and aborted oospore development. This appears to be the first description of paragynous antheridia in *P. cinnamomi* and the second observation of oogonia with both paragynous and amphigynous antheridia in a heterothallic *Phytophthora* species. Moreover, the development of both paragynous and amphigynous antheridia with an oogonium is rare in *Phytophthora*, as is the development of multiple antheridia. Antheridial variation is a characteristic to be taken into account in isolate identification. Nuclei from paragynous antheridia appear able to fertilize oospheres and therefore, have a role in sexual reproduction.

While the plant pathogen *Phytophthora cinnamomi* Rands is known worldwide and has had a devastating impact on many Australian ecosystems to which it has been introduced (Wills, 1993; Weste, 1994; Shearer & Dillon, 1995), its sexual behaviour has not been elucidated fully. Although the pathogen produces oospores in naturally infested soil and roots (Mircetich & Zentmyer, 1966), most understanding of sexuality in *P. cinnamomi* is derived from paired mating studies on agar media. These studies demonstrated that *P. cinnamomi* is heterothallic and, therefore, requires the presence of opposite mating types, designated A1 and A2, to form oospores (Galindo & Zentmyer, 1964; Haasis, Nelson & Marx, 1964; Savage *et al.*, 1968). However, A2 isolates may be homothallic (Zentmyer, 1980; Gerrettson-Cornell, 1989).

Current understanding is that heterothallic species of *Phytophthora* form amphigynous antheridia, while homothallic species form paragynous and occasionally amphigynous antheridia on the same culture plate (Savage *et al.*, 1968; Brasier, 1983; Gerrettson-Cornell, 1989; Stamps *et al.*, 1990). However, some heterothallic species of *Phytophthora*, such as *P. eriugena* Clancy & Kavanagh, *P. erythroseptica* Pethybr., and

P. richardiae Buisman, are exceptions and form both types of antheridia on the same culture plate (Gerrettson-Cornell, 1989; Stamps *et al.*, 1990). The only two species of *Phytophthora* that have both types of antheridia associated with an oogonium are *P. hibernalis* Carne (heterothallic) and *P. porri* Foister (homothallic). It is not known whether there is a relationship between amphigyny and heterothallism, and indeed what purpose amphigynous as opposed to paragynous antheridia would perform in heterothallic species (Brasier, 1983). Other phytopathogenic heterothallic species of Oomycotina, such as *Bremia lactucae* Regel, have only paragynous antheridia (Tommerup, 1988), suggesting that amphigyny may be efficient for sexual reproduction and that it may be of no special advantage *in planta*.

Paragynous antheridia were observed in 1994 during an examination of mating type in 72 new isolates of *P. cinnamomi* from a region of Western Australia (WA). A set of 10 isolates that ranged in ability to form paragynous antheridia were selected for analysis of incidence of paragynous antheridia, variation in their ontogeny and possible functional role in fertilization. Additionally, the incidence, development and possible function of paragyny was investigated in crosses amongst 11 isolates varying in pathogenicity and geographical distribution in Australia and eight crosses amongst Papua New

* Corresponding author.

Guinea (PNG) isolates. Neither paragyny nor multiple antheridia in *P. cinnamomi* have been described previously.

MATERIALS AND METHODS

Isolates

Eleven isolates of *P. cinnamomi* from an Australian-wide culture collection (CSIRO, Canberra) which have a known range of pathogenicity, isozyme type and mating type (Dudzinski, Old & Gibbs, 1993), 14 isolates from PNG (Old, Moran & Bell, 1984) and 10 recent isolates from the northern jarrah (*Eucalyptus marginata* Donn ex Sm.) forest in the southwest of WA were used in this study. The Australian-wide isolates (four A1 and seven A2 mating types) and 14 PNG isolates (six A1 and eight A2 mating types) represent a wide range of isozyme types (Old *et al.*, 1984) or electrophoretic types called CINN (Oudemans & Coffey, 1991). Matings involved isozyme types A1 (1), A2 (1) and A2 (2) called respectively electrophoretic type CINN 2, CINN 4 and CINN 5; and CINN 6 and CINN 7 for which no isozyme type was named although the isozyme variants were described by Old *et al.* (1984). The WA isolates were from Cape Hope (1) and Alcoa of Australia Limited minesites at Willowdale (4), Jarrahdale (4) and Huntly (1). The A1 isolate from Cape Hope was crossed against the nine A2 isolates. The A2 isolates were selected after observations for mating type behaviour of 72 isolates from this region. The isolates were confirmed as *P. cinnamomi* using an isozyme type analysis (Hardy, pers. comm.).

Development and behaviour of paragynous antheridia

The medium for all count data of antheridial types contained 10% cleared V8-juice, 0.01% CaCO₃, 0.002% β -sitosterol, 2% Difco bacteriological agar (V8A), and was prepared as described by Byrt & Grant (1979). From the set of 72 isolates, seven which formed paragynous antheridia and two which formed only amphigynous antheridia were paired individually with the Cape Hope A1 isolate on V8A in Petri dishes and replicated four times. After 14 d incubation at 24 ± 1 °C in the dark, scrapings were taken from the interface of both mating types, mounted on slides, gently squashed, and examined at $\times 1000$ magnification. Numbers of amphigynous and paragynous antheridia associated with each oogonium were recorded for 30 randomly selected mature oogonia for each cross. Antheridium, oogonium and oospore diam. also were recorded. In 26 matings of 11 Australian-wide isolates, the proportion of oogonia with either only amphigynous, only paragynous or both types of antheridia were recorded for 500 oogonia per replicate in a transect along the zone of interaction of the mating isolates. In eight matings of PNG isolates, antheridial associations were recorded as in the 26 Australian-wide matings for 300 oogonia per replicate. There were two replicate plates (values for duplicates were similar). Observations of the presence of paragynous antheridia in all the pairings were repeated 4–7 times.

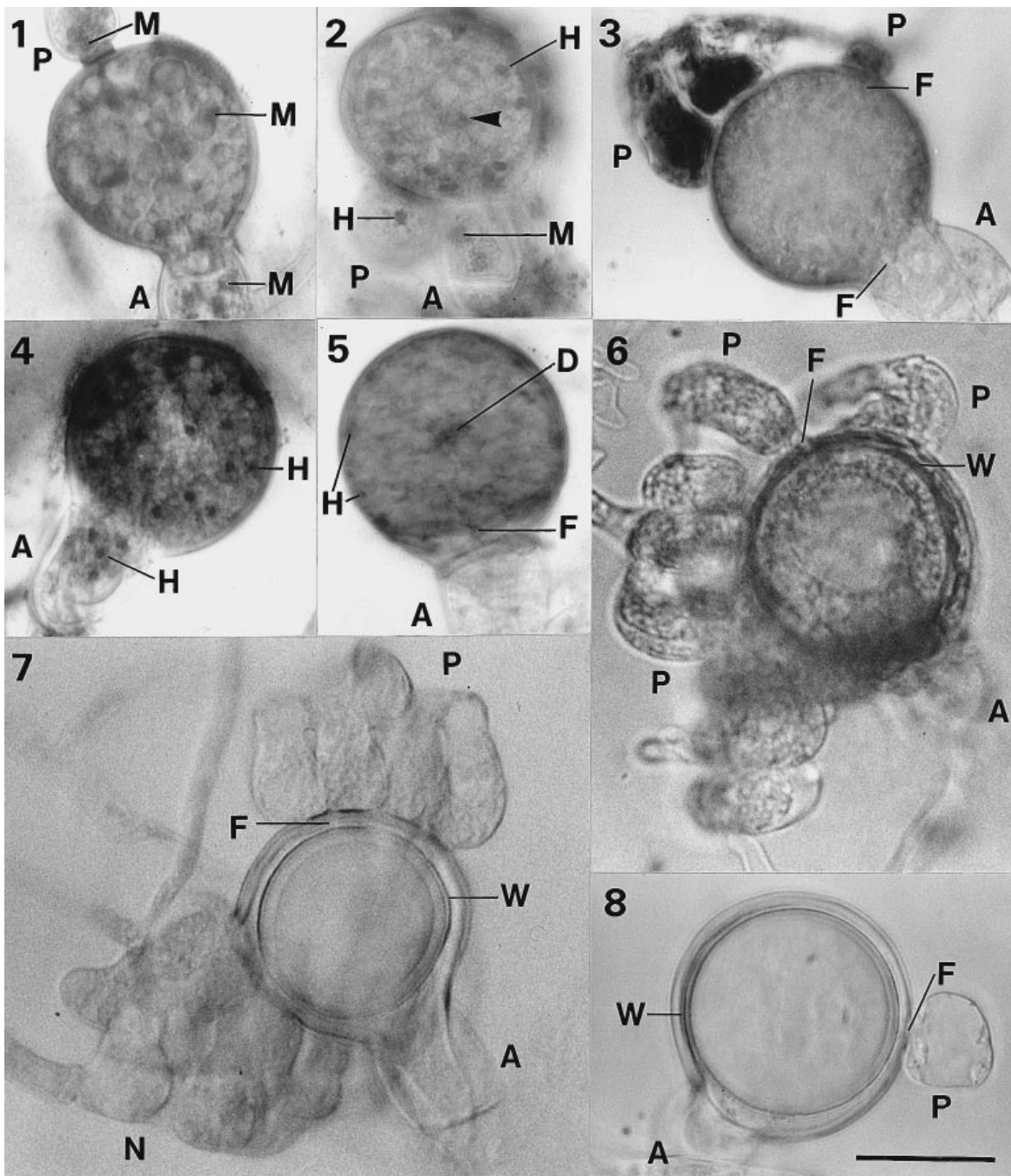
Development of antheridia was examined in living and fixed material. Pieces of agar were cut from 7-d-old pairings in regions containing gametangia and developing oospores. For

fixed material they were incubated in iced water for 1 h prior to fixing for 16 h with acetic acid/100% ethanol (1:3, v/v) and stored in 70% ethanol at 4° for 1–14 d before staining. Trypan blue in lactic acid-glycerol solution was used to reveal structural changes (Tommerup, 1984). Nuclei in antheridia and oogonia were stained by a modification of the technique used by Tommerup (1988). Stored agar segments were suspended in two changes of 60% aqueous acetic acid for 5 min at 24° and for 15 min at 60°, stained immediately in 2% orcein in 60% acetic acid for 2 h at 60° and for 24 h at 20°, destained and mounted in 60% acetic acid and examined at up to $\times 1000$ magnification. The sequence of stages of meiosis (Tommerup, Ingram & Sargent, 1974; Brasier & Sansome, 1975; Sansome, 1987; Brasier, 1992) were confirmed by a preliminary time course examination of gametangial development involving a series of gametangial initials in 5 d pairings, identified gametangia were fixed at daily intervals, and then stained to show nuclei. The vegetative nuclei of *P. cinnamomi* are probably mostly diploid (Brasier & Sansome, 1975) and therefore the post-meiotic nuclei are designated as haploid and the fusion nuclei as diploid (Brasier, 1992).

RESULTS

Diameters of antheridia, oogonia and oospores for the nine WA pairings ranged from 14.9–15.7 μ m, 34.5–39.8 μ m and 28.3–33.4 μ m respectively. The presence of paragynous antheridia did not change the size range of the three characters. Oospores formed in all pairings of these, the 11 Australian-wide and the 14 PNG isolates were plerotic, whether antheridia were amphigynous-only or a combination of amphigynous and paragynous. In all pairings, 93–98% of gametangia formed on the A1 side of the zone where the two colonies met.

All oogonia examined had amphigynous antheridia that enveloped the oogonial stalk (Figs 1–8). No oogonia had only paragynous antheridia. In seven of nine WA pairings, 3–10% of oogonia had paragynous and amphigynous antheridia (Fig. 9). Of these pairings, five had oogonia with multiple (2–13) paragynous antheridia. In the 26 Australian-wide and PNG pairings, all had some oogonia with paragynous and amphigynous antheridia. However, most had fewer than 0.5% with two pairings having more than 5% with paragynous antheridia (Fig. 9). The proportions of oogonia with paragynous antheridia from the nine WA pairings are consistent with these data as in some of the 26 pairings more than 300 oospores had to be examined before a paragynous antheridium was found. Two different A1 isolates and two different A2 isolates were involved in the two pairings having about 35% of paragynous antheridia (Fig. 9). Those isolates in other pairing combinations had less than 5% paragynous antheridia. The number of paragynous antheridia per oogonium ranged from one to at least 13 (Figs 1–3, 6–8) and in extreme cases they completely obscured the oogonium. Meiosis occurred in both types of antheridia and oogonia in all pairings. Paragynous antheridia formed in association with young oogonia in which meiosis had just commenced (Fig. 1) and occasionally they continued to develop after fertilization was completed (Fig. 7).



Figs 1–8. Developmental sequence of paragynous and amphigynous antheridia of *Phytophthora cinnamomi*. **Figs 1, 2, 4, 5.** Fixed and stained in orcein. **Fig. 3.** Fixed and stained in trypan blue. **Figs 6–8.** Living gametangia. A, amphigynous antheridium; D, diploid fusion nucleus; F, fertilization tube; H, haploid nuclei; M, meiotic nucleus; N, non-functional paragynous antheridium; P, paragynous antheridium; W, oospore wall. **Fig. 1.** Adherence of paragynous antheridium to oogonial wall and near synchronous meiosis in nuclei at prophase I. **Fig. 2.** Haploid nuclei moving towards periphery leaving one nucleus (arrowhead) in the centre of the oogonium and in the paragynous antheridium and meiotic nuclei in the amphigynous antheridium (nuclei in anaphase I). **Fig. 3.** Developing fertilization tubes from an amphigynous and a paragynous antheridium. **Fig. 4.** Near synchronous meiosis in an amphigynous-only association showing haploid nuclei. **Fig. 5.** Amphigynous-only association with fertilization tube, a diploid fusion nucleus and peripheral haploid nuclei. **Fig. 6.** Multiple paragynous antheridia one of which may have contributed a nucleus to fertilize the oosphere. **Fig. 7.** Thirteen paragynous antheridia which formed post-fertilization and appeared not to have participated in fertilization. **Fig. 8.** Synchronous development of paragynous and amphigynous antheridia indicated by practically acytoplasmic antheridia. Note the developing oospore wall and fertilization tube from the paragynous antheridia. Bar, 20 μm .

The earliest paragynous interaction observed was at the stage when the amphigynous antheridium diam. was equivalent to that of the oogonium. When paragynous antheridia adhered to the expanding oogonial wall at this early stage, the

meiotic division was synchronous in both antheridial types and almost synchronous with that in the oogonium (Fig. 1). Meiosis in paragynous antheridia sometimes preceded that in the amphigynous antheridia (Fig. 2). After meiosis had

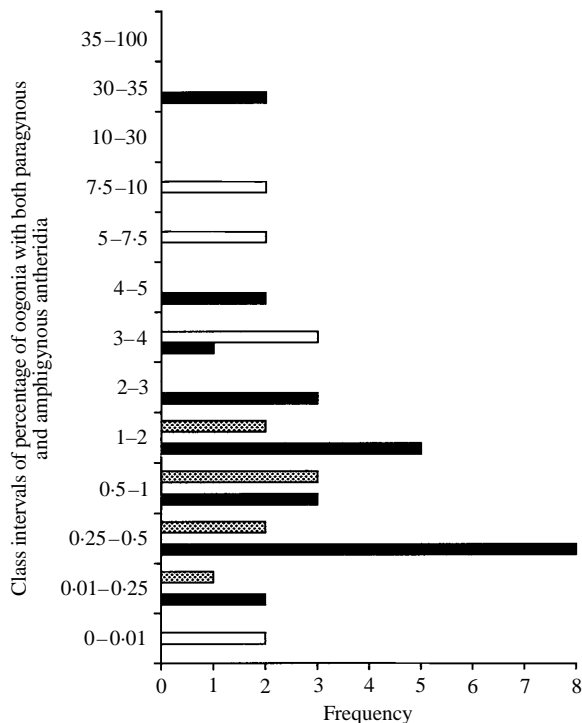


Fig. 9. Frequency distribution of the percentage of oogonia with both paragynous and amphigynous antheridia for 43 pairs of isolates of *Phytophthora cinnamomi* from Western Australia (□), Australian-wide (■) and Papua New Guinea (▨) collections. Note that oogonia with only paragynous antheridia have not been found.

occurred in an oogonium, all except one nucleus appeared to migrate to the periphery (Fig. 2). When nuclei were evident around the periphery of the oogonium, a single nucleus remained in the centre suggesting that the oosphere was delimited by a membrane or a very thin wall at that stage and that it developed where the outer oospore layer would form later. When oogonia were at this stage, fertilization tubes were in the process of developing from antheridia (Fig. 5). Fertilization tubes developed prior to the oospore wall being obvious and the wall of the fertilization tube was very thin when first visible at $\times 1000$ magnification. They were 2–7 μm long for paragynous and 5–15 μm for amphigynous antheridia. Fertilization tubes from amphigynous antheridia had 1–3 nuclei and only one nucleus was seen from paragynous antheridia. Only one fertilization tube was observed per antheridium.

After fertilization tube development, only two nuclei were evident in the oosphere, indicating that the nucleus of only one antheridium fertilized the oosphere in single or multiple antheridial interactions. Moreover, only one nucleus from multinucleate fertilization tubes entered the oosphere. It appeared that those paragynous antheridia where meiosis and the fertilization tube developed synchronously with the amphigynous antheridium had an equal chance of fertilizing the oosphere (Figs 1, 2, 6). However, when meiosis in the paragynous antheridium lagged behind that of the amphigynous antheridium and the oogonium, fertilization by a nucleus from a paragynous antheridium probably did not occur (Fig. 7). The fertilization tube appeared either not to complete development or to remain external to the developing

oospore wall whether there were zero or many paragynous antheridia (Figs 1, 2, 4). After fertilization, oospore development in paragynous fertilized oospheres was the same as in amphigynous fertilized ones.

Asynchrony in meiosis among gametangia may have contributed to oosphere or oospore abortion. Fertilization appeared to fail when meiosis in the two types of gametangia was asynchronous, i.e. haploid nuclei in one gametangium and diploid in the other. The cytological evidence for failure of fertilization was that firstly, the oogonial cytoplasm had 'oil' globules when its meiosis was later than that of the antheridium, i.e. early prophase nuclei in the oogonium and haploid in the antheridium. These large 'oil' globules were not present in synchronized associations. Secondly, when meiosis in the antheridium was behind that of the oogonium, i.e. haploid nuclei in the oogonium and early prophase in the antheridium, an oospore wall was evident prior to meiosis in the antheridium. Such behaviour was associated with 'oospores' in which the cytoplasm was autolysing. They may have been the oospores seen in living material that became acytoplasmic when the cytoplasm lysed. Oospores with thinner walls developed in many aborting oogonia. Thirdly, when oogonial meiosis preceded that of the antheridia by an even longer time, the oogonial cytoplasm appeared to have begun autolysing at the time when prophase was beginning in the antheridia. They may have been some of the oogonia which in living cultures were observed to autolyse and not develop oospores. These three types of developmental failure occurred at all stages in paired colony interactions from 7 to 28 d. In the 26 pairings, abortion was 0.01–0.8% when it was measured as equal to 50–100% degradation of oospore or oogonial cytoplasm.

DISCUSSION

This is the first known report of the occurrence of single or multiple paragynous antheridia in *P. cinnamomi* (Gerrettson-Cornell, 1989; Stamps *et al.*, 1990). Additionally, it is, to our knowledge, the second record of a heterothallic species of *Phytophthora* having both paragynous and amphigynous antheridia associated with an oogonium, the other being *P. hibernalis*. The only other example of this gametangial behaviour in *Phytophthora* is in *P. porri*, a homothallic species (Waterhouse, 1970; Ho, 1981; Gerrettson-Cornell, 1989). Also *P. porri* appears to be the only other species reported to have multiple paragynous antheridia, but it is not recorded as having the large numbers that have developed for *P. cinnamomi*. The capacity to form paragynous antheridia is widespread in Australian *P. cinnamomi* isolates as 94% of pairings had paragynous antheridia and they involved five A1 and 16 A2 isolates. Paragynous antheridia also formed in the eight PNG matings. These results suggest that the capacity to form paragynous antheridia was 'switched' off in most sexual interactions as overall 2.4% of oogonia had paragynous antheridia and most interactions had fewer than 0.5%. Such 'switchable' characters are important to understanding morphogenetic variability, to complete taxonomic descriptions and in descriptions of the whole oomycete used for isolate identification. Switchable characters are not of value to

systematics when their expression is as low as in this case. Some taxonomic issues will be addressed in a subsequent paper. This study has shown that paragynous antheridia are by no means restricted to attaching near the base of the oogonium as Hemmes (1983) found for other *Phytophthora* species. The results imply that the site of adherence on the oogonium does not affect the chances of oosphere fertilisation by an antheridium, it is the timing factor that governs this as discussed below.

There are three possible combinations of gametangia for oospore development in *P. cinnamomi* in paired A1 and A2 cultures; an A1 oogonium and A2 antheridium, A1 antheridium and A2 oogonium, A2 antheridium and A2 oogonium (Brasier, 1975; Brasier & Sansome, 1975; Zentmyer, 1979). There was no evidence that paragynous antheridia derived from only A1 or A2 isolates. Count data from pairings suggest that the incidence of paragynous antheridia was the result of an interaction between isolates. No one isolate was more likely than any other to be involved in interactions producing paragynous antheridia.

No multiple oospores were observed and all other evidence indicated that only one antheridium was involved in fertilization of an oosphere. Oospore ontogeny indicated that one antheridial and one oogonial nucleus were involved in oospore development although, more than one nucleus was present in some fertilization tubes and more than one fertilization tube penetrated some oogonia. Other investigations of oospore development in *Phytophthora* also have indicated that two nuclei capable of fusion are required for functional oospore development (Brasier & Sansome, 1975; Brasier & Brasier, 1978; Jiang *et al.*, 1989). Pre-zygotic nuclei are usually haploid but this needs to be confirmed by unequivocal genetic analysis (Shaw, 1991; Brasier, 1992). Gametangial pre-meiotic or post-zygotic nuclei are mostly diploid although partial polyploids and polyploids have been identified in *Phytophthora* (Brasier & Sansome, 1975; Sansome, 1987; Shaw, 1991; Brasier, 1992). Oospores with more than two pre-zygotic nuclei appear unable to germinate and therefore are non-functional propagules (Jiang *et al.*, 1989). In our study, entry of other nuclei once fertilization had occurred appeared to be prevented by some mechanism possibly controlled by the fertilized oosphere or oogonium. This would explain why fertilization did not occur in cases where paragynous antheridia penetrated the oogonial wall once the oospore was developing although, the oospore wall was sometimes still very thin.

Either an amphigynous or a paragynous antheridial nucleus was transferred to an oosphere. When development of both antheridia was synchronous, they had an equal chance of success. The timing in antheridial development and oogonial receptiveness may be important in oosphere fertilization and the same factors may determine which type of antheridium contributed a nucleus. At the light microscope level, oospores apparently resulting from fertilization by a nucleus from a paragynous antheridium were indistinguishable from apparently viable ones formed in amphigynous associations. Moreover, preliminary experiments show that oospores from paragynous associations can germinate (Tommerup & Catchpole, unpublished data). Abortion of oospores and

oogonia was associated with asynchrony in gametangial meiosis. However, these phenotypes may also be caused by other, as yet unidentified, lethal factors.

Whether oospores are a product of selfing or hybridization needs to be confirmed by analysis of F₁ populations using diagnostic genetic markers such as DNA markers that are being identified in *P. cinnamomi* (Tommerup, 1995; Dobrowolski, Tommerup & O'Brien, 1996). If selfing occurs, then paragyny may be a mechanism of sex among A2 isolates in the field. Selfing and segregation would provide one explanation of the variation in morphology, physiology and pathogenicity we have seen among the 72 A2 isolates and in other localized populations from south eastern Australia (K. Old & M. Dudzinski, pers. comm.; Tommerup, 1995; Dobrowolski *et al.*, 1996).

It has been suggested that amphigyny plays some role in heterothallism since heterothallic species of *Phytophthora* are predominately amphigynous (Brasier, 1983). Amphigyny may be a mechanism to facilitate outbreeding of two potentially antagonistic individuals or it may be that it is efficient and has therefore been reinforced by selection. Interestingly no oospores in *P. cinnamomi* formed from only paragynous interactions *in vivo*. Heterothallic downy mildew fungi have paragynous antheridia, but they reproduce only in plant tissue (Tommerup, 1981). The discovery of paragyny in Australian and PNG isolates indicates it is a widespread character in *P. cinnamomi* and raises the question whether this occurs in other isozyme/electrophoretic types of *P. cinnamomi*. It also raises questions additional to those of Brasier (1983) about current understanding of incompatibility systems in *P. cinnamomi*.

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